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Expansion Microscopy on *Saccharomyces cerevisiae*

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Abstract

The unicellular eukaryote *S. cerevisiae* is an invaluable resource for the study of basic eukaryotic cellular and molecular processes. However, due to its small size compared to other eukaryotic organisms the study of subcellular structures is challenging. Expansion microscopy (ExM) holds great potential to study the intracellular architecture of yeast, especially when paired with pan-labelling techniques visualising the full protein content inside cells. ExM allows to increase imaging resolution by physically enlarging a fixed sample that is embedded and cross-linked to a swellable gel followed by isotropic expansion in water. The cell wall present in fungi – including yeast – and Gram-positive bacteria is a resilient structure that resists denaturation and conventional digestion processes usually used in ExM protocols, resulting in uneven expansion. Thus, the digestion of the cell wall while maintaining the structure of the resulting protoplasts are crucial steps to ensure isotropic expansion. For this reason, specific experimental strategies are needed, and only a few protocols are currently available. We have developed a modified ExM protocol for *S. cerevisiae*, with 4x expansion factor, which allows the visualisation of the ultrastructure of the cells. Here, we describe the experimental procedure in detail, focusing on the most critical steps required to achieve isotropic expansion for ExM of *S. cerevisiae*.

Keywords: expansion microscopy, yeast, *Saccharomyces cerevisiae*, super-resolution

Introduction

The unicellular eukaryote *S. cerevisiae* represents an invaluable resource for the study of basic eukaryotic cellular and molecular processes. The combination of a high genetic amenability, numerous genetic tools, and vast genomic resources makes it one of the most versatile model organisms, employed in a wide range of basic research disciplines. However, its small size compared to other eukaryotic organisms has limited its use for the study of sub-cellular structures. The diameter of unbudded yeast cells ranges approximately between 4 µm in haploids and 6 µm in diploids (Milo and Phillips, 2015), complicating conventional diffraction-limited light microscopy approaches.

The last decade witnessed the rise of super-resolution (SR) techniques that enable sub-diffraction resolution fluorescence imaging of cellular structures. Expansion microscopy (ExM), for instance, allows for increasing imaging resolution by physically enlarging a fixed sample that is embedded and cross-linked to a swellable gel and is then expanded isotropically in water (Wassie *et al.*, 2019). By doing so, fluorescently-labelled structures that previously would be too small or too close together to be distinguished can be resolved in 3 dimensions. The increased z-resolution can be exploited by z-sectioning, using confocal or light-sheet microscopy. In addition, ExM can also be used in combination with other super-resolution methods, such as airyscan confocal microscopy and Structured Illumination Microscopy (SIM), further enhancing resolution. ExM holds great potential to study the cellular structures of yeast, especially when paired with pan-labelling techniques to visualise total protein content inside cells. ExM protocols present a robust tool to study cellular organelles, obtaining super-resolution results with the advantage of employing inexpensive and straightforward equipment compared to other techniques that require more complex and expensive setups, such as optical SR approaches and EM.

ExM can be applied in various organisms and cellular models, offering outstanding outcomes with expansion factors ranging from 4x to 20x (Faulkner *et al.*, 2020; Truckenbrodt *et al.*, 2018). However, certain cellular organelles

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are resistant to these processes, such as the cell wall of fungi and Gram-positive bacteria. These organisms present a cell wall composed of peptidoglycans or glycoproteins that are highly resistant to denaturation and conventional digestion processes. The digestion of the cell wall and the maintenance of the structure of the resulting protoplasts are crucial steps to achieve isotropic expansion. For this reason, specific experimental strategies are needed, and only a few protocols are currently available. In particular, only one study describes an ExM protocol applied to *S. cerevisiae*, showing different yeast structures in high resolution (Chen *et al.*, 2021).

Here we describe an adapted and extended ExM protocol for *S. cerevisiae* (Figure 1a) with a final expansion factor of 4x. Samples are stained with DAPI and the pan-labelling reagent NHS-Ester BODIPY FL, allowing an overview of the ultrastructure of the cells. Detailed step-by-step instructions are provided.

Briefly, the first day begins with the preparation and fixation of yeast cells, which includes the digestion of the cell wall with zymolyase. The generated protoplasts are mounted on the coverslip, and linking takes place overnight, during which linker molecules will attach to the sample's proteins, acting as anchors for the next step. On the second day, the first step is gelation, where the sample is embedded in a monomer solution that polymerases and forms a matrix to which the anchor molecules will be connected. This is followed by the denaturation step, where the sample structures are disintegrated. Then, the samples are left expanding overnight in water, swelling the polymerised gel, which will push the anchored molecules apart in an isotropic manner. Finally, on the third day, staining with DAPI and NHS-Ester takes place. Samples are left again expanding overnight, and they can be imaged from the next day onwards.

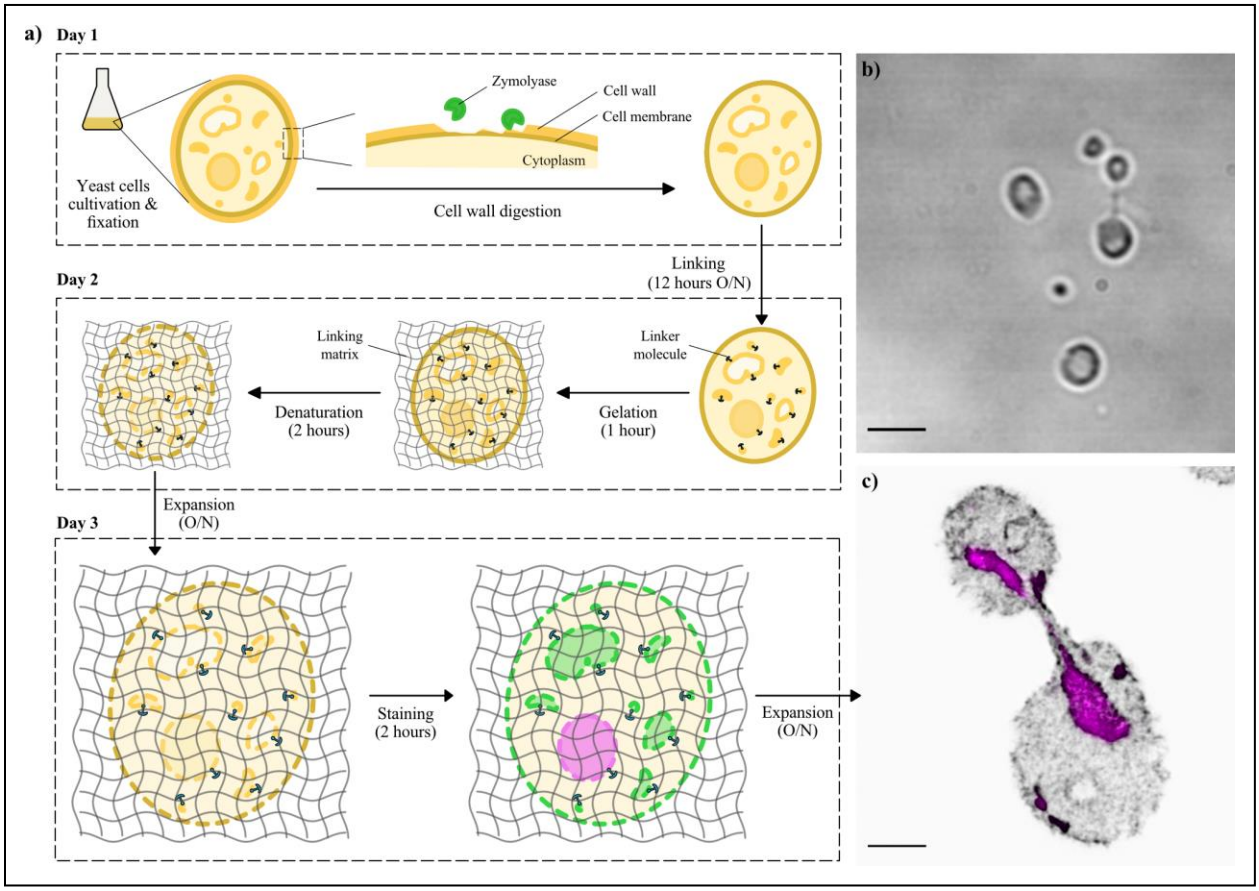

Figure 1. ***a)*** *Scheme of expansion microscopy protocol workflow.* ***b)*** *DIC image of yeast, acquired with a Nikon HCS using a 100x/1.45 oil objective. Scale bar: 5 µm.* ***c)*** *Maximum intensity projection of fluorescence image of post-expanded yeast, acquired with a Zeiss LSM 980 using a 40x/1.1 water objective and airyscan detection. Signals of DAPI and NHS-Ester are represented in magenta and grey, respectively. Scale bar: 5 µm.*

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Methods

Preparation of stock solutions:

Sorbitol buffer:

- 1.2M sorbitol solution in 0.1M KH_2PO_4.

Linking solution:

- 0.1 mg/mL acryloyl X-SE in PBS. Store in aliquots at -20 ºC.

Monomer solution:

Stocks:

- 38% sodium acrylate (w/w, diluted with ddH_2O): 25 g in 65,79 ml, store at -20°C.
- 40% acrylamide stock: 20 g in 50 ml ddH_2O, store at -20°C.
- 2% N,N'-Methylenebisacrylamide : 0.2 g in 10 ml ddH2O, store at -20°C.

Reagent	Final concentration
PBS	1x
Sodium acrylate*	19 g/100mL
Acrylamide	10 g/100mL
N,N'-Methylenebisacrylamide	0.1 g/100mL

Store in 493µl aliquots at -20 ºC.

*Sodium acrylate is provided with variable quality levels. One should test it before using it: the reagent should be fully dissolved and not show impurities in the solution, in the form of precipitates or discolouring.

Denaturation buffer:

Reagent	Final concentration
ddH2O	-
SDS	200 mM
NaCl	200 mM
Tris*	50 mM

Store in falcon tubes at -20 ºC.
*Adjust pH to 9.

Coverslip cleaning

Notes: i) This procedure should be performed under a fume hood, and one should use safety gloves when handling chloroform. ii) The chloroform and NaOH solutions can be reused. iii) The preparation of NaOH is an exothermic reaction, thus the NaOH stock solution should be prepared on an ice bath.

1. Arrange single coverslips in a coverslip rack.
2. Place a glass container in an ultrasonic bath and fill the bath with water.

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3. Place the rack in the glass container.
4. Fill the glass container with chloroform and sonicate for 1 hour.
5. Remove the rack with a tweezers/forceps and place it in an empty glass container. The coverslips will dry in a few minutes as the chloroform evaporates quickly.
6. Pour the remaining chloroform back into the bottle through a funnel.
7. Reinstall the rack in the glass container in the ultrasonic bath.
8. Fill the glass container with a 5 M NaOH solution and sonicate for 1 hour.
9. Pass the coverslips into a beaker with ddH_2O and transfer the NaOH solution back into the bottle.
10. Wash the coverslips three times in a volume of 500 mL fresh ddH_2O.
11. Pre-dry the coverslips with a nitrogen or filtered air gun and then place them in a drying cabinet until they are completely dried.
12. Store in 100 % ethanol in a glass Petri dish.

Coverslip poly-L-lysine coating

1. Dry the previously cleaned coverslips.
2. Place the coverslips in a clean plastic plate, for example, in a 6-well plate.
3. Add 300 µl of 0.1 mg/ml poly-L-lysine on each coverslip and incubate at room temperature (RT) for 10 minutes.
4. Add water to each well until the coverslip is covered and incubate at RT for 5 minutes.
5. Dry the coverslips.
6. Store coverslips on a rack at 4 °C until needed for cell/gel mounting in needed.

Day 1

Yeast cells cultivation and fixation

The *Saccharomyces cerevisiae* BY4741 strain (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) was used in this protocol.

1. Culture yeast cells in the conditions required to assess the relevant biological question.
2. Count yeast cells with a hemocytometer and calculate the volume required to collect $6x10^7$ cells
3. Centrifuge cells at 4,000 rpm for 2 minutes in a Falcon tube.
4. Discard the supernatant and resuspend in 3mL of PBS 1X.
5. Centrifuge cells at 4,000 rpm for 2 minutes in a Falcon tube.
6. Resuspend the cells in 3mL of 4% paraformaldehyde (PFA), and fix them at 4 °C for 30 minutes in a multi-rotator.
7. Centrifuge the fixed yeast cells at 4,000 rpm for 2 minutes.
8. Discard the supernatant and resuspend in 3mL of PBS 1X.
9. Resuspend in 6mL sorbitol buffer.

Cell wall digestion

1. Treat 200µl of fixed cells solution ($2x10^6$ cells) with 0.0075U/µl of the zymolyase enzyme and incubate the reaction at 30ºC for 3 minutes*.
2. Place the samples on ice to block the enzymatic reaction.

* The length of the reaction provided is optimised for a population of exponentially growing cells. Samples collected in other conditions may require an adjusted treatment to optimise the cell wall digestion.

Mounting and fixating the cells on the coverslip

1. Place the poly-L-lysine coated coverslips on a 6-well plate.
2. Add 200 µL of treated cells to each coverslip and incubate them at RT for 10 minutes to allow the sample to sediment.
3. Under a fume hood, add enough of the 4% PFA/1% Glutaraldehyde (GA) fixative to cover the sample and incubate at RT for 10 minutes.
4. Wash 3x with PBS.

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5. Inspect the sample under a microscope to confirm that the cells are attached (i.e., cells are immobile and not floating).

Linking

1. Remove PBS, add 1 mL of 0.1 mg/mL acryloyl X-SE solution in PBS to each well and incubate for 12 hours (overnight) at RT.

Day 2

Gelation

Note: Proceed quickly through steps 3 to 5, as after the gelation solution is prepared, it will polymerise in minutes. For this reason, it is recommended to proceed with these steps with a maximum of four samples each time. Reagents and slides are maintained on ice to prevent premature polymerisation.

1. Cool the reagents (monomer solution, TEMED, and APS) and the gelation chamber slides (see supplementary figure 1) on ice for at least 10 minutes.
2. Remove the excess solution from the coverslip with a lint-free wipe and place it next to the slide on which it will be mounted (Note: the coverslip side with the sample should be facing up).
3. Prepare the gelation solution: Add first the TEMED to the monomer solution, then APS and vortex. (Note: it is important to follow this order, as when APS is added the polymerisation reaction starts).

Volume	Reagents	Stock concentration	Final concentration
493 µL	Monomer solution	-	-
5 µL	APS	50%	0.5%
2.5 µL	TEMED	99%	0.5%
500 µL	Final Volume	-	-

4. Add 120 µL of gelation solution in each gelation chamber, place the coverslip on top, with the side with the sample facing the solution, and gently press it. Carefully remove excess with a lint-free wipe.
5. Keep the slides for 5 more minutes on ice.
6. Incubate for 1 hour at 37 ºC in a humidified chamber.

Denaturation

1. Preheat a heating block to 95°C.
2. Thaw the denaturation buffer in a warm water bath and proceed only when the solution is clear.
3. Carefully remove the coverslip from the slide and transfer the polymerised gel to a new 6-well plate (Note: sometimes the coverslip comes attached to the gel, and it can be transferred along).
4. Add 2 mL of denaturation buffer to each well and incubate for 15 minutes at RT.
5. Prepare an 1.5 mL Eppendorf tube for each sample and add 1 mL of fresh denaturation buffer in each of them.
6. Transfer the gel into the Eppendorf tube with a metal spatula and incubate at 95 ºC for 90 minutes.

Expansion

1. Carefully pour the gel from the Eppendorf tube to a big clean plastic plate (e.g., Petri dish with a diameter of at least 60 mm) and remove the excess of denaturation buffer solution.
2. Add ddH_2O to Petri dish until the gel is completely submerged.
3. Change ddH_2O 2x after 30 minutes.
4. Expand in ddH_2O overnight at RT.

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Day 3

NHS and DAPI staining

1. Remove ddH_2O and measure the width and length of the gel after expansion (to then calculate the macro-expansion factor).
2. Cut gel in small pieces using the custom cutter shape (see supplementary figure 2) and a razor blade.
3. Select the pieces to be stained, cut the correct corner for reference of the gel orientation (see supplementary figure 1), and transfer them to a new 6-well plate with a metal spatula.
4. Wash 2x for 15 minutes with PBS. (Note: the gels will shrink by 50%.)
5. Prepare 10µg/mL NHS Ester and 300 µM DAPI in PBS (Note: confirm that the NHS and DAPI solution is completely homogenised).
6. Add 2 mL to each well, wrap it in aluminium foil and incubate for 90 minutes at RT on a rocking platform.
7. Wash 3x for 15 minutes with PBS+Tween-20 0.1%.
8. Add ddH_2O to each well until the gel is completely submerged.
9. Change ddH_2O 2x after 30 minutes.
10. Expand in ddH_2O overnight at RT.

Imaging day

Immobilisation of the gel

1. Place a poly-L-Lysine coated coverslip in an Attofluor™ Cell Chamber (or another comparable mounting device).
2. Carefully dry a piece of gel on a spatula using lint-free wipes and transfer it to the coverslip, with the sample facing the coverslip (use cut corner as reference of gel orientation).
3. Incubate at RT for 7 minutes. Confirm if the gel is immobilised on the coverslip.
4. Add ddH_2O until the gel is completely submerged.

Information related to troubleshooting steps is provided in Supplementary Table 1.

Reagents

Table 1. List of reagents and materials used with corresponding information on the supplier and the article number.

Reagent	Supplier	Article number
Acrylamide	Sigma	A9099
Acryloyl-X SE	Invitrogen	A20770
APS - Ammonium Persulfate	Roth	9592.3
DAPI	Invitrogen	D1306
DMAA - N,N'-Methylenebisacrylamide	Sigma	M7279-25G
GA - Glutaraldehyde	Sigma	G5882-10X1ML
KH2PO4 - Potassium dihydrogen phosphate	Sangon Biotech	A100781
NaCl	Roth	HN00.2
NHS-Ester	Thermo Fisher Scientific	D2184
PFA - Paraformaldehyde	Thermo Fisher Scientific	43368
Poly-L-Lysine	Sigma	P8920

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SDS - Sodium Dodecyl Sulfate	Sigma	L3771
Sodium acrylate	Sigma	408220
Sorbitol solution	Sangon Biotech	A100691
TEMED - N,N,N′,N′-Tetramethylethylenediamine	Sigma	T9281
Tris	Carl Roth	5429.1
Tween-20	Roth	9127.1
Zymolyase enzyme	Zymo research	E1004
Material	**Supplier**	**Article number**
Attofluor™ Cell Chamber	Thermofisher Scientific	A7816
Coverslip (24 mm round #1.5)	Marienfeld	117640
Coverslip rack	Diversified Biotech	WSDR-1000
Razor blade	Carl Roth	CK08.1

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Author Contributions: RH and MF conceptualised the experiment; AGK and LM performed the experiments and wrote the original draft; MDR and HSH supervised the project, processed the imaging and verified the data curation; All authors contributed to the final version of the manuscript.