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

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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).**