Video Article

The Use of Chemostats in Microbial Systems Biology

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Abstract

Cells regulate their rate of growth in response to signals from the external world. As the cell grows, diverse cellular processes must be coordinated including macromolecular synthesis, metabolism and ultimately, commitment to the cell division cycle. The chemostat, a method of experimentally controlling cell growth rate, provides a powerful means of systematically studying how growth rate impacts cellular processes - including gene expression and metabolism - and the regulatory networks that control the rate of cell growth. When maintained for hundreds of generations chemostats can be used to study adaptive evolution of microbes in environmental conditions that limit cell growth. We describe the principle of chemostat cultures, demonstrate their operation and provide examples of their various applications. Following a period of disuse after their introduction in the middle of the twentieth century, the convergence of genome-scale methodologies with a renewed interest in the regulation of cell growth and the molecular basis of adaptive evolution is stimulating a renaissance in the use of chemostats in biological research.

Video Link

The video component of this article can be found at http://www.jove.com/video/50168/

Introduction

The growth of cells is regulated by complex networks of interacting genetic and environmental factors[1,2]. The multifactorial regulation of cell growth necessitates a system-level approach to its study. However, the rigorous study of regulated cell growth is challenged by the difficulty of experimentally controlling the rate at which cells grow. Moreover, in even the simplest experiments extracellular conditions are frequently dynamic and complex as cells continuously alter their environment as they proliferate. A solution to these problems is provided by the chemostat: a method of culturing cells that enables experimental control of cell growth rates in defined, invariant and controlled environments.

The method of continuous culturing using a chemostat was independently described by Monod[3] and Novick & Szilard[4] in 1950. As originally conceived, cells are grown in a fixed volume of media that is continually diluted by addition of new media and simultaneous removal of old media and cells (**Figure 1**). Coupled ordinary differential equations (**Figure 2**) describe the rate of change in cell density (x) and the concentration of a growth-limiting nutrient (s) in the chemostat vessel. Importantly, this system of equations predicts a single (nonzero) stable steady-state (**Figure 3**) with the remarkable implication that at steady-state, the specific growth rate of the cells (*i.e.* the exponential growth rate constant) is equal to the rate at which the culture is diluted (D). By varying the dilution rate it is possible to establish steady-state populations of cells at different growth rates and under different conditions of nutrient limitation.

The experimental control of growth rate using chemostats was critical to the development of an understanding of how cell physiology changes with rates of growth[5,6]. However, this former mainstay of microbiological methods became increasingly obscure during the explosion in molecular biology research during the late twentieth century. Today, renewed interest in growth control in both microbes and multicellular organisms and the advent of genome-scale methods for systems-level analysis has renewed motivation for the use of chemostats. Here, we describe three applications that capitalize on the precise control of cell growth rates and the external environment that are uniquely possible using chemostats. First, we describe the use of chemostats to investigate how the abundance of thousands of biomolecules - such as transcripts and metabolites - are coordinately regulated with growth rate. Second, we describe how chemostats can be used to obtain precise estimates of growth-rate differences between different genotypes in nutrient-limited environments using competition experiments. Third, we describe how chemostats can be used to study adaptive evolution of cells growing in constant nutrient-poor environments. These examples exemplify the ways in which chemostats are enabling systems-level investigations of cell growth regulation, gene by environment interactions and adaptive evolution.

Protocol

The principle of continuous culturing using a chemostat can be realized in a variety of implementations. In all chemostats it is essential to have 1) methods for maintaining sterility of all components, 2) a well-mixed culture, 3) appropriate aeration of the culture vessel and 4) a reliable means

of media addition and culture removal. Here, we describe the use of a Sixfors bioreactor (Infors Inc) as a chemostat using methods that can be readily adapted to alternative setups.

1. Assembling the Chemostat Vessels

1. Turn on the Sixfors using the main switch.
2. Thoroughly rinse the chemostat vessel, stirrer assembly, and attached tubing with deionized (DI) water. Check all the tubing and o-rings and replace any worn out looking pieces.
3. Make sure the drive shaft support base is facing upward in the glass vessel and that the top of the drive shaft is snapped into its housing on the stirrer assembly. Set the stirrer assembly into the glass vessel ensuring the bottom of the drive shaft is seated in the drive shaft support. Use the clamp to secure the stirrer assembly to the glass vessel.
4. Fill the vessel with around 300 ml of DI water.
5. Remove the caps from a dissolved oxygen (dO_2) probe and check the electrolyte level by unscrewing the bottom casing and making sure that the bottom casing is filled halfway with electrolyte solution. Rescrew the bottom casing and insert into the port on the chemostat vessel. Screw until finger tight.
6. Take a pH probe and remove it from its storage buffer (3 M KCl). Remove the pH probe cap and attach to fermentor 1. Using the Sixfors control screen, navigate to the fermentor parameter menu and select "calibrate pH". Place the pH probe into a standard pH 7 buffer and record reading as High Read. Repeat with pH 4 buffer and record reading as Low Read. Detach the pH probe from fermentor, wash with DI water and insert the probe into the port on the chemostat vessel. Screw until finger tight.
7. Place the chemostat vessel in the rack. Note on the vessel which fermentor (1-6) the pH probe was connected to and calibrated. Place the pH probe cap on the pH probe. Using foil tightly cover the top of the dO_2 probe.
8. Fold foil over the ends of the tubing attached to the chemostat vessel.
9. Repeat steps 1.2-1.8 for all vessels.
10. Sterilize the vessels by autoclaving them for 15 min on a liquid cycle.

2. Preparing the Media

1. Establish the limiting range of concentrations for a nutrient by growing batch cultures in different nutrient concentrations. The nutrient is limiting in the range where the final cell density is a linear function of the initial nutrient concentration (**Figure 4**). Select a nutrient concentration well within the limiting range. Examples of standard media composition for studies with *Saccharomyces cerevisiae* are available in [7-11].
2. Autoclave an empty carboy that is capped with a rubber plug containing an air inlet and media outlet after first ensuring that the end of the media tubing is covered in foil.
3. In a separate vessel prepare 10 L of media.
4. Attach a 500 ml filter cup to a 100 ml media bottle. Remove the cotton filter with forceps sterilized in ethanol and immediately attach to the filtration port on the media carboy.
5. Attach the media carboy to a vacuum source and filter sterilize media by adding it to the filter cup.
6. When filtration of media is complete, close off the filtration port with a metal clamp.

3. Calibrating dO_2 Probes and Setting up Chemostat

1. After the chemostat vessels have been autoclaved and allowed to cool down place the vessel in its corresponding heat jacket. Connect the temperature probe, pH probe, and dO_2 probe. Let the vessel sit with the power on for at least 6 hr to allow the dO_2 probes to polarize.
2. Place the end of each effluent tube into a separate collecting receptacle. Connect the air supply via an autoclaved filter and turn on airflow. The water in each vessel should flow out of the effluent tubes, which indicates that all seals are properly formed.
3. Adjust the height of the effluent tube according to the desired working volume (*e.g.* 300 ml). We use a ruler that is marked with tube placements calibrated to different working volumes.
4. Connect the media carboy to the chemostat vessel. Use ethanol in order to keep tube ends as sterile as possible. Thread the pump tubing through the pump and open clamp. Manually press the pump until the media starts to flow into the chemostat vessel. Release tubing from pump, media should flow freely into chemostat vessel. When the media reaches the effluent tube, reattach tubing to pump and clamp.
5. Start running the basic program with impeller set to 400 rpm and the temperature set at 30 °C.
6. In order to calibrate dO_2 probes, turn off air supply and switch to nitrogen gas. Wait at least one hour and record measure value as "low read". Switch back to air supply (*i.e.* containing ambient oxygen concentration), wait at least one additional hour and record measurement as "high read".
7. Initiate data recording using Iris software.

4. Inoculation

1. Use 70% ethanol to sterilize the top of the culture vessel.
2. Remove screw on vessel top and pipette 1 ml of an overnight culture into the chemostat vessel. Retighten screw.
3. Indicate the time of inoculation in Iris.
4. Wait approximately 24 hr for cultures to reach early stationary phase. As the culture grows, dissolved oxygen and pH will decrease. In the case of glucose-limiting media, dissolved oxygen will return to ~100% in stationary phase. For other nutrient limitations dissolved oxygen will remain <100% in stationary phase.

5. Initiating Pumps and Attaining Steady State

1. The dilution rate is calculated by dividing the flow rate (how much media flows into the vessel per hour) by the culture volume. For example, using a volume of 300 ml a dilution rate of 0.1 means that 30 ml of media is added to the culture every hour. Because the system is under positive pressure the same volume is removed from the vessel ensuring that the culture is continuously diluted. A range of dilution rates (*D*) can be used that do not exceed the maximum growth rate (μ_{max}) of the cells, above which cells will be washed out of the chemostat.
2. Choose pump settings, which specify the number of seconds the pump is on and off, to establish the desired flow rate. The pump delivers media at a rate of ~0.11 ml/sec.
3. Define a program using the Sixfors interface that specifies the pump timing, temperature and impellor speed. Start the program.
4. Empty the collecting receptacles of liquid and record the time.
5. After at least two hours, use a graduated cylinder to measure how much media has been removed from the vessel. This will equal the volume that has been added to the chemostat vessel. Calculate the dilution rate ($D = V_{effluent}/V_{culture}/time$). Adjust pump settings if calculated dilution rate does not match desired dilution rate.
6. At steady state, cell density in the chemostat does not change over time. This can be measured without perturbing the culture by sampling the outflow. We operationally define attainment of steady state as identical cell density measurements that are separated by at least one population doubling. Reaching steady state will take approximately eight culture generations after initiation of culture dilution. At steady state, cells grow exponentially (*i.e.* constant growth rate over time) in nutrient-limited conditions. The doubling time of the population (generation time) is $ln(2)/D$.
7. Continue to periodically measure effluent volumes for the duration of the experiment to ensure that a constant dilution rate is maintained.

6. Application 1: Studying Cells Growing at Different Rates in Steady-state Conditions

1. At steady state in the chemostat, the growth rate of the population of cells is equal to the dilution rate. By systematically altering the dilution rate it is possible to grow cells at different rates. This enables the systematic study of physiological parameters that vary with growth rate including cell volume, cell cycle stage and stress resistance. In addition, steady-state profiles of global mRNA, protein and metabolite levels can be assayed in cells growing at different rates. There are two ways to acquire samples:
2. Small samples can be passively obtained by placing the end of the effluent tube into an 1.5 ml or 15 ml tube. A sample of 1-5 ml can be obtained in a few minutes (depending on the flow rate). Many physiological parameters can be measured from these samples.
3. Gene expression, or metabolite analyses require larger samples that must be obtained as quickly as possible. Place the end of the effluent tube into a 15 ml conical tube. Release the screw holding the metal end of the effluent tube and push down gently. A ~10 ml sample will rapidly fill your tube. Keep in mind that the volume in the chemostat vessel has changed. If many consecutive samples are taken it may be necessary to reduce flow to maintain a constant dilution rate.

7. Application 2: Precise Measurement of Differences in Growth Rates Between Genotypes in Controlled Environments Using Flow Cytometry-based Competition Assays

1. Chemostats can be used to accurately quantify the effect of nutrient concentration on differences in growth rates (*i.e.* fitness) between different genetic backgrounds. By co-culturing strains labeled with different fluorescent proteins the rate of change in relative abundance during exponential growth is determined. Performing this assay at different dilution rates (*D*) allows the study of effects of nutrient concentration (*s*) on fitness differences.
2. Establish steady state cultures of the two strains in separate chemostat vessels with identical dilution rates and a volume of 300 ml.
3. Passively sample 1 ml from each vessel. Spin down cells, resuspend in phosphate buffered saline (PBS) and place at 4 °C. These samples contain homogenous cell samples, which are controls for the subsequent flow cytometry analysis.
4. Place the effluent tube from one vessel into an autoclaved graduated cylinder. Release the screw holding the metal end of the effluent tube and gently push down to expel cells from the chemostat. When the volume reaches 150 ml, return the metal end of the effluent tube to its original position (300 ml). Repeat with the second vessel, using a different graduated cylinder.
5. Use 70% ethanol to maintain sterility while removing screw on the top of the chemostat vessel. Place funnel in opening and pour 150 ml from the other culture into the chemostat vessel. Retighten screw. Repeat with the second vessel, using the second funnel. Each chemostat vessel now contains a mixture of the two strains.
6. Obtain a 1 ml sample from each vessel using passive sampling every 2-6 hr. Spin down cells, resuspend in PBS and store at 4 °C. Continue taking samples for 2-3 days carefully recording the time each sample was taken.
7. At the end of the experiment, sonicate and dilute samples to ~2×10^6 cells/ml. Using flow cytometry, measure the proportion of the two strains in each sample. As both strains are growing exponentially, the relative growth rate difference is determined by linear regression of $ln(strain1/strain2)$ against time (measured in generations). The slope of the regression is the proportional difference in growth rate (*i.e.* the fitness advantage) of one strain relative to the other.
8. As the mixed culture can take some time to attain a new steady-state, early time points may fit poorly to the regression. This issue is best solved by allowing 2-3 generations to elapse before commencing sampling or removing early points that are clear outliers. Conversely, once one strain has outcompeted the other the data are no longer useful and these points should be excluded.

8. Application 3: Experimental Evolution

1. Experimental evolution performed in chemostats selects for mutants that are increased in fitness in the nutrient-limited environment. Selection is generally performed over hundreds of generations.
2. Establish a steady-state chemostat culture using a strain of known genotype (and preferably known genome sequence) and a defined nutrient-limitation.

3. To maintain a "fossil record" of the adapting population passively sample chemostat every 2-3 days and store the sample in 15% glycerol at -80 °C.
4. Monitor the media reservoir and replenish with fresh media as necessary.
5. Maintain the exponentially growing culture for several hundred generations.
6. After completion of the selection plate a sample of cells on solid agar plates. After cells have grown into colonies, pick an unbiased sample of colonies using toothpicks and inoculate clones into individual wells of a 96-well plate containing 100 µl of media. Allow clonal samples to grow overnight, add 100 µl of 30% glycerol and store in -80 °C.
7. Characterize clones by whole genome sequencing and performing phenotypic assays including assessment of fitness, using a common fluorescently labeled reference strain, as described in Application 2.

Representative Results

A major advantage of chemostats is the ability to control the growth rate of cells experimentally by varying the dilution rate. In the budding yeast, Saccharomyces cerevisiae, the morphology of a cell is informative of its phase in the cell division cycle. Populations with higher growth rates contain a higher proportion of actively dividing cells as determined by measuring the fraction of unbudded cells (**Figure 5A**). Analyses of global mRNA expression in chemostat cultures has shown that the expression of many genes are differentially expressed as a function of growth rate (**Figures 5B** and **Figure 5C**).

The relative fitness of different genotypes can be determined by conducting competition assays in chemostats using fluorescently labeled cells and flow cytometry analysis (**Figure 6A**). **Figure 6B** shows a representative result in which a mutant strain was competed against a fluorescently tagged wildtype strain. In this example, the mutant strain has a 40% growth rate advantage per generation. By comparison, an untagged wildtype strain competed against the fluorescently tagged wildtype strain does not differ in its rate of growth.

Chemostats provide a means of exerting a defined and continuous selective pressure on cells. Whole genome sequence analysis can be used to identify acquired mutations in cells with increased fitness. Adaptive evolution experiments in yeast cells in different nutrient limited chemostats have identified copy number variants as a frequent and repeated mechanism by which adaptive mutations are generated. For example, in independent adaptive evolution experiments performed in nitrogen-limited chemostats using different nitrogen sources, multiple copy number variants (CNVs) that include the *GAP1* gene were identified [11] (**Figure 7**).

Chemostats pose some unique challenges that are not otherwise encountered in standard microbiological methods. Potential problems and solutions specific to chemostat experiments can be found in **Table 1**.

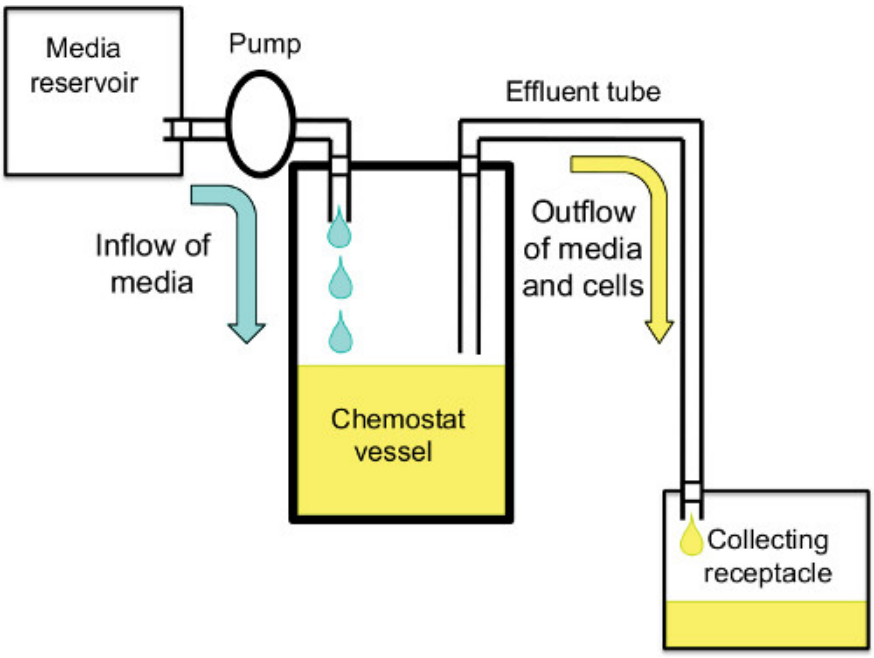

Figure 1. Diagram of a chemostat. The chemostat comprises a media reservoir, pump, a chemostat vessel, an effluent tube, and a collecting receptacle.

$$1) \quad \frac{dx}{dt} = \mu_{max} \frac{s}{K_s + s} x - Dx$$

$$2) \quad \frac{ds}{dt} = DR - Ds - \frac{x}{Y} \mu_{max} \frac{s}{K_s + s}$$

Figure 2. Mathematical model of the chemostat. Differential equation 1 describes the change in cell density (x) in the chemostat over time, which is the result of cell growth and cell removal by dilution (cell death is assumed to be negligible). Monod proposed [3] that cell growth (μ) depends on external nutrient concentration according to a Michaelis-Menten type relationship that includes the variables of maximal growth rate (μ_{max}) and a half-maximal growth rate constant (K_s). The dilution rate (D) is determined by the rate of media addition and culture removal. Differential equation 2 describes the rate of change in the limiting nutrient concentration (s) in the chemostat vessel. The change in concentration of the limiting nutrient in the chemostat vessel is dependent on its concentration in the inflowing media (R), its dilution by outflow (D) and consumption by the cells, which is dependent on the cell parameters μ_{max}, K_s and a yield constant, Y. For simplification, Y is assumed to be constant at different growth rates. The variables, and their typical units, in the coupled ordinary differential equations are x - cell density (cells/ml), s - limiting nutrient concentration (μM), μ_{max} - maximal growth rate (hr^{-1}), K_s - nutrient concentration at which the growth rate equals $\mu_{max}/2$ (μM), Y - yield (cells/ml/μM), D - dilution rate (hr^{-1}), R - nutrient concentration in media reservoir (μM).

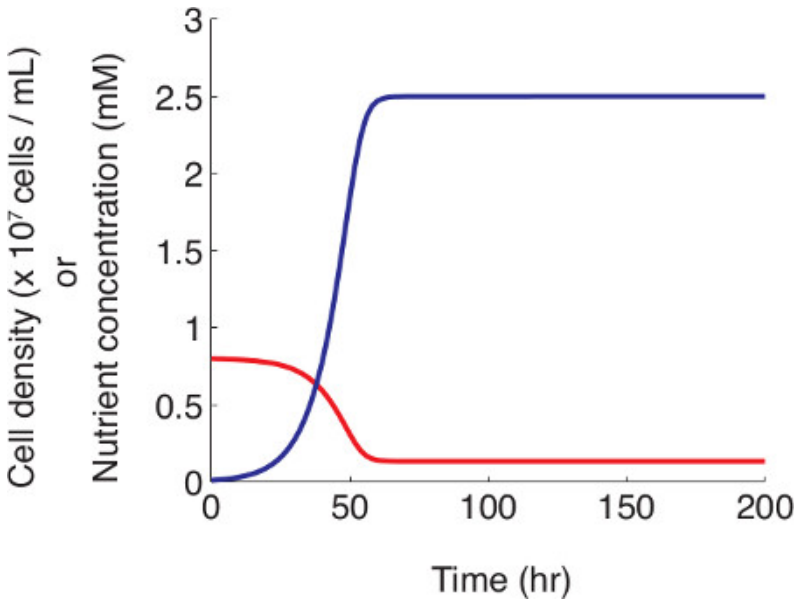

Figure 3. Approach to steady-state as predicted by the mathematical model. Nutrient concentration (red) and cell density (blue) attain non-zero steady states.

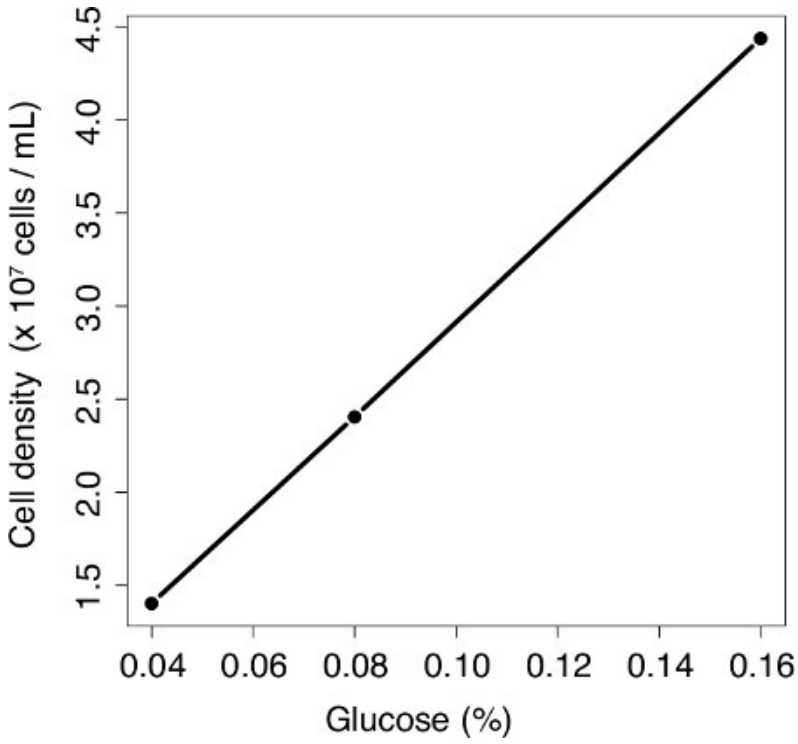

Figure 4. Establishing the limiting range of a nutrient. A haploid strain of *Saccharomyces cerevisiae* was grown at different concentrations of glucose and the final density determined. A linear relationship indicates that the nutrient concentration is in the limiting range.

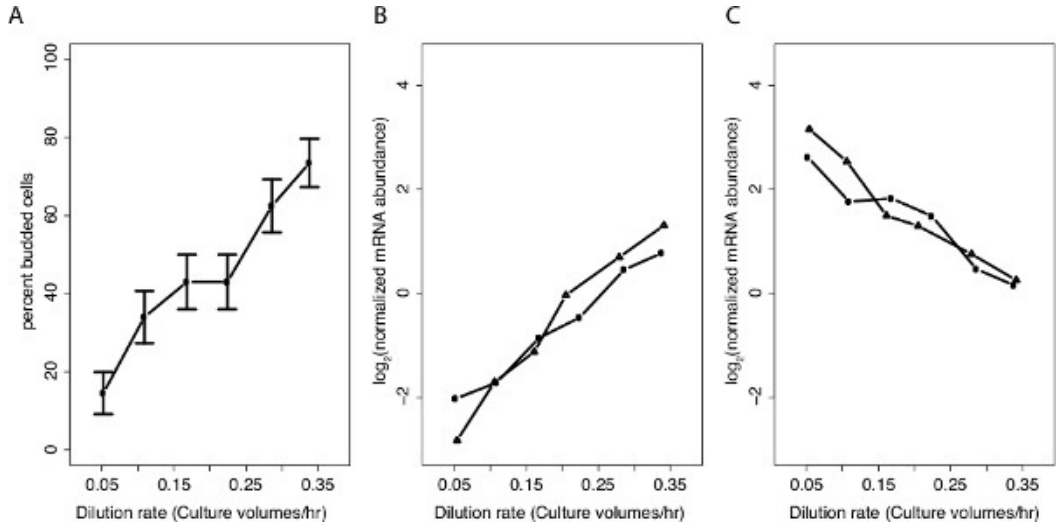

Figure 5. Cell physiology varies with growth rate. The chemostat enables controlled studies of the relationship between growth rate and A) cell division as assayed on the basis of cell morphology (*i.e.* the fraction of budded cells). The abundance of ~25% of yeast mRNAs depends on growth rate: for example the gene B) *UTR2* increases in expression as growth rate increases in both glucose- (o) and nitrogen-limited (Δ) chemostats and the gene C) *ASM1* decreases in expression level as growth rate increases in glucose- (o) and nitrogen-limited (Δ) chemostats [10]. Click here to view larger figure.

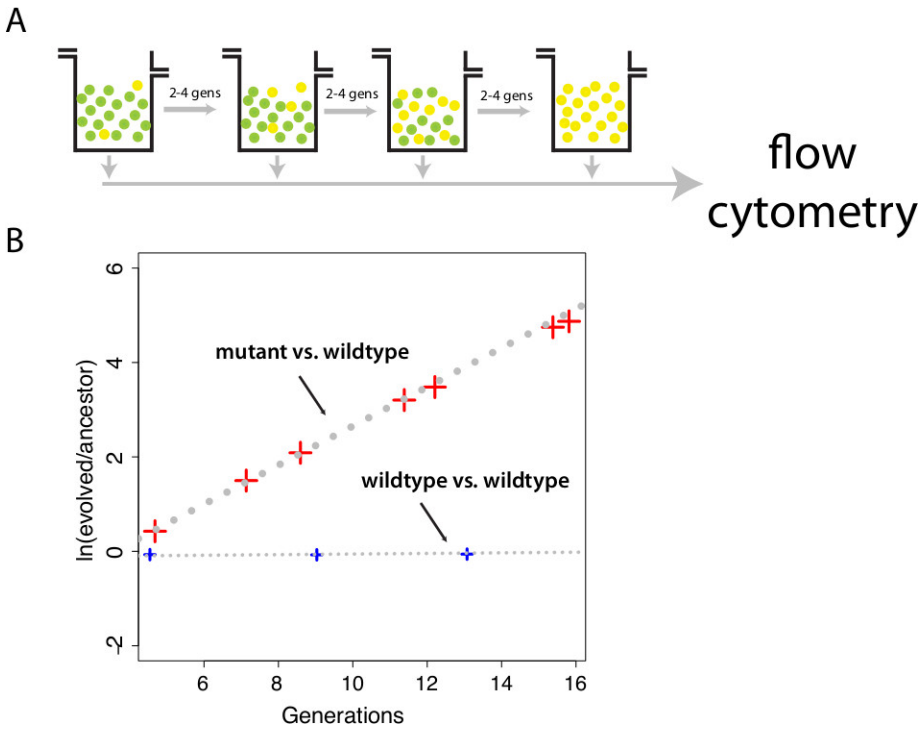

Figure 6. Strain competition assays in chemostats. A) Two strains that are differentially labeled by constitutively expressed fluorescent proteins are co-cultured in a single chemostat and the rate of change in the relative abundance of the two strains is determined every 2-4 generations using flow cytometry. **B)** The relative fitness of a strain is determined by linear regression of the natural log (ln) of the ratio of the two strains against time measured in generations. Click here to view larger figure.

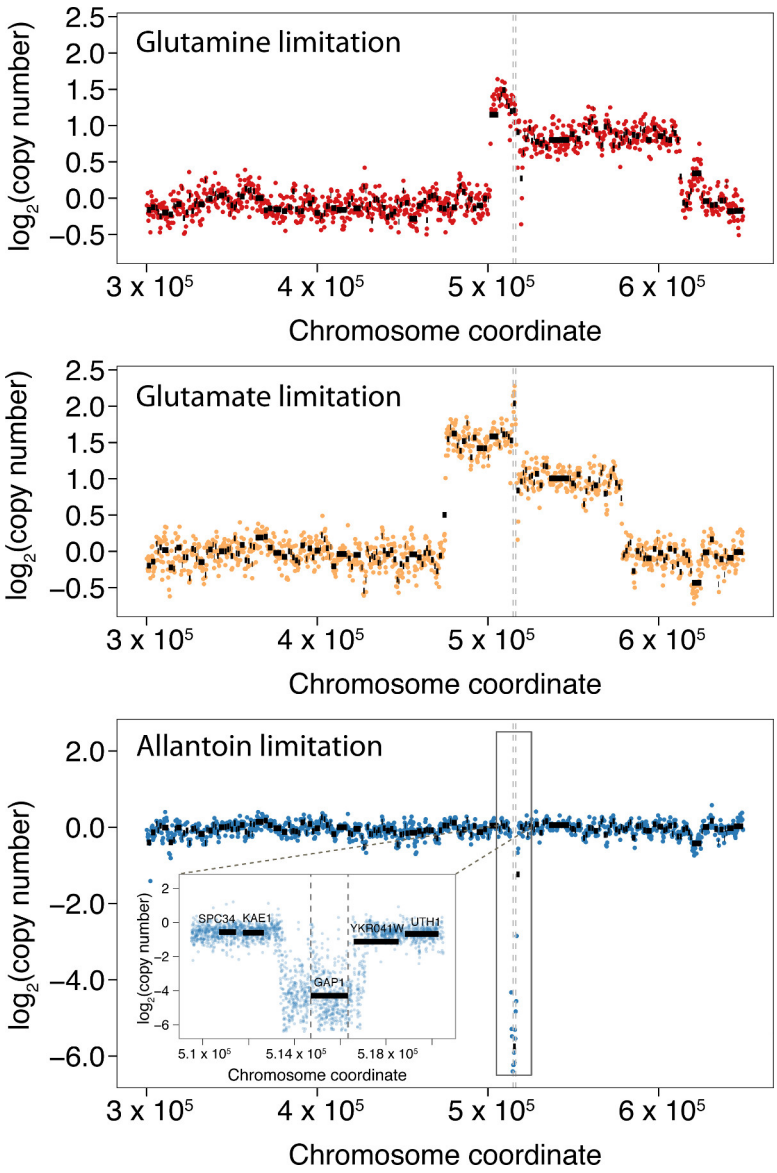

Figure 7. Long-term selection in chemostats efficiently selects for mutants with increased fitness. Genome-scale analysis of mutants has identified frequent chromosomal rearrangements and copy number variation (CNV) in mutants with increased fitness. Independent adaptive evolution experiments in different nitrogen-limiting conditions results in selection for different CNV alleles at the *GAP1* locus (demarked by gray dotted lines), which encodes the general amino acid permease. Each data point is the ratio of DNA copy number in the evolved strain compared with the ancestral strain measured using a DNA microarray that simultaneously analyzes every gene (black).

Table 1. Table of potential problem and solutions.

Problem	Solution
Low pH in culture vessel.	pH can be monitored in real time and controlled by automated addition of acid/base. Alternatively, buffered media can be used.
Flocculant cells and biofilms in culture vessel.	Keep culture well mixed by running impellor at > 400 rpm.
Growth in media feed lines.	The use of filters at the inlet port reduces the potential for colonization of media feed lines.
Cell synchronization and stable dO_2 oscillations in carbon limited chemostats.	These can be avoided by using higher dilution rates and avoiding prolonged periods of starvation before initiation of culture dilution.

Discussion

Chemostats enable the cultivation of microbes in growth-controlled steady-state conditions. The cells grow continuously at a constant rate resulting in an invariant external environment. This is in contrast to batch culture methods in which the external environment is continuously changing and the rate of cell growth is determined by the complex interaction of environment and genotype. Thus, a major advantage of culturing microbes in chemostats over batch cultures is the ability to experimentally control the growth rate of cells.

The rate at which a cell grows is the result of interactions between myriad cellular processes including nutrient sensing, signal transduction, macromolecular synthesis and metabolism. Using chemostats in combination with global analytical methods allows investigation of how the rate of growth impacts fundamental processes in the cell and conversely how the cell regulates and coordinates cellular process with its rate of growth. Studies in wildtype cells have shown that cellular concentrations of RNA and protein are profoundly impacted by rates of cell growth[6] and more recently it has been shown that transcriptome[10,12,13] and metabolome[8] are dramatically impacted by cell growth rates.

The study of mutant behavior in chemostats provides a potentially powerful means of studying the pathways that are important for growth rate regulation[14]. Using high throughput sequencing of molecular barcoded collections of thousands of mutants[15] it is now feasible to multiplex these assays enabling systematic studies of the genetic requirements for growth in nutrient-limited environments. It should be noted, however, that one of the limitations of chemostats is that they do not address the underlying heterogeneity in individual cell growth rates that can be assessed using single cell microscopy methods[16].

Chemostats also provide an ideal system for studying microbial evolution. Nutrient limitation is an ecologically relevant selective pressure and growth rate is a major component of microbial fitness. The chemostat provides a means of precisely controlling the selective pressure and studying how molecular networks evolve. Identifying the genetic loci that are targets of selection and proving their adaptive benefit in the same nutrient-limited environment[11,17-20] holds the promise of understanding the functional basis of adaptive evolution.

Chemostats are increasingly being used in new areas of research including the study of transcriptional dynamics[21,22] and metabolic oscillations[23-26]. Their application in ecology has proved useful in the study of predator-prey dynamics[27]. A renewed interest in mammalian cell growth regulation, and its impairment in human disease, may motivate a return to the study of mammalian cells in chemostats using cells that can be cultured in suspension[28].

Disclosures

The authors declare that they have no competing financial interests.

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References

1. Ingraham, J. L., Maaloe, O., & Neidhardt, F.C. *Growth of the Bacterial Cell*. Sinauer Associates, Inc, (1983).
2. Hall, M. N., Raff, M. C. , & Thomas, G. eds., *Cell Growth: Control of Cell Size*. CSHL Press, (2004).
3. Monod, J. La technique de culture continue, theorie et applications. *Ann. Inst. Pasteur.* **79**, 390-410 (1950).
4. Novick, A. & Szilard, L. Description of the chemostat. *Science.* **112**, 715-716 (1950).
5. Kjeldgaard, N.O., Maaloe, O., & Schaechter, M. The transition between different physiological states during balanced growth of *Salmonella typhimurium. J. Gen. Microbiol.* **19**, 607-616 (1958).
6. Maaloe, O. & Kjeldgaard, N.O. *Control of macromolecular synthesis*. W.A. Benjamin, (1966).
7. Saldanha, A.J., Brauer, M.J., & Botstein, D. Nutritional Homeostasis in Batch and Steady-State Culture of Yeast. *Mol. Biol. Cell.* **15**, 4089-4104 (2004).
8. Boer, V.M., Crutchfield, C.A., Bradley, P.H., Botstein, D., & Rabinowitz, J.D. Growth-limiting intracellular metabolites in yeast growing under diverse nutrient limitations. *Mol. Biol. Cell.* **21**, 198-211, doi:10.1091/mbc.E09-07-0597 (2010).
9. Boer, V.M., de Winde, J.H., Pronk, J.T., & Piper, M.D. The genome-wide transcriptional responses of Saccharomyces cerevisiae grown on glucose in aerobic chemostat cultures limited for carbon, nitrogen, phosphorus, or sulfur. *J. Biol. Chem.* **278**, 3265-3274 (2003).
10. Brauer, M.J., *et al.* Coordination of growth rate, cell cycle, stress response, and metabolic activity in yeast. *Mol. Biol. Cell.* **19**, 352-367, doi:E07-08-0779 [pii]10.1091/mbc.E07-08-0779 (2008).
11. Gresham, D., *et al.* Adaptation to diverse nitrogen-limited environments by deletion or extrachromosomal element formation of the GAP1 locus. *Proc. Natl. Acad. Sci. U.S.A.* **107**, 18551-18556, doi:10.1073/pnas.1014023107 (2010).
12. Regenberg, B., *et al.* Growth-rate regulated genes have profound impact on interpretation of transcriptome profiling in Saccharomyces cerevisiae. *Genome Biol.* **7**, R107, doi:10.1186/gb-2006-7-11-r107 (2006).
13. Castrillo, J.I., *et al.* Growth control of the eukaryote cell: a systems biology study in yeast. *J. Biol.* **6**, 4 (2007).
14. Cipollina, C., *et al.* Revisiting the role of yeast Sfp1 in ribosome biogenesis and cell size control: a chemostat study. *Microbiology.* **154**, 337-346, doi:10.1099/mic.0.2007/011767-0 (2008).
15. Gresham, D., *et al.* System-level analysis of genes and functions affecting survival during nutrient starvation in *Saccharomyces cerevisiae. Genetics.* **187**, 299-317, doi:10.1534/genetics.110.120766 (2011).

16. Levy, S.F., Ziv, N., & Siegal, M.L. Bet hedging in yeast by heterogeneous, age-correlated expression of a stress protectant. *PLoS Biol.* **10**, e1001325, doi:10.1371/journal.pbio.1001325 (2012).
17. Kao, K.C. & Sherlock, G. Molecular characterization of clonal interference during adaptive evolution in asexual populations of *Saccharomyces cerevisiae*. *Nat. Genet.* **40**, 1499-1504, doi:10.1038/ng.280 (2008).
18. Gresham, D.*et al.* The repertoire and dynamics of evolutionary adaptations to controlled nutrient-limited environments in yeast. *PLoS Genet.* **4**, e1000303, doi:10.1371/journal.pgen.1000303 (2008).
19. Wenger, J.W., *et al.* Hunger Artists: Yeast Adapted to Carbon Limitation Show Trade-Offs under Carbon Sufficiency. *PLoS Genet.* **7**, e1002202, doi:10.1371/journal.pgen.1002202 (2011).
20. Dunham, M.J., *et al.* Characteristic genome rearrangements in experimental evolution of Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 16144-16149 (2002).
21. Ronen, M. & Botstein, D. Transcriptional response of steady-state yeast cultures to transient perturbations in carbon source. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 389-394 (2006).
22. Kresnowati, M.T. A.P., *et al.* When transcriptome meets metabolome: fast cellular responses of yeast to sudden relief of glucose limitation. *Mol. Sys. Biol.* **2**, 49, doi:10.1038/msb4100083 (2006).
23. Tu, B.P., Kudlicki, A., Rowicka, M., & McKnight, S.L. Logic of the yeast metabolic cycle: temporal compartmentalization of cellular processes. *Science.* **310**, 1152-1158, doi:10.1126/science.1120499 (2005).
24. Tzur, A., Kafri, R., LeBleu, V.S., Lahav, G., & Kirschner, M.W. Cell Growth and Size Homeostasis in Proliferating Animal Cells. *Science.* **325**, 167-171, doi:10.1126/science.1174294 (2009).
25. Conlon, I. & Raff, M. Size control in animal development. *Cell.* **96**, 235-244 (1999).
26. Conlon, I.J., Dunn, G.A., Mudge, A.W., & Raff, M.C. Extracellular control of cell size. *Nat. Cell Biol.* **3**, 918-921, doi:10.1038/ncb1001-918 (2001).
27. Fussmann, G.F., Ellner, S.P., Shertzer, K.W., & Hairston, N.G. Crossing the hopf bifurcation in a live predator-prey system. *Science.* **290**, 1358-1360 (2000).
28. Cohen, E.P. & Eagle, H. A simplified chemostat for the growth of mammalian cells: characteristics of cell growth in continuous culture. *J. Exp. Med.* **113**, 467-474 (1961).

