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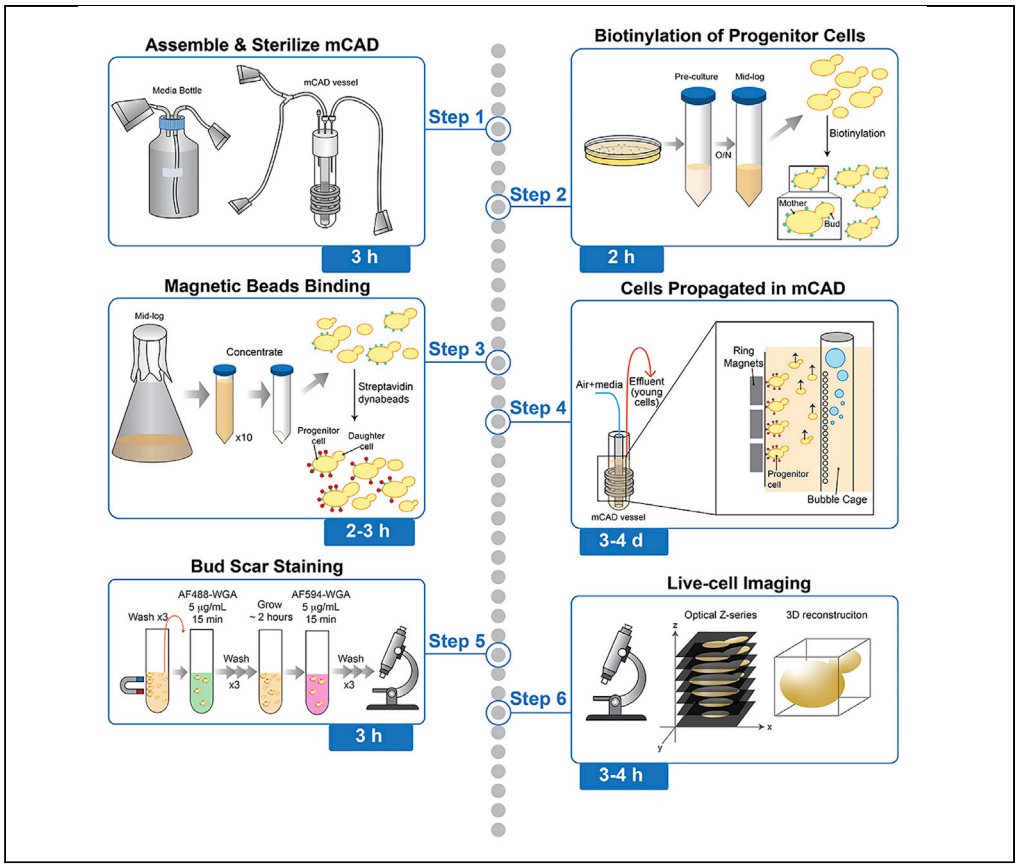
Protocol

Enrichment of aging yeast cells and budding polarity assay in *Saccharomyces cerevisiae*



Replicative lifespan, a measure of the number of times that a yeast cell can divide before senescence, is one model for aging. Here, we provide a protocol for enrichment of yeast as a function of replicative age using a miniature chemostat aging device (mCAD). This protocol allows for isolation of quantities of cells that are sufficient for biochemical or genomic analysis. We also describe an approach to assess bud site selection, a marker for cell polarity, during the aging process.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Step-by-step protocol to assemble a mini-chemostat aging device (mCAD)

Protocol to use the mCAD to isolate yeast as a function of replicative age

Characterization of basic aging phenotypes of cells isolated using the mCAD

Protocol to analyze budding polarity in young and old yeast cells

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Enrichment of aging yeast cells and budding polarity assay in *Saccharomyces cerevisiae*

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SUMMARY

Replicative lifespan, a measure of the number of times that a yeast cell can divide before senescence, is one model for aging. Here, we provide a protocol for enrichment of yeast as a function of replicative age using a miniature chemostat aging device (mCAD). This protocol allows for isolation of quantities of cells that are sufficient for biochemical or genomic analysis. We also describe an approach to assess bud site selection, a marker for cell polarity, during the aging process. For complete details on the use and execution of this protocol, please refer to Yang et al. (2022).

BEFORE YOU BEGIN

Assemble and autoclave miniature chemostat aging device (mCAD)

Timing: 3 h

To isolate aging yeast cells in bulk, the cell wall of a population of mid-log phase yeast cells, which are largely young cells, is biotinylated. Biotinylated cells are then immobilized in the mCAD using streptavidin-coupled magnetic beads and allowed to age under uniform environmental conditions. Daughter cells produced from immobilized cells are removed by continuous media exchange, and cells of defined replicative age are harvested from the mCAD. Cells isolated from the mCAD exhibit aging phenotypes, including an increase in cell size and a decrease in viability, as a function of the time of propagation in the mCAD. This protocol is adapted from a previously described method (Hendrickson et al., 2018). The ministat or miniature chemostat system was developed by the Dunham lab (Miller et al., 2013). For more information, please also refer to the Ministat manual from the Dunham lab (https://dunham.gs.washington.edu/2017MinistatManual.pdf).

The protocols described are for the construction and use of a single mCAD device with a capacity of 40 mL of yeast cell culture (Figures 1 and 2). Each mCAD consists of 3 components: (A) The mCAD vessel and associated tubing. (B) The air pump and air pressure gauge assembly. (C) The media bottle and associated tubing (Figure 3). Each component is assembled, prepared for sterilization, and autoclaved prior to assembly of the mCAD.

1. Prepare tubing for the mCAD vessel (Figure 3A).
   a. Assemble media/air tubing:
      i. Cut small Tygon tubing into 3 pieces: 16″ (40.7 cm), 20″ (50.8 cm), 1″ (2.5 cm).
      ii. Connect 16″, 20″, and 1″ pieces of tubing with a Y-connector.
      iii. Insert a male luer into the 1″ piece. This will be connected to the mCAD glass vessel.

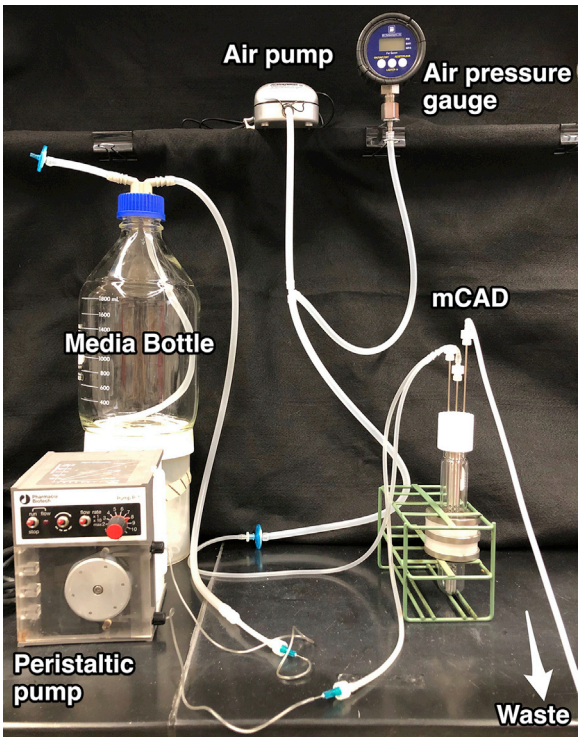

Figure 1. Photograph of a fully assembled miniature chemostat aging device (mCAD)

iv. Insert a male luer into the 20″ piece. This will be connected to the peristaltic pump.
v. Insert an air filter into the free end of the 16″ piece.

b. Assemble effluent tubing:
i. Cut small Tygon tubing into 1–36″ (91.4 cm) piece. The length of this tubing is determined by where the waste flask will be placed relative to the mCAD vessel in the fully assembled mCAD.
ii. Insert a male luer into one end of the ~36″ tubing.

c. Assemble the peristaltic pump tubing:
i. Cut small Tygon tubing into 2 12″ (30.5 cm) pieces.
ii. Insert female luers or blunt-ended needles into one end of piece of 12″ tubing.
iii. Remove the tubing from the peristaltic pump and connect the ends of that tubing to the free ends of each piece of 12″ tubing.

Note: The length of the pieces of small Tygon tubing and the type of connectors used (e.g., luers or blunt-ended needles) vary depending on the peristaltic pump used.

2. Assemble Air Pump and Pressure Gauge Assembly (Figure 3B):
a. Cut medium Tygon tubing into 3 pieces: 1.5″ (3.8 cm), 5″ (12.7 cm) and 10″ (25.4 cm).
b. Connect all 3 pieces of tubing with a T-connector.
c. Connect the air pump to the free end of the 1.5″ tubing.
d. Connect the air pressure gauge to the free end of the 5″ tubing.
3. Assemble the media bottle and associated tubing (Figure 3C):
a. Place a GL45 cap containing 2 hose connectors on an autoclavable 2 L media bottle.
b. Cut medium Tygon tubing into 3 pieces: 13″ (33 cm), 6–10″ (15.2–25.4 cm) and 2″ (5 cm).
c. Insert the 6–10″ tubing into the inner port of a hose connector in the media bottle cap. Adjust the length of the tubing inside the media bottle to ensure that it extends to the bottom of the media bottle.

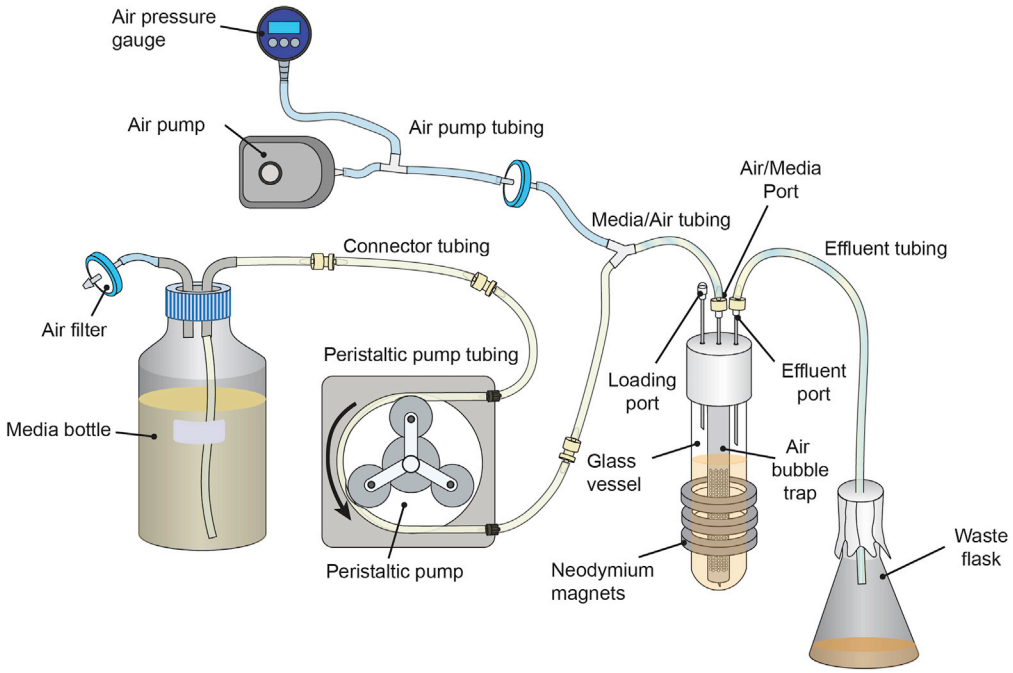

Figure 2. Schematic of the fully assembled mCAD

d. Insert the 13″ tubing into the outer port of the hose connector described in 3c.
e. Insert the 2″ tubing into the outer port of the free hose connector in the media bottle cap and connect an air filter to the free end of that tubing.
f. Cut small Tygon tubing into a 2–10″ (5–25.4 cm) piece. This is the ″connector tubing″.
g. Use the ″reducing adaptor″ to connect the medium-sized 13″ tubing to the small-sized the 2–10″ connector tubing.
h. Adjust the length of the 2–10″ connector tubing to ensure a smooth connection between the media bottle and peristaltic pump. This adjustment is especially important when multiple mCADs are used at the same time.
i. Insert a male luer into the free end of a 2–10″ connector tubing.

4. Assemble the mCAD vessel (Figures 2 and 3A):
a. Insert a silicone disc gasket into the Teflon cap for the glass vessel.
b. Screw the Teflon cap onto the glass vessel.
c. Insert a long, 7″ (17.78 cm) needle into the central hole of the cap. During insertion, the needle pierces through the silicon gasket in the Teflon cap. This is the air/media port.
d. Insert 2 short (5″, 12.7 cm) needles into the other two holes in the cap. Both needles will pierce through the gasket. These needles are effluent and loading ports.
e. Unscrew the Teflon cap and remove debris from the needles and glass vessel. Remove debris from the needles using a water-filled syringe.
f. Fill the glass vessel with ~40 mL water.
g. Assemble the air bubble trap: screw the cap of the trap into the air bubble tube.
h. Place the air bubble trap into the mCAD glass vessel.
i. Use a Sharpie to mark the media level when the mCAD vessel contains 40 mL of fluid.
j. Place the Teflon cap and its associated needles onto the glass vessel and adjust the needles in the vessel, as follows:
i. The long needle, the air/media port, should be in the center of the air bubble trap and extend to the bottom of the glass vessel.

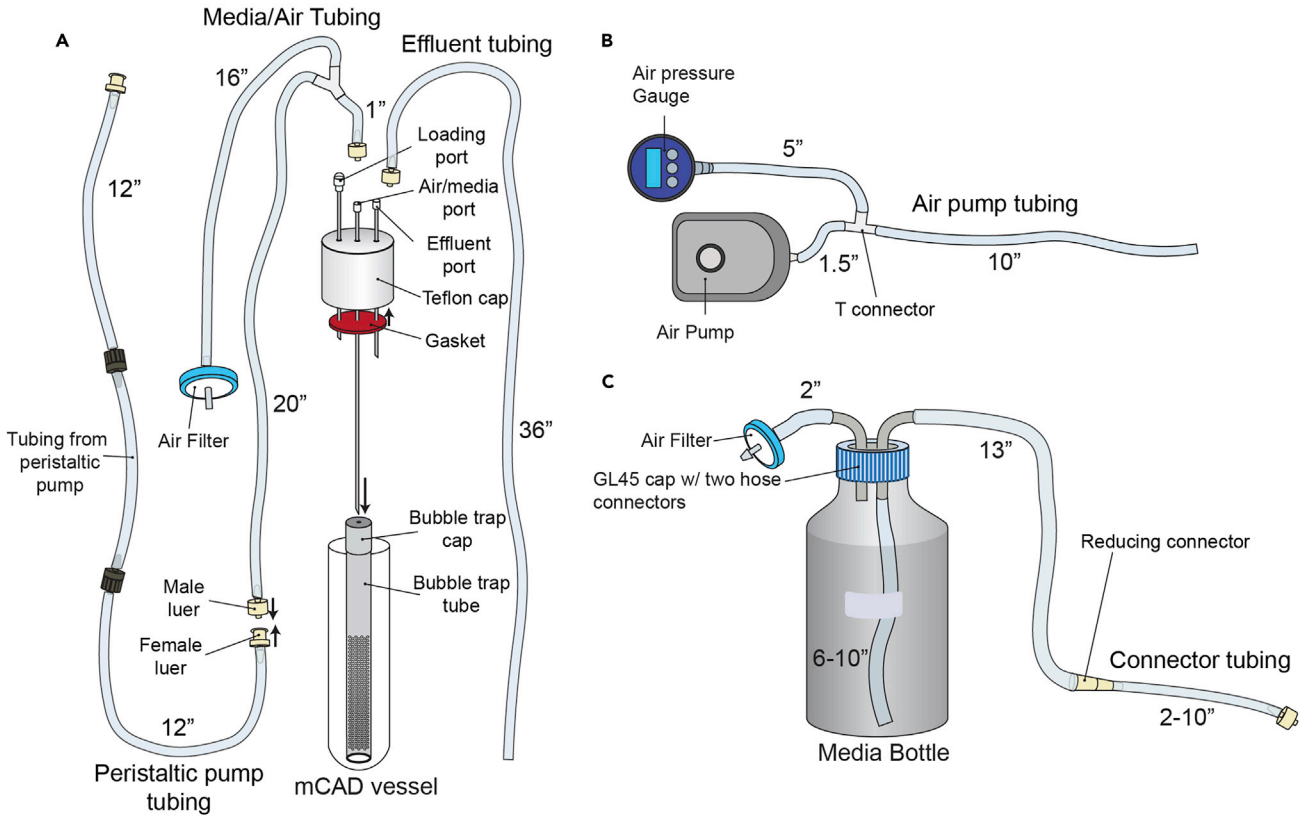

Figure 3. mCAD components

(A) mCAD vessel and associated tubing.

(B) Air pump and air pressure gauge assembly.

(C) Media bottle and associated tubing. Assembly of each component is described in “before you begin”.

ii. One short needle, the effluent port, should extend into the glass vessel until the tip of the needle is just above the level of the water in the glass vessel. The position of this needle is important to maintain the culture volume at 40 mL.

iii. The other short needle, the loading port, should extend into the glass vessel until the tip is ~0.5 cm above the water level.

k. Insert a male plug into the tip of the loading port needle on the outside of the mCAD vessel to block it.

l. Remove Teflon cap and decant water from the mCAD vessel.

5. Attach the tubing assemblies described in steps 1a–c to the mCAD vessel (Figures 2 and 3A):

a. Connect the male luer on the 1″ tubing of the media/air tubing assembly to the long needle in Teflon cap of the mCAD vessel.

b. Attach the male luer on the 20″ tubing of the media/air tubing to one end of the peristaltic pump silicone tubing.

c. Connect the other end of the peristaltic pump tubing to one end of the connector tubing.

d. Connect the effluent tubing to the effluent port in the Teflon cap of the mCAD vessel.

6. Autoclave the components of the mCAD (Figure 4).

a. 2 components are autoclaved:

i. The media bottle with cap and tubing connected to both hose connectors.

ii. The mCAD vessel (glass vessel, air bubble trap, Teflon cap with long and short needles), and tubing connected to the mCAD vessel (media/air tubing with air filter and effluent tubing).

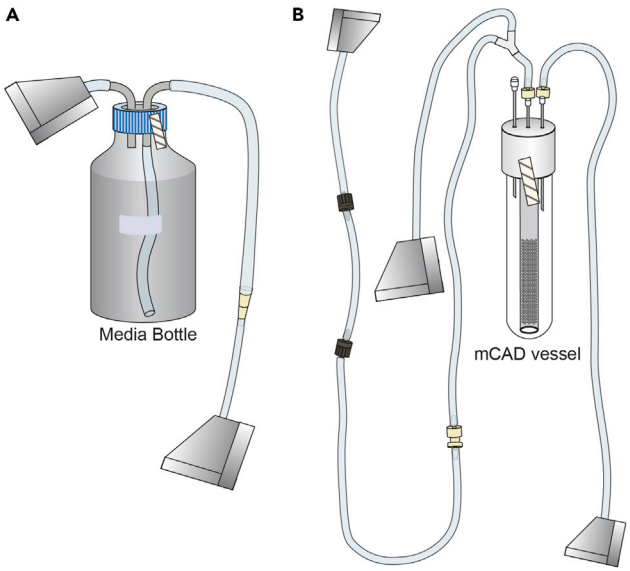

Figure 4. Preparation of mCAD components for autoclaving
(A and B) Using origami method to autoclave media bottle assembly (A) and mCAD device with associated tubing (B).

b. Place the GL-34 cap to the media bottle and its associated tubing on the media bottle (Figure 3C). Tighten the cap to secure it to the media bottle, but do not completely tighten the cap. Use autoclave tape to connect and secure the cap to the media bottle (Figure 4A).
c. Wrap all air filters and the free ends of all tubing with aluminum foil. The foil origami method described in the Dunham lab Chemostat Manual (pg. 19) works well.
d. Autoclave the mCAD vessel and media bottle assemblies using the liquid cycle for 20 min.
e. Instructions for the final assembly of the mCAD are described below (step-by-step method details, step 30).

⚠ CRITICAL: Do not autoclave media in the media bottle with its associated tubing. Sterile media is added immediately before use (see below).

⚠ CRITICAL: Plastic connectors, luers, and plugs can be deformed by autoclaving using the dry cycle, which can lead to leaks in the mCAD.

⚠ CRITICAL: Remove items immediately after the autoclave cycle is completed.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Wheat Germ Agglutinin, Alexa Fluor™ 488 Conjugate	Thermo Fisher Scientific	#W11261
Wheat Germ Agglutinin, Alexa Fluor™ 594 Conjugate	Thermo Fisher Scientific	#W11262
EZ Link Sulfo-NHS-LC-LC-Biotin	Thermo Fisher Scientific	#21338
Bacto Yeast Extract	BD Difco	#DF0127179
Bacto Peptone	BD Difco	#DF0118170
Bacto Agar	BD Difco	#DF0145170
Yeast nitrogen base (YNB) containing ammonium sulfate without amino acids	BD Difco	#DF0919073

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Adenine sulfate	Sigma-Aldrich	#A9126
Uracil	Sigma-Aldrich	#U1128
L-tryptophan	Sigma-Aldrich	#T8941
L-histidine hydrochloride	Sigma-Aldrich	#H5659
L-arginine hydrochloride	Sigma-Aldrich	#A8094
L-tyrosine	Sigma-Aldrich	#T8566
L-leucine	Sigma-Aldrich	#L8912
L-lysine hydrochloride	Sigma-Aldrich	#L8662
L-phenylalanine	Sigma-Aldrich	#P5482
L-methionine	Sigma-Aldrich	#M9625
Dextrose (D-(+)-Glucose)	Sigma-Aldrich	#G8270
Experimental models: Organisms/strains		
BY4741 (*MATa his3Δ0, leu2Δ0, met15Δ0 and ura3Δ0*)	Open Biosystems (Huntsville, AL)	
Software and algorithms		
FIJI	(Schindelin et al., 2012)	https://imagej.net/Fiji; RRID: SCR_002285
Other		
Dynabeads MyOne Streptavidin C1 beads	Thermo Fisher Scientific	#65001
Spectrophotometer	Beckman	BU530
Wide-field fluorescence microscope	ZEISS	Axioskop 2
Objective lens	ZEISS	100×/1.4 Plan-Apochromat
CCD camera	Hamamatsu	Orca-ER
LED illumination system	CoolLED	pE-4000
Excitation filter (GFP)	Chroma	ET470/40×
Excitation filter (mCherry)	Chroma	ET572/35×
Emission filter (eGFP/mCherry)	Chroma	59222
Falcon 50-mL conical centrifuge tube	Corning	352098
Microscope slides	Thermo Scientific	3050
Microscope coverslips	Thermo Scientific	3406
Carl Zeiss™ Immersol™ Immersion Oil	Carl Zeiss	444960
Incubator Shaker	New Brunswick Scientific	E24
DynaMag-2 Magnet	Thermo Fisher Scientific	12321D
2 L bottle with GL45 opening	PYREX	13952L
DURAN™ Screw Cap GL 45 with 2-hose connector	DWK Life Sciences	293102807
Tygon Sanitary Silicone Tubing, 3/32″ Inner Diameter, 7/32″ Outer Diameter, 1/16″ Wall Thickness	Saint-Gobain	ABW00005
Tygon Sanitary Silicone Tubing, 3/16″ Inner Diameter, 5/16″ Outer Diameter, 1/16″ Wall Thickness, 50′ Length	Saint-Gobain	ABW00012
Masterflex Fitting, Polypropylene, Y connector, hose barb union 3/32″ ID	Cole-Parmer	EW-40726-42
Masterflex Fitting, Polypropylene, Tee, hose barb union connector, 3/16″ ID	Cole-Parmer	EW-40627-70
Masterflex Fitting, Polypropylene, Straight, Hose Barb Reducer, 1/4″ ID × 1/8″ ID	Cole-Parmer	EW-30616-55
Male Luer to 1/8″ L Barb Adapter, PP	Cole-Parmer	EW-30800-24
Female Luer to 1/8″ L Barb Adapter, PP	Cole-Parmer	SK-30800-08
Male Luer Lock Plug, PC	Cole-Parmer	UX-45504-56
Nalgene Syringe Filter, PES, 0.45 µm	Thermo Fisher Scientific	725-2545
Pharmacia peristaltic pump variable speed P-1 with silicone tubing	Pharmacia	
Hydrofarm AAPA3.2L 2-Watt 3.2-LPM Active Aqua Air Pump with 1-Outlet	Hydrofarm	
Digital pressure gauge, 300 psi	SSI Technologies	MGA-300-A-9V-R
Hold 20–25 mm tubes, 80 slots. Fisherbrand™ Poxygrid™ Test Tube Racks	Fisher Scientific	S71785
PYREX 55 mL Screw Cap Culture Tubes	Corning	9825-25
16 gauge with Kel-F Hub, Specs: Length: 7 inch / Point Style: 4 / Angle: 45 °	Hamilton	7750-07

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
16 gauge with Kel-F Hub, Specs: Length: 5 inch / Point Style: 4 / Angle: 45 °	Hamilton	7750-07
Air bubble trap tube/cap	Zera Development	SBT001REV1
Teflon cap	Zera Development	SBT001REV1
Silicon gasket	Zera Development	SBT001REV1
Neodymium Magnet 2″ OD × 1″ ID × $^1/_4$″ thick, grade N42	Applied Magnets	NR019
BD Luer-Lok 1-mL syringe	BD	309628
2 L flask	PYREX	4980-2L

Alternatives: Tubing and tubing connectors can be substituted with similar products from other sources, as long as the substituted items are autoclavable (e.g., Polypropylene [PP], Teflon [PTFE], silicone or polycarbonate [PC]).

Alternatives: The 2-L media bottle and bottle cap with 2-hose connectors can be substituted with a carboy and rubber stopper (e.g., Kimble KIMAX Aspirator Bottles with Tubulation [Fisher cat# 02-973-1D] and Twistit Rubber Stoppers, No 6 [Fisher cat# 14-132D]).

MATERIALS AND EQUIPMENT

Yeast Extract Peptone Dextrose (YPD) yeast agar plates		
Reagent	Final concentration	Amount
Bacto Yeast Extract	1% (w/v)	10 g
Bacto Peptone	2% (w/v)	20 g
Bacto Agar	2% (w/v)	20 g
Dextrose	2% (w/v)	20 g
ddH_2O	N/A	To 1 L
Total	**N/A**	**1 L**

Autoclave the mixture with a magnetic stir bar using a liquid cycle (121°C and 0.5 bar) for 20 min. Stir the solution gently at 20°C–22°C until it cools to ~55°C. Pour ~25 mL per plate in a sterile environment (near a Bunsen burner or in a biological hood.) Leave plates at 20°C–22°C overnight (8–16 h). Store plates in plastic bags with media side up at 4°C. The shelf life of YPD plates is 1–2 months at 4°C.

Synthetic complete glucose-based yeast liquid media (SC)		
Reagent	Final concentration	Amount
Difco Yeast nitrogen base without amino acids	0.67% (w/v)	16.75 g
Dextrose	2% (w/v)	50 g
Adenine sulfate (2 mg/mL stock in 0.05 M HCl)	20 mg/L	25 mL
Uracil (2 mg/mL stock in 0.5% $NaHCO_3$)	20 mg/L	25 mL
L-Tryptophan (2 mg/mL stock in H_2O)	20 mg/L	25 mL
L-Histidine hydrochloride (1 mg/mL stock in H_2O)	10 mg/L	25 mL
L-Arginine hydrochloride (1 mg/mL stock in H_2O)	10 mg/L	25 mL
L-Tyrosine	30 mg/L	75 mg
L-Leucine (10 mg/mL stock in H_2O)	100 mg/L	25 mL
L-Lysine hydrochloride (3 mg/mL stock in H_2O)	30 mg/L	25 mL
L-Phenylalanine (5 mg/mL stock in H_2O)	50 mg/L	25 mL
L-Methionine (2 mg/mL stock in H_2O)	20 mg/L	25 mL
ddH_2O	N/A	To 2.5 L
Total	**N/A**	**2.5 L**

Dissolve dry components in 800 mL of ddH_2O and adjust pH to 5.5 with $NaHCO_3$ (5% w/v). Bring volume to 1 L with ddH_2O and sterilize by autoclaving (liquid cycle: 121°C and 0.5 bar for 20 min) Store for several weeks at 20°C–22°C.

⚠ CRITICAL: To avoid caramelization of dextrose in media, remove all media from the autoclave immediately after the autoclave cycle is completed.

⚠ CRITICAL: YPD cannot be used in the mCAD because it contains large amounts of biotin, which will quench the streptavidin beads.

Note: If the strain of interest contains a plasmid with an auxotrophic marker, drop-out SC media should be used to maintain the plasmid.

Phosphate-Buffered Saline (1× PBS, pH 7.4)		
Reagent	Final concentration	Amount
NaCl	137 mM	8 g
KCl	2.7 mM	0.2 g
Na_2HPO_4	8 mM	1.14 g
KH_2PO_4	1.5 mM	0.2 g
ddH_2O	N/A	To 1 L
Total	**N/A**	**1 L**

Dissolve all dry components in 800 mL of ddH_2O and adjust pH to 7.4. Bring volume to 1 L with ddH_2O and sterilize by autoclaving (liquid cycle: 121°C and 0.5 bar for 20 min) 1× PBS can be filter-sterilized.

Polyethylene glycol (PEG) 3350, 50% (w/v)		
Reagent	Final concentration	Amount
PEG 3350	50% (w/v)	50 g
ddH_2O	N/A	To 100 mL
Total	**N/A**	**100 mL**

Dissolve PEG in 50 mL of ddH_2O and bring volume to 100 mL with ddH_2O. Sterilize by autoclaving (liquid cycle: 121°C and 0.5 bar for 20 min) 50% PEG3350 can also be filter-sterilized.

STEP-BY-STEP METHOD DETAILS

Generation of mid-log phase yeast cultures for biotinylation

Timing: 3–4 days

Several days before

Timing: 1 day

1. Streak out the strains of interest on YPD plates from frozen stocks.
2. Seal plates with parafilm and incubate media side up at 30°C for 2–3 days, or until colonies are evident.
3. Cells, which are freshly prepared from frozen stock, should be used for mCAD-based studies.

Note: If strains of interest harbor plasmid(s), cells should be streaked on selective media.

Note: Plates can be stored, sealed in parafilm, at 4°C for up to 4 weeks.

1 day before

4. Prepare liquid pre-culture.

a. In the morning, inoculate several colonies of yeast cells into 5 mL of synthetic complete glucose-based (SC) liquid media in a 50-mL Falcon tube. Loosen the cap on the Falcon tube to ensure proper aeration during growth.
b. Incubate in an orbital shaker with rotation at 200 rpm at 30°C for 7–8 h.
c. In the evening of the same day, measure the OD_{600} of the preculture. It should be mid-log phase (OD_{600} = 0.1–0.3).

5. Prepare liquid culture for mCAD.
   a. Calculate the amount of the pre-culture needed to generate a mid-log phase culture (~0.5–1 × 10^7 cells/mL, or OD_{600} = 0.1–0.3) after propagation overnight (8–16 h). For SC media, the doubling time is roughly 2 h. The volume (V) of pre-preculture is calculated by the formula listed below:

$$\text{Final } OD_{600} \times 25 \text{ ml} = \text{Preculture } OD_{600} \times 2^{growth\ time/doubling\ time} \times V$$

   b. Inoculate 25 mL of SC medium in a 100 mL sterile shake flask with the appropriate amount of the pre-culture.

Note: The doubling time of wild-type yeast cells in SC liquid media is typically 2 h. To obtain 25 mL mid-log phase culture (OD_{600} = 0.2) after a 16-h (overnight) incubation, add 65 μL of a pre-culture (OD_{600} = 0.3) to 25 mL SC.

$$Final\ 0.2\ OD_{600} \times 25\ ml = 0.3\ OD_{600} \times 2^8 \times Volume$$

   c. Grow at 30°C with orbital shaking at 200 rpm for 16 h.

Biotinylation of mid-log phase progenitor cells

Timing: 10 h

Below, we describe a protocol to biotinylate the cell wall of mother cells and their associated buds from the mid-log phase culture prepared as described above. Daughter cells generated from biotinylated yeast will not have biotin on their surface. Biotinylated progenitor cells are isolated using streptavidin-conjugated magnetic beads.

6. Allow EZ-Link Sulfo-NHS-LC-LC-Biotin, which is stored at −20°C, to come to 20°C–22°C.
7. Weigh 2 mg of Sulfo-NHS-LC-LC-Biotin and dissolve it in 0.5 mL of PBS in a 1.5 mL Eppendorf tube. Rotate the tube at 20°C–22°C until the biotin is fully dissolved (5–10 min).

Note: Adjust the amount of Sulfo-NHS-LC-LC-Biotin based on the OD_{600} of cells. For >4 OD_{600} of cells, use 0.5 mg Sulfo-NHS-LC-LC-Biotin per 1 OD_{600} of cells.

Note: Sulfo-NHS-LC-LC-Biotin is moisture-sensitive. Seal the cap of the jar containing the Sulfo-NHS-LC-LC-Biotin with parafilm and store it in a sealed container with desiccant at −20°C. Keep the Sulfo-NHS-LC-LC-Biotin container sealed and within the dessicant-containing storage container when it is brought to 20°C–22°C to reduce moisture condensation.

⚠ CRITICAL: Sulfo-NHS-LC-LC-Biotin does not readily dissolve in aqueous solution. Thus, cells should be transferred to the tube containing biotin-PBS solution to allow any residual undissolved Sulfo-NHS-LC-LC-Biotin to dissolve during sample biotinylation.

8. Measure the OD_{600} of the overnight pre-culture. Calculate the volume of culture corresponding to 4 OD_{600}-mL (e.g., if the OD_{600} is 0.2, use 4/0.2 = 20 mL of culture). Transfer 4 OD_{600} of cells to a fresh 50 mL Falcon tube or a sterile SS34 centrifuge tube.

9. Concentrate cells by centrifugation at 1,500 × *g* in a Sorvall ST 16 centrifuge (or equivalent) for 5 min at 20°C–22°C. Remove supernatant carefully with a P1000 pipet.
10. Wash cells twice with 5 mL of 1× PBS containing 0.25% PEG3350.
    a. Add 5 mL 1× PBS containing 0.25% PEG3350 to the Falcon tube and resuspend cells thoroughly.
    b. Concentrate cells by centrifugation and remove supernatant as in step 9.
    c. Repeat steps 10a–b.
    d. Resuspend final cell pellet in 0.5 mL of 1× PBS.
11. Add resuspended cells to biotin solution from step 7.
12. Rotate the biotin-cell mixture in an orbital mixer for 30 min at 20°C–22°C.
13. Concentrate biotin-treated cells by centrifugation at 3,600 × *g* in a microcentrifuge for 1 min.
14. Carefully remove the supernatant.
15. Wash cells once with 1 mL of 1× PBS containing 100 mM glycine. The glycine quenches any residual Sulfo-NHS-LC-LC-biotin.
    a. Add 1 mL of 1× PBS containing 100 mM glycine to the cell pellet obtained in step 14.
    b. Repeat steps 13–14.
16. Wash cells twice with 1 mL of 1× PBS containing 0.25% PEG3350 as described in step 15.
17. Resuspend biotin-labeled cells in 1 mL of growth media (SC).
18. Transfer resuspended cells to 500 mL of SC media in a 2-L sterile shake flask.
19. Grow cells at 30°C with orbital shaking at 200 rpm for 8–14 h. Cells should be in the mid-log phase and the culture should not exceed OD_{600} = 0.3.

Note: Calculate how much time is required to propagate biotin-labeled cells to the desired density in step 19. We typically use 4 OD_{600}-mL of cells for biotinylation, and obtain 50–100 OD_{600}-mL of cells after propagating biotinylated cells in 500 mL SC for ~10 h.

20. Harvest biotin-labeled cells:
    a. Sterilize surroundings and gloves with 70% ethanol. Use a Bunsen burner to maintain a sterile environment.
    b. Transfer the 500 mL of liquid culture to 10 50-mL Falcon tubes or the appropriate number of sterilized reusable centrifuge tubes/bottles.
    c. Concentrate cells by centrifugation at 1,500 × *g* in a Sorvall ST 16 centrifuge (or equivalent) for 5 min at 20°C–22°C.
    d. Carefully decant supernatant.
    e. Resuspend each cell pellet in 2 mL of SC and pool all cell suspensions in a 50-mL Falcon tube. The total volume of the pooled cells should be 20 mL.

⚠ CRITICAL: When handling yeast cell culture, use sterile techniques (step 20a).

Isolation of biotinylated progenitor cells using streptavidin-coupled magnetic beads

Timing: 2 h

21. Wash Dynabeads MyOne Streptavidin C1 3 times with growth media (SC).
    a. Transfer 108 µL of Streptavidin Dynabeads to a 1.5-mL Eppendorf tube.
    b. Add 1 mL of SC and vortex for >30 s.
    c. Place the tube in a magnetic test tube rack (e.g., DynaMag-2) for 1 min to immobilize magnetic beads.
    d. Discard the supernatant.
    e. Repeat steps 21b–d 2 times.

Note: According to the manufacturer's instructions, 0.9 µL of beads are used per 10^6 labeled cells. We typically start with 4 OD_{600}-mL cells (1.2 × 10^8 cells) and use 108 µL of beads.

Note: Dynabeads MyOne Streptavidin C1 auto-fluorescence upon illumination under conditions that excite GFP, which may interfere with weak green fluorophores. We did not encounter this issue in the budding polarity assay. However, to reduce interference by autofluorescence, the dynabeads can be photobleached before binding to yeast cells (Roth et al., 2019).

⚠ CRITICAL: Take steps to ensure that the area around the mCAD is sterile (steps 22–28).

22. Resuspend the washed Streptavidin Dynabeads in 0.5 mL of SC.
23. Transfer resuspended beads to the biotinylated cell suspension in the 50-mL Falcon tube (step 20, "Biotinylation of mid-log phase yeast").
24. Incubate the 50 mL Falcon tube in a rotary mixer for 15 min at 20°C–22°C.
25. Place ring magnets between the middle and top tiers of a poxygrid rack suitable for 50 mL Falcon tubes (Figure 1).
26. Transfer cells to a sterile mCAD glass vessel. Place the vessel within ring magnets in the poxygrid tube rack and let stand for 5–10 min to ensure that magnetic streptavidin beads and their associated cells are immobilized.

Note: The ring magnet and poxygrid test tube rack can be substituted with a magnetic test tube rack that holds 50-mL Falcon tube and has a magnet of sufficient strength for this application (e.g., DynaMag-50, Invitrogen).

Note: To promote binding of the magnetic beads to the ring magnet, gently tap and rotate the glass vessel.

27. Carefully remove all material that is not bound to the ring magnet using a 10-mL pipet.
28. Wash immobilized cells twice with SC.
    a. Remove the glass vessel from the ring magnets and add 40 mL of SC to the glass vessel.
    b. Place the glass vessel back into the ring magnets. Let stand for 5–10 min and then carefully remove media from the glass vessel using a 10-mL pipet.
    c. Repeat steps 28a–b.
29. Resuspend cells in 1–2 mL of SC media.

Note: Association of biotinylated cells with magnetic streptavidin beads can be monitored by light microscopy (Figures 5 or 7D).

Setting up the mCAD

Timing: 30 min for setting up; 3–4 days for running a typical aging study

Here, we describe assembly of the mCAD and test running the assembled mCAD before inoculating beaded progenitor cells to check for leaks. The mCAD should be assembled and filled with media before the progenitor cells are incubated with streptavidin-dynabeads and dynabead-bound cells are isolated. Dynabead-bound cells should be introduced into the mCAD immediately after isolation.

Note: We operate the mCAD in at room temperature (20°C–22°C) in a temperature-controlled room. Since strong magnets are used in the mCAD, do not use heat blocks for temperature control.

30. Assemble the mCAD (Figures 1 and 2).
    a. The mCAD should be assembled on laboratory bench (ca 2.5 × 2.5 ft) that has a shelf above the bench for the air pump/pressure gauge assembly (Figure 1).

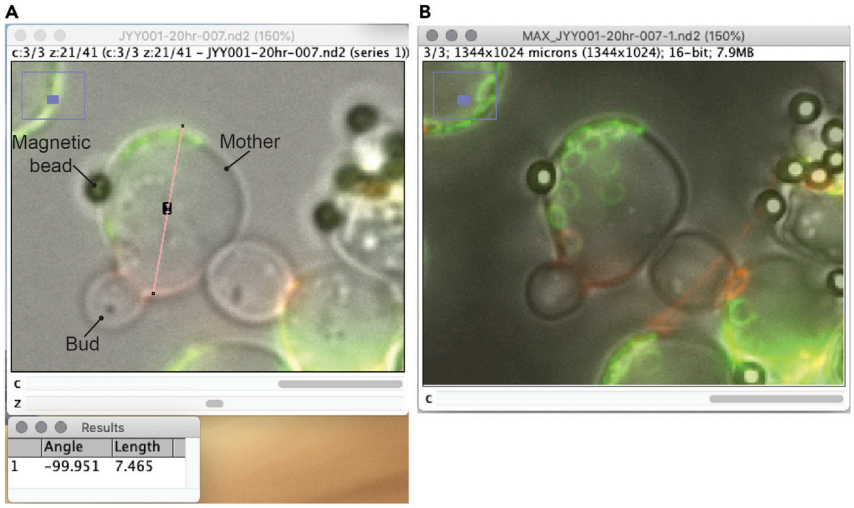

Figure 5. Analysis of cell size and replicative age (bud scars quantitation)

(A) Screenshots of steps 67 (b) and (e). The length of a mother cell is measured by straight line tool labeled with pink outline.

(B) Screenshots of step 67 (c). A maximum projected view of the cell in (A).

b. Wear gloves. Sterilize gloves and the bench with 70% ethanol. Use a Bunsen burner to maintain a sterile environment.

c. Place ring magnets between the middle and top tiers of a poxygrid tube rack suitable for 50 mL Falcon tubes (Figure 1).

d. Remove all aluminum foil and autoclave tape from sterilized mCAD components (Figure 4).

e. Carefully open the sterile 2-L media bottle and add 2 L of sterile SC media. Place the GL45 cap and its associated tubing on the media bottle and tighten it.

f. Tighten the Teflon cap on the mCAD vessel and place the sterilized mCAD vessel into the ring magnets in the poxygrid rack. The ring magnets should be 1–2 cm below the top of the media level in the mCAD vessel (Figure 1).

g. Place the 2 L waste flask on a surface at least 1 ft (30.5 cm) below the bench to ensure that effluent media from the mCAD vessel flows into the waste flask. Insert the free end of the effluent tubing from the mCAD vessel into the waste flask and secure the tubing to the flask with tape.

h. Connect the air filter on the 16″ tubing of media/air tubing on the mCAD vessel (Figure 3A) to the free end of the 10″ tubing on the air pump and gauge assembly (Figure 3B).

i. Connect the female luer on the free end of the peristaltic pump tubing (Figure 3A) to the 2–10″ connector tubing on the media bottle (Figure 3C).

j. Insert the peristaltic pump tubing into the peristaltic pump such that media flows from the media bottle through the peristaltic pump into the mCAD vessel (Figure 1).

k. Tighten the caps on the media bottle and mCAD vessel.

31. Test the mCAD system.

a. Turn on the peristaltic pump (~250 mL/h, speed 7, flow rate x10 for a GE Healthcare peristaltic pump P-1) and air pump. Media should flow from the media bottle through the peristaltic pump to the mCAD vessel. After the mCAD vessel fills with ca 40 mL of media, media should flow from the mCAD vessel out the effluent port into the waste flask.

b. After the mCAD is filled, set the flow rate to ~ 25 mL/h (speed 7, flow rate x1.).

⚠ CRITICAL: Make sure that there are no leaks at connections or caps and that there is consistent positive pressure within the mCAD. If not, there will be no effluent and excess SC will leak from other parts of the set-up. When the system is set up correctly, the total volume of media in the mCAD vessel should be 40 mL.

32. Prepare the mCAD for cell transfer.
    a. Turn off the air and peristaltic pumps. Clamp the effluent tubing with a tubing clamp.
    b. Transfer the mCAD vessel from the ring magnets in the poxygrid rack to a magnet-free location in the poxygrid rack.
    c. Remove the cap from the loading port on the mCAD vessel.
33. Use a 1-mL syringe to transfer streptavidin beads and their associated biotinylated cells from the 50-mL Falcon tube (step 29) to the loading port. After the transfer, flush some air into the loading port using a sterile syringe to ensure that all cells are in the medium within the mCAD vessel.
34. Place the cap on the loading port.
35. Place the mCAD vessel into the ring magnets and let stand for 5–10 min to immobilize the magnetic beads and their associated biotinylated progenitor yeast cells.

Note: Occasionally tapping and rotating the glass vessel or turning on the air pump at its lowest setting promotes circulation and binding of magnetic beads and their associated cells to the ring magnets in the mCAD vessel.

36. Remove the clamp from the effluent tubing and ensure that the caps on the media bottle and mCAD vessel are tight closed.
37. Turn on the air and peristaltic pumps.
    a. The air pump should be set to its highest pressure (0.7–0.8 PSI).
    b. Set the flow rate on the peristaltic pump to ~ 25 mL/h (For a GE Healthcare-peristaltic pump P-1: speed 7, flow rate x1.).
38. Let the system run for 24–56 h. Check the system daily for leaks and ensure that there is sufficient media in the media bottle.

Note: With a flow rate of 25 mL/hr, ~600 mL of media is used each day. Thus, 2–2.5 L SC media is needed for a 3-day experiment.

Harvest desired populations of yeast cells

Timing: 1 h

Harvesting daughter cells

39. When harvesting the daughter cells, use a fresh 50-mL Falcon tube to collect young cells from the effluent tubing.

Harvesting an aliquot of biotinylated mother cells from the mCAD

40. Turn off both pumps. Clamp the effluent port with a tubing clamp.
41. Remove the mCAD vessel from the ring magnet and swirl to resuspend cells in the media.
42. Remove the cap from the loading port and tilt the vessel to submerge the needle tip of the loading port into the media. Use a 5- or 10-mL Luer-lock syringe to withdraw cells from the mCAD vessel.

Note: The amount of culture drawn from mCAD depends on how many time points are needed in a time-course experiment. For a 3-day-long experiment, we recommend collecting 13 mL of cells on the first day, 20 mL on the second day, and 40 mL on the last day.

43. Transfer harvested cells to a fresh sterile glass vessel. Insert the vessel into the ring magnets and allow the system to stand for 5–10 min.
44. Carefully remove all material that is not bound to the ring magnet using a 10 mL transfer pipet.
45. Wash immobilized cells twice with 40 mL of SC.

46. Remove the glass vessel from the ring magnets. Wash the walls of the glass vessel and resuspend cells in 1–2 mL of SC media.

Harvesting all biotinylated cells from the mCAD

47. Carefully remove the Teflon cap, tubing, and bubble cage from the mCAD glass vessel without disturbing cells that are immobilized within the vessel.
48. Remove media from the mCAD glass vessel using a 10 mL pipet.
49. Remove the ring magnets and resuspend cells in the glass vessel with 30 mL of SC media.
50. Place the glass vessel in the ring magnets and let stand for 5–10 min to immobilize biotinylated mother cells.
51. Repeat steps 48–50 at least 4 times.
52. Resuspend washed cells in 1 mL of SC media.

Note: The purity of the biotinylated mother cells can be monitored by staining with AlexaFluor-conjugated WGA and light microscopy (Figure 5). When the purity of the biotinylated mother cells is high, the majority (50%–70%) of cells recovered should be old cells.

Bud scar staining with AlexaFluor-conjugated WGA

Timing: 3 h

Budding yeast undergoes asymmetric cell division to produce progeny (mother and daughter cells) which are not identical. During this process, a bud site is selected on the surface of a mother cell, and the cytoskeleton is polarized to that site to ensure the transfer of all cellular constituents from the mother cell to the developing daughter cells. Bud site selection is one hallmark of cell symmetry breaking during polarity establishment in asymmetric cell division. In haploid yeast cells, which undergo axial or unipolar budding, new bud sites form adjacent to the bud site from the previous round of cell division (Chant and Pringle, 1995). The bud scar, a chitinous ring on the cell wall of the mother cell that forms where buds separate from mother cells, is both a landmark of bud site selection during polarity establishment in asymmetric cell division and a marker for the number of times a mother cell divides (i.e., replicative age). Here, we describe a method to determine the polarity of bud site selection during the yeast aging process by staining bud scars generated during consecutive rounds of cell division with two colors of AlexaFluor-conjugated WGA. This method is inspired by the TrackScar labeling technique (Maxwell and Magwene, 2017).

To monitor age-associated changes in cell polarity during asymmetric cell division, we monitor bud site selection in cells of defined replicative age isolated using the mCAD. Cells are initially stained with AlexaFluor 488-WGA to label all bud scars. They are then propagated for 1 round of cell division (2 h) and stained with AlexaFluor 594-WGA to label the 2 newest bud scars. For haploid cells, bud site selection is scored as polarized if the 2 newest bud scars are adjacent to each other, and random if those bud scars are not adjacent.

53. Transfer 0.5–1 OD_{600}-mL of isolated yeast cells in 1 mL of SC medium to a fresh 1.5-mL Eppendorf tube.
54. Add AlexaFluor 488-WGA (2 μL of 1.09 mM stock in 1 mL), mix and stain cells for 15 min at 20°C–22°C.
55. Concentrate cells by centrifugation at 3,600 × *g* in a microcentrifuge for 1 min at 20°C–22°C.
56. Carefully remove the supernatant.
57. Wash cell pellet 3 times with SC media.
    a. Resuspend cell pellet in 1 mL of SC.
    b. Concentrate cells by centrifugation at 3,600 × *g* in a microcentrifuge for 1 min.
    c. Carefully remove the supernatant.

d. Repeat steps a-c 2 times.
e. Resuspend the washed cell pellet in 1 mL of SC.

58. Transfer cells to a 50 mL Falcon tube and dilute cells to a final volume of 5 mL.
59. Grow cells at 30°C with orbital shaking at 200 rpm for 2 h.
60. Concentrate cells by centrifugation at 1,500 × *g* in a Sorvall ST 16 centrifuge (or equivalent) for 5 min. Remove supernatant and resuspend cells in 1 mL SC.
61. Stain cells with AlexaFluor 594-WGA for 15 min, wash, and resuspend as for step 57.

Note: Bud scars can be stained by blue-emission WGA-AlexaFlour 350 or far-red-emission WGA-AlexaFlour 647. However, WGA conjugated with different colors of AlexaFlour must be used for sequential staining. If replicative age is the only desired factor to measure, bud scars can be stained with calcofluor white (Pringle, 1991).

Live-cell imaging

Timing: 3–4 h

62. Concentrate stained cells by centrifugation at 3,600 × *g* for 1 min at 20°C–22°C.
63. Remove supernatant, leaving 10–20 μL supernatant in the tube.
64. Resuspend the pellet in the residual medium.
65. Transfer 1.8 μL of the cell suspension to a microscope slide, and cover with a #1.5 coverslip.

⚠ CRITICAL: For optimal imaging, cells must be resuspended in the appropriate volume of supernatant to ensure that cell density is low enough to distinguish individual budding yeast cells and high enough to capture 10–15 cells per image during image acquisition (ca 0.2–0.4 OD_{600} per mL or 3,000–6,000 cells per μL). Please refer to (Liao et al., 2020b) for an illustration of the appropriate liquid: pellet ratio.

⚠ CRITICAL: Each slide should be imaged for 10 min or less. In the method described, cells are suspended in a small volume of medium (1.8 μL) for imaging; therefore, the preparation may dry out or nutrients may be exhausted by cells within a short period of time (~10 min). For long-term imaging, cells can be mounted in a culture dish and covered by an agar pad, as described in (Liao et al., 2020a).

66. To image bud scars, locate a field of cells and acquire a z-series using the following settings and excitation/emission filters:
    a. WGA-AlexaFluor 488: excitation/emission 470/507 nm, 50% power, typical exposure time: 300 ms.
    b. WGA-AlexaFluor 594: excitation/emission 561/610 nm, 50% power, typical exposure time: 300 ms.
    c. 1 × 1 binning (If the camera chip has sufficient pixels, 2 × 2 binning may be used. Final pixel dimensions should be < 0.1 μm.).
    d. 21–27 z sections with 0.3 μm spacing (a total depth of 6–8 μm should be sufficient to capture the entire cell for young yeast cells. Since yeast cells increase in size with age, the number of optical sections per z-series should be increased for older cells.).
    e. Acquire AlexaFluor 594 and AlexaFluor 488 z-stacks sequentially for greater speed and stability.

Note: Power settings and exposure times need to be adjusted based on signal intensity. We typically acquire 2–3 images per slide, and 5–6 slides per sample to image 100 cells.

67. Analyze cell size, age, and cellular polarity. The method described uses ImageJ (Schindelin et al., 2012) for these measurements.

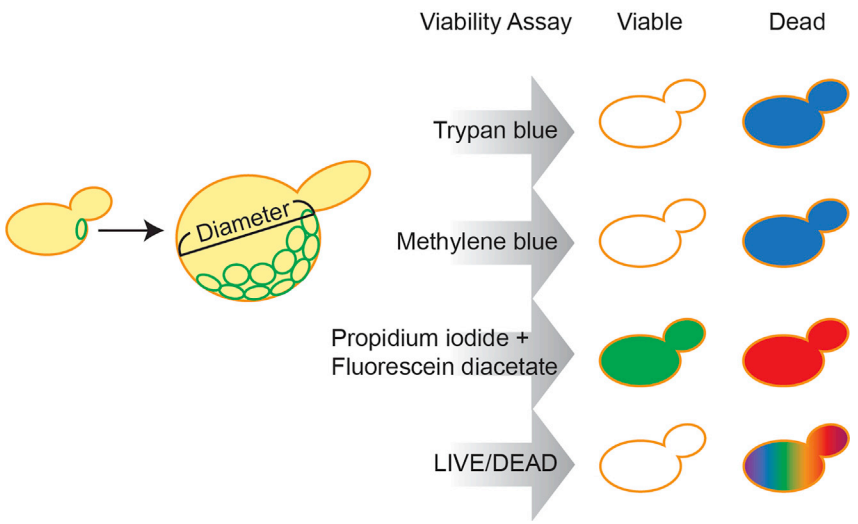

Figure 6. Methods to determine cell viability

Cells can be stained by trypan blue, methylene blue, propidium iodide + fluorescein diacetate, or LIVE/DEAD dye. The outcome from the viability is illustrated on the right panel.

a. Open microscopic images in FIJI using the Bio-Formats importer: ***Plugins*** → ***Bio-Formats*** → ***Bio-Formats Importer.***
b. Make composite: ***Image*** → ***Color*** → ***Make Composite.*** Adjust visible channels by ***Channel tool***… (Figure 5A).
c. Make a maximum projection image: ***Image*** → ***Stacks*** → ***Z project***… Select ***Max intensity.*** (Figure 5B).
d. Define the unit: ***Analyze*** → ***Set Scale…*** In our system, 1 pixel = 0.0645 μm.
e. Use the ***Straight line*** tool to measure the diameter of the mother cell. ***Analyze*** → ***Measure*** (Figure 5A).
f. Use Z-projected image and Z stack image to count bud scars. Score at least 50 cells for each age group per genotype.

EXPECTED OUTCOMES

The recovery of cells of defined replicative age from a single mCAD device can be >5 million cells (0.5 $O.D._{600}$). The efficacy of the mCAD can be verified by determining the age, size, and viability of isolated cells. Biotinylated progenitor cells isolated from the mCAD exhibit aging phenotypes including increased abundance of bud scars, increased cell size and decreased cell viability, as a function of the time of propagation in the mCAD (Figures 7A–7C) (Yang et al., 2022). The viability of yeast cells can be determined by trypan blue, methylene blue, propidium iodide/fluorescein diacetate, or LIVE/DEAD staining and counted by hemacytometer (Figure 6) (Kwolek-Mirek and Zadrag-Tecza, 2014). WGA-AlexaFlour or Calcofluor White can be used to assess buds scars and replicative aging or age-associated declines in polarity (Figure 7E) (Yang et al., 2022). Isolated cells can be analyzed by light microscopy (Sing et al., 2022; Yang et al., 2022) as well as transcriptomic, proteomic or epigenetic approaches (Hendrickson et al., 2018).

LIMITATIONS

Dynabeads MyOne Streptavidin C1 is auto-fluorescent upon illumination with blue light used for excitation of GFP. This autofluorescence may interfere with weak green fluorophores. To reduce the autofluorescence and increase the signal-to-noise ratio, beads can be photobleached before the experiments (Before step 21) (Roth et al., 2019).

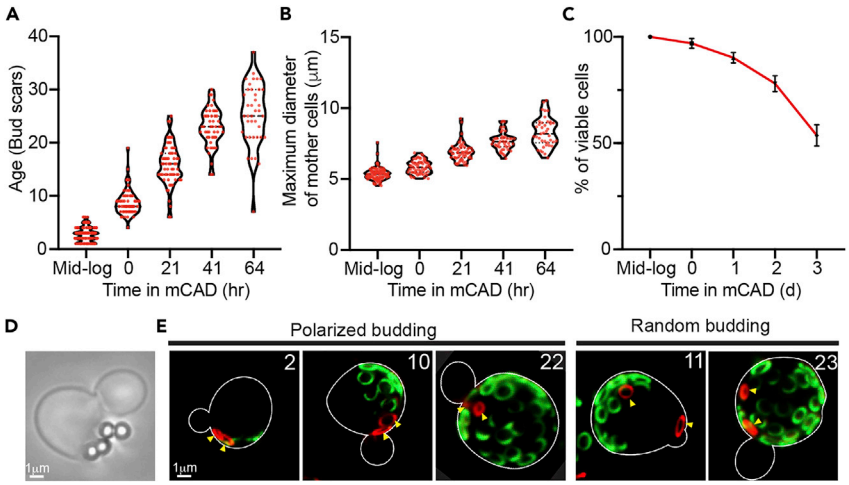

Figure 7. Isolation of cells of defined replicative age using the miniature chemostat aging device

(A) Age of mother cells during a 64-h mCAD time course. Age was determined by counting bud scars stained by AlexaFluor-WGA. Average age ± SEM of mother cells at different time points: 2.75 ± 0.136 (mid-log), 8.79 ± 0.294 (0 h), 15.83 ± 0.467 (21 h), 22.84 ± 0.505 (49 h), 25.26 ±0.937 (64 h). n > 40 for each time point.

(B) Diameter of mother cells during mCAD time course. Average diameter (μm) ± SEM of mother cells at different time points: 5.34 ± 0.041 (mid-log), 5.85 ± 0.057 (0 h), 6.90 ± 0.082 (21 h), 7.66 ± 0.0942 (45 h), 8.3 ± 0.162 (64 h). n > 40 for each time point.

(C) Average percentage viability of mother cells from 3 independent mCAD experiments. Average percentage ± SEM of viable cells: 96.93 ± 2.313 (0 d), 90.24 ± 2.415 (1 day), 78 ± 3.675 (2 d), 53.7 ± 4.975 (3 d).

(D) Representative images of magnetic beads attached to a mother cell.

(E) Representative images of the budding pattern of cells of different replicative age. Replicative age (number in right corner of each image) was determined by scoring the number of WGA-stained bud scars. Older bud scars were labeled with Alexa488-WGA (green). Yellow arrowheads: new Alexa594-WGA-stained bud scars (red). n = 3 independent trials. Figure is adapted from (Yang et al., 2022).

For preparation of slides for imaging, cells are maintained in a small amount of medium (1.8 μL). This small volume of media is sensitive to desiccation and may be depleted of nutrients with long-term imaging or incubation. Therefore, these conditions can only be used for the short-term imaging conditions described. For long-term imaging, please refer to the agar pad method described in (Liao et al., 2020a).

The mCAD method to enrich aged yeast cells is based on a method to non-invasively biotinylate yeast cells and to use magnetic streptavidin-coupled beads to separate the biotinylated progenitor cells from their young progeny (Sinclair and Guarente, 1997). To isolate yeast of advanced replicative age (>20 generations), the isolation protocol must be repeated several times to obtain enough aged cells. Another system described in (Janssens et al., 2015) immobilizes progenitor cells in a magnetic column supplied with constant media flow to wash away daughter cells. However, this method cannot control cell density and aeration during the aging process, and is not amenable to cell sampling during the aging process. The mCAD-based method is very robust and scalable, and cells age in environment in which aeration as well as the levels of nutrients and cellular by-products are carefully controlled.

TROUBLESHOOTING

Problem 1

The volume of media in the mCAD volume is too high (step 31 or steps 37 and 38). Air pressure may be too low or the effluent tubing may be clogged.

Potential solutions

- Check the effluent tubing for kinks or any blockage. Effluent tubing should be straight and led to a waste flask that is lower than the mCAD. Replace the tubing if the blockage cannot be removed.

- Check all luer connections, ports in the caps of the mCAD vessel or media bottle, and caps of the mCAD vessel or media bottle for blocks or leaks. Spray 70% ethanol around the suspected leaky areas: bubbles should be evidence at leak sites. Tighten caps or connectors or replace the tubing/luers, as necessary.

Problem 2

The volume of media in the mCAD volume is too low (step 31 or steps 37 and 38). The media tubing/bottle may be clogged by contamination or the peristaltic pump tubing may be leaking or clogged.

Potential solutions

- Check all luer connections, ports in the caps of the mCAD vessel or media bottle, and caps of the mCAD vessel or media bottle for blockage/leakage of liquid.
- Check the media bottle and all media tubing for contamination.
- Stop the peristaltic pump and remove the tubing to inspect any leakage. Replace with sterile pump tubing if necessary.

Problem 3

Insufficient aeration of the mCAD when the air pump is on (step 31 or steps 37 and 38). There may be an air leak at connections between the air pump and the mCAD vessel. Alternatively, the air filter may be wet.

Potential solutions

- Check all luer connections, the Teflon cap of the mCAD vessel, and the luer cap of the loading port. Spray 70% ethanol around the suspected leaks and look for bubbles at leak sites. Tighten connections, remove kinks or replace the tubing/luers, as necessary.
- Check the air filter on the air tubing. Replace it, if necessary.

Problem 4

Contamination of media reservoir during an experiment (step 38). The media may be contaminated prior to or during transfer to the media bottle, or mCAD device may not be fully sterilized. Alternatively, cells backflow into tubing after inoculation of the mCAD vessel may contaminant the media reservoir.

Potential solutions

- Use greater attention in sterile technique during media preparation and mCAD assembly.
- Maintain a positive pressure in the mCAD system during use. Be mindful of the backpressure and backflow when air/media pumps are not on.

Problem 5

Low yield of cells of defined replicative age from the mCAD (steps 39–51). The Sulfo-NHS-LC-LC-Biotin may have lower activity, or magnetic beads may have dried and lost binding activity. Wash conditions may be too stringent.

Potential solutions

- Use fresh Sulfo-NHS-LC-LC-Biotin and magnetic beads.
- Reduce the number of washes during purification.

Problem 6

Contamination by young cells in the mother cell samples isolated using the mCAD (steps 39–51).

Potential solution

- Increase the number of washes during purification.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Liza A. Pon (lap5@cumc.columbia.edu).

Materials availability

All strains or plasmids generated in this study are available from the lead contact.

Data and code availability

This study did not generate any unique datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, E.J.Y. and L.A.P.; validation, E.J.Y.; formal analysis, E.J.Y.; investigation, E.J.Y.; writing – original draft, E.J.Y.; writing – review & editing, E.J.Y. and L.A.P.; supervision, L.A.P.; project administration, L.A.P.; funding acquisition, L.A.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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