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Protocol

Monitoring Yeast Intracellular Ca^{2+} Levels Using an In Vivo Bioluminescence Assay

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This protocol describes the use of the jellyfish *Aequorea victoria* aequorin protein to measure Ca^{2+} levels in living yeast cells. All yeast strains to be analyzed must express the *A. victoria* apoprotein of the aequorin calcium biosensor, to be reconstituted into fully active aequorin by association with its cofactor, coelenterazine, which cannot be synthesized by yeast itself. The simplest way to achieve reconstitution is to transform yeast cells with a vector driving apoaequorin expression, and then supply commercially available coelenterazine cofactor in the medium. Coelenterazine is a hydrophobic molecule and is able to permeate yeast cells.

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MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

$CaCl_2$

Coelenterazine (1 mg/mL in methanol; Invitrogen)

Store at −20°C in the dark. Coelenterazine is extremely sensitive to oxidation and light. Check that the solution is yellowish and not orange; the change in color indicates oxidation. Native coelenterazine and all five analogs are available together in a Sampler Panel for comparison and evaluation.

Culture medium appropriate for yeast strain of interest

EGTA

MES/Tris buffer (optional; see Step 10)

Prepare a 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) solution and adjust the pH to 6.5 with 1 M Tris base.

Resuspension buffer (RB) appropriate for the stimulus used to induce calcium signaling

- For hypotonic shock, use YPD medium (2% glucose, 2% tryptone, 1% yeast extract) or the same medium used to grow cells. Hypotonic shock can be realized by sample dilution with five volumes of deionized water.

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- For hypertonic shock, use YPD medium (2% glucose, 2% tryptone, 1% yeast extract) or the same medium used to grow cells. Hypertonic shock is usually induced by addition of 1 M NaCl or 1 M KCl.
- For glucose-induced calcium signaling, cells should be starved for a carbon source for 1.5 h before coelenterazine loading. In this case, the RB should be a carbon source-free solution (e.g., 100 mM MES/Tris buffer [pH 6.5] or 0.67% yeast nitrogen base [YNB]). The calcium concentration should be controlled/varied by buffering with $CaCl_2$/EGTA.

Triton X-100 (optional; see Step 10)

Yeast strain of interest containing either multicopy or integrative aequorin-expressing plasmids, such as the following:

pEVP11-Aeq (*ADH1* promoter, *LEU2* gene as selectable marker; Batiza et al. 1996)
pDB617 (*ADH1* promoter, *URA3* gene as selectable marker; Kellermayer et al. 2003)
pVTU(W)-AEQ (*ADH1* promoter, *URA3* or *TRP1* marker; Trópia et al. 2006)
pYX012(KanMX4)AEQ (integrative plasmid; *TPI* promoter, *URA3* and *KanMX4* markers; Tisi et al. 2002)
pYX212-AEQ (*TPI* promoter, *URA3* marker; Tisi et al. 2002)
pYX242-AEQ (*TPI* promoter, *LEU2* marker; Tisi et al. 2002)

Equipment

Benchtop centrifuge
Microcentrifuge
Luminometer tubes

Store in the dark.

Nitrocellulose filters (0.2-µm pores) (optional; see Step 2)

Cellulose acetate filters can also be used, but they are not recommended because they can release fibers in the sample that can interfere with luminescence detection.

Sample collection system (optional; see Step 2)

MicroFunnel Plus Filter Funnels (Pall Corporation) is an example, but any system which allows recovery of the filter after use is suitable.

Tube or multiwell plate luminometer (e.g., Berthold Lumat LB 9507 or superior)

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METHOD

1. Inoculate yeast strains containing either multicopy or integrative aequorin-expressing plasmids in a flask with the appropriate culture medium and incubate with vigorous mixing until mid-log phase ($2–8 \times 10^6$ cells/mL).

   Cells are generally grown at 30°C overnight, but temperature-sensitive cells should be cultivated at 24°C. In order for cells to reach exponential phase, at least six duplication cycles should be allowed. Culture volume depends on the number of variables to be tested and strength of signal to be detected (see Step 7).

   See Troubleshooting.

2. Harvest cells by centrifugation in a benchtop centrifuge at 4000 rpm (~2000*g*) for 10 min or filtration on nitrocellulose filters with a sample collection system and wash them with cold resuspension buffer (RB).

   Add additional wash steps if the chosen RB is completely different from the medium.

3. Resuspend cells in fresh medium or in RB at a density of 2.5×10^9 cells/mL.

   Any experimental pretreatment that takes longer than 10 min should be done in this step, before the addition of coelenterazine, to avoid any significant consumption of the reconstituted aequorin before the phase of calcium-related signal detection is started. After treatment, adjust cell density before proceeding with Step 4.

4. Add 0.02 mg/mL (final concentration) coelenterazine and incubate at room temperature in the dark for 20 min.

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5. Centrifuge cells at 7000 rpm for 2 min. Discard the supernatant to eliminate the excess coelenterazine.
6. Wash cells three times with RB (200 µL/wash/treatment; see Step 7) and collect by centrifugation at 7000 rpm for 1–2 min.
7. Resuspend the cells in an adequate volume of RB (200 µL/treatment).

   The number of cells that are suitable for each treatment depends on the strength of the signal you are detecting, and can vary from 1 to 6×10^7 cells per treatment. The correct number should be assessed in preliminary experiments.

8. Transfer the cellular suspension into the luminometer tubes (200 µL each), and monitor light emission with the luminometer at intervals of 1–10 sec, both before (until the signal is stable) and after the stimulus. Light emission (L) is reported in relative luminescence units/seconds (RLUs/sec).

   Under normal conditions, L values should not exceed 1% of the maximal light emission capacity, to allow proper detection of the signal without aequorin exhaustion. The timing and intensity of the signal can vary according to the stimulus and the conditions used (see examples in Fig. 1).

   See Troubleshooting.

9. Add appropriate experimental stimulus. For example, in the presence of 1 mM EGTA, vary $CaCl_2$ from 0 to 10 mM. Alternatively, add desired compounds or inhibitors to the appropriate final concentrations. Continue to monitor light emission.

   Free calcium concentration influences calcium influx in yeast cells and should be considered in each experiment. Note that yeast media contain a certain amount of calcium, which is quite difficult to evaluate, but is in the order of 0.2–0.9 mM. To evaluate the effect of calcium presence/absence, calcium chelators—such as EDTA, EGTA, and BAPTA—can be used to subtract calcium, and progressive addition of calcium chloride allow the evaluation of dose–response correlation. For evaluation of free calcium concentration in specific experimental condition, free software is available at Stanford University (http://maxchelator.stanford.edu/).

10. At the end of each experiment, test aequorin expression and activity to normalize the signals. Use one of the following two methods for normalization:

    - Lyse cells with 0.5% Triton X-100 in the presence of 10 mM $CaCl_2$ (use a stock solution of 100 mM $CaCl_2$ in 100 mM MES/Tris buffer [pH 6.5]) and monitor total light emission (L_{max}) (Tisi et al. 2002).

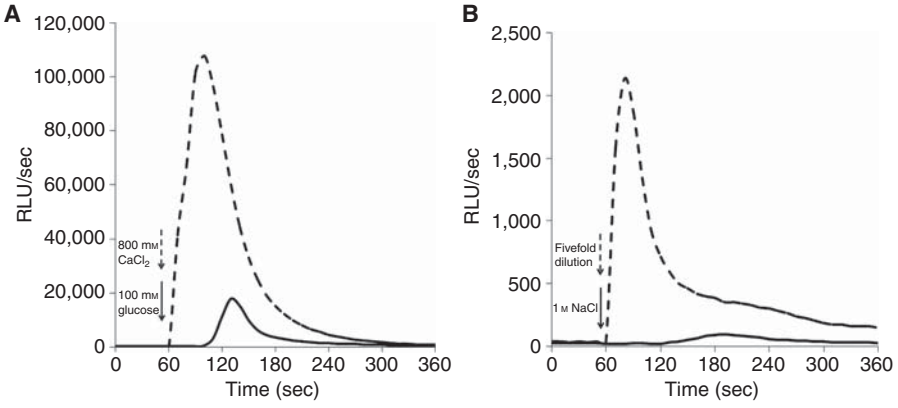

FIGURE 1. Calcium signaling can have different shapes and timing in yeast. The curves represent aequorin light emission in RLU/sec (RLUs per second) from a W303-1A wild-type strain carrying pVTU-AEQ plasmid, grown in YPD medium, loaded with coelenterazine and challenged with the following stimuli: (*A*) solid line, 100 mM glucose addition (in the presence of 1 mM $CaCl_2$ in the resuspension medium) after 2 h nutrient starvation; dashed line, 800 mM $CaCl_2$ addition; (*B*) solid line, hypertonic shock with 1 M NaCl addition in the presence of 1 mM $CaCl_2$; dashed line, hypotonic shock by fivefold dilution in deionized water in the presence of 1 mM $CaCl_2$. Relative luminescence emission was normalized to an aequorin content giving a total light emission of 3×10^6 RLU in 18 min after lysing cells with 0.05% Triton X-100.

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- Measure maximal light emission from crude cell extracts on Ca^{2+} addition (L_{max}) as described in detail by Batiza et al. (1996).

  The maximum intensity is used to normalize light emission according to the amount of aequorin expressed. See Troubleshooting.

11. After measuring maximal light emission on Ca^{2+} addition (L_{max}), plot L/L_{max} values on the appropriate standard curve to estimate the free cytosolic calcium concentrations, as described in **Using Targeted Variants of Aequorin to Measure Ca^{2+} Levels in Intracellular Organelles** (Granatiero et al. 2014).

TROUBLESHOOTING

Problem (Step 1): Yeast cells do not grow.

Solution: Apoaequorin-expressing plasmids usually do not cause any growth defect even in very sick strains. Therefore, consider the following:

- Check the medium requirements for the strain auxotrophies.
- If you are using a mutant strain, check for growth defects requiring medium supplementation.
- Check the marker of the plasmid you are using and if you are using the appropriate selective medium.

Problem (Step 8): The RLUs are not higher in the aequorin-expressing strain than in the negative control.

Solution: Consider the following:

- Total amount of aequorin is very low: See next problem.
- The amount of cells used is too low to reveal the signal. Repeat with more cells per sample.

Problem (Step 10): The estimation of total aequorin is too low (i.e., similar to the value obtained for the negative control, a strain that does not express apoaequorin, loaded with coelenterazine).

Solution: Consider the following:

- Cells do not express enough apoaequorin because they are not exponentially growing or metabolically active, or the strain is very sick and inefficient in protein synthesis. Try to use better culture conditions (richer medium, lower temperature).
- Cells may have lost the plasmid due to genetic instability or incorrect selection. Check cells for the presence of the aequorin expressing vector.

  Note that only 20% of cells in a population will lose a multicopy plasmid after overnight growth even in the total absence of selection (i.e., in rich medium).

- Coelenterazine did not permeate the cells: Check cell density in Step 4; coelenterazine is hydrophobic and permeates cells better if the suspension is very thick.

REFERENCES

Batiza AF, Schulz T, Masson PH. 1996. Yeast responds to hypotonic shock with a calcium pulse. *J Biol Chem* **271:** 23357–23362.

Granatiero M, Patron M, Tosatto A, Merli G, Rizzuto R. 2014. Using targeted variants of aequorin to measure Ca^{2+} levels in intracellular organelles. *Cold Spring Harbor Protoc* doi: 10.1101/pdb.prot072843.

Kellermayer R, Aiello DP, Miseta A, Bedwell DM. 2003. Extracellular Ca^{2+} sensing contributes to excess Ca^{2+} accumulation and vacuolar fragmentation in a *pmr1*Δ mutant of *S. cerevisiae*. *J Cell Sci* **116:** 1637–1646.

Tisi R, Baldassa S, Belotti F, Martegani E. 2002. Phospholipase C is required for glucose-induced calcium influx in budding yeast. *FEBS Lett* **520:** 133–138.

Trópia MJ, Cardoso AS, Tisi R, Fietto LG, Fietto JL, Martegani E, Castro IM, Brandão RL. 2006. Calcium signaling and sugar-induced activation of plasma membrane H^+-ATPase in *Saccharomyces cerevisiae* cells. *Biochem Biophys Res Commun* **343:** 1234–1243.

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