Assay of lipid mixing and fusion pore formation in the fusion of yeast vacuoles.

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Abstract

Fluorescence de-quenching can be used to analyze membrane lipid mixing during an *in vitro* fusion reaction. Here we describe a method to measure lipid mixing using vacuolar membranes purified from the yeast *Saccharomyces cerevisiae*. Labeling the isolated organelles with rhodamine-phosphatidylethanolamine allows to reveal ATP-dependent lipid mixing through fluorescence de-quenching in a spectrofluorometer. Combining this assay with content mixing indicators, such as the fusion-dependent maturation of a lumenal vacuolar phosphatase, then permits the detection of hemifusion intermediates and the analysis of the requirements for fusion pore opening.

Running head: Measuring vacuole fusion

Key words: membrane fusion, lipid mixing, yeast, vacuole

1. Introduction

Membrane fusion is a fundamental process in cell biology that is at the heart of important physiological processes, such as transport between intracellular organelles, secretion of hormones and neurotransmitters, fertilization, or pathological processes such as virus invasion and metastasis [1-4]. Before reaching completion, many fusion events transit through an intermediate hemifusion state, in which two engaged membranes mix lipids but leave their contents separated [5]. Although this situation has usually been considered only as a very transient moment during SNARE-driven membrane fusion, a growing number of *in vitro* and *in vivo* observations suggest that this intermediate could be more stable than expected [1, 6-10].

While hemifusion has been readily detectable in artificial membrane systems, its detection and analysis in physiological SNARE-dependent fusion events, both in vitro and in vivo, has remained much more challenging. This is a crucial limitation to the further elucidation of the fusion pathway because it is critical to study these intermediates not only in synthetic lipid systems but also in their physiological membrane environment. The composition of synthetic SNARE-dependent membrane systems can be chosen at will, which can grossly vary their fusion properties, including the occurrence of intermediate states, and allows to optimize them for the study of precisely defined, mechanistic questions. The advantage of studying a physiological membrane system is that here the parameters are much more constrained, i.e. the lipid composition and the density of the fusion proteins is set by the cell. Since these parameters are critical determinants of the fusion pathway [11-17], the analysis of these complex systems remains necessary to judge the physiological lifetime and relevance of fusion intermediates, and their potential regulation by the cell.

A systematic characterisation of the factors contributing to the formation of this intermediate and its progression to full fusion requires experimental systems and assays that allow a robust detection of the hemifused state and the characterisation of protein interactions that accompany it. Both conditions are met by the lysosome-like vacuoles of yeast, which can be isolated in large

quantities and good purity [18, 19]. They represent a physiological, SNARE-dependent membrane fusion system [20, 21]. Here, we describe a simple method to measure lipid mixing during the fusion of purified yeast vacuoles that can be combined with a content mixing assay in order to reveal a hemifused state [10, 22-25].

Yeast vacuoles allow separate assay of lipid and content mixing. Content mixing is measured through the activation of a luminal vacuolar enzyme, pro-alkaline phosphatase (contained e.g. in strain BJ3505) by the vacuolar protease Pep4 (contained e.g. in strain DKY6281), which is enclosed in the other fusion partner [26]. Assay of content mixing thus requires the separate preparation of two vacuole populations, followed by their mixing in vitro. (Fig. 1A). To measure lipid mixing, one of the fusion partners is labeled with rhodamine-phosphatidylethanolamine (Rh-PE) at a self-quenching concentration [22]. Upon lipid mixing between the donor membranes, Rh-PE dilutes over the acceptor membrane, causing an increase in the fluorescence signal by de-quenching (Fig. 1C and D). Membrane proximity (Fig. 1B, "docking") is not sufficient to achieve this de-quenching [10, 22, 27]. Upon fusion, the contents of both fusion partners are mixed and the protease *pep4* cleaves the pro-peptide from pro-alkaline phosphatase (*p-pho8*), maturing it into the form *m-pho8* (Fig. 1E). The activity of this enzyme provides a readout for content mixing and full fusion. It is measured by a simple colorimetric assay, the conversion of the colorless p-nitrophenol phosphate into the yellow p-nitrophenol (Fig. 1F). Since vacuole fusion reactions proceed for up to 90 min in vitro, fusion samples can be split at numerous intermediate time points. Comparison of the lipid and content mixing signals then allows to identify bona-fide hemifusion intermediates as a state in which Rh-PE dequenching occurs but alkaline phosphatase remains immature. This approach has allowed to dissect the function of numerous fusion factors on the vacuolar membranes and assign their activities to distinct stages of the fusion reaction [10, 22-25, 28-30].

2. Materials.

1. DTT buffer: 9 ml of 1M Tris HCl pH 8.9, 0.45 g of DTT and H_2O up to 300 ml.
2. YP medium: 400g of yeast extract + 800g of polypeptone are dissolved in 36L of ddH_2O, aliquoted in flasks and bottles, sterilized and stored at room temperature.
3. Spheroblasting buffer: 15 ml of 4 M Sorbitol, 10 ml of 500 mM KPi pH 7.5 and 75 ml of YP medium containing 0.2% glucose. Mix, remove 12-18 ml and fil up with 12-18 ml of 0.1 mg/ml lyticase preparation [22]. Mix and leave at room temperature. Other, commercially available spheroblasting enzymes, such as zymolyase 100T, can also be used.
4. Rotor with 6 swing-out buckets, such as SW40 (Beckman).
5. 3 mM Rhodamine-phosphatidylethanolamine (Rh-PE, Molecular Probes) is dissolved in DMSO. The solution stored at -20°C in aliquots of 60-80 µl.
6. Bradford solution diluted 1 to 5 is kept at 4°C.
7. PS buffer: 10 mM PIPES/KOH pH 6.8, 200 mM sorbitol. This buffer is sterile filtered if longer storage is desired.
8. 3 x 15 ml Falcon tubes with 0%, 4% and 15% of Ficoll-400 in PS buffer is freshly prepared and kept at 4°C.
9. 10 ml 5% (w/v) of milk powder in ddH_2O is kept at 20-25°C.
10. 3 M KCl, 10 mM $MnCl_2$, 20% (w/v) Triton TX-100, 1 M glycine pH 11.5 and 1 M Tris/HCl pH 9.0 are kept at room temperature.
11. An ATP regenerating system is prepared by mixing ATP, creatine phosphate (CP) and creatine kinase (CK) in the following ratio: 50µl of 100 mM ATP + 25µl of 50mg/ml CK + 200µl of 1M CP + 275µl Ficoll 0%.

- 100 mM ATP: 60.5 mg of ATP is dissolved in a solution containing 100 µl of 1 M $MgCl_2$, 100 µl of 500 mM PIPES pH6.8, 52.5 µl of 4M KOH and 747.5 µl of ddH_2O. Aliquots were stored at -20°C.
- 50 mg/ml creatine kinase: 25 mg of creatine kinase is dissolved in a solution containing 0.5 ml of 10 mM PIPES/KOH pH 6.8 and 50% (v/v) of glycerol. Aliquots are stored at -20°C. Note that creatine kinase slowly loses activity over several months at -20°C. Once this activity drops below a critical threshold, the ATP regeneration system mixed from an aged stock suddenly stops working.

- 1 M creatine phosphate: 1.31 g of creatine phosphate is dissolved in 4 ml ddH_2O, aliquoted and stored at -80°C.

12. 100 mM p-nitrophenyl-phosphate (PNP) is prepared by dissolving 461.4 mg in 10 ml ddH_2O. The solution is aliquoted and stored at -20°C.
13. Phosphate assay mix: 100 µl of 1 M $MgCl_2$, 100 µl of 100 mM PNP, 200 µl of Triton TX-100 20% (w/v), 2.5 ml of 1 M Tris/HCl pH 8.9 and 7.2 ml of ddH_2O. This solution is sufficient for 20 reactions and has to be freshly prepared before use.
14. Protein inhibitor cocktail (PIC), 1000x stock: 200 mM pefablock (Carl Roth), 5 mg/ml leupeptin (Bachem), 500mM o-phenanthroline (Sigma-Aldrich) and 5 mg/ml pepstatin A (Bachem). Aliquots are stored at -20°C.
15. Fusion Buffer: 120 mM KCl and 0.33 mM $MnCl_2$ in PS buffer. The mix is kept on ice.
16. Non-coated black 96-well plate (NUNC).

3. Methods.

Handle all vacuole-containing samples with pipette tips with a wide orifice (e.g. cut open with scissors) to avoid membrane rupture by shearing.

3.1. Vacuole isolation

1. Cells are incubated overnight in 1L YPD, using baffled 2l Erlenmeyer flasks at 30°C at 150 rpm. Inoculate the cultures such that they are in logarithmic phase at the time of harvesting the next morning, with an OD_{600nm} ranging between 1 and 1.5. Growth to higher densities should be avoided because it lowers fusion activity. Re-dilution of overgrown cultures for 1-2 h before harvesting does not cure the adverse effects of growth at higher cell density. Once the cultures have been taken out of the incubator, harvest the cells immediately and do not let the cultures stand for longer times without shaking.
2. Harvest 330 ml of culture at 2500 x g for 2-3 minutes at 4°C. Preferably, a centrifuge with high acceleration and deceleration rates should be chosen.
3. Discard supernatant and resuspend cells in 50 ml of DTT buffer by vortexing and incubate in the water bath at 30°C for 5-6 minutes.

4. Centrifuge at 2500 x g for 2-3 minutes, discard supernatant, resuspend the cells in 15 ml of spheroblasting buffer by vortexing and incubate in water bath at 30°C for 25-30 minutes.
5. Transfer the suspension into 30 ml Corex tubes and harvest spheroblasts by centrifugation at 4°C, 2500 x g for 2 minutes. Discard supernatant, taking care that the pellet, which is quite loose, is not lost.
6. Resuspend spheroblasts in 2 ml of ice-cold 15% Ficoll-400 in PS buffer by gentle vortexing or stirring with a rod.
7. Add 150-250 µl of ice-cold DEAE-dextran solution, mix by gentle shaking and leave tubes on ice for 2 minutes before incubating them in a water bath for other 2 minutes at 30°C. The amount of DEAE dextran to be added must be optimized according to the strain background, growth conditions and spheroblasting enzyme used.
8. Cool the suspension on ice and transfer it into an SW40 tube. Make discontinuous gradients by overlaying the suspension with steps of 8%, 4% and 0% Ficoll-400 in PS buffer, such that the tube is filled up to the top.
9. Spin in an ultracentrifuge at 2°C, 150000 x g for 90 minutes.
10. Remove lipids from the top of the tubes by using an aspiration pump and harvest vacuoles from the 0-4% Ficoll interface using cut pipette tips. Transfer the organelles into ice cold reaction tubes. For optimal fusion results, the organelles should be used within an hour after harvesting. Longer storage leads to loss of activity.

3.2. Vacuole membrane labeling

1. A Rh-PE aliquot is thawed by incubating it at 37°C for 20 minutes under strong agitation (14'000 rpm).
2. During this period of time, incubate non-coated black 96-well plate with 200 µl of 5% milk powder (w/v) per well at 20-25°C. This coats the wells with protein and reduces the adhesion of vacuoles and proteins to the plastic.
3. Centrifuge Rh-PE for 15 minutes at 12'000 x g in a table top centrifuge to sediment non-dissolved material.

4. Meanwhile, collect vacuoles from the Ficoll gradient and determine their protein concentration using Bradford solution and BSA as a standard.
5. 560 µg of DKY6281 vacuoles are mixed with 800 µl of PS buffer in a siliconized 2 ml reaction tube and equilibrated for 40 seconds at 32°C under gentle agitation (500 rpm).
6. Rh-PE is slowly injected (3 x 17 µl) into the vacuole suspension under continuous vortexing at 500 rpm. After that, vacuoles are incubated in a water bath for 30 seconds at 27°C (see Note 1).
7. Add 500 µl of pre-warmed 15% Ficoll buffer and gently mix by inverting the tubes 4 times. After a short spin, put the sample on ice.
8. Prepare small discontinuous density gradients in 2 ml reaction tubes by overlaying vacuoles with 300 µl of pre-warmed 4% Ficoll buffer and 400 µl of pre-warmed PS buffer, taking care to create sharp interfaces.
9. Transfer the mini-gradients in a pre-cooled centrifuge equipped with a swing-out rotor. Spin for 7 minutes at 3°C and 11'700 x g, using slow acceleration and deceleration.
10. Stained vacuoles can be harvested from the 4% Ficoll-PS interface by careful aspiration with a pipette. The organelles are kept on ice and their protein concentration is determined by Bradford assay.
11. Discard milk from the 96-well plate, wash the wells with ddH_2O and take care to remove all traces of water before proceeding to the next step.

3.3. Lipid mixing assay

1. After labeling with Rh-PE and determination of protein concentration, vacuoles from BJ3505 and DKY6281 are mixed at a ratio of 5 to 1.
2. A standard lipid mixing reaction contains:
    - 36 µg of vacuoles (30 µg non-labelled BJ3505 + 6 µg labelled DKY6281)
    - 112 mM KCl
    - 0.33 mM $MnCl_2$
    - 60 µl Fusion Buffer
    - 9.5 µl of ATP regeneration system
    - PS buffer up to 120µl

3. Add 100 µl of each reaction mix into the corresponding wells of a 96-well plate pre-cooled on ice, reserving the remaining 90 µl for the parallel content mixing assay.
4. Put the plate into a microplate fluorescence reader with temperature control (SpectraMax Gemini XS) and let it to equilibrate at 27°C for 2-5 minutes before starting the measurements.
5. Samples are excited at 544 nm and fluorescence changes are measuring at 590 nm every 2 minutes for a total period 32 minutes (from F_t=0 min to F_t=32min). An emission cutoff filter (590nm) is used.
6. After 32 minutes, add to every well 100 µl of 1% Triton TX-100/Ficoll 0%, mix and continue acquisition for the next 10 minutes taking measurements every 30 seconds. The corresponding average values will $F_{(TX100)}$, the fluorescence expected upon maximal de-quenching of the vacuole-associated Rh-PE (see Note 2).

3.4. Content mixing assay

1. Use the remaining 90 µl of every reaction for the content mixing assay. Incubate the samples in the water bath at 27°C for 90 minutes.
2. After 90 minutes, assay the generated activity of alkaline phosphatase by adding 0.5 ml of pre-warmed phosphate assay mix to every tube and continue the incubation at 27°C for further 5 minutes.
3. Stop the reactions by adding 440 µl of 1M glycine pH 11.5 and read the absorbance at 405nm (Fig. 1F), using a vacuole-free sample as a reference (see Note 3).

3.5. Analysis of Lipid Mixing Data

All 17 fluorescence measurements (F_t) taken between t=0 min and t=32 min are divided by $F_{(TX100)}$ and the degree of de-quenching is calculated, $(F_t - F_{0min})/F_{(TX100)}$. For representing the values on a graph, the 0 min value is set to 0.01 and the values of all other time-points are normalized to it. This operation facilitates comparisons between individual samples, even if their absolute starting values vary slightly (Fig. 1D). After the 30 min timepoint, the samples continue to show a slow increase in fluorescence, which typically is identical

in slope for all samples, even for those incubated under conditions that do not support vacuole fusion. Therefore, we consider this as an unspecific background signal that is independent of vacuole fusion.

4. Notes

1. Membrane labeling with Rh-PE represents the critical point of the experiment. Rh-PE must be incorporated into the existing vacuole membrane at the right concentration to obtain strong fluorescence self-quenching, such that any dilution of the probe by fusion of these vacuoles with an un-labeled vacuole can dilute the probe enough to result in a significant decrease in self-quenching.
2. In order to directly compare lipid and content mixing signals, the two assays must be calibrated against each other. This is best achieved by titrating fusion inhibitors that inhibit very early reaction stages, such as the Rab-GTPase inhibitor Gdi1 or antibodies to vacuolar SNAREs or NSF/Sec18, which interfere with membrane docking [23]. This allows to optimize the Rh-PE concentrations for vacuole labeling such that the lipid mixing signals titrate in correspondence to the content mixing signals. This condition must be met in order to allow the identification of hemifusion states, which are defined as states in which lipid mixing occurs whereas content mixing is impaired.
3. Limitations of the assay. In this protocol, content mixing is detected by the transfer of an >30 kDa protease from one fusion partner into the other. This requires a fusion pore of sufficient size to let this protein pass, which should be >2.5 nm. The approach could not detect fusion pores that are narrower than this or remain open only for very short periods of time that would not suffice to transfer sufficient amounts of the protease to the other fusion partner. Thus, very small or flickering fusion pores, which can be detected by electrophysiological methods, may be missed by this approach.

Figure legends

Fig. 1: *In vitro* assay for content and lipid mixing.

A) Purified donor vacuoles expressing the protease *pep4* are labeled with the lipid probe rhodamine-phosphatidylethanolamine (Rh-PE) at self-quenching concentration and mixed in the fusion reaction with purified acceptor vacuoles expressing the precursor of alkaline phosphatase *p-pho8*. B) Membrane juxtaposition brings vacuoles in very close proximity (docking) without causing lipid mixing. C) Hemifusion allows lipid exchange between the outer leaflets of causing a reduction of local Rh-PE concentration in the vacuolar membranes. D) Rh-PE de-quenching results in a fluorescence signal enhancement in a time and ATP-dependent manner. E) Inner leaflet mixing allows content mixing and conversion of *p-pho8* by *pep4* to produce the mature form *m-pho8*. F) The ATP-dependent formation of *m-pho8* is measured through its enzymatic activity, using a colorimetric assay.

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