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Review

Identification of vacuole defects in fungi

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ABSTRACT

Fungal vacuoles are involved in a diverse range of cellular functions, participating in cellular homeostasis, degradation of intracellular components, and storage of ions and molecules. In recent years there has been a significant increase in the number of studies linking these organelles with the regulation of growth and control of cellular morphology, particularly in those fungal species able to undergo yeast-hypha morphogenetic transitions. This has contributed to the refinement of previously published protocols and the development of new techniques, particularly in the area of live-cell imaging of membrane trafficking events and vacuolar dynamics. The current review outlines recent advances in the imaging of fungal vacuoles and assays for characterization of trafficking pathways, and other physiological activities of this important cell organelle.

Contents

1. Introduction

Vacuoles are the largest organelle in fungi (Gow, 1997). They participate in a wide range of processes and play a key role in the regulation of cellular homeostasis. The basic functions of fungal vacuoles, common to mammalian lysosomes and plant vacuoles, include degradation of cell components, storage of ions and metabolites and various aspects of cellular homeostasis (see reviews in Klionsky et al., 1990; Weber, 2002; Li and Kane, 2009). In this context, many studies have focused on yeast vacuoles, and have generated novel protocols to elucidate mechanisms of membrane trafficking and vacuolar inheritance, aspects of which are often conserved from yeast to humans (Roberts et al., 1991; Conibear and Stevens, 1998; Weisman, 2006). The methodology relating to vacuole biology is however dispersed across organismal and discipline boundaries. This review

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integrates and evaluates this information, and presents a critical evaluation of the state of the art in vacuole methodologies.

Certain specialized functions of fungal vacuoles are not conserved across kingdoms and are adapted to the particular requirements of the fungal life style in the context of the niche of individual organisms (reviewed in Veses et al., 2008). The kingdom Fungi includes saprophytic, symbiotic and pathogenic species. Many of these species alter their cellular morphology in response to environmental triggers and when the fungi encounter a potential host. Vacuoles play a fundamental role in the regulating of this morphological plasticity and niche-specific adaptation. For example, altered vacuolar morphology and physiology have been linked with impairment of hyphal growth and virulence in human pathogens (such as *Candida albicans*) and plant pathogens (such as *Ustilago maydis* and *Magnaporthe grisea*) (Veses et al., 2008; Steinberg et al., 1998; Weber et al., 2001). We provide in this work an overview of the techniques available to characterize different aspects in vacuolar physiology, from staining methods for the characterization of trafficking pathways, vacuole acidification, macromolecule and ion storage and hydrolytic activities.

2. Vacuole morphology

Because vacuoles often occupy a significant fraction of the overall cell volume they can usually be seen directly using conventional microscopical techniques such as phase contrast and differential interference contrast (DIC) microscopy (Gow, 1997). When they are viewed by phase contrast imaging differences in the refractive index of the watery vacuole and the protein-rich cytosol generates obvious differences in their refractivity (Fig. 1A). Viewed with DIC vacuoles are again easily distinguished by their size and boundary shape (Fig. 1B). The visibility of vacuoles viewed by light microscopy can be enhanced by the addition of a mounting medium solution of 20–50% (w/v) bovine serum albumin (BSA). Because BSA has a high molecular weight high concentrations of BSA do not result in a significant alteration in the osmotic potential of the mountant and this does not result in cell plasmolysis. However, the change in the refractive index of mounting medium becomes similar to the refractive index of the cytoplasm and consequently the lower refractive index of the vacuole becomes phase-bright. Fig. 1C shows a *C. albicans* cell viewed in phase contrast with 35% (w/v) BSA applied directly to slides just before the cover slip is added. This contrasts with the hypha shown in Fig. 1A, which does not have BSA in the mounting medium (Gow and Gooday, 1982). Addition of polyvinylpyrrolidone can also be used to aid visualisation of *Saccharomyces cerevisiae* vacuoles (Gomes de Mesquita et al., 1991). Visualisation of vacuoles has also been studied by transmission electron microscopy (TEM; Chanda et al., 2009) and high resolution scanning electron microscopy (HRSEM; Isola et al., 2009).

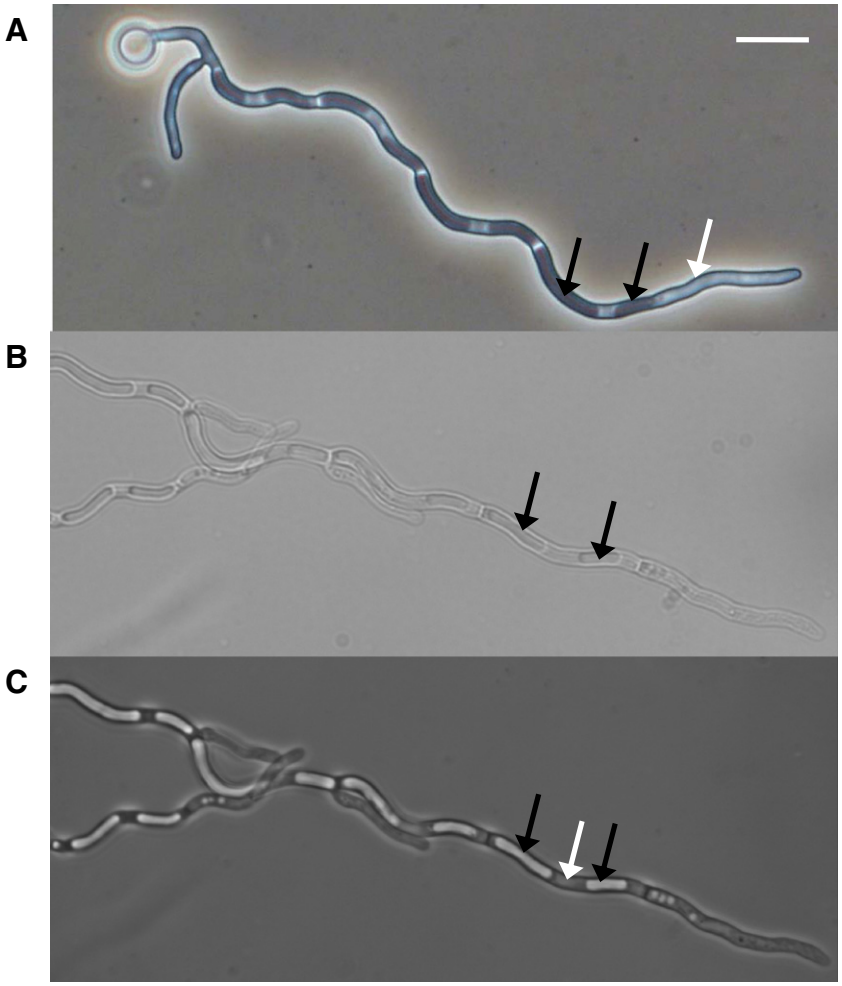

Fig. 1. Appearance of vacuoles using phase contrast and differential interference contrast microscopy. A) Phase contrast image of a *C. albicans* hypha. Vacuoles appear darker compared to the cytosol. Dark vacuoles are indicated by black arrows and the lighter cytosol by a white arrow within the tip cell. B) Differential interference contrast image of part of *C. albicans* hyphae. Vacuoles appear as hollows. Black arrows indicate some vacuoles. C) Phase contrast image of *C. albicans* hyphae with 35% (W/V) Bovine serum albumin (BSA). Black arrows indicate vacuoles, now brighter compared to the cytosol, since BSA changes the refractive index. Scale bar 10 μm.

Vacuoles are dynamic organelles which undergo extensive expansion and remodelling during morphogenetic transitions, such as germ tube formation of *C. albicans* (Gow, 1997); appressorium formation of *M. grisea* (Weber et al., 2001), formation of dikaryotic hyphae of *U. maydis* (Steinberg et al., 1998). These morphogenetic transitions involve modifications in the cell cycle programmes during the alteration of cell shape (Berman and Gow, 2004). This is accompanied by divergent patterns of vacuolar inheritance between the various cellular morphologies (Veses and Gow, 2008). A wide range of techniques have been made available in recent years to follow vacuolar dynamics associated with these morphological transitions.

2.1. Use of vacuolar markers

The majority of vacuolar marker molecules take advantage of the intrinsic acidity of this organelle. Cells are usually incubated with non-fluorescent compounds that, upon reaching the vacuole, undergo a chemical reaction generating a fluorescent derivative that is trapped in the vacuolar lumen. For example, chloromethyl coumarin-derived Cell Tracker vacuole markers, including CMAC (7-amino-4-chloromethylcoumarin; Molecular Probes) and CMFDA (5-chloromethylfluorescein diacetate), have been used to visualise vacuoles in a range of fungi such as *Aspergillus nidulans* (Leeder and Turner, 2008), *Aspergillus parasiticus* (Chanda et al., 2009), *Aspergillus oryzae* (Ohneda et al., 2005; Shoji et al., 2006; Tatsumi et al., 2007), *U. maydis* (Steinberg et al., 1998), *Cryptococcus neoformans* (Liu et al., 2006), *Pisolithus tinctorius* (Cole et al., 1997, 1998) and *Phanerochaete velutina* (Zhuang et al., 2009). These compounds readily diffuse across cell membranes due to their hydrophobic nature. The chloromethyl group reacts with thiols such as glutathione producing fluorescent conjugates that are membrane impermeable and so are retained within the vacuole lumen (Haugland, 1995; Cole et al., 1997). Similarly, a set of fluorescent vacuole markers based on 6-carboxyfluorescein diacetate (CFDA) and its derivatives (CDCFDA, 5-[and 6-] carboxy-2′, 7′-dichlorofluorescein diacetate, cDFFDA, and Oregon Green 488 carboxylic acid diacetate) have been used to visualise vacuoles in a range of fungi such as *C. albicans* (Veses et al., 2009b), *P. tinctorius* (Hyde et al., 2002), *Paxillus involutus* (Tuszynska, 2006), *P. velutina* (Darrah et al., 2006; Fricker et al., 2008; Zhuang et al., 2009), *A. nidulans* (Peñalva, 2005), *Gigaspora margarita* (Saito et al., 2004), *S. cerevisiae* (Shiflett et al., 2004), *U. maydis* (Torralba and Heath, 2002) and *C. neoformans* (Harrison et al., 2002). They work by the same principle as CMAC, and are colourless non-polar compounds which readily diffuse across cell membranes. Once within the vacuole the compounds are hydrolysed by intracellular esterases and become desterified yielding a fluorescent product that is polar and unable to re-cross the vacuolar membrane by diffusion. Transport into the vacuole is mediated by an anion transport mechanism across the vacuole membrane and no such mechanism operates in the opposite direction. Therefore the fluorescent product is sequestered and retained within the vacuole lumen (Roberts et al., 1991; Slayman et al., 1994; Cole et al., 1997, 1998; Weber, 2002).

A variant of these compounds is Neutral Red, a dye that also accumulates within any membrane bound compartment with an acidic lumen (Weber, 2002). Neutral Red has been used successfully in studies of *Botrytis cinerea* (Weber et al., 1999), *M. grisea* (Weber et al., 2001), *Trichophyton mentagrophytes* (Naka et al., 1995) and *Colletotrichum graminicola* (Schadeck et al., 2003). The compound is non-toxic, is visible using bright field light microscopy, and is not prone to photobleaching, the major problem when using fluorescent vacuolar markers (Hickey et al., 2004). This information is summarized in Table 1. Another staining method that does not involve the use of fluorescent molecules is the use of acid phosphomonoesterase, a substrate of vacuolar acid phosphatase (Weber et al., 2001). The hydrolysed substrate forms a coloured product, highlighting the vacuole lumen. The disadvantage of this technique is the lack of specificity, since acid phosphatase is also secreted, particularly at the growing hyphal apex, and therefore the stain may be seen in the endomembrane system throughout the cytoplasm (Weber and Pitt, 1997).

Table 1
Summary of available techniques for vacuolar studies.

Objective	Method	Target/mechanism
Vacuolar morphology	*Fluorescence microscopy*	
	— CMAC/CMFDA	Lumen
	— CFDA (and derivatives)	Lumen
	— Neutral red	Lumen
	— FM-464	Membrane
	— (G/C/Y)FP-fusion proteins	Various
	— Mono/Polyclonal Immunohistochemistry (See Table 2)	Peripheral and integral membrane proteins/soluble lumen proteins
Vacuolar size	*2D software*	
	— CalMorph	Determination of pixels associated with vacuolar fluorescence
	— OpenLab	
	3D software	Analysis of z-stack fluorescence microscopy images combined with pixel complexity algorithms
	— V3D	
Fusion analysis	*Enzymatic assays*	
	— Pho8/Pep4 assay	Fusion events are detected by generation of a coloured metabolite
	— *E. coli* β lactamase/ carboxypeptidase Y fusion protein	Fusion events are detected by restoration of β lactamase activity
	Fluorescence microscopy	Docking site proteins
Vacuolar acidification	*Fluorescence microscopy* with Quinacrine	Acidic vacuolar lumen
	Flow cytometry with BCECF	Alkaline vacuolar lumen
Protein sorting in vacuoles	CPY detection	See Table 3
	Immunodetection	
	— ALP detection	Antibody-mediated detection of ALP
	— API detection	Antibody-mediated detection of API
Macromolecule	*Radiolabelled* C^{14} time course experiments	Arginine transport and storage
	Microscopy	
	— Toluidine Blue O staining	Polyphosphate transport and storage
	— Fluorescently tagged antibody mediated	Polyphosphate transport and storage
	— SEM	Polyphosphate transport and storage
	Colourimetric spectrophotometry	Quantification of vacuolar polyphosphate

A used vacuole stain is FM4-64 (N-[triethylammoniumpropyl]-4-[p-diethylaminophenylhexatrienyl] pyridium dibromide; Molecular Probes) — a lipophilic styryl compound. It does not permeate cell membranes, but inserts into the plasma membrane, and becomes fluorescent upon doing so. It is taken up by the cell via endocytosis, staining compartments of the endocytic pathway, and ultimately accumulates in the vacuole membrane (Vida and Emr, 1995; Steinberg et al., 1998; Fischer-Parton et al., 2000). FM4-64 has been used to visualise vacuoles in a range of fungi such as *C. albicans* (Veses and Gow, 2008; Veses et al., 2009b), *U. maydis* (Steinberg et al., 1998), *A. nidulans* (Peñalva, 2005), *A. oryzae* (Shoji et al., 2006; Tatsumi et al., 2007), *S. cerevisiae* (Meaden et al., 1999; Shiflett et al., 2004; Ogita et al., 2010), *Yarrowia lipolytica* (Nazarko et al., 2005) and *C. neoformans* (Liu et al., 2006). FM4-64 is virtually non-fluorescent in water and more photostable than CMAC and CMFDA. This range of characteristics makes it very suitable for live cell imaging since it can be added directly to the medium

without the need of washing out steps and has a higher resistance to photobleaching (Hickey et al., 2004). However because the dye follows membrane internalisation via the endocytosis pathway, vacuoles are not revealed up shorter incubation times and pilot experiments need to be performed to determine when the vacuole becomes stained with this dye. In yeast cells pulse-chase staining with FM4-64 results ultimately in staining of the vacuole but, in hyphae of filamentous fungi, FM4-64 eventually accumulates in the secretory vesicles in the hyphal tip that defines the Spitzenkörper (Hickey et al., 2004).

A limiting factor in the use of all of these techniques is the optimization of the methodology to enable the fungus to be visualised in the microscope in a flat focal plane. Cells have successfully been immobilised on glass surfaces treated with (i) poly-L-lysine slides or (ii) on microscope slides coated with thin films of agar. These techniques were used to show differences in vacuole segregation during cell division in yeast and filamentous growth forms of *C. albicans*. In this fungus the presence of a vacuole segregation structure was revealed in pseudohyphae and true hyphae whereas in yeast cells a stream of vacuole vesicles was observed during vacuolar inheritance (Veses and Gow, 2008). Slide cultures using agar prevent desiccation and therefore longer time lapse sequences can be imaged. Poly-L-lysine-coated slides have proved useful when media need to be perfused or exchanged within the system — for example to trigger morphogenetic transitions or to study vacuolar responses in response to nutritional changes. One of the possible inconveniences of using FM4-64 can be the non-desired staining of other membranes. This can be helped by introducing a second co-staining agent that targets the vacuolar lumen. Combining CDCFDA and FM4-64 has been used successfully in this way — they fluoresce at different wavelengths and they localise to the vacuole lumen and vacuole membrane respectively. Addition of a third dye, Calcofluor White, which stains fungal cell walls (Pringle, 1991) can improve further the visualisation of the vacuolar dynamics by defining the cell boundary (Veses et al., 2008). At high concentrations Calcofluor White has been shown to increase chitin synthesis and interfere with cell growth (Roncero and Duran, 1985; Pringle, 1991) but can be used as a benign live cell stain at low concentrations (around 1.5 mM) (Hickey et al., 2004). Fig. 2 shows merged images of CDCFDA (yellow) and FM4-64 (red) stained vacuoles, in addition to cell wall staining (blue) with Calcofluor White in *C. albicans* yeast and pseudohyphal (A) and hyphal (B) cells, grown in poly-L-lysine. More co-staining protocols have been developed for other fungi, such as *A. nidulans*, to trace the endocytic pathway (Peñalva, 2005).

2.2. Fusion proteins

Molecules such as Green Fluorescent Protein from *Aequorea victoria* and modified versions (Cormack et al., 1997; Gerami-Nejad et al., 2001) have been used to tag vacuolar proteins, including proteins targeted to the lumen and integral vacuole membrane proteins (Table 1). The vast majority of studies which use vacuolar proteins fused to GFP or derivatives YFP, RFP, etc., have been used to investigate the functions of unknown gene products and confirm subcellular localisations (in combination with other fluorescent fusion proteins or vacuole stains). Examples include studies of a range of GFP-tagged vacuolar proteins (the alkaline phosphatase Pho8, components of the HOPS complex Vps39 and Vps33, small G protein Ypt7, V-ATPase component Vma11) to show enrichment of proteins at docking sites (Wang et al., 2002). Fluorescent protein-fusions have also been used to demonstrate the role of the vacuolar transporter chaperone (VTC) complex in microautophagy. GFP tagged versions of the Vtc components were localised to the vacuole using GFP-Pho8 as a marker control (Uttenweiler et al., 2007). In *Neurospora crassa* heterokaryons of RFP and GFP tagged proteins have been used to identify calcium transporters located on

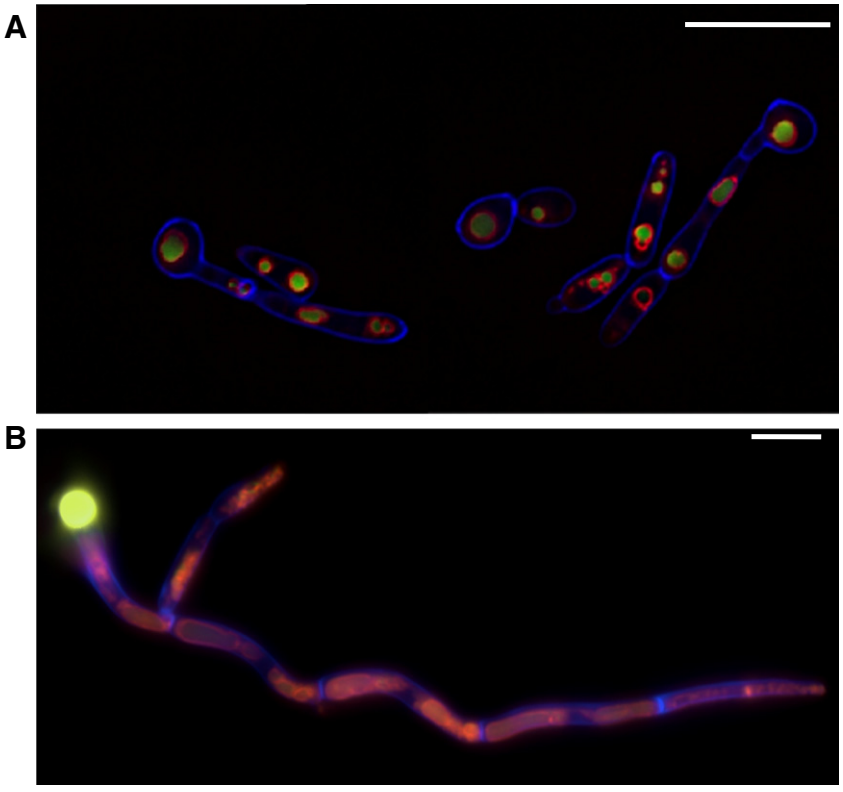

Fig. 2. Co-staining vacuoles using fluorescent markers. A) Triple staining (FM4-64 + CDCFDA + CFW) in yeast and pseudohyphae of *C. albicans*. B) Triple staining ((FM4-64 + CDCFDA + CFW) in hyphae of *C. albicans*). FM4-64 stains vacuolar membranes (in red); CDCFDA stains vacuolar lumen (in yellow); CFW stains cell walls (in blue). Scale bar 10 μm.

vacuole membranes. For example, heterokaryons containing the calcium transporter NCA2-GFP and the vacuolar membrane SNARE protein RFP-VAM3 exhibited co-localisation indicating that Nca2 is located at the vacuole membrane (Bowman et al., 2009). Vacuole membrane fluorescent stain FM4-64 was used in conjunction with GFP to localise Rab7 homologue Ypt71 to vacuole membranes in fission yeast *Schizosaccharomyces pombe* (Kashiwazaki et al., 2009). All these reports however, focus on pinpointing characteristics of particular proteins, and the number of studies that use GFP fused to vacuolar proteins as tool for live cell imaging are relatively low, despite the fact that fluorescent protein fusions allow stable, high-resolution prolonged observation of vacuole dynamics at different developmental stages. Examples include studies of the recombinant protein Vam3-GFP in *A. oryzae*, to study vacuolar morphology (Shoji et al., 2006), and Vph1, a component of the vacuolar ATPase in *S. cerevisiae*, that was used to examine the vertex ring during vacuole fusion (Wang et al., 2002). The infrequent use of GFP technology in vacuolar studies may be due to the availability of convenient dyes which, although less photostable, do not require construction and testing of protein fusion reporters. Additionally, a clear disadvantage of GFP fusions is the possible reduction of fluorescence intensity caused by changes in pH, reported in *A. oryzae* when using a GFP-vacuolar enzyme carboxypeptidase Y (CPY) (Ohneda et al., 2002; Tatsumi et al., 2007). This problem can be offset by the use of alkaline growth medium at around pH 8.0. However, there are obvious limitations of this approach for fungi that do not exhibit growth or morphogenesis in more alkaline pH media.

GFP fusions have also been a key tool in the characterization of protein trafficking pathways converging in the vacuole. Although Kunze and coworkers have described the common mislocalisation of many secretory proteins to the vacuole in *S. cerevisiae* (Kunze et al., 1999), this problem has been overcome by preferentially using COOH-terminal fusions rather than amino terminal fusion, since targeting signals are usually within the NH_2-terminal sequences of proteins (Veses et al., 2005). In many studies both COOH and NH_2 fusions have been constructed, to ensure that the vacuolar localisation is not an artefact caused by the construction of the fusion protein. This approach has proved useful in establishing the role of the vacuolar enzymes in the synthesis of aflatoxins produced by *Aspergillus* spp., such as *Nor-1* and *Ver-1* in *A. parasiticus* (Hong and Linz, 2008, 2009).

2.3. Immunohistochemistry

There are several antibodies specific for vacuolar proteins available for use in immunohistochemistry, western blot and ELISA protocols. Some of them are specifically reactive against yeast vacuole proteins, but others are reactive against human or mouse cells and their reactivity against fungal vacuoles should be assessed before use. The range of antibodies includes polyclonal antibodies made ad hoc for specific studies and monoclonal antibodies raised against vacuole membrane proteins and soluble proteins located in the vacuolar lumen (Table 1).

Monoclonal antibodies are commercially available from Molecular Probes (Invitrogen), and their characteristics are described in Table 2. Detection normally involves the use of a secondary antibody, coupled to a colourimetric enzymatic assay, or fluorophore. Antibodies attached to a wide variety of fluorescent dyes are commercially available (e.g. Texas Red, FITC), and new generation fluorophores such as Alexa Fluor (Molecular Probes) or Dylight Fluor (Thermo Scientific) which exhibit superior brightness and photostability are being used increasingly. For antibodies targeting specific vacuolar proteins which are not commercially available, an immunological epitope such as hemagglutinin (HA) or Myc, can be used to tag the protein, allowing recognition with a secondary antibody. A range of companies such as Sigma Aldrich, Aviva Systems Biology or Pierce (Thermo scientific) all generate bespoke antibodies against purified epitopes.

Table 2
Monoclonal antibodies available for immunohistochemistry of vacuolar components.

Antigen	Protein	Location	References
V-ATPase 69 kDa subunit	Vma1	Vacuole membrane	Doherty and Kane (1993)
V-ATPase 100 kDa subunit	Vph1	Vacuole membrane	Doherty and Kane (1993)
V-ATPase 60 kDa subunit	Vma2	Vacuole membrane	Doherty and Kane (1993)
Alkaline phosphatase	Pho8	Vacuole membrane	Nothwehr et al. (1996)
Carboxypeptidase Y	Prc1	Vacuole lumen	Piper et al. (1994)

2.4. Quantification of vacuolar size

As mentioned previously vacuoles are the largest organelle in fungal cells. In mycorrhizal fungi tubular vacuoles expand over many cellular compartments, creating a complex network with a key role in long-distance transport of nutrients. In fungal plant pathogens such as *Puccinia* and *Ustilago*, vacuoles undergo dramatic expansions to support fungal extension without increasing anabolic demands whilst growing in leaf surfaces (reviewed in Veses et al., 2008). Similar expansions occur prior to hyphal development in *C. albicans* (Gow, 1997) and prior to appresorium formation in the rice pathogen *M. grisea* (Weber et al., 2001). In this context it has been useful to generate 3D images showing location, shape and size of vacuoles to further elucidate the physiological mechanisms that govern expansions and partitioning of fungal vacuoles in response to environmental and internal cues.

Most developments in quantitative analysis have facilitated enumeration of organelle abundance and are based on the development of new software (Table 1), imaging equipment (confocal laser scanning microscope) and improved fluorescent probes (such as photoactivatable proteins) (reviewed extensively in van Zutphen and van der Klei, 2011). Specific software was used to obtain volumetric measurements in the study by Barelle and co-workers (Barelle et al., 2003), focused on vacuolation of *C. albicans* germ tubes using a digital image analysis system (3D-DIAS) (Soll et al., 2003). Images were captured through optical sections on the z-axis. Cell perimeters were outlined with pixel complexity algorithms and vacuoles outlined manually. With these measurements the programme composes a composite 3D reconstruction of a cell and its vacuoles although smaller vacuoles cannot easily be resolved (Barelle et al., 2003), and the generation of 3D data is time consuming. Several recent software packages (V3D software; copyright Howard Hughes Medical Institute, Janelia Farm Research Campus) have been developed making 3D image analysis faster and capable of analysing multidimensional and multi-gigabyte data image sets (Peng et al., 2010). Two dimensional measurements of vacuole size, based on 2D medial optical images of cells, are more frequent in research studies. These provide fast and reliable datasets that allow quick comparisons between different experimental conditions or sets of mutants. Available software for these 2D measurements includes:

i) CalMorph (Ohtani et al., 2004), a multi-dimensional high throughput image processing software that makes measurements of cell parameters using fluorescent images of ellipsoidal budding yeast cells (Ohtani et al., 2004). Using this software, vacuolar measurements are based on the area of fluorescence determined by pixel counting. Calmorph has been used to study vacuole morphology in the budding and mitotic stages of cell division of *S. cerevisiae* (Negishi et al., 2009).
ii) Openlab (Improvision, UK). This software makes manual measurements in which the overall cell or vacuole volume is expressed within different fluorophore-delimited compartments.

The total cell volume is expressed as the total number of pixels within Calcofluor White stained fungal cells and the vacuole space is traced manually to include the pixels associated with the fluorescence of a particular vacuolar stain such as CDCFDA. This method proves useful in the generation of ratios "total cell volume to vacuolar size" in *C. albicans* hyphal compartments (Veses et al., 2009b).

3. Characterization of trafficking pathways

Fungal vacuoles have proved to be valuable models for studies of membrane trafficking processes. In particular this has been studied in *S. cerevisiae*, which has served as a model for mammalian lysosome biology (Weisman, 2006). Vacuoles are essential organelles for fungal viability (Weisman, 2003). They can be formed by fission of pre-existing vacuoles, or synthesised de novo (Warren and Wickner, 1996; Fagarasanu and Rachubinsky, 2007). Organelle fusion can also occur between vacuoles, leading to a decrease in organelle number (reviewed in Richards et al., 2010). Many genes governing vacuolar biogenesis and homotypic membrane fusion have been identified, including the *VAM* genes, involved in vacuole morphology and biogenesis (Wada et al., 1992; Wang et al., 1996), and *VAC* genes, involved in vacuole inheritance (Weisman et al., 1990; Weisman and Wickner, 1992). Several biochemical-based protocols have been developed to purify vacuoles and study vacuolar fusion in vitro (Table 1). The process of vacuole fission is less well understood. In vitro assays of vacuole fission have not yet been described, in particular with respect to the dynamin-like Vps1 protein (Peters et al., 2004). Purified vacuoles have been used to begin elucidating components and the order of events occurring during vacuolar fission (Peters et al., 2004; Baars et al., 2007).

3.1. Purification of vacuoles

Protocols for the purification of vacuoles from yeast cells have been reviewed (Cabrera and Ungermann, 2008; Ostrowicz et al., 2008). The procedure involves creating spheroplasts from yeast cells, breakage and separation of vacuoles from the rest of the cellular debris. Separation is achieved by flotation of vacuoles in a discontinuous gradient of Ficoll via density gradient centrifugation. Vacuoles can be purified using this procedure in around 4 h and can be stored frozen in glycerol (Conibear and Stevens, 1998). This procedure has been used successfully to isolate vacuoles from yeast cells (Perzov et al., 2002; Ogita et al., 2010). A similar process can be used in filamentous fungi —for example protoplast formation followed by centrifugation on a high-density sucrose cushion has allowed vacuole isolation in *A. parasiticus* (Chanda et al., 2009). Purity of the isolated vacuole fraction was confirmed using specific markers for vacuoles, mitochondria and the cytosol and by microscopic methods (Chanda et al., 2009).

3.2. Vacuole fusion assays

In vitro vacuolar fusion assays have used complementary combinations of strains to elucidate the components of fusion stages and the order of events (Conradt et al., 1994; Haas et al., 1994). One common assay is the Pho8/Pep4 test. Pho8 (alkaline phosphatase; ALP) is synthesised as an inactive precursor form that is transported to the vacuole via the Golgi. Proteinase A (Pep4) converts Pho8/ALP into its mature active form within the vacuole lumen. The assay requires vacuoles purified from two different strains — one lacking Pep4 which accumulates inactive Pho8/ALP within the vacuoles; and the other strain lacking Pho8 but containing active Pep4. When purified vacuoles from opposite strains fuse, Pep4 activates Pho8/ALP whose enzymatic activity can be measured via a colourimetric reaction (Haas et al., 1994; Haas et al., 1995; Cabrera and Ungermann, 2008). Another assay that is based on the same rationale uses vacuoles purified from two strains in which each contains a different portion of *Escherichia coli* β-lactamase, which is targeted to the vacuole by fusion with a fragment of carboxypeptidase Y (CPY). On fusion β-lactamase activity is restored (Jun and Wickner, 2007). Other approaches do not require the use of specific background strains, making possible the study of specific stages of the vacuole fusion process such as docking. Docked vacuoles aggregate and form clumps, which can be observed microscopically. This assay has been used in conjunction with labelled proteins to localise proteins to docking sites (Mayer and Wickner, 1997; Wang et al., 2003; Fratti et al., 2004), to assay *trans*-SNARE complex assembly by addition of various trans-acting factors (Ungermann et al., 1998; Collins and Wickner, 2007), and to analyse palmitoylation of the membrane protein Vac8 on vacuole membranes (Veit et al., 2001; Dietrich et al., 2004, 2005).

4. Vacuole function

4.1. Acidification

The pH within the vacuole lumen is acidic (around pH 6.0) compared to the neutral cytosol (Preston et al., 1989; Yamashiro et al., 1990; Plant et al., 1999; Martínez-Muñoz and Kane, 2008). This is due to active transport of protons from the cytosol into the vacuole lumen by the vacuolar H^+-ATPase (Kane, 2006). Many vacuolar functions are dependent on its acidity — including vacuole lumenal and membrane enzymes (Yamashiro et al., 1990), the efficient sorting of soluble proteins (Klionsky et al., 1992a, 1992b), transport of ions and metabolites across the vacuole membrane (Ohsumi and Anraku, 1981; Booth and Guidotti, 1997; MacDiarmid et al., 2002; Poltermann et al., 2005) and vacuolar fission and fusion (Peters et al., 2001; Bayer et al., 2003; Baars et al., 2007). Several methods have been used to assess vacuolar acidity, and to screen for vacuolar acidification mutants (*vph*) and potential V-ATPase inhibitors (Preston et al., 1989; Johnson et al., 2010). The simplest method takes advantage of fluorescent dyes with emission spectra sensitive to pH, such as quinacrine, which cross membranes by diffusion, concentrating in acidic vacuole compartments. This is not visible in vacuoles with increased pH (Weisman et al., 1987). Quinacrine staining has been widely used in yeast-like fungi, including *S. cerevisiae* (Sambade et al., 2005) and *C. neoformans* (Harrison et al., 2002), filamentous fungi, such as *N. crassa* (Bowman et al., 1992) and *A. niger* (Schachtschabela et al., 2012) and pleomorphic fungi, such as *C. albicans* (Veses et al., 2009a), in all cases for direct microscopic visualisation (Table 1). For high-throughput screening of vacuole acidification inhibitors, Johnson and co-workers used the carboxyfluorescein derivative 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) fluorescent signal in flow cytometry assays in living yeast cells (Johnson et al., 2010). This fluoresces more intensely when the vacuole is alkalinized, therefore providing a convenient screen for potential V-ATPase inhibitors (Table 1).

4.2. Vacuolar protein sorting

Various routes operate in the transport of proteins of the vacuole to the lumen and membrane. Vacuolar proteins traverse the secretory pathway but then are sorted away from the rest of the secretory traffic and diverted to the vacuole via: (a) — the carboxypeptidase Y (CPY) pathway, which involves transit from the late Golgi through an endosomal/prevacuolar compartment (Stevens et al., 1982; Piper et al., 1995; Conibear and Stevens, 1998), (b) — the alkaline phosphatase (ALP) pathway, which transits via a Pep12p- and Vps45p-independent route, in contrast to the CPY pathway (Klionsky and Emr, 1989; Raymond et al., 1992; Cowles et al., 1997), — or the (c) cytoplasm to vacuole targeting pathway (Klionsky et al., 1992c; Harding et al., 1996; Scott et al., 1997), which is used to transport aminopeptidase (aminopeptidase I, API) and a mannosidase. This route partially

Table 3
Available methods for detection of mis-sorted CPY.

Technique	Detection method	Reference
Growth on CPY esterase plates	Direct visualisation	Rothman and Stevens, 1986; Rothman et al., 1989
CPY-invertase fusion protein	Enzymatic	Robinson et al. (1988)
CPY specific substrate (N-benzoyl-L-tyrosine p-nitroanilide; NTPNA)	Colourimetric	Jones, 2002; Palmer et al., 2003
Immunoprecipitation + SDS PAGE	Immunologic	Rothman and Stevens, 1986; Bonangelino et al., 2002
Colony immunoblot assay	Enzymatic	Bonangelino et al. (2002)
CPY-eGFP fusion	Fluorescence microscopy	Ohneda et al. (2005)

overlaps with the autophagy route, induced in yeast in response to starvation and is linked to morphogenic processes in some fungal pathogens (Weber et al., 2001).

Identification of new genes involved in these vacuolar protein sorting pathways has been investigated by genetic screens that select for mis-sorting of the potential cargoes. This has involved detecting proteins that would normally be targeted to the vacuole, but have been mis-sorted to the cell surface and secreted into the medium. The most extensively studied of these is the CPY pathway. The vast majority of available techniques for CPY mis-sorting assays take advantage of the fact that after the precursor of CPY is mis-sorted, it is converted into its active form in the periplasm (Table 3). Immunological methods for detection of ALP and aminopeptidase I (a cargo protein of the cytoplasm to vacuole targeting pathway) were used to characterize mutants defective in these pathways. In both cases purified antisera raised against ALP and API respectively were used to immunolocalize or immunoprecipitate the mis-sorted ALP or API (Table 1; Raymond et al., 1992; Scott et al., 1997).

4.3. Storage of macromolecules

Fungal vacuoles participate actively in the storage of a range of ions, amino acids and phosphate (reviewed extensively in Klionsky et al., 1990). The proton gradient generated by the vacuolar ATPase is the primary driving force for the transport of these metabolites. Seven different H^+/amino acid antiport systems have been described for arginine, arginine–lysine, histidine, phenylalanine-tryptophan, tyrosine, glutamine–asparagine and isoleucine–leucine in *S. cerevisiae* (Sato et al., 1984a, 1984b), along with an additional arginine–histidine exchange mechanism (Sato et al., 1984b). The majority of studies on amino acid transport have focused on arginine, which has three transport systems. Assays for determination of arginine transport and storage into the vacuole have been based on the accumulation of ^{14}C-arginine in vacuolar vesicles (Kim et al., 2005).

Regulation of ion concentration in the cytosol is crucial for a cell, and the fungal vacuole participates actively in the regulation, accumulating cations such as Sr^{2+}, Co^{2+} Pb^{2+} Ca^{2+} and Zn^{2+} whose concentration in the cytosol must be controlled for physiological reasons (reviewed in Klionsky et al., 1990). Calcium has been proposed to play a key role in regulating growth at the hyphal tip (Silverman-Gavrila and Lew, 2003). However, routine tests to assess Ca^{2+} transport into the vacuole have not been developed. Recently Bowman and coworkers described four calcium transport proteins, regulating intracellular calcium levels in *N. crassa*, including *Cax*, a protein which encodes a Ca^{2+}/H^+ exchange protein in vacuolar membranes (Bowman et al., 2011).

Inorganic polyphosphate (polyP) is the only macromolecular anion found within the vacuole. During growth large amounts of polyP accumulate within vacuoles as granules that move by Brownian motion, but little is known about its metabolism mobilisation under conditions of phosphate limitation (Hürlimann et al., 2007). Visualisation techniques of polyP storage are summarized in Table 1, including the use of Toluidine Blue O, a metachromatic dye that has been used in the plant pathogen *U. maydis* (Boyce et al., 2006). Recently a new technique has been developed which exploits the affinity of *E. coli* exopolyphosphatase to polyP. A recombinant polyphosphate binding domain (RPBB) of the exopolyphosphatase is tagged with an epitope to allow detection via immunohistochemistry. Cells are labelled with the RPBB-epitope then localised with antibodies conjugated with fluorophores or colloidal gold. This allows more specific detection with fluorescence or electron microscopy. It has been used successfully in yeast vacuoles and tubular vacuoles of filamentous fungi (Saito et al., 2005, 2006; Kuga et al., 2008). Werner and coworkers have developed an assay to determine spectrophotometrically the amount of polyP in vacuoles. PolyP is extracted, purified, and specifically digested with *S. cerevisiae* exopolyphosphatase. To quantify the released phosphate, ammonium heptamolybdate and malachite green are added. Then the malachite green solution is quantified by spectrophotometry (Werner et al., 2005). This assay provides a systematic method to compare polyP storage for example across panels of mutants of a particular fungal species or when comparing different environmental conditions.

5. Conclusions

Vacuoles are complex and versatile organelles in fungi, as indicated by their diverse range of cellular functions. Their involvement in cellular homeostasis has been widely studied in yeast, generating a vast collection of protocols. Recent developments in the field of live-cell imaging have contributed to deepening the knowledge of such fields and have given rise to new series of roles for fungal vacuoles, as mediators of morphogenetic changes, which are particularly relevant in pathogenic species. This review aims to highlight the available repertoire of resources for investigation of vacuolar morphology, physiology and classical functions for maintenance of the cellular homeostasis.

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