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Purification of G1 daughter cells from different *Saccharomyces* species through an optimized centrifugal elutriation procedure

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Abstract

Centrifugal elutriation discriminates cells according to their sedimentation coefficient, generating homogeneous samples well suited for genomic comparative approaches. It can, for instance, isolate G1 daughter cells from a *Saccharomyces cerevisiae* unsynchronized population, alleviating aging and cell-cycle biases when conducting genome-wide/single-cells studies. The present report describes a straightforward and robust procedure to determine whether a cell population of virtually any yeast species can be efficiently elutriated, while offering solutions to optimize success. This approach was used to characterize elutriation parameters and S-phase progression of four yeast species (*S. cerevisiae*, *Candida glabrata*, *Lachancea kluyveri* and *Pichia sorbitophila*) and could theoretically be applied to any culture of single, individual cells.

Keywords: yeast, Ascomyceta, elutriation, synchronization, replication, daughter cells

Introduction

DNA-related processes can be investigated using a combination of quantitative *in vivo* assays, ranging from fluorescence real-time imaging to molecular and biochemical assays eventually coupled to deep sequencing. Ideal studies in the field combine these complementary approaches, resulting data being processed through statistical analysis aimed at resolving stochastic cell-to-cell variability from significant variations. Reaching the highest level of homogeneity regarding the original biological sample is therefore an important starting point of these studies. Indeed, important information can be attenuated by background resulting from population heterogeneity. For instance, cellular differentiation can generate differences in replication timing [Hiratani et al. 2010], cell-cycle will affect chromosome three-dimensional organization (through changes in condensation state), or aging can lead to alternative responses to a stimulus [Laun et al. 2005].

Fission and budding yeasts are unicellular eukaryotes that multiply through clonal divisions. The size and compactness of their genomes proved ideally suited to the genomic era: from the beginning of genome-wide applications, they have conveniently been used in a number of DNA-metabolic processes studies, including gene expression, replication-timing profiling, ncRNA mapping, chromatin architecture analysis, etc. [Spellman, Sherlock, Zhang, Iyer, Anders, Eisen, Brown, Botstein, and Futcher 1998; Raghuraman et al. 2001; Pan, Yuan, Xiang, Wang, Sookhai-Mahadeo, Bader, Hieter, Spencer, and Boeke 2004; Dujon et al. 2004; Neil, Malabat, d'Aubenton-Carafa, Xu, Steinmetz, and Jacquier 2009; Duan et al. 2010]. In recent years, a growing number of complete genomes of Ascomycota, among the thousand of so identified so far, have become available. Yeast clades present broad variations in term of amino-acid divergence, chromosome number, cell size or chromosome architecture, and span very long evolutionary times [Dujon 2010]. Comparison between DNA-metabolic pathways and genome architecture among divergent species offers the unique opportunity to understand evolutionary mechanisms that may be at play for other eukaryotic lineages [Keeling, Burger, Durnford, Lang, Lee, Pearlman, Roger, and Gray 2005]. For instance, genome-wide nucleosome positioning was recently characterized for 13 *Saccharomyces* species during exponential growth and led to the identification of lineage-specific variability in the use of binding sites, broadening the understanding of the rules of chromatin organization [Tsankov, Yanagisawa, Rhind, Regev, and Rando 2011].

Experimental work using different species involves using conditions that allow the recovery of cell sample as homogeneous as possible to facilitate comparison. One well-suited option is to use synchronized yeast cells. Different methods are available, with sometimes outcomes difficult to interpret [Marguerat, Jensen, de Lichtenberg, Wilhelm, Jensen, and Bähler 2006; Spellman et al. 1998; Cooper and Shedden 2003]. Pheromone treatments or biochemical perturbations have proved effective in this regard. For instance, the use of the pheromone peptide α -factor from *S. cerevisiae* induces a G1

arrest in ~80% of the cells of a *Lachancea kluyveri* population, a phylogenetically “not-so-distant” species [Payen, Fischer, Marck, Proux, Sherman, Coppée, Johnston, Dujon, and Neuvéglise 2009]. However, this approach is not necessarily always successful. For instance, *Candida glabrata* does not respond to sexual pheromones [Muller, Hennequin, Gallaud, Dujon, and Fairhead 2008]. Synchronization can also be achieved through the use of temperature sensitive mutations of proteins involved in the control of cell-cycle progression. However, such approaches can be tedious and time consuming to transpose to species that have diverged from the two well-studied organisms *S. pombe* and *S. cerevisiae*. Overall, these treatments can introduce biases such as cellular/nuclear deformations or physiological responses that should be avoided if possible. Finally, and most importantly, none of these induced cell-cycle synchronization methods allow discriminating cells according to their age. As a result, daughter cells, although representing circa half of the population, will be mixed with mothers, grand-mothers, etc. and will generate an additional layer of complexity.

Such caveats can be alleviated with the use of centrifugal elutriation (CE), that discriminate cells according to their sedimentation coefficient (*i.e.* linked with their mass and shape). Asymmetric cell division of *S. cerevisiae* is associated with a size discrepancy between the daughter and mother cell. The resulting change in the sedimentation coefficient has been shown to be discriminated by CE, which has long been used to isolate G1 daughter cells from a *S. cerevisiae* population [Diamond 1991; Laun, Pichova, Madeo, Fuchs, Ellinger, Kohlwein, Dawes, Fröhlich, and Breitenbach 2001; Lesur and Campbell 2004]. In this article, we describe the steps needed for the optimal elutriation of several yeast species exhibiting different cell sizes and limited cell-to-cell adhesion. We show that ideal elutriation conditions need to be determined through the careful analysis of cell size variability during the cell-cycle, and that growth conditions are an amenable parameter to regulate cellular size changes if needed. We show that the populations obtained are highly homogeneous in both age and cell-cycle stage, and that the G1 elutriated fractions isolated through this procedure can either synchronously proceed through S-phase or, if cross-linked before elutriation, be directly treated through adequate protocols. Our approach allows for reproducible generation of homogeneous, high-quality biological yeast samples, and could also be used to identify some of the elutriation properties of cellular cultures from any species.

Material and Methods

Strains and Culture

We used the strains of *S. cerevisiae* (S288C and W303), *Candida glabrata* (CBS138), the derivative 1AKI001 of *Lachancea kluyveri* [Payen et al. 2009] and *Pichia sorbitophila* (CBS7064). Cultures of the various species are performed in YPD (glucose rich) medium, at 30°C, under agitation.

Elutriation

Elutriation is performed in a Beckman elutriation system (Avanti J-26 XP centrifuge combined with a JE-5.0 elutriator rotor and a 40 ml elutriation chamber, as well as standard elutriation accessories) using a method similar to the one described in [Manukyan, Abraham, Dungrawala, and Schneider 2011] with a few modifications. For exponential and stationary cultures, a colony is inoculated in 5ml of YPD medium and incubated overnight. The next day, for exponential cultures these 5 ml are transferred to 800ml of fresh medium, and grown overnight. For stationary phase culture, about $8 \cdot 10^{10}$ cells are directly pelleted, washed in 1X PBS and suspended in a final volume of 50 ml PBS 1X before being loaded in the elutriator flow chamber. For exponential culture, 2 x 2l of pre-warmed medium are inoculated at OD600 = 1 ($\sim 7 \cdot 10^6$ cells/ml in our conditions) and incubated for approximately 1.5 – 2 generations. Once OD600 ~ 3-4 ($2.1 - 2.8 \cdot 10^7$ cells/ml) about $8 \cdot 10^{10}$ cells are pelleted, washed in 1X PBS and resuspended in a final volume of 50 ml PBS 1X. The elutriation chamber is loaded with the cell suspension at a flow rate of ~ 25 ml/min (see Table 1) and a rotor speed of 2,500 rpm. Flow rate is gradually increased until cells reach the top of the chamber. Equilibrium is let to settle in the chamber for one hour, and then the flow rate is increased by increments of 2 ml/min, with 1l of cell suspension being recovered between each increment (fraction 1, 2... until 8). Fractions are then centrifuged, and cell pellets resuspended in 10 ml PBS 1X. Aliquot are collected for microscopy and cytometry analyses. Pellets are pooled, frozen in liquid nitrogen and stored at -80C. Elutriation parameters for all species are described in Table 1.

FACS analysis

About $5 \cdot 10^6$ cells are fixed in 2ml ethanol 70% and stored at 4°C for a maximum of 20 hours. Cells are then pelleted, washed with 2 ml 50mM sodium citrate (pH 7.4) and then resuspended in 0.2 ml 50mM sodium citrate (pH 7.4). 20 µl of RNase A (20mg/ml) are added to each tube and cells are incubated at 37°C for 1 h. Next, 0.8 ml of propidium iodide (50 µg/ml dissolved in 50mM sodium citrate [Sigma Aldrich]) is added and cells are incubated for an additional 30 minutes at 4°C. Cells are pelleted, resuspended in 2 ml 50mM sodium citrate (pH 7.4) and sonicated as for *S. cerevisiae*. Flow cytometry is performed on a MACSQuant Analyzer (Miltenyi Biotec) and data is analyzed using FlowJo software (Tree Star).

Calcofluor staining

About 10^6 cells are washed in 150 μ l H₂O, pelleted and resuspended in 100 μ l of Calcofluor solution (100 μ g/ml final, suspended in water). After 5 min of incubation at room temperature cells are washed twice with H₂O and finally resuspended in 20 μ l of H₂O. Cells are observed through fluorescence microscopy (Nikon inverted TI).

Replication restart

Fractions recovered in PBS 1X are centrifuged, and cell pellets are resuspended in 10 ml PBS 1X. Fractions are pooled and the amount of cells is determined from the cellular concentration measured through optical density (OD₆₀₀) and/or cell counting. In order to obtain a population as synchronized as possible it is recommended to discard the first fraction (that should not contain more than a $\sim 1-3 \times 10^8$ cells) and not to pool more than two fractions. Cells are subsequently pelleted and finally resuspended in 50 ml pre-warm YPD (30°C). Cells are transferred to a volume of pre-warm YPD (30°C) in order to reach a concentration of 7×10^6 cells/ml and incubated at 30°C under constant agitation. To perform S-phase time course 600 μ l of cell culture are collected every 10 minutes during 100 to 160 minutes and resuspended in 1.4 ml of 100% ethanol. Cell populations are then analyzed by flow cytometry.

Results and Discussion

1. Growth conditions allow to discriminate G1 vs. S + G2 cells according to cell shape

We used FACS analysis to quantify the G1 cells likely to be recovered through elutriation. Briefly, a rough estimation of the number of G1 and G2 cells was defined by splitting the propidium iodide (PI) fluorescence intensity histogram between the two peaks corresponding to these two populations. The PI value corresponding to that intensity was defined as gate 1 (Figure 1A, left panel). The G1 and G2 populations determined by gate 1 were then plotted according to their forward scatter (FSC) parameter (*i.e.* cell size) (Figure 1A, middle panel). The two distributions overlap over a large fraction of the FSC scale. However, for small FSC values up to an upper limit (defined as gate 2) a non-overlapping area representing small cells from the G1 population can be identified (darker area on the distribution). By reporting gate 1 and gate 2 values on the PI vs. FSC graph (cell density plot), one can therefore identify an area corresponding most likely of G1 cells that could be elutriated with minimal G2-S cells contamination (Figure 1A, right panel, shaded area 4). This area was determined on the cell density plots of exponentially growing cultures of *S. cerevisiae*, *L. kluyveri*, *C. glabrata*, and *P. sorbitophila*, four yeast species exhibiting different cell sizes and somehow different shapes (see Figure 2). The amount of cells present in area 4 was then quantified. While these supposedly pure G1 populations account for 9.5 % and 6.9 % of all *S. cerevisiae* and *L. kluyveri* cells, respectively, it drops to 1.9 % and 2.3% for *C. glabrata* and *P. sorbitophila* populations (Figure 1B, shaded areas). We performed an elutriation on these asynchronous cultures (Figure 1B, lower panel; Table 1). The first fraction recovered was highly enriched in G1 cells (>99 %; add area 3 and 4 on Figure 1B lower panel) for both *S. cerevisiae* and *L. kluyveri*, while we obtained a much lower enrichment rate for the two later species (*C. glabrata* ~ 81.8 % and *P. sorbitophila* ~ 92.3 % of G1 cells). Given the first fractions are the purest one (data not shown), the enrichment drops significantly lower and prevents the recovery of synchronized populations. Therefore, elutriation is able to segregate very efficiently G1 cells from *L. kluyveri* and *S. cerevisiae* but not from *C. glabrata* and *P. sorbitophila* populations (see also a direct application of elutriation to *L. kluyveri* in [Agier, Romano, Touzain, Lagomarsino, and Fischer 2013]). A simple flow cytometry analysis helps assessing the feasibility of elutriating G1 cells during exponentially growing culture of yeast species. If for a species of interest the proportion of G1 elutriable cells appears similar to those of *S. cerevisiae* or *L. kluyveri* then direct proceeding with elutriation can be envisioned. For other species, improving the efficiency of elutriation involves determining conditions where the size distribution of G1 cells would be temporarily modified.

2. Stationary phase cultures enrich the G1 population with cells exhibiting large size differences.

After a period of exponential growth in rich medium, glucose becomes limiting and yeast cells enter stationary phase. Most *S. cerevisiae* cells arrest in G1. We performed direct flow cytometry analysis on saturated cultures. Under these conditions, the fraction of G1 cells indeed significantly increases for all four species (Figure 1C, upper panel; cells under gate 1). Interestingly, the size of G1 cells appears to be affected as well by the limiting environment: an important shift towards smaller sizes was observed for *S. cerevisiae*, *L. kluyveri* and *P. sorbitophila*. For that later species, the proportion of cells now fitting within the gate defined previously now represent ~9 % of the total population (from 2.3 %). Surprisingly, the *C. glabrata* saturated culture now present a strong enrichment in small G1 cells, representing 25 % of the total population. To alleviate the potential effect of stationary phase on the metabolism, the cells were resuspended in fresh YPD and incubated at 30°C. Cells can grow for one hour without shifting significantly back to normal size distribution (data not shown). Elutriation performed at this moment allows the recovery of large, highly enriched fractions of G1 cells of *P. sorbitophila* (99.3% G1, from ~92.3%; Figure 1B, lower panel, add area 3 and 4) and *C. glabrata* cells (99.8% G1, from ~81.8%). The amount of cells recovered is indicated in Table 1.

3. For each species, G1 cells recovered in the first fraction consist in daughter cells.

The fluorescent dye Calcofluor binds and stains cellulose and chitin of fungi cell walls, allowing visualization of bud scars, hence determination of cell age. As expected, G1 cells from the first fractions recovered during *S. cerevisiae* elutriation consist almost exclusively of daughter cells, *i.e.* cells that have just separated from their mother and that represent 50% of the overall asynchronous population (Figure 2; Table 1). Indeed, these cells are smaller than G1 mother cells. For each of the first fractions recovered through elutriation, G1 cells of *P. sorbitophila*, *C. glabrata* and *L. kluyveri* where stained with calcofluor (Figure 2). Staining revealed that 80 to 96% of the recovered cells from these species exhibit a single scar, and therefore consist of daughter cells (Table 1). This observation shows that the approach described here is well suited for the recovery of yeast populations highly homogeneous in age and cell-cycle stage.

4. G1 cells recovered through elutriation proceed synchronously through S-phase.

Upon recovery, G1 fractions are pooled to the appropriate concentration and either stored at -80°C, processed through chromatin-related purification protocols (immunoprecipitation, capture of chromosome conformation, Chia-PET, etc.), or suspended in rich medium at 30 °C to proceed through S-phase. Replication occurs in a highly synchronous way when using the first $\sim 3 \times 10^9$ cells recovered as starting material (Figure 3A). Among the three new species tested here, only *C. glabrata* exhibits a small asynchrony in the restart of the G1 cells, although the overall S-phase progression remains

highly synchronous to a level never reached before. If more cells are needed we recommend discarding the first fraction recovered, which contains the most babyish cells. Indeed, given stochastic delays in G1 cell growth rates these smaller cells are likely to proceed through the cell cycle in an asynchronous manner [Talia, Skotheim, Bean, Siggia, and Cross 2007].

An alternative option is to pursue elutriation past the first G1 fractions to recover replicating cells (Figure 3B – see fraction 4 to 6). This alternative option is of special interest in case large amounts of cells are necessary, or if a species appears especially reluctant to replication restart. A similar approach has for instance been used to recover older G1 cells in the past (for instance, [Laun et al. 2001]). However, using this alternative approach will give much more heterogeneity in the population recovered than with S-phase restart, as seen from the overlapping distributions of the cells at each fraction (compare Figure 3A with Figure 3B). An advantage is that one can then cross-link cells prior elutriation during exponential growth, allowing highly samples reproducibility.

Centrifugal elutriation is a technique that conveniently isolates sub-populations of cells grown in liquid conditions based on small discrepancies in their physical properties. As shown above, this technique appears well suited for cells growing through asymmetric divisions such as budding yeasts. Using a systematic approach to analyze cell cultures of different species exhibiting different cell sizes, we showed that as for each tested species highly homogenous population of daughter G1 cells can be recovered. However, this may necessitate changes in growth / metabolic conditions to affect the cell sizes. These cells can then progress through S-phase in a highly synchronous manner. The approach presented here could also be used to identify whether and which subpopulations of cell cultures from species belonging to other clades could be isolated through CE.

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Table legends

Table 1 Parameters and settings for centrifugal elutriation experiments of the four hemiascomycetous yeast species, with expected recovery for each of the four tested yeast species.

(ND : Not Done)

Table 1

	Culture conditions					Elutriator loading settings			Cells recovery (fraction 1)		
	Culture	vol	inoculation (cells/ml)	incubation at 30°C	stop growing (cells/ml)	centrifuge speed (rpm)	pumping flow rate (ml/min)	total cells	Cells Total recovery	G1 cells	Single scar cells
<i>S. cerevisiae</i>	exponential	4l	7.10 ⁶	±3h30	2 – 3.10 ⁷	2,5	26	8 – 12.10 ¹⁰	3 – 5.10 ⁹	97%	96%
	stationnary (ND)										
<i>C. glabrata</i>	exponential	2L	8.10 ⁶	±3h	4 – 5.10 ⁷	2,5	26	8 – 10.10 ¹⁰	2 – 4.10 ⁹	1- 2%	ND
	stationnary	1L	8.10 ⁷	1H	8 – 10.10 ⁷	2,5	26	8 – 10.10 ¹⁰	2 – 4.10 ⁹	80%	80%
<i>L.kluyverii</i>	exponential	4l	7.10 ⁶	±3h30	2 – 3.10 ⁷	2,5	26	8 – 12.10 ¹⁰	2 – 4.10 ⁹	95%	92%
	stationnary (ND)										
<i>P.sorbitophila</i>	exponential	4l	7.10 ⁶	±4H	2 – 3.10 ⁷	2,5	26	8 – 12.10 ¹⁰	1 – 2.10 ⁹	6- 8%	ND
	stationnary	4L	2-3.10 ⁷	1H	2 – 3.10 ⁷	2,5	26	8 – 12.10 ¹⁰	1 – 2.10 ⁹	96%	87%

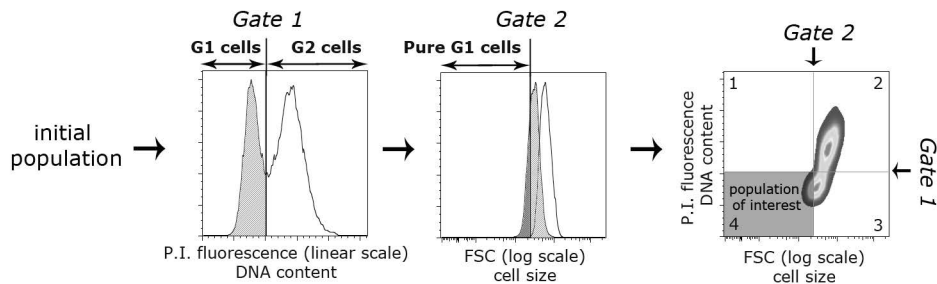
Figure Legends

Figure 1 Determination of the feasibility of yeast elutriation. (A) Determination of the gates used to quantify the amount of G1 cells elutriable from a population. Left panel: histogram of propidium iodide (PI) fluorescence, reflecting DNA content, of an asynchronous population of *S. cerevisiae*. Gate 1 corresponds to the PI value in-between the two peaks reflecting the distribution of G1 and G2 populations. Middle panel: distribution, according to their FSC, of the G1 and G2 populations defined by gate 1. Gate 2 corresponds to the FSC value under which the FSC of the G1 population defined by gate 1 does not overlap with the FSC of the G2 population. Right panel: flow cytometry diagrams of the same population, with the X axis corresponding to the FSC and Y axis represents PI fluorescence. The two gate values mark four areas, labeled from 1 to 4. Area 3 and 4 contains the G1 cells as defined by Gate 1, but only area 4 (shaded) represents the G1 population whose cell size does not overlap with sizes of cells present in the G2 population (including most S-phase cells). For the four species *S. cerevisiae*, *L. kluyveri*, *C. glabrata* and *P. sorbitophila*, flow cytometry diagrams of an asynchronous (B, upper panel) and a stationary phase (C, upper panel) populations were plotted, with gates 1 and 2 characterized as described above. Grey areas represent the G1 cells whose sizes do not overlap with G2 population and therefore elutriable with minimal contamination. The proportion of cells within each area is indicated. Flow cytometry diagrams of the first elutriated fraction for each population are plotted in the lower panels. Each scatter plot is divided into four areas.

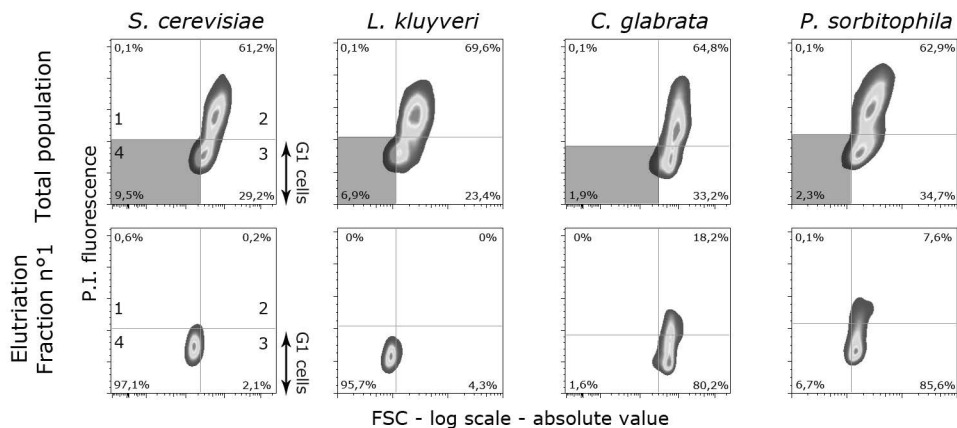
Figure 2 Fluorescent imaging of the cell wall and bud scars of an asynchronous population (A) and the first elutriated fraction (B) for the four species. Scale bars = 5 μm .

Figure 3 Flow cytometry histograms of elutriated cells. X axis correspond to PI intensity fluorescence (reflecting the DNA content). C stands for 1 genome DNA content (haploid or diploid), 2C for 2 genome DNA content. (A) Elutriated G1 daughter cells as they proceed through the S phase for the four species. Histograms of the asynchronous (AS) populations used to realize the different elutriation are indicated. Time points (in minutes) are indicated, with 0' corresponding to restart at 30°C in YPD. (B) Flow cytometry histogram of asynchronous (AS) population, and the corresponding elutriation first fractions (labeled from F1 until F6) for the four species.

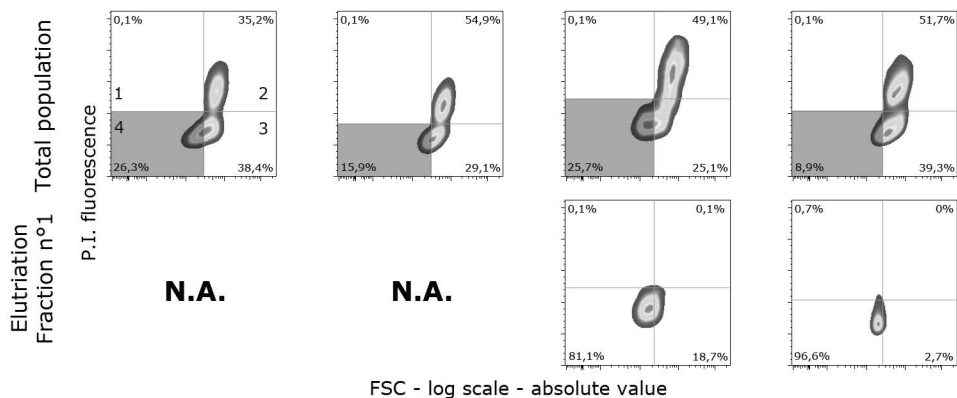
A Gate determination

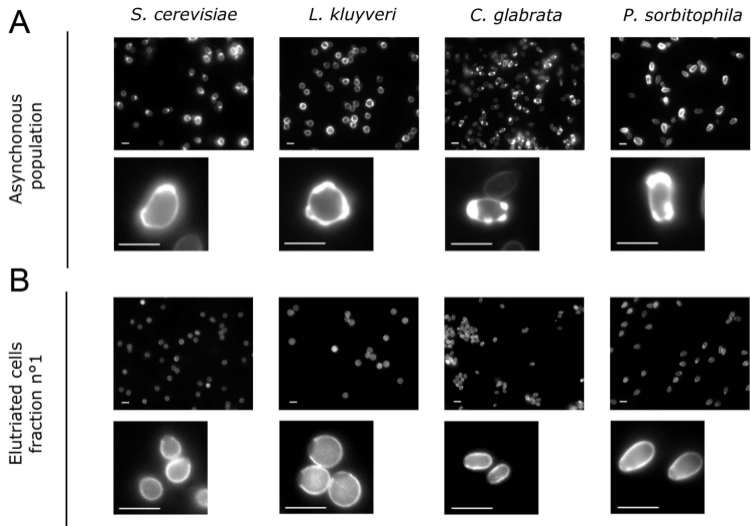


B Asynchronous populations



C Saturated cultures





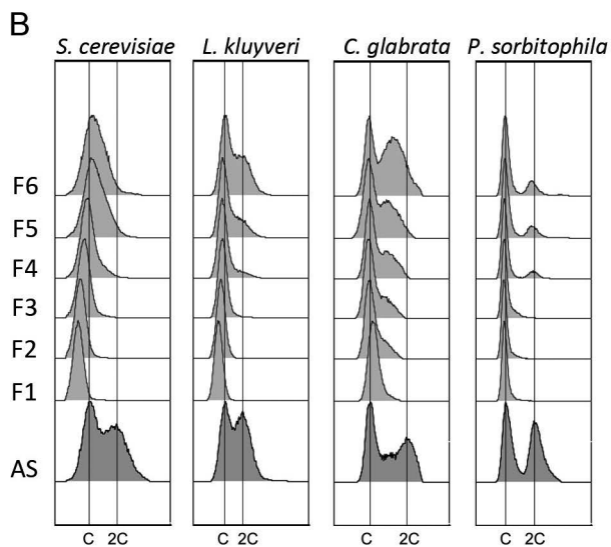
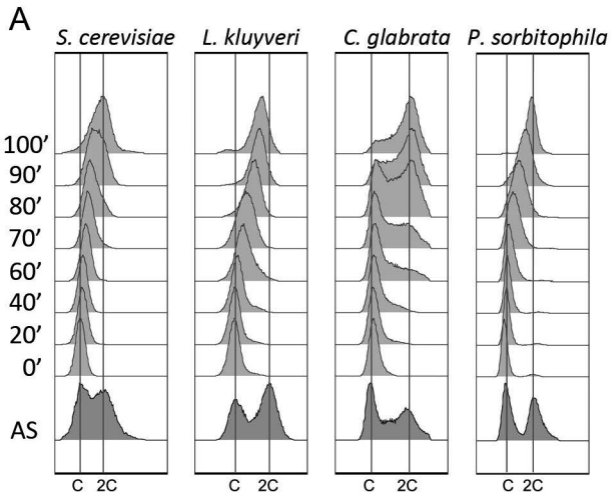


Figure 3 - Marbouty *et al.*