HIGH-THROUGHPUT MICROFLUIDIC ACOUSTIC SYSTEM TO GENERATE YEAST CELLS FOR AGING STUDIES

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

High-Throughput Microfluidic Acoustic System to Generate Yeast Cells for Aging Studies.
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The mechanisms and causes of aging, the greatest risk factor of disease, are poorly understood. For aging at the single-cell level, this lack of knowledge results from limitations in the methods traditionally used in cell aging studies, including inefficiencies of manually culturing cells and constraints in the number of cells that can be analyzed. Microfluidic technologies solve these deficiencies that have prevented researchers from fully understanding cell aging; therefore, in this study we develop a high-throughput microfluidic system that generates billions of replicatively-aged yeast cells for subsequent biochemical analyses. Our system leverages size-based differences of acoustic forces to remove smaller, younger daughter yeast cells and isolate a large quantity of bigger, aged mother cells. With this tool, researchers can potentially generate a large, highly pure population of aged yeast cells without the need for time-consuming, error-prone purification steps or genetic modification. Experiments with such a system may reveal new insights into aging at the cellular and molecular levels. This improved understanding of aging can be used for treating age-related diseases, including sarcopenia, osteoporosis, macular degeneration, neurodegeneration, and cancer.

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NOMENCLATURE

RLS	Replicative Life Span
CLS	Chronological Life Span
YPD	Yeast Peptone Dextrose
MEP	Mother Enrichment Program
FOV	Field-of-View
FACS	Fluorescence-Activated Cell Sorting
MACS	Magnetic-Activated Cell Sorting
PBS	Phosphate Buffered Saline

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Molecular causes of aging are poorly understood

Conventional cell aging studies must rely on limited, cumbersome cell analysis techniques, and this deficiency presents one reason why the molecular causes of aging are not well-understood.[1] In other words, traditional methods of culturing and studying yeast cells have not allowed researchers to draw collective conclusions about aging, which is currently the greatest risk factor for disease.[2]

As examples illustrating the ambiguities in cell aging studies, researchers have not been able to explain the relationships between environmental conditions, genetics, oxidative damage,[2] apoptosis[3] and replicative life span of yeast cells. Also, studies have shown that chronological life span of yeast may be related to oxidative stress, dysfunction and reactive oxygen species, reduced autophagy, nuclear DNA damage, mutagenesis, replicative stress, metabolic alterations, extrinsic stress, and other factors.[2] The diversity of and lack of agreement about factors that influence replicative and chronological life spans of yeast underscore the needs for more studies in this field and improved instruments to perform the analyses.

Single-cell studies gather data unobtainable by studying higher organisms

Understanding aging at the single-cell level is important for two main reasons. First, characteristics and functions of individual cells contribute to overall well-being of an organism.

Second, studying individual cells is necessary to understand cell physiology of certain processes such as those that occur asynchronously or on limited timescales.[4]

Yeast aging studies can reveal pathways that influence aging in humans

Aging studies of unicellular eukaryotic organisms have revealed several pathways that influence aging in more complex organisms, including mammals. For this reason, simple organisms like yeast have emerged as ideal model systems for understanding aging in humans and other multicellular species.[3] Researchers have published >50,000 articles in which they used yeast as a model system. Benefits of studying yeast include short replication time (~90min), amenable to straightforward, inexpensive, high-throughput experimental approaches, and similarities with mammalian cells.[5] Additionally, yeast has a well-characterized genome and its genetics can be manipulated relatively easily,[2] especially compared to complex rodents and humans.[6] Recently, researchers hypothesize that aging is influenced to some degree by ancestral evolutionary origins, which makes yeast an even more promising tool with which to gain a better understanding of aging in more complex organisms.[3]

Several researchers are trying to determine which characteristics of yeast aging have analogs in mammals and other multicellular eukaryotes,[2] which shows the usefulness of yeast as model organisms to understand aging. Already, studying yeast has identified several mammalian genes and signal pathways that impact aging (e.g. the sirtuin pathway and the TOR signaling pathway).[2]

Yeast aging models

Three models are commonly used to study aging of yeast cells: replicative life span, chronological life span, and clonal senescence.[3,7] Replicative life span (RLS) is defined as the number of daughter cells produced by a mother cell before senescence. A single mother yeast cell divides 20-25 times (produces ~25 daughters) and then enters a postreplicative state followed by lysis.[8] Fig. 1 shows a schematic of RLS as well as a time-lapse yeast culture. Throughout the aging process, mothers accumulate damage, illustrated in Fig. 1 by circular rings that appear after each cell division. Note the differences in mother and daughter sizes in the time-lapse culture, a characteristic often exploited in yeast aging studies. The most common method of studying yeast RLS, first used in 1959 [Mortimer], involves manually separating daughter cells from mothers with a standard tetrad dissection microscope and micromanipulator. We discuss this and alternative methods of studying RLS in sections below.

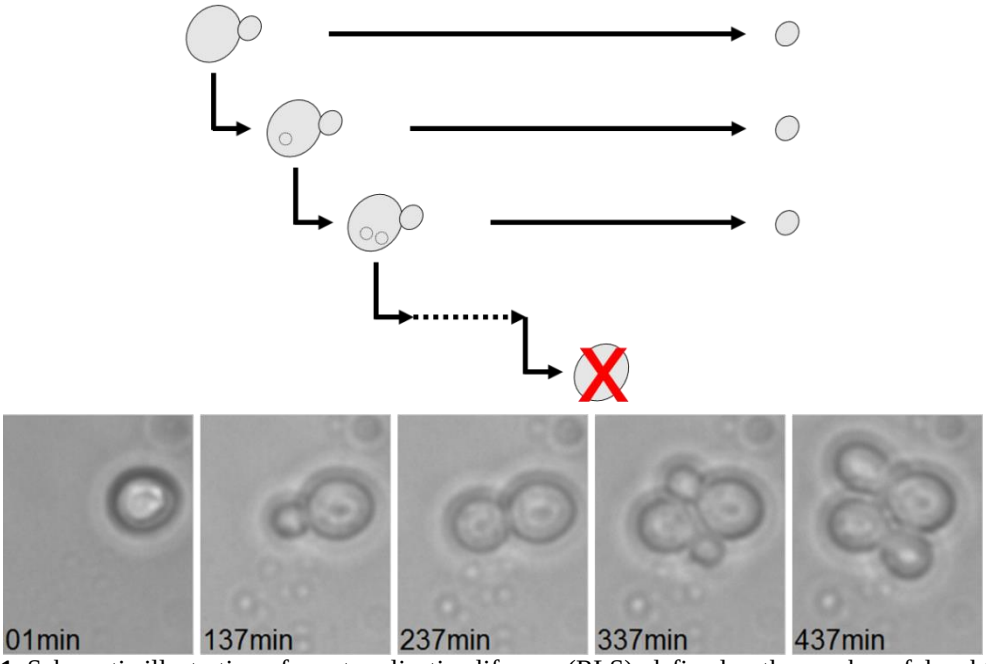


Figure 1. Schematic illustration of yeast replicative lifespan (RLS), defined as the number of daughter cells produced by a mother before senescence.

Chronological life span (CLS) experiments examine how long a yeast cell survives after exiting the cell cycle, that is, the length of time a yeast cell survives in stationary (non-replicating) phase. Yeast exit the cell cycle and enter stationary phase after the postdiauxic state, which occurs when cells deplete extracellular glucose, decrease growth, and metabolize using mitochondrial respiration ~24hr after a yeast culture begins.[2] Yeast cells that have stopped dividing once they deplete their environment of nutrients and enter the postdiauxic phase can survive a few days to several weeks. Environmental conditions including temperature, nutrients, pH, acetic acid concentration and genotype can affect CLS.[5] Three methods are commonly used to study yeast CLS, all of which monitor cell survival but differ in growth conditions. In one method, cells are grown in 2% glucose through the postdiauxic phase and into stationary phase. The second technique is similar to the first except cells are transferred to water during the postdiauxic phase. In the third well-established method, cells are cultured on 2% glucose agar plates containing all nutrients except tryptophan.[2]

Clonal senescence of yeast cells, which is similar to the Hayflick limit observed in mammalian cell cultures, is indicated by the finite number of times a cell population can divide. Clonal senescence, also known as clonogenic survival, is quantified by colony-forming units (CFU). CFUs are calculated by scoring the ability of each yeast cell from a pre-determined number of cells to divide/reproduce and form a mini-colony on day 3 of a culture, known as the "100% survival point."[5] Other approaches to measure clonogenic survival are also used, such as the FUN-1 assay that measures metabolic activity of a population.

Researchers hypothesize that RLS studies may reveal insight into aging of mitotic/stem cells or cells with finite RLS (such as fibroblasts and lymphocytes) in multicellular eukaryotes like humans, and that CLS studies can be used to understand aging of post-mitotic tissues, non-dividing cells (e.g. neurons), and cells having long stationary phases.[5] Researchers most commonly study RLS and CLS in yeast cells, and these models complement each other. Furthermore, both aging models are needed to understand aging completely.[2]

Aging studies can improve how we treat age-related diseases

The primary motivation of this project stems from the fact that large quantities of replicatively-aged mother cells are needed to analyze molecular and biochemical factors related to cell aging.[9] We discuss below how conventional and microfluidic methods currently cannot produce a sufficient quantity of cells. Without a large-scale tool to generate aged yeast cells, our knowledge about the molecular causes of aging is limited and incomplete. The new knowledge gained from studies of aged yeast cells can be used for treating age-related diseases, including sarcopenia, osteoporosis, macular degeneration, neurodegeneration, and cancer.[10]

Challenges of generating a large, highly-pure population of aged yeast cells

Isolating a large number of aged cells is challenging for several reasons. Offspring of mother cells exponentially grow in number such that they can impact environmental conditions of mothers and impact normal growth/aging. In other words, daughter cells quickly overtake the majority of nutrients and space in the small regions around a mother cell (see Fig. 9). Because the number of yeast cells in a growing culture exponentially increases, a very small fraction of the total population will be very old mothers (>12 generations).[9] Identifying and isolating the

small number of aged mothers is already challenging. As mentioned above, a sufficient quantity of aged cells is required to obtain reliable results in subsequent biochemical analyses; therefore, several groups are trying to solve this problem of generating large numbers of replicatively-aged yeast cells.

Yeast phenotypes relevant to replicative lifespan

The phenotypes of yeast cells change throughout their replicative lifespans. Researchers have observed bud scar accumulation, enlargement of cell size, decreasing cell cycle and fertility rates,[9] and thickening of cell wall[6] as effects of replicative aging. We exploit size-based differences between mother and daughter cells to achieve our overall aim of isolating large quantities of aged mother cells. We count bud scars, or circular chitin deposits on cell walls resulting after cell division,[6] to confirm the efficiency of our system.

Of particular importance in our experiments, a mother yeast cell keeps most of its cell wall throughout its lifetime, whereas the daughter cell wall is newly synthesized.[7] In other words, none of a mother's cell wall is inherited by its daughter; the daughter is grown with an entirely new cell wall. We utilize this characteristic to verify our system's ability to separate mothers from daughters.

Micromanipulation and purification to study yeast replicative lifespan

Researchers use two methods to study replicatively-aged cells: micromanipulation and purification. Micromanipulation has been used for more than 50 years. Recently, researchers have turned to higher-throughput purification methods including selectively killing daughter

cells and using microfluidics to remove daughter cells from mothers; however, these alternatives have not replaced the manual dissection method as the "gold-standard life span assay"[2] for reasons discussed below.

The most common method of studying yeast RLS, first used in 1959,[11] leverages the fact that daughter yeast cells are smaller in size than their mother cells.[2] Experimenters must manually separate daughter cells from mothers using a standard tetrad dissection microscope with micromanipulator. Specific instructions for performing such a micromanipulation experiment are discussed elsewhere (e.g. Park[9]). In general, though, a researcher skilled at using a micromanipulator cultures ~40 virgin cells arrayed on an agar plate. Because of significant variations among individual cells, 40 or more cells must be studied to obtain reliable data. As each cell divides, the researcher must identify the daughter cell and carefully remove it with a glass pipette tip attached to the micromanipulator. Removal of the daughter cell is important so that the mother cell is not obstructed visually and to prevent the mother's offspring, which increase in number exponentially, from stealing nutrients and influencing the mother's normal life span. If each cell produces ~25 daughters, then ~1,000 cells must be removed in a single experiment, which takes 10-14 days.[6,8] Although 40+ cells can be analyzed to obtain meaningful results, larger quantities of cells (thousands to millions) simply cannot be studied in a single aging experiment because of limitations in keeping track of all mother cells.

Although micromanipulation to study RLS in yeast cells is most accurate especially when precise cell ages in number of cell divisions are needed, this conventional method has several disadvantages. Any contamination may ruin the RLS study, so researchers must practice extreme

caution when handling the cultures so extensively.[9] They must also work diligently not to dry-out the cultures during removal of daughter cells.[6] Only a limited number (~40) of cells can be studied and they are isolated on an agar plate, so the aged mothers cannot be used in subsequent immunofluorescence or biochemical experiments.[9] This approach to study RLS is low-throughput, labor intensive, and time-consuming.[8] Micromanipulation may damage cells and invalidate experimental data. Because most of the RLS experiments are not carried out continuously, cells must spend time in cold storage to prevent a surplus of daughter cells around each mother.[5] Some studies claim incubation at low temperatures does not significantly impact RLS; however, growth rate varies (e.g. mother cells bud on average once every ~2hr at 30°C, once every 12hr at 10°C, and stop dividing at 4°C),[9] and growth rate may impact RLS. Natural variations in growth rate (those not induced by temperature or other environmental conditions) also complicate the experiment because researchers must then conform to the varying replication schedule of cells. For example, cells at the very beginning of their life spans divide once every ~90min at 30°C, while aged mother cells sometimes may not divide for several hours—resembling replicative senescence—and then continue proliferation. This phenomenon complicates determining whether a mother cell is finished dividing.[9] Much experience is required for the manual micromanipulation RLS experiments, not only for operating the equipment but also recognizing subtle aspects of the yeast lifespans. For example, differentiating between mother and daughter cells is difficult during the first few cell divisions before the mother has grown very large. Finally, aged mother cells are especially fragile, and cells should not be dissected too often because over-dissection may shorten lifespan.[6]

Recall one significant disadvantage of using micromanipulation to study RLS in very old yeast (>15 generations) is that this technique does not produce a sufficiently large quantity of replicatively-aged cells for subsequent molecular and biochemical analyses. Large-scale purification methods have been developed, however, and studies using these isolation techniques have revealed important factors associated with cell aging, including nucleolar fragmentation, movement of the Sir complex from telomeres to nucleolus, and accumulation of rDNA circles.[9]

Several researchers have developed methods of isolating cells based on replicative age. In one method,[12] a "baby machine" generates virgin daughter cells, but it cannot produce aged mothers. Another method uses centrifugal elutriation,[13] which utilizes differences in mother/daughter sizes to isolate larger mother cells and remove their daughters. To produce very old cells, the elutriation must be repeated multiple times. Another procedure using sucrose gradient centrifugation produces only a modestly pure population of aged cells (~90%) even after repeated purifications. Centrifugation is not amenable to high-throughput operation and may harm cells.[14]

One of the most successful methods of obtaining a large, pure population of aged mother cells requires tagging the mother cell surface with a label that is not passed to daughter cells. The cell membranes of daughter cells are newly synthesized, so no parts of the previously synthesized mother cell membrane are passed to any of its daughters. Using this property, researchers can isolate labeled mother cells after any number of cell divisions. One such labeling system described by Smeal et al.[15] produces a large number of aged mother cells by selectively coating mothers with biotin. The biotin remains attached to the mother's cell wall even after cell division

because the daughter cell wall is newly synthesized. Isolation of biotin-labeled mother cells for subsequent analysis is accomplished using either fluorchrome-conjugated avidin and fluorescence-activated cell sorting (FACS) or streptavidin-coated paramagnetic iron beads and magnetic-activated cell sorting (MACS). The technique employing FACS produces an aged population that is >99% pure; however, expensive instruments are needed to accomplish this isolation, and the population of aged cells is relatively small (10^4 cells). The magnetic sorting method separates a much larger quantity of cells (10^8) that are 7-12 generations old. Unfortunately with these techniques, several manipulation and sample processing steps are needed, especially to obtain a very pure population and one that is older than 12 generations. In addition to the repeated sorting steps, increasing risks of contamination that can ruin the experiment decrease the attractiveness of this method.[9] Although FACS and MACS are considered high-throughput techniques, they may damage cells and are not label-free.[14]

One disadvantage of these purification methods is that the exponential proliferation of daughter cells limits the replicative age of cells that can be obtained from such techniques. Purifying a cell culture once produces cells that are only 7-12 generations old. Other drawbacks of these methods are low yield, contamination by daughter cells, and manual, time-consuming experimental steps such as several sample washes. Repeating the purification steps can produce cells older than 7-12 generations; however, the additional sample processing and greater risk of adverse effects (e.g. contamination) reduce the usefulness of these purification techniques.[7]

Other purification schemes rely on genetically modifying yeast cells to aid in isolation of aged mother cells. In one genetic manipulation approach that arrests proliferation of daughter cells,

thereby helping to isolate aged mother cells,[16] the RLS of mother cells is significantly decreased.[7] This major drawback precludes the use of this technique for our target application.

Another approach termed the Mother Enrichment Program (MEP) genetically modifies daughter cells by removing essential genes and stopping cell division.[7] Therefore, in a cell population experiencing MEP, genetically modified daughter cells do not divide, and so a mother cell's offspring do not limit the mother's nutrients or impact normal aging. In other words, RLS of mother cells is not changed, but replicative ability of daughter cells is turned off. Due to these genetic changes, overall cell viability becomes a function of RLS. With the MEP, researchers can reliably study genetic and environmental factors that affect RLS. The MEP can also produce a sufficient quantity of cells at any point in their replicative lifespans for subsequent biochemical analyses. MEP has several drawbacks, however, because it relies on genetic selections. A researcher must genetically modify a strain of interest. Mutations of modified strains and cells that escape genetic modification both decrease the effectiveness of MEP and introduce contamination into the aged-mother populations. In numbers, MEP selectively kills all but ~8% of daughter cells.[6] Additionally, daughter cells in MEP cultures can remain metabolically active in M-phase and grow for ~24hr before lysis.[7] Metabolically active daughters can impact normal environmental and growth conditions of mothers. Their effects on mothers are likely emphasized when the mothers are older than ~20 generations because the replication rate of the mother slows and the mother experiences more adverse effects of old age. These drawbacks interfere with the ability of MEP to generate large numbers of very old mother cells (>20 generations).

In summary, biochemical and genetic analyses of factors that influence yeast aging are limited by the inability to isolate large numbers of replicatively-aged cells. Micromanipulation, which requires costly equipment and skilled professionals, cannot yield a sufficient quantity of cells. Several methods have been developed to replace micromanipulation. These techniques suffer from several drawbacks, including the inability to produce cells older than 7-12 cell divisions due to nutrient depletion and needing to purify a culture in several steps such that risks become significantly concerning. For example, purifying a culture repeatedly can result in contamination and low yield/viability of aged cells.[7] Even for techniques like the Mother Enrichment Program (MEP), which does not have some of the drawbacks like extensive sample processing, the resulting isolation of aged mother cells is only modestly pure (~8% of daughter cells are not killed by the MEP). The drawbacks associated with conventional micromanipulation along with the disadvantages of techniques aiming to improve micromanipulation have sparked the developments of further-improved systems utilizing microfluidics.

Microfluidics to study yeast aging

Two disadvantages of traditional cell culture techniques motivated researchers to introduce microfluidics into cell aging studies. First, during cell culture, a single mother cell divides into two daughter cells, the two daughter cells divide into four cells, and so on. This exponential increase in the number of daughter cells quickly makes individual cell tracking challenging, if not impossible. Due to the exponential increase in daughter cells, researchers cannot acquire high-resolution time-lapse images throughout replicative cell aging. Second, researchers cannot reliably and efficiently study individual cell responses to different outside stimuli when the cells are in dense cultures. Instead, researchers must resort to averaging their findings over the entire

cell population, which may hide important clues about aging and limit the variety of data that can be collected. Microfluidics promises to solve these problems.

Microfluidics is the science and technology of manipulating small quantities of liquids and gases using devices with small dimensions.[17] Microfluidic devices dedicated to culturing cells have already provided significant advantages over conventional cell culture techniques, including increased automation, higher-throughput, and greater reliability and repeatability.[18-27] The systems in these particular works, however, cannot be applied to generating large quantities of aged mother cells for obvious reasons.

To our knowledge, three research groups have applied microfluidics to the study of aging, revealing new knowledge in the field of cell aging.[23,28,29] Lee et al. and Xie et al. developed systems to automate yeast RLS studies and capture high-resolution images of cells throughout their replicative lifespans. The microfluidic systems trap mother cells and wash away smaller daughter cells after each budding. Lee et al.[28] observed age-related changes in cell phenotypes, and Xie et al.[29] found a molecular marker that is a "good predictor" for the lifespan of cells. With further development, these microfluidic systems may enable high-throughput studies,[8] but as of recently, the systems still have not replaced the conventional micromanipulation method or any of the purification methods discussed above. This is likely due to functional imperfections such as trapping too many mother cells under one trap or failing to remove offspring of a trapped mother cell. An additional disadvantage of these systems involves through-put having sole dependence on geometry of the devices. The number of trapping sites determines the max number of cells that can be studied in a given experiment, that is, to study 1,000 cells, >1,000

trapping sites are required (because not all of the sites will successfully trap a cell throughout its lifetime), and each site must be analyzed. Additionally, differences in experimental results of the microfluidic devices show they must have some bias. For example, mean replicative lifespan results obtained by Lee et al. (25 cell divisions), Xie et al. (18 cell divisions), and micromanipulation methods (27 cell divisions) all differ.[8]

The most significant disadvantage of these microfluidic systems—relative to our work—is the inability to generate large numbers of old cells for biochemical tests. The mother-enrichment program (MEP), in which daughter cells are genetically altered so that they cannot divide, is a better method for these types of tests;[8] however, as discussed above, even MEP suffers from drawbacks such as failing to kill ~8% of daughters cells, which can impact aging of mother cells.

To sum up, aging studies need large quantities of replicatively-aged mother cells (>15 generations) to perform biochemical experiments of age-associated phenotypes. Without a system to provide the mother cells needed for true large-scale screening in replicative aging studies, we cannot fully understand the molecular causes of aging.

The conventional micromanipulation technique, although the "gold standard life span assay"[2] for over 50 years, cannot generate a large number of aged mother cells and suffers from numerous other disadvantages. Purification techniques, such as labeling the mother cells for subsequent separation or genetically modifying daughter cells so that they cannot replicate, still do not provide researchers with an efficient and straightforward method of isolating a large number of

aged mother cells. Finally, the microfluidic systems aimed at studying yeast aging are not well-suited for generating aged cells for subsequent analyses.

Size-based acoustic separation of mothers and daughters

To combat the problems of the techniques discussed above, in this project we develop an acoustic microfluidic system that separates aged yeast cells for large-scale aging studies. Gentle, efficient, label-free separation of mother/daughter yeasts cells within a microchannel is enabled by acoustic forces that depend on cell density, compressibility, and volume. In our system, we exploit differences in mother/daughter size to isolate mother yeast cells. We evaluate the effectiveness of acoustic deflection at isolating aged mother cells and quantify the acoustic separation resolution for differentiating between cells of different replicative age. In acoustic deflection, acoustic forces alter the trajectory of a cell as it flows in a microchannel across a region in which acoustic waves are applied. The trajectories of all cells are influenced by acoustic forces; the magnitude of influence is theoretically greater for larger cells, all other characteristics being equal.

The acoustic force on a cell in a microchannel is described by

$$F_a = 4\pi a^3 k_0 E_0 \left[\frac{\rho_c+\frac{2}{3}(\rho_c-\rho_m)}{2\rho_c+\rho_m} - \frac{1}{3}\left(\frac{\kappa_c}{\kappa_m}\right)\right] \sin(2k_0 z) \quad (1)$$

where a is cell radius, k_0 is wavenumber, E_0 is acoustic energy density, z is a position coordinate, ρ is density, and κ is compressibility.[30] Subscripts c and m indicate cell and medium, respectively. In this work, because mother/daughter yeast cells generally differ in volume and $F_a \sim a^3$, we can utilize acoustic forces to isolate larger mother cells. We assume that density and

compressibility do not vary considerably for mother and daughter yeast cells, and instead exploit the large dependence of acoustic force on cell size.

Advantages and anticipated problems of an acoustophoresis approach

By using acoustic forces to remove smaller daughter cells from larger mother cells, we are able to apply several advantages of acoustophoretic separation to yeast aging studies. Because acoustic forces are not affected by pH, salt concentration, and other medium properties, the isolation of mother cells in our experiments is not affected by variations in medium or cell environment. In other words, reagents and cell types (e.g. different strains of yeast) can change without affecting acoustic separation efficiency. Also, acoustic forces acting perpendicular to the liquid flow impose minimal stress on the cells; therefore, acoustophoresis is ideal for experiments with delicate cells, especially replicatively-aged mother cells.[14]

Other groups have shown acoustophoresis is useful for large-scale separation.[14,30-40] For example, Yang and Soh[14] separate viable mammalian cells from a mixture also containing apoptotic cells. Because live and dead cells differ in volume but have similar density and compressibility, acoustic forces are much stronger on viable cells.

For our particular application, none of the acoustophoresis devices from these groups can be used because the microfluidic channels are not configured appropriately. In particular, none of the systems implement a recirculation mechanism to isolate and age a large population of mother cells continuously throughout an experiment. Additionally, the parameters driving the acoustic radiation in these works are not optimized for purifying aged mother cells from a mixture of

yeast cells because none of the previous acoustophoresis works have attempted isolating aged mother cells using acoustic forces. As mentioned above, a large, highly-pure population of aged cells is needed for biochemical analyses so that we can better understand the molecular causes of aging, which influence how we treat age-related diseases. For these reasons, we pursue acoustophoresis for this yeast aging application.

Our acoustophoretic approach for aging studies enables straightforward, high-throughput processing to generate a large quantity of replicatively-aged mother cells without labeling or extensive sample manipulation. As mentioned above, acoustic forces acting perpendicular to the liquid flow impose minimal stress on the cells;[14] therefore, acoustophoresis is ideal for experiments with delicate, aged mother cells. An additional advantage of our approach is that it inherently removes "petites," one source of error that can distort RLS data. "Petites," smaller yeast cells in a population that may have a longer lifespan in some strains, must be removed at the beginning of a RLS study. Otherwise, RLS data from these outlier cells may impact the experiment data obtained.[9]

We also can leverage the numerous advantages of microfluidics in biochemical studies. Microfluidic technologies allow precise control over cells and liquids at the microscale and are amenable to high-throughput operation with parallel microchannels. Precise control over cells allows researchers to acquire high-resolution images throughout the entire RLS.[28,29] Microfluidics need not have skilled technicians for operating the devices, in contrast to the micromanipulation method discussed above to study RLS. Another advantage of a microfluidics approach is that the environment can be kept constant throughout the entire experiment. The

researcher also has the ability to alter environmental conditions to study biochemical reactions. This versatility and control is especially important in aging experiments because environmental conditions of yeast cell cultures can significantly impact RLS and CLS studies.[2,5,9] In other words, microfluidics allows automated, efficient methods of performing biological tests, such as exposing individual cells to the various external stimuli that may influence aging. Microfluidics also eliminates the need to move mother cells from one culture medium to another, which costs time, consumes reagents, and increases risks of contamination. Contamination is a significant concern for the purification methods discussed above and becomes increasingly problematic whenever purification steps must be repeated to obtain a very old yeast cell population (>15 generations). On the other hand, with a microfluidic system performing all sample handling and processing, the risks of contamination are drastically reduced. Researchers only need to handle the cells once when loading the microfluidic device, and a biocompatible and contamination-free environment can be maintained easily throughout the experiment.

We identify three potential problems for our acoustophoresis approach of isolating a large quantity of aged mother cells. First, acoustic forces may impact the normal aging of yeast cells. This concern is insignificant in our application because cells are not continuously exposed to acoustic radiation. Also, we can define the amount of time cells experience acoustic forces by adjusting the flow rates in our system, which means we can minimize this time if needed. Second, determining the precise replicative age of cells is challenging because we are not continuously monitoring the cells like Xie et al.[29] and Lee et al.[28] do with their systems; however, we use Calcofluor white staining of cell walls to count the bud scars manually under a microscope to estimate replicative age of cells. Therefore, although we cannot directly observe

the cells divisions, we can still determine replicative age of yeast cells in our system. A third potential problem for our acoustic separation experiments that may worsen the efficiency of isolated aged cells results from similarities between mother and daughter cell sizes at the beginning and end of the mothers' lifespans.[9] Mother yeast cells are generally larger than their daughters except at the beginning and end of the mothers' lifespans. Similarities in mother/daughter cell sizes at the beginning of the mothers' lifespans do not significantly impact the effectiveness of our approach, because if we are unable to remove the 1^{st} or 2^{nd} generation daughters, then the final cell population after the experiment can still have very old cells (20-22 generations). This symmetry in budding during the last few cell divisions is more concerning for our studies; however, even if the mother/daughter cells are similar in size when the mother is very old, we can still use acoustics to separate them. As described above in equation 1, the acoustic force is determined by cell size, density, and compressibility. Because an aged mother's cell wall has bud scars and thickens throughout its RLS, the compressibility of its cell wall will likely differ from that of a newborn daughter, which has a newly synthesized cell wall. Whenever the original mother cells are very old, we can adjust the acoustic settings of our system to separate cells based on differences in cell compressibility instead of size. Thus, acoustic-based separation can potentially mitigate this problem when mother cells undergo their last few divisions. This concern of mothers and daughters having similar sizes affects all of the size-based separation techniques discussed above, including centrifugation and microfluidic aging observation platforms; however, these techniques do not have built-in solutions like acoustic-sorting does.

CHAPTER II

METHODS

Acoustophoresis and on-chip recirculation to enrich the mother cell population

We investigate size-based acoustic manipulation of cells to achieve our goal of generating a large quantity of aged mother cells for subsequent analyses. The basic idea is to utilize acoustic deflection within a microfluidic device to separate larger cells into a different outlet channel than the one into which smaller cells flow (Fig. 2).

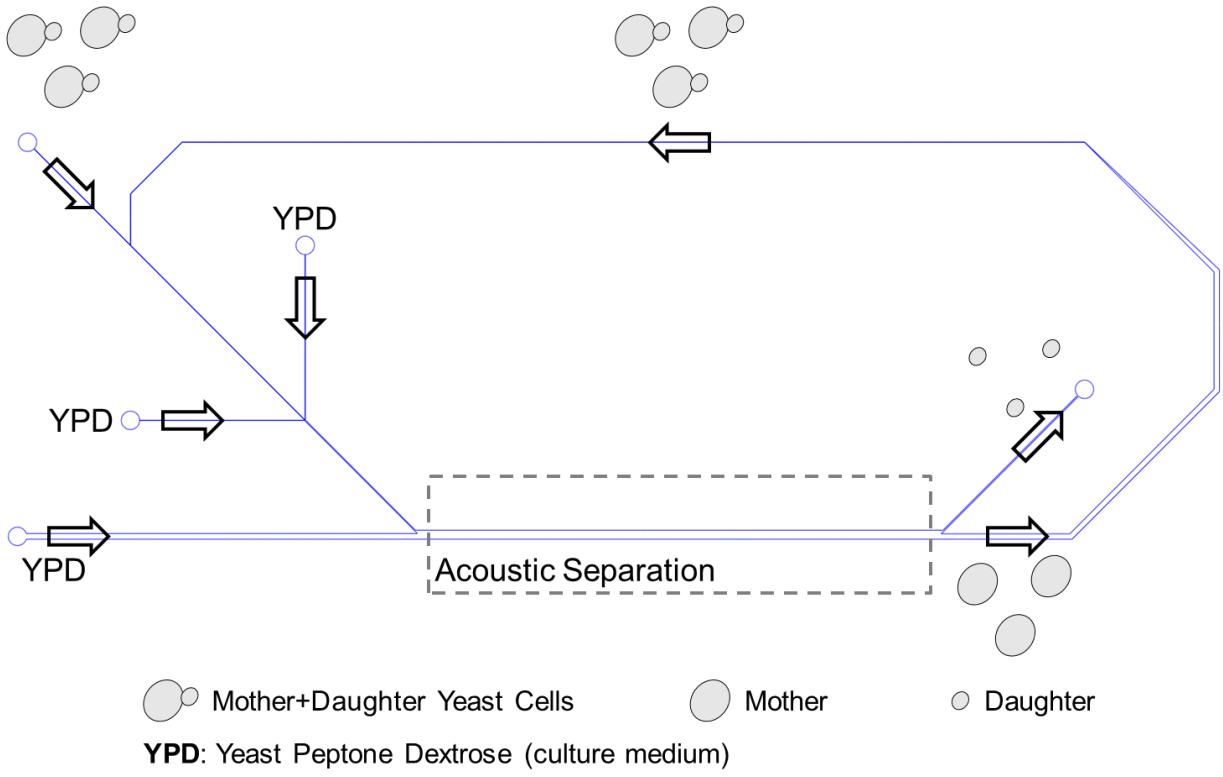


Figure 2. Schematic of microfluidic device utilizing size-based acoustic separation to remove smaller daughters and continuously age the larger mother cell population.

Cells enter the microfluidic device through the center inlet at top left of the image and are hydrodynamically focused by two streams carrying culture medium (YPD)—one on either side of the center channel. Fig. 3 shows how we can adjust the flow rates of the focusing channels to adjust the width of the center channel carrying yeast cells. Laminar flow, which describes the phenomena whenever adjacent fluid streams flow without mixing (turbulence) in a microchannel, produces the well-defined interfaces between water and red-colored dye in the images of Fig. 3.

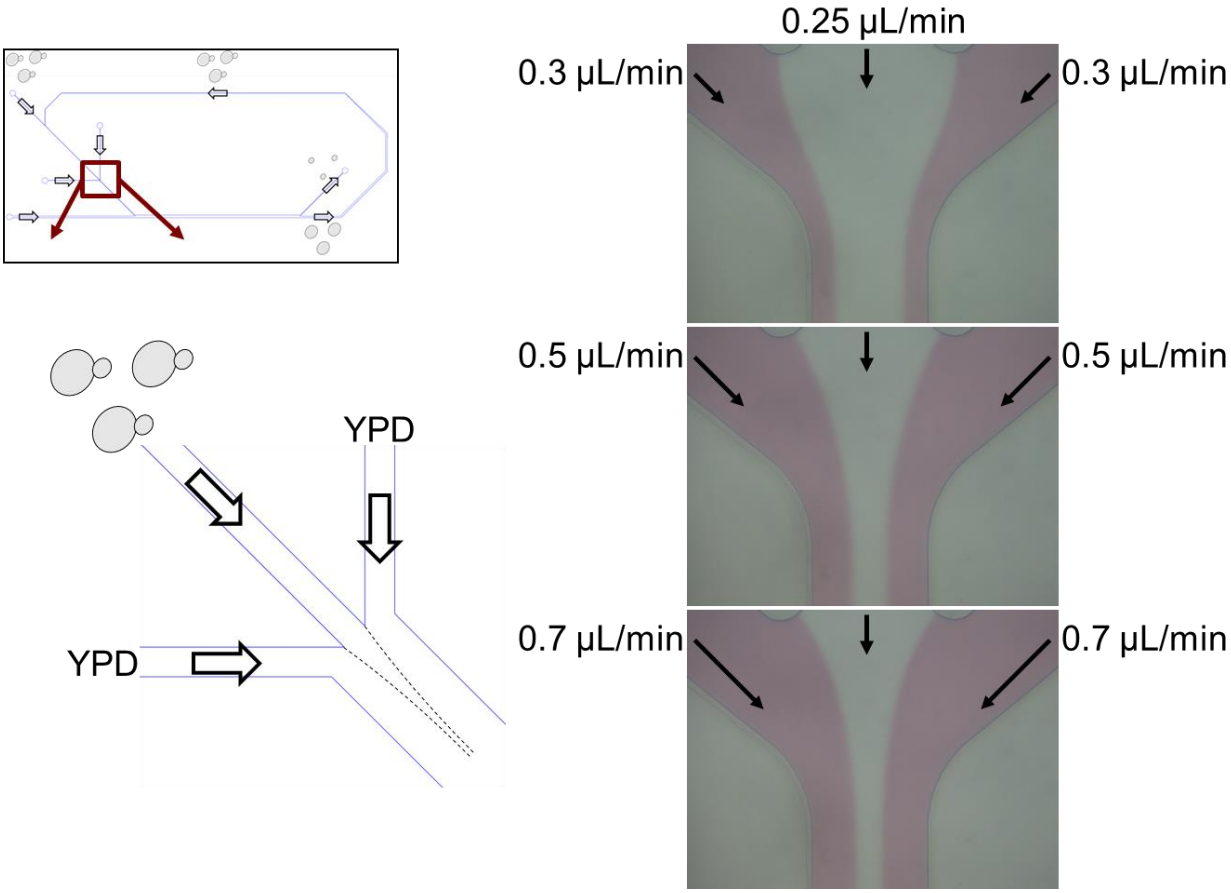
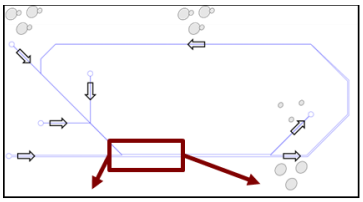


Figure 3. Hydrodynamic flow focusing, which results from laminar flow in a microchannel, helps ensure that all yeast cells enter the acoustic separation region at approximately the same location. DI water flows from top to bottom at 0.25µL/min in the images at right, while the flow rate of the red-colored dye increases from 0.3µL/min to 0.7µL/min. Adjusting the side channel flow rates focuses the center channel.

This focusing of the center channel helps ensure that all cells enter the acoustic separation region at approximately the same spatial coordinates so that the cell trajectories as they travel through the main channel depend only on size and do not vary by start position. As cells flow from left to right through the main channel with acoustic radiation off, laminar flow keeps them at approximately the same vertical position through the entire channel length; diffusion is the only mechanism deflecting them downward. If acoustic radiation is applied, cells experience a force pushing them to the center of the channel where an acoustic node is developed (Fig. 4). The positions of acoustic nodes and antinodes depend on the frequency chosen for the piezoelectric transducer. We choose the frequency so that a standing wave is formed within the channel and an acoustic node is located at the channel's center.

The cell trajectories are determined by the liquid flow rate and the acoustic force. Higher flow rates produce less diffusion between adjacent streams as they travel through the main channel. Less diffusion means less downward movement of cells. Acoustic force on yeast cells depends on size, compressibility, and density (equation 1). We assume density and compressibility of all yeast cells are approximately equal, and thus utilize size-based differences in acoustic force to separate the cells. The smaller cells consist of young daughters and petite mothers, and they travel into a waste outlet because laminar flow keeps them confined vertically in line with this outlet (Fig. 5). The acoustic forces experienced by these smaller cells are not large enough to deflect them below this outlet. Larger cells, which are primarily mother cells, experience sufficient acoustic force to deflect them below the waste outlet and are directed into a channel that recirculates them back to the original inlet.



$$F_a = -\left(\frac{\pi p^2 V_p \beta_m}{2\lambda}\right)\Phi(\beta,\rho)\sin(2kx) \qquad \Phi(\beta,\rho) = \frac{5\rho_p - 2\rho_m}{2\rho_p + \rho_m} - \frac{\beta_p}{\beta_m}$$

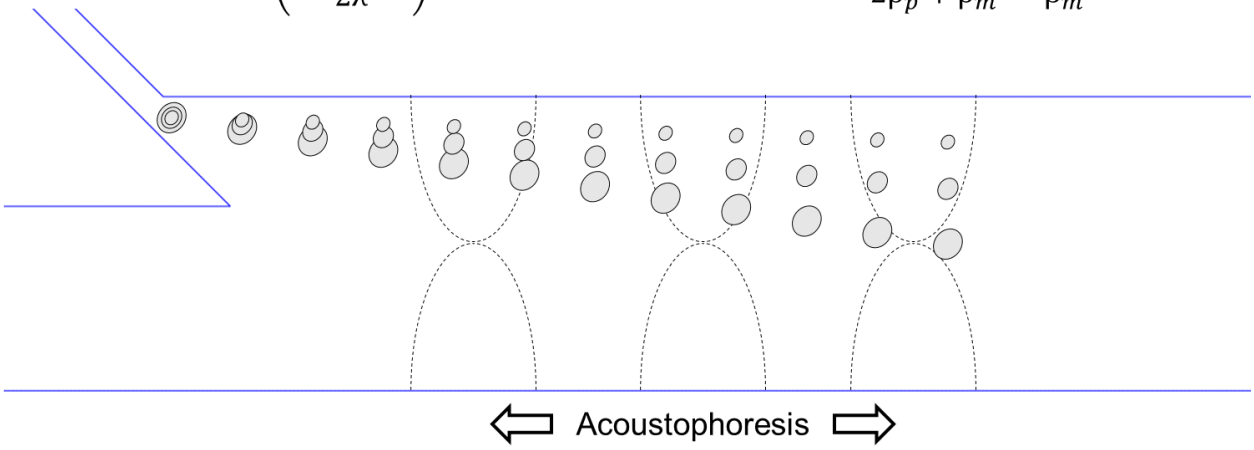


Figure 4. Acoustic separation region in which larger yeast cells are deflected more strongly to the center of the channel. $\boldsymbol{V_p}$ is particle volume, $\boldsymbol{p}$ is pressure amplitude, $\boldsymbol{\rho_p}$ and $\boldsymbol{\rho_m}$are densities of particle and medium with corresponding compressibilities $\boldsymbol{\beta_p}$ and $\boldsymbol{\beta_m}$. $\boldsymbol{k}$ is wavenumber and $\boldsymbol{\lambda}$ is wavelength.[30]

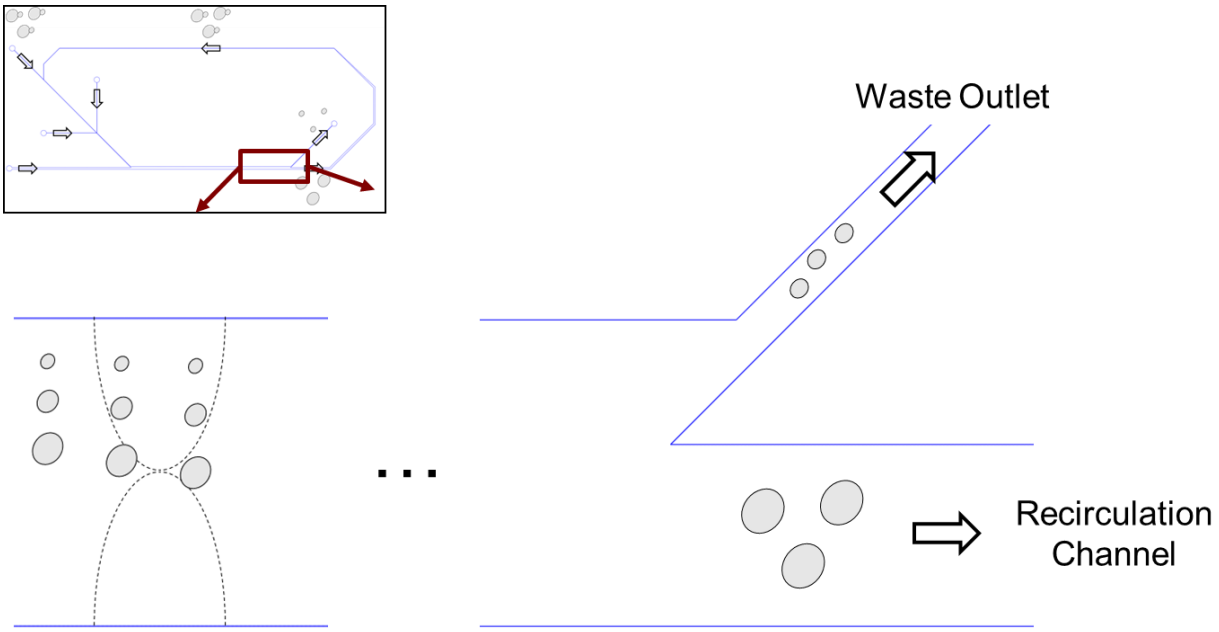


Figure 5. After acoustic separation, smaller daughter cells travel into waste outlet, and larger mother cells enter recirculation channel. In recirculation channel, mother cells divide and produce daughters, after which they repeat acoustic separation.

As mother cells travel in the recirculation channel, they ideally bud and produce smaller daughter cells. The aging mothers and new daughters return to the acoustic separation region where larger mother cells again experience acoustic forces sufficiently large to deflect them into the recirculation channel while smaller daughters travel into the waste outlet. In this way, our acoustic separation device ages a population of cells by continuously removing smaller daughter cells and enriching the aged mother cell population.

Acoustic power is optimized by experimenting with several voltages

Once our microfluidic device geometries are set, we must define several experimental parameters for removing daughter yeast cells and enriching the mother population: acoustic frequency, acoustic power, and flow rates (of four inlets). The acoustic power is the most crucial

parameter because it significantly affects the cut-off size between yeast cells we keep (those that are deflected into the recirculation channel) and cells we discard (those that do not experience sufficiently large acoustic force and exit the device through the waste outlet). To determine these parameters, we use the device shown in Fig. 6. This device is very similar to the one we use for removing daughter cells (Fig. 2) except it is missing the recirculation channel and the flow focusing side channels combine to a single inlet for convenience. We design our main and test devices to have very similar geometries so that the results obtained from using the test device can easily transfer to the main device.

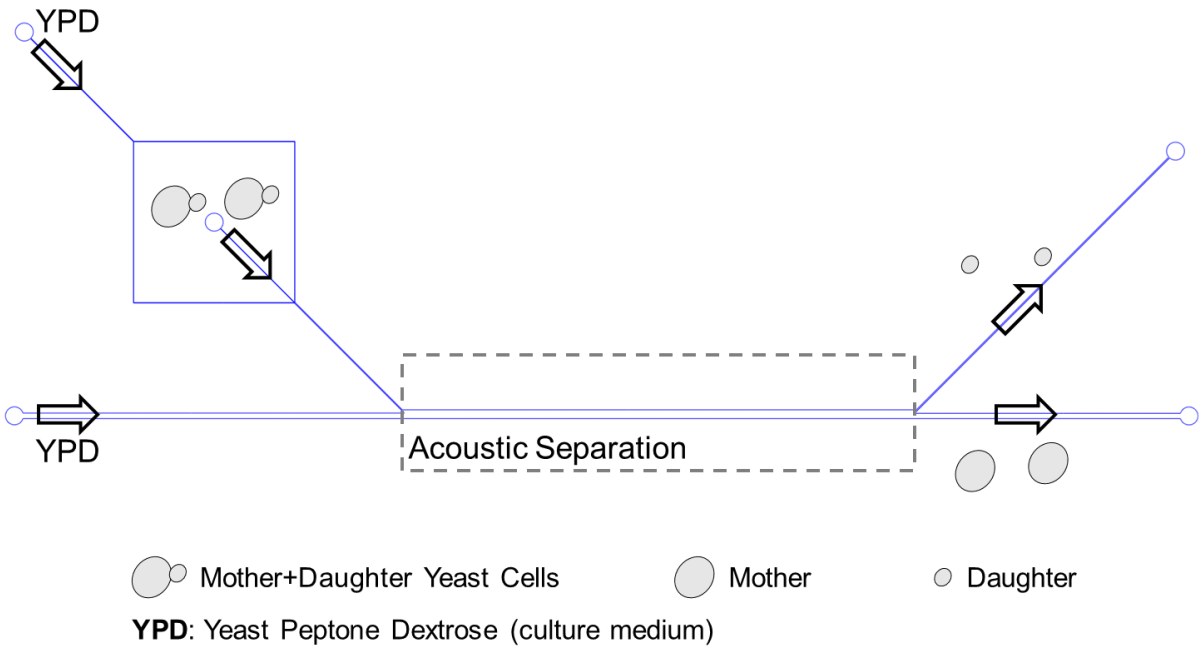


Figure 6. After acoustic separation, smaller daughter cells travel into waste outlet, and larger mother cells enter recirculation channel. In recirculation channel, mother cells divide and produce daughters, after which they repeat acoustic separation.

We determine optimal acoustic power and analyze size-based resolution of our system by adjusting the function generator voltage that drives the acoustic radiation while keeping constant

the flow rates of the culture medium carrying yeast cells. As shown in equation 1 for acoustic force F_a, the acoustic energy density E_0 directly determines F_a, and E_0 depends on the voltage driving the piezoelectric transducer. Thus the applied voltage largely impacts the distances that cells are deflected in the acoustic region shown in Fig. 6. To investigate the influence of voltage on acoustic manipulation, we sweep peak voltage between V_{low} and V_{high} (both measured in mV) and observe the acoustic effects on cells. V_{low} and V_{high} are determined by experimentation. That is, with the rightmost portion of the main channel where it splits into two outlets focused under a microscope, we set V_{low} as the smallest voltage at which cells just begin to deflect into the bottom outlet and V_{high} as the highest voltage at which most cells deflect into the bottom outlet.

An alternative approach for optimizing acoustic power is simulating microparticle acoustophoresis in COMSOL Multiphysics using the Acoustics, Computational Fluid Dynamics (CFD), and Particle Tracing Modules. We decided the more straightforward approach described above would more accurately determine the optimal acoustic power with fewer iterations.

Acoustophoresis effectiveness is evaluated by labeling/staining mother cells

We can compare several yeast cell characteristics before and after experiments to analyze and quantify aging. These properties include number of cell divisions, cell size/growth, bud scars, cell wall thickness, presence of biotin tagged on cell walls, culture growth, clonogenicity, intracellular reactive oxygen species, cell death, and metabolic activity. Mirisola et al. provide an excellent review for these approaches used to study cell aging and death.[5]

For our experiments, we evaluate the effectiveness of isolating aged mother cells by leveraging the fact that a mother retains most parts of its cell wall throughout its RLS while the cell walls of its daughters are all newly synthesized. We utilize a biotin-avidin labeling system. Sulfo-NHS-LC biotin is cross-linked to the initial population of mother cells before loading them into the microfluidic devices. The biotin remains selectively attached to cell walls of mothers because daughter cell walls are newly synthesized. After completing the acoustic experiments, we stain the separated cells with fluorescein-avidin and use microscopy and flow cytometry to determine which cells belong to the initial mother population.

To determine the replicative age of the isolated mother cells, we count bud scars that are highlighted after staining cells with calcofluor dye.

To sum up, with our particular acoustophoresis approach we observe how acoustic power and cell size affect trajectory through the main channel (acoustic separation region) of the microfluidic device (Fig. 6). We use the results of these first experiments to define the acoustic parameters for testing the device with a recirculation channel (Fig. 2). An advantage of acoustic separation using our system is that cells are not continuously exposed to acoustic forces—only when cells are in the separation region do they experience acoustic radiation. Additionally, with our approach we can potentially analyze whether cell characteristics other than size affect acoustic path. For example, we can investigate the impact of cell compressibility-based differences on acoustic force. (Compressibility of mother cells likely varies from that of daughter cells as a result of aging and the accumulation of bud scars on mothers' cell walls.) One disadvantage of our system is that cells cannot be imaged continuously because acoustically

separated cells must recirculate and undergo repeated filtering. This inability means that we cannot directly observe the number of cell divisions so we must use alternative methods of confirming the replicative age of cells, such as counting bud scars.

Microfluidic device design and fabrication

Our acoustophoresis device designs resemble a very common architecture used by several groups.[14,31,38,39,41] We arrange the microchannels so that laminar flow keeps cells at desired positions during device operation and acoustic forces separate cells based on size into either a recirculation channel or waste outlet. Devices are designed in DWGeditor (SolidWorks) and simulated in COMSOL Multiphysics. Our main goal in the simulations is to verify sufficient flow in the recirculation channel (Fig. 2), that is, we ensure that the liquid exiting the main channel does not immediately flow into the waste outlet. Fabrication details for our silicon-glass microfluidic acoustic devices are described in Wang et al.[42]

We fabricate Polydimethylsiloxane (PDMS) devices for our time-lapse yeast cultures (discussed in RESULTS section) using a Professional Laser Series (PLS) 6.120D Laser Engraving and Cutting System (Universal Laser Systems, Inc.). A 10:1 resin-to-curing agent composition is degassed, poured into a petri dish, and cured in an 80°C oven overnight (>8hr) to create a thin slab of PDMS (~4mm thick). The PDMS slab is engraved using the laser micromachine so that the resulting pattern has an inlet, main chamber, and outlet. Inlet/outlet holes are punched, the PDMS is cleaned using DI water and IPA, and the slab is bonded to a glass slide using oxygen plasma treatment. We fabricated this particular device for the yeast cultures because it is more

similar to our acoustophoresis devices than are microscope slides. It also allows for a larger quantity of cells and culture medium than what can be placed between two microscope slides.

Yeast culture and device preparation

Wild-type yeast cultures (CEN.PK) are prepared by first combining Yeast Peptone Dextrose medium (YPD) with a small streak of yeast colonies from a culture stock grown on an agar plate (obtained from a TAMU collaborator, Dr. Michael Polymenis). The cells and media are incubated (Thermo Scientific MaxQ6000) at 27°C, 5% CO_2, 95% humidity (no shaking) overnight (>12hr) to stationary phase. For experiments not requiring labeled yeast cells, the overnight culture is transferred to fresh YPD and grown for ~3hr in an incubator immediately before the experiment. This allows cells to resume exponential growth, at which time they can be placed in additional fresh, warmed YPD and loaded into the microfluidic device for testing. To introduce cells into the device, the cell culture is placed in a syringe (BD with Luer-Lok Tip) with flat needle tip that is then inserted into the plastic tubing of the microfluidic inlet channels. Before loading cells, the microfluidic device is primed by sterilizing it in UV light for >15min and by flowing ethanol, DI water, and warmed culture medium (in that order) for at least 5min each.

For experiments in which cells must be tagged with biotin, the overnight culture is diluted in fresh YPD and grown for ~5hr in the incubator. The cells are then harvested and re-suspended with ~0.5mL of phosphate buffered saline (PBS). To tag these mother cells with biotin, sulfo-NHS-LC-biotin (Pierce) is first warmed in the incubator. 8mg sulfo-NHS-LC-biotin is dissolved in 0.3mL PBS, and this mixture is added to the cell culture. The cells and biotin are incubated for

~15min, pelleted with a centrifuge (Eppendorf Centrifuge 5702) at <6000rpm for 2min, and washed with 1mL PBS to remove excess biotin. Pelleting and washing are repeated at least 3 times, after which cells are re-suspended in 1mL YPD. Cells can then be loaded into a syringe and introduced into a primed microfluidic device.

Protocol to quantify acoustophoresis size-based resolution

As discussed above, we investigate cell size-based resolution and effectiveness at isolating aged mother cells in our acoustic deflection system. For analyzing size-based resolution of acoustic deflection, we first pre-load the microfluidic device with warmed YPD to prime the device and remove air bubbles. A syringe containing a low density of cells is attached to the device inlet, and the device is placed onto the stage of an upright microscope (Nikon Eclipse LV100) with camera (Digital Sight DS2Mv) and supporting software (NIS-Elements Br Microscope Imaging Software). Cells are prepared as described above. In an additional step after culturing cells overnight to stationary phase, we allow cells to resume exponential growth by transferring them into fresh growth medium and incubating for 3hr before the experiment. Cells flow into the device through the center inlet at a rate of 25µL/hr by fixing the syringe to a syringe pump (Harvard Apparatus). The flow rates for side focusing channels are set to 25µL/hr and for main channel 370µL/hr. Acoustic excitation frequency for a sinusoidal wave is set to 2.08MHz on a function generator (Tektronix AFG 3021B Single Channel Arbitrary/Function Generator). The microscope is adjusted so that the main channel where it splits into 2 outlets is focused in the field-of-view (FOV). Once cells begin passing the FOV, acoustic radiation is applied by triggering 100mV at the function generator and passing the signal through a 50dB RF Power Amplifier (E&I). Voltage is increased until cells just start deflecting into the bottom outlet. This

voltage is V_{low}. Voltage is increased until most cells are deflected into the bottom outlet (V_{high}). We choose 5 voltage (or power) levels in between V_{low} and V_{high}. For each power level, approximately 8,000 cells pass into either outlet (a quantity sufficient for using a Beckman Coulter Z2 Particle Count and Size Analyzer), and videos of the cell trajectories are captured as cells pass through various regions of the acoustic deflection zone. After each power level, the cell populations at both outlets are immediately placed on ice to hinder cell growth and division. Alternatively, the resulting cell separations can be sonicated, centrifuged, and placed in 70% ethanol in a 4°C refrigerator (VWR). After experiments with the first power setting are completed, we take one sample from the original cell culture and place this on ice. This sample serves as a control for subsequent cell size measurements. After the experiment, videos are processed in MATLAB (MathWorks) to extract the cell sizes and trajectories. After sweeping through all voltage levels, the resulting cell populations are inserted into the Beckman Coulter Z2 Particle Count and Size Analyzer with Z2 AccuComp Software to determine all cell sizes.

Protocol to evaluate acoustophoresis effectiveness

We pre-load the microfluidic device with warmed YPD media to prime the device and remove air bubbles. Additional warmed culture media is loaded into a syringe with part of the biotin-labelled yeast culture so that the syringe has a relatively low density of yeast cells. Approximately 10% of this initial mother cell population is separated to serve as a control and cultured in an incubator throughout the acoustic experiment. The microfluidic device is positioned on a custom hot-plate fixed on the stage of the upright microscope with camera. The syringes are connected to a syringe pump that keeps the flow rates of the inlet focusing channels at 25μL/hr and of the main channel at 370μL/hr. Once cells are introduced in the device from the

center inlet channel, the flow from this channel is stopped and replaced by the recirculating channel. The acoustic radiation is started before cells enter the device by applying a 2.08MHz sinusoidal wave from the function generator through the power amplifier driving the piezoelectric transducer. Function generator voltage is determined from the optimization experiment described above. Cells are loaded into the device through the center inlet at 25μL/hr, and the side, flow-focusing inlet syringes have only warmed YPD (no cells). The waste outlet is connected to a flask chilled with ice. This experiment proceeds for 36hr with time-lapse images of the outlet taken every 1min using the Nikon software. After 36hr, the flow and acoustics are turned off. The first outlet flask that has captured all filtered cells throughout the experiment is removed and placed in a 4°C refrigerator. A new flask is placed at the waste outlet, and the cells remaining in the device are flushed into this flask that is then placed in the refrigerator. The control flask with part of the initial cell population is also placed in the refrigerator to halt cell growth. We use a Beckman Coulter Z2 Particle Count and Size Analyzer on all cell populations to measure cell sizes. Time-lapse images are processed in MATLAB to measure cell size and trajectory.

To determine which cells are from the initial, biotin-tagged mother population, the cell culture is stained with fluorescein avidin, which binds to biotin. The cells of interest are centrifuged at 5000rpm for 10min at 4°C and resuspended in chilled PBS. The cells are washed twice with 10mg fluorescein avidin (Sigma Aldrich) mixed in 1mL chilled PBS, and the mixture sits chilled for 2-3hr for avidin to bind to biotin. Cells are loaded into a fluorescence-activated cell sorter (FACS) to count the number of cells with biotin-avidin. Alternatively, cells can be placed in PBS

on a microscope slide with a coverslip, and UV fluorescence images of the cells can be captured to determine the purity of mother cells.

Counting bud scars to determine replicative age

To measure replicative age of separated mother cells, cells are stained with Calcofluor white M2R (fluorescent brightener 28, Sigma), which highlights the bud scars that remain on the mother cell wall after the birth of each daughter cell. 10mg of Calcofluor white M2R is dissolved in 1mL PBS, and this mixture is centrifuged at 8000rpm for 5min. We use only the top portion of the resulting supernatant. After washing the cells in 1mL PBS and pelleting them at 6000rpm for 1min, the prepared Calcofluor solution is added to the cells, and the solution is incubated for at least 5min. The cells are washed in PBS, pelleted at the same centrifugation settings, and resuspended in 20µL PBS. The stained cells are placed on a microscope slide with a coverslip, and the bud scars on each cell illuminated with UV fluorescence are counted. The z-axis focus is adjusted to count bud scars over the entire cell.

Confirming acoustic radiation does not affect cell viability

Although acoustic radiation does not adversely impact cell viability in experiments cited in the literature, we confirm this in our experiments by plating processed cells with fresh YPD on a microscope slide with coverslip. The slide is placed on a hotplate fixed to a microscope stage, and we capture images of the cells every 1min for at least 6hr to measure the ability to proliferate and form mini-colonies.

Image processing algorithm to measure cell size differences

Fig. 7 shows the steps used to calculate the volume percent difference between mother and daughter yeast cells. First, specific regions-of-interest are chosen and cropped from the overall image. The cropped image is gray-thresholded so that the cells can be fit to a circle using a built-in MATLAB function from the Image Processing Toolbox.

Similar image processing techniques are used for other analyses of images. We leverage built-in MATLAB functions and the software's ability to manipulate several hundred images in a straightforward way.

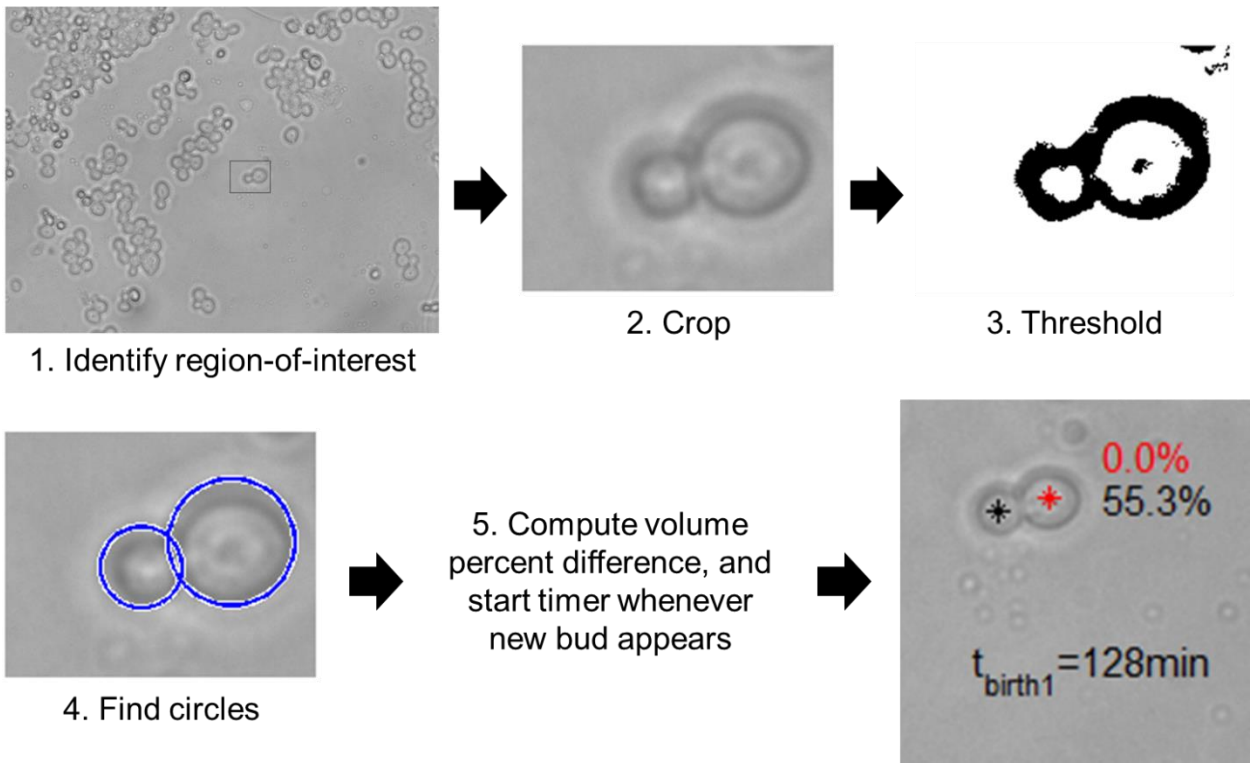


Figure 7. Image processing algorithm to quantify differences in volume between mothers and daughters and analyze proliferation rate of daughters.

CHAPTER III

RESULTS

In this section, we present results from time-lapse cultures of yeast cells and from simulations of our on-chip recirculation design. We then discuss problems of an experiment to optimize acoustic power for a future experiment. Because of the problems we experienced during this power optimization experiment, we did not complete our on-chip recirculation/purification experiment as discussed in the METHODS.

Comparing mother and daughter yeast sizes

Our first experiment was a yeast culture time-lapse to observe differences between mother and daughter wild-type yeast cells cultured under known conditions (culture medium, temperature, time) in our lab. We aimed to determine the mother and daughter sizes immediately before they enter our microfluidic device and to observe the proliferation of daughter cells. Fig. 8 shows sequences of images taken at various points in time for 2 separate cultures. The times are measured from the beginning of the time-lapse experiment, that is, 0min corresponds to the time after culturing cells overnight, loading them into the test device, focusing them in the microscope, and starting the time-lapse. For the time-lapse images shown in Fig. 8B, the culture medium evaporated and prevented the cells from being imaged in a single focal plane. This problem is solved by continuous flow-through and replenishment of medium, which we plan to use for the on-chip recirculation/purification experiment.

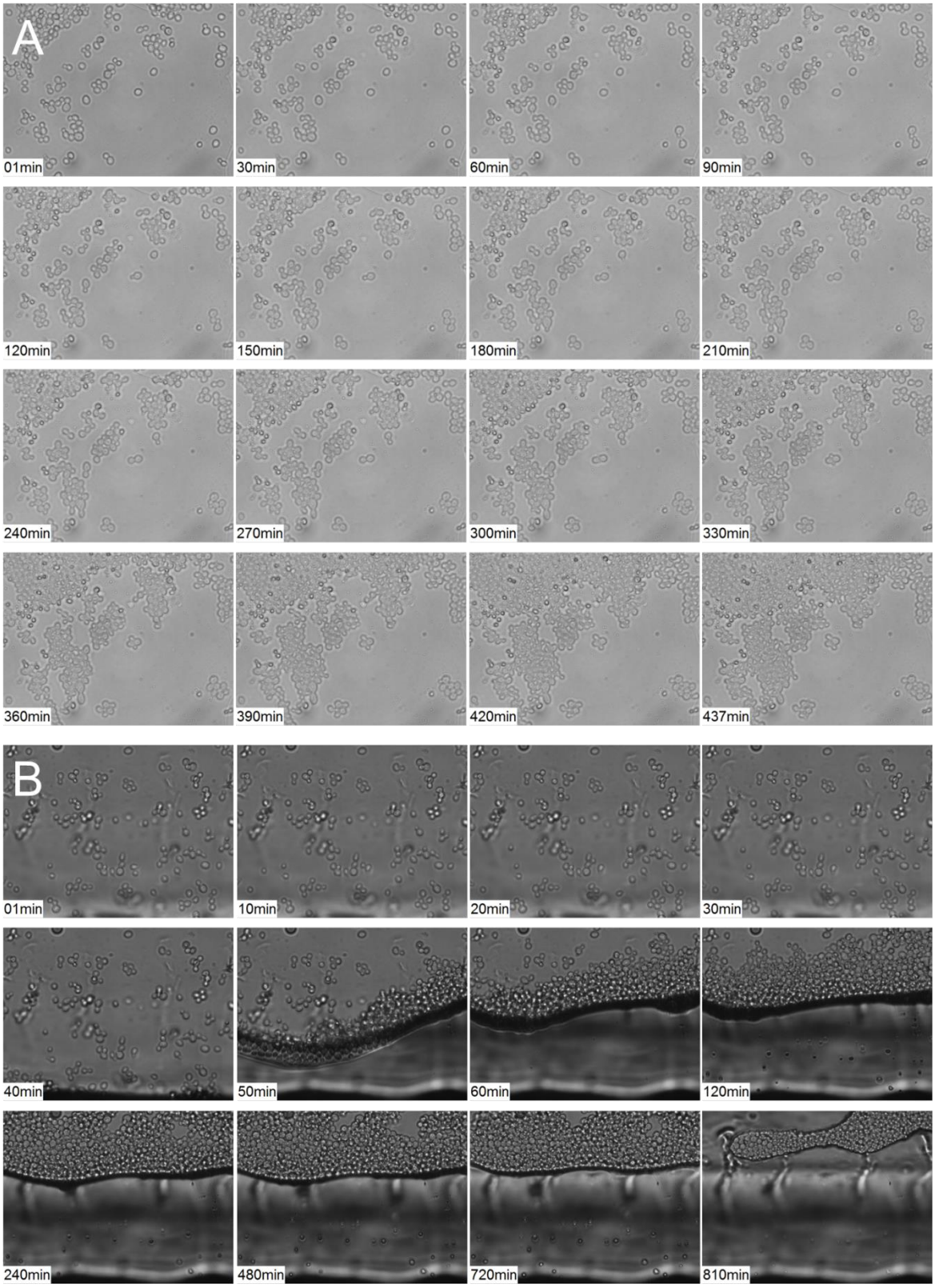


Figure 8. Time-lapse yeast cultures. Daughter cells increase exponentially in A, showing how daughter cells impact the growth environment of mothers. In B, the culture medium evaporated unexpectedly during the time-lapse.

Fig. 9 magnifies 2 mother cells in one of the time-lapse sequences and demonstrates how daughter cells quickly crowd the mothers and can impact the mothers' normal growth environments. The times shown in each image measure the elapsed time after the time-lapse sequence was started using the microscope software. Fig. 10 shows a similar sequence along with 1) volume percent difference between mother and daughter and 2) lifetime of daughter cells measured in minutes. The colors of asterisks and percentages match, and all volume percent differences are measured relative to the cell in the image with 0% volume difference (0% difference relative to itself). The timers counting upward as time progresses indicate the time since a bud first appears.

In Fig. 11, we show the evolution of yeast sizes extracted from the time-lapse images using MATLAB. The overall trend of these plots is an increase in the number of smaller cells identified in the image. A histogram of diameters for a wild-type yeast culture is shown in Fig. 12 for comparison. The overall mean of 394,533 total cells from a wild-type, haploid culture is 4.61μm, and 90% of all cells have diameters below 5.68μm. The mean birth size of 3.45μm is calculated as the mean of the lowest 10% of cells, which is a definition commonly used in the field.

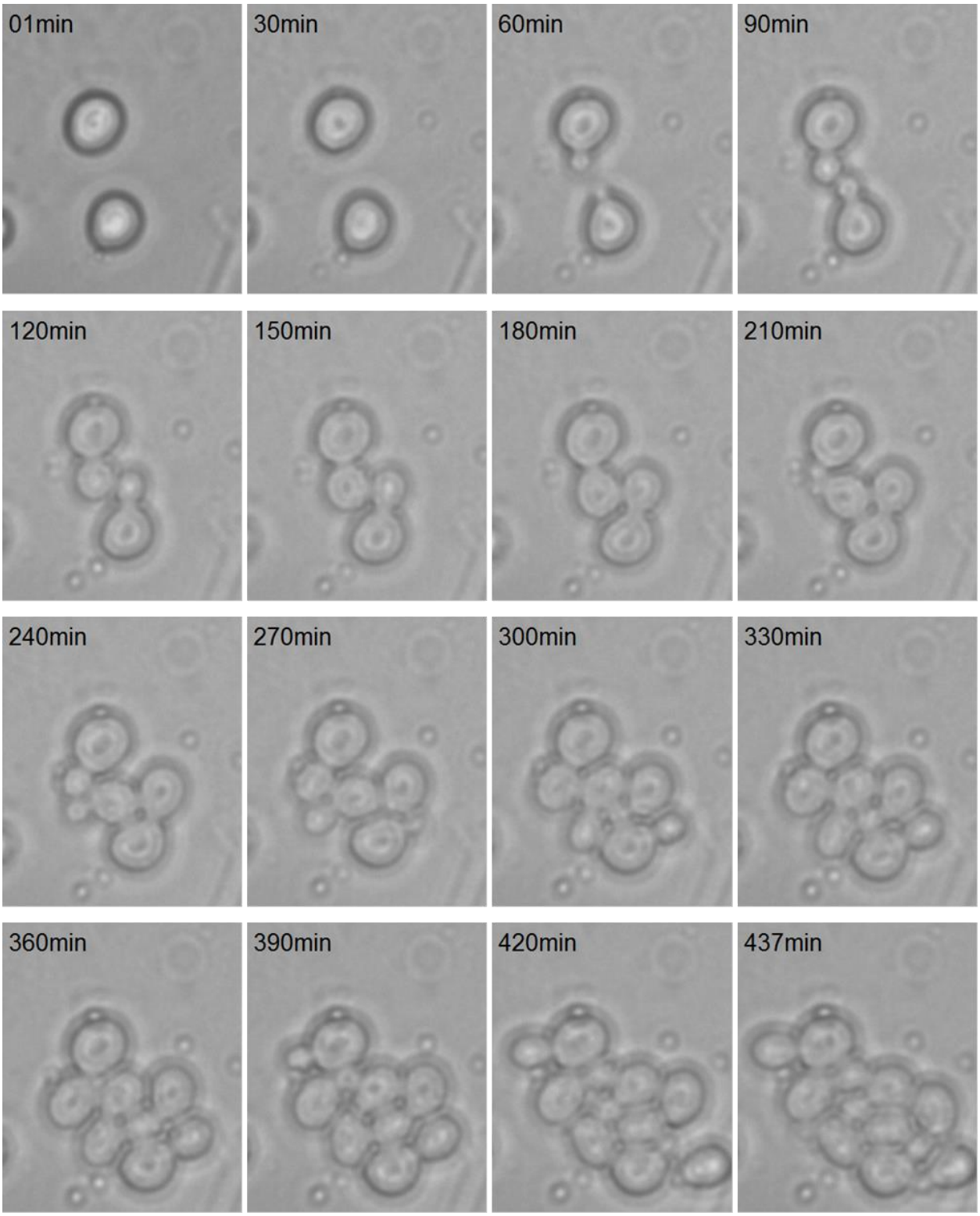


Figure 9. Magnified region of yeast culture in Fig. 8A shows how daughter cells quickly crowd the mother cells and impact the mothers' growth conditions.

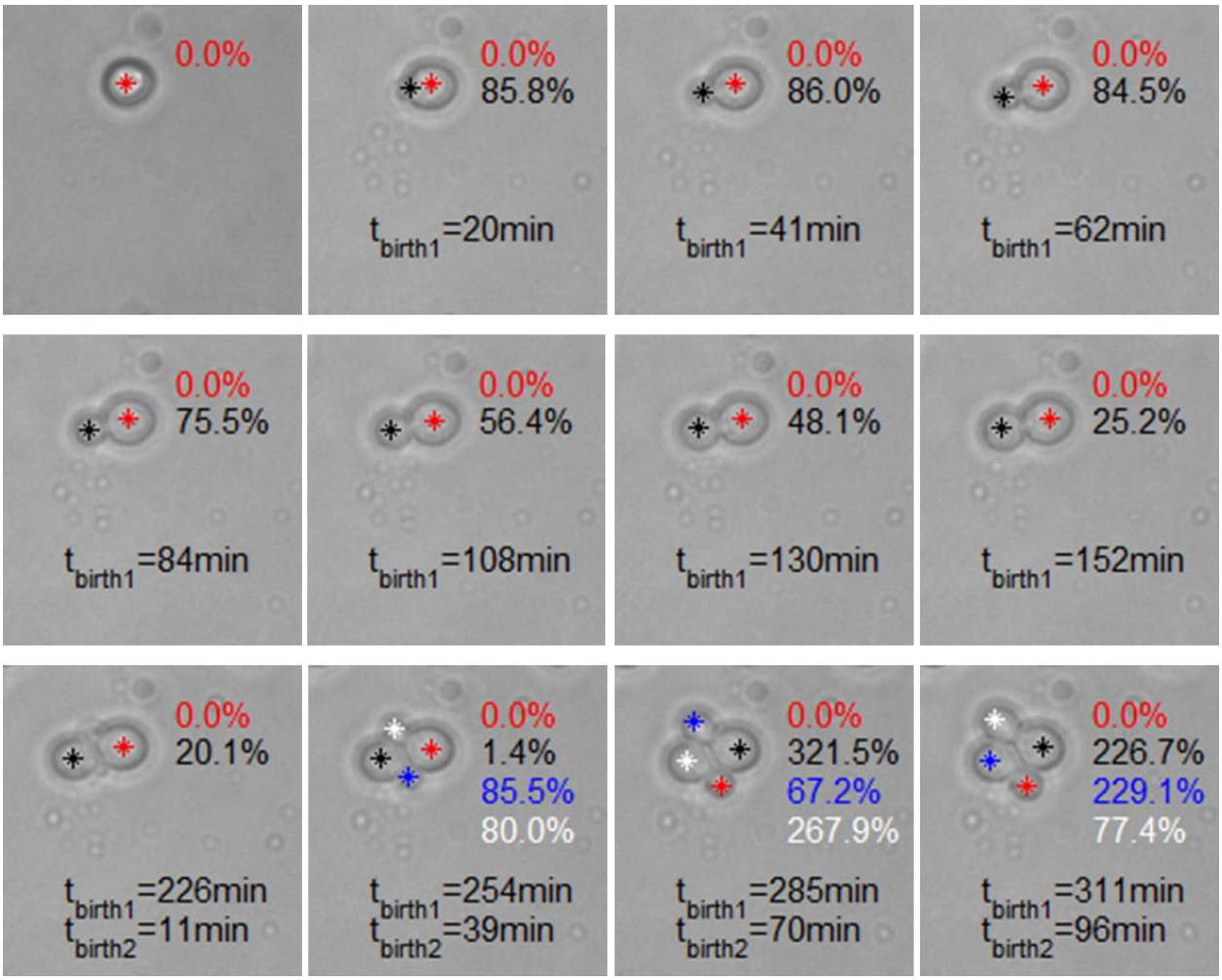


Figure 10. Time-lapse showing volume percent differences of all yeast cells within the image (relative to one cell) and time since a bud first appears. The colors of asterisks and percentages are matched such that, for example, in the top right image the cell marked by a black asterisk has 84.5% volume difference relative to the cell marked by a red asterisk, which has 0% volume percent difference relative to itself.

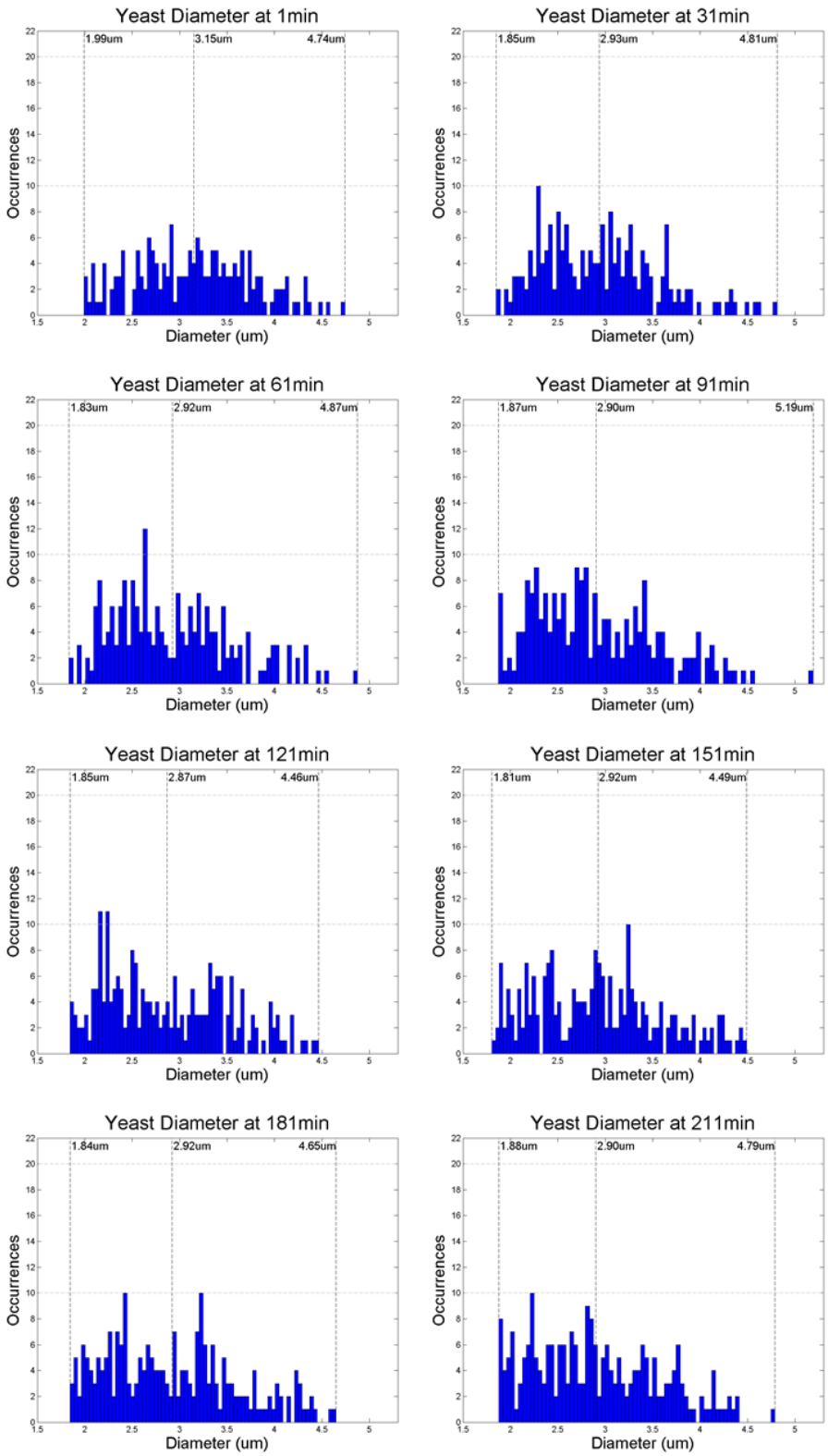


Figure 11. Evolution of yeast sizes shows a steady increase in the number of smaller yeast cells. Because yeast cells grow as they age, smaller yeast cells are mostly younger daughter cells, and larger cells are mostly older mother cells. Plots show time from 1min to 211min.

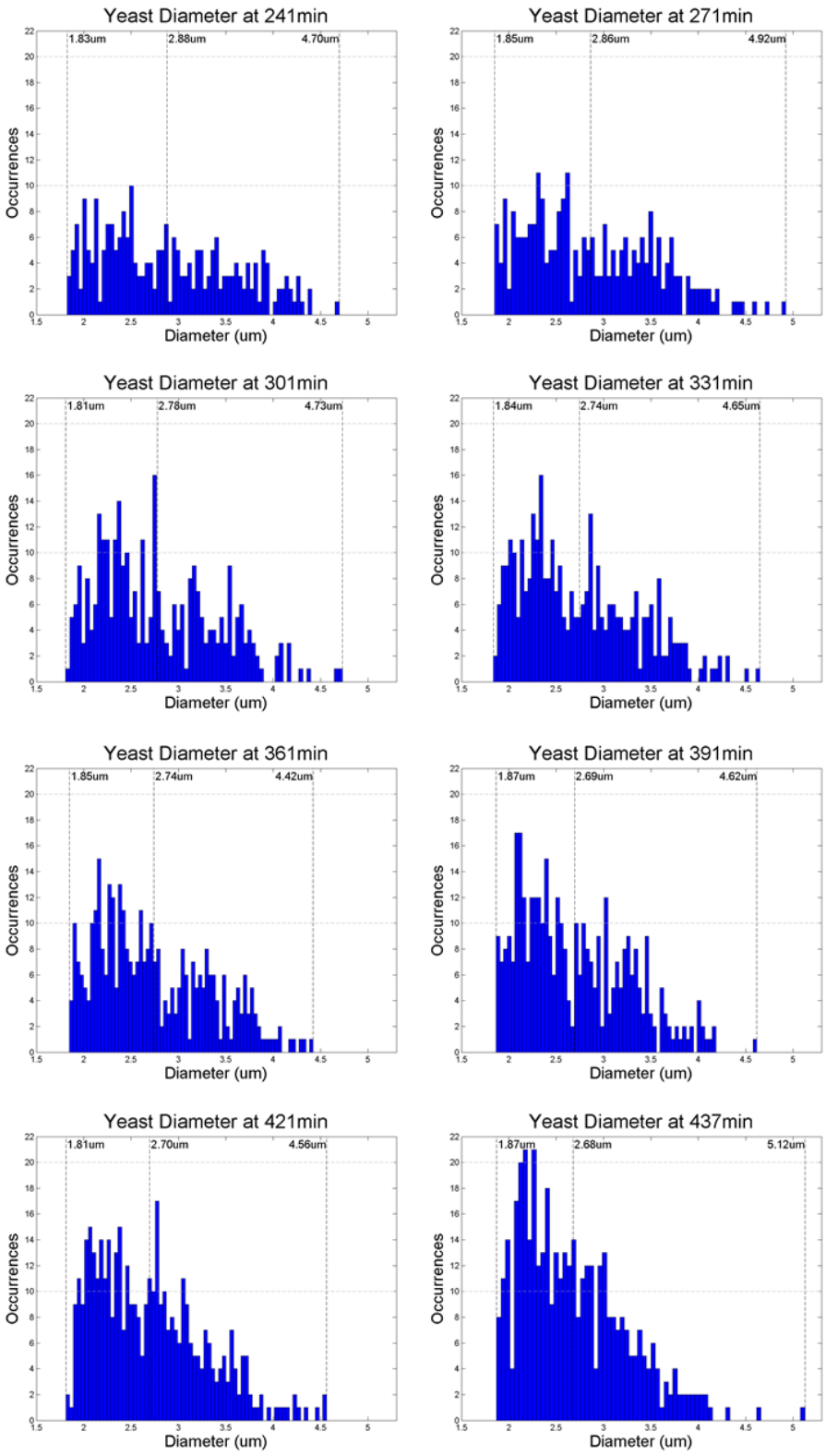


Figure 11. Continued. Plots show time from 241min to 437min.

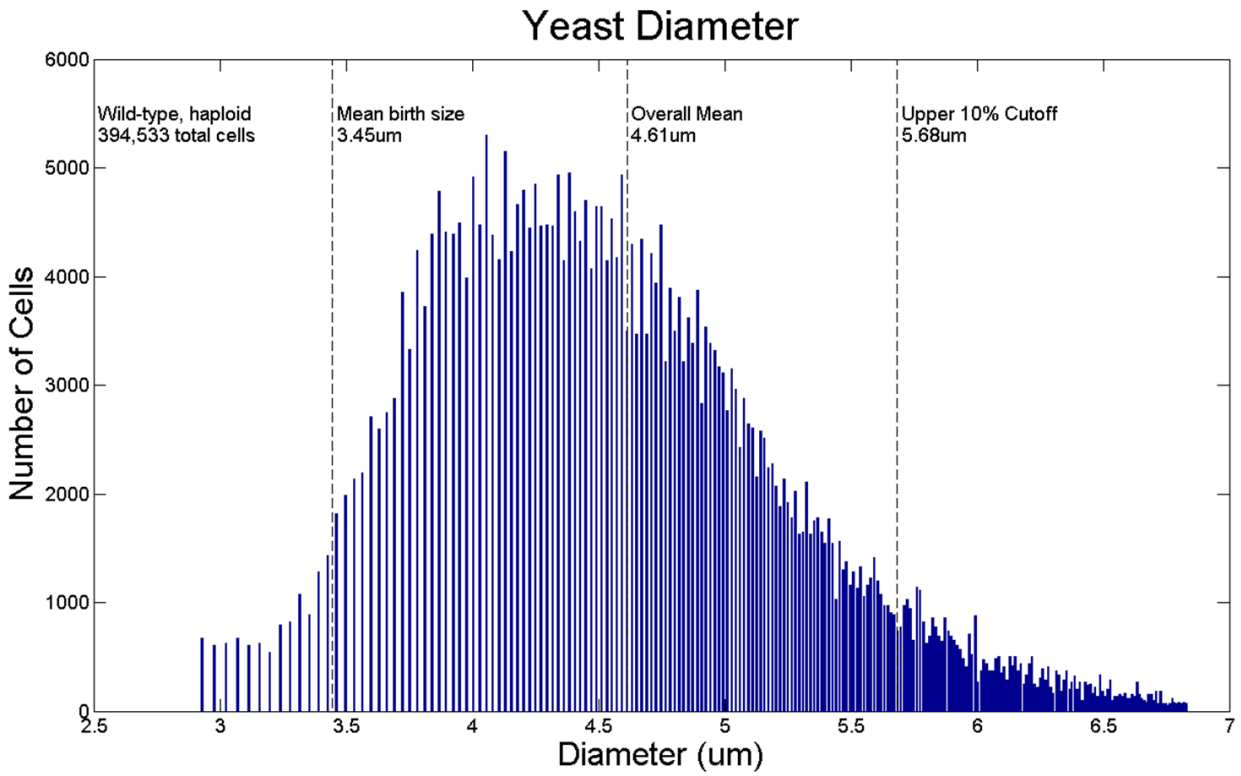


Figure 12. Histogram of wild-type yeast diameters.

Solution to import CAD files from design software into simulation toolkit

We attempted to import our CAD file created by DWGeditor and containing the on-chip recirculation design directly into COMSOL but experienced several problems with this transfer. Directly importing the CAD file into COMSOL ensures we simulate the exact dimensions of our microfluidic device and eliminates the need to define the channel geometry within COMSOL, which does not offer the same drawing functions that are available in DWGeditor. Two-dimensional CAD file import failed because COMSOL could not recognize/convert some of the channel geometries. After converting the CAD file from DWGeditor into .STL format using SolidWorks (which can be imported into COMSOL relatively easily), we imported this 3D model into COMSOL. COMSOL could recognize the geometry; however, fluid flow simulations

failed because COMSOL could not mesh the 3D channels into triangles suitable for the simulation. Adjusting the mesh size and other simulation parameters did not resolve this problem. Additionally, a 3D simulation is not necessary to determine whether the recirculation channel has sufficient flow (our overall goal of the simulation), and the locations of inlets/outlets for a 3D simulation may differ from those that actually exist in the fabricated device because the definition in the simulations is somewhat arbitrary. We finally resolved the issue with importing a 2D CAD file by scaling the design in DWGeditor by 1000, importing into COMSOL, and scaling the design by 0.001 to return it to its original dimensions. This solution indicates that COMSOL cannot import geometries smaller than a certain size. Additionally, COMSOL did not immediately recognize which closed regions of the design comprise the channels and which are blank spaces. In particular, COMSOL identified the entire design as a single solid object. To resolve this problem, we imported two files: one file containing the outer design boundaries and one containing the inner boundaries. COMSOL reads both of these files as solid objects. We subtract the inner boundaries from the outer ones to obtain our device geometry with the exact dimensions that we designed in DWGeditor. Using this sequence (scaling in DWGeditor, unscaling in COMSOL, and subtracting inner and outer boundaries), we are able to design the microfluidic channels in DWGeditor while leveraging the drawing functions it offers, and then simulate the design in COMSOL.

Figs. 13-19 show fluid flow simulations through several designs. The differences between each simulation are either 1) varying flow rates for inlets or 2) additional inlets/outlets. The geometry and inlet flow rates for each design are shown in the upper half of each figure, and the 3D velocity plots (surface with height) in the lower half show the resulting velocities in all channels.

The height and color of the channels indicate the velocity magnitude of water flowing in the channels. All simulations show insufficient fluid flow through the recirculation channel, and most of the flow is directed into the outlet(s) near the lower right of the designs. Neither adjusting inlet flow rates nor adding inlets/outlets helps direct fluid into the recirculation channel. Fig. 19 shows that increasing the lateral dimensions of the recirculation channel also fails to distribute flow more evenly between the outlet and recirculation channel. Low flow through the recirculation channel indicates that the fluidic resistance looking into this channel is much greater than the resistance of the outlet(s).

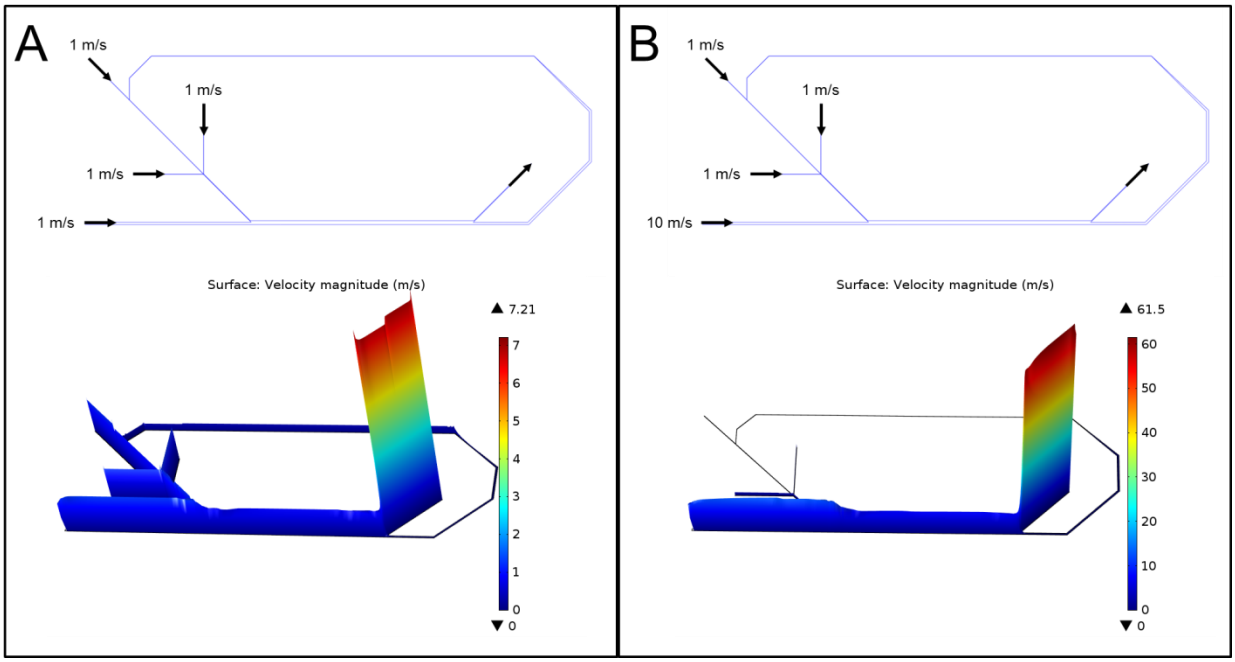


Figure 13. Velocity flow profiles (3D surface plot with height) in channels of the on-chip recirculation design. The design schematic shows the direction and magnitude of inlet velocity and direction of outlet flow. Recirculation channel has much smaller flow compared to inlet channel. Magnitude of main inlet channel flow rate increases from A to B.

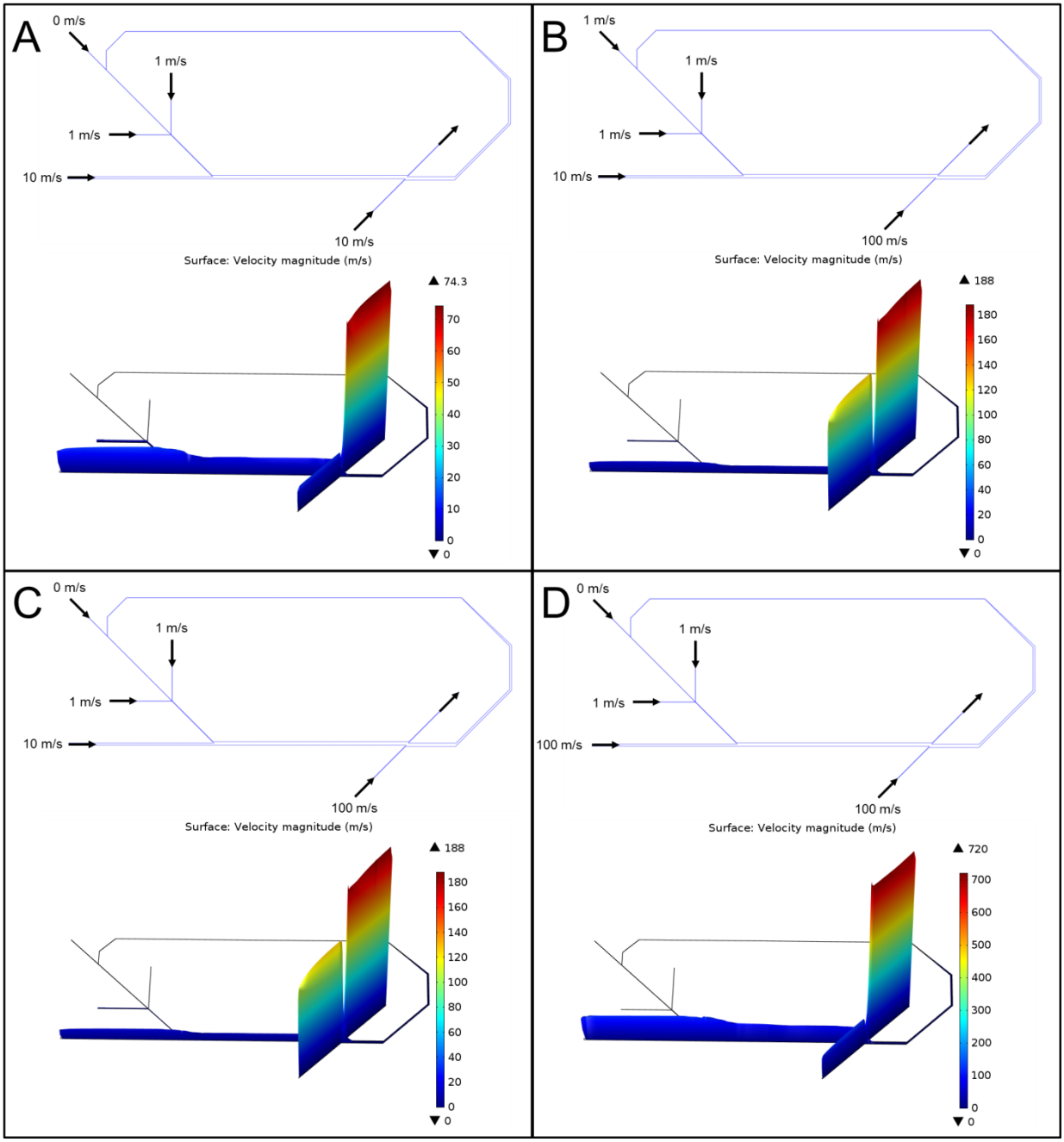


Figure 14. Velocity flow profiles in channels of the on-chip recirculation design. Channel geometry is the same for each case (A, B, C, D) but inlet flow rates vary.

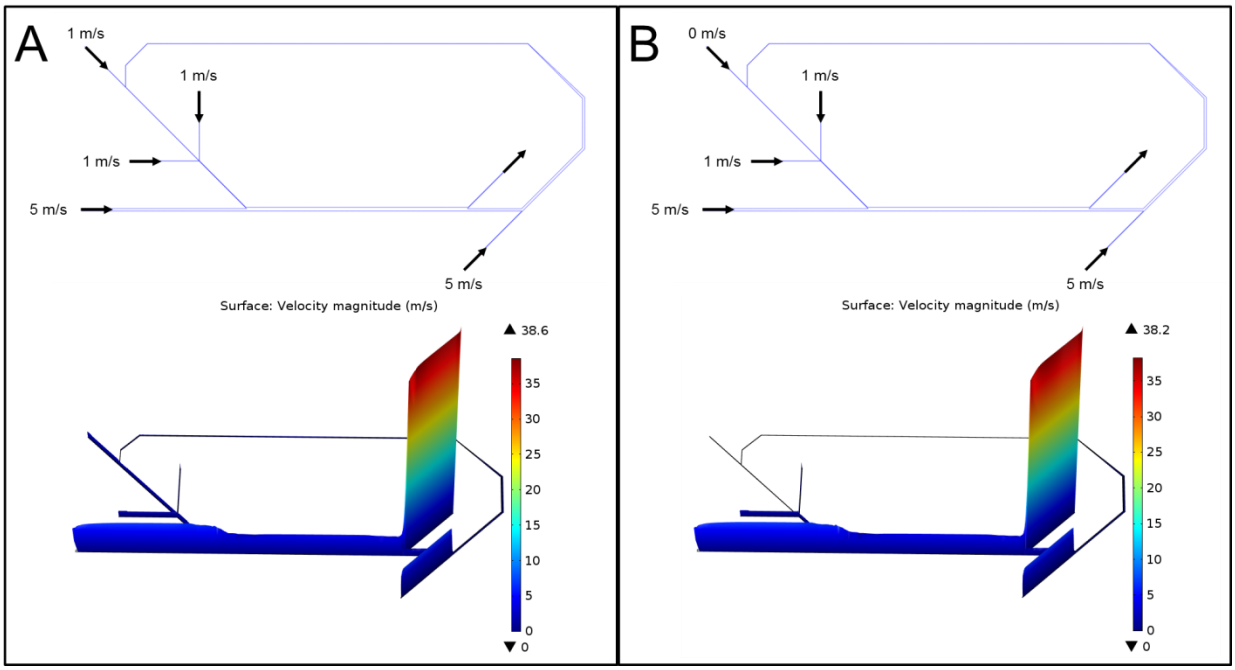


Figure 15. Velocity flow profiles in channels of the on-chip recirculation design. Additional outlet at bottom right of schematic is shifted counterclockwise along the recirculation channel compared to Fig. 14. Top-left inlet flow rate magnitude decreases from A to B.

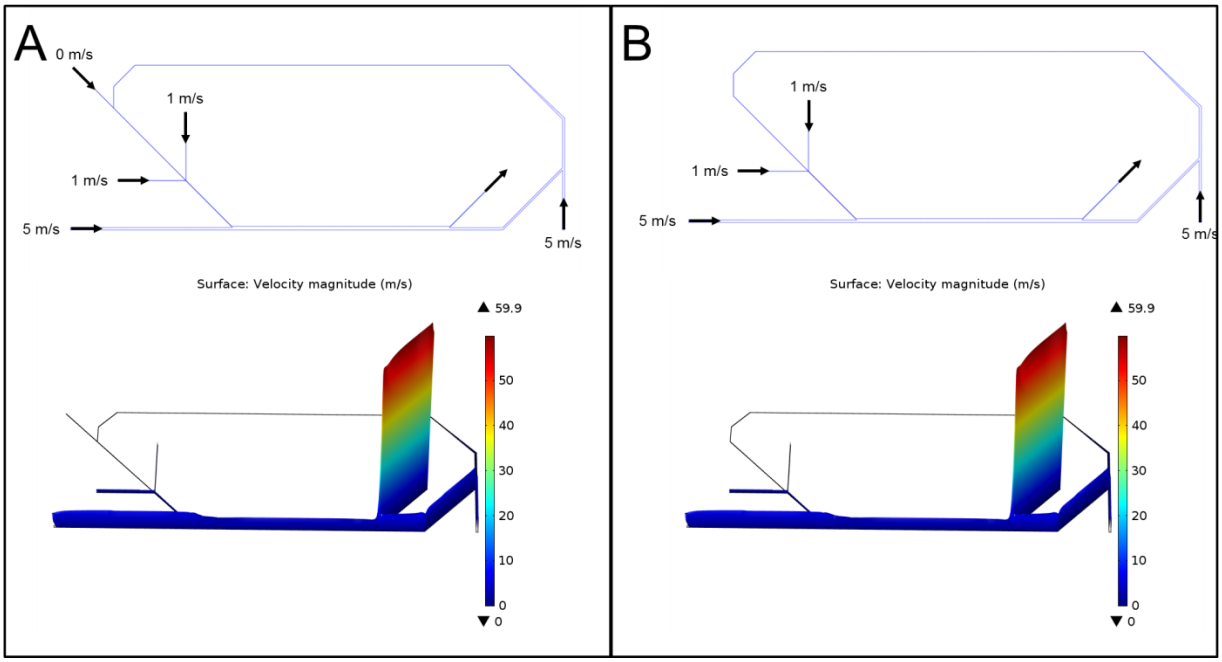


Figure 16. Velocity flow profiles in channels of the on-chip recirculation design. The additional outlet at bottom right of the schematic is shifted counterclockwise along the recirculation channel compared to Fig. 15. Top-left inlet is removed from A to B. The other flow rates are the same for both cases (A and B).

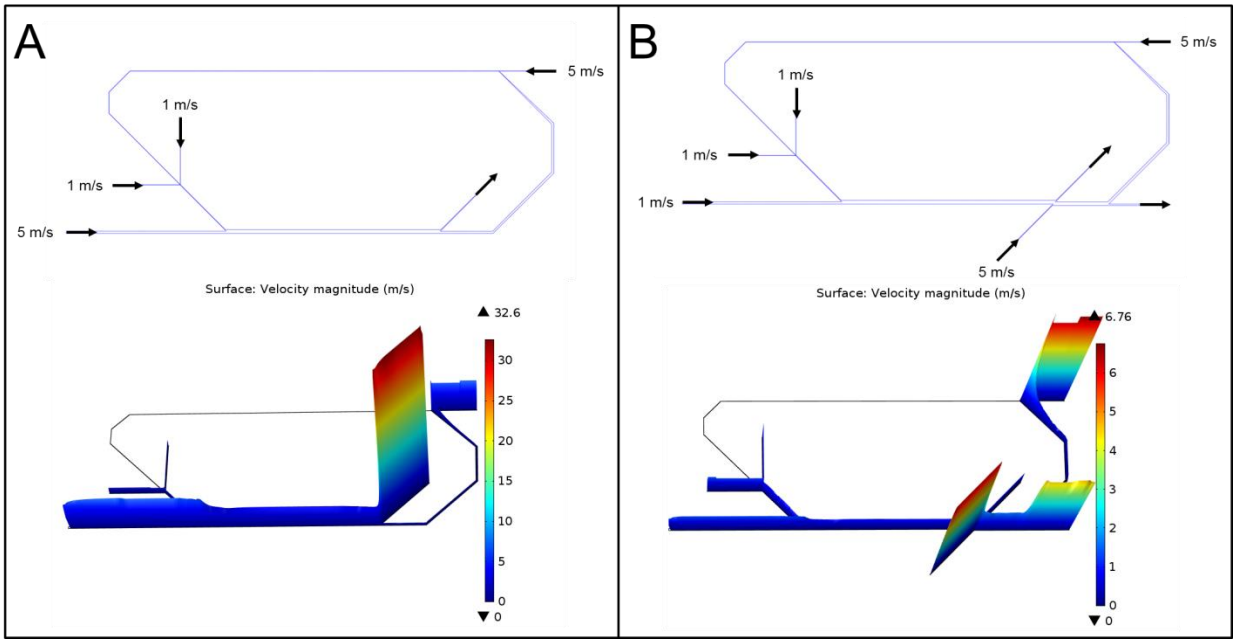


Figure 17. Velocity flow profiles in channels of the on-chip recirculation design. An extra inlet and outlet are added in B near the entrance to the recirculation channel.

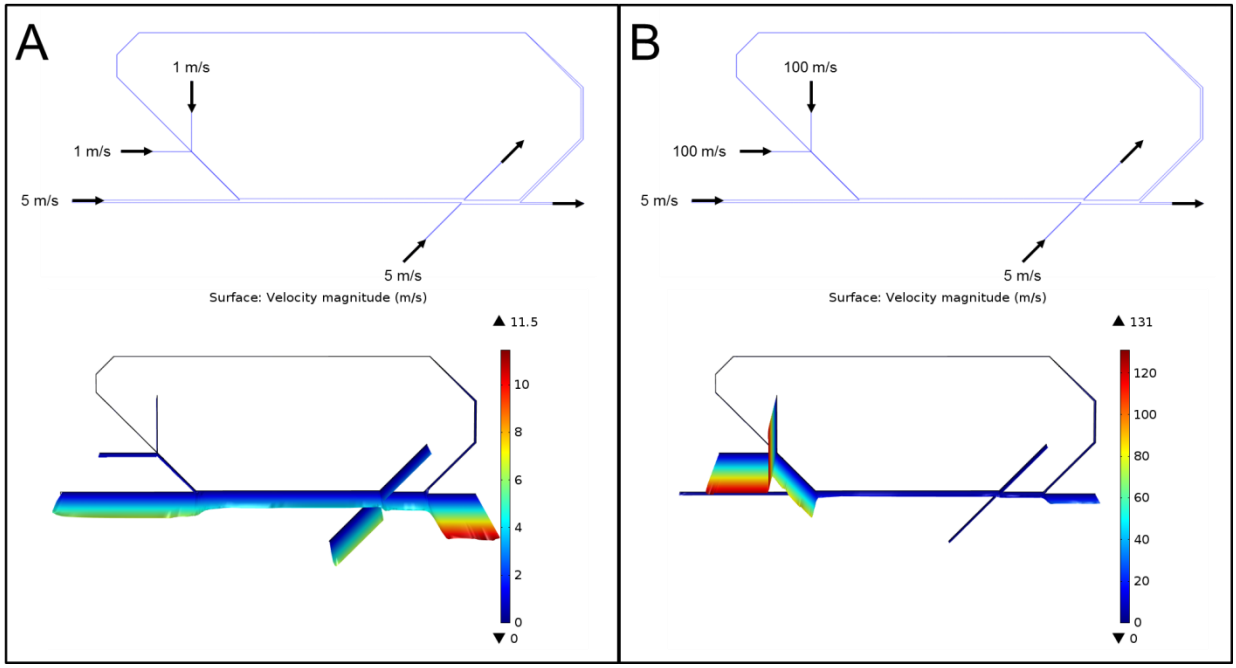


Figure 18. Velocity flow profiles in channels of the on-chip recirculation design. The flow rates of the side inlet channels are increased from A to B.

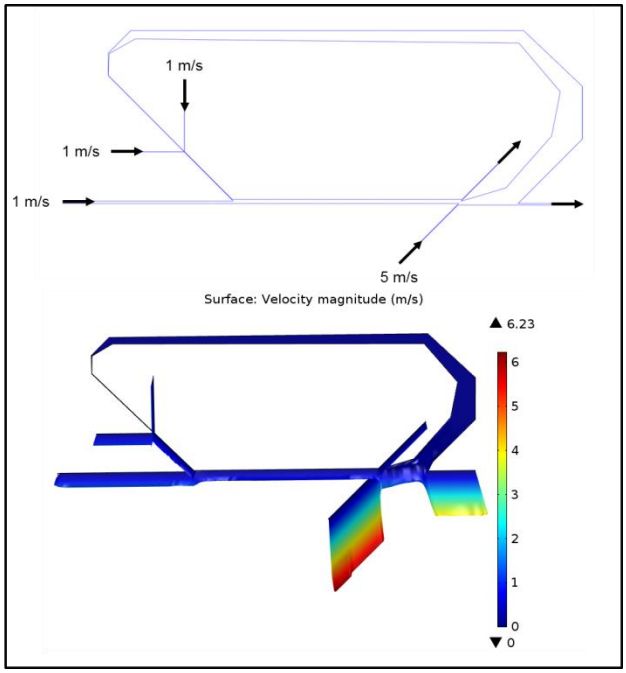


Figure 19. Velocity flow profile in channels of the on-chip recirculation design. The recirculation channel width is increased compared to the cases in Figs. 13-18. Flow rate in recirculation channel is much smaller than the outlet flow rates.

Problems with experiment to optimize acoustic power

Fig. 20 shows the fabricated microfluidic acoustic device for the experiment to optimize acoustic power for separating mother and daughter yeast cells. The image was taken before integrating fluidic connections.

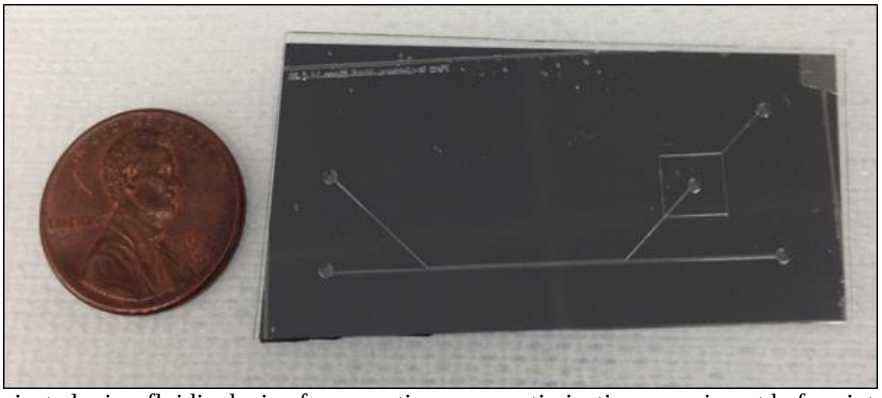


Figure 20. Fabricated microfluidic device for acoustic power optimization experiment before integrating fluidic connections to inlets/outlets.

We experienced several problems during this experiment. First, we observed an excess of bubbles in syringes with culture medium (no cells) and with medium+cells. Sporadic bubbles are undesirable because they interrupt the designed flow of cells in the main channel, which worsens separation resolution. The bubbles in syringes with only culture medium (no cells) are likely the result of incubating the solutions for 3hr before beginning the experiment. Incubation also produced bubbles in the syringes with cells, and this problem was worsened by the natural gas byproducts of yeast cell growth. We can reduce the unwanted effects of bubbles by degassing the syringes before connecting them to the tubing. Degassing is known to be safe for yeast cells.

Another issue we observed was the inability to produce a typical acoustic resonant wave in the main channel of our microfluidic device. We definitely noticed cells being deflected toward the center of the main channel whenever the acoustic radiation was applied. But instead of cells being focused to the channel center, the acoustic force pushed cells past the center and toward the opposite channel wall. We experimented with several combinations of frequencies and voltages on the function generator but were not able to achieve the standing wave we expected. This problem may have been caused by non-ideal ratios of flow rates among all inlets, such that the inlets delivering cells to the acoustophoresis region (within the main channel, Fig. 6) pushed the cells past the channel center. Another cause may be due to differences in main channel dimensions due to uneven etching, that is, etching that varies by position along the length of the channel. Differences in dimensions would require that we use different frequencies along the channel to produce standing waves because resonant frequency is determined by the channel width.

Our third problem was not being able to achieve a well-defined, stable line of yeast cells entering the acoustic radiation region. Even after adjusting the ratio of flow rates (for main channel, focusing inlets, and cell inlet), we were not able to ensure that all cells entered the acoustic separation region at approximately the same location. Cells entering the acoustic separation region at varying locations worsens the acoustophoresis size-based resolution because differences in starting points can impact differences in final positions of the cells after acoustic separation. As long as the starting points of cells 1) are different or 2) do not have a well-defined dependence on size, the resolution may not be good enough to separate mothers and daughters.

Another concern that exacerbated the previous problem (the inability to ensure all cells entered the acoustophoresis region at the same location) is having a non-ideal cell density within the syringe that introduced cells into the microfluidic device. We observed cells entering the acoustic separation region either at very high or very low concentration, both from a single syringe. Having an overly high concentration of cells complicates the task of achieving a single, well-defined line of cells entering the acoustic separation region. Having an overly low concentration decreases throughput. Having both high and low cell densities requires our system to operate back and forth between the extremes, which worsens separation resolution and decreases efficiency of the system.

Once this experiment is completed successfully, the results will be used as input (frequency, voltage, flow rate) to some of the instruments (function generator, syringe pump) we will use in our on-chip recirculation/purification experiment. In particular, after acoustically separating cells and measuring the sizes of cell populations from both outlets of the device (Fig. 6) for a range of

function generator voltages, we can determine the optimal acoustic power for separating mothers and daughters. Plots of cells sizes as a function of acoustic power for waste outlet and beginning of recirculation channel will reveal the optimal function generator voltage. The function generator frequency and syringe pump flow rates used in this experiment will be applied with little-to-no modification in the on-chip recirculation/purification experiment. The only reason to adjust the frequency from this experiment to the next is if the main channel dimensions are different between the test device (Fig. 6) and the on-chip recirculation/purification device (Fig. 2). We may need to modify the flow rates because the fluidic resistances at the main channel outlets could be different for our two microfluidic devices (Fig. 2 and Fig. 6) because one device has an outlet leading off-chip while the other has the recirculation channel.

On-chip recirculation to remove daughters and enrich the mother population

We have not completed the on-chip recirculation/purification experiment to remove daughter cells continuously and generate a large number of replicatively-aged mother yeast cells. The acoustic parameters for this experiment depend on results of our experiment to optimize the acoustic power, which is currently ongoing. Once completed, though, this experiment will determine how effective our approach is for generating a large quantity of replicatively-aged yeast cells.

CHAPTER IV

DISCUSSION

Overall project motivation

Researchers already have tools that allow them to study single yeast cells in high resolution throughout their replicative lifespans. What researchers need is a method to generate a large quantity ($>10^6$) of very old yeast cells (>15 generations). The most successful approaches to accomplish this rely on 1) labeling cells and extensive sample processing or 2) genetically modifying daughter cells to arrest cell division. All of these approaches suffer from several disadvantages, including high risks of contamination and time-consuming, manual processing steps. Therefore, generating a large, highly-pure population of replicatively-aged yeast cells (>15 generations) is a significant innovation because existing instruments cannot extract cells older than 8-12 generations without repeated purification. A large number of aged yeast cells can be used in subsequent biochemical analyses to gain a better understanding of the molecular causes of aging, which can improve treatments for age-related diseases.

Conclusions from time-lapse yeast cultures

To achieve our goal of efficiently generating a large quantity of aged yeast cells, we are developing a microfluidic acoustophoresis system that continuously removes daughters and enriches the mother population. First, we cultured wild-type yeast cells to investigate the size differences between mothers and daughters and the proliferation of daughters from a single mother. Figs. 8, 9, and 10 illustrate the challenges of isolating a large number of very old cells

(mother yeast cells) from the culture. These challenges arise from the exponential increase of daughter cells that crowd, steal nutrients from, and impact normal growth conditions of the mothers. Our results support the well-known requirement that daughter cells be removed for yeast aging studies. In addition to motivating the need to remove daughter cells, the time-lapse images suggest size-based separation can accomplish this task. That is, we see in Fig. 10 how the daughter cells are much smaller in size than the mother at the times when the daughter cells bud from the mother, which is consistent with results in the literature. We leverage the size differences between mother and daughter in our acoustophoresis system to enrich the mother cell population.

The size data obtained by processing images in MATLAB (Figs. 10 and 11) varies from the histogram produced by the Beckman Coulter Counter (Fig. 12). The overall trend in the evolution of yeast sizes agrees with expectations: as time passes, the proportion of smaller cells grows and the overall mean diameter of the population decreases. This behavior results from the exponential proliferation of daughter cells and indicates a healthy culture. The values of diameters extracted from MATLAB, however, differ from those obtained from the commercial particle counter. The results from the commercial counter agree very well with expectations for a healthy wild-type, haploid yeast culture; therefore, our image processing technique must have some error. The discrepancies may be the result of capturing only a limited number of cells within the microscope camera field-of-view (FOV) such that the cells within the FOV are not accurately representative of the entire cell population. More likely causes of the differences between size data measured in MATLAB and in the commercial counter are imaging cells slightly out of focus and fitting a circle to an oval-shaped yeast cell. Imaging the cells out of

focus will blur the cell boundaries, which must be clear and well-defined for MATLAB to detect the boundary accurately. Fitting a circle to a non-circular shape will obviously produce differences between the observed and actual cell sizes. Another cause of this size data discrepancy results from how we convert yeast size data reported by the commercial counter. The Beckman Coulter Counter measures yeast cell volume, and we convert volume to diameter for our analyses by approximating the yeast cells as spheres. As shown in Figs. 8-10, yeast cells are not perfectly spherical, and our approximation may produce differences between data obtained in MATLAB and in the cell counter. We must therefore consider the limitations of calculating cell size using MATLAB whenever precise size data is needed, such as when calibrating the size-based resolution of our system.

Overall, this size data quantifies the differences between mothers and daughters immediately as they enter our microfluidic device. It also defines a size cut-off for which we will aim when removing daughters in our system.

As mentioned above, Fig. 12 shows very typical size data for a wild-type yeast culture (CEN.PK). We realize, however, that yeast size varies depending on strain. Therefore, if the acoustophoresis size-based resolution in our system is inadequate for separating mothers and daughters in the wild-type strain, we can investigate another strain that is larger in size overall or has a larger size difference between mothers and daughters.

COMSOL simulations require that recirculation channel is redesigned

All COMSOL simulations in Figs. 13-19 show insufficient flow through the recirculation channel. Adjusting the inlet flow rates and adding inlets/outlets do not seem to resolve this problem, which results from having larger fluidic resistance in the recirculation channel than in the outlet(s).

We simulated several variations of our initial design (some even with very minor changes) because we want our acoustic power optimization device and on-chip recirculation/purification device to be as similar as possible. Similarities between these devices help achieve a more direct transfer of acoustic settings (frequency, power) and flow rates from one device to the next, which increases chances of success in our main on-chip recirculation/purification experiment. We are currently investigating other variations of the design in COMSOL, including applying reverse pressure at the outlets and modifying the dimensions of the recirculation channel (Fig. 2). Applying pressure at the outlets could be used as part of a somewhat complicated sequence ultimately to direct mother cells back into the acoustic separation region. This scenario is not ideal because it requires a more elaborate experimental setup that is not as straightforward as the on-chip recirculation. As long as the fluidic resistance of the waste outlet is smaller than that of the recirculation channel, fluid will flow preferentially to the waste outlet. Fluidic resistance is primarily defined by the channel width and length. Modifying the recirculation channel dimensions so that its fluidic resistance equals that of the waste outlet will therefore more evenly distribute flow between these two paths. If this change does not solve the recirculation problem, we can pursue more drastically changing the overall device geometries. Although a more drastic change complicates the transfer of experimental parameters from our acoustic power

optimization device to this on-chip recirculation/purification device, it may be simpler than programming a complicated experimental protocol for ultimately recirculating the mother cells through the acoustic separation region.

Because of this uneven flow between outlet and recirculation channel, we are not justified in building the on-chip recirculation/purification device with our current design. That is, we must redesign the device and verify it before proceeding with the next steps of this project.

Lessons learned from acoustic power optimization experiment

The negative results of our experiment to optimize acoustic power help us identify the problems we must mitigate before beginning a long-term on-chip recirculation/purification experiment. Most of these problems can be solved relatively easily. For example, by degassing the syringes containing culture medium and cells we can eliminate bubbles that would worsen the acoustic separation resolution. Also, specialty lab instruments can be used to control the cell density within a solution precisely, which would alleviate the problems associated with overly high or low cell concentrations within the syringes that load cells into our microfluidic device. Some of these problems we experienced are very common in microfluidics, so they likely affect the other microfluidic systems applied to yeast aging studies discussed in the INTRODUCTION. These problems, and others, are perhaps why these microfluidic systems have not replaced the conventional micromanipulation method as the standard technique for RLS studies. Thus, for our microfluidic system to have a significant impact on aging studies, we must implement reliable fixes for the problems we experienced and may experience in future experiments. We must

design our microfluidic acoustic system so that it can be used by all types of scientists and not by only a specialized technician.

Future work and solutions to potential problems

We must modify the recirculation channel of our design and verify it in simulations before we fabricate the device for on-chip recirculation/purification. We must also resolve the problems we experienced during our experiment to optimize acoustic power. After completing these tasks, we can proceed to test on-chip recirculation/purification and evaluate its efficiency. We can then compare this approach to the conventional and other microfluidic methods applied to yeast aging studies to determine the technological merit of our microfluidic acoustic system.

If we experience problems with having only a very small number of aged yeast cells after completing the on-chip recirculation/purification experiment, we can purify the initial yeast mother population immediately before loading into our microfluidic device. Centrifugal elutriation can yield mother cells that are up to 8 generations old, which means these cells have grown to much larger sizes compared to their sizes at birth. Instead of loading an unfiltered population of mothers and daughters into our device, we can remove some of the daughter cells using centrifugal elutriation. In this way, our system would operate on a greater number of mother cells that are larger in size compared to cells that are only 1-2 generations old. Although this particular approach relies on a conventional purification method (centrifugal elutriation) before loading cells into our device, the results of such an experiment still improve on existing techniques because the existing techniques cannot generate a large number of very old yeast cells in a less error-prone manner than can our microfluidic system.

Because the acoustic force on a cell in a microchannel depends on compressibility (see equation 1), our system can potentially separate yeast cells of similar sizes and densities but varying compressibility. Compressibility-based acoustic separation may be useful for separating very old mothers (>20 generations) from their daughters. Researchers have found that mother yeast cells are similar in size to their daughters at the very beginning and end of their replicative lifespans, so size-based separation is not appropriate at these times in the RLS of the mothers; however, other differences exist between mothers and daughters near the end of the mothers' replicative lifespans. As yeast cells grow and age, their cell walls thicken. Also, mothers accumulate damage from aging, especially birth scars consisting of rings of chitin deposits at the sites of each daughter bud. Daughter cells, on the other hand, acquire a newly synthesized cell wall at birth, free of birth scars and thinner than the cell walls of mothers. The cell wall thickening, birth scars, and other effects of aging can alter the compressibility of the mother cells resulting in compressibility-based differences between mothers and daughters. Therefore, even though mother and daughter sizes are similar at the end of the mothers' replicative lifespans, compressibility-based acoustic separation can still be utilized for separating mothers and daughters. Furthermore, the shift from size-based acoustophoresis to compressibility-based separation requires no changes in our system design and minimal-to-no changes in system operation.

Multi-frequency acoustophoresis can potentially improve the separation resolution between mother and daughter yeast cells in our device.[43] This technique involves alternating between frequencies as cells travel in the acoustic separation region. For example, by switching between

the 1^{st} and 3^{rd} harmonics at well-defined intervals, the differences in final positions of mothers and daughters after acoustic separation would be much larger compared to the differences obtained when using only the 1^{st} harmonic. Thus, future work for this project can include comparing single- and multi-frequency acoustophoresis to determine which method produces a more pure population of mother cells.

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