Density separation of quiescent yeast using iodixanol

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As yeast are starved of nutrients, they enter G_0, a quiescent state. Quiescent yeast (Q) cells retain viability for extended periods of time and resume growth following supplementation of missing nutrients. As such, Q cells have become a valuable model for studying longevity and self-renewal of chronologically aged cells. Traditional isolation of Q cells involves a relatively long centrifugation time through a continuous density gradient. Here, we describe a rapid and cost-effective Q-cell isolation technique that uses a single-density, one-step gradient prepared from media containing iodixanol.

Cells undergo phenotypic changes in response to external and internal stimuli. Yeast grow exponentially when nutrients are abundant and enter stationary phase (SP) when nutrients become scarce. Under nutrient-limited conditions, a clonal population of haploid budding yeast differentiates into quiescent (Q) and nonquiescent (NQ) cells (1). Q cells are dense, unbudded daughter cells that are arrested in G_0 but are capable of re-entering the mitotic cell cycle upon nutrient replenishment. In contrast, NQ are unable to enter G_0 and consist of mainly replicatively older mother cells. Q cells can survive up to 3 weeks longer in SP than NQ cells (1) and are thus used as a model for chronological aging (2). Furthermore, Q cells possess stem cell–like properties and are used to examine re-entry into the cell cycle and self-renewal (3).

One important characteristic of Q cells is their resistance to a multitude of environmental stresses (4). Unlike dividing cells, Q cells are thermotolerant (5) and possess cell walls resistant to zymolyase (6). NQ cells, on the contrary, are less resistant to these treatments and are prone to genomic instability (5,7,8). Because of their short life cycle and the extensive availability of molecular tools, budding yeast are an excellent model in which to study the unique biology of quiescence (9). Rapid and inexpensive methods for separating NQ and Q yeast populations would therefore facilitate future studies.

Various strategies have been employed to isolate Q cells, including cell elutriation (10) and fluorescence activated cell sorting (FACS) (6). However, these techniques require expensive equipment and specialized training. Presently, the most common method for isolating Q cells uses density-based Percoll gradients (1), which are both labor- and time-intensive. The nontoxic density gradient medium iodixanol has been used to enrich for blood monocytes (11) and motile sperm cells (12), separate peroxisomes from other organelles (13), and harvest retroviruses (14). Here, we describe an inexpensive and efficient Q-cell separation technique using a single-density, one-step gradient prepared from a solution of iodixanol (Figure 1A).

Materials and methods

Strains, media, and culture conditions

The haploid prototrophic *Saccharomyces cerevisiae* FY4 *MAT*a strain (derived from S288C) was used for Q-cell isolations (15). Sterile technique was used for all methods, and at least four replicates of each procedure were performed. Yeast cultures were grown in shaking incubators at 250 rpm and 30°C. Briefly, FY4 was streaked from freezer stocks using sterile toothpicks onto YPD (1% yeast extract, 2% peptone, 2% dextrose) agar plates and grown to single colonies. Single colonies were used to start overnight cultures in 3 mL YPD medium in 14 mL polypropylene tubes. Cells from independent overnight cultures were used to inoculate 7 mL fresh YPD medium in 18 × 150 mm borosilicate glass test tubes at a starting OD_{600} of 0.01. Cultures were grown with shaking for 7 days. The 7-day old cultures were then used for density gradient separation analysis.

Generating iodixanol solutions for enrichment of Q cells

Density step-gradients were created from a 60% (v/v) solution of iodixanol (OptiPrep Density Gradient Medium; Axis-Shield PoC AS, Oslo, Norway), and stocks of Tris-HCl and NaCl to give final concentrations of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 10%–40% v/v iodixanol. A linear relationship exists between iodixanol solution concentrations and density as measured by an Anton Paar Density Meter (DMA 4100M; Anton Paar GmbH, Graz, Austria) (Supplementary Figure S2).

METHOD SUMMARY

A five-minute centrifugation of stationary-phase yeast through a one-step iodixanol gradient enables recovery of yeast quiescent (Q) cells in the pellet, while nonquiescent (NQ) cells remain at the boundary between the culture medium and the iodixanol.

Rapid enrichment of Q cells using iodixanol

Five-hundred microliters of a 30% iodixanol solution was transferred directly to a microfuge tube. Cells from SP (500 µl, containing ~2 × 10^8 cells) were layered on top of this solution and centrifuged at 2500 × *g* on a table-top micro-centrifuge for 2.5 min. The microfuge tube was rotated 180 degrees and then centrifuged for another 2.5 min. Q cells were recovered in the pellet, while NQ cells remained at the SP buffer/iodixanol boundary (Figure 1B). NQ and Q cells were washed twice with resuspension (RS) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and resuspended in 1 mL RS buffer for further analysis.

Percoll gradient formation and Q-cell isolation

Percoll gradient separations were adapted from previously described methods (7). Briefly, Percoll (GE Healthcare Bio-Sciences, Piscataway, NJ) was diluted 9:1 with 1.5 M NaCl and vortexed thoroughly to mix. A 1.75-mL aliquot of the Percoll solution was transferred to 2-mL microfuge tubes and spun at 19,250 × *g* for 15 min on a table-top microcentrifuge to generate a continuous gradient. Approximately 2 × 10^8 SP cells from 7-day old cultures were resuspended in 100 µl RS buffer and then layered on top of these pre-formed Percoll gradients. Cells were separated by centrifugation at 400 × *g* for 1 h in a swinging bucket rotor. Cells from the top and bottom fractions were isolated (Supplementary Figure S1), washed twice with RS buffer, and resuspended in 1 mL RS buffer.

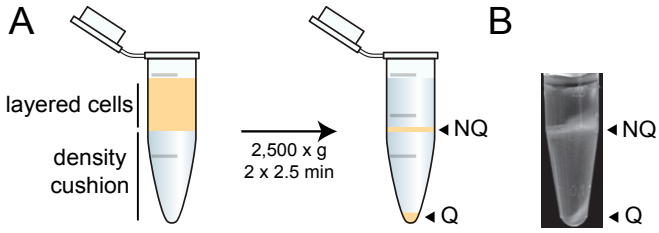

Figure 1. Iodixanol gradient density-based separation of quiescent (Q) yeast. (A) Yeast cells are layered onto preformed iodixanol density cushions and centrifuged to separate populations of different densities. (B) Iodixanol gradient separation of nonquiescent (NQ) and Q yeast cells in a microfuge tube.

Cell wall staining

Cell populations in RS buffer from iodixanol and Percoll isolations were stained for 30 min with Calcofluor White M2R (Fluorescent Brightener 28; Sigma-Aldrich, St. Louis, MO) at a final concentration of 100 µg/mL. Cells were then washed twice with RS buffer. Cells were imaged with a Zeiss Axioimager M1 Epifluorescence microscope (Carl Zeiss Microscopy, Thornwood, NY) using 365 nm excitation and 445/50 nm emission filters.

Acute stress treatments

Suspensions of log-phase and SP cultures as well as NQ and Q cell populations isolated via Percoll or iodixanol gradients were diluted 50-fold with water, and 10 µl of this dilution was loaded onto Countess cell counting chamber slides (Thermo Fisher, Carlsbad, CA). Raw counts were acquired using a Countess

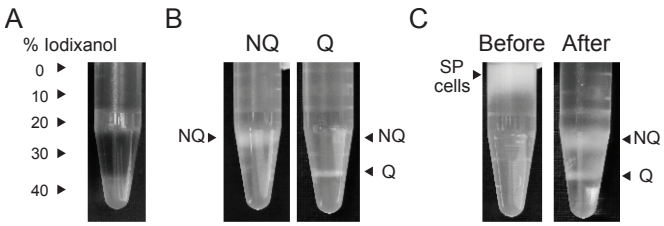

Figure 2. Quiescent (Q) and nonquiescent (NQ) yeast cells migrate to different boundaries on an iodixanol step-gradient. (A) A five-step iodixanol gradient for simple identification of working density. For illustration purposes, the 10% iodixanol step was dyed with 1% phenol-red, while the 30% iodixanol step was dyed with 1% bromophenol blue. (B) Q cells isolated independently from Percoll gradients were found to migrate to the boundary between 30% and 40% iodixanol, while NQ cells from Percoll gradients were found to migrate to the boundary between 20% and 30% iodixanol. (C) Stationary phase (SP) cultures migrate in a similar fashion to isolated NQ and Q fractions on a five-step gradient. Therefore, a single 30% iodixanol gradient is sufficient for separation of NQ and Q yeast cells.

II Automated Cell Counter (Thermo Fisher) and adjusted for dilution factor to determine the original concentration. Yeast cells (3×10^6) from each population mentioned above were resuspended in 450 µl RS buffer. From each of these populations, 3 equal volumes (1×10^6 yeast cells/150 µl each) were aliquoted and used for stress treatments or as a no-treatment control.

Temperature stress: Cells (150-µl cell suspension) from each population were incubated for 1 h at 50°C. A 100-µl aliquot of room temperature RS buffer was added to lower the temperature, and the total volume (250 µl) was transferred to a single well of a 96-well plate.

Cell wall stress: Zymolyase (40 U) (E1005; Zymo Research, Irvine, CA) in 50 µl digest buffer (50 mM Tris-HCl, pH 7.5, 40 mM β-mercaptoethanol) was added to 150 µl of cell suspension from each population and incubated for 2 h at room temperature. A 50-µl volume of 5% SDS in RS buffer (1% final concentration) was then added to further promote cell lysis. The total volume (250 µl) was transferred to a single well of a 96-well plate.

No treatment control: RS buffer (100 µl of) was added to 150 µl of cell suspension, and the total volume (250 µl) was transferred to a single well of a 96-well plate.

Stress-treated yeast and control yeast were 5-fold serially diluted in a 96-well plate with RS buffer and spotted on YPD plates using a 48-pin replicating tool (Sigma-Aldrich). Plates were incubated at 30°C for 2 days and photographed to assess growth.

Results and discussion

Gradient separation is a valuable technique for isolating distinct populations of cells or organelles. Density gradients made from polyvinylpyrrolidone-coated colloidal silica (e.g., Percoll) have been heavily used for research and clinical applications for more than three decades. However, for precision separations there are several disadvantages to Percoll. For example, the formation of the initial gradient is time-consuming and a likely source of sample-to-sample variation (e.g., variations in centrifugal force or brake speed). Iodixanol step-gradients present advantages in efficiency, simplicity, and cost savings over Percoll gradients (Table 1). Here, we demonstrate the utility of a one-step gradient (the iodixanol solution acts as a cushion), which does not require the problematic gradient formation step typical of continuous Percoll gradients. This enables larger numbers of cells to be separated using lower volumes of costly gradient solutions. Requiring ~5 min to complete, iodixanol gradient separation is also more than 10 times faster than traditional Percoll gradient separation (Table 1).

First, we used a five-step gradient generated by layering decreasing iodixanol solutions on top of one another to determine the density at which NQ and Q cells are differentially sedimented (Figure 2A). Boundaries between steps were stable during centrifugation, as determined by the immobility of dye across steps. NQ and Q cells were initially isolated from Percoll gradients and then added to five-step iodixanol gradients to ascertain cell sedimentation patterns (Figure 2B). Subsequent separation of SP cultures on iodixanol confirmed that NQ and Q cells sedimented to densities equivalent to their pre-isolated fractions (Figure 2C). Since a single-step 30% iodixanol gradient separated NQ and Q cells from the FY4 yeast strain in both cases, we used a single-step gradient for all subsequent experiments. This optimization process is simple and modular (by increasing the number, size, or volume of the gradient steps) and can be used to find an optimal density for enriching Q cells or dense cells in any yeast species or strain.

To validate our approach and ascertain the characteristics of Q cells isolated from iodixanol gradients, we subjected isolated cells to a battery of stresses and measured both viability and morphological features of Q cells. For example, Q cells are unbudded daughter cells (1) that are thermotolerant and zymolyase-resistant (4,7). Therefore, we evaluated the stress-tolerance characteristics of NQ and Q cell populations isolated from both iodixanol and Percoll gradients. Cells were acutely stressed with heat (50°C) and cell wall disruption (zymolyase + SDS) (4,7). Survival was assessed by a spotting assay on YPD plates (Figure 3A). SP and log-phase populations were also treated to determine the efficacy of treatment. The log-phase population was sensitive to heat and zymolyase, as expected, whereas the SP population had a mixture of stress-resistant and non-resistant (Q and NQ) cells. The NQ population from Percoll gradients was more sensitive to treatments than the NQ population isolated from iodixanol. We attribute this observation to some Q cells getting trapped at the boundary NQ fraction during the iodixanol separation. Regardless, the Q populations isolated from both gradients were equally robust against heat and zymolyase treatment. Based upon these results, we conclude that the cells isolated by a 30% iodixanol one-step gradient possessed quiescent properties.

Table 1. Comparison of quiescent (Q) yeast isolation using Percoll and iodixanol density gradients.

Characteristic	Percoll		Iodixanol	
Tube size	*2 mL*	*15 mL*	*2 mL*	*15 mL*
Minimum volume	1 mL	10 mL	0.5 mL	4 mL
Maximum cells	2×10^8	2×10^9	4×10^8	5×10^9
Cost per sample*	$0.69	$6.90	$0.32	$2.56
Gradient preparation time	15 min		–	
Centrifugation time	60 min		5 min	
Total time	75 min		5 min	

Brief comparison outlining some of the advantages of iodixanol gradients with respect to time, cost, and experimental scale.
*Based on 2017 academic pricing in U.S. dollars

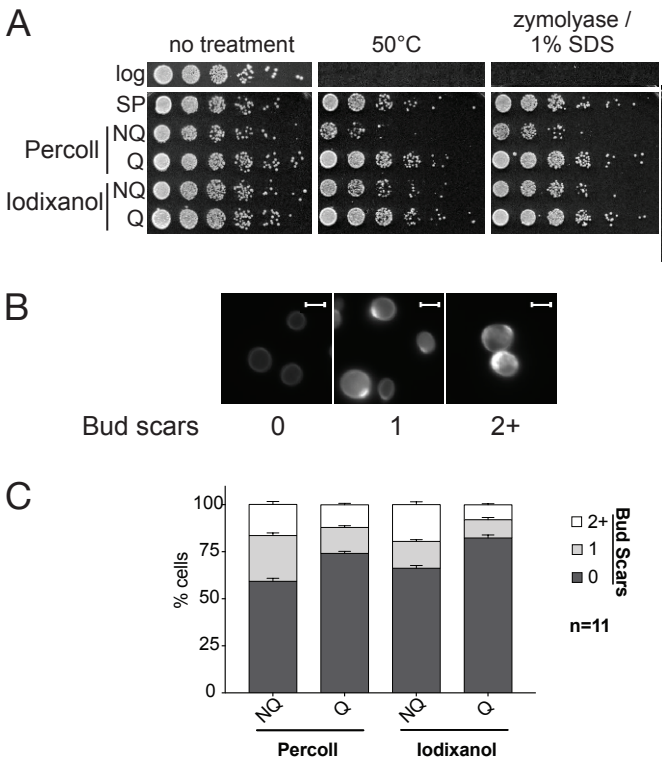

Figure 3. Iodixanol density-separated quiescent (Q) cells are thermotolerant and resistant to zymolyase. (A) Yeast cells [Q and nonquiescent (NQ) cells] isolated from Percoll or iodixanol gradients were exposed to acute heat (50°C for 1 h) and cell wall disruption (zymolyase followed by 1% SDS). These cells, as well as stationary phase (SP) and log-phase controls, were then spotted as 5-fold serial dilutions onto YPD agar and grown for 2 days at 30°C. (B) The pictures show how cells stained with Calcofluor White M2R were scored for replicative age: virgin daughters (0 bud scars), 1 division (1 bud scar), and 2 or more divisions (2+ bud scars). Scale bars denote 5 µm. (C) Yeast cells (Q and NQ) isolated from Percoll or iodixanol were stained using Calcofluor White M2R and scored for replicative age. The iodixanol Q-cell fraction had the highest number of virgin daughters and the lowest number of replicatively older mother cells.

Next, we compared the yield of cells in the NQ and Q fractions between the iodixanol and Percoll gradients. We found that although the yield of Q cells using iodixanol gradients (when separating 2×10^8 cells in a microfuge) was less than that of Percoll gradients (Supplementary Figure S3), the yield is more reproducible (smaller SE). Again, we attribute the difference in yield to the stringent density cutoff used to isolate Q cells. The iodixanol density could be refined to maximize yield, but this would likely increase the risk of carrying some NQ cells into the Q fraction.

Lastly, we analyzed the morphological features of isolated cells. Previous studies determined that Q cells are unbudded (arrested in G_0) and possess fewer bud scars than NQ cells (1). Therefore, we quantified and compared the budding index and replicative age of the yeast cells isolated in the iodixanol Q fraction. Calcofluor White M2R was used to stain NQ and Q cells isolated from iodixanol and Percoll gradients (Figure 3B). Approximately 250–500 cells from NQ and Q fractions isolated via iodixanol or Percoll across 11 trials were visually scored for buds and bud scars; a representative field of view is shown in Supplementary Figure S4. When NQ and Q cells separated by an iodixanol gradient were quantitatively compared with

Table 2. Budding index of nonquiescent (NQ) or quiescent (Q) yeast isolated using either a Percoll or an iodixanol density gradient.

Gradient	Fraction	% budded
Percoll	NQ	9.9 ± 1.6
	Q	3.2 ± 0.7
Iodixanol	NQ	2.2 ± 0.5
	Q	0.6 ± 0.2

Comparison of the number of budded cells in iodixanol and Percoll gradient-isolated populations. Data are represented as the mean ± SEM (n = 11).

NQ and Q cells separated by a Percoll gradient, the iodixanol-separated Q population had the lowest number of budded cells (Table 2). Similarly, the iodixanol Q population had the lowest number of replicatively older cells (2+ bud scars) and the highest number of virgin daughter cells (0 bud scars) (Figure 3C). These data suggest that iodixanol gradients enrich for unbudded daughter cells (Q cells) with greater reliability than Percoll gradients.

We have demonstrated not only the feasibility but also the practicality of using iodixanol step-gradients to isolate Q cells. In addition to its value for studies of quiescence, we think this approach can have wider-ranging applications, from aging analysis in diverse yeast species to long-term cryostorage. Our technique would facilitate the harvesting of large numbers of Q cells for biochemical and genetic research. Similar separation systems can also be prepared as multiple step-density gradients (16). These stable systems have been shown to enable the enrichment of desired populations from complex samples based on very small differences in density (17,18). Such an approach could find additional applications in the study of biochemical differences within quiescent cell populations. In summary, iodixanol step-gradients are time- and cost-saving options for researchers interested in chronological aging, regeneration, or G_0 homeostasis in yeast model systems.

Author contributions

I.Q. contributed to the conception of the study, acquisition of data, analysis and interpretation of data, drafting of the article, and critical revision of the manuscript. C.J.L. contributed to the determination of iodixanol densities and the editing and revision of the manuscript. C.R.M. contributed to the conception of the study and the editing and revision of the manuscript. S.M.F. contributed to the conception of the study, analysis and interpretation of data, drafting of the article, critical revision of the manuscript, and overall supervision of the project.

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Competing interests

The authors declare no competing interests.

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