Journal of Visualized Experiments

High-throughput live imaging of microcolonies to measure heterogeneity in growth and gene expression

--Manuscript Draft--

Article Type:	Invited Methods Collection - Author Produced Video
Manuscript Number:	JoVE62038R2
Full Title:	High-throughput live imaging of microcolonies to measure heterogeneity in growth and gene expression
Corresponding Author:	Mark Siegal UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	mark.siegal@nyu.edu
Order of Authors:	Federica Sartori
	Cassandra Buzby
	Yevgeniy Plavskin
	Mark Siegal
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)
Please specify the section of the submitted manuscript.	Biology
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)

TITLE:

High-throughput live imaging of microcolonies to measure heterogeneity in growth and gene expression

AUTHORS:

Federica M. O. Sartori, Cassandra Buzby, Yevgeniy Plavskin, Mark L. Siegal

Center for Genomics and Systems Biology, Department of Biology, New York University, NY, NY

federica.sartori@nyu.edu
cb4097@nyu.edu
eugene.plavskin@nyu.edu
mark.siegal@nyu.edu

Keywords:

growth rate, *Saccharomyces cerevisiae*, nongenetic heterogeneity, high-throughput microscopy, natural variation

Summary:

Yeast growth phenotypes are precisely measured through highly parallel time-lapse imaging of immobilized cells growing into microcolonies. Simultaneously, stress tolerance, protein expression, and protein localization can be monitored, generating integrated datasets to study how environmental and genetic differences, as well as gene-expression heterogeneity among isogenic cells, modulate growth.

Abstract:

Precise measurements of between- and within-strain heterogeneity in microbial growth rates are essential for understanding genetic and environmental inputs into stress tolerance, pathogenicity, and other key components of fitness. This manuscript describes a microscope-based assay that tracks approximately 10^5 *Saccharomyces cerevisiae* microcolonies per experiment. After automated time-lapse imaging of yeast immobilized in a multiwell plate, microcolony growth rates are easily analyzed with custom image-analysis software. For each microcolony, expression and localization of fluorescent proteins and survival of acute stress can also be monitored. This assay allows precise estimation of strains' average growth rates, as well as comprehensive measurement of heterogeneity in growth, gene expression, and stress tolerance within clonal populations.

Introduction:

Growth phenotypes contribute critically to yeast fitness. Natural selection can efficiently distinguish between lineages with growth rates differing by the inverse of the effective population size, which can exceed 10^8 individuals[1]. Furthermore, variability of growth rates among individuals within a population is an evolutionarily relevant parameter, as it can serve as the basis for survival strategies such as bet hedging[2–6]. Therefore, assays that allow for highly accurate measurements of growth phenotypes and their distributions are pivotal for the study

of microorganisms. The microcolony growth assay described here can generate individual growth-rate measurements for ~10^5 microcolonies per experiment. This assay therefore provides a powerful protocol to study yeast evolutionary genetics and genomics. It lends itself particularly well to testing how variability within populations of genetically identical single cells is generated, maintained, and contributes to population fitness[7–10].

The method described here (**Figure 1**) uses periodically captured, low-magnification brightfield images of cells growing in liquid media on a 96- or 384-well glass-bottom plate to track growth into microcolonies. The cells adhere to the lectin concanavalin A, which coats the bottom of the microscope plate, and form two-dimensional colonies. Because the microcolonies grow in a monolayer, microcolony area is highly correlated with cell number[7]. Therefore, accurate estimates of microcolony growth rate and lag time can be generated with custom image-analysis software that tracks the rate of change of the area of each microcolony. Furthermore, the experimental setup can monitor the abundances and even the subcellular localizations of fluorescently labeled proteins expressed in these microcolonies. Downstream processing of data from this microcolony growth assay can be achieved by custom analysis or by existing image-analysis software, such as Processing Images Easily (PIE)[11], an algorithm for robust colony area recognition and high-throughput growth analysis from low-magnification, brightfield images, which is available via GitHub[12].

[Place Figure 1 Here]

Because growth-rate estimates derived from the microcolony-growth assay are generated from a large number of single-colony measurements, they are extremely accurate, with standard errors several orders of magnitude smaller than the estimates themselves for a reasonably sized experiment. Therefore, the power of the assay to detect growth-rate differences between different genotypes, treatments, or environmental conditions is high. The multiwell-plate format allows numerous different environment and genotype combinations to be compared in a single experiment. If strains constitutively express different fluorescent markers, they may be mixed in the same well and distinguished by subsequent image analysis, which could increase power further by allowing well-by-well data normalization.

Protocol

1. Preparation of Randomized Plates (Prior to Experiment Day)

1.1. Plan the strains and conditions to be tested with the growth assay. At this point, randomly assign strains and conditions to any well.

NOTE: When considering plate setup, it is advisable to include more than one replicate per strain and growth condition on a single plate to account for well-related noise in measurements. See Discussion for more details.

1.2. Computationally randomize the location of each strain and environmental condition for plate replicates that will be run on different days.

1.3. Grow all cells that will be used in the experiment to saturation in yeast extract-peptone-dextrose (YEPD; 2% glucose) medium in a shaker at 30 °C (or any other appropriate temperature).

1.4. Create the randomized stock plates either manually or with a liquid-handling robot. Add 10 µL of the designated saturated cells to each well of a sterile U-bottom tissue culture plate. If multiple strains will be tested in a single well, do not combine them at this point; this combining will be done just prior to cell dilutions on the day of the experiment to ensure all strains are at the correct concentrations when plated as microcolony founder cells.

1.5. Add 10 µL of 30% glycerol to each well of each plate. Pipet up and down so that the cells and the glycerol become well mixed.

1.6. Seal each plate with a foil cover and freeze down immediately at -70 °C until ready to use.

NOTE: It is important to create all randomized plates on the same day, and freeze them, so that the pre-growth conditions of the cells in each plate will have been identical and will not generate technical variation in the growth-rate assay.

2. Pre-Growth of Yeast

NOTE: Typically, this starts prior to experiment day and is highly dependent on the experimental question. See Discussion for details.

2.1. Remove a stock plate (10 µL yeast, 10 µL glycerol per well) from the -70 °C freezer and add 180 µL of the media to be used for the experiment. If the experiment will be conducted using nutrient-limiting media, do not pre-grow yeast to saturation in the nutrient-limiting media as sporulation of yeast can occur. Instead, pre-grow in non-limiting media.

2.2. Grow yeast while shaking at 30 °C. Consider whether to run the assay starting with cells in log phase or in stationary phase to determine if diluting the cells multiple times prior to the experiment will be necessary. If the yeast strains or conditions in the assay are expected to have significantly different growth rates, then a two-day pre-growth period will be necessary in order for all the different conditions to reach stationary phase.

3. Microscope Setup

3.1. Microscope plate preparation

3.1.1. Ensure that the microscope incubator is on and heating the microscope chamber to the desired growth temperature for the experimental conditions. For standard experiments using

Saccharomyces cerevisiae cells, the incubator should heat the microscope chamber to 30 °C to ensure that the growth conditions for the cells will be correct during the growth rate assay.

3.1.2. Sanitize the workbench, pipettes, and other tools with 70% ethanol. Retrieve a microscope plate and place it on the bench on top of a lint- and static-free wipe.

NOTE: Never touch the bottom of the microscope plate, even with gloves on, and always set the microscope plate down on top of a lint- and static-free wipe any time it touches any surface. This prevents smudges or scratches from impeding growth-rate measurements once the experiment is being imaged.

3.1.3. Thaw 5 mL of 5x concanavalin A solution, dilute to 1x with water, and filter sterilize through a syringe fitted with a 0.2-µm filter.

3.1.4. Filter sterilize all other liquids that will be used in the assay with a 0.2 µm filter, including experimental media, to remove any crystals or debris that may have materialized in the solutions. The presence of crystals would reduce the quality of the microscopy images.

3.1.5. Pipet 200 µL of concanavalin A solution into each well of the microscope plate.

3.1.6. Centrifuge the plate for 2 min at 411 x gravity (*g*) with a lint- and static-free wipe under the plate, to ensure that the concanavalin A solution evenly covers the bottom of each well and that there are no air bubbles.

3.1.7. Cover the plate with its lid and let it sit for 1–2 h. The precise time the plate sits is flexible, but it is important to be consistent between different runs of the experiment.

3.1.8. Remove all of the concanavalin A solution from the plate either by suction or by forcefully discharging it out into the sink or a receptacle. Be careful not to touch the glass part of the plate. It is acceptable if some drops of concanavalin A solution remain in the wells.

3.1.9. Wash the microscope plate wells by adding 400 µL of sterile water. Remove the water as done with the concanavalin A in the previous step**. Do not let the plate sit dry.**

3.1.10. Immediately add 185 µL experimental growth media into the plate. 15 µL of **correctly diluted** cells will be added to this plate.

3.2. Yeast Cell Dilution

NOTE**:** The steps below describe a dilution of yeast from a saturated culture (approximately 10^8 cells/mL) 400-fold to achieve a concentration of 250,000 cells/mL, 15 µL of which will be diluted into 400 µL in the glass-bottom plate, giving a final number of approximately 4000 cells per well in a 96-well plate. If using a 384-well plate the final number of cells per well should be approximately 700 and the dilutions should be adjusted accordingly. This ratio should be adjusted

for cells collected in log phase, growing in richer or poorer pre-growth media, or from different strains. The final density of cells per well should be reduced when running the growth rate assay for time periods longer than 10 h.

3.2.1. Set up two 96-well culture plates for serial dilutions: label as plate 1 and 2, and add 90 µL experimental growth media (i.e., the media that the yeast will grow in on the microscope) to each serial dilution plate.

NOTE: Regardless of what final dilution is used, at least two serial dilutions of cells are recommended, in each of which a small volume of yeast is pipetted into a bigger volume of experimental media and then a large volume of experimental media is mixed in vigorously with a pipet (as in steps 3.2.5 and 3.2.6 below).

3.2.2. Retrieve the plate of cells from pre-growth and centrifuge the plate for 2 min at 411 x *g*.
NOTE: It is very important not to cross-contaminate different wells in the plate. The purpose of this centrifugation step before removing the foil covering from plates is to ensure that yeast-filled droplets from one well do not fly off the foil and end up in other wells. Be careful never to tilt or agitate the plates to avoid yeast coming into contact with the foil covering after centrifugation.

3.2.3. Carefully peel back the foil and resuspend cells by vigorously pipetting cells with a pipet set to approximately one-half the total volume in the plate while moving the pipet around the well to mix. Check that all cells have been resuspended from the bottom of the wells.

3.2.4. If multiple strains within individual wells will be used, strains should be mixed at this time at the ratio necessary for the experiment. If a reference strain will be used to generate growth-rate measurements, the ratio of reference to test strain should be 1:1.

3.2.5. Pipet 10 µL of yeast from growth media into dilution plate 1. Add 100 µL of experimental growth media to each well to a final volume of 200 µL per well. Pipet up and down vigorously.

3.2.6. Pipet 10 µL of yeast from plate 1 into plate 2. Add 100 µL of experimental growth media to each well, and pipet up and down vigorously to mix.

NOTE: These dilution steps are critical to help separate clusters of yeast that are stuck together at the end of the pre-growth stage and ensure that approximately equal numbers of yeast cells end up in each well. Having consistent numbers of yeast in each well helps remove experimental noise and biases in growth-rate measurements (see Representative Results).

3.3. Sonication

NOTE: **Sonication is optional**, and only needs to be performed for yeast strains that have a high propensity to adhere to one another (e.g., some wild strains). For lab strains, sonication is generally not necessary and may be skipped by proceeding to step 3.4.

3.3.1. Sanitize a 96-pin sonicator head with 70% ethanol by placing it in a 96-well plate filled with 70% ethanol and dry with a lint- and static-free wipe.

3.3.2. Set a sonication program that is sufficiently strong to break apart flocculated yeast cells, but does not kill cells or cause elevated stress responses. Some testing may be required to identify the best sonication program for a given experiment. The sonication program used in this experiment is: amplitude = 10, process time = 10 s, pulse-on = 1 s, pulse-off = 1 s. This exact program is likely not applicable to all sonicators so testing is suggested prior to the experiment day.

3.3.3. Mix the yeast in serial dilution plate 2 once more by pipetting up and down vigorously five times.

3.3.4. Place dilution plate 2 on the platform and secure it with the sonicator pins in the cell suspension but not touching the bottom of the plate. Run the sonication program using appropriate ear protection.

3.3.5. After the program runs, clean the sonicator head with 70% ethanol and then with water, and then **immediately** proceed to microscope plate preparation so that the cells do not flocculate again.

3.4. Prepare Plate for Microscope:

3.4.1. Pipet 15 µL of yeast from serial dilution plate 2 into the microscope plate to a volume of 200 µL. Add 200 µL of experimental growth media to each well to a final volume of 400 µL per well, and pipet up and down vigorously to mix.

3.4.2. Cover the plate with a breathable membrane. It is important to seal the plate well with this membrane, for example using a rubber roller.

3.4.3. To adhere the yeast cells to the concanavalin A on the glass surface, centrifuge the plate with a lint- and static-free wipe beneath it for 2 min at 411 x *g*.

3.4.4. At the microscope, wipe the top and bottom of the plate with a lint- and static-free wipe, and blow compressed air onto the plate to get rid of debris.

3.4.5. Place the plate onto the microscope, making sure it is level and that the A1 well is in the top left corner.

4. Time-lapse Microscopy Growth-rate Measurements

NOTE: During time-lapse microscopy the following features are computer controlled: x, y, and z position, shutters, and fluorescence filters. A hardware-based auto-focus system is optimal to

prevent focal plane drift during time-lapse imaging. Alternatively, a software-based auto-focusing loop can be used. To maintain humidity in the microscope chamber, it is advised to keep a beaker with purified water in the chamber throughout the duration of the experiment.

4.1. Create a list of positions (x,y) to image, so that each microscope-plate well is fully imaged. Avoid overlapping images so that no cell is analyzed multiple times.

4.2. Image in brightfield with diascopic illumination (DIA) at a magnification of 15x. Set exposure to ~5 ms.

4.3. Zoom in on the image digitally so that cells are clearly visible. Use the focusing knobs to identify the ideal focus for the experiment in the four wells on the corner of the plate and in one well at the center of the plate. Focus in such a way as to get maximum contrast of the cells.

4.4. Set the z position (or autofocus position) for the experiment to be an average of the z/autofocus positions identified for each of these wells. If the microscope plate is well made and the glass bottom does not have defects, the ideal focus positions should be similar for each well.

NOTE: When analyzing images with the Processing Images Easily (PIE) image analysis pipeline[11, 12], it is helpful for the cells to be slightly out of focus on the microscope so that there is a dark rim outside of the cell and a light-colored interior, which aids in accurate colony recognition and size estimates.

4.5. If using fluorescent strains, identify the channels and the exposures with which to image, ensuring that no pixels are overexposed. When setting exposure time for fluorescent channels, turn off the "live capture" mode on the microscope to avoid exposing cells to fluorescence excitation for long periods of time, as this can both photobleach the cells and cause stress.

4.6. Set up the time-sequence acquisition to capture images at the desired time interval for the desired length of time.

4.7. Run the experiment.

Representative Results

The novelty of this protocol is that growth rate can be calculated for individual cells within a population by tracking their growth into microcolonies through time-lapse imaging (**Figure 2A**). Because microcolonies grow for many hours in a planar manner due to the presence of concanavalin A, their areas can be tracked throughout the experiment, and a linear fit to the change in the natural log of the area over time can be used to calculate growth rate for each individual colony observed [7, 9, 10, 13]. Differences in microcolony growth rate for individual isogenic cells in the same environment are clearly recorded (**Figures 2A, 2B**). Image-analysis software should be used to automatically track changes in microcolony size and fluorescence (**Figure 2C**); the colony tracking shown in **Figure 2A** was done using PIE[11, 12].

[Place Figure 2 here]

Typically, ~10^5 microcolony growth rates per experiment can be collected using this assay. These data can be used to observe both differences between strains/growth conditions, and variation across genetically identical microcolonies grown in a shared condition. For example, Figure 3A shows the distributions of growth rates for ~12,000 colonies from a mutation-accumulation line (MAH.44)[14] and GFP-marked reference strain grown in the same wells, with both differences between the strains and high within-strain variability visible. In Figure 3B, individual growth rates and summary statistics for 10 mutation-accumulation strains, paired with their in-well GFP-marked controls, are shown; the collected data allow precise calculation of small average growth-rate differences.

[Place Figure 3 here]

The microcolony growth assay can also be used to simultaneously measure growth and gene expression by imaging both brightfield and fluorescence channels. In the experiment shown in Figure 2, fluorescent imaging of GFP expression after the completion of the growth phase of the experiment allowed identification of colonies belonging to a GFP-marked in-well reference strain with weak GFP expression (Figure 2C); however, measurement of intercolony differences in gene expression levels across timepoints is also possible (see Discussion).

A number of common pitfalls can prevent collection of accurate growth-rate data or the correct analysis of these data. One key point is that growth-rate measurements rely on the formation of immobile, two-dimensional microcolonies from single founder yeast cells. Concanavalin A interacts noncovalently with polysaccharides on the surfaces of yeast cells to immobilize microcolonies. The bond between concanavalin A and yeast cells can be reversed by competition with sugars or by low pH[15]. Therefore, strongly acidic media or media containing lectin-binding components such as yeast extract (YEPD) or phthalate (Edinburgh Minimal Media), cannot be used for this assay without modifications to the immobilization technique (**Figure 4A**).

If the growth assay is being conducted with yeast strains that flocculate, the optional sonication step should be used to break apart aggregated cells so single cells are immobilized on the microscope plate at the beginning of the experiment (see **Figure 4B** for an example of flocculating cells post-plating if the sonication step is omitted). Any microcolonies that are founded by a cluster of multiple cells should be excluded in downstream analysis, as growth-rate measurements are no longer derived from a single founder cell, and cells may not be growing in two dimensions. Microcolonies that are founded by a budding cell are admissible. The formation of two-dimensional microcolonies is impeded by yeast that flocculate very strongly, even if colonies are founded by a single cell, and therefore the ability of a yeast strain to grow in a single layer on concanavalin A should be tested before conducting a growth assay.

Another important set of considerations comes during both the planning and analysis stages of the experiment, when determining how many timepoints to include in analysis. First, it is important to include data for enough timepoints across a sufficient period of time to accurately

track growth: it is recommended that assays are run in such a way that yeast have time to go through ~5 doublings, with at least 10 timepoints collected over that period. However, simply including every collected timepoint in the growth-rate calculation will result in a bias that artificially lowers growth rate for many colonies. This bias can occur when cells go through a lag phase before beginning growth (**Figure 3C**). Pre-growth lag of microcolonies is common and varies between experimental conditions and strains[9, 13]. Experimental analysis methods must be able to differentiate timepoints in the "active growth" period from pre-growth lag; one approach is to use a pre-set number of timepoints in a window and find the window that corresponds to the highest growth rate (**Figure 4D**, solid line)[11, 13].

Finally, one of the most critical steps of the microcolony growth assay is the yeast-cell dilution step. The concentration of cells and the ratio of different genotypes within microscope-plate wells must be carefully controlled. For statistical analysis it is important that experiments be balanced such that approximately equal numbers of cells for each genotype and condition are tested and compared[16]. In addition, useful growth rates typically cannot be measured after neighboring colonies merge because the growth rates of the individual colonies can no longer be discerned; therefore, more densely plated cells will yield growth data from fewer timepoints (Figure 4D). Importantly, if cell density is high or uneven at the start of an experiment, filtering out merged microcolonies will disproportionately exclude faster growing microcolonies from downstream analyses because fast growers will merge more frequently than slow growers. Therefore, final growth measurements will be biased towards slower-growing sub-populations. Additionally, different numbers of colonies may be filtered out for different treatments, precluding a balanced experiment. It is recommended to plate around 4000 cells per well in a 96-well plate (or 700 cells per well in a 384-well plate). **Thorough pipet mixing throughout the yeast-dilution portion of the protocol is imperative to ensure that the correct number of cells is present in each well, and that cells are evenly dispersed throughout the well.** It is also advisable to remove any microcolonies from analysis whose centers are within ~10 cell diameters of each other.

[Place Figure 4 here]

Figure 1: Schematic representation of the protocol. This protocol follows two main steps, which are the preparation of the experimental plate and the preparation of the cells to image. Randomization of plates and growth of cells should be conducted before and leading up to the experiment day. Repeated mixing of cells at each step during dilution is imperative in the steps until plating, and therefore preparing the experimental plate first is recommended so that it is ready for plating immediately upon the completion of cell dilution.

Figure 2: Quantification of growth rate and fluorescence in yeast microcolonies
(A) A portion of a single imaging field showing microcolonies growing at the start of the experiment, after 3 h, and after 6 h, showing colony tracking using the PIE colony tracking software. Colonies are visibly growing in a monolayer for the duration of the experiment. (B) Change in ln(area) over time for the tracked colonies in panel A. If sufficient timepoint data exist for a colony, its growth rate and pre-growth lag time can be calculated. (C) Image showing GFP

fluorescence intensity for the colonies in panel A, taken after the growth portion of the experiment is complete, with the colony outlines from the 6-h timepoint overlaid. Here, Scw11P::GFP marks a reference strain included in every experimental well. Calculating the GFP fluorescence level of each colony allows determination of which colonies originate from the reference strain, and which from the non-reference strains in each well. Each scale bar is 50 μm.

Figure 3. Large sample sizes of individual microcolony growth rates allow for precise quantification of within-strain and between-strain growth variation

(A) The distributions of growth rates of ~12,000 colonies from two strains. Note differences between the modes of the distributions, as well as the long tail of slow-growing colonies present in each one; the latter is only detectable due to individual microcolony measurements.

(B) Distributions of individual microcolony growth rates (black dots) and summary statistics (boxplots showing median, as well as lower and upper 25% quantiles) for ~120,000 colonies from 11 strains. As in (A), each strain was grown in an individual well with a spiked-in GFP-marked reference strain; data shown here represent 14 experimental wells per MAH-reference strain pair.

Figure 4. Experimental pitfalls

(A) Colonies growing in YEPD media, which prevents efficient binding of cells to concanavalin A. The appearance of new cells far from any colony (arrowheads), as well as the appearance of many out-of-focus cells at the edge of the colony, are the result of cells failing to adhere to the glass surface and drifting away from the colony during growth. (B) Cells from a flocculating strain right after plating without sonication; notice the presence of large numbers of clusters of multiple cells (arrowheads), and cells within the clusters in different focal planes. (C) Change in ln(area) over time for a colony with a long lag phase. Note that if all timepoints are used for growth-rate estimation, the estimated growth rate is significantly depressed; an accurate measurement is produced only when the subset of *n* timepoints (here *n*=6) that results in the highest growth rate estimate is used. (D) Cells that were plated too densely shown at the start of the experiment and after 7 h of growth. Colors track individual colonies until they merge with neighbors; here, by the 7-h mark, the majority of colonies have merged, with only a small number of individual slow-growing colonies remaining. (Tracking for a small subset of the colonies shown here is lost for reasons unrelated to colony merging.) Each scale bar is 50 μm.

Discussion:

The protocol described here is a versatile assay that allows cell growth and gene expression to be monitored simultaneously at the level of individual microcolonies. Combining these two modalities yields unique biological insights. For example, previous work has used this assay to show a negative correlation between expression of the *TSL1* gene and microcolony growth rate in isogenic wildtype cells by measuring both simultaneously[7, 10]. It is also possible to monitor the relationship between growth rate and subcellular localization dynamics of fluorescently tagged proteins with the described assay. For example, a negative relationship between growth rate and the nuclear occupancy of RFP-tagged transcription factor Msn2 was identified by imaging fluorescence in cells every minute for 30 min before initializing the growth assay[10]. Finally, this protocol allows monitoring of cell responses to environmental stresses and perturbations.

Treatments such as heat shock can be administered part-way through the growth assay. Such studies have revealed, for example, that slow-growing cells expressing high levels of Tsl1 are more tolerant of heat shock [7, 10].

In addition to the common pitfalls described in the Representative Results section (Fig 4), several key factors must be considered when designing a growth-assay experiment. The phenotypic variation that is measured with the assay is affected by multiple factors, some of which are repeatable and of inherent interest, such as genotype or environment, whereas others are the result of technical variation[13]. Therefore, the first important consideration when designing a microcolony assay is to carry out the experiment in a way that facilitates the separation of effects of interest from the effects resulting from technical variation; key to this separation are randomizing treatments or strains on experimental plates, and including controls in every experiment. Appropriate statistical methods, such as linear mixed modeling (LMM), must be applied to data after they are collected, to account for different sources of variation in downstream analysis[17, 18].

In order to employ statistical methods to separate variation in growth phenotypes due to experimental variables of interest from variation due to random factors such as batch effects, it is necessary to run multiple experiments on different days and with randomized well locations of genotype and growth conditions. In addition, to increase the power of the experiment to detect growth differences between strains or treatment conditions, it is also important to include multiple replicates of each tested genotype or growth condition, on a single experimental plate where possible.

One key advantage of the microcolony growth assay lies in the amount of data it can collect: a single experimental plate typically generates data for ~10^5 individual microcolonies, which is several orders of magnitude greater than typical experiments using microfluidic devices instead of multiwell plates. Software that automatically tracks colonies and calculates relevant data (e.g. lag times, growth rates, and mean fluorescent intensities) is key to taking full advantage of this assay. This software should not only robustly identify colony bounds and track colonies through time, but also correctly measure growth in colonies with a lag phase[13]. One option developed for this purpose is PIE, open-source software that tracks colonies over time (including accounting for shifts in colony position), calculates growth rates, and allows integration of fluorescent imaging data into experimental measurements[11, 12].

The microcolony growth assay can be used to gain insight into fundamental questions relating to yeast fitness and evolution, including growth phenotype variability, changes in different environments or in response to stress, and the relationship between growth and protein expression or subcellular localization. The assay has been used extensively in studies of both laboratory and wild strains of *S. cerevisiae*[7, 9, 10, 13].

Disclosures

The authors have nothing to disclose.

Acknowledgements

We thank Naomi Ziv, Sasha Levy and Shuang Li for their contributions to developing this protocol, David Gresham for shared equipment, and Marissa Knoll for help with video production. This work was supported by National Institutes of Health grant R35GM118170.

References:

1. Geiler-Samerotte, K.A., Hashimoto, T., Dion, M.F., Budnik, B.A., Airoldi, E.M., Drummond, D.A. Quantifying condition-dependent intracellular protein levels enables high-precision fitness estimates. *PloS one*. **8** (9), e75320 (2013).
2. Kussell, E., Leibler, S. Phenotypic diversity, population growth, and information in fluctuating environments. *Science*. **309** (5743), 2075–2078 (2005).
3. Thattai, M., van Oudenaarden, A. Stochastic gene expression in fluctuating environments. *Genetics*. **167** (1), 523–530 (2004).
4. King, O.D., Masel, J. The evolution of bet-hedging adaptations to rare scenarios. *Theoretical population biology*. **72** (4), 560–575 (2007).
5. Acar, M., Mettetal, J.T., van Oudenaarden, A. Stochastic switching as a survival strategy in fluctuating environments. *Nature genetics*. **40** (4), 471–475 (2008).
6. Avery, S.V. Microbial cell individuality and the underlying sources of heterogeneity. *Nature reviews. Microbiology*. **4** (8), 577–587 (2006).
7. Levy, S.F., Ziv, N., Siegal, M.L. Bet hedging in yeast by heterogeneous, age-correlated expression of a stress protectant. *PLoS biology*. **10** (5), e1001325 (2012).
8. van Dijk, D. *et al.* Slow-growing cells within isogenic populations have increased RNA polymerase error rates and DNA damage. *Nature communications*. **6**, 7972 (2015).
9. Ziv, N., Shuster, B.M., Siegal, M.L., Gresham, D. Resolving the Complex Genetic Basis of Phenotypic Variation and Variability of Cellular Growth. *Genetics*. **206** (3), 1645–1657 (2017).
10. Li, S., Giardina, D.M., Siegal, M.L. Control of nongenetic heterogeneity in growth rate and stress tolerance of Saccharomyces cerevisiae by cyclic AMP-regulated transcription factors. *PLoS genetics*. **14** (11), e1007744 (2018).
11. Plavskin, Y., Li, S., Ziv, N., Levy, S.F., Siegal, M.L. Robust colony recognition for high-throughput growth analysis from suboptimal low-magnification brightfield micrographs. doi: 10.1101/253724.
12. https://github.com/Siegallab/PIE
13. Ziv, N., Siegal, M.L., Gresham, D. Genetic and nongenetic determinants of cell growth variation assessed by high-throughput microscopy. *Molecular biology and evolution*. **30** (12), 2568–2578 (2013).
14. Hall, D.W., Joseph, S.B. A high frequency of beneficial mutations across multiple fitness components in Saccharomyces cerevisiae. *Genetics*. **185** (4), 1397–1409 (2010).
15. Saleemuddin, M., Husain, Q. Concanavalin A: a useful ligand for glycoenzyme immobilization--a review. *Enzyme and microbial technology*. **13** (4), 290–295 (1991).
16. Geiler-Samerotte, K.A., Bauer, C.R., Li, S., Ziv, N., Gresham, D., Siegal, M.L. The details in the distributions: why and how to study phenotypic variability. *Current opinion in biotechnology*. **24** (4), 752–759 (2013).
17. Nakagawa, S., Schielzeth, H. Repeatability for Gaussian and non-Gaussian data: a practical guide for biologists. *Biological reviews of the Cambridge Philosophical Society*. **85** (4), 935–956

(2010).
18. Bolker, J.A. Exemplary and surrogate models: two modes of representation in biology. *Perspectives in biology and medicine*. **52** (4), 485–499 (2009).

Figure 1

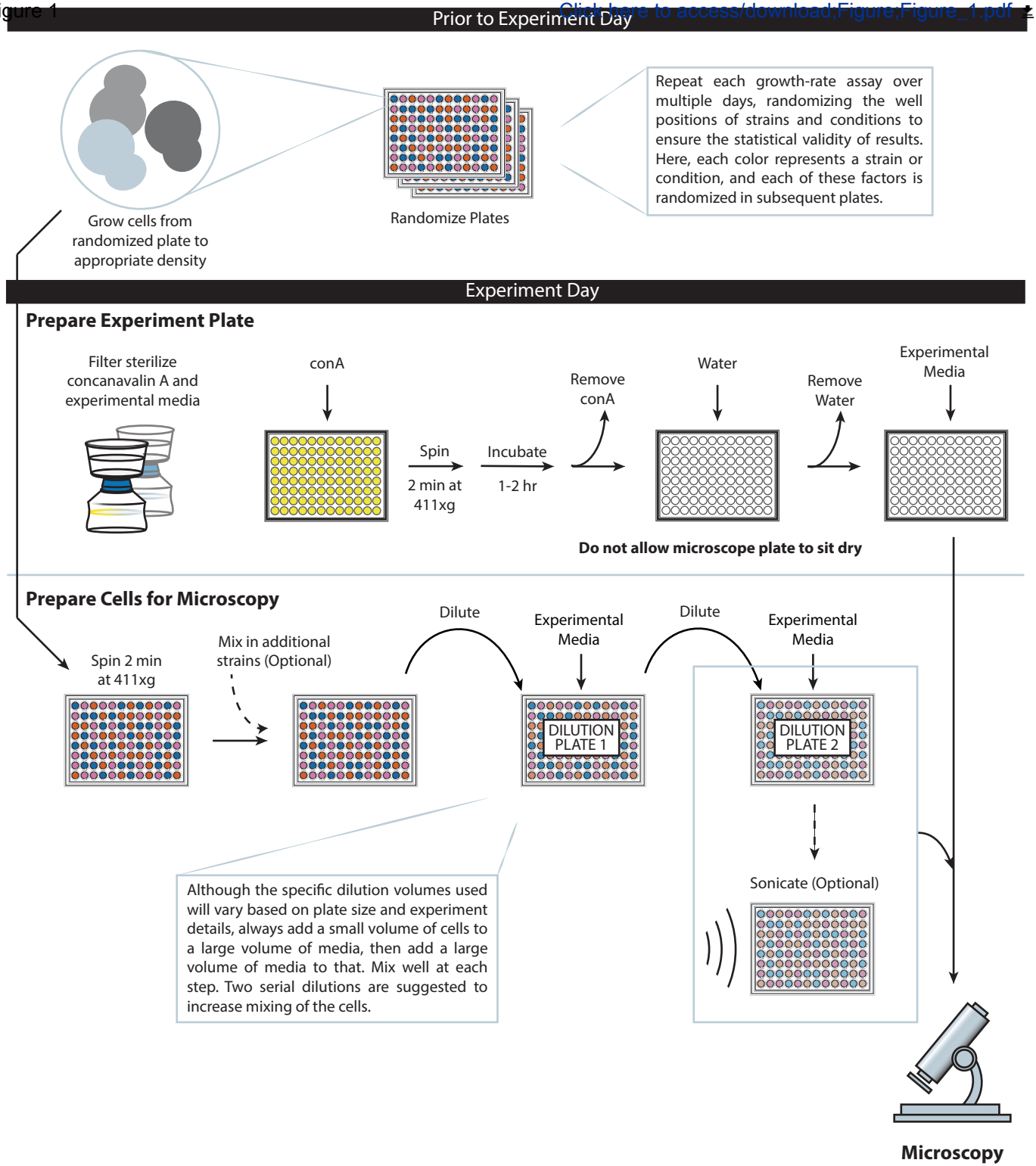
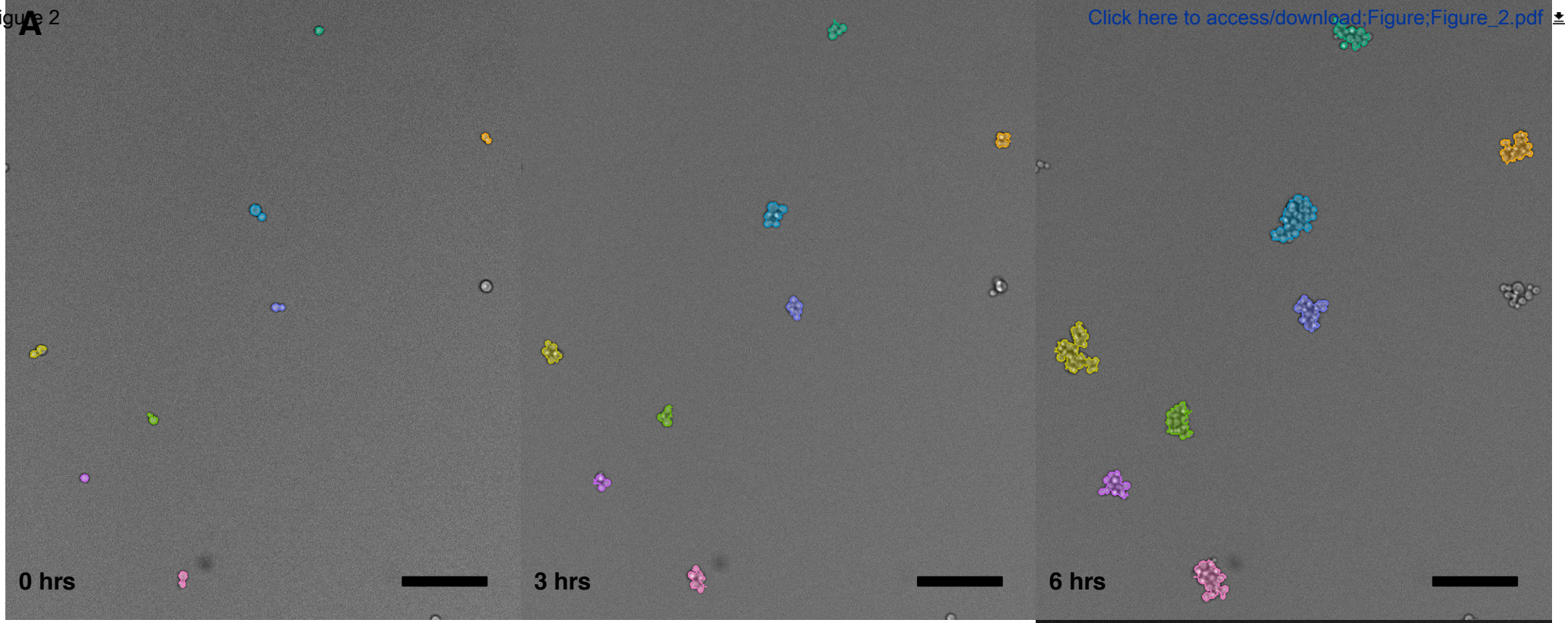
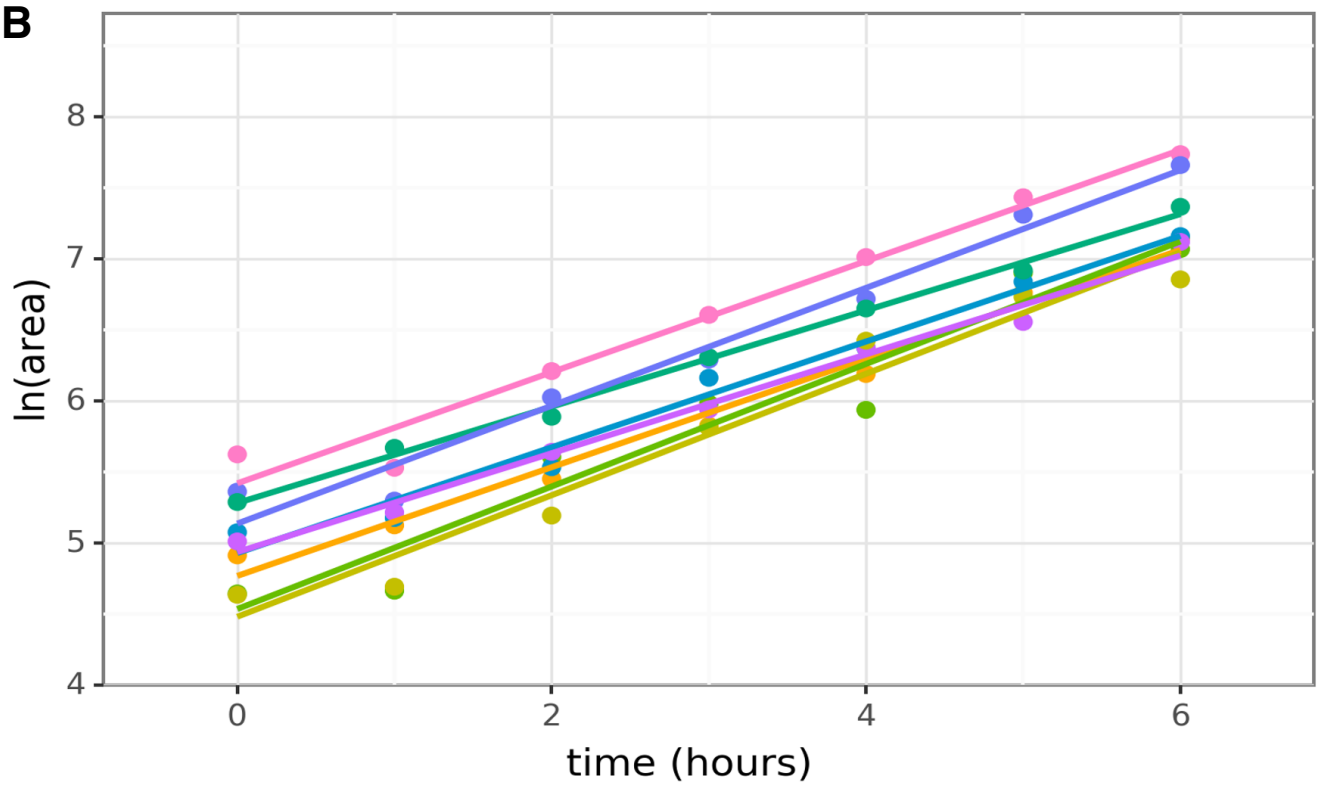


Figure 2

Click here to access/download:Figure;Figure_2.pdf





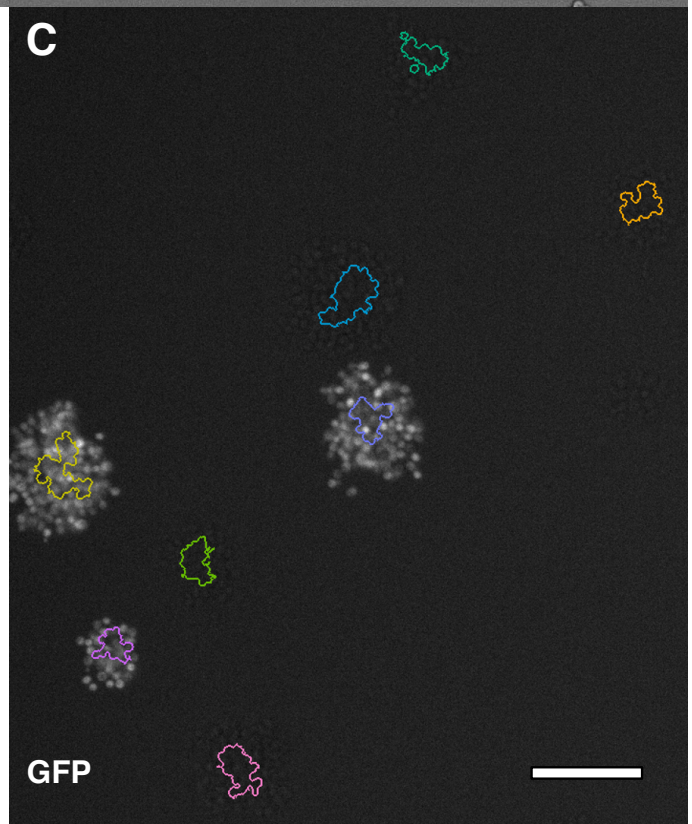


Figure 3

Click here to access/download;Figure;Figure_3.pdf

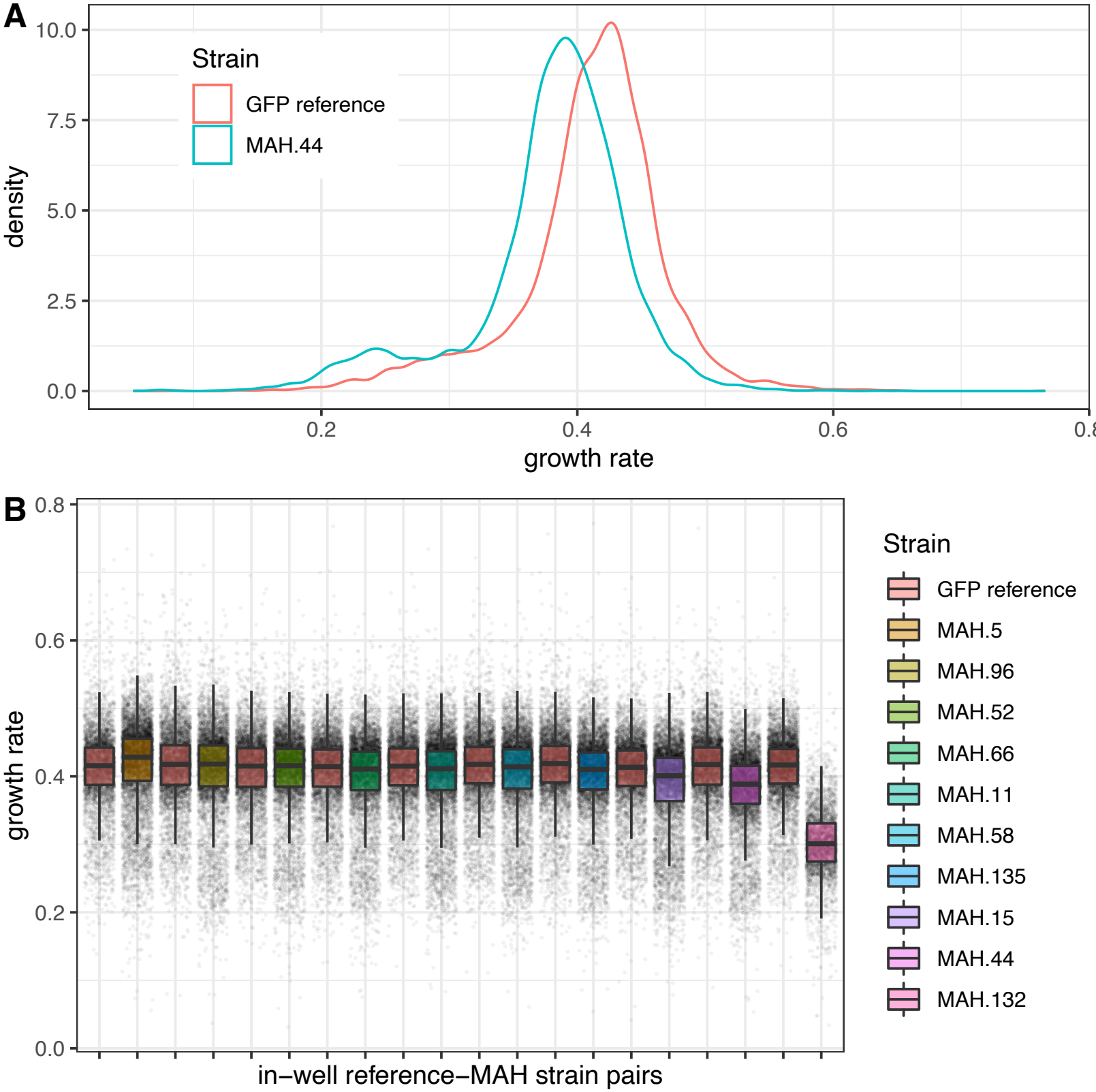


Figure 4

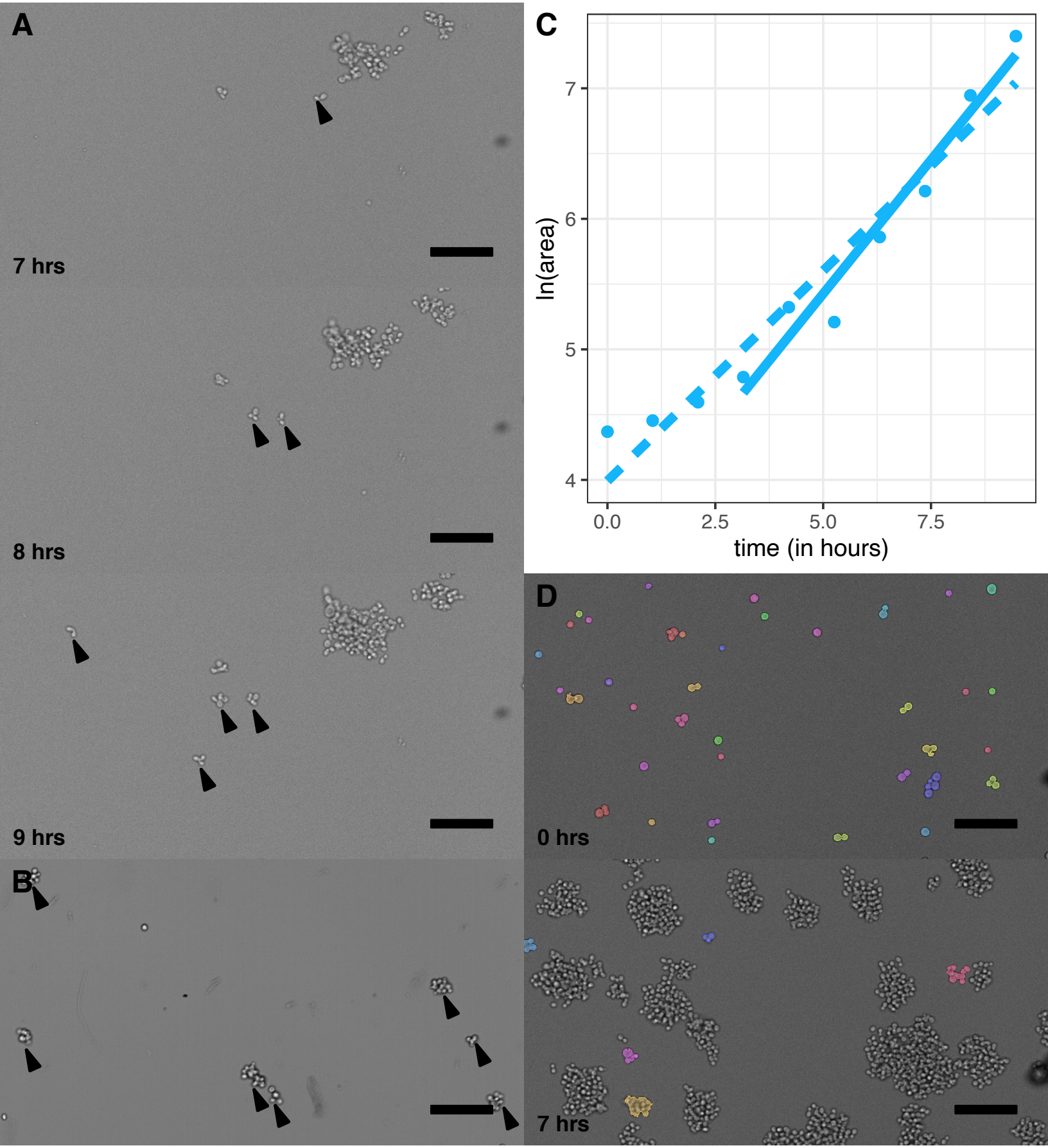


Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
General Materials			
500 mL Bottletop Filter .22 µm PES	Fisher	CLS431154	used to filter the media
BD Falcon*Tissue Culture Plates, microtest u-bottom	Fisher	08-772-54	96-well culture tubes used to freeze cells, pre-grow cells, and dilutions
BD Syringes without Needle, 50 mL	Fisher	13-689-8	Used to filter the Concanavalin A
Costar Sterile Disposable Reagent Reservoirs	Fisher	07-200-127	reagent reservoirs used to pipette solutions with multichannel pipette
Costar Thermowell Aluminum Sealing Tape	Fisher	07-200-684	96-well plate seal for pre-growth and freezing
lint and static free Kimwipes	Fisher	06-666A	lint and static free wipes to keep microscope plate bottom free of debris and scratches
Nalgene Syringe Filters	ThermoFisher Scientific	199-2020	0.2 µm pore size, 25 mm diameter; used to filter concanavalin A solution
Media Components			
Minimal chemically defined media			alternative microscopy media used for yeast pre-
Synthetic Complete Media (SC; 2%			microscopy media used for yeast pre-growth and
Yeast extract-peptone-dextrose (YEPD; 2% glucose) medium			cell growth prior to freezing down randomized plates
Microscopy Materials			
Breathe-Easy sealing membrane	Millipore Sigma	Z380059-1PAK	breathable membranes used to seal plate during
Brooks 96-well flat clear glass bottom	Dot Scientific	MGB096-1-2-LG-L	microscope plate
Concanavalin A from canavalia	Millipore Sigma	45-C2010-1G	Make 5x concanavalin A solution and freeze 5ml of
Strains Used			
MAH.5, MAH.96, MAH.52, MAH.66, EP026.2A-2C			Haploid mutation accumulation strains in a Progeny of the ancestral Hall and Joseph 2010 mutation accumulation strain, transformed with YFR054cΔ::Scw11P::GFP
Equipment			
Misonix Sonicator S-4000 with 96-pin			Sonicator https://www.labx.com/item/misonix-inc-s-

Nikon Eclipse Ti-E with Perfect Focus

Inverted microscope with automated stage and

NYU COLLEGE OF ARTS & SCIENCE
Office of the Dean | College Core Curriculum
100 Washington Square E, Suite 903
New York, NY 10003
P: (212) 998-8119
F: (212) 995-4055
cas.vicedean@nyu.edu

Mark L. Siegal, Ph.D.
Professor and Vice Dean of General Education

Department of Biology
12 Waverly Place, 8th Floor
New York, NY 10003
P: (212) 998-7908
F: (212) 995-4266
mark.siegal@nyu.edu

Center for Genomics and Systems Biology
New York University

January 29, 2021

Dear Editors,

Please find attached our revised submission, “High-throughput live imaging of microcolonies to measure heterogeneity in growth and gene expression.” We thank you and the reviewers for the helpful comments, which we feel we have addressed fully in our revision and which we believe have made it a stronger contribution. We have included a point-by-point response to editorial, production and reviewer comments.

We hope you agree that the manuscript and associated video are now suitable for publication in *JoVE*, and we look forward to hearing from you.

Sincerely,

Below we provide point-by-point responses (in **bold**) to editorial, production and reviewers' comments.

Editorial and production comments:
Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done.

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

Done.

3. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Details were added in protocol step 1.3 (line 90), step 1.5 (lines 100–101), and step 3.2.6 (line 200).

4. 3.1.1: What cells are used and what temperature is used? Please provide an example.

We added this information in lines 127–129: "For standard experiments using *Saccharomyces cerevisiae* cells, the incubator should heat the microscope chamber to 30 °C to ensure..." Additionally, cells that have been used in past studies are described in line 466.

5. What happens after centrifugation throughout? Aspiration?

The purpose of centrifugation in our protocol often is to avoid cross-contamination between different wells on the same 96-well plate. Therefore, after the centrifugation step, cells are simply resuspended without any other action (such as aspiration or change of media). To make this more clear in the manuscript we edited the "NOTE" after the centrifugation step in 3.2.2 (lines 183–186), to say "The purpose of this centrifugation step is…" and we edited step 3.2.3, line 188, to state up front that resuspension of the cells is the primary goal of the mixing steps described.

Changes to be made by the Author(s) regarding the video:
1. Text
• 00:01, 14:55 Text is too close to the edge of the frame. Please allow more room between the text and the frames edge.
• 00:10, 00:40, 14:35 There's no person caption. Please add text with the person's name and affiliated university.

The suggested Text changes have been made.

2. Pacing
I noticed a lot of gaps where there’s no narration, which feels a bit much. I also noticed some shots are not in sync with the narration. By cutting out certain parts of the shots, the video doesn't need to drag on like it is without narration.

For example, we're seeing pipette tips being put on, or caps being taken off the tubes when we don't need to see that; and when the pipetting actually happens, it happens after they stop talking, instead of over the narration when it's mentioned

So by re editing these shots, the gaps would be a lot less and the video will sync with the audio much better:
01:52 - 01:56, 02:00 - 02:03, 02:50, 03:21, 04:19 - 04:32, 06:03 - 06:07, 06:13 - 06:22, 06:27 - 06:30, 06:38 - 06:42, 06:47, 06:50, 06:57, 07:01 - 07:11, 07:42 - 07:54, 08:26 - 08:31, 08:29 - 08:40, 08:37 - 08:45, 08:53 - 08:56, 08:58, 09:07 - 09:12, 11:15 - 11:19, 11:24 - 11:31, 11:46 - 11:52, 12:05 - 12:13

• 01:58 - 01:58 I would hold on this shot a bit longer. It shows up too quick.
• 02:05 - 02:05 The centrifuge shot should start at the same time the narration mentions it. Please trim the clip so it's in sync with the narration.
• 02:18 - 02:23 The shot holds on the centrifuge after it's been closed for too long.
• 03:54 - 03:54 I would suggest removing this cross dissolve and just do a straight cut.
• 04:06 - 04:06 I would recommend removing this cross dissolve and doing a straight cut. I would also make sure the following shot matches the action of closing the lid in the previous shot.
• 07:16 - 07:16 Please remove this cross dissolve and just do a straight cut.
• 07:19 - 07:19 The cross dissolve in the previous edit should go here instead.
• 08:49 - 08:49 I recommend extending this shot to cover the rest of this sentence.
• 09:18 - 09:18 Please remove cross dissolve and just do a straight cut.
• 09:19 - 09:38 Please remove this LONG gap of no narration.
• 10:03 - 10:03 The shot cuts from the person approaching the microscope, to using compressed air to clean. To avoid this awkward jump cut, I would remove the previous shot and start this shot earlier.

> **Specific changes to video transitions, videos of non-specific lab techniques (i.e. adding tips to a pipette), and large gaps in narrative have been made per suggestions. These changes often solved the video-audio sync in terms of pacing, and where they did not, further edits were made.**

3. Audio
• 05:19 - 05:19 I can hear the narrator breath in but the audio gets cut off. I would recommend cleaning this up so you can't hear any breaths.
• 05:58 - 05:58 The first word gets cut off here. Please adjust this edit so we can hear the full word being said.

The suggested Audio changes have been made.

4. Composition
• 10:38 - 10:38 For screen capture footage, I would recommend zooming in to the panels that the mouse pointer is manipulating so we can see more detail.
• 12:18 - 12:18 I really like the results sections visuals, but I would recommend leaving more space around the edge of the frame for the figures on the right.
12:40 - 12:40 Please resize the figure so it's not so close to the edge of the frame.

The suggested Composition changes have been made.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This paper shows us the protocol for high-throughput measurement of yeast growth rate and protein expression level by fluorescence in budding yeast, using the microcolony tracking and microscopy assay pioneered by the Siegal lab. In the first part, the authors provide detailed steps for setting up the experiment. In the second part, the author presents their results according to the protocol in the first part.

The manuscript is detailed and practical, especially offering many notes in the protocol, and potential pitfalls. This adds some nice additional information, in comparison to the methods sections of the group's previous papers.

Major Concerns:
none

Minor Concerns:
A few minor comments:

Section 3.2 is very wordy. While the detail is appreciated, a few sentences at the top will help orient the reader. Possibly something like:
The steps below describe a dilution of yeast from a saturated culture (10^8) to a target cell density of 10^4 cells per ml, 15ul of which will be diluted into 400ul in the glass bottom plate, giving a final population of 1000 cells per well.

The suggested change was made in lines 166–169.

Section 3.4.1
Is the final volume 400ul per well? Please state the final volume.

Yes, the final volume is 400 µL. Statements of all volumes after the addition of cells and media were included in lines 233–235 to be more clear.

Section 3.2.5 : add "final volume is 200ul per well"
These serve as a check to make sure the reader/experimenter is doing things correctly.

> **We added “to a final volume of 200 µL per well” in line 198.**

For how many generations are they really 2D?
Do you have a good idea of the number of cells, or colony area, at which time enough cells are out-of-plane that the area based microcolony growth rate is no longer reliable?

> **Although some previous observations suggested that colonies ceased to grow in two dimensions after extended imaging, this question from the reviewer prompted us to systematically examine our data for examples. We found that through 7–8 cell doublings (the largest amount of growth for which multiple colonies are recorded in our datasets), microcolonies continue to grow in a single plane, with no decrease in the rate of change of colony area over time. We amended the manuscript in lines 371–373 to remove references to a loss of two-dimensional growth, although the potential pitfall of fitting a single growth rate slope across all collected areas still applies due to the commonly observed pre-growth lag.**

"lectin-binding components such as yeast extract (YPD) or phthalate (EMM), cannot be used for this assay without modifications to the immobilization technique"
Have you figured out a way to use YPD or EMM?

> **We are aware of reagents besides Concanavalin A that can be used to adhere cells to surfaces. For example, Cell-Tak has been shown to work with a broad range of organisms and seemingly does not lose adhesive properties in LB media (https://doi.org/10.1038/s41598-019-55798-0), which (like YPD) includes yeast extract. However, we have not tested this method (or others) in the context of the assay described here.**

Is there a github repository for the PIE software? Make it clear how the reader should get the latest version of the code.

> **PIE is now available on github (https://github.com/Siegallab/PIE), with details of how the software works available in the documentation and the cited bioRxiv preprint. We have added a reference (reference 12) with the link where PIE is discussed in the paper, which is cited in line 57.**

Reviewer #2:
Manuscript Summary:
It is known that yeast population displays strong cell to cell heterogeneity in growth and stress resistance, these two features being anti-correlated. However, extra experiments are needed in order to assess this relationship across different yeast strains and environmental conditions. For

such purpose, different microfluidic tools have been designed. However, there are actually different chip design available, as well as several image analysis tools. This paper presents a new experimental pipeline allowing the analysis of the growth of a high amount of yeast micro-colonies, as well as the determination of gene expression level and stress resistance.
The protocol is clear and well described, however there are some flews that has to be addressed before publication.

Major Concerns:
- We have actually a lot of microfluidics tool at hand allowing the cultivation of microbes in "liquid" culture. This paper is focused on the analysis of yeast colonies growing on agar slide. Liquid culture allows for example for rapid switching between stress conditions, whereas it is more difficult to control the switch on agar slide. This have to be discussed. Additionally, it is more challenging to induce monolayer on agar slide. These considerations make difficult to justify the use of agar slide by comparison with liquid microfluidics.

> **The assay we describe focuses on immobilized yeast growing in liquid culture, not on agar. In order to clarify this from the beginning of the manuscript we stated this fact explicitly in the abstract, in line 23, by saying "automated time-lapse imaging of yeast immobilized in a multiwell plate."**
>
> **We restructured the introduction (lines 45–57) to include the details of the yeast growth and immobilization earlier on in order to bring attention to the growth conditions.**

- A sonication step is proposed in option. The drawback is that cells can be damaged during the process. What is the meaning of the sonication program proposed ? Is it transposable to all sonicators ?

> **The goal of the sonication proposed is solely to break apart flocculated cells in order to allow single cell measurements at the start of the imaging protocol. We state this in the note above the sonication step in the protocol in lines 209–211, present a figure of cells that need to be sonicated in the experimental pitfalls section of the representative results (Figure 4B), and discuss this in the Representative Results section (lines 353–362).**
>
> **In the protocol, step 3.3.2 we also state that sonication can cause elevated stress responses in cells and also kill cells, and we indicate that "testing may be required to identify the best sonication program for a given experiment" (line 216–217) so that cells are not killed or that their growth patterns are not altered as a result of a sonication-induced stress response (unless the experimental question calls for this).**
>
> **The sonication program we describe in step 3.3.2 is likely not transposable to all sonicators and, in order to clarify this, we added "This exact program is likely not**

applicable to all sonicators so testing is suggested prior to the experiment day" in lines 219–220.

- The protocol that has been proposed is focused on the analysis of microcolonies and not single cell (no access to cell lineage). This has to be clearly stated (the title is eloquent, I do agree).

We refer to the assay as "microcolony growth assay" as a way to ensure that it is clear that this is not a single-cell assay throughout the manuscript, the abstract, the summary, and (as the reviewer stated) the title. Additionally, the key word "single-cell" was removed from the Keywords list.

- You relate fluorescence and growth based on the analysis of microcolonies. What about very heterogeneous processes where GFP negative and GFP positive cells are present within the same colony?

We do not perform this kind of analysis currently, so we do not mention it in the text, but one could analyze fluorescence intensities of pixels within the colony outline.

- What about the determination of stress resistance as it has been announced in the abstract/introduction ?

To maintain this manuscript relatively brief and clear we describe and show basic experiments that can be done with the growth-rate assay. Our lab has, however, published more complicated experiments using this growth-rate assay. In the first paragraph of the discussion we describe some of the more involved growth-rate assay experiments from previous publications, and how they were used to answer specific questions. One of these examples is the determination of stress tolerance. We state "Finally, this protocol allows monitoring of cell responses to environmental stresses and perturbations. Treatments such as heat shock can be administered part-way through the growth assay. Such studies have revealed, for example, that slow-growing cells expressing high levels of Tsl1 are more tolerant of heat shock" (lines 426–429). We include this reference to allow interested readers to find out more about how growth-rate assays can be used for the specific purpose of determining variation in stress tolerance.

Reviewer #3:

Manuscript Summary:

This manuscript provides a written/video overview of a microscopy based protocol for measuring microcolony growth rates for the budding yeast, Saccharomyces cerevisiae. Microscopy based analyses of microcolonies are a sensitive assay for measuring growth rates,

and thorough description of key steps for using these methods will be a useful resource for the yeast genetics community.

Major Concerns:
Overall, this manuscript is well written and the video is informative but both lack critical details for what is perhaps the most challenging step of this protocol -- accurate, automated computational analysis of time series images. The paper refers to the a paper available on bioRxiv, also from the Siegal lab, that describes an algorithm called "Processing Images Easily" (PIE) that they report is suitable for this task. However, this paper isn't peer reviewed/published and the software tools for applying the PIE algorithm don't seem to be available (or at least I couldn't find a reference to a software repository in the bioRxiv PDF). My recommendation is therefore that the authors revise the manuscript to include discussion and details of these critical image analysis steps, and include appropriate links to the PIE software implementation.

> **We agree that software for high-throughput determination of growth rate based on imaged colonies forms an important part of this protocol, and the approach we prefer is our lab's PIE software. We have now made a GitHub repository with the full PIE code available online (https://github.com/Siegallab/PIE) linked in reference 12, which is cited in the manuscript in line 57. We are in the process of revising the manuscript describing PIE and submitting it for publication to a peer-reviewed journal. Importantly, detailed instructions for use of the software are available in the documentation in the GitHub repository, and technical aspects of the software's function are explained in the bioRxiv preprint. Therefore, rather than expanding on the discussion of PIE in this manuscript, we have added a link to the PIE repository (see also response to Reviewer 1's final comment).**