Flow cytometry as a novel tool for structural and functional characterization of isolated yeast vacuoles

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The yeast vacuole is functionally analogous to the mammalian lysosome. Both play important roles in fundamental cellular processes such as protein degradation, detoxification, osmoregulation, autophagy and apoptosis which, when deregulated in humans, can lead to several diseases. Some of these vacuolar roles are difficult to study in a cellular context, and therefore the use of a cell-free system is an important approach to gain further insight into the different molecular mechanisms required for vacuolar function. In the present study, the potentialities of flow cytometry for the structural and functional characterization of isolated yeast vacuoles were explored. The isolation protocol resulted in a yeast vacuolar fraction with a degree of purity suitable for cytometric analysis. Moreover, isolated vacuoles were structurally and functionally intact and able to generate and maintain electrochemical gradients of ions across the vacuolar membrane, as assessed by flow cytometry. Proton and calcium gradients were dissipated by NH₄Cl and calcimycin, respectively. These results established flow cytometry as a powerful technique for the characterization of isolated vacuoles. The protocols developed in this study can also be used to enhance our understanding of several molecular mechanisms underlying the development of lysosome-related diseases, as well as provide tools to screen for new drugs that can modulate these processes, which have promising clinical relevance.

INTRODUCTION

The vacuole is the most prominent and acidic organelle in yeast cells, occupying up to one quarter of the total intracellular volume (Premsler et al., 2009; Wiederhold et al., 2009). It is a membrane-bound organelle and functionally equivalent to the plant vacuole and mammalian lysosome. This functional analogy has led to the use of yeast to study important features of this organelle, namely to elucidate biosynthetic and endocytic pathways of all eukaryotes, as well as the mechanisms underlying vacuole/lysosome inheritance.

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Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; AO, acridine orange; DiBAC₄(3), bis-(1,3-dibutylbarbituric acid) trimethine oxonol; EGFP, enhanced green fluorescence protein; FM 1-43, N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide; NAO, acridine orange 10-nonyl bromide.
well as to analyse the membrane and luminal proteome (Michaillat et al., 2012; Sarry et al., 2007; Wiederhold et al., 2009). Recent examples were the use of this cell-free system to identify novel genes involved in lysosomal vacuole function and morphology and to study the mechanisms underlying the regulation of vacuolar size and number (Michaillat et al., 2012; Ricarte et al., 2011).

In the present study, we intended to explore the potentialities of flow cytometry as a powerful tool for the structural and functional characterization of isolated yeast vacuoles. This approach provides the possibility of performing a cell-based quantitative analysis of thousands of cells in a few minutes and offers considerable advantages over classical biochemical analysis, namely the assessment of the homogeneity/heterogeneity of the sample and a much more robust statistical analysis. Therefore a set of staining protocols for structural and functional characterization of isolated vacuole populations by flow cytometry, which were validated by fluorescence microscopy and spectrofluorimetry, was developed. These protocols may constitute valuable tools for understanding the role of the vacuole/lysosome in different biological processes, as well as for high-throughput assessment of vacuole-specific effects using drug libraries.

**METHODS**

**Reagents.** The yeast vacuole membrane marker MDY-64, N-(3-triethylammoniumpropyl)-4-((3-dibutylamino)styryl) pyridinium dibromide (FM 1-43), the Fluoro-4 AM cell permeant, LysoSensor Green DND-189, bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC4(3)), acridine orange 10-nonyl bromide (NAO) and 9-amino-6-chloro-2-methoxyacridine (ACMA) were purchased from Sigma–Aldrich. Zymolyase 20T was purchased from Seikagaku. Difco, Calcimycin, concanamycin A, amino acids and Ficoll PM400 were purchased from Becton Dickinson. Zymolyase 20T was purchased from Seikagaku.

**Yeast strains and plasmids.** The Saccharomyces cerevisiae wild-type strain W303-1A (MATa ade2 his3 leu2 trp1 ura3 can1) was used throughout this study. p416 ADH-PEP4-EGFP was used for Pep4p overexpression (Mason et al., 2005). Strains were transformed by the lithium acetate method (Gietz & Woods, 2006) and the resulting transformants were grown in selective media lacking the appropriate amino acid.

**Isolation of intact yeast vacuoles.** For vacuole isolation, W303-1A cells were grown in YPD (1 % yeast extract, 1 % peptone and 2 % glucose) and W303-1A Pep4–EGFP cells were grown in SC (0.67 % yeast nitrogen base, 2 % glucose and 0.1 % of all required amino acids) to OD660 0.7–1.0. Cells were collected, washed twice with cold distilled water, resuspended in washing buffer (5 % glucose, 10 mM Tris/HCl, pH 6.5) and incubated in an orbital shaker at 30 °C for 30 min. Cells were then incubated with digestion buffer (1.35 M sorbitol, 10 mM citric acid, 30 mM Na2HPO4, 1 mM EGTA, 30 mM DTT, pH 7.5) for 15 min at room temperature and converted to spheroplasts by incubation with 2 mg zymolyase ml⁻¹ in digestion buffer without DTT. Cell wall digestion was monitored by phase-contrast microscopy. The spheroplasts were pelleted by centrifugation at 4500 g for 5 min, washed with digestion buffer without DTT, resuspended in 12 % Ficoll (w/v) and homogenized in a Potter–Elvehjem grinder to disrupt the cell membrane while preserving vacuole integrity. The vacuolar fraction was then recovered by gradient centrifugation. For this purpose, the resulting homogenate was centrifuged at 4500 g for 3 min. Approximately 10 ml supernatant containing a crude fraction of vacuoles was collected, while the remaining pellet was resuspended in 10 ml 12 % Ficoll (w/v) and homogenized with a hand grinder. This second homogenate was also centrifuged at 4500 g for 3 min and 10 ml of the supernatant was added to that collected in the first centrifugation. The gradient was prepared by adding 12 ml 8 % Ficoll (w/v) solution to 20 ml crude fraction of vacuoles in 12 % Ficoll (w/v). Centrifugation was performed at 26000 r.p.m. for 30 min in a Beckman SW28 rotor. The white fraction at the top of the gradient, containing highly purified vacuoles, was collected and used in subsequent studies. Protein concentration was determined by the Lowry method (Lowry et al., 1951), using BSA as a standard.

**Preparation of vacuolar membrane vesicles.** The vacuoles were converted to vesicles by transferring the vacuole fraction (6 mg protein) to 20 ml resuspension buffer containing 15 % glycerol (w/v), 20 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM PMSF and 1 mM DTT. After homogenization with a Potter–Elvehjem grinder, the vesicles were sedimented at 100 000 g for 45 min in a Beckman 70Ti rotor. The pellet was resuspended in 300–500 μl resuspension buffer without PMSF and DTT. Protein concentration was determined as described above.

**Purification of vacuole fractions – silicone oil technique.** The silicone oil floating filtration technique was performed according to the protocol of Tohge et al. (2011) with some modifications. A 10 μl vacuole aliquot (20 μg protein) was pipetted into an appropriate 400 μl tube (Sarstedt) and mixed with 90 μl incubation buffer [40 % (w/v) Percoll, 0.45 M sorbitol and 30 mM HEPES pH 7.4]. Wackman silicone oil AR-200 (200 μl) was layered on top of this mixture and 60 μl vacuole buffer (0.4 M mannitol and 0.01 M HEPES pH 7.4) was placed on top of both layers. After centrifugation for 1 min in a Beckman Microfuge B at 14 000 g, 50 μl vacuole buffer containing highly purified vacuoles was recovered.

**Mitochondria preparation.** For mitochondrial preparation, W303-1A cells were grown in YPG (1 % yeast extract, 1 % peptone and 2 % galactose) to OD660 0.7–1.0. Cells were collected and processed according to the protocol described in Silva et al. (2011).

**Staining protocols for structural and functional analysis of isolated vacuoles.** Several fluorescent probes were used to evaluate the integrity and functionality of yeast vacuoles and vacuolar membrane vesicles. To assess vacuolar membrane integrity and purification, the yeast vacuole membrane markers MDY-64, FM4-64 and NAO were used. Isolated vacuoles were stained with 10 μM MDY-64, with 5 μM FM4-64 and 0.2 μM NAO and incubated in the dark for 10 min at room temperature. Spheroplasts were also stained with 10 μM MDY-64.

Vascular membrane potential was assessed by DiBAC4(3) staining. Isolated vacuoles were stained with 13 μM DiBAC4(3) and incubated for 5 min in the dark at room temperature. Dissipation of membrane potential was monitored after addition of increasing concentrations of NH4Cl to vacuole samples pre-stained with DiBAC4(3).

Assessment of calcium accumulation in the vacuolar lumen was performed with Fluo-4 AM Fluo-4 fluorescence is enhanced by increasing calcium concentrations. Isolated vacuoles were stained with 5 μM Fluo-4 AM and incubated for 1 h in the dark at room temperature. Dissipation of calcium accumulation in the vacuole was monitored after addition of 300 μM of the calcium ionophore calcimycin.
Vacuolar lumen acidity was assessed with the two pH-sensitive probes AO and LysoSensor Green. Vacuoles were stained with 30 μM AO or 5 μM LysoSensor Green and incubated for 10 min in the dark at room temperature. Before staining, vacuolar samples were prepared by adding 20 or 100 μg isolated vacuoles to 1 or 0.05 ml staining buffer [1 mM MOPS-Tris (pH 7.2) and 100 mM KCl] for flow cytometry or fluorescence microscopy analysis, respectively.

**Fluorescence and confocal microscopy.** Fluorescence microscopy analysis of the different samples was performed with a Leica Microsystems DMLM-5000B epifluorescence microscope with appropriate filter settings. Images were acquired with a Leica DFC350FX digital camera and processed with LAS AF Leica Microsystems software. Confocal microscopy analysis of the different samples was performed with an Olympus confocal laser scanning biological microscope, Fluoview FV10i, with the appropriate filter settings.

**Flow cytometry.** Flow cytometry analysis of isolated vacuoles was performed in an Epics XL Beckman Coulter flow cytometer equipped with an argon-ion laser with a beam emitting at 488 nm at 15 mW. Green fluorescence and red fluorescence were collected through a 525 nm band-pass filter and a 620 nm band-pass filter, respectively. For most samples, 20,000 events were analysed at a low flow rate with the exception of vacuoles purified with the silicone oil method, where 5000 events were analysed. Data were analysed with Flowing Software 2.0.

**Proton-pumping activity of V-H⁺-ATPase in intact vacuoles and membrane vesicles.** Proton-pumping activity was determined by measuring the fluorescence quenching of ACMA using a PerkinElmer LS-5B spectrophotometer. The excitation wavelength was set at 415 nm and the emission wavelength was set at 485 nm. After the addition of intact vacuoles (~20 μg protein) to 1.5 ml buffer containing 1 mM MOPS-Tris 7.2, 2 mM ACMA, 1 mM MgCl₂ and 100 mM KCl, the reaction was started by addition of ATP at appropriate concentrations, and the rate of initial fluorescence quenching was recorded. All experiments were performed at 25 °C. Addition of 1 mM CaCl₂ abolished the gradient formed by ATP hydrolysis. Pre-incubation of reaction mixtures with 0.05 μM concanamycin A was used to inhibit V-H⁺-ATPase pumping activity. The initial rates of ACMA fluorescence quenching were regarded as the initial rates of H⁺-transport activity [Δ% F min⁻¹ (μg protein)⁻¹] and the results were analysed by computer-assisted non-linear regression analysis (GraphPad Prism software). Using this method, proton-pumping kinetics best fitting the experimental initial acidification curves, corresponding to the quenching of ACMA fluorescence, were determined and estimates for the ka were obtained. Proton-pumping measurements of vacuolar membrane vesicles were also performed, using approximately 20 μg protein.

**RESULTS**

**The isolated fraction is enriched in functional vacuoles**

In order to assess the functionality of the vacuoles isolated, the V-H⁺-ATPase activity of this fraction was monitored. For this purpose, vacuoles were incubated with the pH-sensitive probe ACMA and fluorescence quenching was monitored by spectrofluorimetry. There was a fluorescence decrease in energized vacuolar suspensions, indicative of V-H⁺-ATPase activity. Addition of CaCl₂ led to dissociation of the proton gradient and confirmed that the intravacuolar acidification was a consequence of the proton-pumping activity of V-H⁺-ATPase (Fig. 1a). This shows that the isolation protocol used in this study resulted in the purification of functional vacuoles. When the specific V-H⁺-ATPase inhibitor concanamycin A was added to the incubation mixture, only the non-specific effect of ATP addition on fluorescence quenching was detected, indicating that the V-H⁺-ATPase was the main proton pump responsible for the formation of the proton gradient (Fig. 1a). This non-specific effect is always observed after ATP addition even in the absence of vacuoles/vesicles and therefore was also seen in the presence of concanamycin A. To estimate the initial velocities of V-ATPase at each ATP concentration the corresponding initial rate of non-specific quenching was discounted.

A similar result was obtained when H⁺-pumping activity was measured in vacuolar vesicles obtained from intact vacuoles (Fig. 1b). Moreover, in this fraction the mitochondrial F-ATPase inhibitor azide (Na₃Az; 100 μM) decreased the activity only approximately 29%, and the plasma membrane P-ATPase inhibitor vanadate (Na₃VO₄; 100 μM) did not affect proton pumping, indicating that the sample is not substantially contaminated with vesicles from the plasma membrane or from the internal mitochondrial membrane (Fig. 1b). This indicated that our samples exhibited a degree of purity suitable for single-particle fluorescence analysis by flow cytometry. The initial velocities of proton-pumping by V-H⁺-ATPase at 0.2–1.2 mM ATP followed Michaelis–Menten kinetics and an apparent Kₘ value of 0.65 mM ATP was estimated by the application of GraphPad Prism software for both vacuoles and membrane vacuolar vesicles, which is in the range of the values described in the literature. Representative Lineweaver–Burk graphs are shown in Fig. 1c, d.

**Characterization of yeast vacuoles by fluorescence microscopy**

Isolated vacuole suspensions were also characterized by phase-contrast and fluorescence microscopy, before and after staining with specific fluorescent probes. In spheroplasts, large intracellular vacuoles were observed by phase-contrast microscopy and the vacuolar membrane by fluorescence microscopy after staining with 10 μM of the structural marker MDY-64 (Fig. 2a). Though this structural marker has been used to visualize the vacuolar membrane in whole cells, it also stained isolated vacuoles, and intra-vacuolar membrane structures could be detected, as observed by confocal microscopy (Fig. 2b). Intra-vacuolar structures were not present in all vacuoles, indicating they can present different degrees of complexity.

To ascertain whether the isolated vacuoles maintained their electrochemical potential DiBAC₄(3) was used. This fluorescent probe is a membrane potential indicator and accumulates on positively charged membranes. Purified vacuoles exhibited intense fluorescence with this probe, when observed by confocal microscopy, indicating that their membrane integrity and function was preserved (Fig. 2c).
Fig. 1. V-H⁺-ATPase activity in intact vacuoles (a, c) and vacuolar membrane vesicles (b, d) purified from yeast. (a) Typical fluorescence signal of the initial velocity of proton pumping by V-H⁺-ATPase in a vacuolar suspension after adding 1 mM ATP (A), and effect of addition of 1 mM ATP to the fluorescence intensity in the absence of vacuoles (B). Inhibition of proton pumping activity by concanamycin A in intact vacuoles (C) and dissipation of the proton gradient by 1 mM CaCl₂ (D). (b) Fluorescence signals of the initial velocities of proton pumping by V-H⁺-ATPase in vacuolar membrane vesicles after addition of 0.5 mM ATP (A) and 0.05 μM concanamycin A (B), 100 μM azide (C) or 100 μM vanadate (D). Representative Lineweaver–Burk plots of the initial velocities of proton pumping by V-H⁺-ATPase as a function of ATP concentration in vacuolar suspensions (c) and vacuolar membrane vesicles (d).

Fig. 2. Microscopy analysis of spheroplasts and vacuoles isolated from yeast. Spheroplasts were observed by phase-contrast microscopy (a) and fluorescence microscopy after staining with the structural dye MDY-64 (insert). Confocal microscopy analysis of vacuoles stained with MDY-64 (b) and DibAC₄(3) (c).
Structural and functional analysis of intact vacuoles and vacuolar membrane vesicles by flow cytometry

Since isolated vacuoles exhibited positive staining with both structural and functional probes, flow cytometry was explored to further characterize and quantitatively analyse populations of isolated yeast vacuoles and vacuolar membrane vesicles. Biparametric histograms [forward scatter (FS) log vs side scatter (SS) log] of a mixture of whole cells, purified vacuoles and vacuole membrane vesicles clearly revealed differences of complexity and size between these three populations (Fig. 3a). Cells appeared more complex and larger than vacuoles and vacuolar membrane vesicles. The latter appeared to be similar in size to the smallest vacuoles but with lower complexity. In accordance with the observations by fluorescence microscopy the vacuole suspensions analysed by flow cytometry revealed more heterogeneity in relative size and complexity than the cell and vesicle populations. However, purification by the silicone oil centrifugation technique resulted in a vacuole population more homogeneous in relative size and complexity (Fig. 3b).

The positive staining of vacuoles with the structural vacuolar membrane probe MDY-64 observed by confocal microscopy was quantified by flow cytometry. The stained vacuolar population exhibited a green positive staining completely discriminated from the autofluorescence (Fig. 3c). Identical staining was obtained with the non-specific membrane fluorescent dye FM1-43 (Fig. 3d). These results indicate that flow cytometry allows for structural characterization of vacuolar suspensions stained with these two probes. However, this staining does not allow assessment of whether the isolation procedure used compromises vacuole integrity. Therefore, vacuoles from a strain expressing the soluble vacuolar protease fused with the enhanced green fluorescence protein (Pep4p–EGFP) were isolated and analysed by flow cytometry. This vacuolar suspension exhibited a higher level of green fluorescence than vacuoles isolated from the wild-type strain (Fig. 3e), confirming that they retained Pep4p–EGFP and thus that vacuolar structure integrity was preserved during the purification procedure.

Flow cytometry also proved useful for monitoring the contamination of the vacuole suspension with other sub-cellular fractions. Fig. 3(f) shows the overlay of the red fluorescence histograms of vacuolar membrane vesicles, purified vacuolar suspensions and purified yeast mitochondria stained with NAO. This comparison indicates that the vacuole sample was not substantially contaminated with intact mitochondria or mitochondrial vesicles, since the fluorescence intensity after staining with NAO, which stains the mitochondrial-membrane-specific lipid cardiolipin, was approximately ten times lower than that of isolated mitochondrial suspensions. These results are in accordance with those reported above, showing only residual activity of the mitochondrial F-ATPase in the vacuole samples.

Incubation of vacuole samples from wild-type cells with several functional dyes resulted in clear positive staining (Fig. 4). AO is a weak base that accumulates in a pH-dependent manner in acidic cellular compartments by an ion-trap mechanism (Cools & Janssen, 1986) and has been used to monitor vacuolar pH (Cohen et al., 1999). LysoSensor Green is another probe that becomes more fluorescent in acidic environments. Vacuoles were highly stained with AO and LysoSensor Green, confirming the acidic nature of the vacuole lumen (Fig. 4a, b). The same results were obtained by fluorescence microscopy using neutral red (results not shown).

Flow cytometric analysis of DiBAC₄(3)-stained vacuoles confirmed the presence of an electric potential across the
vacuole membrane (Fig. 4c), also detected by confocal microscopy (Fig. 2c). Addition of NH$_4$Cl, which dissipates the electrical potential, led to a concentration-dependent decrease in fluorescence intensity of DiBAC$_{4}(3)$-stained vacuoles, as assessed by flow cytometry (Fig. 4d). This suggests that changes in fluorescence intensity reflect changes in vacuolar membrane potential, and validates DiBAC$_{4}(3)$ staining to monitor electrical potential of the isolated organelles.

Vacuoles are important calcium reservoirs in the cell. The calcium-sensitive probe Fluo-4 AM was therefore used to monitor vacuolar calcium content in isolated vacuoles. Results showed that isolated vacuoles were highly stained with Fluo-4 AM, indicating that they store high amounts of calcium (Fig. 4e). In agreement with this, incubation with the calcium ionophore calcimycin caused a 30% reduction in fluorescence intensity 25 min after treatment (Fig. 4f), and the addition of calcimycin 30 min prior...
to Fluo-4 AM prevented positive staining (results not shown).

**DISCUSSION**

In order to understand the function and structure of yeast vacuoles, several studies have been performed over recent decades using intact cells, purified vacuoles or vacuolar membrane vesicles (reviewed by Li & Kane, 2009). In particular, purification of yeast vacuoles has been a valuable tool to characterize the V-H\textsuperscript{+}-ATPase (Arata et al., 2002), determine the vacuole proteome (Wiederhold et al., 2009), study the vacuolar fusion and fission mechanisms and characterize its involvement in different cell processes (Wickner, 2002). Like lysosomes, yeast vacuoles have recently been implicated in programmed cell death (Sousa et al., 2011), and thus the use of isolated vacuoles may also contribute to unveiling their role in this process. Until now, the study of these organelles relied on biochemical, spectrofluorimetric, spectrophotometric, light and fluorescence microscopy techniques associated with the use of several fluorescent probes. However, these techniques only allow determination of mean values and disregard the possible heterogeneity of the samples. Results from standard biochemical techniques used to functionally characterize these fractions are therefore less informative, and for instance the contribution of soluble contaminants, particularly of enzymes, is impossible to eliminate.

In the present study, we took advantage of the wide variety of fluorescent probes now available to monitor distinct structural and functional vacuolar features to perform a quantitative and statistically robust analysis of the structure and function of isolated yeast vacuoles under different experimental conditions by flow cytometry. The main advantage of this technique is the possibility of performing single-particle fluorescence analysis to assay functional features of intact vacuolar membrane vesicles and suspensions without interference from soluble contaminants.

The protocol used in this study for the isolation of yeast vacuoles resulted in highly purified vacuolar samples, in line with previous studies based on a similar procedure (Wiederhold et al., 2009). Inhibition studies with concanamycin A, NaN\textsubscript{3} and Na\textsubscript{3}VO\textsubscript{4} suggested that the vacuole membrane V-H\textsuperscript{+}-ATPase is the main proton pump operating in the sample. This is in accordance with flow cytometry and fluorescence microscopy studies showing that a purified fraction of intact vacuoles was obtained. In particular, the introduction of an additional centrifugation step with silicone oil resulted in a vacuole population much more homogeneous in relative size and relative complexity, as monitored by flow cytometry.

Flow cytometry also proved adequate to monitor important functional characteristics of the isolated vacuole suspensions. The fluorescent dyes MDY-64 and FM1-43 had been used only to visualize vacuolar membranes in whole cells (Cochilla et al., 1999; Etzen et al., 2002). In the present study, both MDY-64 and FM1-43 stained isolated vacuoles, indicating that these probes can be used in structural characterization of isolated vacuoles, not only by fluorescence microscopy techniques but also by flow cytometry. Several intra-vacuolar vesicles were observed in some preparations by confocal microscopy. Though we could not identify the nature of these vesicles, it is conceivable they can be formed during the endocytic processes, or result from the involvement of the vacuole in autophagy and mitophagy.

The staining protocols with MDY-64 and FM1-43 did not allow assessment of whether the isolation procedure compromised the integrity of the vacuole membrane, but flow cytometric analysis of isolated vacuoles from cells expressing Pep4p-EGFP clearly confirmed that. Thus, the isolation protocol did not compromise vacuole membrane integrity, in accordance with previous results showing that vacuoles isolated from yeast cells expressing Pep4p–EGFP exhibit a strong green fluorescence under the fluorescence microscope (Sousa et al., 2011).

Flow cytometry also proved adequate to monitor important functional characteristics of the isolated vacuole suspensions. Analysis of isolated non-energized vacuoles stained with AO, LysoSensor Green and DiBAC\textsubscript{4}(3) confirmed that intact vacuoles were able to maintain an acid pH together with a H\textsuperscript{+} electrochemical gradient across the membrane, in agreement with confocal microscopy analysis. V-H\textsuperscript{+}-ATPase is responsible for building up a proton gradient across the vacuole membrane, as shown in the present study with the probe ACMA in intact vacuoles and vacuolar membrane vesicles, but other transporters, such as exchangers of Na\textsuperscript{+}/K\textsuperscript{+}, cotransporters of weak acids/bases, H\textsuperscript{+} leak pathways (H\textsuperscript{+} cotransporters and antiporters), membrane potential shunts (K\textsuperscript{+}, chloride transporters) and proton leak, may also contribute to vacuolar pH (Brett et al., 2005; Brett et al., 2011; Plant et al., 1999). Furthermore, it has recently been shown that phospholipid flippases and ergosterol play a role in pH regulation (Brett et al., 2011; Zhang et al., 2010).

The probe Fluo-4 AM proved very efficient for monitoring intra-vacuolar calcium levels by both flow cytometry and fluorescence microscopy. Accordingly, calcium promoted the immediate dissipation of the H\textsuperscript{+}-dependent gradient generated by V-H\textsuperscript{+}-ATPase across the vacuolar membrane (Fig. 1a). This accumulation is probably mediated by the vacuolar Ca\textsuperscript{2+}/H\textsuperscript{+} antiporter Vcx1p, which is a regulator of cytosolic calcium (Cunningham & Fink, 1996; Miseta et al., 1999). One of the many vacuolar functions is the storage of ions and metabolites. Under normal conditions, small amounts of calcium are released to the cytosol, but loss of vacuolar acidity prevents calcium movement into the vacuole and increases the reverse transport from the vacuole to the cytosol, allowing calcium release and reutilization (Forster & Kane, 2000). Since vacuolar calcium concentration is critical for the function of this...
organelle, the cell-free system described here can thus be exploited to study mechanisms underlying vacuolar calcium exchanges and to screen for drugs that can interfere with calcium homeostasis.

The ability to control lysosomal-like vacuole pH homeostasis is an essential cellular function, which when affected leads to specific disorders and numerous vacuolar storage diseases. Our findings show that the isolation procedure used in this study results in vacuoles with preserved electrochemical potential, maintaining a pH and calcium gradient. This can constitute a powerful instrument to further elucidate mechanisms underlying vacuolar ion homeostasis, as well for high-throughput analysis of vacuolar function in response to several drugs with impact on the treatment of diseases related to lysosomal dysfunctions. Indeed, the set of staining protocols developed in this study to characterize the structure and function of isolated vacuoles by flow cytometry provides a sophisticated tool for future research into these organelles, by allowing evaluation of their physiological characteristics on a single-particle basis and thus gaining further insight on the role of the vacuole/lysosome in eukaryotic cells.

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