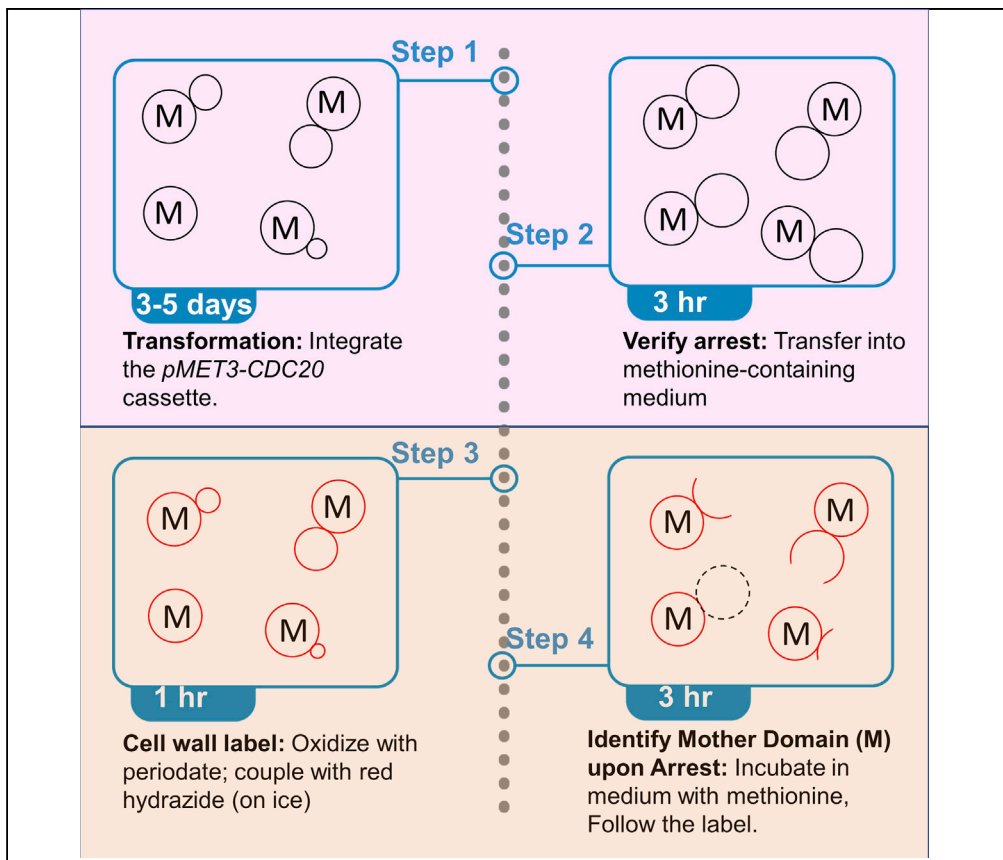


## Protocol

# Cell cycle arrest of *S. cerevisiae* in conjunction with labeling of the cell wall



*S. cerevisiae* can be arrested in metaphase by depleting Cdc20. We describe (1) how to achieve this arrest and verify it, (2) how to label cell surface glycans covalently to distinguish mother from bud, and (3) how to detect the nucleolus and learn that it remains in the mother domain upon arrest.

Alan Michael

Tartakoff

amt10@case.edu

### Highlights

Efficient cell cycle arrest results from depletion of Cdc20

Upon arrest, the size of the bud is comparable to the size of the mother cell

Cell walls can be pre-labeled with a fluorescent lectin or reactive fluorophore

Tartakoff, STAR Protocols 2, 100646

September 17, 2021 © 2021

The Author.

[https://doi.org/10.1016/](https://doi.org/10.1016/j.xpro.2021.100646)

[j.xpro.2021.100646](https://doi.org/10.1016/j.xpro.2021.100646)



## Protocol

Cell cycle arrest of *S. cerevisiae* in conjunction with labeling of the cell wallAlan Michael Tartakoff<sup>1,2,3,\*</sup><sup>1</sup>Pathology Department and Cell Biology Program, Case Western Reserve University, Cleveland, OH 44106, USA<sup>2</sup>Technical contact<sup>3</sup>Lead contact\*Correspondence: [amt10@case.edu](mailto:amt10@case.edu)<https://doi.org/10.1016/j.xpro.2021.100646>

## SUMMARY

*S. cerevisiae* can be arrested in metaphase by depleting Cdc20. We describe (1) how to achieve this arrest and verify it, (2) how to label cell surface glycans covalently to distinguish mother from bud, and (3) how to detect the nucleolus and learn that it remains in the mother domain upon arrest.

For complete details on the use and execution of this protocol, please refer to Tartakoff et al. (2021), Rai et al. (2017), and Zapanta Rinonos et al. (2014).

## BEFORE YOU BEGIN

Different organelles and macromolecular complexes are inherited at different points in the cell cycle (Knoblauch and Rachubinski, 2015). It is also of interest to study the exchange of molecules and organelles across the bud neck when the cell cycle has been arrested (Tartakoff et al., 2013). To investigate these matters, it is necessary to distinguish the mother cell from buds. This distinction can be difficult to make when the bud has already become as large as the mother.

It is therefore useful to prelabel the mother cell. One also can follow cells by time-lapse imaging; however, cumulative photobleaching can become prohibitive. Furthermore, it may not be feasible to maintain cells in conditions that are optimal for both growth and imaging during several hours. The protocols described below make it possible to impose a metaphase arrest on essentially any strain of *S. cerevisiae* and to distinguish the mother vs bud domains by prelabeling the cell wall of the mother. The labeling procedures should be applicable to other organisms with carbohydrate-rich cell walls.

**Note:** The covalent labeling procedure that we have used to tag cell surface carbohydrates might be problematic for any cells that are especially sensitive to the preliminary oxidation step that is used. For wildtype yeast, we find that cell growth continues after labeling, as judged both by noting the presence of unlabeled newly synthesized cell wall (see below) and by replating cells that have been labeled.

The second labeling procedure involves binding of a fluorescent lectin to the cell surface (Tkacz and Lampen, 1972).

In both cases, the protocol involves pulse labeling followed by a regrowth interval. Since the labeled cell surface carbohydrates do not traverse the bud neck, newly synthesized wall can be distinguished by its relative absence of label. Since many lectins (including Concanavalin A) bind glucose, covalent labeling may be preferable if such sugars are present. Nevertheless, we find that Concanavalin A can be used in normal growth medium that includes 2% glucose.



Cells can be synchronized in a variety of ways (Amon, 2002). Metaphase-like arrest is often imposed by adding nocodazole to eliminate microtubules, although this arrest can be unstable (Jacobs et al., 1988). Arrest can also be imposed using temperature-sensitive strains, e.g., (Palmer et al., 1989). This has the disadvantage that temperature increase to 37°C elicits a heat shock response that inhibits ribosome genesis and alters the appearance of the nucleolus (Warner, 1989).

When cycling cells are first shifted to medium with methionine (as in the procedure that we describe), some cells will be close to metaphase while others will be near the beginning of the cell cycle. Taken together with the possibly variable rate of depletion of Cdc20 in different cells, we can achieve ~ 75% arrest, but 100% arrest is not expected over 3 h. Moreover, among the large-budded cells that do accumulate, some will have been in this condition for longer than others - as for most cell cycle arrest protocols. It is not obvious that Cdc20 depletion results in a higher percentage arrest than other synchronization procedures, but it avoids depolymerization of the microtubule cytoskeleton, addition of DMSO and temperature increase.

We construct strains that allow conditional loss of the activator of the anaphase promoting complex, Cdc20 (Lim et al., 1998, Amon, 2002). Since Cdc20 normally turns over rapidly throughout the cell cycle (Prinz et al., 1998), its titer can be reduced within a couple of hours by interrupting its synthesis. Depletion of Cdc20 has been used in various studies over the past decades (Sullivan et al., 2004, Nasmyth, 2001).

**Note:** Strains that carry the *met15Δ* deletion can be grown in synthetic medium lacking methionine. This may be due to traces of methionine that are present in commercial sources of leucine.

### Recurrent procedures

⌚ Timing: 2–10 days for step 1

⌚ Timing: 3 days for step 2

⌚ Timing: 1–2 days for step 3

Unless indicated otherwise, all incubations can be conducted either at 23°C or at 30°C. Cells are routinely sedimented for 5 s at 5,000 × g in a table-top microfuge. All cell incubations and washes use 0.5 mL volumes. Unless specified otherwise, typical procedures involve processing the entirety of the 0.5 mL.

#### 1. Strain construction.

##### a. Strategy 1.

- i. Restrict the plasmid that encodes CDC20 under control of a MET3 methionine-repressible promoter, using Msc1.
- ii. Purify the product using a Qiagen spin column, according to procedures recommended by New England Biolabs and Qiagen.
- iii. Transform cells with the cut plasmid using a lithium-acetate based protocol (Amberg et al., 2005). Transformants are collected on plates lacking methionine and leucine or tryptophane, as appropriate.

##### b. Strategy 2.

- i. Cross a strain that already carries the *MET3-CDC20* cassette with a second strain, i.e., mix the two parental strains (MAT a, MAT α) on the surface of a plate lacking methionine and incubate for 10 h at 30°C.

- ii. Select for diploid cells by streaking the mixed sample onto a doubly selective plate (e.g., lacking both leucine and uracil if one parent was *leu2 Ura+* and the other was *Leu+ ura3*). Allow to grow for 10 h.
  - iii. Culture the diploids in liquid medium.
  - iv. When the optical density reaches about 1.0, sediment the cells, wash once with water and transfer to sporulation medium (1% potassium acetate, 0.1% bacto-yeast extract, 0.05% glucose) lacking methionine and supplemented with nutrients at 5µg/mL to cover any auxotrophic deficiencies (Amberg et al., 2005).
  - v. After 3–7 days being shaken at 23°C, when tetrads can be readily detected in an inverted microscope with a 40× objective, remove 100 µL samples for random sporulation.
  - vi. Add an equal volume of diethyl ether, incubate 10 min at 4°C–10°C, wash with water and then plate serial dilutions on plates that lack methionine and the marker that is linked to the *MET3-CDC20* cassette (leucine, tryptophane). Additional components can be omitted to select for spores of interest.
  - vii. After 1–3 days at 30°C, sample 20–50 of the smallest colonies under a dissecting microscope equipped with an illuminator ring using toothpicks. Establish 50 µL microcultures in methionine-free synthetic medium (Amberg et al., 2005) in a 96-well plate.
2. Genotype verification.
    - a. Transfer samples of microcultures with a multi-prong replicator device into a set of 50 µL microcultures with complete synthetic medium (i.e., including methionine) (Amberg et al., 2005).
    - b. Shake the cultures for 3–5 h and then observe them at 40× in an inverted brightfield microscope to see whether a preponderance of the cells have large buds whose size approximates that of the mother cell (Tartakoff et al., 2021).
    - c. Retain and expand clones if >50% of cells have large buds.
  3. Cell pre-growth, e.g., for ATY7671 that carries the *MET3-CDC20* cassette.
    - a. Transfer samples of cells from –80°C into synthetic medium lacking methionine (Amberg et al., 2005). Shake for 1–2 days until the cells reach  $OD_{600} = 0.5–1.0$ .
  4. Reagent preparation.

Keep low molecular weight fluorescent reagents as small aliquots of 100× stocks in DMSO at –20°C. Freeze 100× concentrated lectin stocks as small aliquots in PBS with calcium and magnesium. Dissolve periodate immediately before use.

#### 5. Preparation of agarose pads for imaging.

Add agarose to medium (1.5% final), bring to a boil, vortex, and apply samples to the surface of microscope slides that are immediately overlaid with a second slide (Zapanta Rinonos et al., 2012). These slides can be kept 1–2 days in a refrigerator.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals		
Media for yeast growth	This laboratory	Cold Spring Harbor Yeast Genetics Course Manual (Amberg et al., 2005)
Rhodamine-Concanavalin A	Vector Labs	RL-1002-25
Texas Red-hydrazide	Invitrogen/Thermo Fisher	T6256
CF488A Aminoxy	Biotium	92152
Agarose	Invitrogen/Thermo Fisher	15510-027
Sodium periodate	Sigma	311448
Dimethyl sulfoxide	Fisher Scientific	D128
37% Formaldehyde	Fisher Scientific	F79-1

(Continued on next page)

**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: organisms/strains		
<i>S. cerevisiae</i> strains and their derivatives	Invitrogen/Thermo Fisher	Yeast Deletion and Yeast GFP Collections
MATa Mid2-GFP Sik1-mRFP	Laboratory stock	ATY1922
MET3-CDC20 Htb2-mRFP [pGAR1-GFP]	Laboratory stock	ATY7671
pMET3-CDC20 (LEU2/Yip22)	F. Uhlmann	p640
pMET3-CDC20 (TRP1/Yip22)	F. Uhlmann	p109
pGAR1-GFP (URA3/CEN)	N. Gas	ZUT3
Software and algorithms		
SoftWorX deconvolution software	Applied Precision, Inc.	<a href="http://www.sussex.ac.uk/gdsc/intranet/pdfs/softWoRx_user_manual.pdf">http://www.sussex.ac.uk/gdsc/intranet/pdfs/softWoRx_user_manual.pdf</a>
Other		
48-Pin multi-prong replicator	V&P Scientific, Inc.	VP481
Illuminator ring for dissecting microscope	OMAX	144 LED Ring Light for Stereo Microscope
Titer Plate Shaker	Thermo	Labline 4625
Sterile toothpicks	Local market	Local market

## MATERIALS AND EQUIPMENT

Cells are routinely cultured in 24-well plasticware dishes shaken in a Thermo Titer Plate Shaker, sedimented using Eppendorf tubes, and observed in an inverted microscope with a 40× phase objective. A Deltavision RT epifluorescence microscope with an automated stage (Applied Precision, Inc) is used for color imaging with a 100× objective.

## STEP-BY-STEP METHOD DETAILS

⌚ Timing: 30 min for step 1

⌚ Timing: 3 h for step 2

⌚ Timing: 30 min for step 3

### 1. Cell Wall Staining to Distinguish Mother and Bud

- Wash the cells twice in ice-cold PBS and then resuspend them in 0.5 mM sodium m-periodate in PBS (freshly prepared). Incubate for 15 min on ice. Wash 2× with ice-cold PBS.
- Incubate on ice for 30 min with 0.5 mg/mL Texas Red-hydrazide or 13 mM CF488A Aminoxy in PBS (diluted from 100× stocks). Wash the cells twice and return them to growth medium ± methionine.  
Procedure 2, Lectin labeling.
- Label the cells in growth medium by adding a 1/100 volume of rhodamine-Concanavalin A stock (1 mg/mL). Incubate for 15 min ice.
- After two washes in growth medium ± methionine, resuspend the cells in medium ± methionine.

### 2. Cell Cycle Arrest

Grow shaking cultures of cells in synthetic medium lacking methionine. When they reach mid-log phase ( $OD_{600} = 0.5-1$ ), sediment samples in Eppendorf tubes and resuspend in an equal volume of complete synthetic medium ([methionine] = 20 mg/liter). Shake for 3 h at 23°C.

### 3. Imaging

- Sediment cell cultures.

- b. Apply 0.5–1.0  $\mu\text{L}$  samples of the pellet to the surface of agarose pads after exposure of the agarose by sliding off the upper slide.
- c. Overlay with a No. 1 thickness coverslip.
  - i. For imaging in excess of 15 min duration, trim the agar to the dimensions of the coverslip and seal with vaseline to reduce drying.
- d. Examine samples in a Deltavision RT epifluorescence microscope with an automated stage (Applied Precision, Inc) and an oil immersion objective (Olympus UPlanApo 100 $\times$ /1.40;  $\infty$ /0.17/FN26.5).
  - i. Capture z-stacks at 0.2–0.5  $\mu\text{m}$  intervals using a CCD digital camera (Photometrics Cool-Snap HQ). % transmission = 100%, exposure time 0.05–1 s, field 1040  $\times$  1040 pixels, binning 1  $\times$  1. Collect through-focal series spanning a total of 6  $\mu\text{m}$ . Remove out-of-focus light using the Softworks deconvolution program (<http://www.sussex.ac.uk/gdsc/intranet/pdfs/softWoRx> user manual.pdf).
  - ii. If it is not convenient to image living cells, fix the cultures by adding formaldehyde to a final concentration of 3.7% from a 37% stock in water, agitate for 5 min and then wash once with growth medium to quench free aldehydes. Store samples in water at this point for 1–2 days at 4°C before examination.

### EXPECTED OUTCOMES

Under optimal conditions (see below) upon transfer to methionine-containing medium,  $\sim 70 \pm 10\%$  of cells should have large buds after 3 h at 23°C. In >95% of these cells, the nucleolus is restricted to the mother domain.

Typical surface labeling using the red hydrazide or green aminoxy reagent are illustrated in the [Figure 1](#). The figures also show how these labeling procedures can be used to detect the sites of continued cell growth.

Examples of lectin labeling have already appeared in the literature, e.g., ([Tkacz and Lampen, 1972](#)).

### TROUBLESHOOTING

#### Problem 1

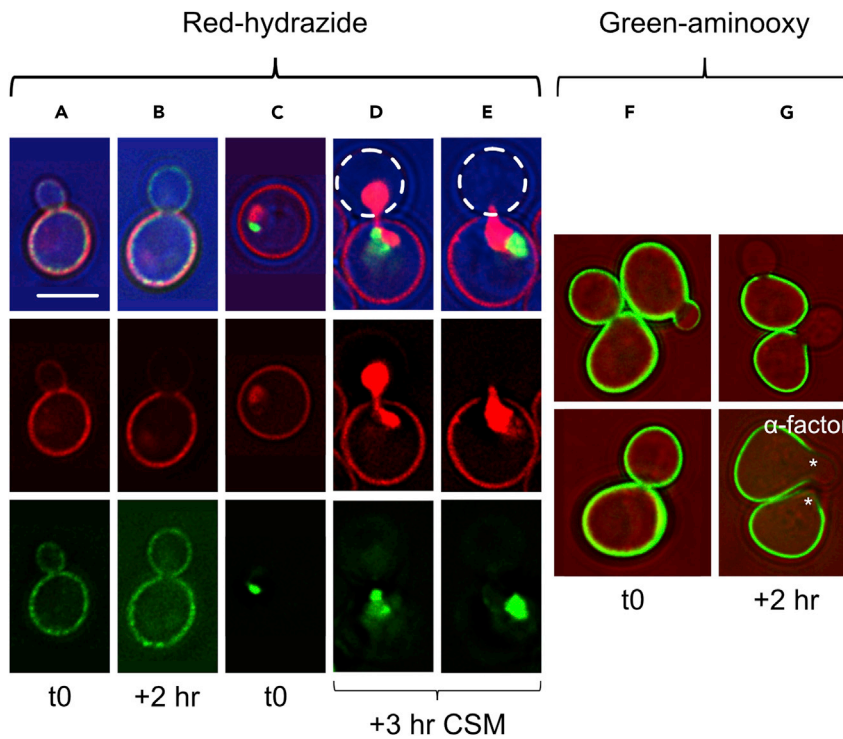
Cells do not arrest efficiently (step 1)

#### Potential solution

The most important consideration is that all the cells be growing rapidly when transferred to methionine-containing medium. It is not advisable to initiate cultures from stored samples and study them without growth for 10 h. If arrest continues to be poor, reclone the cells by "streaking-to-singles." This is achieved by using three successive toothpicks to streak the cells orthogonally onto a plate lacking methionine. The streaking results in an array of colonies since the first streak is horizontal at the top of the plate, the second streaks are vertical (intersecting the initial streak), and the third back-and-forth streaks are again horizontal, intersecting the second streaks. The plate is then incubated 1–2 days. When colonies appear, grow up several clones, test them individually, and verify their genotypes.

To ensure nutritional sufficiency, transfer of cells into medium with methionine should be made by sedimenting cells and resuspending them in fresh medium, rather than merely adding methionine from a concentrated stock.

Cell cycle arrest proceeds satisfactorily after covalent labeling of the cell surface. We expect that the dose of periodate could be reduced, but have not explored this issue.



**Figure 1. Examples of cell surface label**

(A) When cycling cells that express the plasma membrane stress sensor, Mid2-GFP (Philip and Levin, 2001), are labeled with the red hydrazide, the red label extends along the entire cell perimeter and is external to Mid2-GFP.

Interestingly, the Mid2-GFP signal is irregular and both Mid2-GFP and the red signal are weaker around the bud surface that along the perimeter of the mother cell. For all figures, each z-section was  $0.2 \mu$  thick and single z-sections are illustrated after deconvolution with Softworks software. The scale bar is  $5 \mu$ . Strain: ATY1922.

(B) When cells are labeled (as in A) and then are reincubated in growth medium at  $23^\circ\text{C}$  for 2 h, note that the new buds lack the red label although Mid2-GFP is present. In other cells, the basal surface of the bud is labeled but the tip is invisible (not shown). Such observations are expected if wall insertion/assembly occurs at the apex of the bud. Strain: ATY1922.

(C) Cycling cells that carry the *MET3-CDC20* cassette are labeled with red hydrazide. They express both the histone, Htb2-mRFP, and the nucleolar protein, Gar1-GFP. As for Panel A, the perimeter of cells is red. Strain: ATY1922.

(D and E) When cells are surface-labeled (as in C) and then are transferred to methionine-containing medium for 3 h, the preexisting surface label remains visible, but the perimeter of the large bud is unlabeled. The perimeter of the bud is approximated by the dashed white circle. The red bulk chromatin - which transits between mother and bud (Palmer et al., 1989) - can be found on one or the other side of the bud neck or stretching across the bud neck. The nucleolus (labeled by Gar1-GFP) remains in the mother domain, as we have discussed elsewhere (Rai et al., 2017, Tartakoff et al., 2021). The same approach can be used to monitor the distribution of single proteins or of other organelles. Strain: ATY7671.

(F) When cells are labeled with green CF488A aminoxy, the entire perimeter is labeled.

(G) When cells are labeled (as in F) washed, and then reincubated  $2 \text{ h} \pm$  addition of alpha factor, the cell surface continues to enlarge. Note that the surface of the buds (upper image) and the shmoo tip (asterisks) are not labeled. Panels F and G suggest that surface label is lost during the reincubation interval, but these images come from separate experiments. As judged by flow cytometry, the surface label is stable for several hours when cells are reincubated in growth medium (Zapanta Rinonos et al., 2014).

If the incubation in methionine-containing medium is extended beyond 3 h, a minority of cells progress through nuclear division. In such cases, according to need, the incubation time could be reduced.

## Problem 2

Endogenous fluorescence is strong (step 3).

### Potential solution

Cells with lesions along the adenine biosynthetic pathway (*ade2* or *ade3* mutants) accumulate fluorescent pigments in their vacuoles and this can interfere with imaging of other structures. In general, the vacuolar signal becomes minimal if adenine is added to 200  $\mu\text{g}/\text{mL}$  during cell growth.

### Problem 3

Reagents have diminished activity in successive experiments (step 2).

### Potential solution

Remake the solutions and always use small frozen aliquots that are thawed only once.

### Problem 4

The surface of some cells is labeled much more than others (step 2).

### Potential solution

Be sure that the cells are always well-mixed and that the culture is growing vigorously. Any dead cells persist and contribute to visual heterogeneity. Cells should routinely be cloned by streaking to singles.

### Problem 5

Surface labeling is faint (step 2).

### Potential solution

The concentration of the labeling reagents can be increased 5 $\times$ .

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. A.M.Tartakoff, [amt10@case.edu](mailto:amt10@case.edu).

### Materials availability

This study did not generate unique reagents.

### Data and code availability

This study did not generate datasets.

## ACKNOWLEDGMENTS

The authors acknowledge funding sources R01GM089872 (A.T.) and the Visconsi family for support. This publication was made possible by the Clinical and Translational Science Collaborative of Cleveland, UL1TR002548 from the National Center for Advancing Translational Sciences (NCATS) component of the National Institutes of Health, and NIH Roadmap for Medical Research. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIH. The authors thank the following collaborators for technical help: Dalya Khalife, Chun-Peng Pai, and Eric Yuen. The authors thank W-K. Huh, F. Kizito, B. Luttge, D. MacDonald, and F. Uhlmann for materials and advice, and the DeltaVision microscope is managed by the Molecular and Cellular Virology Core of the CFAR P30CA43703.

## AUTHOR CONTRIBUTIONS

A.T. conceived the project and conducted the experiments.

## DECLARATION OF INTERESTS

The author declares no competing interests.



## REFERENCES

- Amberg, D., Burke, D., and Strathern, D. (2005). *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual*, 2005 Edition (Cold Spring Harbor Press).
- Amon, A. (2002). Synchronization procedures. *Methods Enzymol.* **351**, 457–467.
- Jacobs, C.W., Adams, A.E., Szaniszlo, P.J., and Pringle, J.R. (1988). Functions of microtubules in the *Saccharomyces cerevisiae* cell cycle. *J. Cell Biol.* **107**, 1409–1426.
- Knoblauch, B., and Rachubinski, R.A. (2015). Motors, anchors, and connectors: orchestrators of organelle inheritance. *Annu. Rev. Cell Dev. Biol.* **31**, 55–81.
- Lim, H.H., Goh, P.Y., and Surana, U. (1998). Cdc20 is essential for the cyclosome-mediated proteolysis of both Pds1 and Clb2 during M phase in budding yeast. *Curr. Biol.* **8**, 231–234.
- Nasmyth, K. (2001). Disseminating the genome: joining, resolving, and separating sister chromatids during mitosis and meiosis. *Annu. Rev. Genet.* **35**, 673–745.
- Palmer, R.E., Koval, M., and Koshland, D. (1989). The dynamics of chromosome movement in the budding yeast *Saccharomyces cerevisiae*. *J. Cell Biol.* **109**, 3355–3366.
- Philip, B., and Levin, D.E. (2001). Wsc1 and Mid2 are cell surface sensors for cell wall integrity signaling that act through Rom2, a guanine nucleotide exchange factor for Rho1. *Mol. Cell Biol.* **21**, 271–280.
- Prinz, S., Hwang, E.S., Visintin, R., and Amon, A. (1998). The regulation of Cdc20 proteolysis reveals a role for APC components Cdc23 and Cdc27 during S phase and early mitosis. *Curr. Biol.* **8**, 750–760.
- Rai, U., Najm, F., and Tartakoff, A.M. (2017). Nucleolar asymmetry and the importance of septin integrity upon cell cycle arrest. *PLoS One* **12**, e0174306.
- Sullivan, M., Higuchi, T., Katis, V.L., and Uhlmann, F. (2004). Cdc14 phosphatase induces rDNA condensation and resolves cohesin-independent cohesion during budding yeast anaphase. *Cell* **117**, 471–482.
- Tartakoff, A.M., Aylyarov, I., and Jaiswal, P. (2013). Septin-containing barriers control the differential inheritance of cytoplasmic elements. *Cell Rep.* **3**, 223–236.
- Tartakoff, C.L., Raghavachari, S., Gitiforooz, D., Dhinakaran, A., Ni, C.L., Pasadyn, C., Mahabaeleshwar, G.H., Pasadyn, V., and Woolford, J.L., Jr. (2021). The nucleolus as a polarized coaxial cable in which the rDNA axis is surrounded by dynamic subunit-specific phases. *Curr. Biol.* **31**, 2507–2519.e4.
- Tkacz, J.S., and Lampen, J.O. (1972). Wall replication in *saccharomyces* species: use of fluorescein-conjugated concanavalin A to reveal the site of mannan insertion. *J. Gen. Microbiol.* **72**, 243–247.
- Warner, J.R. (1989). Synthesis of ribosomes in *Saccharomyces cerevisiae*. *Microbiol. Rev.* **53**, 256–271.
- Zapanta Rinonos, S., Rai, U., Vereb, S., Wolf, K., Yuen, E., Lin, C., and Tartakoff, A.M. (2014). Sequential logic of polarity determination during the haploid-to-diploid transition in *Saccharomyces cerevisiae*. *Eukaryot. Cell* **13**, 1393–1402.
- Zapanta Rinonos, S., Saks, J., Toska, J., Ni, C.L., and Tartakoff, A.M. (2012). Flow cytometry-based purification of *S. cerevisiae* zygotes. *J. Vis. Exp.* <https://doi.org/10.3791/4197>.