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Real-Time Fluorescence Detection of Calcium Efflux During Vacuolar Membrane Fusion

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Abstract

During in vitro homotypic yeast vacuole fusion Ca^{2+} is transported into and out of the organelle lumen. In vitro, Ca^{2+} is taken up from the medium by vacuoles upon the addition of ATP. During the docking stage of vacuole fusion Ca^{2+} is effluxed from the lumen upon the formation of *trans*-SNARE complexes between vesicles. Here we describe a real-time fluorescence-based assay to monitor the transport of this cation using purified organelles. Extraluminal Ca^{2+} is detected when the cation binds the low-affinity fluorescent dye Fluo-4 dextran. This allows for the use of a 96-well microtiter plate to be read in a fluorescence plate reader. Thus, in addition to a curve of calibrated Ca^{2+} standards, up to 91 experimental conditions can be monitored in a single microplate using this method.

Keywords

Membrane fusion; SNARE; Membrane trafficking; Ca^{2+} efflux; Fluorescence; Yeast vacuole

1 Introduction

The role of Ca^{2+} as a regulator of cellular processes is well established in cell biology [1]. In the context of SNARE-mediated fusion, Ca^{2+} stimulates synaptic vesicle fusion with the plasma membrane. SNARE bundles on synaptic vesicles are locked in a partially zippered state by synaptotagmin-1 to prevent fusion. Upon membrane depolarization, Ca^{2+} is taken up by the cells from the extracellular space after which these cations interact with the C2 domain of synaptotagmin-1. Ca^{2+} binding causes synaptotagmin-1 to undergo a conformation change that releases it from SNAREs, thus permitting the completion of zippering and fusion of the bilayers [2].

During *Saccharomyces cerevisiae* vacuolar fusion a similar influx of Ca^{2+} into the cytoplasm is observed [3]. The major reservoir of Ca^{2+} in *Saccharomyces cerevisiae* is the lumen of vacuoles/lysosomes [4]; therefore, the influx of Ca^{2+} into the cytoplasm is commonly described as an efflux of Ca^{2+} from the vacuole lumen. Release of Ca^{2+} during vacuolar membrane fusion is triggered by *trans*-SNARE pairing [5] (Fig. 1). While the trigger of Ca^{2+} efflux is known, the mediators of this event are poorly understood though several studies have addressed the need for Ca^{2+} in fusion [3, 6, 7]. Additionally, the ubiquity of Ca^{2+} as an intracellular signal makes analysis of a specific event difficult. In

order to accomplish a detailed study of Ca^{2+} efflux during vacuolar fusion it is therefore useful to isolate vacuoles and study fusion in vitro.

Here we describe a real time to detect *trans*-SNARE-triggered Ca^{2+} efflux during in vitro vacuolar fusion. Extraluminal Ca^{2+} is detected when bound to the low-affinity fluorescent Ca^{2+} probe Fluo-4 dextran. The vacuole fusion pathway is initiated by the addition of ATP. An initial influx of Ca^{2+} is immediately observed upon the addition of ATP and is followed by an efflux of Ca^{2+} during the docking stage. The Ca^{2+} efflux assay protocol included here has been used by our lab to characterize the effect of multiple fusion inhibitors and genetic deletions on *trans*-SNARE complex formation [8–11].

2 Materials

Prepare all solutions using ultrapure water. PS buffer, Ficoll solutions, DEAE-dextran Solution, and $10 \times$ fusion salts can be made in advance and stored at 4 °C. ATP regeneration buffer can be made in advance and stored in aliquots at –80 °C. Wash buffer and spheroplasting buffer should be made fresh before each experiment.

2.1 Vacuole Isolation

1. YPD: 10 g/L Yeast extract, 20 g/L peptone, 20 g/L dextrose.
2. 0.2% YPD: 10 g/L Yeast extract, 20 g/L peptone, 2 g/L dextrose.
3. 1 M-PIPES-KOH, pH 6.8: Weigh 151 g of PIPES and mix with 300 mL of water. Adjust pH with 10 M KOH; the solution will appear milky until the pH is close to 6.8. Once the pH is 6.8 dilute to 500 mL with water. Filter sterilize and store at 4 °C.
4. 4 M Sorbitol: Add 364 g sorbitol to 200 mL warm water. To ensure mixing begin stirring water with a magnetic stir bar prior to addition of sorbitol. Once solution is clear dilute to 500 mL with water. Store at room temperature.
5. 1 M Potassium phosphate buffer, pH 7.5: Mix 1 M H_2HPO_4 with 1 M KH_2PO_4 to reach a pH of 7.5.
6. Wash buffer: 10 mM Tris-HCl, pH 9.4, 1 mM DTT. Add 5 mL of 1 M Tris-HCl, pH 9.4 and 500 μL of 1 M DTT to a 100 mL graduated cylinder. Add water to a volume of 50 mL.
7. Spheroplasting buffer: 50 mM Potassium phosphate buffer, 0.8 $\times$ 0.2% YPD, and 0.6 M sorbitol. Add 0.75 mL of 1 M potassium phosphate buffer, and 2.25 mL of 4 M sorbitol to a graduated cylinder. Add 0.2% YPD to a volume of 15 mL.
8. Oxalyticase (see Note 1).

¹.We purify oxalyticase as described [12]. Purified oxalyticase is stored in 20–25 mL aliquots at –80 °C or kept at 4 °C for short-term use. Commercially available versions may be used.

9. PS buffer: 20 mM PIPES-KOH, pH 6.8, and 200 mM sorbitol. Add 20 mL of 1 M PIPES-KOH, pH 6.8, and 50 mL of 4 M sorbitol to a graduated cylinder. Dilute to 1 L with water. Store at 4 °C.
10. 15% Ficoll solution: Add 45 g Ficoll PM400 to 200 mL warm water. To ensure mixing begin stirring water with a magnetic stir bar prior to addition of Ficoll. Once solution is clear add 3 mL of 1 M PIPES-KOH, pH 6.8, and 15 mL of 4 M sorbitol. Dilute to 300 mL with water. Store at 4 °C.
11. 8% Ficoll solution: Add 21 mL of 15% Ficoll to 18.4 mL PS buffer.
12. 4% Ficoll solution: Add 20 mL of 8% Ficoll to 20 mL PS buffer.
13. DEAE-dextran solution: Add 100 mg of DEAE-dextran to 10 mL of 15% Ficoll in a 15 mL conical tube. Allow dextran to sit for 10 min before vortexing into solution. Store at 4 °C.
14. Temperature-controlled water-bath.
15. Ultracentrifuge with SW-41 or compatible rotor.
16. Low-speed centrifuge with JLA 10.500 and JA-20 rotors, or compatible rotors.

2.2 Calcium Efflux

1. ATP regeneration buffer: 6 mg/mL ATP, 10 mg/mL creatine kinase, 95 mg/mL creatine phosphate, 10 mM MgCl₂, 10 mM PIPES-KOH, pH 6.8, and 200 mM sorbitol. Weigh 60 mg of ATP and dissolve in 1 mL of water. Weigh 100 mg of creatine kinase and dissolve in 2 mL of water. Weigh 950 mg of creatine phosphate and dissolve in 3 mL of water. Mix 2 mL of water with 100 µL of 1 M PIPES-KOH, pH 6.8, and 100 µL 1 M MgCl₂. In the following order add creatine kinase, creatine phosphate, and ATP solutions into the PIPES-KOH solution (*see* Note 2). Add 500 µL of 4 M sorbitol. Adjust pH to 6.8 with 1 M KOH. Dilute to 10 mL with water. Store in aliquots at -80 °C.
2. 10× Fusion salts: 1.25 M KCl, 50 mM MgCl₂, 20 mM PIPES-KOH, pH 6.8, and 200 mM sorbitol. Mix 41.6 mL of 3 M KCl, 5 mL of 1 M MgCl₂, 2 mL of PIPES-KOH, pH 6.8, and 5 mL of 4 M sorbitol in a graduated cylinder. Dilute to 100 mL with water. Store at 4 °C.
3. 10 µM Fluo-4 dextran low affinity (*see* Note 3).
4. Calcium calibration buffer kit.
5. Black, half-volume 96-well flat-bottom microplate.
6. Microplate fluorescence reader.

²-Addition in the wrong order can cause components to precipitate out of solution.

³-We have utilized Fluo-4 dextran low affinity for our studies. This product has since been discontinued for commercial sale. We have used Cal-520-dextran conjugate MW 10,000 and have found it to reproduce results obtained with low-affinity Fluo-4 dextran. Alternatively, Aequorin luminescence can be utilized [3, 5]; be aware that in our hands the Aequorin detection system has shown sensitivity to DMSO and thus is not suitable for studies involving reagents diluted in DMSO.

3 Methods

3.1 Vacuole Isolation

1. Cells are grown overnight in 1 L of YPD in a 2 L Erlenmeyer flask to an $OD_{600} = 0.6\text{--}0.8$ (*see* Note 4).
2. Harvest 900 mL of culture by two rounds of centrifugation at $3000 \times g$ for 5 min at 4 °C. Discard supernatant.
3. Resuspend the pellet in 50 mL of wash buffer by vortexing (*see* Note 5).
4. Pellet the culture by centrifugation at $3000 \times g$ for 5 min at 4 °C. Discard supernatant (*see* Note 6).
5. Resuspend the pellet in 15 mL of spheroplasting buffer by vigorously swirling. Once in solution add 600–750 μL of oxalyticase and incubate at 30 °C for 35 min in a water bath (*see* Note 7).
6. Transfer spheroplasted cells to pre-chilled Oak Ridge tubes and centrifuge at $900 \times g$ for 10 min at 4 °C. Discard supernatant by aspiration (*see* Note 8).
7. Resuspend spheroplast pellet in 2.0 mL of 15% Ficoll solution by gentle swirling (*see* Note 9).
8. Add 35–74 μL of DEAE-dextran solution to lyse resuspended spheroplasts. Incubated on ice for 4 min followed by incubation at 30 °C for 3 min in a water bath. After incubation at 30 °C place lysate on ice (*see* Note 10).
9. Transfer 4 mL of lysate to a pre-chilled high-speed centrifugation tube on ice (*see* Note 11).
10. Overlay the lysate with 3 mL of 8% Ficoll solution followed by 3 mL of 4% Ficoll solution. Finally fill the tube to within 5 mm of the top with PS buffer (*see* Note 12).

⁴Cells at lower or higher densities than recommended show lower fusion activity.

⁵Pellet should resuspend easily. If the pellet does not easily resuspend this is commonly a sign of contamination in the culture.

⁶The pelleted culture will quickly go back into solution after centrifugation; therefore it is recommended to discard the supernatant immediately following centrifugation.

⁷Add 0.45–0.55 g of prepared oxalyticase per liter of culture. The required concentration of oxalyticase is strain specific and therefore will need to be independently determined for any new strain. To assess whether the chosen concentration of oxalyticase is correct, ensure that after pelleting the spheroplasting buffer the pellet is loose.

⁸After centrifugation the resulting pellet will be loose; therefore ensure that the Oak Ridge tube is angled with the pellet at the bottom to avoid resuspension.

⁹During resuspension of the spheroplasted cells a resistant pellet will be left; this should not be resuspended but rather left out of solution.

¹⁰The required volume of DEAE-dextran solution per liter of culture is strain specific and therefore will need to be independently determined. To assess whether the chosen concentration of DEAE-dextran solution is correct, ensure that layers form during the organelle flotation step indicating that organelles were released from spheroplasts.

¹¹Volume of lysate will vary between 3 and 4 mL depending on starting OD_{600} of cells. It is beneficial at this step to pipet air into the lysate creating bubbles at the top of the layer. This will make formation of subsequent layers less prone to disruption. Additionally, it is recommended to submerge all but the top 1/2 in. of the tube in ice so it does not move during gradient formation.

¹²It is useful to hold the tube out of the ice while layering with a light source behind in order to monitor the layer interface. If the interface begins to move this indicates that you are adding in buffer too quickly and need to slow down. To control the flow of buffer addition we utilize a 21 g 1–1/2 in. needle with a 3 mL syringe. If a layer fully mixes you should remove all but the lysate layer and restart the gradient. Ensure that tubes are at least 90% full; otherwise they may collapse during high-speed centrifugation.

11. Centrifuge gradients in a high-speed centrifuge in a swinging bucket rotor at $96,000 \times g$ for 90 min at 4 °C.
12. Place gradients back on ice following centrifugation (*see* Note 13).
13. Harvest vacuoles from the interface between the 0% Ficoll solution and 4% Ficoll solution interface. Collect vacuoles in a 1.5 mL Eppendorf tube placed on ice (*see* Note 14).
14. Quantitate concentration of vacuoles harvested by Bradford assay (*see* Note 15).

3.2 Calcium Efflux Assay

1. Prepare calcium efflux reactions by mixing 6 μ L of 10 $\times$ salts, 0.6 μ L of 1 mM CoA, 0.24 μ g of IB₂, 20 μ g of vacuoles, and 0.9 μ L of 10 μ M Fluo-4 dextran low affinity per reaction in an Eppendorf tube (*see* Notes 16 and 17).
2. Bring volume of mix up to 56 μ L per reaction with PS buffer (*see* Notes 18 and 19).
3. Add 56 μ L of Ca²⁺ efflux reaction to the chosen well of a pre-chilled microplate on ice. Repeat for all conditions to be tested.
4. Prepare Ca²⁺ standards in the microplate by mixing 30 μ L of standard solution, 0.9 μ L of 10 μ M Low-Affinity Fluo-4 Dextran, and 29.1 μ L of PS buffer (*see* Note 20).
5. Load the plate into a microplate fluorescence reader set to 27 °C.
6. Measure Fluo-4 Dextran Low-Affinity fluorescence by excitation at 485 nm and detecting emission at 520 nm every minute for a period of 90 min (*see* Note 21).
7. Take an initial measurement and pause the fluorescence plate reader.
8. Add 6 μ L of ATP regeneration buffer or PS buffer to each well (*see* Note 22).

13. To avoid mixing of the gradient place the high-speed centrifugation tube in a premade hole in the ice. This will ensure that the tube is unable to tip.

14. To harvest vacuoles, use a p200 pipette tip with the tip cut off, or a wide-bore p200 pipette tip. Remove 150 μ L of vacuoles at a time ensuring to minimize the amount of buffer collected. If done slowly the vacuoles typically will clump making it easier to harvest without diluting.

15. If left on ice vacuoles can begin to settle to the bottom of the Eppendorf; therefore ensure that vacuoles are always resuspended by gently inverting the tube before using. Useable vacuole concentrations are 0.4–1.5 mg/mL. We have found vacuoles below this range fuse poorly, and higher concentrations while possible generally indicate contamination in the vacuole layer.

16. We purify IB₂ as described [13]. Alternative names include LMA2, Pbi2, and I₂^B.

17. For the most accurate results it is preferable to create a master mix by scaling up the mix for the number of conditions to be tested.

18. If additional reagents are to be tested in the Ca²⁺ efflux assay these can be added directly to the microplate. Ensure that all wells have an equal volume prior to Ca²⁺ efflux reaction addition by adding PS buffer to control wells. Compensate for the pre-loaded volume in the microplate by adding less PS buffer to the master mix. The amount of additional reagents able to be added to a calcium efflux assay is dependent on the concentration of harvested vacuoles; this volume is typically 4–15 μ L.

19. Additional reagents may be added during the course of the assay. These should be treated the same as reagents added at the start of the assay by compensating for their volume in the master mix. Additionally, when adding reagents during the assay ensure that all wells to be analyzed together receive an equal volume by compensating with PS buffer in control wells.

20. Ca²⁺ standards are created according to the manufacturer's specifications. We utilize the Ca²⁺ calibration buffer kit #1 from Thermo Fisher. Ca²⁺ levels during the assay are in the range of 0.75–0.1 μ M; therefore at least 4 Ca²⁺ standards spanning this range should be used in addition to a 0 M Ca²⁺ standard.

21. We have found that our results are best when gain is set to 75% of a No ATP well, 10 flashes per measurement are used at minimum, and the plate undergoes 100 rpm orbital shaking.

22. A no-ATP regeneration buffer well is run in order to ensure that changes in Fluo-4 Dextran Low-Affinity signal are due to an ATP-triggered fusion reaction and used to normalize data. As signal rapidly changes upon ATP addition it is recommended to utilize a

9. Continue measuring Fluo-4 Dextran Low-Affinity fluorescence for 90 min (*see* Note 23).

3.3 Calcium Efflux Analysis

1. Background fluorescence is subtracted from all wells at all time points as determined by the corresponding time point in the 0 calcium standard well.
2. Fluorescence measurements are normalized by dividing by the average fluorescence signal of a well lacking ATP regeneration buffer.
3. Calcium efflux is calculated by subtracting the amount of fluorescence at max influx by the amount of fluorescence at maximum efflux (*see* Notes 24 and 25).

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multichannel pipettor when adding ATP regeneration buffer or PS Buffer. These buffers can be loaded in the microplate ahead of time in empty wells. Finally, when adding buffers ensure that excess air is not pipetted into wells as this may create bubbles which will alter fluorescence signal.

²³. As vacuolar fusion causes an initial Ca^{2+} influx followed by Ca^{2+} efflux we have it useful to assess the effects of reagents on both separately. Addition of reagents at the start of the reaction can alter Ca^{2+} influx whereas addition of reagents following influx will only alter the following efflux event.

²⁴. Ca^{2+} influx typically plateaus at 6–10 min and calcium efflux typically takes 15–30 min to plateau. The rate of efflux appears to be highly dependent on time from vacuole harvest to the start of the Ca^{2+} efflux assay. It is recommended to start the assay within 30 min of harvesting vacuoles.

²⁵. Ca^{2+} influx can also be calculated by subtracting the fluorescence signal at maximum influx from the initial fluorescence reading.

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Fig. 1.

Ca^{2+} efflux is not affected on *dgk1* vacuoles. Fusion reactions ($2 \times$) were prepared with 20 μg of wild-type or *dgk1* BJ3505 variants in the presence of 150 nM low affinity Fluo-4 dextran in the presence or absence of ATP. A subset of reactions were treated with 0.5 μM Gyp1-56. Fluo-4 fluorescence was normalized to wild-type vacuoles without ATP at the initial time point (reproduced from [11] with permission from John Wiley and Sons)