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BRIEF REPORT

# Intracellular dry mass density increases under growth-induced pressure

[version 1; peer review: 2 approved with reservations]

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## Abstract

Cells that proliferate in confined environments develop mechanical compressive stress, referred to as growth-induced pressure, which inhibits growth and division across various organisms. Recent studies have shown that in these confined spaces, the diffusivity of intracellular nanoparticles decreases. However, the physical mechanisms behind this reduction remain unclear. In this study, we use quantitative phase imaging to measure the refractive index and dry mass density of *Saccharomyces cerevisiae* cells proliferating under confinement in a microfluidic bioreactor. Our results indicate that the observed decrease in diffusivity can be at least attributed to the intracellular accumulation of macromolecules. Furthermore, the linear scaling between cell content and growth-induced pressure suggests that the concentrations of macromolecules and osmolytes are maintained proportionally under such pressure in *S. cerevisiae*.

## Plain language summary



Cell proliferation in confined environments leads to the buildup of mechanical pressure. Mechanical pressure has been associated with the decreased motion of intracellular nanoparticles, but the physical basis for this slowdown has not been revealed. In this study, we measure the change in dry mass density of budding yeast growing in a confining microfluidic chamber using quantitative phase imaging. The dry mass density of cells increases linearly with pressure, which can be explained by continued mass accumulation in constrained cell volume. Our results suggest that the accumulation of mass beyond cellular homeostasis may be the physical driver of the restricted growth of cells in a confined space.

## Keywords

macromolecular crowding, microfluidic, growth-induced pressure, quantitative phase imaging, refractive index, dry mass density

## Open Peer Review

Approval Status ? ?

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## Introduction

Living cells proliferating in confined spaces eventually build up mechanical compressive stress exerted onto themselves and their surroundings. This growth-induced pressure (GIP) has the potential to decrease cell growth in all kingdoms of the living, including bacteria<sup>1,2</sup>, fungi<sup>3,4</sup>, plants<sup>5,6</sup> or mammals<sup>7-9</sup>. It has recently been shown in the budding yeast *Saccharomyces cerevisiae* and in mammalian cells that GIP is accompanied by a decrease in the diffusion of genetically-encoded tracer nanoparticles<sup>10</sup>. This decreased diffusion has been attributed to an increase in intracellular density through a mechanism where the production of macromolecules with limited cell volume expansion leads to increased biomass within the cytoplasm. However, the accumulation of macromolecules has yet to be observed experimentally, as this decrease in diffusion could also be attributed to other effects, such as a decrease in biochemical activity which is known to fluidize the cytoplasm<sup>11,12</sup>.

The dry mass density of a living cell can be a direct proxy of macromolecular concentration. Numerous methods exist to measure the dry mass density of living cells, but their applications to confined cells developing GIP are inexistent. To develop GIP, cells are proliferating inside a confining microfluidic chamber and become densely packed<sup>3,7</sup>. The experimental conditions restrict access to several measurement techniques, such as suspended microchannel resonator (SMR) measuring single cells' buoyancy suspended in media<sup>13,14</sup>, or cryoelectron tomography requiring cell fixation<sup>15,16</sup>. On the other hand, quantitative phase imaging (QPI) is an alternative technique that can non-invasively measure the dry mass density of optically transparent biological cells or tissues, which does not require sample preparation<sup>17,18</sup>.

QPI can quantify the refractive index (RI) distribution of a biological sample by detecting the phase difference of light passing through the sample with the surrounding media. This measured RI is directly proportional to the dry mass density of a biological sample<sup>19</sup>. Here, we investigated the changes in the dry mass density of the budding yeast *Saccharomyces cerevisiae* growing within a confined space using QPI. We observed that confined growth was associated with increased dry mass density. We measured the concentration of a fluorophore inside the cell and observed that the increase in RI was proportional to the increase in fluorophore concentration. A linear extrapolation between GIP and RI down to the refractive index of water predicted the nominal intracellular osmotic pressure of the cell.

## Methods

### Cell culture conditions

*Saccharomyces cerevisiae* strains were grown and maintained on a Synthetic Complete (DCS0019, Formedium) + 2% dextrose (SCD) media agar Petri dishes. Next, a single colony was inoculated in a fresh liquid SCD liquid media and incubated with orbital shaking at 200 rpm at 30°C overnight. Exponentially growing culture at OD = 0.3 was then loaded into the microfluidic chamber.

### Microfabrication of PDMS devices

The mold with two layers of different heights is fabricated in a cleanroom at LAAS-CNRS using classical photolithography with negative photoresists. The first layer at a height of 0.8 μm defining the culture media channels was prepared with 2ml of negative photoresist (SU8 3000.5, Kayaku Advanced Materials), and the second layer at a height of 9.2 μm defining the cell growth chamber and the main cell-loading channel was prepared with 2ml of another negative photoresist (HARE-SQ10, Kemlab). After the lithography process, perfluorodecyltrichlorosilane was supplied in 100 sccm for 10 seconds and grafted with O<sub>2</sub> plasma under 40 Torr of pressure onto the wafer surface using an SPD (Memsstar Technology) machine to improve the hydrophobicity and non-stiction. PDMS elastomer was prepared by mixing the base and curing agent in a 10:1 ratio, poured onto the mold, and cured overnight at 60°C. Both PDMS and a glass coverslip were activated with oxygen plasma treatment (Diener PICO; gas, oxygen; pressure, 0.3 mbar; power, 100%; activation, 20 s), and bonded to each other immediately after surface activation. The PDMS/glass chip was then baked for ≥5 h at 60°C.

### Microfluidic device operation

The cell suspension was introduced to the chambers using a syringe. We next injected the SCD media through the main inlet channel using a Fluigent MFCS pressure control system. During the cell incubation and imaging, the pressure at the culture media inlet was maintained at ~1 bar. The chips were placed in a microscope environmental chamber at 30°C (TempControl-37, Leica Microsystems).

### Refractive index measurement of bulk liquid/solid samples

Liquid and solid samples were measured using an Abbe refractometer (2WAJ, OPL) with white light at 30°C. Culture media (SCD) and distilled water samples were measured in spread, like a thin film, between the main and secondary prisms. A cured PDMS sample was prepared into a film of about 500 μm thickness and was carefully placed between the two prisms' surfaces without any air bubbles. The RI of each sample was consistent for three independent measurements, and errors were not stated because the variances were smaller than the refractometer's measurement resolution (10<sup>-4</sup>).

### Bright field and fluorescent microscopy

All imaging acquisition in this study was conducted on an inverted microscope (DMi8, Leica Microsystems). The lateral deformation of PDMS chambers was measured to infer growth-induced pressure through bright-field microscopy simultaneously with the QPI. The relationship between pressure and chamber deformation was calibrated as done in previous work<sup>10</sup>, giving a value of 8.2 μm.MPa<sup>-1</sup>.

To measure GFP accumulation, cytosolic GFP expressed from *HIS3* promoter were measured by fluorescent z-stacking (0.5 μm interval) with a spinning-disk confocal scanner unit (CSU-X1, Yokogawa). We acquired z-stack of the first cell layer of each chamber using the same way of measurement for the

chamber height with ET525/50 nm red emission filter, a dichroic mirror (ZT405/488/561/638rpc, Chroma) and Hamamatsu scientific complementary metal–oxide–semiconductor camera (ORCA-Flash4.0 v3, Hamamatsu Photonics). In the same way, we imaged the cells without any GFP labeling to subtract the cellular autofluorescence which can be detected from GFP channels. The GFP expression of each cell was calculated by summing of pixel values across all z-stack slice of a chamber in ImageJ/Fiji<sup>20</sup>.

### Refractive index and dry mass density measurement of cells with QPI

For transmission QPI, the microscope was equipped with conventional LED Köhler transillumination and a condenser of maximum numerical aperture NA = 0.55. To acquire QPI data, we imaged the samples with a 20x 0.8 NA objective (Leica) and a quadriwave lateral shearing interferometry (QWLSI) system (SID4 sc8, Phasics) mounted on the microscope's lateral camera port. The QWLSI measures the local phase shift, also called optical path difference (OPD), introduced by a specimen placed under a microscope. Depending on the sample's thickness and the difference of sample's refractive index from the background material, the OPD was depicted in grayscale on the image, the so-called phase image.

To estimate RI of cells ( $n_{\text{cell}}$ ) on a culture plate, every cell was segmented from a phase image, which set the culture media as a background baseline, using Otsu algorithm method by CellProfiler<sup>20</sup>. The  $n_{\text{cell}}$  is estimated by the relation,  $n_{\text{cell}} = n_{\text{background}} + \text{OPD}/d_{\text{cell}}$ , where the OPD is difference between cell and background and  $d_{\text{cell}}$  is the height of the cell. Since cells were assumed to be prolate ellipsoids, the  $d_{\text{cell}}$  was taken as the length of the minor axis of the cell in the phase image. The corresponding OPD was taken as the brightness of the upper 5% of the intensity distribution within the cell from the phase image in which the background was subtracted.

To measure the average RI of cells within a microfluidic chamber, the averaged OPD value along the chamber area was measured, comparing to surrounding PDMS. To minimize the influence of local and variable OPD gradients occurring in phase images of cells within the PDMS microfluidics chip, we took the average value of the PDMS area in all directions surrounding the cell sample as the background baseline of the measurement. The chamber height under pressure was determined by the OPD of the calibrated culture medium of chambers expanded by predefined hydraulic pressure:  $d_{\text{chamber}} = \text{OPD}/(n_{\text{PDMS}} - n_{\text{medium}})$ . Measurement of chamber's height at GIP = 0 MPa perfectly matched the measurement of the SU8 mold measured with a profilometer. For GIP > 0 MPa, the cellular RI was measured in comparison to PDMS:  $n_{\text{cell}}(P) = n_{\text{PDMS}} + \text{OPD}(P)/d_{\text{chamber}}(P)$ .

The dry mass density of a yeast cell is directly calculated from the mean RI value since the RI in biological samples is linearly proportional to the dry mass density inside cells as  $n_{\text{cell}} = n_{\text{media}} + \alpha\rho$ , where  $n_{\text{cell}}$  is the average RI of each cell,  $n_{\text{media}}$  is the RI of the surrounding media which was obtained

with Abbe refractometer ( $n_{\text{media}} = 1.336$ ),  $\alpha$  is an RI increment ( $\alpha = 0.190$  ml/g for proteins and nucleic acids<sup>19</sup>), and  $\rho$  is the dry mass density inside cells.

### Data and statistical analysis

All image analysis was performed using ImageJ (National Institutes of Health, US). Statistical analysis was performed on Python with Statsmodels modules<sup>21</sup>.

## Results

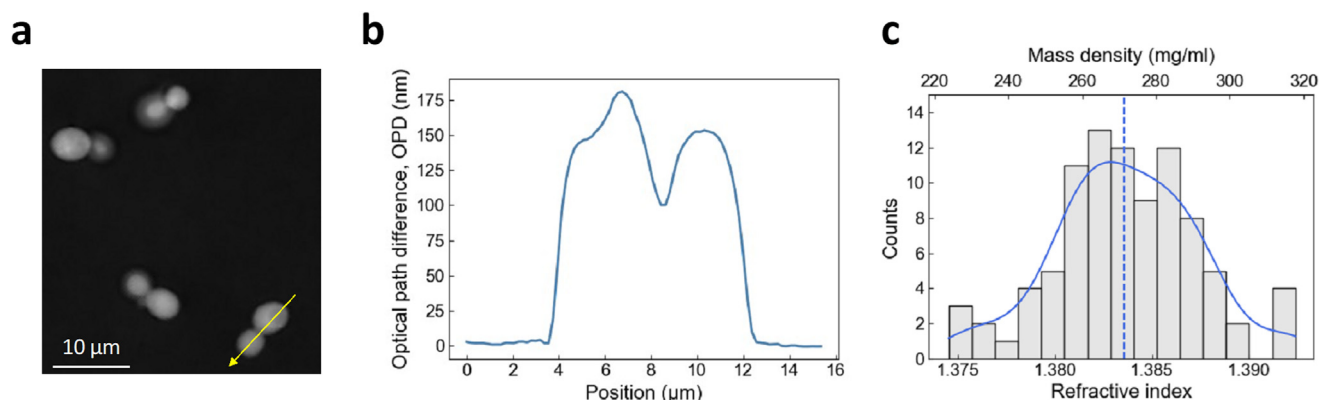
### Refractive index and dry mass density of *S. cerevisiae* determined with QPI

We measured the RI and dry mass density of living yeast *S. cerevisiae* on a culture plate using QPI, which measures the phase difference between a sample and a reference in the image. Phase images displayed the optical path difference (OPD) between cells and the surrounding culture media in grayscale (Figure 1a). The OPD was the difference in RI of a sample with regard to a reference (here, the culture media), times the height of the sample. The pixel value along the cross section of a cell was related to the physical thickness and the local RI of different parts of living yeasts (Figure 1b). We measured the maximum OPD of each cell with respect to the culture media to be  $180 \pm 20$  nm. We estimated the average cellular height to be  $3.94 \pm 0.50$   $\mu\text{m}$  (see Materials and Methods). We determined the mean RI and dry mass density of an asynchronous population of yeast *S. cerevisiae*,  $n_{\text{cell}} = 1.384 \pm 0.004$  and  $\rho_{\text{cell}} = 271 \pm 19$  mg/ml (Figure 1c), which lied within the range of the RI measured for various yeast cells<sup>22–26</sup>.

### Increased intracellular density under GIP

We investigated the change in intracellular dry mass density as a function of growth-induced pressure (GIP). We used microfluidic chambers to grow *S. cerevisiae* within a confined space to investigate the cellular RI under GIP (Figure 2a). To prevent nutrient depletion and exchange media, chambers were supplied with nutrients through microchannels on both sides. After the cells filled the space through proliferation, they developed GIP by pushing against their neighbors and onto their surroundings. GIP was measured through the deformation of the PDMS elastic wall.

We acquired phase images of cells in a chamber under GIP using QPI to examine cellular RI (Figure 2a). We measured that the average OPD of the cells with respect to the surrounding PDMS decreased linearly with pressure (Figure 2b). As PDMS has a higher RI than the cells on average,  $n_{\text{PDMS}} = 1.405$ , the decrease in OPD implied that the RI of a cell approached the value of PDMS with increased GIP. We measured the effective height of the deformed confining chamber using OPD of the chamber filled with calibrated culture medium and pressurized by a measured hydraulic pressure (Figure 2c). We then extracted, assuming that the chamber was fully filled with cells, the mean cellular RI as a function of GIP (Figure 2d). We showed that RI increased roughly linearly with increased GIP under confinement.



**Figure 1. Measurement of cellular refractive index and dry mass density using QPI.** (a) Optical path difference (OPD) image of budding yeast cells in SCD medium. (b) Profile plot drawn from (a) following the yellow arrow line. (c) Histogram of Refractive index (RI) and dry mass density measurements of budding yeast cells grown at 30°C in SCD medium. A kernel density estimation was plotted (blue) with the median value (dashed line).

We noted that the RI of cells without pressure in the chambers was underestimated (gray points in Figure 1c, compared to the blue point measured outside of the device). We attributed this underestimation to the fact that cells were not tightly packed in the chamber, lowering the effective RI due to culture medium at a non-negligible volume fraction in the chamber.

#### Proportional increase in GFP production with intracellular density under GIP

We measured the mean fluorescence intensity of a GFP expressed from the *HIS3* promoter. We showed a linear increase of GFP concentration in the cell as a function of GIP (Figure 2e). Interestingly, we observed that the increase in mass density was proportional to the increase in GFP concentration (Figure 2e, inset), suggesting that the linearity between RI and protein concentration was kept in these conditions. Importantly, intracellular density extrapolated to 0 when the fluorescence intensity did. These results together demonstrated that the cellular biomass and GFP concentration increase roughly proportionally to GIP.

#### Estimation of the nominal intracellular osmotic pressure

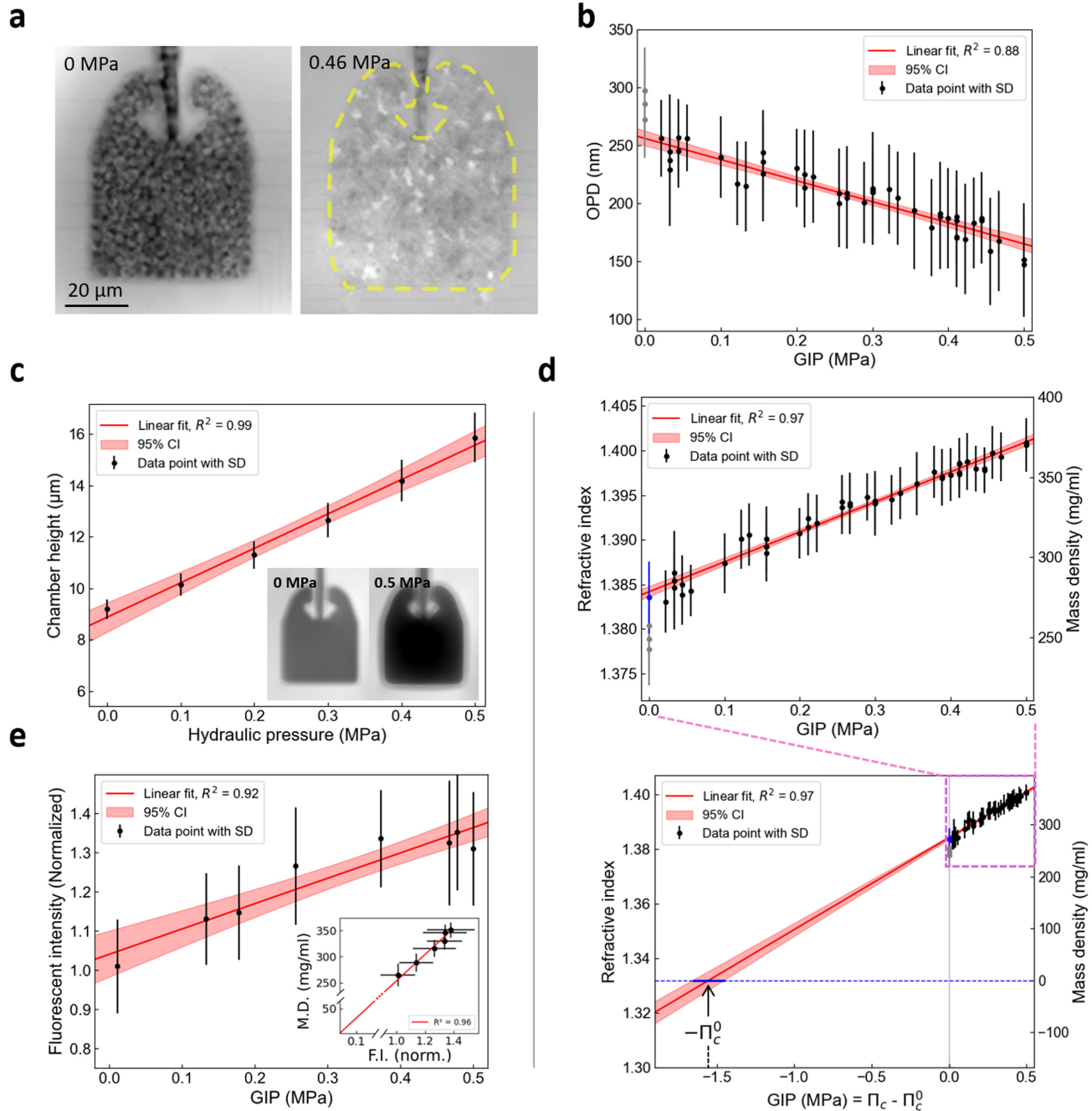
The intracellular RI is proportional to the dry mass of the cell. Figure 2d showed that mass and intracellular osmotic pressure increased proportionally under confined growth. We performed a linear extrapolation of the pressure to a RI matching the one of water at 30°C ( $n_{\text{water}} = 1.332$ ), without considering the points at GIP = 0 MPa measured in the microfluidic chip, but considering the RI of cells measured in culture medium (blue point). We assumed that when the cell would have the RI of water, it would be empty of its constituents, and its intracellular pressure would then be null,  $\Pi_c = 0$  MPa. When the RI would match the RI of water, it would thus correspond to a point where the cell would be “empty” of macromolecules. The corresponding GIP, denoted  $P_{n_{\text{water}}} = \Pi_c - \Pi_c^0$ , corresponding

to the pressure difference between the intracellular osmotic pressure cell,  $\Pi_c$ , and the nominal intracellular osmotic pressure,  $\Pi_c^0$ , allowed us to estimate the nominal intracellular pressure of the cell:  $\Pi_c^0 = -P_{n_{\text{water}}}$  (Figure 2d). We found in this case a nominal pressure of 1.55 MPa and the 95% confidence interval ranges from 1.46 to 1.66 MPa, comparably larger than the 0.95 MPa estimated for the same *S. cerevisiae* in previous studies, with a different method<sup>10</sup> (see Discussion).

#### Conclusion

In this study, the dry mass density under pressure was estimated by quantitative phase microscopy, and increased with GIP. The previous measurement of intracellular density under GIP has only been estimated indirectly by tracking fluorescently tagged tracer nanoparticles. It was assumed that the decrease in particle diffusion occurred due to the reduction of the free volume of the cytoplasm<sup>10</sup>. However, the macromolecular diffusion within the cytoplasm, a highly heterogeneous active media, can be limited not only by the accumulation of macromolecules but also by a change in macromolecule size distribution<sup>27</sup>, molecular electrostatic interactions<sup>28</sup>, or active random force<sup>29</sup>. Therefore, our results experimentally verify that the physical basis of the crowding-induced reduction in particle diffusion is compatible with an increase in dry mass density.

The previous theoretical model relating nanoparticle tracer diffusion with GIP assumed that the concentrations of osmolytes and macromolecules were produced proportionally<sup>10,33</sup>, but this was not confirmed experimentally. The linear relation between GIP and RI shown in our results indicated that the production ratio of macromolecules and osmolytes was kept constant under pressure in the yeast *S. cerevisiae* (Figure 2d). However, the nominal osmotic pressure of budding yeast estimated through linear extrapolation, 1.55 MPa, was slightly larger than the value obtained from the previous study’s theoretical



**Figure 2. Intracellular dry mass density of budding yeast increases linearly with growth-induced pressure.** (a) OPD images of budding yeast cells growing within the confining space of a PDMS microfluidic chamber. Cells do not experience growth-inducing pressure until their growth fills the entire volume within the chamber (left, GIP = 0 MPa). After, confined growth leads to the build-up of growth-induced pressure (GIP), deforming the elastic chamber, where the expanded boundary is labeled with a yellow dashed line (right, GIP = 0.46 MPa). (b) Change in OPD of cells under GIP measured relative to surrounding PDMS. (c) The effective height of the PDMS chamber linearly increased with the GIP level, which is proportional to the OPD between the chamber and surrounding PDMS (inset). (d) The RI and dry mass density increase linearly as a function of GIP. The value estimated outside of the device previously is presented in a blue point. The measurements within the device at GIP = 0 are presented in grey, which were excluded from the regression as they are assumed to be underestimated. The nominal intracellular osmotic pressure ( $\Pi_c^0$ ) is estimated through linear extrapolation. An estimated  $\Pi_c^0$  value is indicated. (e) Fluorescence intensity (F.I.), corresponding to GFP expression level, linearly increases along GIP ( $n = 20$ ). Dry mass (D.M.) density is proportional to fluorescence intensity (inset). The values in the inset graph is denoted by mean  $\pm$  standard deviation in both the x and y-axis direction.

model, 0.95 MPa<sup>10</sup>, but remained in the same order of magnitude.

## Ethics and consent

Ethical approval and consent were not required.

## Data availability

### Underlying data

Zenodo: Data of the subfigures of article “Intracellular dry mass density increases under growth-induced pressure”, <https://doi.org/10.5281/zenodo.13842648><sup>30</sup>

- This project contains the following underlying data: [Raw\\_data.xlsx](#) (Each excel file tab corresponds to the data plotted in the different sub-figures.)

Data is available under the terms of the Creative Commons Attribution 4.0 International

### Author contributions

HK, BA, NC, JR and MD did the experiments. HK and MD analyzed the data and wrote the manuscript. All authors proofread the manuscript.

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- Kim H, Delarue M: **Data of the subfigures of article “Intracellular dry mass density increases under growth-induced pressure”**. Zenodo. 2024. <http://www.doi.org/10.5281/zenodo.13842648>



# Open Peer Review

Current Peer Review Status: ? ?

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## Version 1

Reviewer Report 07 November 2024

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**Anahit Shirvanyan**

Biochemistry, Microbiology and Biotechnology, Yerevan State University, Yerevan, Armenia

The manuscript presents a detailed investigation into how growth-induced pressure (GIP) affects the intracellular dry mass density of *Saccharomyces cerevisiae* using quantitative phase imaging (QPI). The study effectively connects these physiological changes to the physical phenomena of macromolecular crowding within confined spaces. However, there are some points which I want to raise before considering accepting the manuscript. With revisions, this manuscript would be a strong addition to the field. Below are my comments:

1. Simplify Language for a Broader Audience: The manuscript is somewhat difficult for first-time readers. Some sentences are long and complex, which may hinder comprehension. Breaking these into smaller, more digestible parts would improve readability.
2. The following sentence in the introduction: 'We observed that confined growth was associated with increased dry mass density. We measured the concentration of a fluorophore inside the cell and observed that the increase in RI was proportional to the increase in fluorophore concentration. A linear extrapolation between GIP and RI down to the refractive index of water predicted the nominal intracellular osmotic pressure of the cell.' I recommend moving the sentence to the Results section to maintain clarity and flow.
3. The introduction clearly describes GIP and its relation to the refractive index (RI) of the cell's dry mass, as well as the novelty of the study. However, I suggest highlighting the potential applications or implications of this study to give readers a clearer understanding of its broader significance.
4. The authors mention several other techniques (e.g., SMR, cryoelectron tomography) but don't provide much detail on why they are inadequate for their purposes. A sentence or two explaining the limitations of these methods in the context of this study would make the argument for QPI even stronger. The last sentence linking RI and osmotic pressure is a nice touch but could use a bit more explanation. For example, why is this important? How does it support the hypothesis or lead to further questions for investigation? A more explicit link between this finding and the larger context of GIP would strengthen the study.
5. In the Methods section, *S. cerevisiae* strains are mentioned, but the specific strains used are not indicated. This should be clarified.
6. If possible, I would suggest adding a diagram or image of the designed PDMS microfluidic

- device (chamber). This would help readers better understand the method and experimental setup.
7. Were the cells pre-inoculated in liquid media? If the inoculation was done directly from plates, it could elongate the lag phase, and some cells may be inhibited or even die. What was the initial cell concentration? This should be clarified.
  8. The manuscript lacks citations for some of the methods used. If the methods are newly designed and described for the first time, this should be explicitly mentioned. If previously used methods are referenced, they should be cited.
  9. In the Methods section, the phrase "Next, a single colony was inoculated in a fresh liquid SCD liquid media and incubated with orbital shaking at 200 rpm at 30°C overnight." contains a repetition of the word "liquid."
  10. The manuscript uses many abbreviations. It would be helpful to include an abbreviation list for readers' reference.
  11. Some abbreviations, such as PDMS and GFP, are not defined in the text. These should be written out the first time they appear in the manuscript.
  12. The sentence "To measure GFP accumulation, cytosolic GFP expressed from HIS3 promoter" requires clarification. How was GFP expressed? Does this mean the authors used genetic engineering? If so, could this influence the growth properties of the strain? If genetic manipulation was used, the methods and plasmids should be described or referenced in the Methods section.
  13. The Results section contains some description of the experimental setup, which should be transferred to the Materials and Methods section. Keeping the Results focused on data interpretation would help readers engage more easily with the study's findings.
  14. Since the cells are growing in confined spaces, aeration of the medium is likely low. This could shift yeast metabolism towards fermentation, which may produce gases and increase internal pressure. Was the outlet for these gases considered in the setup? How was this accounted for in the study?
  15. Can the results obtained with SCD medium be generalized to other media, such as YPD? Do the growth characteristics depend on the medium used?
  16. It would be helpful to present growth differences between confined and non-confined conditions. This could help readers understand how GIP influences the growth properties of the cells.
  17. If intracellular osmotic pressure rises, water from the surrounding medium would enter the cells to balance the osmotic pressure. This would likely lead to cell swelling, which could influence the optical path difference (OPD). Was this taken into consideration?
  18. In the abstract, the sentence: "the observed decrease in diffusivity can be at least attributed to the intracellular accumulation of macromolecules" could be more assertive. If the authors have evidence supporting macromolecule accumulation as the primary cause, this could be emphasized more strongly.
  19. The authors briefly mention that statistical analysis was performed using Python with Statsmodels, but there is no further description of the specific tests or methods used. It would be helpful to clarify which statistical tests were applied.
  20. The explanation of GFP imaging and processing in ImageJ is clear, but it would help to mention how the authors handle potential variations between cells (e.g., differences in GFP expression or autofluorescence). Are the cells normalized in some way? Is there any quality control on the imaging process?
  21. The results mention several figures (e.g., Figures 1a, 2a, 2d), but the description of the figures themselves is minimal. While it's clear that the figures are central to understanding

the results, it might be helpful to briefly describe the key observations or trends in each figure within the text. For example, "Figure 2b shows a clear linear decrease in OPD with increasing GIP..." This makes it easier for readers to follow without needing to cross-reference the figures repeatedly.

22. While the authors mention that the nominal osmotic pressure you estimated (1.55 MPa) is slightly higher than the 0.95 MPa from the previous model, they don't elaborate much on why there's a difference. Is this difference due to the experimental method you used (QPI) compared to the theoretical assumptions in the previous study? Are there any potential sources of error or variation in these measurements?

**Is the work clearly and accurately presented and does it cite the current literature?**

Partly

**Is the study design appropriate and does the work have academic merit?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Yeast Stress Biology, Biochemistry, Biophysics, Physiology, Bioenergetics

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Reviewer Report 06 November 2024

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**Marco Eigenfeld**

Medical University of Graz, Graz, Austria

### Summary of the main findings:

The authors effectively demonstrate the link between dry cell density, as measured through refractive index changes, and the stress induced by growth in *Saccharomyces* yeast cells. The findings observed in normal yeast strains are further validated by incorporating GFP-expressing yeast, which supports the study's primary objectives.

### Recommendations to

#### Introduction:

The introduction would benefit from an initial list of confined spaces, which would set the context more effectively. I suggest removing the brackets and abbreviation "SMR" since it is not reused elsewhere in the text. Additionally, please introduce *S. cerevisiae* by following its full name with the abbreviation in parentheses for clarity. In the third section of the introduction, the full name is used again, as well as in the methods section; please use the abbreviation consistently throughout.

At the end of the first section, the authors refer to "other effects" in plural, but only mention a single effect. Either change this to singular or provide an additional example to maintain consistency. A transition between the first and second sections would help improve the flow, as the text currently shifts abruptly from cytoplasmic fluidity effects to dry mass density.

If "numerous methods" are mentioned in plural, please list some specific methods along with references to avoid a potentially unsupported assertion. Currently, these methods are discussed following an unrelated sentence, "To develop GIP, cells are proliferating inside a confining microfluidic chamber and become densely packed," which could disrupt readability.

#### M&M section:

In the methodology, please add the flow speed at which the cell suspension was introduced via syringe for completeness. Additionally, correct the typo "inversemicroscope" in the microscopy section. Clarify how many cells were measured for the refractive index (RI) and microscopy; this information would be helpful for assessing the robustness of the data.

The inclusion of GFP expression measurement is commendable. However, details regarding the expression mechanism and yeast strain used are currently missing. Please incorporate a brief paragraph in the methods section that specifies the strain and describes how GFP expression is regulated.

#### Results:

On page 4, revising the last section for readability would be beneficial. Presently, nearly every sentence begins with "we," and introducing variation here would improve style and readability. On page 5, there is an incomplete sentence: "Importantly, intracellular density extrapolated to 0 when the fluorescence intensity did," which requires revision for clarity.

#### Discussion:

Lastly, there appears to be some mixing of results and discussion in the section "Refractive index and dry mass density of *S. cerevisiae* determined with QPI." In this section, the authors contextualize their findings within existing literature, which would typically suit a discussion section. However, the later sections only describe findings, fitting the results format. To enhance

consistency, I suggest either maintaining a combined results and discussion structure or introducing a distinct discussion section to compare results with literature values. The second paragraph of the conclusion also seems more aligned with a discussion, and moving this content could strengthen both sections.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and does the work have academic merit?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Partly

**Are the conclusions drawn adequately supported by the results?**

Yes

***Competing Interests:*** No competing interests were disclosed.

***Reviewer Expertise:*** Investigating the mechanisms of yeast aging and yeast physiology, with a focus on the interplay between aging and protein synthesis.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

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