Video Article

# Flow Cytometry-based Purification of *S. cerevisiae* Zygotes

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## Abstract

Zygotes are essential intermediates between haploid and diploid states in the life cycle of many organisms, including yeast (**Figure 1**) [1]. *S. cerevisiae* zygotes result from the fusion of haploid cells of distinct mating type (MATa, MATalpha) and give rise to corresponding stable diploids that successively generate as many as 20 diploid progeny as a result of their strikingly asymmetric mitotic divisions [2]. Zygote formation is orchestrated by a complex sequence of events: In this process, soluble mating factors bind to cognate receptors, triggering receptor-mediated signaling cascades that facilitate interruption of the cell cycle and culminate in cell-cell fusion. Zygotes may be considered a model for progenitor or stem cell function.

Although much has been learned about the formation of zygotes and although zygotes have been used to investigate cell-molecular questions of general significance, almost all studies have made use of mating mixtures in which zygotes are intermixed with a majority population of haploid cells [3-8]. Many aspects of the biochemistry of zygote formation and the continuing life of the zygote therefore remain uninvestigated.

Reports of purification of yeast zygotes describe protocols based on their sedimentation properties [9]; however, this sedimentation-based procedure did not yield nearly 90% purity in our hands. Moreover, it has the disadvantage that cells are exposed to hypertonic sorbitol. We therefore have developed a versatile purification procedure. For this purpose, pairs of haploid cells expressing red or green fluorescent proteins were co-incubated to allow zygote formation, harvested at various times, and the resulting zygotes were purified using a flow cytometry-based sorting protocol. This technique provides a convenient visual assessment of purity and maturation. The average purity of the fraction is approximately 90%. According to the timing of harvest, zygotes of varying degrees of maturity can be recovered. The purified samples provide a convenient point of departure for "-omic" studies, for recovery of initial progeny, and for systematic investigation of this progenitor cell.

## Video Link

The video component of this article can be found at https://www.jove.com/video/4197/

## Protocol

### 1. Haploid Cell Growth

1. Grow *S. cerevisiae* cells from the S288C background (BY4741) under standard conditions [10].
2. Transform haploids to express either of two conspicuous fluorescent markers [10]. The markers of choice are soluble GFP expressed in the cytoplasm (pEG220, URA3/YIp, linearized with BglII [11]) and a single ribosomal protein, RPL25, fused with mCherry (pAJ1661, URA3/CEN, from A. Johnston). We expect that equivalent two-color purification protocols could make use of other brightly fluorescent proteins that are synthesized by the haploid parental cells. Alternatively, the cell wall could be labeled with fluorescent lectins.
3. On the day preceding zygote purification, inoculate 5 ml cultures of each cell type and grow them with shaking overnight at 23 °C to reach A600 not exceeding 1.0. Grow GFP transformants (ATY4442) in complete synthetic medium including 2% glucose (CSM-glucose) and RPL25-mCherry transformants (ATY4552) in synthetic selective medium lacking uracil [10]. Maintain parallel cultures of each cell type with shaking at room temperature.

### 2. Conducting a Cross

1. Adjust the optical density (A600) of each sample to 1.0 in fresh medium. [1 Optical Density Unit ~ 3 x $10^7$ cell/ml]
2. Mix equal volumes of the two cultures, vortex briefly 3 times, followed by one longer vortex for 10 sec.
3. Prepare CSM-glucose plates. The plates are identical to those used for routine growth and have been dried 45 min after pouring and solidification of the agar [10].

4. Distribute as many as twenty 40-ml droplets of the mixture 1 cm from the edge of the surface of CSM-glucose plates. When the droplets are first applied to the surface start the timer to measure time elapsed. Once the liquid has been absorbed (approximately 30 min), cover the plates and leave them undisturbed at room temperature.

## 3. Harvesting Preparations

1. Harvest samples after 1.75-2.5 hr at 23 °C by repeatedly washing individual dried droplets off the plate using 150 ml cold CSM-glucose, keeping the plates on ice. Repeatedly wash the droplet area 10-20 times with the same medium to maximize recovery. Collect in 15 ml Falcon tubes on ice.
2. Probe sonicate the samples 10x at 60% amplitude with a Fisher FB120 sonicator (120Watt, 120Volt, Freq 20KHz) on ice.
3. Verify in a phase microscope that few or no aggregates remain. 15-50% of the cells are expected to have formed zygotes at this point.
4. Leave the tube of cells in a vertical position undisturbed on ice for 10 min as a further precautionary measure (to allow aggregates to settle).
5. Recover the topmost 90% of the volume and filter it through a nylon mesh (BD Falcon Polypropylene tube with strainer cap (Ref 352235)). In a typical experiment, $10^7$ cells recovered from one plate were at this point diluted into 4 ml of CSM-glucose.

## 4. Flow Cytometry

1. Sort cells using the Sony iCyt Reflection Model Flow Cytometer, (Highly Automated Parallel Sort) HAPS1 with a 100 micron tip. For GFP excitation, use the blue argon-ion, 250 milliwatt laser at 488 nm. For mCherry excitation, use the 100 milliwatt laser at 561 nm. Although we do not have experience with other machines, we expect that Becton Dickinson FACSAria, Becton Dickson Influx or Beckman Coulter MoFlo XDP would be fine when equipped with the correct lasers.
2. Base sorting parameters on a forward vs. back scatter plot in order to gate on viable cells. Establish an emission plot of 615 +/- 30 nm bandpass filter (mCherry) versus 525 +/- 25 nm bandpass filter (GFP) to divide the events into four quadrants, "Red+ Green-", "Red+ Green +", "Red- Green-", and "Red- Green+" (**Figure S2**). Use nonfluorescent cells as a standard for determination of the "Red- Green-" quadrant.
3. Sort cells with a sheath pressure of 21.5 psi, a droplet frequency of 43,400 drops/sec, and a sample pressure of 20.5 psi.
4. **Initial Sort ("Recovery Mode"):** Collect samples into chilled Eppendorf tubes containing 250 ml of cold CSM-glucose. Sediment 10 sec at top speed in a refrigerated microfuge (Tomy MRX-150, 15,000 rpm) and aspirate the flow solution (Flow cytometry sheath solution (PBS with 1 mM EDTA) BioSure Catalog No. 1027). Resuspend in 500 ml cold CSM-glucose.
5. **Second Sort ("Purity Mode"):** Conduct the sort as for the initial sort, using the "purity mode" option. Sediment the purified cells and resuspend the pellets in 500 ml-1.0 ml CSM-glucose. Shake for 15 min in 24-well plates at 23 °C to allow metabolic recovery.

## 5. DeltaVision Microscopy

1. Prepare agarose pads as follows: Bring a suspension of 1.5% Agarose (UltraPure Agarose, Invitrogen, Catalog # 15510-07) in CSM-glucose to a boil, vortex briefly, and then apply 0.3 ml samples of the agarose solution to the surface of horizontal standard glass slides and cover them at once with a second slide (without applying pressure).
2. After cooling for 10 min, gently remove the upper slide by sliding it horizontally, taking care to leave a smooth surface and not to disturb the Agarose layer. (When the upper slide has been removed, the sample of cells should be applied within 1-2 min. When kept in a humid chamber at room temperature without removing the upper slide, pads can be stored for up to a day before use.)
3. Sediment samples and deposit 1 ml aliquots of the pellet on agarose pads (without touching the surface). Immediately cover the sample with a square No. 1 coverslip. Trim away excess agarose with a single-edged razorblade. Seal the preparations with Vaseline dispensed from a syringe.
4. Image using Deltavision (Applied Precision) with a 100x oil immersion objective without binning (Olympus UPlanApo 100x/1.40; ∞/0.17/ FN26.5).
5. Deconvolve z-stacks using the **softWoRx** 5.0.0 program (http://www.api.com/softworx.asp) and process minimally. Successive z-planes were generally collected at 0.2-0.4 micron intervals.

## 6. Representative Results

**Fluorescence intensity levels in transformed haploid cells:** The level of fluorescence in the haploids is illustrated in **Figure S1**. Before the cross, epifluorescent examination demonstrated that essentially all GFP transformants were green and that all the mCherry transformants, although often fainter, were red. To ensure that all cells used for the cross were indeed fluorescent, we used the subpopulation with the brightest fluorescent signal.

**Yield:** After the initial sort, the Red+ Green+ sample (**Figure S2**) typically includes 8-20% of the input number of cells and an excessive number of haploid cells, perhaps due to persistent cell-cell adhesion. Since high enrichment is not achieved with a single sort, the partially purified sample should be probe sonicated twice at 60% amplitude followed by re-sorting. (Recovery sorts typically last between 1-2 hr, while the second sort requires only 15 min.) Of the cells subjected to the second sort, the Red+ Green+ samples included 23-52% of the input cells.

**Phase microscopic examination:** Examination by phase contrast microscopy showed that the purity of the final zygote preparation averaged approximately 90% over 21 preparations (ranging from 86% to 91%). The contaminants were either green or red haploids, or pairs of haploids that were in contact with each other. **Figure 3** tabulates the percent of zygotes that have no bud, medial buds (Med), lateral buds (Lat) or terminal buds (Term), the number of paired haploids of the same color ("2Same") or different color ("2Hap"), as well as individual haploids (Hap). When cells were harvested after 1.75 hr, more than 50% were unbudded and the other subcategories (with medial, lateral, or terminal buds) were equally represented. After 2.5 hr there were approximately equal numbers of zygotes in each of four categories (unbudded, with medial, lateral or terminal buds).

**Microscopic Observations (Figure 4):**

The structure of zygotes appears to remain unchanged during purification. According to the duration of the cross, the population had different proportions of unbudded and budded zygotes, as explained above. Since both the green and red haploids showed considerable variation in fluorescent signal intensity, some zygotes were predominantly red while others were predominantly green, as is observed by comparing the single-color images to the combined images in **Figure 4**. Note also that the GFP (MW 26kDa) enters the nucleus and can be found in the nucleus even when the nuclei have not fused. Vacuoles stand out as black non-fluorescent spheres.

The end product of purification yields a diverse population at various stages of development. Zygotes labeled A have not undergone fusion. Type B zygotes are unbudded. Type C have a small bud. Type D have a larger bud, and Type E zygotes are about to undergo cytokinesis. Note also that many of the earlier zygotes still exhibit a non-fluorescent medial division line (*), and that the vacuolar "inheritance structure" (I) [12] can often be seen to extend from the zygote into the bud. In some cases, the green signal has redistributed into the partner to a much greater extent that the red signal. This reflects the delay of transfer that is characteristic of polysomes (Tartakoff, in preparation).

Starting with a pair of 5 ml logarithmic cultures of haploid cells, we recover approximately 1-2 x $10^6$ zygotes, which is sufficient for many immunoblots, using chemiluminescent detection procedures. Using phenol-chloroform isolation protocols we extract approximately 1.5 mg of RNA from this number of cells.

Since the purified zygotes have been chilled, prior to further analysis we recommend incubation at 23 °C in growth medium.

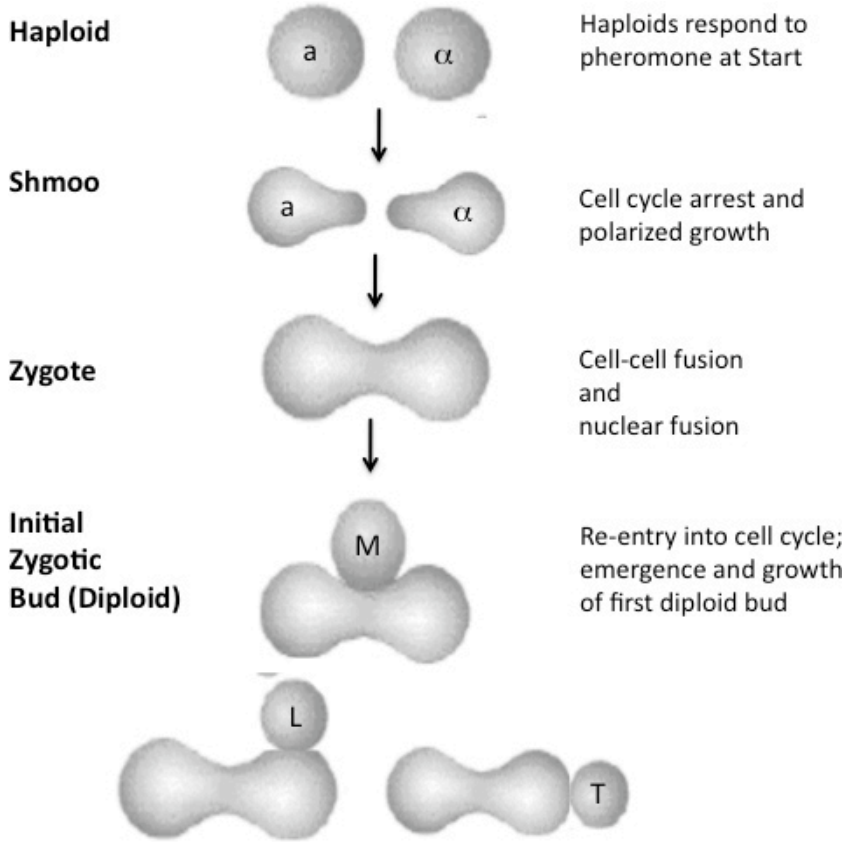


**Figure 1. Chronology of zygote formation.** Zygotes as an intermediate between haploids and diploids. This diagram shows the classical activation (shmooing) of haploids, their fusion, and the formation of the initial bud. Buds can be either medial (M), lateral (L) or terminal (T).

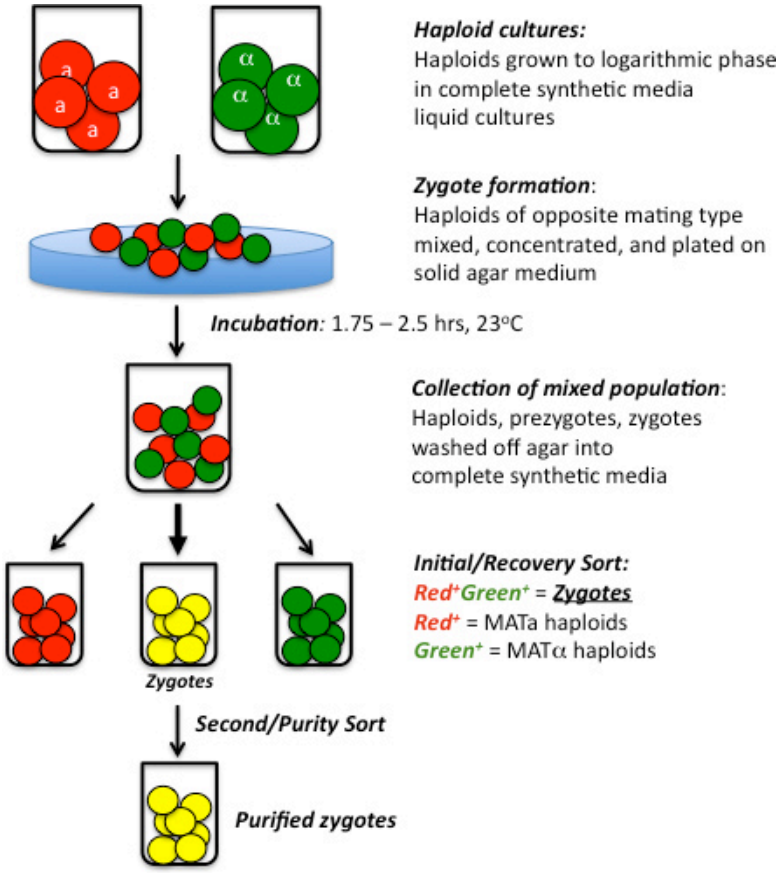


**Figure 2. Schematic of the zygote purification protocol.** After incubation on the surface of agar plates in complete medium with glucose, the cell mixture was recovered by washing and then subjected to an **initial sort** in "recovery mode" followed by a **second sort** in "purity mode." The yellow color signifies that both mCherry and GFP signals were visible in the zygotes.

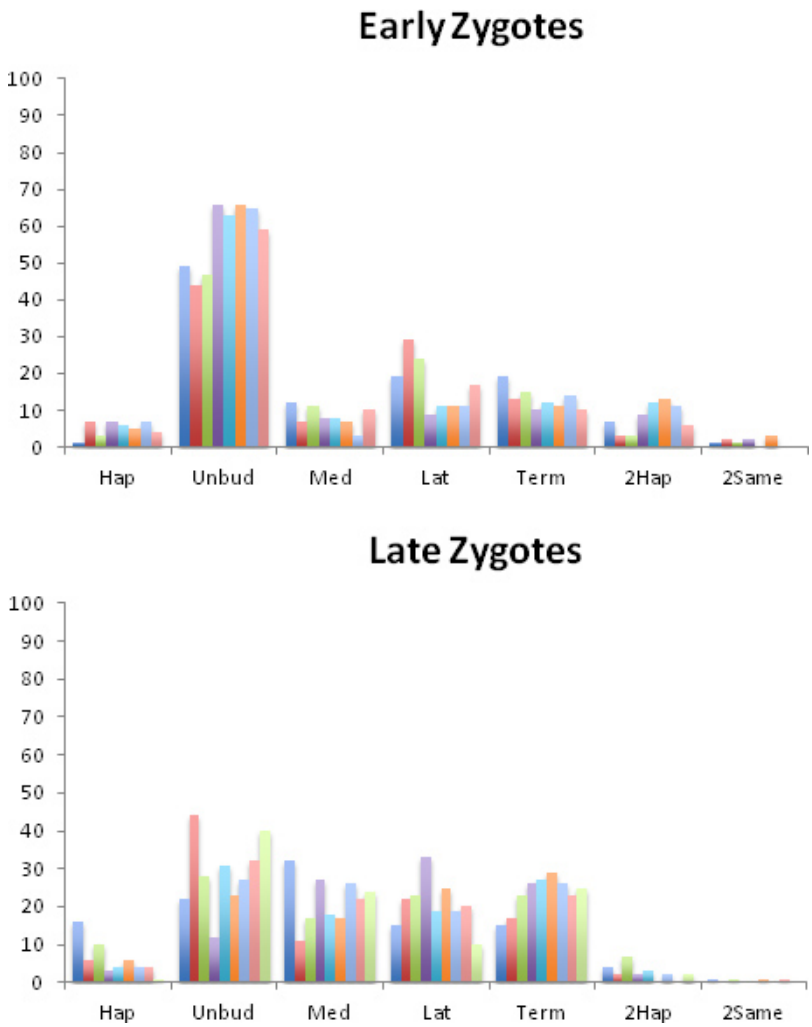


**Figure 3. Varieties of zygotes in the purified fractions.** Mating mixtures were harvested after 1.75 or 2.5 hr to recover fractions enriched in relatively early vs. relatively more mature zygotes. Eight independent experiments, designated by the distinct colors, were performed for each time point. The graphs indicate the relative numbers of zygotes that have spatially distinct patterns of bud formation. Given the brief duration of the incubation (less than 2.5 hr at room temperature) all buds observed are initial zygotic budding events.

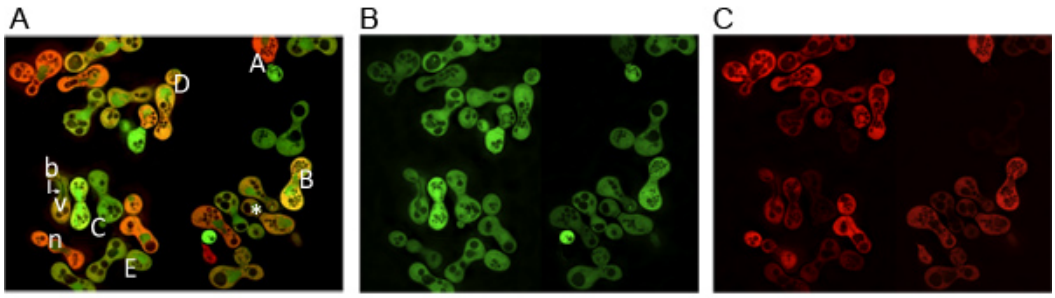


**Figure 4. DeltaVision examination of enriched fractions.** A typical field was examined by Deltavision microscopy. The illustrations show either both red and green or single colors. The red corresponds to the polysomal label (Rpl25p-mCherry) and the green to cytosolic GFP. Note the high enrichment of zygotes, and the designated subcellular features: the vacuoles(v), buds(b), nucleus(n), vacuolar "inheritance structure"(i), and the partition(*) which appears to separate the parental domains in relatively early zygotes. Their variable overall color reflects the relative intensity of the haploids that fused. In cases in which the two parental domains retain a distinct color, equilibration of the markers (cytoplasmic GFP, mCherry-tagged polysomes) was incomplete. Time-lapse studies in progress show that soluble GFP redistributes prior to polysomes. Click here to view larger figure.

**Supplementary Legends**

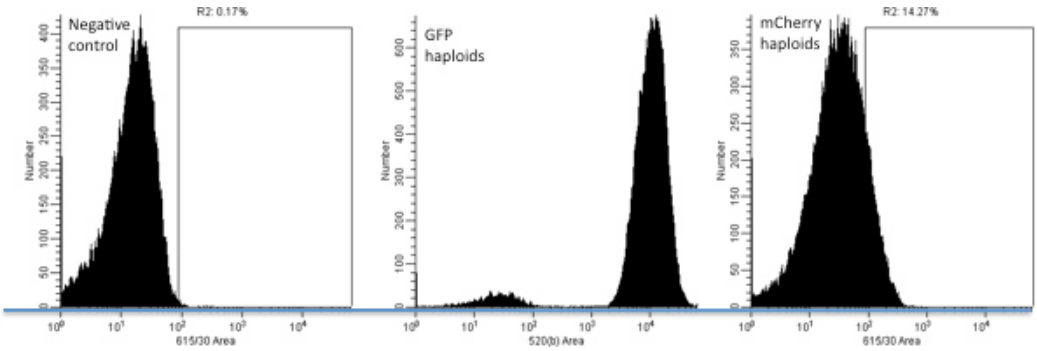


**Figure S1. Fluorescence of haploid cells.** Far left: Cells expressing mCherry-tagged polysomes were examined to detect a green signal. The rectangular window was set to avoid the negative cells. Middle graph: Cells expressing cytosolic GFP were analyzed, showing the distinctly bright major population of positives. Far right: Cells expressing mCherry-tagged polysomes were analyzed. The rectangle (identical to that on the far left) includes the brightest cells and allows all negatives to be avoided. The designation "R2" indicates the percentage of cells recovered within the indicated rectangle.

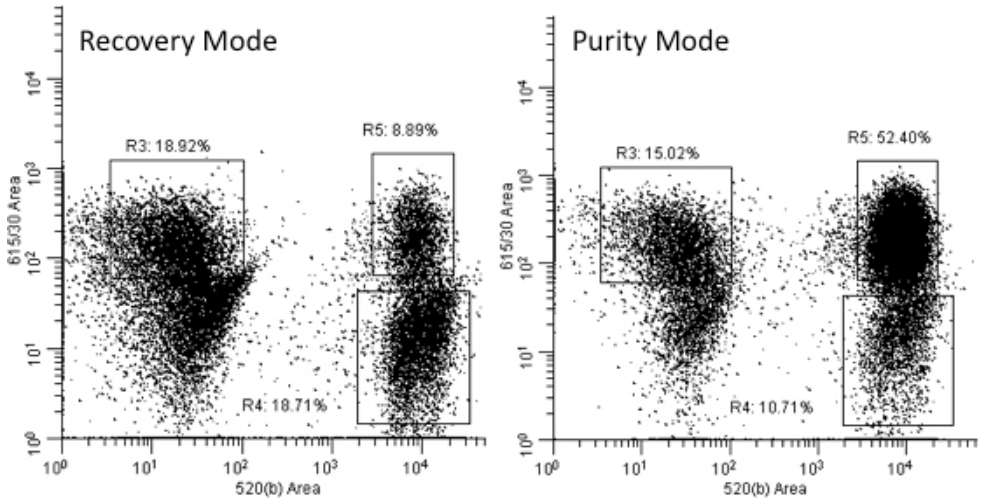


**Figure S2. Cell distribution in first and second sorting.** These two-color plots indicate the proportion of Green+ Red+ cells in the upper right quadrant (R5). This proportion increases to 52% in the second sort. Horizontal axis: green signal. Vertical axis: red signal.

## Discussion

The availability of purified zygotes should facilitate biochemical studies including transcriptomics, proteomics and lipidomics, as well as investigation of mechanisms by which zygotes undergo cell cycle re-entry, cell wall remodeling, budding and inheritance characteristics, etc. Alternatively, zygotes could be generated starting with strains carrying characteristic mutations in one or both parents. Moreover, the purified zygotes, as for haploid or diploid cells, can be frozen in medium containing 15% glycerol and later thawed to monitor aspects of recurrent budding.

Preliminary experiments using other fluorescent markers suitable for microscopic purposes (*e.g.* tagged histones or mitochondrial proteins) have not allowed reliable recovery of zygotes; however, it is likely that comparable purification could be achieved starting with haploid cells whose cell wall has been coupled to red or green fluorophores. We have avoided such procedures since they could impose additional stress and affect early stages of zygote formation.

## Disclosures

No conflicts of interest declared.

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