Isolation of yeast vacuoles and assays of in vitro homotypic vacuole fusion

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Short abstract

Yeast lysosomal vacuoles undergo homotypic fusion in vivo and in cell-free reactions, making them a premier system for understanding membrane docking and fusion. This protocol demonstrates how to isolate intact vacuoles from *Saccharomyces cerevisiae* and quantitatively assay two stages of fusion: lipid bilayer continuity and mixing of aqueous lumenal contents.

Abstract

Membrane traffic in eukaryotic cells requires that vesicles dock and fuse with target membranes specifically and efficiently. Trafficking events in the secretory and endocytic pathway rely on a highly conserved system of Rabs, tethering factors and SNARE proteins to mediate membrane recognition and fusion in a complex reaction cascade [1,2]. Significant advances in our understanding of membrane fusion have been obtained in analyses of the homotypic fusion of *S. cerevisiae* lysosomal vacuoles [3-6]. Yeast vacuoles undergo fission and homotypic fusion *in vivo*, are readily purified, and fuse efficiently *in vitro.* Monitoring fusion *in vitro* permits precise control over reaction conditions while still using intact organelles that contain a full complement of fusion regulators. In addition, vacuoles isolated from mutant cells can be used to examine endolysosomal trafficking processes and to analyze the roles of specific gene products in vacuole fusion. There exist quantitative assays of several critical reaction milestones. Here, we describe isolation of vacuoles from cultured yeast and quantitative assays of outer-leaflet lipid mixing and content mixing (Figure 1).

The content mixing assay (Figure 2; [3,4,7] is based on the maturation of alkaline phosphatase (ALP, product of the *PHO8* gene) in the vacuole lumen. ALP is normally

synthesized as an inactive proenzyme (pro-ALP) and trafficked to the vacuole lumen, where the resident vacuolar proteases A and B cleave and thereby activate ALP [3]. To monitor content mixing *in vitro*, ALP activation is linked to homotypic fusion. Vacuoles are separately purified from two strains of yeast: *pho8*Δ yeast which harbor only the activating proteases, and *pep4*Δ yeast which lack activating protease activities but contain inactive pro-ALP. *In vitro* fusion between vacuoles of opposite type exposes the pro-ALP to the proteases, which rapidly cleave and activate the ALP. The total amount of fusion in a single reaction is quantified by measuring ALP enzyme activity using a colorimetric assay (Figure 3).

Prior to lumenal content mixing, outer leaflet lipids of the membrane bilayers mix in a kinetically faster sub-reaction [8-11]. To quantify passage through this transient intermediate, a sub-population of vacuoles is labeled selectively in the outer leaflet with a fluorescently-tagged lipid (Rhodamine-PE) at a self-quenching concentration. When a rhodamine-labeled vacuole fuses with an unlabeled vacuole, the rhodamine-PE label flows across the limiting membranes of two vacuoles, resulting in probe dilution, de-quenching and increased fluorescence (Figure 4). Fluorescence intensity thus provides a quantitative real-time measure of the amount of outer-leaflet lipid mixing in the population.

Strains

In this example the *pep4*Δ reporter strain was BY4742-*pep4*Δ (MATalpha *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *pep4*Δ::*KanMx4*), and the *pho8*Δ effector strain was BY4742-*pho8Δ* (*pho8*Δ::*KanMx4*). These strains may be obtained from EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/index.html) or from the American Type Culture Collection (http://www.atcc.org/). If purifying vacuoles from a wild-type strain (neither protease- nor phosphatase-deficient), use the conditions for *pho8*Δ strains as a starting point.

Vacuole Isolation

1. Generating spheroplasts

1.1 Grow a 1 L culture of each strain to a density of ~2.6×10^7 cells / mL in YPD (an OD_{600nm} of ~1.3 using our lab's spectrophotometer). Cultures are grown in 2 L shake flasks at 30° C, 225 rpm.

1.2 Harvest the cells by sedimentation in a preparative centrifuge (we use 10-20 min, 4500 × g, room temperature. The time may be shortened depending on the equipment used).

1.3 Decant YPD. Resuspend each cell pellet in 50 mL alkaline wash buffer and transfer to a 50 mL conical tube.

1.4 Incubate cell suspensions at 30°C for 10 min.

1.5 Recover cells by sedimentation (5 min, 3200 × g, room temperature).

1.6 Decant alkaline wash buffer and resuspend each cell pellet in 15 mL spheroplasting buffer by vigorous shaking or by repeated aspiration with a 25 ml glass pipet.

1.7 Add lyticase enzyme, mix by inversion, and incubate cells at 30°C for 25 min with periodic gentle agitation. See reagents section for a discussion of lyticase dosages.
1.8 Recover spheroplasted cells by centrifugation (3200 × g, 3 min, 4°C). Place pellets on ice. Spheroplasted cells are delicate; handle the pellet gently.
1.9 Remove supernatant by aspiration.
1.10 Add 2 mL of ice cold PS-15% Ficoll to each spheroplast pellet and resuspend on ice with gentle agitation (swirl tube). A small fraction of the pellet may resist resuspension; this will not significantly affect the assay and may safely be ignored.
1.11 Add DEAE-dextran solution to the spheroplast suspension. Mix gently on ice. Use 20 µL DEAE-dextran solution for *pho8*Δ strains and 36 µL for *pep4*Δ strains. This step lyses yeast cells while maintaining the integrity of organelles including lysosomal vacuoles.
1.12 To lyse spheroplasts, transfer cells to 30°C for 3 min with occasional gentle agitation. After this heat shock step, return the lysates to ice.

2 Purifying vacuoles by equilibrium flotation

2.1 Transfer up to 4 mL of spheroplast lysate to the bottom of a Beckman Ultra-Clear SW-40 or SW-41 centrifuge tube, pre-chilled on ice.
2.2 Overlay each lysate with ~3 mL of ice cold PS-8% Ficoll. Add or withdraw small volumes of PS-8% Ficoll to ensure that paired tubes hold equivalent volumes as gauged by eye.
2.3 Gently overlay each sample with 3 mL of ice cold PS-4% Ficoll.
******The interface between each density step should be visible and sharp.***
2.4 Gently overlay each sample to within 3 mm of the top of the tube with ice cold PS buffer (0% Ficoll).
2.5 Balance paired tubes with small volumes of PS buffer.
2.6 Centrifuge samples (30,000 rpm = 160,000 × g, 90 min, 4°C in pre-chilled SW40Ti or SW41Ti rotor).
2.7 Harvest vacuoles from the 4%-0% Ficoll interface with a wide-bore 200 µL pipet tip. Avoid recovering 4% Ficoll solution. Volumetric yield is generally 300 - 500 µL.
2.8 Quantify vacuole yield by Bradford protein assay using bovine serum albumin to generate a standard curve. Typically, concentrations of 0.8 – 1.2 mg/mL are obtained, with total yield of 400 – 800 µg per L of liquid cell culture.
2.9 Freshly isolated vacuoles may be used directly in content mixing assays (described in text), solubilized and analyzed for protein content or protein-protein interactions, or doped with fluorescent lipid for use in lipid-mixing assays.

3. Content Mixing Assay

******Standard fusion reactions have a total volume of 30 µL and contain 6 µg of vacuoles (generally 3 µg pho8Δ vacuoles and 3 µg pep4Δ vacuoles). Each standard***

reaction contains: 20 mM piperazine-N,N-bis(2-ethanesulfonic acid) (PIPES)-KOH pH 6.8; 125 mM KCl; 5 mM $MgCl_2$; 200 mM sorbitol; 10 µM Coenzyme A; 3.2 µg/mL IB_2; 1× ATP regenerating system (1 mM ATP, 1 mg/mL creatine kinase, 9.5 mg/mL creatine phosphate). Alternatively, fusion can be driven in the absence of ATP by adding recombinant Vam7 and 10 mg/mL BSA and omitting the ATP regenerating system (Figure 3). In general, all reaction components are prepared and stored in PS buffer. Reaction components such as salts, exogenous proteins, and small molecules are usually prepared as concentrated stocks in PS buffer and combined in appropriate proportions immediately prior to the assay. Consideration should be given to order of addition of reaction components; generally vacuoles should be added last and fusion should be initiated as soon as possible after vacuole addition. Some reaction components cannot be prepared in PS or other fusion-compatible buffers. Always include buffer-only control reactions when adding a component suspended in a non-standard buffer.

3.1 On ice, prepare a concentrated "master mix" containing all common reaction components. See text protocol for reaction composition details.
****Prepare master mix from stock solutions of 10× Salts/PS, 10× ATP regenerating system, CoA/PS, IB_2/PS, 30× BSA/PS and PS buffer.*

3.2 Add the master mix to each reaction tube and place on ice.

3.3 Add any non-common reaction components, such as recombinant Vam7 or fusion inhibitors, to individual reaction tubes.

3.4 On ice, pre-mix vacuoles from each strain in PS buffer.
****This vacuole suspension will be added to each reaction to bring the final volume to 30 µL and the vacuole concentration to 0.2 µg/µL (6 µg/reaction).*

3.5 Add the mixed-vacuole suspension to each reaction tube.

3.6 Briefly and gently vortex the reaction tubes, then place them in a 27°C circulating water bath for 90 min.
****For Vam7-driven reactions, this can be shortened to 70 min.*

3.7 After 90 min, raise the temperature of the water bath to 30°C.

3.8 Add 500 µL of Developer Solution to each tube in succession at regular, timed intervals.

3.9 Each reaction should develop for exactly 5 min. To terminate development, add 500 µL of Stop Solution to each tube at regular, timed intervals.

3.10 Measure the absorbance of each reaction at 400 nm (A_{400nm}).
****A sample with only Developer Solution and Stop Solution but no fusion reaction should always be used to blank the spectrophotometer. If the absorbance of a sample exceeds the linear range of the spectrophotometer, dilute the sample 1:2, using a mixture of equal parts Developer and Stop Solution to permit measurement within the instrument's linear range.*

Lipid mixing assay

4. Labeling Vacuoles with Rhodamine-PE

4.1 Thaw one 80 µL aliquot of rhodamine-PE per labeling reaction. Tubes containing rhodamine solutions should be dark amber plastic. Handle solutions in subdued light.
4.2 Bath sonicate each tube of rhodamine-PE for 5 - 10 min. at room temperature and vortex vigorously to resuspend.
4.3 Pellet unsolubilized rhodamine-PE at (20,000 × g, 15 min, room temperature).
4.4 Place 560 µg of freshly isolated vacuoles in 800 µL PS buffer (0.7 mg/mL) on ice.
4.5. Warm vacuoles to 27° C for 1 – 2 min.
4.6 Add 60 µL of clarified rhodamine-PE suspension to vacuoles. Mix by swirling pipet tip and by inverting tube.
4.7 Incubate vacuoles with rhodamine-PE for 30 sec at 27°C, then return to ice.
4.8 Using a wide-bore 1000 µL pipet tip, transfer vacuole suspension to the bottom of a Beckman Ultra-Clear thin-walled SW-50.1 centrifuge tube on ice.
4.9 Add 1140 µL of PS-15% Ficoll to the vacuole suspension and mix by gentle pipetting with a wide-bore 1000 µL pipet tip.
4.10 Overlay the vacuole suspension with 2 mL of PS-8% Ficoll.
4.11 Overlay the 8% layer to the top of the tube with PS buffer (PS-0% Ficoll).
4.12 Centrifuge the gradient in an SW-50.1 rotor (30,000 rpm = 108,000 × g, 45 min, 4°C). The vacuoles will float to the 8%-0% interface.
4.13 Harvest labeled vacuoles from the 8%-0% interface.
4.14 Quantify vacuole yield by Bradford protein assay, using bovine serum albumin (BSA) to generate a standard curve.

5. Lipid mixing assay

****Lipid mixing reactions differ from content mixing assays in the following ways. Lipid mixing reactions are conducted on a larger scale, typically 6× the scale of standard content mixing assays (180 µL and 36 µg vacuoles). Lipid mixing assays are set up in a 96-well black-bottom conical micro-plate. The vacuole mixture is 4 µg rhodamine-PE-labeled pep4Δ vacuoles and 32 µg pho8Δ vacuoles per reaction. For Vam7 driven reactions, 1 mg/mL creatine kinase is used as a carrier protein in place of the 10 mg/mL BSA, which has the potential to extract lipids and interfere with the fluorescence assay. Prior to the assay, the dynamic range of the fluorimeter can be set by comparing 4 µg of rhodamine-PE labeled vacuoles to 4 µg of rhodamine-PE labeled vacuoles diluted into 200 µL of a 0.33% Triton X-100 detergent solution. The Triton-solubilized sample is a proxy for infinite probe dilution and provides a measure of maximally dequenched fluorescence for the sample.*

5.1 Place a 96-well black conical bottom plate on ice.
5.2 To each well, add master mix, individual reaction components, and premixed vacuoles. See text for details.

5.3 Incubate the microplate in a thermostatted fluorimeter at 27° C for 90 min, shaking the plate and measuring rhodamine fluorescence at 1 to 5 min. intervals.

5.4 After the standard 90 min incubation at 27°C, transfer the microplate to ice. Remove 30 µL aliquots to individual microfuge tubes on ice, and assay for ALP activity as described in the content mixing assay procedure.

Reagents

Vacuole Isolation

1. Alkaline wash buffer [0.1 M Tris-HCl pH 9.4, 10 mM DTT]. Add DTT powder immediately prior to use.
2. Spheroplasting buffer [50 mM potassium phosphate pH 7.8, 8% (v/v) YPD, 600 mM sorbitol]. Prepare fresh each time or prepare as 1× stock solution, sterile filter through 0.2 µm membrane, and handle aseptically to avoid growth of contaminating microbes.
3. Lyticase – We purchase commercial enzyme and then further purify it by cation exchange chromatography. Alternatively, recombinant enzyme can be prepared [12]. Dissolve Zymolyase-20T into 50 mM potassium phosphate pH 7.5 at 1 g / 25 mL. Dialyze the sample into 50 mM sodium acetate pH 5.0 (3500 mwco membrane). Clarify the dialysate at (5000 × g, 20 min, 4°C). Pass clarified dialysate over a column (5 × 20 cm) of CM-Sepharose Fast Flow pre-equilibrated in 50 mM sodium acetate pH 5.0. Wash the column with several volumes of 50 mM sodium acetate pH 5.0, then elute bound lyticase with 50 mM sodium acetate pH 5.0 + 150 mM NaCl. This eluate is used directly for the spheroplasting step. It can be frozen in aliquots and stored indefinitely at -80°C. 1 unit of lyticase is defined as the quantity of enzyme required to reduce the OD_{600nm} of a sample of alkaline-washed *pep4*Δ yeast suspended at OD_{600nm} = 1.0 in 50 mM potassium phosphate pH 7.6 by 0.1 OD_{600nm} units in 30 min at 30° C. Based on this assay, we typically use 3200 U of lyticase per L of *pho8*Δ or Wild-type yeast and 7300 U of lyticase per L of *pep4*Δ yeast. However, the activity assay is rather non-linear and should be used only as a rough guide of enzyme activity. For each batch of lyticase, optimization of the proper amount of lyticase to use per L of cells must be assessed empirically. Use the smallest amount of lyticase that produces a maximal yield of high quality (fusogenic) vacuoles.
4. DEAE-dextran solution [10 mg / mL DEAE-dextran in PS-15% (see #6 below)].
5. PS buffer [20 mM PIPES-KOH pH 6.8, 200 mM sorbitol] (store at 4° C)
6. PS-15% Ficoll [15% Ficoll (w/v) in PS buffer] (store at 4° C)
7. PS-8% and PS-4% Ficoll – mix appropriate volumes of PS-15% Ficoll and PS buffer to make these solutions.

Content Mixing Assay

1. 10× Salts / PS [1.25 M KCl, 50 mM $MgCl_2$, 20 mM piperazine-N,N′-bis(2-ethanesulfonic acid)(PIPES)-KOH pH 6.8, 200 mM sorbitol] (store at 4°C)

2. 10× ATP regenerating system [10 mM ATP, 10 mg/mL creatine kinase, 95 mg/mL creatine phosphate, 10 mM $MgCl_2$, 20 mM PIPES-KOH pH 6.8, 200 mM sorbitol]
   -Separately dissolve 100 µmol ATP in 1 mL dH_2O, 100 mg creatine kinase in 2 mL dH_2O and 950 mg creatine phosphate in 3 mL dH_2O. In order, mix 2 mL dH_2O, 200 µL 1 M PIPES-KOH pH 6.8, 100 µL 1 M $MgCl_2$, creatine kinase solution, creatine phosphate solution, ATP solution, and 0.5 mL 4 M sorbitol. Bring to pH 6.8 with 1 M KOH. Add dH_2O to bring volume to 10 mL. Aliquot and snap freeze over liquid N_2. Store at -80°C.
3. 30× BSA / PS [300 mg/mL BSA, PIPES-KOH pH 6.8, 200 mM sorbitol] (store at 4°C)
4. CoA / PS [1.2 mM coenzyme-A, 20 mM PIPES-KOH pH 6.8, 200 mM sorbitol] (store at -80°C)
5. IB_2 / PS [purified IB_2 protein, 20 mM PIPES-KOH pH 6.8, 200 mM sorbitol] (store at -80°C) IB_2 (or Pbi2) is a membrane-impermeant protease inhibitor that prevents signal derived from vacuole lysis [13].
6. Recombinant Vam7 is purified as described [14].
7. Developer Solution [250 mM tris-HCl pH 8.5, 10 mM $MgCl_2$, 0.4% Triton X-100 (v/v), 1 mM paranitrophenylphosphate (PNPP; added just before use)]
8. Stop Solution [1 M glycine-KOH pH 11.5]

Labeling Vacuoles with Rhodamine-PE

1. Rhodamine-PE – Resuspend rhodamine-PE to 3 mM in DMSO. Overlay with argon. Incubate for 1 hour at 37°C, vortexing every 5 – 10 minutes. Divide into 80 µL aliquots, overlay with argon and store at -80°C.

Discussion

The quality of the vacuole preparation generally correlates with the protein concentration. A typical high-quality preparation yields ~ 1 mg/mL protein by Bradford assay. Vacuole yield and quality is rather sensitive to the cell density at harvest. To improve yield the harvest density of the culture can be modified, though cultures grown to > 3.2 * 10^7 cells / mL (>OD_{600nm} 1.6 with our laboratory's spectrophotometer) tend to not yield high-quality vacuole preparations. It is inadvisable to back-dilute cultures that have overgrown to more than twice the target harvest density. A second common cause of low-quality vacuole preparations is proteolysis. Proteolysis can result from rupture of vacuoles containing functional luminal proteases, such as those purified from wild type or *pho8*Δ yeast strains, or from contaminating proteases that are present in commercial Zymolyase preparations. Vacuole rupture is minimized by gentle handling of spheroplasts, cell lysates, and purified vacuoles. Contaminating proteases are removed by re-purification of commercial Zymolyase, and by removal of all excess Zymolyase via

thorough removal of all spheroplast buffer at the end of the Zymolyase digestion (Procedure 1.9).

Every content mixing fusion assay should include at least two standard conditions to assess the background (fusion-independent) signal: first, an uninhibited, ATP-driven reaction incubated at 27˚ C; and second, an ice control which is held in ice water at (0 to 4˚ C) while all other reactions are incubated at 27˚ C. The ice control is warmed to 30˚ C immediately prior to the alkaline phosphatase activity assay. To compare data from multiple separate assays, recorded absorbances (A_{400nm}) are normalized to the standard condition according to the equation:

$$F_n = (A_{sample} - A_{Ice})/(A_{ATP} - A_{Ice})$$

where F_n = Normalized fusion signal; A_{sample} = absorbance for sample; A_{Ice} = absorbance of the ice control; A_{ATP} = absorbance of the uninhibited ATP-driven reaction. The signal from fusion should be well above the background signal for the assay. Optimized experiments typically have A_{Ice} = 0.02 to 0.05 A_{400nm} units, and A_{ATP} of ~1.0 A_{400nm} units, *i.e.*, a signal-to-background ratio of ~25. Experiments with A_{ATP} ≤ 0.6 absorbance units should be optimized. Variables that can negatively affect the assay include the quality of the vacuole preparation, pipetting technique, the temperature at which vacuoles and reactions are held during preparation, and the quality of the reagents.

It is critical to note that certain small molecule inhibitors artifactually inhibit the coupled assay of content mixing, either by directly interfering with ALP activity or by impairing the proteolytic maturation of ALP by vacuolar proteases. For example, the protein phosphatase inhibitor microcystin-LR, initially reported to inhibit fusion [15,16], was subsequently demonstrated to impair ALP activation but not fusion [4,8]. Detergent lysates of vacuoles from Δ*pep4* and Δ*pho8* cells undergo pro-ALP maturation without fusion, and can be used to evaluate potentially interfering effects of small molecule inhibitors on the ALP reporter system [4]. To assess fusion in the presence of small molecule inhibitors that impair ALP maturation or activity, an alternative content mixing assay based on beta-lactamase complementation was developed [8].

Every lipid mixing assay should include three standard conditions for normalization: first, a reaction that is unstimulated (lacking both Vam7 and ATP) or fully-inhibited (using, for example, an anti-Vam3 antibody) to measure the fusion-independent background dequenching; second, an uninhibited fusion reaction (in which efficient fusion is permitted through, for example, the presence of ATP); and third, a reaction containing vacuoles in 0.33% Triton X-100 to measure the fluorescence of fully-dequenched rhodamine-PE. For the Triton-containing reaction, measure F_{ttx100} as the mean of the final 10 measurements made during the assay run. For each reaction, record F_0, the fluorescence at time = 0. For each fluorescence measurement at t > 0 (F_t), calculate the relative fluorescence change $\Delta F = (F_t - F_0)/F_{ttx100}$. For each time -point, calculate the change in fluorescence due to lipid mixing by subtracting from the sample's ΔF_t the ΔF_t from an unstimulated or fully-inhibited reaction. To estimate total lipid mixing for a given condition, a lipid-mixing time series can be modeled by non-linear curve fitting using the Gompertz equation,

which is well suited for description of time-dependent, irreversible processes. End-point lipid mixing values extrapolated from the curve-fitting analysis (F_∞) can then be normalized to a standard condition for comparison amongst multiple independent assays.

In each assay, vacuoles that harbor the signal-generating molecule (pro-ALP or fluorescent lipid) are referred to as "reporter" vacuoles, while the vacuoles that allow the reporter to generate signal upon fusion are referred to as "effector" vacuoles[4]. In the case of the content mixing assay, a single reporter-effector fusion event will proteolytically mature all ALP within the reporter. In the case of the fluorescence dequenching assay, dilution increases the inter-probe distance. Self-quenching of the fluorescent lipid is an isomolecular Förster energy transfer process, with quenching efficiency varying as a function of the mean inter-probe distance [17]. Hence, the amount of dequenching depends on the dilution factor, which is determined by the relative areas of reporter and effector membranes. When effector membranes are in excess, on average they fuse with one another prior to undergoing fusion with a reporter. Under this condition the degree of dilution for an average reporter's first fusion event will be larger, and the fluorescence yield for the reporter's first round of fusion will be greater. Consequently, for both lipid and content mixing assays, when effectors are in excess the assay is biased to report only the first round of fusion. In contrast, when reporters are in excess, each reporter may undergo several rounds of fusion with other reporters prior to fusion with an effector. Hence, with reporters in excess over effectors, the assay is biased to report multiple rounds of fusion [4,18]. By systematically varying the reporter:effector ratio is possible to quantitatively infer both fusion efficiency and the number of rounds of fusion that have occurred; analogous quantitative approaches have been presented for lipid dequenching assays [18] and for the vacuole content mixing assay [4] .

In order to meaningfully compare lipid mixing versus content mixing results for comparable experimental interventions, it is critical that the reporter:effector ratios of fluorescent:unlabeled and *pep4*Δ:*pho8*Δ vacuoles in each assay should be similar. When this condition is satisfied the lipid and content mixing assays yield highly correlated results (Fig. 5; ref. 14). If this condition is not satisfied, results from the lipid and content-mixing assays cannot be usefully compared. For example, if the lipid mixing assay is biased to report the first round, and the content mixing assay is biased to report later rounds of fusion (e.g., ref. 19), it is not possible to distinguish between the apparent accumulation of a hemifusion intermediate, and reduced efficiency of subsequent rounds of fusion after an efficient first round.

The techniques presented here are suitable for measuring membrane fusion both in the specific case of vacuoles, and in the broader context of the endocytic pathway. Indeed, similar methods have been used to design assays for heterotypic fusion events including endosome-vacuole fusion (C. Brett, personal communication and ref. 20). Proteins with a candidate role in vacuolar fusion may be added to this assay to reveal whether they enhance or inhibit fusion in these *in vitro* conditions. Vacuoles may also be isolated from yeast strains containing a null or mutant form of a protein of interest, and then the protein may be added back to the reaction to test its effect.

The present procedures result from many years' optimization in several laboratories, and with variations can facilitate the study of numerous sub-reactions occurring at the vacuolar membrane. Due to the high degree of conservation of membrane fusion proteins, results from experiments in this system are often broadly applicable to other SNARE-mediated fusion events occurring in eukaryotic cells. An important advantage of this *in vitro* system is that fusion conditions can be controlled to an extent impossible within intact cells. To augment results obtained from experiments with intact cells and isolated organelles, reconstituted systems employing purified proteins and lipids have been developed, along with quantitative assays of membrane tethering, content mixing, content leakage, and inner and outer leaflet membrane mixing. [11,18,21,22,22-27]

Representative Results

To generate the example data series, lipid-mixing assays were conducted with variable concentrations of recombinant Vam7 to drive variable amounts of fusion (in the absence of ATP). After measurement of the lipid mixing time-series, single reaction aliquots were transferred to Eppendorf tubes and assayed for content mixing. Lipid mixing data sets were plotted without background subtraction (Fig. 5A) to illustrate the appearance of the raw data due to the fusion-independent dequenching. After subtracting the fusion-independent dequenching signal from each data-series, the fusion-dependent dequenching is observed to plateau within the normal 70 minute incubation period (Fig. 5B). A comparison of the normalized, averaged end-point values for each condition in both the outer-leaflet lipid mixing and content mixing assays reveals a tight correlation between the values measured by each assay (Fig. 5C). A scatter-plot of the normalized end-point values for the two assays for many individual reactions further illustrates the tight correlation between total outer-leaflet lipid mixing and content mixing measurements (Fig. 5D). Typical endpoints for a single assay under various inhibitory conditions are shown in Fig. 5E.

Tables and Figures

Figure 1. Diagram of lipid and content mixing.
Figure 2. Diagram of content mixing assay.
Figure 3. The role of Vam7 in catalyzing fusion reactions.
Figure 4. Diagram of lipid mixing assay
Figure 5. Representative results for content and lipid mixing assays of fusion.

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Disclosures

The authors declare that they have no competing financial interests.

Reagents and equipment

Reagent	Company	Catalogue number	Comments
PIPES buffer	Sigma-Aldrich	P1851	
Sorbitol	Sigma-Aldrich	S1876	
Ficoll	GE Healthcare	17-0300-10	
Coenzyme A	Sigma-Aldrich	C3019	
ATP	Roche Applied Science	10519987001	
DEAE-dextran	Amersham	17-0350-01	
Creatine phosphate	Roche Applied Science	10621714001	
Creatine kinase	Roche Aplied Science	10746988001	
Rhodamine-PE	Molecular Probes	L-1392	
Zymolase-20T	Seikagaku Corporation/Amsbio	120491-1	See preparation under "Reagents"
Cary 50 Bio UV/Visible Spectrophotometer	Varian (Agilent)		
SW 40 Ti rotor centrifuge tube	Beckman Coulter	344060	14 × 95 mm, 14 mL volume
SW 41 Ti rotor centrifuge tube	Beckman Coulter	344059	14 × 89 mm, 13.2 mL

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Captions for figures

Figure 1. There are two steps in vacuolar fusion. First, the outer leaflets of the membrane bilayer fuse (A), and then the inner membranes fuse and the vacuolar contents mix (B).

Figure 2. The content mixing assay measures fusion by monitoring the cleavage-dependent activation of alkaline phosphatase (ALP). Vacuoles deficient in ALP are mixed with vacuoles deficient in the activating lumenal protease (represented by

scissors). Upon content mixing the protease cleaves pro-ALP into activated ALP, and when the reaction is developed ALP dephosphorylates the synthetic substrate PNPP to produce the yellow reaction product PNP (right).

Figure 3. Vam7 is a soluble SNARE protein required for vacuole fusion. Recombinant Vam7 protein catalyzes on-pathway fusion by complexing with native SNAREs on purified vacuoles. In the absence of ATP each fusion reaction traps Vam7 in an assembled SNARE complex. Alternatively, ATP activates SNARE disassembly factors Sec17 and Sec18, releasing endogenous Vam7 from purified vacuoles, thereby allowing native Vam7 to drive fusion.

Figure 4. The lipid mixing assay uses vacuoles labeled with Rhodamine- PE and unlabeled vacuoles. In the labeled vacuole population the Rhodamine-PE is at a high enough concentration to quench all fluorescent signal (upper left). Upon the fusion of the outer leaflets the rhodamine diffuses (center), dequenching and emitting fluorescence.

Figure 5. Representative data for lipid and content mixing assays driven by variable amounts of recombinant Vam7. A. Raw fluorescence intensity measurements for reactions driven by the indicated concentration of recombinant Vam7 in the lipid mixing assay. B. Background subtracted fluorescence intensity measurements for the same datasets plotted in (A). C. Comparison of the average end point values for both the lipid mixing and content mixing assays for reactions driven by variable amounts of recombinant Vam7, normalized as described above (n=3). D. Scatter plot showing the relationship between measured total lipid mixing and measured content mixing for each individual reaction in this dataset. E. Lipid and content mixing over a range of concentrations of GDI, an inhibitor of Rab G-protein signaling. F. Control conditions for a typical content mixing assay.

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