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Luminescence as a Continuous Real-Time Reporter of Promoter Activity in Yeast Undergoing Respiratory Oscillations or Cell Division Rhythms

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Abstract

This chapter describes a method for generating yeast respiratory oscillations in continuous culture and monitoring rhythmic promoter activity of the culture by automated real-time recording of luminescence. These techniques chiefly require the use of a strain of *Saccharomyces cerevisiae* that has been genetically modified to express firefly luciferase under the control of a promoter of interest and a continuous culture bioreactor that incorporates a photomultiplier apparatus for detecting light emission. Additionally, this chapter describes a method for observing rhythmic (cell cycle-related) promoter activity in small batch cultures of yeast through luminescence monitoring.

Keywords

Saccharomyces cerevisiae; Luciferase; Bioluminescence; Continuous culture; Bioreactor; Yeast respiratory oscillation

1. Introduction

The bioluminescent reaction catalyzed by the enzyme firefly luciferase has become a useful genetic reporting system for monitoring rhythmic promoter activity in circadian studies of mammals (1, 2), insects (3), plants (4), and filamentous fungi (5). In addition, our work introduced the use of luciferase as a genetic reporter of respiration and cell cycle rhythms in the budding yeast *Saccharomyces cerevisiae* (6). Luciferase from fireflies emits light when the 62-kDa protein catalyzes the oxidation of a bioluminescent substrate “luciferin” (in the presence of O₂, ATP, and Mg²⁺) into oxyluciferin (and ADP and CO₂) (7). The emitted light is, therefore, an immediate and measurable indication of the enzyme’s activity. The relatively short half-life of luciferase (~30 min for destabilized luciferase (6)) allows its expression to dynamically reflect transcription on a faster time scale than longer-lived reporters such as βGal, CAT, or GFP (7, 8). Additionally, luciferase does not need excitation from an external light source as does GFP and other fluorescent reporters. Therefore, issues of photobleaching, autofluorescence, phototoxicity, and biological responses to an intense excitatory illumination can be avoided with a luciferase reporter.

Much of our research on the adaptation of the luciferase reporter system to yeast was the study of the yeast respiration oscillation (YRO) in bioreactors (6). A bioreactor (sometimes called a fermentor or chemostat) is a continuous culture apparatus that maintains a microorganism culture in a near steady-state level of exponential growth in which one component of the medium is the growth-limiting factor (9, 10). Within the reactor’s vessel, a specified volume of aerated medium sustains yeast growth in much the same way batch

growth occurs, but unlike batch growth, the growth environment (including pH, temperature, nutrition, biomass, and metabolic byproducts) is kept relatively constant by continually monitoring and adjusting variables such as pH and temperature in addition to constantly introducing fresh media at a steady rate while removing culture (i.e., media, cells, and byproducts) from the vessel at the same rate. As a result of these conditions, an inoculated culture grows to a concentration that is limited by the depletion of some component(s) of the medium and from that time onward, the growth rate is determined by the rate at which fresh medium is supplied (10).

Under a range of specific conditions of glucose-limited, aerobic continuous culture in bioreactors, spontaneous perturbations of the steady-state can lead to oscillations in various metabolite concentrations in the medium that are sometimes accompanied by (and possibly reinforced by) subpopulations of synchronously dividing cells (11). The most easily observed oscillating metabolite in the continuous culture is the dissolved oxygen (DO) concentration, which reflects the culture alternating between respiration and fermentation (Fig. 1) (12–14). We call this phenomenon as the yeast respiratory oscillation (YRO), but it also goes by other names including the yeast metabolic cycle (YMC) (13) and the energy metabolism oscillation (EMO) (14). Rhythmic transcription of many genes has been shown to occur at different phases of the YRO using microarrays (13, 15) and northern blots (14), but these methods are time consuming and ultimately limited by the frequency and number of samples taken from the culture. Bioluminescence monitoring of a promoter-coupled luciferase reporter in yeast is a good way to monitor rhythmic transcription continuously over the course of several days as well as to observe transcriptional responses to various treatments in real time.

In addition to having luciferase expressed in a desired strain of yeast, oxygen and luciferin are two requirements for the light emitting reaction that may become limiting during growth (and cause light levels to decrease regardless of luciferase expression). In particular, the researcher must be aware that during times of severe hypoxia, the luminescence signal may not represent the expression level of the promoter coupled to the luciferase gene (as shown during recurring hypoxic periods in Fig. 1, corresponding to the gray highlighted portions of Fig. 1b and the similar but not highlighted portions of Fig. 1a). During these hypoxic “masks,” no quantification of promoter activity can be obtained with the luciferase reporter regardless of its expression level. However, we have shown (by immunoblotting with anti-Luc) that levels of luciferase expressed from a constitutive promoter (*ACT1*) remain high during the hypoxic mask (as expected) and once this period of oxygen depletion subsides, luminescence from the reporter returns as a reliable indicator of promoter activity (6). A similar problem occurs, if the luciferin concentration is allowed to become limited. If this occurs, luminescence will decrease. Nevertheless, by keeping these limitations in mind and being aware of when they occur, using a luciferase reporter of promoter activity can be a useful tool in yeast. Corrections for hypoxia can be undertaken by using the P_{ACT1} -LUC reporter in an equivalent culture or experiment to that of the reporter of interest to indicate, if cultures are becoming hypoxic as discussed previously (6).

2. Materials

2.1. Yeast Inoculum Preparation for Continuous Culture

1. *S. cerevisiae* strain CEN.PK113-7D (containing luciferase reporter stably transformed into the genome, if bioluminescence is to be monitored).
2. YPD: 1% yeast extract, 2% peptone, and 2% D-glucose (anhydrous).
3. 50 mL Flask.

2.2. Generation of the YRO in Continuous Culture

1. 3 L New Brunswick Scientific Bioflo 110 or 115 Bioreactor with water jacket and direct drive agitation equipped with two Rushton-type impellers, condenser, pH probe, and DO probe.
2. Pressurized air supply capable of at least 4 L/min.
3. Bioreactor medium: 10 g/L anhydrous glucose, 5 g/L ammonium sulfate, 0.5 g/L magnesium sulfate heptahydrate, 1 g/L yeast extract, 2 g/L potassium phosphate, 0.5 mL/L of 70% v/v sulfuric acid, 0.5 mL/L of antifoam A, 0.5 mL/L 250 mM calcium chloride, and 0.5 mL/L mineral solution A. (Mineral solution A consists of 40 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 20 g/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 10 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2 g/L $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, and 20 mL/L 75% sulfuric acid.) (see Note 1).
4. Tubing: Silicone tubing i.d. 3/16 in., o.d. 9/32 in.; Norprene A-60-G tubing i.d. 1/16 in., o.d. 3/16 in. (see Note 2).
5. Reduction Couplers, sizes 1/16–1/8, 1/8–3/16, and 3/16–1/4 (see Note 2).
6. 2 N NaOH.
7. Media Bottles (10 L, 1 L, and 250 mL) with filter-vented cap and liquid exit port (see Note 3).
8. 30 mL Syringe and 21 g 1.5 in. needle.
9. 1 L Graduated Cylinder (suitable for autoclaving).
10. Chiller (circulating chilled water bath).
11. Waste collection container of choice (bucket, flask, or bottle) with at least a 4 L capacity.
12. Two (or more) 0.2 μM autoclavable air filters.

2.3. Luminescence Monitoring in Continuous Culture

1. Beetle luciferin (potassium salt).
2. 1 mL Syringe w/needle.

¹Make 10 L of bioreactor medium in a 10 L bottle. Mark the level on the 10 L bottle for 10 L of ddH₂O at room temperature with tape and/or permanent marker before mixing the medium. Remove at least 200 mL of the water and then combine all components of the bioreactor medium *except antifoam A and mineral solution A* before autoclaving. Mix, stir, or shake as needed to dissolve all components. Add ddH₂O until the volume of media is close to the 10 L mark. Cover the mouth of the bottle with a loose fitting cap and/or aluminum foil and autoclave the medium for 45 min (sterilization time). Let it cool overnight. Add antifoam A and mineral solution A after the medium has cooled, and then bring the volume to the 10 L mark with sterile ddH₂O.

²Tubing of different materials and sizes are needed for different tasks. The tubing that goes through peristaltic pumps needs to be both pliable and durable. The tubing that carries culture for luminescence monitoring needs to be flexible, sturdy, and transparent. The small 3/16 in. (o.d.) Norprene tubing is ideal for peristaltic pumps because it is both pliable and durable. The larger 9/32 in. (o.d.) silicone tubing is also pliable enough for peristaltic pumps, but is not as durable as the Norprene tubing and should be inspected for wear between uses. When possible, the Norprene tubing should be used in peristaltic pumps. However, because the ports on the bioreactor and media bottles may not permit the smaller Norprene tubing to attach, it may be necessary to use tubing of a different size to make the connections and join the different sized tubing with plastic reduction couplers. This is also true for joining the types of tubing needed for monitoring luminescence during continuous culture. If different tubing sizes or types are used besides the ones recommended here, make sure that they possess the necessary characteristics for their purposes (see Fig. 2).

³Bottles containing liquids that are to be added to the bioreactor need a filtered gas vent in their caps to reduce the risk of contaminating the continuous culture by air entering the bottle to replace displaced liquid. In addition, these bottles need a tube or pipe that penetrates the cap and extends to nearly the bottom of the bottle, through which the liquid in the bottle is removed and added to the bioreactor (usually by a peristaltic pump). These caps can be made using an appropriately sized two-hole rubber stopper with glass or metal tubes penetrating the holes with tight seals. If needed, the stopper can be held firmly in the bottle by an appropriately sized plastic cap that contains a wide hole drilled in the top to accommodate the glass or metal tubes coming through the stopper and large enough to allow the cap to screw down onto the bottle.

3. 60 mL syringe.
4. 16 gauge 1 in. needle.
5. Harvard Apparatus syringe pump.
6. Tubing: Clear plastic tubing i.d. 1/8 in., o.d. 1/4 in. (Nalgene); PTFE tubing i.d. 0.012 in., o.d. 0.03 in.; Silicone tubing i.d. 1/32 in., o.d. 3/32 in. (see Note 2).
7. Reduction Couplers, sizes 1/16–1/8, 1/8–3/16, and 3/16–1/4 (see Note 2).
8. Cole-Parmer Masterflex L/S Standard Drive 600 rpm Peristaltic Pump.
9. Black box (see Note 4).
10. Hamamatsu HC135-01 photomultiplier.
11. Two ring stands and clamps small enough to fit inside black box.
12. 50 mL plastic conical tube.
13. Aluminum foil.
14. Binder clip (1/2 in.).
15. Black cloth (2 × 10 ft, dimensions can vary).
16. Computer with data logger software (e.g., BioCommand by New Brunswick Scientific).
17. Computer with luminescence monitoring software (e.g., PMTMON by Tom Breeden, U. Virginia).

2.4. Luminescence Monitoring in Small Batch Culture

1. *S. cerevisiae* strain of choice (containing luciferase reporter stably transformed into the genome).
2. YPD: 1% yeast extract, 2% peptone, and 2% D-glucose (anhydrous).
3. Beetle luciferin (potassium salt).
4. 50 mL Flask.
5. Magnetic micro stirbar (~10 mm length).
6. Magnetic stirrer.
7. Styrofoam cup (see Note 5).
8. Black box (see Notes 4 and 5).
9. Hamamatsu HC135-01 photomultiplier.

⁴The light emitted from bioluminescent yeast is very dim compared with the amount of light in the environment. Even a room that is dark to the eye has enough stray photons from various sources to flood a sensitive light detecting photo-multiplier with noise that can conceal the true bioluminescent signal. Therefore, luminescent measurements must be made in an enclosure that totally excludes light from the environment. These enclosures are often painted black inside and out (to absorb stray photons) and so are sometimes called “black boxes.” A black box can be constructed from plywood and should include light-tight ports to permit tubing and wires to pass into and out of the box (see Fig. 3). Light-tight ports can be easily constructed out of black PVC elbows that are connected so that there are “corners” around which incident light cannot pass.

⁵The motorized magnetic stirrer used to stir the yeast culture in the black box generates some heat. Depending on the application, this heat may be useful to warm the culture to an optimum growth temperature for yeast. However, if this heat is undesirable for the application or too much heat is generated from the stirplate, excess heat can be dissipated from the culture by using a fan-cooled black box and/or elevating the 50 mL culture flask off of the magnetic stirrer by using an inverted Styrofoam cup cut to the desired height. (If fan cooling is used, a light-tight pathway for airflow must be constructed as mentioned in Note 4. For example, in Fig. 3, the small box on the left of the black box can serve as a light-tight path for airflow when a small fan is attached.)

10. Ring stand and clamp small enough to fit inside black box.
11. Computer with luminescence monitoring software (e.g., PMTMON by Tom Breeden, U. Virginia).

3. Methods

3.1. Yeast Inoculum Preparation for Continuous Culture and Bioluminescence Monitoring

1. In a 50 mL flask, prepare a 20 mL starter culture of yeast in YPD medium that will be used to inoculate the bioreactor. Inoculate 20 mL of YPD with a match-head-sized yeast colony or a scraping from an YPD plate (see Note 6).
2. Grow the starter culture for 20–30 h at 28–30°C with agitation.

3.2. Establishment of Respiratory Oscillations During Continuous Culture

Respiratory oscillations spontaneously arise in continuous cultures of certain strains of yeast when grown under a specific range of conditions. However, for this to occur, the culture must be sufficiently dense so that oscillations reinforce themselves. The quickest way to achieve this critical cell density is to inoculate the bioreactor with a starter culture of yeast and grow that bioreactor culture in batch overnight before beginning continuous culture the next day.

3.2.1. Bioreactor Setup

1. Prepare the Bioflo 110 (without baffles) for batch and continuous culture. Adjust two Ruston-type impellers on the agitator so that one is below the media level and one is at the air–media interface (when the vessel contains ~850 mL).
2. Autoclave the bioreactor and the necessary accessories (see Note 7).
3. Fill the bioreactor vessel with 850 mL sterile bioreactor media. A sterile 1 L graduated cylinder and sterile 1 L bottle with filter-vented cap can be used to measure and add the medium to the bioreactor.
4. Attach the loose end of tubing from the 250 mL bottle that has the filter-vented cap to a port of the bioreactor. Add 200 mL of sterile 2 N NaOH to the 250 mL bottle and load the Norprene tubing from the bottle's cap into the peristaltic pump that controls the culture's pH, but do not turn on the pump at this time (see Note 8).
5. Attach an autoclaved 0.2 μ M air filter to the sparger inlet and connect the other end of the filter to a regulated pressurized air supply. Introduce filtered air into the

⁶This protocol will reproducibly generate respiratory oscillations for the MAT-a yeast strain CEN.PK113-7D (from Peter Kötter, U. Frankfurt, Germany), but other strains of CEN.PK may work as well. Other strains of yeast such as S288C (14) and IFO 0233 (15) also manifest robust YROs under certain conditions of continuous culture but may not be suited to the precise conditions described here. If bioluminescence will be monitored, then the appropriate strain containing the desired luciferase reporter should be used. If the luciferase reporter has been stably integrated into the genome of the yeast strain, continued selection with an antibiotic is not needed during the establishment of respiratory oscillations.

⁷In addition to the autoclaved bioreactor, it is helpful to have the following items sterilized by autoclave and cooled before proceeding: one 0.2 μ M air filter connected to ~3 in. of silicone tubing, one 250 mL bottle with a filter-vented cap and the outflow tube (see Note 3) connected to ~6 ft of Norprene A-60-G tubing (i.d. 1/16 and o.d. 3/16), one 1 L bottle with a filter-vented cap and the outflow tube connected to ~6 ft of Norprene A-60-G tubing, one separate filter-vented cap (that fits the 10 L bottle) with the outflow tube connected to ~6 ft of Norprene A-60-G tubing, one 1 L graduated cylinder, ~6 ft of silicone tubing (i.d. 3/16 and o.d. 9/32), and a ~10 ft length of the same silicone tubing. The exposed ends of all the tubing should be covered with aluminum foil before autoclaving. Also, the separate filter-vented cap that fits the 10 L bottle should be autoclaved in a covered beaker or completely wrapped in foil. It will be added to the 10 L bottle of medium later.

⁸Attaching the bottle of NaOH at this time serves to keep the used tri-port inlet covered by sterile tubing. And it is important to install the tubing into a peristaltic pump (that is off) at this time to prevent back flow of the pressurized air from the bioreactor into the NaOH bottle.

bioreactor's media through the sparger at a flow rate of 0.9 L/min. Begin agitation at 550 rpm.

6. If the bioreactor has a water jacket, attach the water jacket and vapor condenser to the circulating water chiller set to operate at 4°C (see Note 9). Turn the bioreactor's temperature control to 30°C and let the media and condenser come to the desired temperatures.
7. Adjust the level of the stainless steel tube in the bioreactor that is to serve as the medium's outflow tube to the level of the media–air interface (see Note 10). Then connect a ~10 ft length of sterile silicone tubing to the media outflow port and load it into a peristaltic pump (turned off) and arrange the rest of the 10 ft tubing to deliver bioreactor waste to a collection container of choice.
8. Adjust (and maintain) the pH of the media to the desired pH (recommended pH 3.4–4) (see Note 11).
9. After the DO probe has polarized (see Note 12) and prior to inoculation, calibrate the DO probe.

3.2.2. Inoculation and Growth

1. Inoculate the bioreactor by injecting the 20 mL culture through the septum with a syringe and a 21 gauge needle. If luminescence from this culture is going to be monitored, see Note 13.
2. Grow the yeast in batch culture overnight. During this time, the DO of the culture gradually drops as the culture becomes denser and total respiration of the culture increases. Once the carbon sources have been consumed, the DO of the culture rises sharply (about 16–18 h after inoculation). Incubate the culture in this starved condition for 4–7 more hours before beginning continuous culture (however, see Note 14).
3. Begin continuous culture at a dilution rate of ~0.085/h (see Note 15). Set the outflow pump for 100% duty cycle to remove media as the level of the culture rises

⁹The same water chiller can be used to cool the bioreactor and the vapor condenser, but the vapor condenser must be plumbed so that it can be continually cooled by the circulating water from the chiller. When the vapor condenser is kept cool (0–4°C), it helps to prevent the bioreactor from drying out as a result of continuously flowing air through the culture. The vent from the vapor condenser can be covered with an air filter to help minimize risk of culture contamination, but the filter sometimes becomes wet over time and air flow through it is reduced. An uncovered length (2–3 ft) of sterile silicone tubing from the condenser's vent works well to prevent culture contamination while permitting unrestricted air flow through the condenser. Also, for the condenser to work properly, all other avenues of gas flow from the bioreactor should be sealed. This includes unused tri-port inlets and other ports in the bioreactor's head plate. A small length of tubing with a knot tied in one end works well for sealing an unused port.

¹⁰Since the volume of the bioreactor should be kept constant at ~850 mL during continuous culture, setting the level of the outflow tube (i.e., the tube to the waste) to the level of the stirred and aerated media at this time will establish the proper volume for the culture (see Fig. 2, "tube at surface of culture").

¹¹The pH of the media will often lag the readout from the probe so one should manually adjust the pH gradually until the desired pH is reached. However, accidentally overshooting the desired pH by less than one pH unit at this point does not noticeably affect the establishment of respiratory oscillations. At times during batch growth, the pH of the culture may rise above the desired pH, but this will not adversely affect the formation of respiratory oscillations once continuous culture begins.

¹²Various DO probes require some length of time to polarize their electrodes before accurate oxygen concentrations can be made. It is recommended to allow 2–6 h (or a length of time specified by the manufacturer) after attaching the DO probe's wiring to the bioreactor before calibrating the DO probe.

¹³The best time to begin moving culture through the closed loop for luminescence monitoring is prior to inoculating the bioreactor (or at least prior to the establishment of respiratory oscillations). The initial change of conditions that occurs when the high rpm pump begins moving culture through the closed loop can perturb oscillations that have already been established. The best way to avoid this perturbation is to have the culture moving through the closed loop from the beginning (during batch growth).

¹⁴Batch growth (including the 4–7 h of starvation) has been found *not to be necessary* for the establishment of respiratory oscillations. One can begin continuous culture immediately after inoculation, but such a method may consume more media before oscillations begin (usually ~24 h after inoculation).

to the level of the removal tube within the bioreactor. Respiratory oscillations often begin about 12 h after the initiation of continuous culture.

3.3. Luminescence Monitoring in Continuous Culture

Luminescence of the continuous culture is constantly monitored by using a high speed peristaltic pump to move culture through a closed loop from the bioreactor, into a dark box, in front of a photomultiplier tube for measurement, and back to the bioreactor (see Fig. 2).

1. Connect a closed loop of autoclaved tubing to the bioreactor for luminescence monitoring. This loop includes a length of Norprene A-60-G tubing (3/16 in. o.d.) that passes through a high rpm peristaltic pump (e.g. Cole-Parmer Masterflex L/S) (turned off) and connects (by a coupler) to a ~10 ft length of transparent Nalgene tubing (1/4 in. o.d.) (see Notes 2 and 16). Connect the free end of the Norprene tubing of the loop to the media sampling port – this is where the circulating loop of culture leaves the bioreactor. Connect the other end of the loop (the free end of the transparent Nalgene tubing) to a port that returns the culture back to the vessel (see Note 16).
2. Pull a portion of the closed loop (comprising the majority of the transparent Nalgene tubing) through a light-tight port of the black box. Wrap the transparent Nalgene tubing of the loop around a 50 mL conical tube (or some other cylinder of approximate size) for several turns (see Note 17). Use a 1/2 in. binder clip to keep the tubing from unraveling from the conical tube (see Fig. 3). Within the black box, use a ring stand clamp to hold the cylinder and coiled tubing near a photomultiplier device so that light from yeast flowing through the transparent tubing can be detected by the photo-multiplier (see Note 18). Close the black box and cover any light leaks with foil or black cloth.
3. Turn on the high rpm peristaltic pump to begin moving culture through the closed loop (see Note 13). The speed of the pump is not critical, but it should not be so slow that the culture is kept away from the bioreactor for more than a minute. A circuit time of about half a minute is preferable, which can be achieved with ~180 rpm.
4. Immediately after the closed loop is filled with culture (and culture from the closed loop can be seen returning to the bioreactor), lower the level of the outflow tube in the bioreactor to the new level of the culture–air interface. This is important in

¹⁵One needs to know the flow rate of the media supply pump in combination with the tubing used to know what pump speed results in a dilution rate of 0.085/h. For an 850 mL culture and media supplied through the pump by Norprene A-60-G tubing (i.d. 1/16 in. and o.d. 3/16 in.), a duty cycle of 34% will achieve a dilution rate ~0.085%. The speed of the outflow pump is not important as long as it removes culture at a faster rate than the supply pump adds medium to the culture. Setting the outflow pump to 100% is recommended.

¹⁶The order of the loop in the direction of culture-flow should be as follows: media sampling port, Norprene tubing (through high rpm peristaltic pump), transparent Nalgene tubing, and inlet port (of choice). It is important that the high rpm peristaltic pump draws the culture from a port that has a stainless steel tube that extends below the surface of the mixed bioreactor culture. The lengths of the Norprene and Nalgene tubings can vary as needed to accommodate distances from bioreactor, pump, and black box. The connection between the Norprene tubing and the Nalgene tubing should be made just downstream of the high rpm peristaltic pump and should remain outside of the black box since a leak at this connection may be difficult to identify if it is within the black box.

¹⁷Wrapping the transparent tubing around a cylinder provides an increased surface exposure of the culture to the light detecting photomultiplier tube. If the luminescence is sufficiently bright, fewer turns around the cylinder are required. The intensity of the luminescence signal can be increased by coating the cylinder with reflective aluminum foil before wrapping the tubing around it and can be further increased by wrapping the cylinder with a double layer of turns of the transparent Nalgene tubing from the loop.

¹⁸If the black box is not completely light tight, background light can still interfere with the luminescent signal. Background light can be further reduced by encapsulating the entire photomultiplier and cylinder with aluminum foil and then covering both with a loose arrangement of black cloth. Also, room light can travel through the transparent Nalgene tubing carrying the culture into and out of the black box (by analogy with optic fibers); therefore, wrapping the exposed Nalgene tubing with foil and keeping the room lights off (or dim) will help to reduce background light. If there is a small amount of unavoidable background room light leak detected by the photomultiplier, it is better to maintain the room light at a stable (dim) level than turning on (and off) the room lights to make adjustments to the apparatus.

order for continuous culture to maintain the same dilution rate since some of the volume of the culture no longer resides in the reactor vessel.

5. Add 5 μM luciferin to the bioreactor's culture during a phase of the respiratory oscillation when dissolved oxygen is decreasing rapidly or near the trough (see Note 19). This can be done by injecting 425 μL of a 10 mM (2,000 $\times$) stock solution of luciferin into the bioreactor through the septum with a 1 mL syringe and needle.
6. Maintain a 5 μM concentration of luciferin in the bioreactor during continuous culture by adding luciferin to the media that feeds the culture or supplying a steady drip of luciferin from a syringe pump (see Notes 20 and 21).
7. Turn on the power to the photomultiplier device and begin recording bioluminescence.

3.4. Luminescence Monitoring in Small Batch Culture

For other applications, where continuous culture is not required, luciferase reporters can be used to monitor promoter activity in small batch cultures of yeast, for example, to monitor promoters for inducible genes such as *GAL1* or cell cycle-related genes such as *POL1*.

1. Synchronize the cell cycle of the bioluminescent yeast strain of choice (see Note 22). Various methods for synchronizing the yeast cell cycle are described elsewhere (6, 16).
2. Transfer a volume (10 mL) of the synchronized cells in the appropriate growth medium (YPD) with 50 μM luciferin to a 50 mL flask containing a micro-stirbar.
3. Place the 50 mL flask containing the culture on a magnetic stirrer within the black box (see Notes 4 and 5) and stir the culture at a medium to fast speed.
4. Use a stand and clamp to position a photomultiplier tube next to the stirred culture in the black box. Angle the photo-multiplier tube so that it can capture the most light from the culture. Aluminum foil may be used to help direct more photons toward the photomultiplier.
5. Close the black box and begin recording (see Note 23).

¹⁹A sudden delivery of luciferin to a bioluminescent strain of yeast in the respiro-fermentative phase can acutely drop the intracellular oxygen concentration, which can result in a phase shift of the oscillation. To avoid affecting the oscillation, charge the culture with luciferin during the respiratory phase of the oscillation when intracellular oxygen levels are already low.

²⁰Because the culture in the bioreactor is constantly being diluted during continuous culture, the concentration of luciferin will gradually decline if not constantly supplied at a concentration and rate that keeps up with the dilution rate of the culture. One easy way to do this (over a short term) is to add 5 μM luciferin to the medium that feeds the continuous culture; however, this method is not recommended for long-term experiments because luciferin degrades in the acidic medium over time. For long-term experiments, where luminescence needs to be measured for more than several hours, use a syringe pump to supply a steady drip of a concentrated stock of luciferin to the bioreactor. For example, a 120 $\times$ stock of luciferin in water (i.e., 600 μM) supplied to the bioreactor at 1/120 of the culture's dilution rate (i.e., 0.6 mL/h) will maintain a constant 5 μM luciferin concentration in the culture without adversely affecting the dilution of the culture. 60 mL of luciferin at this concentration and pump speed can supply the bioreactor for more than 4 days. The stability of the luciferin in the syringe can be increased by shielding the luciferin from light and by chilling the syringe with several wraps of tubing carrying cold water from the bioreactor's condenser.

²¹It can be difficult to regularly drip luciferin into the culture at slow pump speeds. If delivered to the bioreactor through one of its normal ports, luciferin can adhere to the inside of the vessel or headplate rather than dripping down into the culture. A steady drip into the culture can be achieved, however, if the luciferin is delivered to the culture through very thin rigid tubing (e.g., PTFE tubing i.d. 0.012 and o.d. 0.03). Use a 16 gauge needle to penetrate the septum of the bioreactor and while the needle is through the septum, thread a few inches of the autoclaved tubing through the needle so that the end of the tubing hangs freely in the reactor's vessel. Gently remove the needle from the septum leaving the tubing in place, held securely by the septum. Attach the other end of the tubing to the syringe of the syringe pump that supplies luciferin to the vessel. The thin PTFE tubing can be connected to the syringe by constructing an adaptor from a cut p200 pipette tip and a short (~1 in.) piece of silicone tubing i.d. 1/32 and o.d. 3/32. This adaptor including the cut pipette tip should be autoclaved while attached to the PTFE tubing prior to use.

²²This protocol describes the steps needed to monitor the rhythms of cell cycle-related promoter activity. If the use of bioluminescence is not to observe cell cycle-related promoter activity, then cell cycle synchronization may not be required.

6. Perform any necessary detrending of the luminescent signal (see Note 24).

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²³During batch culture, yeast will eventually begin respiring and consuming oxygen at a high rate. As a result, luminescence can decline due to limited oxygen. Luminescent reporters of promoter activity are not accurate once oxygen becomes limited. Oxygen limitation can be monitored with a parallel culture of luciferase driven by a strong constitutive promoter such as actin (*ACT1*).

²⁴The number of cells in a batch-grown culture increases over time. As a result, the total bioluminescence from the culture increases as well. To observe rhythmic promoter activity from a culture in which bioluminescence increases with cell density, it may be necessary to subtract from the luminescent signal the trend of luminescence that results from the increase in cell density. There are several methods to accomplish a trend correction. One procedure is to generate a polynomial trendline that best represents the growth of the culture and use this formula for baseline subtraction of the luminescence signal. Another method is to repeat the experiment using a parallel culture of the same strain that has not been synchronized; luminescence from this nonsynchronized culture can be used as a baseline for cell growth that can be subtracted from the luminescent trace from the synchronized culture.

Fig. 1.

Examples of the yeast respiratory oscillation monitored by dissolved oxygen (DO) and simultaneously plotted with luminescence measurements from two different luciferase reporters. **(a)** Seven cycles of a yeast respiratory oscillation are shown for 35 continuous hours by monitoring the dissolved oxygen concentration (*dashed black line*) and bioluminescence from yeast transformed with a destabilized luciferase driven by the promoter for *POL1* (a cell cycle-regulated promoter whose activity peaks near the G1/S boundary; *gray line*). This particular culture has a period of about 5 h for the YRO. Dissolved oxygen is measured as percent saturation by atmospheric oxygen of the medium. The brackets above the first oscillation labeled R-F and R show the respiro-fermentative phase and respiration phase of the oscillation, respectively. **(b)** Six cycles of a yeast respiratory oscillation from a separate experiment are shown for 22 continuous hours by monitoring the dissolved oxygen concentration (*dashed black line*) and bioluminescence from yeast transformed with luciferase driven by the promoter for *ACT1* (a constitutive promoter under these conditions; *gray line*). This particular culture has a period of 3.75 h for the YRO. Note that during times of recurring hypoxia (indicated by the *gray highlighted regions*), the luminescence signal drops to nearly zero until adequate oxygen levels return.

Fig. 2.

A schematic diagram showing the setup for continuous monitoring of bioluminescence during continuous culture. Pump A is the peristaltic pump that supplies medium to the bioreactor. Pump B is the peristaltic pump that removes culture from the bioreactor. Pump C is the high speed peristaltic pump that moves culture from the bioreactor into the black box for luminescence monitoring and back to the bioreactor through the closed loop. The *large arrows* indicate the direction of flow through the different types of tubing indicated in *parentheses*. The coupler shown in the closed loop of pump C indicates the junction of Norprene tubing (needed to withstand the action of the high speed peristaltic pump C) and Nalgene tubing (needed because it is transparent). PMT is the photomultiplier tube.

Fig. 3.

A diagram showing the setup within the black box for continuous monitoring of bioluminescence during continuous culture. The front panel has been removed in this diagram to show the box's interior. The small box on the *left* and the pipe on the *right* of the black box are light-tight ports through the black box for wires and tubing, respectively. Within the box on the *left*, a ring stand and clamp hold a photomultiplier tube positioned to collect light. On the *right*, a ring stand and clamp support a 50 mL conical tube around which Nalgene tubing (from the bioreactor) is wrapped and held in place by a 1/2 in. binder clip.