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Microfluidic Platforms for Yeast-Based Aging Studies

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Abstract

The budding yeast *Saccharomyces cerevisiae* has been a powerful model for the study of aging and has enabled significant contributions to our understanding of basic mechanisms of aging in eukaryotic cells. However, the laborious low-throughput nature of conventional methods of performing aging assays limits the pace of discoveries in this field. Some of the technical challenges of conventional aging assay methods can be overcome by use of microfluidic systems coupled to time-lapse microscopy. One of the major advantages is the ability of a microfluidic system to perform long-term cell culture under well-defined environmental conditions while tracking individual yeast. Here, recent advancements in microfluidic platforms for various yeast-based studies including replicative lifespan assay, long-term culture and imaging, gene expression, and cell signaling are discussed. In addition, emerging problems and limitations of current microfluidic approaches are examined and perspectives on the future development of this dynamic field are presented.

1. Introduction

Aging studies are becoming more prominent in biomedical research because aging is a primary risk factor for many diseases, including cardiovascular diseases, diabetes, and neurodegenerative disease.^[1–7] Therefore, a breakthrough in the study of aging that results in successful retardation of aging or a delay in the onset of age-associated diseases would have a tremendous impact on quality of life.^[8] However, the underlying molecular mechanisms of aging and their contributions to the development of age-associated diseases remain poorly understood. Research into the fundamental mechanisms of aging may uncover the secrets of longevity and enable the development of interventions to promote longevity and healthy aging.

Because humans are complex organisms with a long lifespan, aging studies are usually performed using various model organisms such as yeast, worms, flies, and mice. The budding yeast *Saccharomyces cerevisiae* is a powerful model organism used in aging-related research. Compared to other model organisms, *S. cerevisiae* has the benefits of a short lifespan, a fully sequenced genome, easy genetic manipulation, and ease of maintenance in

the lab. Because many proteins in yeast are similar in sequence and function to those found in humans,^[9–11] yeast have been used to uncover fundamental mechanisms, molecular pathways, and enzymatic activities that are conserved among all eukaryotic cells.^[12–15] For instance, the aging regulators *TOR1* and *SIR2* were both first discovered and studied in yeast, and their orthologs exist in all eukaryotes including humans.^[16]

Yeast replicates by asymmetric cell division, in which a mother cell produces a smaller daughter cell, referred to as a bud. Generally, aging research in yeast requires removal of daughter cells from their mother cells, which are grown on agar plates. This removal of daughter cells has been accomplished by dissecting daughter cells away from mother cells manually under a microscope equipped with a dissection needle.^[17–20] To conduct traditional yeast aging studies, cells are taken from logarithmically growing liquid cultures and they are then transferred at low density to fresh medium to grow at 30 °C for approximately 3 hours. Daughter cells are separated from mother cells and moved to the agar plate for virgin daughter cell selection. All buds produced by these daughter cells are isolated with a dissection needle every 1–2 generations for analysis of lifespan. At least 50 cells are typically necessary to obtain reliable lifespan for a single strain and each experiment is carried out at least twice. Based on the average lifespan of 25 generations for the wild-type strain, this work would take a few weeks to accomplish by the conventional microdissection method. Such methods have not changed appreciably over last 50 years since their initial discovery in 1959.^[21]

However, traditional assays of yeast aging, including microdissection methods, have technical challenges; for instance, the methods are low-throughput and the experimental procedures are laborious. An experiment typically lasts four to six weeks on average, and requires overnight storage of the assayed cells at 4 °C to pause replication throughout the course of the experiment. This tedious procedure has substantially hindered progress in the field of aging research. While high-resolution imaging is needed for a mechanistic understanding of cellular aging and death in yeast, the use of a thick, opaque agar plate impairs visualization. Thus, it is difficult to monitor organelle morphology and track molecular markers in individual cells during aging.

As an alternative to the conventional microdissection technique, microfluidic technologies have recently been developed to study yeast aging. These technologies provide the advantage of both scalability and precise fluid control and, therefore, have become increasingly attractive for long-term culture and monitoring of yeast cells in precisely controlled environments. Another important feature of microfluidic technologies is the ability to track single cells in real time using high-resolution fluorescence imaging, thus enabling the study of cell cycle control, organelle morphology, and a wide variety of other molecular processes. These types of measurements are critical to understanding the interplay between various cellular processes and aging.

To date, many papers have reviewed microfluidic technologies for various biological, biomedical, and clinical applications.^[22–27] Very recently, a mini-review has discussed seven microfluidic devices that allow whole-lifespan tracking of budding yeast cells.^[28] There is, however, no review article addressing microfluidics applications to various yeast-based

aging studies, even though many promising microfluidic platforms have been developed. In this article, we discuss recent advancements in the microfluidic systems for yeast-based aging studies including replicative lifespan assay, long-term culture and imaging, gene expression, and cell signaling. We also investigate the emerging problems and limitations of current microfluidic systems and provide perspectives on the future development of this dynamic field.

2. Yeast Replicative Lifespan Assay

The replicative lifespan in yeast, which refers to the number of daughter cells produced by a mother cell prior to senescence, is an important metric used in the discovery of new genes and pathways that contribute to aging. The genetic determinants and molecular mechanisms of the replicative lifespan in yeast may provide important insights into the underlying mechanisms of aging in higher eukaryotes as well. For example, reduction in calorie intake by restricting nutrients such as glucose, amino acids, and proteins extends the replicative lifespan of yeast and also increases organismal longevity in higher eukaryotes.^[29–32]

The microdissection technique is the conventional method with which to assay the replicative lifespan of yeast. However, this method has many technical challenges and limitations as outlined above. Many microfluidic systems have been developed for long-term culture and monitoring of yeast cells; however, they do not allow researchers to perform replicative lifespan assays due to a lack of ability to remove daughter cells from their mother cells.^[33–39] To date, seven microfluidic platforms have successfully demonstrated the ability to track the entire yeast replicative lifespan.^[40–46] Here we discuss these microfluidic platforms for the automated whole-lifespan tracking of yeast cells in terms of working principle, design, and capability of each device.

2.1. Working Principle

The microfluidic platforms that are capable of automated whole-lifespan tracking of yeast cells have similar working procedures. First, young mother cells are immobilized in the microfluidic device. Then, the immobilized mother cells begin to grow and bud, producing daughter cells. As cytokinesis is completed, detached daughter cells are washed away automatically from their mother cells by a continuously flowing medium. By combining this technology with time-lapse microscopy, microfluidic platforms enable researchers to track and monitor the trapped mother cells over an entire lifespan under stable nutrient and temperature conditions. These platforms are able to take complete replicative lifespan measurements in three days, rather than the few weeks required for conventional microdissection methods.

Xie et al. developed the first microfluidic device that allowed automated tracking of yeast aging for a replicative lifespan assay (Figure 1A).^[40] The glass surface of the device was coated by biotinylated bovine serum albumin (BSA) followed by neutravidin, and mother cells were labeled with biotin. The mother cells were immobilized to the glass surface by biotin-avidin bonds. The daughter cells were separated from mother cells by medium flow because the newly generated cell walls of the daughters did not inherit the biotin label. This microfluidic device was also capable of continuous observation of cell cycle dynamics and

morphologies. Using this method, Xie et al. found significantly enhanced stress responses during the last few cell divisions in both WT cells and the long-lived mutant, *fohl*. In addition, cell death patterns were characterized by different terminal morphologies depending on the lifespan of individual cells, and the expression level of Hsp104 was identified as a longevity marker for yeast replicative aging. However, cell surface labeling and chemical modification of the device could present technical challenges for device fabrication and the required modifications to the yeast cells may introduce complicating effects on replicative lifespan.

To address this problem, the same group developed a different microfluidic device that eliminated the requirement of cell surface labeling and glass surface modification (Figure 1B).^[41] In this device, mother cells were physically trapped in the gap between the pensile columns and the glass surface while the daughter cells were not trapped due to their smaller size, resulting in automated removal by the flowing medium. The microfluidic device demonstrated its capability to automatically measure replicative lifespan for the short-lived mutant *sir2* and long-lived mutant *fohl*. By observing mother-daughter cell pairs in the device, they discovered that the ratio of stress response reporter level between mother and daughter cells is dependent on mother cell age, demonstrating asymmetric partitioning.

Another microfluidic dissection platform was introduced by Lee et al. to track single yeast cells over their entire replicative lifespan with high-resolution live-cell imaging (Figure 1C).^[42] This design was similar to the device of Zhang et al.,^[41] in which the application of hydrostatic pressure lifted micropads in the microfluidic chamber and mother cells were captured in the gap under the micropads when hydrostatic pressure was released. The emerging daughter cells were washed away by a continuous medium flow because the micropads were unable to hold the daughter cells due to the size difference between mother and daughter cells. This platform provided not only replicative lifespan measurement in a less laborious manner, but offered continuous high-resolution fluorescence imaging of aging yeast cells.

Fehrmann et al. developed a microfluidic chip, called a cell loaded into a cavity (CLiC) device, to monitor successive cell division and mitochondrial membrane potential in individual cells throughout their replicative lifespan (Figure 1D).^[43] In the CLiC device, cells were loaded into a trapping area with attached elongated cavities. Subsequent budding of the loaded cells led to confinement of budded cells at the dead-end tips of the elongated cavities. The successive cell divisions of the confined virgin cells were then monitored over their entire lifespan. This microfluidic technique allowed tracking of cells from the virgin stage in which they had not yet generated a daughter cell, marking the beginning of the replicative lifespan. This selection of virgin cells was not possible in the previously developed microfluidic devices. Using the CLiC device, Fehrmann et al. found that yeast cells undergo a sudden transition to enter senescence, and the loss of mitochondrial membrane potential is not a hallmark of aging but, instead, is independent of cell age.

To track a large number of aging yeast cells, Crain et al. developed a microfluidic device called A Long-term Culturing and TRapping System (ALCATRAS) (Figure 2A).^[44] The microfluidic chamber in ALCATRAS included an array of over 1500 trap structures which

consisted of two pillars placed in a V-shape. Mother cells were captured between the two pillars and were immobilized by a pressure force induced by continuous medium flow against the two pillars. Upon budding, daughter cells were released with the medium flow through the small gap between the two pillars placed in a V-shape. The device was capable of tracking up to 1000 individual yeast cells during cell aging with fast-changing media. ALCATRAS provided not only the ability to measure lifespan simultaneously for a large number of yeast cells, but also the ability to observe stress response due to changing external stimuli.

Recently, Jo et al. developed a microfluidic system called the high-throughput yeast aging analysis chip (HYAA-chip) for high-throughput analysis of yeast replicative aging (Figure 2B).^[45] The HYAA-chip contains 16 parallel microfluidic channels grouped into four separated modules. Each module consists of four microfluidic chambers. In each microfluidic chamber, there are 520 single cell-trapping structures, which are cup-shaped with a small outlet opening through the center. The mother cells in each microfluidic channel are immobilized by the trapping structures, and the newly budded daughter cells are removed with continuous fluid flow of media via the small outlet opening of the cup-shaped structure. The HYAA-chip enables automated tracking of the entire lifespan of up to 8000 individual yeast cells of 16 different strains in a single experiment, with high spatiotemporal resolution. The HYAA-chip is the first microfluidic platform that demonstrates massively parallel replicative lifespan measurement in a high-throughput manner. The continuous fluorescence imaging by the HYAA-chip also provided high-throughput visualization of subcellular protein localization in organelles and structures including the nucleolus, vacuolar membrane, late Golgi, lipid particle, actin filaments, and mitochondria, over the lifespan of single yeast cells. This system was also used to discover specific lifespan-related changes in critical cell size, terminal morphology, and calorie restriction.

Most recently, the multiplexed microfluidic device called the Yeast Replicator has been developed by Liu et al. for high-throughput lifespan experiments in distinct growth environments (Figure 2C).^[46] The Yeast Replicator device is composed of ten separated modules that can perform ten independent experiments with different strains of yeast and/or different growth media. Initially, yeast cells are immobilized between two bars outside the replicator unit, which is composed of three bars in an equilateral triangle shape. Virgin cells budded from the immobilized cells are confined randomly into the replicator units, and then the virgin cells are monitored over their entire lifespan. Daughter cells produced from these confined cells are washed away through the gap between two bars. The Yeast Replicator device enables researchers to track the replicative lifespan by starting from virgin cells similar to the CLiC device.^[43] Liu et al. determined replicative lifespan in different media conditions and found that the lifespan of cells grown in a galactose environment was 16.8% shorter than the cells grown in glucose.

2.2. Comparison Between the Microfluidic Platforms

Each microfluidic platform has strengths and limitations in design and capability with regard to yeast replicative lifespan assays. Table 1 summarizes the differences between the seven microfluidic platforms based on trapping mechanisms, design, experimental conditions, and

capabilities. To immobilize mother cells, all microfluidic devices confine mother cells vertically in the trapping structures. The height of the trapping structures in all devices range from 3.3 to 6.0 μm , comparable to yeast cell size. Some devices have non-uniform heights due to multilayer structures,^[40–43] whereas other devices have a single layer and thus a uniform height of all microfluidic channels and trap structures.^[44–46] The single layer design is easier to fabricate and operate as compared with the multilayer channel design.

As presented above, all devices use the size difference between mother and daughter cells to remove daughter cells while mother cells age. The opening size for automated removal of daughter cells with continuous medium flow is slightly smaller than the typical yeast cell size, and was between 3.0 and 4.0 μm in the devices being compared. Although the devices have a similar opening size for the removal of daughter cells, the retention rate of the initially trapped mother cells, which can be continuously monitored over the course of the experiment, is quite different among the devices. The retention rate is critical for high-throughput lifespan analysis of yeast cells. A low retention rate may restrict statistical significance of the lifespan analysis due to small sample sizes. For example, the micropad trapping method only achieves a retention rate of 15–30% of the initially immobilized cells due to insufficient pressure holding the mother cells and low efficiency in removing daughter cells.^[42] On the other hand, the unique and well-optimized trap design of the HYAA-chip allows for stable immobilization of the initially trapped mother cells with a significantly higher retention rate of up to 92% over a time course of 96 hours.^[45]

Two of the seven microfluidic platforms developed for yeast replicative aging studies guarantee that the trapped and monitored cells are virgin cells at the beginning of the experiment. However, in a typical log phase culture of yeast cells, the majority of cells will either be virgin (about 60%) or have only budded once (about 27%). Therefore, the lack of virgin cell trapping should not have a significant effect on the replicative lifespan of the yeast cells.^[47,48]

In addition, each microfluidic platform has different experimental conditions and capabilities. For example, devices with a lower flow rate of media for culturing mother cells and removing daughter cells may conserve culture media and enable longer experiments. This longer monitoring time is preferred to track longer-lived mutants or slower growing cells. Some of the platforms can be used to observe a larger number of strains in a single experiment, which is required for high-throughput analysis of yeast replicative aging.

For all seven microfluidic platforms, medium flow is provided continuously to the devices during the entire experiment to keep mother cells immobilized, to wash away daughter cells, and to maintain constant fresh growth condition. The flow rate of the medium is critical factor for high efficiency in pressure holding mother cells initially trapped and for high efficiency in removing daughter cells from their mothers, resulting in high retention rate of the tracked mother cells during the entire experiment. The flow rate is precisely controlled with a programmable syringe pump and is optimized in terms of giving a sufficiently high trapping efficiency, minimal chance of clogging, and high retention rate. The optimized flow rate is different for each microfluidic platform due to different working principles and geometric difference in the trap structures and microfluidic channels. Applying higher flow

rate to yeast cells for a long time might affect their phenotypes such as morphology, division time, and replication. To date, studies have not performed to investigate the effect of medium flow rate on aging yeast cells in the microfluidic platforms. Such studies would lead to the wider adoption of microfluidic technologies for yeast-based aging research.

Conventional materials for the microfluidics are silicon and glass. Silicon has excellent chemical resistance and good mechanical properties. Glass has several attractive properties including hydrophilic surface properties, transparency in the visual range, and electrical insulation. However, silicon is a relatively expensive material, needs high-cost manufacturing processes, and is not transparent conflicting with optical characterization. Glass has disadvantages such as brittleness, opacity in UV light, and limited integration capability with other materials. The material of all microfluidic devices for yeast aging studies is polydimethylsiloxane (PDMS), which is transparent in visible/UV ranges and gas- and vapor-permeable elastomer. PDMS has several features including the relatively inexpensive and easy fabrication, the ability to modify surface properties, the capability for bonding to various materials, and the easy replication.^[25]

In the microfluidic platforms using PDMS for yeast aging studies, cells are immobilized by the gap or space in geometric confinement comparable to the size of initial young cells. The size increase of the mother cells with aging might result in deformation of the cells and influence their replicative lifespan. However, the elasticity and flexibility of PDMS allow it to prevent the effect of cell deformation on the viability and replication of yeast cells. A few studies with the microfluidic platforms demonstrated that the replicative lifespan obtained using the microfluidic devices are very comparable to those obtained using the conventional microdissection methods with identical preculture and culture medium conditions.^[42,43,45] It should be noted that the size and shape of geometric confinement using even PDMS could affect the replicative lifespan for particular strains. A further discussion of such influences will be presented in the Summary and Outlook section.

3. Gene Network Dynamics in Yeast

In addition to replicative lifespan assays, microfluidic devices are also valuable tools with which to track the dynamics of gene networks in single cells under various environmental conditions. Microfluidic platforms with time-lapse fluorescence microscopy allow researchers to examine signaling pathways and the dynamics of gene regulation in yeast cells. The ability of the microfluidic platform to rapidly exchange culture media and generate time-dependent fluctuations and spatial gradients of media concentrations are essential to study cellular responses to environmental perturbations and changes. In the following sections, we begin by presenting long-term culture and microscopic imaging of yeast cells using microfluidic platforms, then discuss important features of various microfluidic devices to study gene expression and cellular signaling in yeast aging.

3.1. Long-Term Culture and Imaging

Long-term culture and high-resolution microscopic imaging of yeast cells over several generations is important for understanding the cellular processes throughout the lifespan of budding yeast. Microfluidic technologies offer high-resolution microscopic analysis of

dynamic phenomena in yeast cells grown in well-controlled environmental conditions for an extended time course. Cell loading and culturing in a microfluidic device are generally performed with particularly designed structures such as microwells and microtraps.^[49–56] Since these methods may have disadvantages such as complicated device fabrication, difficult environment control, and high flow shear, Luo and colleagues developed a cell loading technique using gas absorption of degassed PDMS.^[57,58] This technique allows for loading of yeast cells into triangular micro-cavities within one minute by degassing-induced aspiration of PDMS and medium change without washing the cells out of the micro-cavities. Long-term imaging of phenotypic changes including doubling time, growth rate, and EGFP expression level were examined in response to copper ion concentration in the medium for 20 hour time periods. Lee et al. integrated the microfluidic device with a standard 96-well plate to enable live yeast cell imaging and to address a lack of commercial product use (Figure 3A).^[59] Such a microfluidic system overcomes certain technical issues of traditional microfluidic devices such as clogging due to air bubbles or complicated operation with multiple fluidic connections. This platform was used with high-resolution fluorescence imaging to monitor and analyze the induction of GFP expression by pheromone exposure.

Cellular morphology analysis is essential to reveal the key factors related to cell cycle progression. Because conventional glass slide methods for morphological cell analysis are laborious, time-consuming, and low-throughput, they have limited application for systematic comparative analyses.^[60–63] Ohnuki et al. developed a microfluidic device for high-throughput and long-term imaging of morphological changes during the cell cycle and aging in yeast (Figure 3B).^[64] Their microfluidic device consists of two layers: the upper layer is a medium-filled main channel and the lower layer is an air-filled control channel. The yeast cells are immobilized and released by the air pressure of the lower control channel. Ohnuki et al. successfully characterized and quantified the cell cycle-dependent changes in cellular morphology of yeast with this microfluidic device.

Recently, Frey and colleagues reported a microfluidic system for long-term microscopy of different cell types with constant or changing media perfusion (Figure 3C).^[65] This microfluidic device is capable of culturing and tracking non-adherent or adherent cells in parallel under various growth conditions, without cross-contamination. In this system, non-adherent cells are trapped under hexagonal pads and cultured with medium flow driven by pressure and diffusion, whereas adherent cells are cultured on the glass substrate of the microfluidic chamber without an additional trapping structure. The device capability for different cell types (e.g., bacteria to mammalian cells) was validated via culturing and monitoring of *S. cerevisiae*, *Schizosaccharomyces pombe*, *Pseudomonas putida*, HepG2 cells, and HCT116 cells. With rapid medium exchange, yeast aging based on different oxygenation levels was tracked and analyzed for 60 hours.

3.2. Gene Expression

Gene expression plays a critical role in cellular processes and provides extremely valuable insights into cell differentiation and mutation. As differential gene expression has been implicated in the aging process, the ability to monitor and track gene expression over several generations of yeast cells could allow researchers to discover new genes that contribute to

aging. Conventional methods, such as microscopy and flow cytometry, have characterized gene expression based on cell population scale, resulting in high expression noise levels due to sample heterogeneity. Additionally, these methods do not allow for the investigation of changes in gene expression over time resulting from aging in individual cells. Microfluidic technologies, on the contrary, enable researchers to measure single-cell gene expression accurately for a large number of cells over multiple generations. In the early development of microfluidics, several groups created microchemostat platforms to examine gene expression and regulation of yeast in constant or dynamically changing environments over multiple cell cycles (Figure 4A).^[66–68] These devices, however, have limitations; as the yeast cells age, microfluidic channels can get clogged and lose the tracked cells, resulting in low efficiency. Ryley and Pereira-Smith successfully demonstrated the ability to measure single-cell gene expression of yeast over time courses of up to 18 hours using a microfluidic device.^[69] The ‘jail’ structures employed in their microfluidic device are capable of discriminating between mother and daughter cells based on their size and location during aging. Expression levels were found to vary significantly for individual yeast cells and gene expression was correlated with their lifespan.

Microfluidic platforms that immobilize single cells by microtraps are technically challenging for lineage analysis of yeast cells because the daughter cells produced are not retained for aging. Rowat et al. introduced a microfluidic device that is capable of tracking several lineages of single yeast cells in parallel.^[70] Single mother cells are immobilized in linear chambers with long channels and narrower constrictions. The cells are grown in the chambers and their lineages can be tracked for up to eight generations. Their work illustrated that phenotypic persistence is protein-specific, and suggested the possibility of studying temporal fluctuations in expression levels. Recently, microfluidics has been exploited for real-time control of gene expression in yeast. Uhlenhof et al. developed a microfluidic platform interconnected to computational tools for open-loop and closed-loop control to provide long-term regulation of gene expression in yeast (Figure 4B).^[71] The average level of reporter gene expression and the differential expression between individual cells were controlled by adjusting the pulse-frequency of an extracellular signal in the microfluidic environment. The open-loop strategies were unsuccessful, but closed-loop strategies using measurement feedback allowed real-time control of gene expression with quantitative accuracy for up to 17 hours.

Previous studies reported that the yeast transcription factor Msn2 responds to external stresses in its translocation dynamics.^[72,73] Hansen et al. developed an automated high-throughput microfluidic system to investigate the response of genes to different Msn2 dynamics (Figure 5A).^[74,75] The integrated system consists of multiplexed microfluidic devices for yeast cell culture, automated programmable medium handling for cell perturbation, and a computational analysis program for time-lapse microscopy data. This microfluidic system allows researchers to conduct up to 20 different experiments and obtain data for thousands of cells in a day. Using this system, Hansen et al. generated oscillations of Msn2 localization and determined the dynamic gene expression of individual genes in single cells.

Microchemostats have emerged as a powerful platform for high-throughput single-cell analysis.^[76–79] These platforms allow for the growth and tracking of strains in a large number of separated chambers under constant conditions by generating a flux of medium. Dénervaud et al. developed a microfluidic microchemostat for high-throughput analysis of the yeast proteome with high spatiotemporal resolution (Figure 5B).^[80] The massively parallel device enables researchers to culture and monitor 1152 different strains in a single experiment. Using this technology, the abundance and localization of 4085 yeast proteins from a GFP library were determined in response to the DNA damage inducing agent methyl methanesulphonate. Such high-throughput capability in a microfluidic device is clearly a technical breakthrough.

Parkinson's disease is one of several age-associated neurodegenerative disorders. The form of α -synuclein (aSyn) protein involved in Parkinson's disease was revealed to also be toxic to yeast cells.^[81–84] However, the cellular effects of different expression and aggregation levels of aSyn in yeast are still uncharacterized. Recently, two groups performed similar studies with the microfluidic-based gradient generators to monitor yeast cells expressing aSyn modulated by the concentration of the galactose-inducible promoter (GAL).^[85,86] They found that aSyn expression is linearly dependent on GAL concentration and increases with age. They also demonstrated that oxidative stress can promote the accumulation of aSyn in yeast.

3.3. Cell Signaling

Cellular signaling is critical in determining the phenotype of a cell or organism. Yeast is a prototypical model for cell signaling because of the ease of genetic manipulation and the availability of reporters. In particular, the yeast pheromone response is one of the best characterized mitogen-activated protein kinase (MAPK) signaling networks in eukaryotic organisms. MAPK signaling plays an important role in cellular decision-making processes, and responds to various robust stimuli such as growth factors, stress, cellular adhesion, and nutrient conditions.^[87,88] The pheromone response in yeast cells enables the tracking of spatial gradients of mating pheromones α - or a-factor, which allow haploid cells to mate with cells of the opposite mating type.^[89–91] Moore et al. investigated spatial gradient sensing of the mating pheromone α -factor in yeast cells using a microfluidic device.^[92] The gradient-generating microfluidic device is capable of tracking spatial gradient sensing and polarization mechanisms of yeast cells. They found that morphologies of yeast cells were strongly affected by α -factor concentrations; lower concentrations caused the cells to form clusters, while higher concentrations caused the cells to become thin 'shmoo-like' shapes. Moore et al. showed that the pheromone gradient and concentration profiles have an important role in regulating the direction and polarization of yeast cells.

Paliwai et al. employed a diffusion-based microfluidic device to study gene expression and pheromone gradient sensing for studying the mating response in individual yeast cells (Figure 6A).^[93] The device offered the generation of stable pheromone gradients in a microchamber connected to sink and source microchannels, and allowed for the monitoring of cell phenotype and gene expression at the same time. They demonstrated the switch-like pheromone activation of gene expression, and the critical role of the MAPK in controlling

transcription factor Ste12. Kss1, one of the MAPK factors activated in the mating pathway, extended the dynamic range of pheromone gradient sensing. A different microfluidic platform has been developed by Hao et al. that can create precise spatial chemical gradients to study the response of yeast cells to different concentrations of mating pheromone (Figure 6B).^[94] In this microfluidic platform, the linear gradient is generated in the microfluidic chamber by passive diffusion between two parallel microfluidic channels that connect the media channels to the microfluidic chamber. For this experiment, one channel lacked pheromone, whereas the other channel included a dose of pheromone sufficient to cause cell division arrest. In the device, three growth states are created based on the concentration of pheromone; vegetative growth at low pheromone levels, chemotropic growth at intermediate pheromone levels, and growth arrest at high pheromone levels. The study found that the two pheromone-responsive MAP kinases, Fus3 and Kss1, affect cell elongation. However, using quantitative measurement of the angle of budding relative to the gradient, they found that only Fus3 could mediate directed growth toward high pheromone channels. The scaffold protein Ste5 dynamically regulates Fus3 activity. Kss1 shows a fast and graded response to the concentration of pheromone, while Fus3 exhibits a slow, switch-like, and ultrasensitive response to the concentration of pheromone.

As presented above, microfluidics has been employed to investigate cellular responses of yeast cells to the presence of various genetic perturbations and chemical environments. However, some methods lacked technologies for high-throughput analysis of a large number of cells under well-controlled and varied-time conditions. To address this problem, Taylor et al. developed a high-throughput microfluidic imaging platform to track the signaling network responses of single yeast cells to hundreds of combined genetic perturbations and stimulant sequences that vary over time (Figure 7A).^[95] Their microfluidic platform featured two layers of microchannels including a flow layer for cells and for introducing reagents, and a control layer for pneumatic valves. The platform enables the researcher to conduct 256 simultaneous time-lapse imaging experiments by integrating programmable on-chip mixing and perfusion, customized image-processing algorithms, and computational tools. In this study, the device captured yeast cells with an array of 2048 trap structures and performed more than 3000 experiments to investigate the mating pheromone response and MAPK signaling in yeast cells under combined genetic perturbations and changing stimulant conditions. Taylor et al. discovered thresholds of morphological switching and new dynamic phenotypes for 11 deletion mutants. The same group has further developed a high-throughput microfluidic imaging platform for enhancing the capabilities of high-resolution imaging and robust cell tracking.^[96] In this platform, yeast cells are immobilized by a combination of mechanical clamping and polymerization in an agarose gel. The microfluidic channels and valves allow fully programmable medium exchange via diffusion to a matrix of 128 integrated agarose gel pads. They demonstrated the quantitative analysis of the pheromone signaling response of over 60 000 individual yeast cells in 8 genotypes exposed to 16 different α -factor concentrations. These high-throughput microfluidic imaging platforms can be used for systems-level studies for quantitative and predictive understanding of cellular decision making.

Cells demonstrate variability in their response to identical external stimuli due to non-genetic differences between individual cells including the localization, abundance, and state

of cellular components. In yeast cells, these non-genetic differences are heritable between successive divisions and dependent on the cell cycle stage and age of cells. Ricicova et al. exploited a high-throughput and fully automated microfluidic platform to investigate the effects of non-genetic heritability and cell cycle on cell-to-cell variability in MAPK signaling (Figure 7B).^[97] The microfluidic platform offered simultaneous tracking of cell-lineage relationships, cell cycle, age, and gene expression under temporally changing medium conditions in single yeast cells. Using 72 microfluidic chambers, the device was used to collect single-cell data for over 25 000 cells and identify over 9000 mother-daughter pairs over 13 hours. Their comprehensive analyses revealed an asymmetric response between daughters and mothers in pheromone stimulation of aging yeast, with daughter cells exhibiting a higher response than their mothers. This asymmetric response was correlated with the replicative lifespan of cells. Two unique phenotypes causing alterations of the cell cycle and age asymmetry were identified, with *fus3* having higher heritability and *ste50* having higher heterogeneity.

4. Summary and Outlook

As discussed in this review, the development of microfluidic platforms is a critical methodological advancement in the study of yeast aging. The combination of other advanced technologies such as time-lapse (fluorescence) microscopy with the capabilities of microfluidics has provided clear benefits for yeast aging research. For example, the microfluidic platform allows automated whole-lifespan tracking with high spatiotemporal resolution and large-scale quantification of single yeast cells, resulting in significant reduction of labor, time, and cost. In addition, the high-resolution fluorescence imaging of yeast cells grown in constant and dynamically changing environments offers the ability to examine the dynamics of gene expression and signaling networks in a high-throughput manner. The quantity and types of data acquired by such microfluidic technologies would be impossible to acquire using conventional research techniques. Even though microfluidics has made significant contributions to the study of yeast aging, there are still some challenges that remain. For discussions regarding general challenges and limitations of microfluidics (such as the choice of materials, operation, and integration), we refer the readers to the several recent reviews.^[98–100] Here we discuss only challenges in microfluidics associated yeast aging research.

For the replicative lifespan assay, the microfluidic devices immobilize mother cells physically in a trap structure and remove daughter cells from their mother cells with media flow. As shown in Table 1, the size of the gap or space in geometric confinement is designed to be comparable to the size of initial young mother cells to capture them efficiently at the beginning of the experiment. However, the size of mother cells significantly increases as they age, at times over 200%. This increase in cell size might result in the squeezing of the cells and affect their replicative lifespan. For example, when the yeast cells of W303 background were tested in the microfluidic device, which have a relatively faster size increase during aging, the physical squeezing caused premature cell death. In this experiment, the maximum replicative lifespan of W303 cells was only seven generations,^[46] while the typical mean replicative lifespan of W303 was 27 generations.^[101] Therefore, the design of the trap structure in the microfluidic device should not only accommodate larger

mother cells, but prevent spatial constraint to cell size increase upon aging. In addition, as the efficient removal of daughter cells depends on the size difference between mother and daughter cells, methods must be established to address cases of diminished size difference between mother and daughter cells (due to, for example, experiments using mutants that have altered cell size or shape). Low efficiency of daughter cell removal results in deficient tracking of the trapped mother cells due to low retention rate of mother cells and clogging in the microfluidic channel.

Calorie restriction (CR), a dietary regimen low in calories without malnutrition, extends lifespan in most model organisms including yeast, worms, flies, and mammals.^[10] CR in yeast is commonly modeled by reducing the glucose concentration in otherwise rich medium.^[29] Interestingly, Huberts et al. reported that CR-induced lifespan extension could not be detected with their microfluidic platform.^[102] They hypothesized that a reduction in the glucose concentration of medium may influence differently the yeast cells grown in the microfluidic platform compared with those on an agar plate for the conventional microdissection method. In contrast, other microfluidic platforms observed robust longevity effect of CR that is consistent with results obtained from the conventional microdissection-based lifespan assay.^[45,46] There is no clear explanation for the discrepancy but the different designs and capabilities of the microfluidic platforms may be a reason of this discrepancy for CR-induced longevity effect. For example, Jo et al. reported one possible explanation that the low retention rate of Huberts' device might have been the cause of the reported lack of lifespan extension by CR.^[45] In their censored data to determine the lifespan curve, the contribution of the lost mother cells to the resultant data was significantly larger than that of the retained mother cells due to the low retention rate of their microfluidic platform. If the working mechanism and device design of the microfluidic platforms could significantly bias the analysis results, the adoption of the microfluidic technologies for yeast aging studies would be challenged. Thus, more studies need to be conducted about yeast's response to various growth environmental factors (i.e., nutrients, pH, and temperature) in the microfluidic platforms to further verify and strengthen the reliability of the microfluidic technologies for yeast aging studies.

Post-processing analysis of images is also a challenging aspect of high-throughput yeast aging research. Microfluidic systems coupled to time-lapse microscopy allows for faster and less laborious yeast aging experiments than tedious conventional methods. However, to examine various aspects of aging (including replicative lifespan, morphology, expression, and signaling), several days' worth of images often must be analyzed. Therefore, the development of automated image-processing tools would further enhance the throughput of aging experiments.

The next goal for microfluidics in the field of yeast aging research should be to further increase the experimental scale and throughput capacity of these devices. These enhanced capabilities would enable large-scale genetic screens that may significantly accelerate the discovery of novel genes and pathways that contribute to aging. Additionally, large-scale high-throughput drug screens for age-associated diseases such as Alzheimer's and Parkinson's diseases using microfluidic devices may accelerate discoveries in these areas, as well. When microfluidic technologies are coupled to system-biology approaches, the ability

to map complex regulatory networks and resolve the dynamics of signal transduction pathways will enhance our understanding of the aging process.

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Figure 1.

Microfluidic platforms with multi-height channels for yeast replicative lifespan assay. A) Photograph and schematic illustration of microfluidic device. Mother cell surface labeling and glass modification are illustrated. Reproduced with permission.^[40] Copyright 2012, John Wiley and Sons. B) Schematic illustration of the microfluidic chip design and the experimental setup. The chip is composed of main microfluidic channel connected with two side channels including arrays of pensile columns. Reproduced with permission.^[41] Copyright 2012, Public Library of Science. C) Schematic illustration of principle of microfluidic dissection. Mother cells are trapped between a PDMS micropad and thin cover glass that allows high-resolution imaging. The images show the GFP-tagged vacuole of the yeast cell during division. Reproduced with permission.^[42] Copyright 2012, National Academy of Sciences. D) Image of the CLiC microfluidic device and tracking mother cells. Sequence images show time course of the aging process for the trapped yeast cell in the elongated cavity with enclosed tip. Reproduced with permission.^[43] Copyright 2013, Elsevier.

Figure 2.

Microfluidic platforms with single-height channels for yeast replicative lifespan assay. A) Overview of the ALCATRAS microfluidic device and a DIC image of the trapped cells. The device contains more than 1500 individual V-shaped traps. Reproduced with permission.^[44] Copyright 2014, Public Library of Science. B) Optical images of the HYAA-chip device and SEM images of cup-shaped trap structures. The device allows automated tracking of whole-lifespan for up to 8000 individual yeast cells of 16 different strains in a single experiment with high spatiotemporal resolution. Reproduced with permission.^[45] Copyright 2015, National Academy of Sciences. C) SEM pictures of an independent module and a single replicator unit in the microfluidic device. Replicative lifespan of yeast is determined by starting from virgin cells. Reproduced with permission.^[46] Copyright 2015, Cell Press.

Figure 3.

Microfluidic system for high-resolution microscopic analysis of dynamic phenomena in yeast cells. A) Schematic illustration of the microfluidic circuit for yeast cell imaging. The cell trapping region contains eight trapping sites and each site is separated from the flow channel by a barrier to prevent crosstalk of cells. Reproduced with permission.^[59] Copyright 2009, BioTechniques. B) Schematic illustration and photograph of the microfluidic device and experiment setting. The monitoring of yeast cells is achieved with four steps including injection, hold, release, and outflow. Reproduced with permission.^[64] Copyright 2009, BioMed Central. C) Schematic top view of the microfluidic chip consisting of three cell culturing areas surrounded by a nutrient supply channel. Hexagonal clamping pads hold cells on the thin glass slide and constrain movement to the focal plane. Reproduced with permission.^[65] Copyright 2015, American Chemical Society.

Figure 4.

Microfluidic platforms to examine the gene expression and regulation in yeast cells in a dynamically changing environment. A) Design and implementation of the microfluidic platform. The monolayer chamber is connected to the switch output channel with the feeding channels. A controllable waveform generator provides sinusoidal perturbations in the glucose concentrations while the galactose levels maintain constant. Reproduced with permission.^[68] Copyright 2008, Nature Publishing Group. B) Microfluidic platform for real-time, closed-loop control of gene expression in yeast. The platform integrates microscopy for tracking gene expression, microfluidic device to change the osmotic environment of yeast cells grown as a monolayer, and original software for automated imaging, quantification, and model predictive control. Reproduced with permission.^[71] Copyright 2012, National Academy of Sciences.

Figure 5.

Microfluidics technologies for high-throughput gene expression analysis of single yeast cells. A) Schematic illustration of the microfluidic platform. The computer-controlled solenoid 3-way electrovalves control whether normal medium or drug medium is delivered to yeast cells grown in five microfluidic channels. Protein translocation dynamics and gene expression responses are tracked by using time-lapse microscopy. Reproduced with permission.^[74] Copyright 2015, Nature Publishing Group. B) Microchemostat array platform for spatiotemporal analysis of the yeast proteome. The platform is capable of culturing and tracking 1152 yeast-GFP strains on the single-cell level. The analysis of entire yeast-GFP library is performed under various environmental conditions. Reproduced with permission.^[80] Copyright 2013, National Academy of Sciences.

Figure 6.

Microfluidic device for pheromone dose-response analysis. A) Microfluidic device to study pheromone gradient sensing for the mating response. Yeast cells are grown in the test chambers of different lengths and exposed to a continuous range of pheromone concentrations presented in gradients of different steepness. Reproduced with permission.^[93] Copyright 2007, Nature Publishing Group. B) Microfluidic device to study yeast cell differentiation induced by different concentrations of pheromone. Gradient in the cell chamber is established by diffusion between m1 and m2 ports. Yeast cells undergo three distinct developmental fates in a pheromone gradient. Reproduced with permission.^[94] Copyright 2008, Elsevier.

Figure 7.

High-throughput microfluidic platforms to study mitogen-activated protein kinase (MAPK) signaling networks in yeast cells. A) High-throughput microfluidic single-cell imaging platform for dynamic analysis of MAPK signaling in yeast cells. The device consists of two layers of channels including a flows structure for cell/reagents loading and a control structure for pneumatic valves. The regions indicated in the device layout are 1) cell-loading ports, 2) experiment matrix, 3) chemical inputs and control, 4) peristaltic pump, 5) fluidic multiplexer and 6) waste outlet. Each column of the device is for a single yeast genotype and each row of the device is for a single experimental condition. Reproduced with permission.

[95] Copyright 2009, National Academy of Sciences. B) Microfluidic device for high-throughput automated lineage tracking of yeast cells in MAPK signaling. An example of lineage tree obtained by image analysis of the indicated chamber area. At the lineage tree, each horizontal line corresponds to single cell and each square in the line shows 12-minutes time point. Vertical lines connect each mother cell with its progeny. The intensity of red color depends on transcriptional response and the gray rectangle shows a period of pheromone induction. Reproduced with permission.[97] Copyright 2013, National Academy of Sciences.

Table 1

Comparison between the microfluidic platforms for yeast replicative lifespan assay.

Trapping mechanism	Height of trapping structure [μm]	Gap or opening size for daughter cell removal [μm]	Medium flow rate	Monitoring time [hours]	Number of strains tracked in a device	Retention rate	Selection of virgin cells	Reference
Cell surface labeling & glass modification	20 (main channel) 4 (trapping chamber)	4	30 μm s ⁻¹	50	8	Not reported	No	[40]
Pensile columns	Not reported (Three layer structure)	4	Not reported	41	4	Not reported	No	[41]
Micropads	15 (main channel) 100 (side channel) 4 (micropad)	4	1–3 μL min ⁻¹	Not reported	1	≈15–30%	No	[42]
Elongated cavities with enclosed tips	40 (main channel) 3.3 (cavity)	3.3	25 μL min ⁻¹	130	1	≈65%	Yes	[43]
V-shaped traps	6 (channel, trap)	3	4 μL min ⁻¹	67	1	≈40–60%	No	[44]
Cup-shaped traps	5 (channel, trap)	3	5–10 μL min ⁻¹	96	16	≈92%	No	[45]
Three bars in triangular-shape	5.3 (channel, trap)	3.2	2 μL min ⁻¹	91	10	Not reported	Yes	[46]