University of Edinburgh

Ph.D. Thesis

Imaging vesicle trafficking and organelle dynamics in living fungal hyphae

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This thesis is dedicated to my family, especially my late uncle, Peter Crombie for introducing me to video, computers and technology.
Abstract
The aims of the research were to develop and apply live cell imaging techniques using confocal microscopy to image and analyse vesicle trafficking, organelle dynamics and molecular localization at high spatial and temporal resolution in filamentous fungi. The dyes FM4-64, and to a lesser extent, FM1-43, were used to analyse vesicle trafficking, and as general probes to image hyphal tip growth, branching, septum formation, hyphal fusion, conidiophore development and the early stages of protoperithecium development. *Neurospora crassa* was the main system studied, although a taxonomically diverse range of other species were also analysed. Uptake of FM4-64 into hyphae was shown to be time- and energy-dependent, consistent with internalisation being mediated by endocytosis. FM4-64 proved to be an excellent vital stain for different cell components including the apical vesicle cluster (AVC), vacuolar network and mitochondria. The dye was used to compare the morphology of the AVC in 15 different species. The green fluorescent protein (GFP) is a recombinant fluorescent probe that can be targeted to organelles and used to label specific proteins within living cells. Confocal microscopy was used to image transformants of *Aspergillus nidulans* expressing GFP targeted to the vacuolar network, mitochondria, nuclei, spindle pole bodies, endoplasmic reticulum, and Golgi cisternae. In addition, a range of vital fluorescent dyes were used to image organelles, and results from these studies were compared to those obtained with GFP transformants. Vacuolar staining revealed an extensive tubular network in actively growing regions of hyphae, whereas in sub-apical regions large spherical vacuoles were observed. Division of a single mitochondrion was imaged in *A. nidulans* expressing GFP targeted to mitochondria. Using the potentiometric dye rhodamine-123, the membrane potential of mitochondria in growing hyphal tips was shown to be higher than that in sub-apical regions. *A. nidulans* expressing GFP targeted to nuclei allowed detailed observations of mitosis and revealed waves of nuclear division within hyphae. Live-cell imaging techniques were then applied to the following morphological mutants of *N. crassa*: (1) cot-1: a temperature-sensitive hyper-branching mutant. FM4-64 allowed a detailed analysis of the morphological changes that occur when the fungus is shifted between permissive and restrictive temperatures. (2) Nkin: a kinesin-deficient mutant that lacks conventional kinesin and exhibits abnormal hyphal morphology. Behaviour and morphology of the AVC was found to be abnormal and mitochondria were abnormally positioned in hyphal tips. (3) spray: a hyper-branching mutant. The AVC behaviour and branching pattern were shown to be abnormal. (4) slime: a combination of three mutations results in cells which lack functional cell walls, and exist as non-polar protoplasts. The endocytic pathway via putative endosomes to the vacuole in *slime* cells was found to be similar to that in hyphae.
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Abbreviations

ATP  adenosine triphosphate
AVC  apical vesicle cluster
carboxy-DFFDA  Oregon Green 488 carboxylic acid diacetate
CLSM  confocal laser scanning microscope
COMOS  confocal mouse operated software
Conc.  Concentration
ER  endoplasmic reticulum
Em  Emission
Ex  Excitation
DH2O  deionised water
DMSO  Dimethylsulphoxide
EtOH  ethanol
FM1-43  N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide
FM4-64  N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino)phenyl)hexatrienyl) pyridinium dibromide
FWHM  full width half maximum
GFP  green fluorescent protein
h  hour(s)
KDa  kilodalton
min  minute(s)
MW  Molecular weight
s  second (s)
uv  ultraviolet
w/v  weight by volume
Chapter 1: Literature review

1.1 Cell polarity and the growth habit in filamentous fungi

Fungi are a unique group of eukaryotic organisms, and an extremely important component of the biosphere. Fungi are heterotrophs, requiring pre-formed organic compounds as sources of energy and carbon (in contrast to auxotrophic plants and bacteria). Fungi, plants and animals probably parted evolutionary company in the Pre-Cambrian era. Phylogenetically, fungi are more closely related to animals than plants or bacteria (see Carlisle et al. 2001). Five basic cellular elements can be recognised in higher fungi: hyphae, yeast cells, spores, conglutinate cells, and a heterogenous group of other determinate cell types (Read 1994). Vegetative fungal cells are most commonly filamentous and individual filaments are termed hyphae. Central to the success of filamentous fungi has been the evolution of hyphae and the developmental plasticity these hyphae possess (Read 1994). Hyphae exhibit highly polar growth, and they branch and fuse to form a complex interconnecting network, the mycelium. Most higher fungi reproduce by spores, which may be formed sexually or asexually.

Hyphae often contain extensive regions along which considerable movement of cytoplasm and organelles can occur, and in most fungi, this movement commonly involves the transport of material through septal pores between adjacent hyphal compartments (Gull 1978). Hyphae may be relatively non-specialized, (e.g. vegetative hyphae), or specialised and sometimes highly differentiated (e.g. asci). The relatively high surface area to volume ratio makes hyphae well adapted for absorption, secretion and excretion but leaves them prone to desiccation. The tubular nature of hyphae, coupled with a tendency to form branched, anastomosed networks provides an efficient means for the interchange of materials and organelles between different regions of a colony (Read 1994).

During tip growth, extension of the hypha is confined to a region occupying only a few micrometers at the hyphal apex (Heath 1995). Apical growth is the predominant method of cell growth in filamentous fungi, and has also evolved in the plant kingdom in a number of specialised cell types including pollen tubes, root hairs and rhizoids. Tip growth represents highly polarised exocytosis and cell wall synthesis and requires the trafficking of secretory vesicles (here termed wall-building vesicles) to the hyphal tip. Secretory vesicles deliver membrane, cell wall precursors and
wall-synthesising enzymes to the hyphal tip, and many extracellular enzymes released into the surrounding medium are also believed to be secreted from this region (Wessels 1990). Hyphal growth rates vary considerably and rates of elongation up to 2 μm per second have been recorded in some species (e.g. *Neurospora crassa*) under optimal conditions in Petri dish culture (Bracker *et al.* 1997). The establishment, maintenance, reorientation and loss of cell polarity occur at different stages during normal fungal morphogenesis. Different examples of cell polarity regulation are summarised in Fig. 1.1.

![Diagram](image)

**Fig. 1.1** Different guises of cell polarity regulation in fungi.

### 1.2 Hyphal tip growth

Many ultrastructural studies have been made on hyphal tip organisation (e.g. Grove and Bracker 1970; Howard 1981; Roberson and Fuller 1988). The hyphal tip region is usually hemispherical to ellipsoid in shape (sometimes referred to as a hyphoid shape, Bartnicki-Garcia *et al.* (1995b) and contains organelles including vesicles, multivesicular bodies, mitochondria, Golgi cisternae and endoplasmic reticulum (ER). The vacuolar system is a dynamic and complex organellar network present throughout hyphae (reviewed by Ashford 1998). Hyphal compartments are multinucleate and sometimes contain several hundred nuclei (Deacon 1997).

Three distinct types of vesicles have been observed in the hyphal tip region: (1) apical vesicles of ~100 nm in diameter (Grove and Bracker 1970; Howard 1981), (2)
microvesicles of ~40 nm (Grove and Bracker 1970; Howard 1981), including chitosomes which contain chitin synthase (e.g. Bracker et al. 1976; Sietsma and Wessels 1994), and (3) filasomes which are a type of microvesicle surrounded by filamentous material containing actin (Howard 1981; Bourett and Howard 1991). Microautoradiographic studies of fungal hyphae supplied with radioactively labelled N-acetylglucosamine showed that wall synthesis primarily occurs in the apical region (Bartnicki-Garcia and Lippman 1969).

Pre-packaged membrane-bounded secretory vesicles are believed to be generated from Golgi-cisternae in the cytoplasm and are translocated towards the apical plasma membrane via the Spitzenkörper (see section 1.3). Vesicles fuse with the apical plasma membrane, resulting in an increase of surface area and localised exocytosis at the tip. Exocytosis is thought to be primarily responsible for the delivery of cell wall synthesizing enzymes and cell wall precursors required for tip growth. The major cell wall synthesizing enzymes (chitin and glucan synthase) involved in hyphal tip growth are integral membrane proteins and thus firmly anchored within the plasma membrane (Heath 1990). The cell wall in the tip region of a growing hypha is plastic but becomes progressively more rigid behind the tip (Wessels 1990; Gooday 1995).

In filamentous fungi, it has been proposed that turgor provides the driving force for hyphal expansion. However, Harold et al. (1996), observed that growth still occurs in hyphae of *Saprolegnia ferax* when turgor is reduced by 95%. Thus, other mechanisms (e.g. actin-based processes) may also be important for cell expansion during tip growth (Money and Harold 1993). Cytoskeletal elements (microtubules and actin) also play an important role in delivering secretory vesicles to the hyphal tip (Gooday, 1995; see section 1.1.4).
1.3 The vesicle supply centre: the "Spitzenkörper"

In higher fungi (i.e. members of the Ascomycota, Basidiomycota and Deuteromycota), vesicle trafficking to the apex during tip growth is highly organised and involves the activity of a specific, multicomponent organelle complex which, in most cases, is called the Spitzenkörper (= 'apical body'). This structure is predominated by secretory vesicles, which make up what is commonly described as the 'apical vesicle cluster' (Grove & Bracker 1970; López-Franco & Bracker 1996). The dynamic behaviour of the Spitzenkörper has indicated that it is intimately associated with the precise growth pattern of the hyphal apex (Girbardt 1957; Bartnicki-Garcia et al. 1995b; López-Franco & Bracker 1996). The Spitzenkörper, originally described by Brunswick (1924), contains a dynamic aggregation of apical vesicles, microvesicles, microtubules, microfilaments and sometimes ribosomes, and is located at the apex, but is apparently not in contact with the plasma membrane (Grove and Bracker 1970; Howard 1981). There is also evidence that the Spitzenkörper contains a microtubule organising centre, from experiments using anti-γ-tubulin antibodies in Allomyces (McDaniel and Roberson 1998). Spitzenkörper have been observed in basidiomycetes and ascomycetes, but not in lower fungi (Grove and Bracker, 1970), with a few exceptions such as Allomyces. However, fungi lacking a visible Spitzenkörper still appear to accumulate vesicles within their hyphal tips.

The Spitzenkörper disappears when a hypha is shocked or stops growing (Girbardt, 1957; Grove and Bracker 1970), and its position influences the direction of growth (Girbardt 1957; Bartnicki-Garcia 1990). A new Spitzenkörper appears at the site of hyphal branch emergence (Girbardt 1957). The exact method of formation and maintenance of the Spitzenkörper is not fully understood, though cytoplasmic movement, interactions with cytoskeletal components and other mechanisms are probably involved.

Smaller, migratory "satellite Spitzenkörper" have been observed in a variety of fungi (López-Franco et al. 1994, 1995; Fischer-Parton et al. 2000). These "satellites" appear as phase-dark ovoid plaques just beneath the plasma membrane, a few micrometers back from the apical pole and then migrate towards the main Spitzenkörper. Satellites fuse with the main Spitzenkörper within 2-15 s following formation. The fusion of satellites with the main Spitzenkörper is followed by a transient increase in tip growth rate, which results in the characteristic pulsed growth
pattern of hyphae. Satellites that linger close to the apex are often associated with the production of lateral bulges in the hyphal profile, further indicating that satellites can provide wall-building vesicles for cell growth (López-Franco et al. 1994).

1.4 Roles of the cytoskeleton and motor proteins in tip growth

Microtubules and actin microfilaments are known to play an important role in organelle positioning, and are thought to be involved in the transport of secretory vesicles for tip growth (Grove and Bracker 1970; Howard 1981; McKerracher and Heath 1987). Microtubules are longitudinally arranged down the length of hyphae and often extend up into the Spitzenkörper (Heath and Skalamera 2001). Actin is found throughout hyphae and fluorescent labelling experiments have shown that it is particularly concentrated at the apices of hyphae (Heath and Skalamera 2001). Behind the hyphal tip, actin plaques are present and have been shown to be associated with filasomes (Roberson 1992; Bourett and Howard 1991).

Motor proteins such as kinesin and dynein play a major role in organelle movement along microtubules, and in the delivery of secretory vesicles to the apex (Steinberg and Schliwa 1993; Lemhler et al. 1997; Seiler et al. 1997). Kinesin has been shown to be important in the movement of organelles towards the distal (+) end of microtubules, whereas dynein is important for moving organelles towards their (-) ends (Mandelkow and Mandelkow 1995). Motor proteins bind tightly to and generate movement along microtubules using energy supplied by ATP hydrolysis. A conventional kinesin null mutant of Neurospora crassa was found to display abnormal hyphal morphology, abnormal Spitzenkörper and to have a reduced growth rate (Seiler et al. 1996). In Nectria haematococca, the conventional kinesin (NhKIN1) has been found to be required for normal mitochondrial positioning in hyphal tips (Wu et al. 1998). These results suggest that the microtubule-based kinesins have a direct role in the apical transport of secretory vesicles and mitochondrial positioning.

Several other motor proteins have been identified in filamentous fungi. These include unconventional myosins and cytoplasmic dynein (Steinberg 2000). In Aspergillus nidulans, GFP fused to cytoplasmic dynein heavy chain was found to localise towards hyphal tips, as "comet-like" structures which appeared to move along microtubules (Liu et al. 2001). Seiler et al. (1999) reported that the
simultaneous mutation of conventional kinesin and dynein genes was not lethal and resulted in an additive phenotype that combined the features of the single mutants. The mutation of kinesin and dynein had opposite effects on the apical and retrograde transport, respectively, and major defects in vacuole formation and dynamics were also evident (Seiler et al. 1999). Myosin and dynamin are actin binding motor proteins that have been shown to be important in endocytosis (Hasson and Mooseker 1995; Yamashita and May 1998). A gene isolated from *Aspergillus nidulans* encoding a novel chitin synthase was recently found to contain a myosin motor-like domain, and the authors suggested that this motor domain may play a role in the transport of chitin synthase (Fujiwara et al. 1997).

### 1.5 The vesicle trafficking network

The vesicle trafficking network includes the secretory and endocytic pathways (Gruenberg and Maxfield 1992; Rothman 1994). In filamentous fungi a reasonable amount is understood about exocytosis, whilst very little is known about endocytosis (reviewed by Read and Hickey 2001). Most of our current understanding of vesicle trafficking in filamentous fungi is concerned with tip growth and is based upon ultrastructural studies (e.g. Grove and Bracker, 1970; Howard, 1981), pharmacological treatments (e.g. Howard and Aist, 1980), analyses of mutants (Wu et al. 1998, Seiler et al. 1999) and mathematical modelling of vesicle trafficking in relation to tip growth (e.g. Bartnicki-Garcia et al., 1989).

A common rate of hyphal extension for *N. crassa* growing in open culture in a Petri dish is $\sim 36 \mu m \ sec^{-1}$. In order for a $10 \mu m$ wide hypha of *Neurospora* to supply sufficient plasma membrane to the hyphal tip to maintain this growth rate, it has been estimated that $\sim 600$ secretory vesicles per second would have to fuse with the apical plasma membrane (Collinge and Trinci, 1974). However, even this magnitude of membrane transport must be a significant underestimate of the amount of vesicle trafficking being undergone within a growing hyphal tip because it does not take account of vesicles moving between organelles or the activity of endocytic vesicles.

### 1.6 Endocytosis

Endocytosis involves the invagination and pinching off of endocytic vesicles from the plasma membrane that are subsequently transported and fuse with other membranes within the cell, and most notably early endosomes in the lytic pathway.
The process of endocytosis is well characterised in animal and yeast cells and to a lesser extent in plant cells (Watts and Marsh 1992; Hawes et al. 1995; Vida and Emr 1995). In animal and plant cells, endocytic vesicles are most commonly clathrin-coated although both clathrin-dependent and clathrin-independent endocytic pathways are known to exist (Geli and Reismann 1998). The rate at which the plasma membrane is internalised varies between types of cells but is usually surprisingly large. In fibroblasts about 2,500 vesicles are endocytosed per minute, and within seconds these shed their clathrin coat and are able to fuse with early endosomes (Alberts et al. 1994; Robinson 1987; Brodsky 1998). Caesar Ton-That et al. (1987) identified a fraction from hyphae of *Neurospora crassa* similar to that of the heavy chain of clathrin, the major coat protein of endocytic vesicles in animal and plant cells (Hawes et al., 1995; Mellman, 1996). However, convincing ultrastructural evidence for clathrin-coated vesicles or pits in fungal hyphae is lacking. The best evidence for endocytosis in filamentous fungi comes from studies using the endocytic marker dye FM4-64 (see section 1.8).

The general organisation of endocytic traffic inside budding yeast cells resembles that of mammalian cells (Vida and Emr 1995). Genetic approaches and the isolation of endocytosis mutants in yeast are helping to show the differences and similarities between yeast and animal cells (Geli and Riezman 1998). In contrast to animal cells, the requirement of actin for endocytic internalisation may be of key importance to the mechanism of endocytic vesicle formation in the budding yeast (Geli and Riezman 1998). Two forces that work against vesicle formation in yeast include surface tension and turgor pressure. Geli and Riezman (1998) have suggested that the forces generated through actin polymerisation and actin associated motors are used to deform the plasma membrane against these forces during endocytic vesicle formation in yeast. Interestingly, it has been found that filasomes, which have been suggested as candidates for endocytic vesicles (Fischer-Parton et al. 2000), are concentrated in the apical zone (Roberson, 1992). Furthermore, clathrin-coated endocytic vesicles have also been shown to be concentrated in a similar region in pollen tubes (Derksen et al. 1995).

1.7 The use of fluorescent dyes to monitor endocytosis

Amphiphilic styryl dyes, such as FM4-64 and FM1-43 (Fig. 1.2) insert into the outer leaflet of the plasma membrane (Fig. 1.3) and are believed not to directly enter intact cells by unfacilitated diffusion (Betz et al. 1996). They have, therefore, been
widely used as fluorescent reporters of endocytosis and other components of the vesicle trafficking network in animal cells (e.g. Betz et al. 1996) and the budding yeast (Vida & Emr, 1995; Rieder et al. 1996). FM4-64 is a styryl dye with rhodamine-like properties and is fluorescent only when it is membrane-bound (Betz et al. 1996). The FM4-64 molecule is composed of three different elements: a hydrophobic tail (which promotes partitioning into membranes), a dicationic head (which prevents passage across membranes), and a body or nucleus (which determines the spectral properties of the dye) (Fig. 1.2). Recently, uptake of FM4-64 by hyphae has provided strong experimental evidence for membrane internalisation by endocytosis in fungal hyphae (Hoffmann & Mendgen, 1998; Fischer-Parton et al. 2000; Read and Hickey, 2001). The main mechanism by which FM4-64 is believed to be internalised by hyphae and distributed to different organelles is shown in Fig. 1.4.

![Chemical structures of (a) FM4-64 and (b) FM1-43 (from Fischer-Parton et al. 2000).](image)

In yeast cells and hyphae of *N. crassa*, FM4-64 initially stains the plasma membrane, then small cytoplasmic compartments (believed to be endosomes), and finally the vacuolar membranes. Prolonged staining with FM4-64 resulted in the staining of most membrane-bound organelles within hyphae (Fischer-Parton et al. 2000; Read and Hickey 2001). FM4-64 internalisation is time-, temperature- and energy-dependent in both yeast cells and fungal hyphae (Vida and Emr 1995; Fischer-Parton et al. 2000).

Fluid phase endocytosis has been monitored in animal cells by adding "dextran-conjugated fluorescein" or other membrane-impermeable probes (e.g. Lucifer
Yellow) to the external medium and following their internalisation. Interestingly, Cole et al. (1997) were unable to observe uptake of any membrane-impermeant fluorescent probes by fluid-phase endocytosis into hyphae of the basidiomycete *Pisolithus tinctorius*. However, recent studies have indicated that the dextran dyes can be internalised by germlings of *Magnaporthe grisea* (Atkinson 2001).

The proposed uptake mechanism of FM4-64 into fungal hyphae (from Read and Hickey 2001).

**1.8 Model of the vesicle trafficking network**

It is now clear that a complex network of vesicle trafficking pathways is present in fungal cells. Based on the interpretation of results in the context of current knowledge in other cell types, a speculative model of the vesicle trafficking network within growing hyphae has been formulated (Read and Hickey 2001; Fig. 1.4).

[1] Plasma membrane $\rightarrow$ endocytic vesicles $\rightarrow$ endosomes $\rightarrow$ vacuole. Dye fluorescence is initially detected within the cytoplasm within 10 sec of adding FM4-64 to hyphae. Ten to fifteen minutes after adding FM4-64 to hyphae the spherical and tubular vacuolar elements become stained.
**[2]** **Endoplasmic reticulum (ER) → Golgi → main Spitzenkörper → apical plasma membrane.** The traditional view of the secretory process involved in tip growth is that proteins synthesized on the ER are transported via vesicles to the Golgi within which they are processed and transported in secretory vesicles to the apical vesicle cluster within the main Spitzenkörper (Howard 1981). These secretory vesicles are then directed to the apical plasma membrane with which they fuse.

![Hypothetical vesicle trafficking pathways in fungal hyphae](image)

*Fig. 1.4* Hypothetical vesicle trafficking pathways in fungal hyphae. (from Read and Hickey, 2001).

**[3]** **ER → Golgi → subapical plasma membrane.** Secretion also occurs from subapical regions of fungal hyphae. This is particularly important during the delivery of wall-building vesicles and cell wall lytic enzymes to new sites of branch formation (Gooday 1995). Other extracellular enzymes are possibly also secreted from subapical locations.

**[4]** **ER → Golgi → satellite Spitzenkörper → main Spitzenkörper → apical plasma membrane.** Satellite Spitzenkörper also appear to contain wall-building vesicles as is indicated by the observation that a bulge in a hypha often appears adjacent to these multicomponent structures (López-Franco et al. 1995; Fischer-Parton et al. 2000). However, it is not clear exactly where these vesicles are generated. One possibility is that at least some are derived from Golgi cisternae.
[5] *Plasma membrane* → *endocytic vesicles* → *satellite Spitzenkörper* → *main Spitzenkörper* → *apical plasma membrane*. It is possible that satellite Spitzenkörper may also contain endocytic vesicles derived from the plasma membrane below which these Spitzenkörper characteristically arise. These endocytic vesicles could play a role in recycling proteins and lipids back to the hyphal tip.

[6] *Endocytic vesicles* → *endosomes* → *main Spitzenkörper* → *apical plasma membrane*. A second pathway which may be important for recycling membrane proteins and lipids back to the growing hyphal tip is via endosomes. A third possible pathway for recycling membrane proteins and lipids may be from endosomes to the main Spitzenkörper via the Golgi.

[7] *ER* → *Golgi* → *endosomes* → *vacuoles*. Proteins (e.g. lytic enzymes) within vacuoles are ultimately derived from ER and then transported via the Golgi and endosomes in which they are respectively processed and sorted (Ashford 1998).

[8] *Retrograde vesicle trafficking*. Retrograde pathways of vesicle trafficking must occur to maintain the correct balance of membrane between different organelles and also to allow the recycling of specific molecules (Ashford 1998).

1.9 *Vesicle docking, recognition and fusion with membranes*

Specific molecules (e.g. v-SNARES and t-SNARES) on the cytosolic surface of membranes guide the targeting of vesicles, ensuring that they recognise, dock and fuse with only the correct organelle compartment, thus dictating the pattern of traffic between one compartment and another, which results in the localised delivery of specific proteins (Alberts *et al.* 1994; Edwardson 1998; Woodman 1998). Little is known about these proteins in filamentous fungi (Gupta and Heath 2000), although the mechanisms of vesicle docking, recognition and fusion have been much studied in the budding yeast (Pelham 1994).

1.10 *Importance of endocytosis for hyphal tip growth*

Endocytosis may play critical roles in hyphal tip growth and this has been discussed by Fischer-Parton *et al.* (2000) and by Read and Hickey (2001). Important functions may include:
(a) **Removal of excess plasma membrane.** Calculations have indicated that in the tips of hyphae and pollen tubes, insertion of new membrane by secretory vesicle fusion results in an excess in apical plasma membrane relative to the amount of cell wall components necessary to maintain tip extension (Picton and Steer 1983; Derksen et al. 1995; Steer 1998; Bracker, personal communication). Endocytosis could provide a mechanism to retrieve this membrane. Studies in which the time course of FM4-64 uptake into hyphae has been followed indicate that initial internalisation of the dye is concentrated in a region 2-20 μm back from the apical pole (see section 3.2.1) (Fischer-Parton et al. 2000; Hoffman and Mendgen 1998; Hickey and Read 2001). Interestingly, it has been found that filasomes, which have been suggested as candidates for endocytic vesicles (Fischer-Parton et al. 2000) are concentrated in this zone (Roberson 1992). Furthermore, clathrin-coated endocytic vesicles have also been shown to be concentrated in a similar region in pollen tubes (Derksen et al. 1995). In chemically fixed hyphae, membranous aggregates associated with the plasma membrane and termed plasmalemmasomes or lomasomes (e.g. see Beckett et al. 1974) often develop. These structures, which in most cases are artefacts generated during the slow process of chemical fixation, may represent the build up of excess membrane that failed to be retrieved normally by endocytosis in the dying hyphae.

(b) **Generation of vacuolar system.** If the assertion is correct that an excess of membrane inserted by secretory vesicles into the apical plasma membrane has to be internalized, it may be that much of this membrane plays an important role in generating vacuolar membranes of the vacuolar system, especially in the apical hyphal compartment. When the vacuolar system in the apical compartments of *N. crassa* is stained using a vacuole-selective dye such as Oregon Green 488 carboxylic acid (Cole et al. 1997), we find that tubular and small, near spherical vacuolar compartments started to appear 10-30 μm back from the apical pole of the hyphal tip. Behind this region the tubular elements got progressively longer (Read and Hickey, 2001). The much larger spherical vacuolar compartments, characteristic of subapical hyphal compartments (Fischer-Parton et al. 2000; Ashford 1998), do not appear until the first septum back from the tip.

(c) **Recycling of membrane proteins.** Endocytosis could provide a mechanism for recycling enzymes and lipids involved in tip growth (e.g. cell wall synthases, ion transporters and possibly membrane lipids involved in signalling).
maintain a concentration of these molecules in the tip region where they are active and where these molecules once recycled can be reused. Besides providing one of probably several mechanisms for polarising the distribution of important proteins and lipids in the tip region, recycling these molecules makes economic sense for a hypha rather than having to synthesise all of these molecules *de novo* which would require a much greater energy expenditure. In the budding yeast there are numerous examples of proteins with a polarised distribution concentrated at the site of growth (i.e. buds). Furthermore, two of the three chitin synthases in budding yeast are recycled via endocytosis (Chuang and Scheckman 1996; Ziman et al. 1996; Ziman et al. 1998; Holthuis et al. 1998). Endocytic recycling of membrane proteins has also been shown to occur in animal cells (e.g. in neurones [Betz and Bewick 1992]).

(d) *Transport of membrane proteins and lipids to the vacuole for degradation.* Besides endocytosis possibly supplying vacuolar membrane, many membrane proteins and lipids may also be transported to vacuoles for degradation. Fungal vacuoles are well established as the main lytic compartments in hyphae (Ashford 1998).

(e) *Uptake of molecules in fluid-phase of endocytic vesicles.* Small molecules may be taken up by endocytosis within the lumen of endocytic vesicles. This has been suggested as a possible mechanism for the uptake of certain small nutrients in budding yeast (Dulic et al. 1991).

(f) *Uptake of signalling molecules involving receptor-mediated internalisation of ligands.* Pheromones and other signalling molecules may be internalised by receptor-mediated endocytosis in the hyphal tip region. In budding yeast, for example, this is the way in which α-factor is internalised (Wendland et al. 1998) and results in yeast cells exhibiting polarized growth in the direction of the highest concentration of the pheromone (Duntze et al. 1994). There are a number of examples in different filamentous fungi where hyphae (e.g. trichogynes in *N. crassa*) exhibit positive chemotropisms towards pheromones. Endocytosis may also be important for the uptake of host signal molecules by hyphae in host-pathogen interactions.
1.11 The Endocytosis-Exocytosis Equilibrium model of hyphal morphogenesis

We have formulated the Endocytosis-Exocytosis Equilibrium (or "Triple E") model of hyphal morphogenesis. The background to this model is based on the following key attributes of fungal hyphae:

(a) They exhibit tip growth (i.e. hyphal extension is limited to a region occupying a few micrometers at the hyphal tip) and this involves polarized secretion (Heath 1990).

(b) The major cell wall synthesizing enzymes (chitin and glucan synthases) involved in hyphal tip growth are integral membrane proteins (Gooday and Gow 1990).

(c) The cell wall in the tip region of a growing hypha is plastic but becomes progressively more rigid behind the tip; changes in hyphal morphology are only possible where the cell wall is plastic (Gooday 1995; Wessels, 1990).

The Triple E model proposes that the amount of cell wall synthesizing plasma membrane encased by plastic cell wall in the hyphal tip region is regulated by the dynamic equilibrium between exocytosis and endocytosis. The predicted effects of the steady state of this equilibrium on hyphal morphogenesis are shown diagrammatically in Fig. 1.5. We further propose that the dynamic equilibrium in exocytosis and endocytosis will vary in different parts of the apical dome of a hypha because of gradients in each of these processes, and this may also influence the precise pattern of hyphal tip morphogenesis. Finally, we propose that the capacity for the endocytosis-exocytosis equilibrium to influence hyphal morphogenesis will be further modulated by the local plasticity of the encasing cell wall which again we would expect to vary in different regions of the hyphal dome.

In relation to possible gradients in exocytosis and endocytosis, it is well established that exocytosis is highly polarized in the growing hyphal tip (Harold, 1997) and various mechanisms have been proposed to explain this phenomenon (e.g. a mobile vesicle supply centre, calcium promoting localized vesicle fusion, and targeted transport along cytoskeletal elements to marked sites on the apical plasma membrane). Indeed, a range of physiological devices may be employed by hyphae to generate gradients in exocytosis (Bartnicki-Garcia 1990). Our preliminary evidence suggests that gradients in endocytosis also exist in the hyphal tip region.
with a concentration of endocytic activity just behind the main Spitzenkörper (Hickey and Read unpublished).

**Fig. 1.5** A diagrammatic summary of the Endocytosis-Exocytosis Equilibrium ('Triple E') model of hyphal morphogenesis showing the effects of three different steady state equilibria between exocytosis and endocytosis in the hyphal tip region. This simplified diagram does not take account of likely gradients in the exocytosis-endocytosis equilibrium, or how gradients in cell wall plasticity, within the hyphal tip region might influence hyphal tip morphogenesis (from Read and Hickey, 2001).
1.12 Live-cell imaging

Developments in microscopy, fluorescent probes, digital imaging and advanced computing have resulted in a dramatic increase in the non-invasive interrogation and analysis of the biology of living cells. These technologies are allowing many novel and dynamic aspects of cell organisation, biochemistry and physiology to be studied in filamentous fungi. Live-cell imaging reduces perturbation to cells and allows a wide range of treatments to be applied to them over extended periods of time. Digital image analysis allows useful information to be extracted from the images obtained (Angleson and Betz 1997, Fischer-Parton et al. 2000).

A diverse range of vital fluorescent probes are commercially available, including dyes that stain nuclei, mitochondria, vacuoles and membranes. The selectivity of dyes for different cell components varies extensively. The majority of dyes have been tested in animal cells and may function differently in other organisms. Many dyes are cytotoxic (i.e. they perturb normal cellular function and may result in cellular death). Cyotoxicity may be reduced by staining hyphae at lower concentrations, or by using dyes with less cytotoxic properties. Phototoxicity occurs when some dyes are irradiated with light, and this often involves the production of free radicals and toxic intermediates, which can cause protein and membrane damage. Phototoxicity may be reduced through lowering dye concentration, and decreasing the time cells are irradiated. Photobleaching is the “fading” phenomenon, occurring when dyes become irreversibly damaged, resulting in reduced fluorescence. Photobleaching usually occurs when dyes are excited for extended periods of time using high levels of illumination. Confocal laser scanning microscopy (CLSM) has the advantage that the cells are exposed to light for very short periods as the laser beam scans across the sample. Relatively few live-cell imaging studies on filamentous fungi using CLSM and vital fluorescent dyes have so far been published (e.g. Knight et al. 1993; Parton et al. 1997; Fischer-Parton et al. 2000).

Prasher (1992) cloned the cDNA for the green fluorescent protein (GFP) gene from the jellyfish *Aequoria victoria*. GFP is a 238 amino acid, 27kDa protein that absorbs light at maxima of 395 nm and 475 nm and emits light at a maximum of 508 nm (Prasher, 1992). The fluorescence of GFP requires ultraviolet or blue light and oxygen. Since GFP does not require cofactors or substrates for activity, *in vivo* observation of GFP expression is possible with individual cells, cell populations or in
whole organisms. Fluorescence is optimal at pH 7.2 to 8.0 (Ward, 1998). Mutant forms of GFP have been developed that have an ability to fold properly at high temperatures, increased solubility, increased fluorescence and reduced photobleaching (Crameri et al. 1996; Cubitt et al. 1995; Siemering et al. 1996). A range of new and modified fluorescent proteins, have been developed, including red, cyan and yellow (Cubitt et al. 1995).

The vast majority of studies utilizing GFP expression in fungi have been with yeast (Cormack, 1998). The first report of successful expression of GFP in filamentous fungi was Ustilago maydis (Spellig et al. 1996) closely followed by Aspergillus nidulans (Suelmann et al. 1997; Fernández-Abalos et al. 1998). Expression of GFP in filamentous fungi requires a GFP variant efficiently translated in fungi, a transformation system and a fungal promoter that satisfies the requirements of a given experimental objective. Presently, GFP expression has been reported in 16 species of filamentous fungi (Lorang et al. 2001).

The GFP protein is extremely stable in vivo and has been fused to the C or N terminus of many proteins without loss of activity, thereby permitting tagging of proteins for gene regulation analysis, protein localization or specific organelle labeling (Ward, 1998). Specific tagging of organelles with GFP localized to the nucleus, mitochondria, ER, peroxisome, vacuole and plasma membrane has been accomplished in yeast (Cormack, 1998). Nuclear-targeted GFP in A. nidulans allowed visualization of nuclear migration and mitosis, detailing for the first time the behaviour of specific nuclei at various stages in mitosis (Suelmann et al. 1997; Fernández-Abalos et al. 1998). GFP has also been targeted to the endoplasmic reticulum (ER) (Fernández-Abalos et al. 1998).
1.13 Introduction to research carried out in this thesis

Very little is known about vesicle trafficking and the dynamic organisation of organelles in living hyphae. The focus of my research has been on analysing these aspects in living hyphae using confocal microscopy and a wide range of vital fluorescent dyes and GFP probes, and to push these techniques to their limits in order to obtain high spatial and temporal resolution with minimal cellular perturbation.

The main aims of chapter 3 were to use the dye FM4-64 as a tool to follow endocytosis and vesicle trafficking in living hyphae, and as a general membrane stain to analyse the cytology of several important processes in fungal hyphae: tip growth, branch formation, septum formation, hyphal fusion and the development of reproductive structures. Staining of the apical vesicle cluster was observed in 15 different species and showed considerable variation in morphology between species. Several phenomena were observed in time-lapse studies with the confocal microscope including retraction of the Spitzenkörper, satellite Spitzenkörper and apical branching. Differential staining of colonies provided a new technique for following the fusion process of living hyphae and showed dramatic changes in cytoplasmic flow between fused hyphae.

The research in chapter 4 was carried out using the techniques developed in the previous chapter. The main aims were to image and analyse the dynamic organisation of the vacuolar network, mitochondria, nuclei, ER and Golgi in living hyphae using vital dyes and GFP probes. GFP targeted to organelles proved to be mostly more specific for organelles, less toxic and more photostable than the vital dyes used. The dynamic nature of the vacuolar network was shown in time-lapse studies and three different morphological types of vacuole compartments were demonstrated. For the first time, mitochondrial division in hyphae was imaged and mitochondrial potentiometric dyes showed a distinct gradient of fluorescence within hyphae, indicating that mitochondria are more active towards the growing hyphal tips. GFP targeted to nuclei allowed the detailed observation of mitosis and revealed waves of division within hyphae.

Confocal microscopy of the BIMG PP1 phosphatase fused to GFP showed that this enzyme is localised in spindle pole bodies, the nucleus and the plasma membrane.
of hyphal tips and septa. GFP fused to COP1 α-coatomer protein allowed imaging of the Golgi in living hyphae for the first time. Golgi often had a donut-shaped morphology, and were more concentrated towards growing hyphal tips.

In chapter 5, four different morphological mutants of *Neurospora crassa* were studied in order to gain a greater understanding into how the mutations affected hyphal growth, branching and the organisation and dynamics of organelles. Confocal microscopy proved excellent for studying the phenotypes of living mutant strains since it allows optical sectioning of samples which, due to their complex morphology, cannot be easily resolved using conventional light microscopy. A colonial temperature-sensitive mutant, cot-1, was shown to exhibit dramatic changes in hyphal morphology following a shift to the restrictive temperature. These changes were followed over time and presented as 3-D (xyz) reconstructions. The hyphae recovered surprisingly quickly following a shift down to the permissive temperature, and the recovery process was shown in 4-D (xyzt) animations. The Nkin mutant lacks conventional kinesin and was shown to have an abnormal AVC with abnormal behaviour, and the mitochondrial positioning was changed in the hyphal tips. Stained with FM4-64, the hyperbranching mutant *spray* was compared to wild type hyphae, and showed a number of differences including the location of septa and nuclei, which were much closer to hyphal tips. The pattern of FM4-64 staining by the wall-less mutant *slime* was similar to that in wild type hyphae and indicative of FM4-64 being transported by the endocytic pathway, through endosomes to the vacuole.

As a supplement to the printed document, a CD-rom has been provided which contains time-lapse movies of many of the confocal sequences presented in the thesis. The movies show details of the research that are difficult to present as still sequences, and reveal the dynamic behaviour of living fungal hyphae.
Chapter 2: Materials and Methods

2.1 Chemicals

Unless stated otherwise, chemicals were purchased from BDH Ltd. (Poole, Dorset, UK), Sigma Chemical Co. (Poole, Dorset UK) or Fluka (UK).

2.2 Fungal material

Experiments were performed using the wild type species, mutants and transformants indicated in Table 2.1.

Table 2.1 Species and strains used.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurospora crassa</td>
<td>Shear and Dodge, wild type</td>
<td>FGSC no. 2489</td>
</tr>
<tr>
<td></td>
<td>(74A) spray</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cot-1</td>
<td>FGSC no. 68</td>
</tr>
<tr>
<td></td>
<td>Nkin01</td>
<td>M. Schliwa</td>
</tr>
<tr>
<td></td>
<td>slime (fs;os;sg-)</td>
<td>FGSC no. 1118</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>(AF-10) wild type, clinical</td>
<td>University of Manchester</td>
</tr>
<tr>
<td></td>
<td>isolate</td>
<td></td>
</tr>
<tr>
<td>Aspergillus nidulans</td>
<td>wild type (R-153)</td>
<td>X. Xiang</td>
</tr>
<tr>
<td></td>
<td>GFP-nuclei</td>
<td>J. Doonan</td>
</tr>
<tr>
<td></td>
<td>GFP-ER</td>
<td>J. Doonan</td>
</tr>
<tr>
<td></td>
<td>GFP-BIMG PP1-phosphatase</td>
<td>J. Doonan</td>
</tr>
<tr>
<td></td>
<td>GFP-mitochondria</td>
<td>A. Brakhage</td>
</tr>
<tr>
<td></td>
<td>GFP-vacuole</td>
<td>K. Kitamoto</td>
</tr>
<tr>
<td></td>
<td>GFP-Golgi cisterna</td>
<td>S. Assinder</td>
</tr>
</tbody>
</table>
### Table 2.1 (continued)

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Botrytis cinerea</td>
<td></td>
<td>R. López-Franco and C. E. Bracker</td>
</tr>
<tr>
<td>Morchella esculenta</td>
<td></td>
<td>Isolated by P. C. Hickey, Edinburgh, 2000</td>
</tr>
<tr>
<td>Mycena citricolor</td>
<td></td>
<td>American Type Culture Collection (ATTC)</td>
</tr>
<tr>
<td>Phycomyces blakesleeanus</td>
<td></td>
<td>Isolated by P. C. Hickey, Edinburgh, 1999</td>
</tr>
<tr>
<td>Rhizoctonia solani</td>
<td>Kuhn</td>
<td>J.F. Tuite</td>
</tr>
<tr>
<td>Sclerotinia sclerotiorum</td>
<td></td>
<td>R. López-Franco and C. E. Bracker</td>
</tr>
<tr>
<td>Ganoderma lucidum</td>
<td></td>
<td>P. Stamets</td>
</tr>
<tr>
<td>Lentinus edodes</td>
<td>Adam</td>
<td>A. Miller</td>
</tr>
<tr>
<td>Basidiobolus ranarum</td>
<td></td>
<td>N. D. Read</td>
</tr>
</tbody>
</table>

#### 2.3 Media

Most species were maintained by regular sub-culturing on solid agar media (Oxoid Agar No. 3, Unipath Ltd., Basingstoke, Hampshire, UK). An agar strength of 2% (w/v) was used because it provides a firm gel and encourages hyphae to grow upon the surface, which is more suitable for imaging. Agar media were prepared in 1 litre flasks, autoclaved for 15 min at 121°C, allowed to cool to ~50°C and poured into 8.5 cm diameter polystyrene Petri dishes (Disposables Media Ltd. UK). Petri dishes were stored at room temperature, to prevent condensation, and used within 2 weeks. Liquid media (for slide culture methods and dilution of dyes) were prepared according to the same recipes, without agar, sterilised and divided into 10 ml vials and stored at 5°C.
2.3.1 Vogel’s sucrose minimal medium

Vogel’s medium was the standard culture medium used to grow *Neurospora crassa* wild type and mutants (Table 2.2). Liquid Vogel’s sucrose medium was prepared according to Table 2.2, but without agar.

Table 2.2 Vogel’s sucrose agar medium.

<table>
<thead>
<tr>
<th>Vogel’s sucrose minimal medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vogel’s 50x salt stock</td>
</tr>
<tr>
<td>Sucrose (Fluka)</td>
</tr>
<tr>
<td>Agar</td>
</tr>
<tr>
<td>Purified water</td>
</tr>
</tbody>
</table>

Table 2.3 Composition of Vogel’s 50x stock solution.

<table>
<thead>
<tr>
<th>Component</th>
<th>Molecular weight</th>
<th>x50 stock contains per litre dH₂O</th>
<th>Final concentration in standard culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₃ Citrate-2(H₂O)</td>
<td>294.1</td>
<td>126.7 g</td>
<td>8.62 mM</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>136.1</td>
<td>250.0 g</td>
<td>36.74 mM</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>80.04</td>
<td>100.0 g</td>
<td>24.98 mM</td>
</tr>
<tr>
<td>MgSO₄-7(H₂O)</td>
<td>246.5</td>
<td>10.0 g</td>
<td>0.81 mM</td>
</tr>
<tr>
<td>CaCl₂-2(H₂O)</td>
<td>147.0</td>
<td>5 g</td>
<td>0.68 mM</td>
</tr>
<tr>
<td>*Trace elements solution</td>
<td>-</td>
<td>5 ml stock</td>
<td>-</td>
</tr>
<tr>
<td>**Biotin solution</td>
<td>-</td>
<td>5 ml stock</td>
<td>-</td>
</tr>
<tr>
<td>Chloroform</td>
<td>-</td>
<td>2-3 ml</td>
<td>-</td>
</tr>
</tbody>
</table>

* see Table 2.4 ** see Table 2.5

Table 2.4 Trace element stock solution

<table>
<thead>
<tr>
<th>Trace elements solution (per litre dH₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid-1(H₂O)</td>
</tr>
<tr>
<td>ZnSO₄-7(H₂O)</td>
</tr>
<tr>
<td>Fe(NH₄)₂)(SO₄)-7(H₂O)</td>
</tr>
<tr>
<td>CuSO₄-5(H₂O)</td>
</tr>
<tr>
<td>MnSO₄-1(H₂O)</td>
</tr>
<tr>
<td>H₃BO₄</td>
</tr>
<tr>
<td>Na₂(MoO₄)-2(H₂O)</td>
</tr>
</tbody>
</table>
Table 2.5 Biotin stock solution.

<table>
<thead>
<tr>
<th>Biotin solution (per litre dH₂O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-biotin</td>
</tr>
<tr>
<td>5 g</td>
</tr>
</tbody>
</table>

2.3.2 Osmotically balanced Vogel’s medium

*Neurospora* “slime” mutant was grown on an osmotically balanced Vogel’s medium (Table 2.6) containing 2% (w/v) mannitol and 1.5% (w/v) agar (Emerson 1963).

Table 2.6 Osmotically balanced Vogel’s medium

<table>
<thead>
<tr>
<th>Osmotically balanced Vogel’s medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vogels 50x salt stock*</td>
</tr>
<tr>
<td>20 ml</td>
</tr>
<tr>
<td>Mannitol (Sigma)</td>
</tr>
<tr>
<td>20 g</td>
</tr>
<tr>
<td>Agar</td>
</tr>
<tr>
<td>15 g</td>
</tr>
<tr>
<td>Purified water</td>
</tr>
<tr>
<td>1.0 litre</td>
</tr>
</tbody>
</table>

* Table 2.3

2.3.3 Complete medium

Malt extract agar (MEA) was used for culturing *Aspergillus spp.* and most other species (Table 2.7). *Aspergillus spp.* grown on malt extract generally had wider, faster growing hyphae and exhibited good sporulation. Liquid malt extract medium was prepared according to Table 2.7, but without agar. Potato dextrose agar was used as an alternative medium (Table 2.8).

Table 2.7 Malt extract agar.

<table>
<thead>
<tr>
<th>Malt extract agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malt Extract (Fluka)</td>
</tr>
<tr>
<td>20 g</td>
</tr>
<tr>
<td>Agar</td>
</tr>
<tr>
<td>20 g</td>
</tr>
<tr>
<td>Purified water</td>
</tr>
<tr>
<td>1.0 litre</td>
</tr>
</tbody>
</table>
Table 2.8 Potato dextrose agar.

<table>
<thead>
<tr>
<th>Potato dextrose agar (PDA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato Dextrose Broth</td>
</tr>
<tr>
<td>Agar</td>
</tr>
<tr>
<td>Purified water</td>
</tr>
</tbody>
</table>

2.3.4 Hyphal fusion medium

A modified form of Vogel’s medium was developed for hyphal fusion experiments (Table 2.9). The weak (10%) carbon/nutrient source resulted in thinner hyphae, and permitted better imaging conditions in the peripheral regions of the colony. The stronger (3% w/v) agar encouraged more mycelium to grow on the surface of the agar.

Table 2.9 Fusion medium.

<table>
<thead>
<tr>
<th>Fusion medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vogels 50x salt stock</td>
</tr>
<tr>
<td>Glucose</td>
</tr>
<tr>
<td>Agar</td>
</tr>
<tr>
<td>Purified water</td>
</tr>
</tbody>
</table>

Table 2.10 Ethanol induction medium.

<table>
<thead>
<tr>
<th>Ethanol induction medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vogels 50x salt stock</td>
</tr>
<tr>
<td>Ethanol</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
</tr>
<tr>
<td>Agar</td>
</tr>
<tr>
<td>Purified water</td>
</tr>
</tbody>
</table>
2.3.5 Ethanol induction medium

Media with ethanol as the sole carbon source (Table 2.10) was used to induce GFP expression in all strains of *A. nidulans* except for the constitutive GFP-mitochondria and GFP-BIMG strains (Table 2.1). Cultures were grown for 48 h, and hyphae were excised from the colony periphery and incubated in liquid media for 3 h prior to imaging. The 3 h recovery period was to ensure hyphae were growing uniformly and as healthily as possible. Hyphae were slightly thinner and slower growing than cultures grown on complete medium (Section 2.3.3).

2.4 Culture conditions

Cultures of *A. nidulans* and *A. fumigatus* were grown at 30°C in darkness. *Neurospora crassa*, mutants and all other species were grown at 25°C in darkness, unless otherwise stated.

2.5 Storage of fungal material

*Neurospora* and *Aspergillus* strains were cultured on agar slants, consisting of universal glass tubes filled with 10 cc agar media, allowed to set at a ~45° angle, and incubated with caps loosely fastened to allow adequate gas exchange. *Neurospora* cultures were incubated for 5-7 days at 25°C, and *Aspergillus* cultures were incubated for 5-7 days at 30°C, until significant sporulation had occurred. Spores were used to inoculate Petri dishes and slants, and stored at 4°C for up to 4 weeks, after which spores were transferred to fresh agar slants. Additionally, agar blocks were cut from sporulating cultures placed in Eppendorf vials, sealed and stored at 4°C. Mycelial cultures were transferred to fresh Petri dishes every 4 weeks, and backup agar slants were stored for up to 6 months at 4°C. The *slime* mutant was cultured on Vogel’s osmotically balanced medium (Section 2.3.2) and sub-cultured every 2 weeks.

2.5.1 Silica gel for long-term storage of spores

For long-term storage, *Neurospora* and *Aspergillus* spores were preserved on silica gel (Davis and de Serres, 1970; Smith and Onions, 1983). Silica gel (1-3 mm particle size) was sterilized at 180°C for 1.5 h and after cooling used to half fill (about 10 g silica gel per vial) sterile 20 ml universal glass vials sealed with polypropylene screw caps. The vials were temporarily closed and put on ice. 2 ml of sterile 7% w/v non-fat dried milk (Marvel, UK) was added, and stirred with a glass
rod to form a suspension of spores. 0.5 ml of spore suspension was added to each vial containing silica gel, closed and shaken vigorously until the spores appeared to be evenly distributed. Vials were quickly put back on ice to dissipate the heat released by wetting the silica gel. Vials were kept sealed at room temperature and cultures were recovered by shaking out a few crystals into fresh agar slant tubes.

2.5.2 Cryopreservation
Agar blocks (~5 mm³ in size) were cut from the leading edge of the colony and dropped into sterile 1 ml polypropylene Eppendorf vials containing 10% w/v glycerol and 90% w/v distilled water. The vials were then sealed and placed in the −70°C freezer. Cultures were recovered by thawing the vials at room temperature, and mycelial blocks were placed upon fresh agar plates. Most cultures recovered and resumed normal growth within 3 days.

2.6 Preparation of specimens for microscopy
When the mycelium had grown 3-5 cm across the Petri plate, peripheral portions of the colony, containing fast growing hyphae, were excised and transferred for imaging. During manipulation and preparation of samples, hyphal tips often stopped growing, but resumed growth within 2-10 min with the formation of one or two new tips. According to Robertson (1958), apical branching of hyphae is typical after treatments that arrest hyphal growth. Usually 10 min of recovery time was allowed before imaging. The 3 major methods for imaging cells are described in sections 2.6.1 - 2.6.3.

2.6.1 Sandwich culture
The agar medium was overlaid with cellophane (525 gauge, uncoated rayophane from A.A. Packaging, Walmer Bridge, Lancashire, UK) and cultures grown at 25°C in the dark. Sandwich slide chambers for microscopic examination were prepared using a similar method to that described by López Franco (1992) and López-Franco and Bracker (1996) (Fig. 2.1). Strips of lithographer's tape 2 mm wide (No. 616, Scotch Brand 3M) were placed on either side of the cover slip to support an overlying 18 x 18mm cover slip. Cover slips were pre-washed in ethanol and distilled water, then coated with poly-L-lysine (Sigma, UK) to aid cell adherence. Liquid Vogel's sucrose medium (Section 2.3.1) was oxygenated prior to experiments by bubbling oxygen through the medium through a Pasteur pipette and shaking by
vortexing for 3 min. A piece of mycelium covered cellophane was cut from the edge of the colony and transferred to 35 μl of 2% sucrose Vogel's medium containing dye, located on the lower cover slip. The mycelium was then floated off the cellophane onto the liquid medium containing stain and the second cover slip was carefully mounted on the supporting strips of tape to cover the mycelium. Silicon grease was applied to keep the cover slip in place. The advantages and disadvantages of using sandwich cultures are summarised in Table 2.11.

Table 2.11 Advantages and disadvantages of sandwich cultures

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Dyes can be washed in from the side and washed out to some extent.</td>
<td>• Hyphae are often damaged during preparation</td>
</tr>
<tr>
<td>• Good optics: hyphae are close to objective</td>
<td>• Poor gas exchange</td>
</tr>
<tr>
<td></td>
<td>• Dries out quickly</td>
</tr>
</tbody>
</table>

Fig. 2.1 Sandwich culture (see section 2.6.1)
2.6.2 Open Culture

Glass cover slips were coated with a very thin layer of Vogel's sucrose medium (Section 2.3.1) made with agarose, to improve the optics (Fig. 2.2). A thin layer is of molten agar spread upon a heated cover slip, using a pipette tip. A 4 x 6 mm wedge of inoculum was transferred to slide chambers, and incubated in a humid chamber for 4 h to recover and grow across the slide. The advantages and disadvantages of using open cultures are summarised in Table 2.12.

Table 2.12 Advantages and disadvantages of open cultures

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Easy to apply dyes externally</td>
<td>Aerial hyphae are prominent</td>
</tr>
<tr>
<td>Good gas exchange</td>
<td>Prone to drying out quickly</td>
</tr>
<tr>
<td></td>
<td>Difficult to prepare perfectly uniform and thin layer of agar</td>
</tr>
<tr>
<td></td>
<td>Distance of hyphae from objective means reduced</td>
</tr>
<tr>
<td></td>
<td>image quality/high background</td>
</tr>
</tbody>
</table>

Fig. 2.2 Open culture (see section 2.6.2)
2.6.3 Inverted agar block

A ~20mm², 5mm thick block of agar was cut from the edge of a colony grown in a Petri dish, and inverted onto a droplet of liquid media (containing dye) upon a glass cover slip (Fig. 2.3; Movie 2.1). Depending on the organism, if hyphae deeply penetrated the agar, a higher agar concentration (3% w/v) was used. A humid Petri dish chamber should be placed above the slide for imaging over extended periods. To perfuse dyes over the sample, the agar block was sometimes propped up on one side using a cover slip, allowing the growing tips more space to grow.

Table 2.13 Advantages and disadvantages of inverted agar block cultures

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good gas exchange due to porous nature of agar</td>
<td>Dyes may leach into or bind to agar</td>
</tr>
<tr>
<td>Agar and liquid media keeps the culture moist and bathed with nutrients</td>
<td>Difficult to wash or re-stain after imaging</td>
</tr>
<tr>
<td>Cultures can be observed for extended periods of time (3-6 h)</td>
<td>Agar block needs to be kept moist</td>
</tr>
<tr>
<td>Hyphae are forced to grow directly between the agar and glass, thus giving the best optics.</td>
<td>Reduced quality of transmitted bright field light images</td>
</tr>
</tbody>
</table>

Fig. 2.3 Inverted agar block culture (see section 2.6.3)
2.6.4 Loading of cell-permeant dyes

The majority of imaging experiments utilised the inverted agar block method with the application of dye in the liquid medium. The different dyes used are summarised in Tables 2.14 and 2.15. To test for fluid-phase endocytosis, agarose medium containing 40 μM calcium green 10 kDa dextran dye (Molecular Probes Inc, Eugene, OR, USA) was applied to slides and cultures were incubated for 4 h before analysis. Most of the dyes used have limited solubility in aqueous solutions and have to be dissolved in organic solvents before they can be diluted into the medium. In all cases the concentration of solvents (DMSO and EtOH) was kept below 0.2% (w/v) in the final loading medium.

Table 2.14 Summary of fluorescent dyes used (data from Molecular Probes Inc).

<table>
<thead>
<tr>
<th>Dye</th>
<th>Selectivity</th>
<th>Conc. (μM)</th>
<th>Ex (nm)</th>
<th>Em (nm)</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oregon Green</td>
<td>Vacuoles</td>
<td>50</td>
<td>488</td>
<td>517</td>
<td>496</td>
</tr>
<tr>
<td>FM1-43</td>
<td>Membranes</td>
<td>25</td>
<td>514</td>
<td>626</td>
<td>512</td>
</tr>
<tr>
<td>FM4-64</td>
<td>Membranes</td>
<td>25</td>
<td>514</td>
<td>734</td>
<td>608</td>
</tr>
<tr>
<td>Mitotracker Green</td>
<td>Mitochondria</td>
<td>10</td>
<td>488</td>
<td>516</td>
<td>672</td>
</tr>
<tr>
<td>Rhodamine-123</td>
<td>Mitochondria</td>
<td>10-65</td>
<td>514</td>
<td>529</td>
<td>381</td>
</tr>
<tr>
<td>Dihydrorhodamine-123</td>
<td>Mitochondria</td>
<td>10-65</td>
<td>514</td>
<td>529</td>
<td>346</td>
</tr>
<tr>
<td>DASPMI</td>
<td>Mitochondria</td>
<td>10-100</td>
<td>488</td>
<td>585</td>
<td>366</td>
</tr>
<tr>
<td>Nile red</td>
<td>Lipid</td>
<td>10</td>
<td>514</td>
<td>636</td>
<td>318</td>
</tr>
</tbody>
</table>

Abbreviations: Conc., concentration; Ex, excitation maximum; Em, emission maximum; MW, molecular weight

Table 2.15 Syto dyes for DNA and RNA that were used (data from molecular probes Inc).

<table>
<thead>
<tr>
<th>Dye</th>
<th>Conc. (μM)</th>
<th>Abs (nm) DNA</th>
<th>Abs (nm) RNA</th>
<th>Em (nm) DNA</th>
<th>Em (nm) RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Syto11</td>
<td>5.0</td>
<td>508</td>
<td>510</td>
<td>527</td>
<td>530</td>
</tr>
<tr>
<td>Syto13</td>
<td>5.0</td>
<td>488</td>
<td>491</td>
<td>509</td>
<td>514</td>
</tr>
</tbody>
</table>

Abbreviations: Conc., concentration; Ex, excitation maximum; Em, emission maximum
2.6.5 Differential staining of adjacent mycelia

A new technique was developed for observing hyphal fusions between stained and unstained hyphae. 5 mm x 20 mm agar blocks were excised from the leading edge of a *Neurospora* colony. The mycelium on the first agar block was stained with a droplet of liquid medium, containing the dye FM1-43, then placed mycelium-side-down on a fresh agar plate. A droplet of liquid medium was applied to the unstained agar block and it was placed opposite the stained agar block, with a gap of 5-10 mm between the two blocks. Petri dishes were incubated for 2-4 h, or until mycelium had grown across the gap. A new agar block was then excised from the area between the 2 blocks. Where mycelia had grown together they were observed for hyphal fusions with the light microscope.

![Diagram](image)

**Fig. 2.4** Differential staining procedure.
2.7 Confocal microscopy

Cells were imaged using confocal laser scanning microscopy (CLSM), because the images have significantly better resolution and contrast than those obtained with a conventional light microscope. The confocal microscope uses an aperture (pinhole) in front of the light detector that rejects out-of-focus blur and allows optical sectioning (Czymmek et al. 1994). Confocal microscopy was performed using a Bio-Rad MRC600 system equipped with a 25 mW Argon ion laser and mounted on a Nikon Diaphot TMD inverted microscope with epifluorescence equipment (all supplied by Bio-Rad Microscience, Hemel Hempstead, UK). The laser power used was 1% or 3% of full intensity.

Image capture and storage was on a Dell Poweredge 2300 computer (Pentium 3 500MHz; 256mb RAM; 18 Gb hard disc) containing a Series 5 framestore, running COMOS (version 7; supplied by Bio-Rad). The laser beam could be regulated via neutral density filters to 1%, 3% or 10% of the full intensity. The MRC600 was equipped with two photomultipliers, PMT1 (Channel 1) and PMT2 (Channel 2) for fluorescence imaging. Alternatively, Channel 2 could be used to collect a transmitted light, bright field image via an optical fibre, which collects light behind the microscope condenser and can be inserted directly to PMT2. Table 2.16 gives an overview of the filter sets used (all supplied by Bio-Rad).

Table 2.16 Filter sets used for confocal microscopy.

<table>
<thead>
<tr>
<th>Filter set</th>
<th>Excitation filter</th>
<th>Dichroic mirror</th>
<th>Emission filter</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>standard BHS</td>
<td>488/10 nm BF</td>
<td>510 nm LP</td>
<td>515 nm LP</td>
<td>Channel 1</td>
</tr>
<tr>
<td>standard GHS</td>
<td>514/10 nm BF</td>
<td>540 nm LP</td>
<td>550 nm LP</td>
<td>Channel 1</td>
</tr>
</tbody>
</table>

Abbreviations: BHS = blue excitation filter set, GHS = green excitation filter set, BF = barrier filter, LP = longpass filter.

FM4-64 and Rhodamine-123 were excited with the 514 nm laser line and their fluorescence was detected using the GHS filter block. DASPMI, SYTO and GFP were excited with the 488 nm laser line using the BHS filter block. Either oil immersion 60x (N.A. 1.4) or dry 40x (N.A. 0.95) plan apochromatic objective lenses were used for imaging. The resolution of objectives, under standard imaging conditions used here is shown in Table 2.17 (Fischer-Parton 1999). Resolution was
calculated in terms of the FWHM (full width half maximum; Cogswell and Larkin, 1995) using green fluorescent 63 μm diameter microspheres (Bangs Laboratories Inc., Carmel, USA) and 514 nm excitation laser line.

Table 2.17 Estimated Resolution of objectives.

<table>
<thead>
<tr>
<th>FWHM</th>
<th>x40 dry plan apo</th>
<th>x60 oil plan apo</th>
</tr>
</thead>
<tbody>
<tr>
<td>lateral (xy)</td>
<td>0.6 μm</td>
<td>0.4 μm</td>
</tr>
<tr>
<td>axial (xz)</td>
<td>1.6 μm</td>
<td>0.8 μm</td>
</tr>
</tbody>
</table>

2.7.1 Confocal settings for fluorescence imaging

Settings for CLSM are a compromise between image quality and a need to minimise stress to cells (Pawley, 1995; Parton and Read, 1999). Table 2.18 lists the conditions employed throughout the study. Cell health was assessed in terms of growth, cytoplasmic streaming and appearance under the microscope. The Bio-Rad MRC 600 offered the possibility of improving the temporal resolution by reducing the box size in vertical orientation. This leads to a decrease in the number of lines necessary to scan one frame. When possible, the hyphae were orientated horizontally relative to the frame width and could be imaged in a half to a quarter of the time used for scanning a full frame. This made averaging of frames more feasible for growing hyphae. The time taken to collect 1 full size image was approximately 3 s with the setting F1. By decreasing the box size to a quarter, one could theoretically obtain an image in just over 1 s. Because of problems with dye photobleaching and phototoxic effects, laser scanning of individual hyphae was kept to a minimum. Generally this initially required ~ 3 s of rapid scanning (using the F4 scan setting which takes 0.25 s to scan a full image frame) to obtain a median section through the cell, and then 1-2 slow scans using the F1 setting (3 s per full image to produce the final image. Kalman filtering (n = 2) was sometimes used to improve the signal-to-noise-ratio (Parton et al. 1997).
Table 2.18 Settings used for confocal microscopy.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Settings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laser Power</td>
<td>1-3% mW Argon ion laser</td>
</tr>
<tr>
<td>Scan speed</td>
<td>F1 = 3 sec per 512 lines</td>
</tr>
<tr>
<td></td>
<td>F2 = 1 sec per 512 lines</td>
</tr>
<tr>
<td>Low signal enhance</td>
<td>Available, but not employed</td>
</tr>
<tr>
<td>PMT gain settings</td>
<td>Dyes 40-70%; GFP 50-80%</td>
</tr>
<tr>
<td>PMT black level settings</td>
<td>To yield dark signal intensity of</td>
</tr>
<tr>
<td></td>
<td>about 10</td>
</tr>
<tr>
<td>Frame averaging</td>
<td>Up to 2 scans Kalman filtering</td>
</tr>
<tr>
<td>Objectives</td>
<td>X10 dry (NA 0.45)</td>
</tr>
<tr>
<td></td>
<td>X20 dry (NA 0.75)</td>
</tr>
<tr>
<td></td>
<td>X40 dry (NA 0.95)</td>
</tr>
<tr>
<td></td>
<td>X60 oil (NA 1.4)</td>
</tr>
<tr>
<td>Confocal apertures</td>
<td>4-5 = ca. 30% open</td>
</tr>
<tr>
<td>Electronic zoom</td>
<td>1-4</td>
</tr>
</tbody>
</table>

2.7.2 Image processing, 3-D rendering and animation

Files were saved directly from the confocal as Bio-Rad .pic format and 2 copies were recorded onto CD-ROMs (1 working copy, 1 archive). The Bio-Rad .pic files contain extra data with important information: time and date, objective used, zoom factor and distance between z-sections.

Images were viewed using the freeware software, Confocal Assistant (version 4.02). 3-D projections and animations were made using Confocal Assistant and exported as Microsoft .avi files. Still images may be cut and pasted directly into standard graphics software (e.g. Paintshop Pro. or Adobe Photoshop) for further processing. Time-lapse .pic sequences must be re-sampled as Microsoft .avi format in order to be recognised by Adobe Premiere as animations.
2.7.3 Animation

Files were imported as separate .avi clips into Adobe Premiere. Clips were then dragged into the timeline monitor window, and set to “maintain aspect ratio”. The default speed of the re-sampled .avi files exported from Confocal Assistant is 4 frames per second (fps). In Adobe Premiere, the imported .avi files may be speeded up to 250% to play at 10 fps, and in proportion, to any other speed up to the limit of 25 fps, the maximum PAL format frame rate. A time sequence was collected at one frame every 5 s, converted to an .avi file (4 fps), then speeded up to 250% (10 fps), giving a final speed which is actually 50x of the original. For slower growing hyphae and low-magnification sequences, animations were speeded up to 5x or more. This faster frame rate produces much smoother animation at 20-25 fps. Contrast may be adjusted by selecting the brightness/contrast filter in Premiere, and animations may also be played backwards or modified using horizontal flip / vertical flip, rotate or zoom functions. Titles and scale-bars were added by superimposing them onto video sequences. Files were exported in a number of different formats: Quicktime, Windows AVI, MPEG-1 and animated GIF. For presentations in Microsoft Powerpoint, movies were imported in .avi or MPEG-1 format. Compression was often necessary, otherwise “stuttering” playback occurred due to the limited data transfer rate (i.e. the time taken to transfer data from hard disk to the video display hardware. The compression codecs used to create animations are listed in Table 2.19.

Table 2.19 Compression codecs used for animations.

<table>
<thead>
<tr>
<th>Codec</th>
<th>Video format</th>
</tr>
</thead>
<tbody>
<tr>
<td>BBmpeg plug-in for Premiere</td>
<td>MPEG-1, MPEG-2</td>
</tr>
<tr>
<td>cinepak (Radius)</td>
<td>.avi / Quicktime</td>
</tr>
<tr>
<td>Indeo 5.02 (Intel)</td>
<td>.avi</td>
</tr>
<tr>
<td>Sorensen 2.0</td>
<td>Quicktime</td>
</tr>
</tbody>
</table>
Chapter 3: Analysis of the vesicle trafficking network

3.1 Introduction

Vesicle trafficking is fundamental to many activities in filamentous fungi, and underlies many of the basic processes involved in their growth and differentiation (see section 1.2.6). Most studies on vesicle trafficking in fungi have been based on the budding yeast *Saccharomyces cerevisiae* (Vida and Emr, 1995) (see section 1.6) and we know relatively little about vesicle trafficking in filamentous fungi. Two different styryl dyes (FM4-64, FM1-43) are commonly employed as indicators of endocytosis (Vida and Emr 1995; Betz et al. 1996; see section 1.8). The overall objective of the work presented in this chapter was to use these dyes as experimental tools to analyse vesicle trafficking and the cytology of living filamentous fungi. More specifically the aims were to:

- Provide time courses of staining in both apical and sub-apical regions of hyphae, and compare the patterns of dye internalisation.
- Show that dye internalisation is energy-dependent using the metabolic inhibitor sodium azide. This would be consistent with dye internalization being via endocytosis and would rule out the possibility of the dyes diffusing into cells.
- Analyze the organization and behaviour of the apical vesicle cluster (AVC) in a wide range of phylogenetically diverse species.
- Using the dyes as general membrane stains, to provide a detailed cytological analysis of the following aspects of fungal biology: hyphal tip growth, branch formation, septum formation, hyphal fusion, development of conidiophores, protoperithecia and clamp connections.

Some of the results described in this chapter have been published in Fischer-Parton *et al.* (2000) and Read and Hickey (2001).
3.2 Results

3.2.1 Uptake of FM4-64 by fungal hyphae

Initial staining of the plasma membrane was followed by staining in the cytoplasm (Fig. 3.1a; Movie 3.1), then successive staining of putative endosomes and other membranous organelles (Fig. 3.1b), the AVC (Fig. 3.1c), vacuolar membranes (Figs. 3.2 and 4.4b) and mitochondria (Fig 3.4). The time course of staining of Neurospora crassa hyphae is summarised in Table 3.1. This time-dependent staining of different organelles is consistent with dye internalization by endocytosis and subsequent staining of different organelles by the distribution of dye via the vesicle trafficking network (see section 1.8). A similar time-dependent staining pattern was observed in subapical regions of N. crassa hyphae (Fig. 3.2; Movie 3.2) and the membranes of large spherical vacuoles became clearly stained within 10 min (also see section 4.2.1). The time-dependent FM4-64 staining shown in N. crassa was also found in other fungi (e.g. Sclerotinia sclerotiorum; Fig. 3.3; Movie 3.3).

Fig. 3.1 Neurospora crassa stained with FM4-64. (a) Pronounced staining of the plasma membrane (30 sec after adding dye). (b) Staining of putative endosomes (1 min after adding dye). (c) Staining of the main Spitzenkörper (30 min after adding dye). Published in Read and Hickey (2001). Bar = 10 μm.

Table 3.1 Timing of staining following application of FM4-64 to Neurospora crassa hyphae. Published in Read and Hickey (2001).

<table>
<thead>
<tr>
<th>Time after adding FM4-64</th>
<th>Cell component stained</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1 sec</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>&lt; 10 sec</td>
<td>Putative endocytic vesicles</td>
</tr>
<tr>
<td>30 sec</td>
<td>Putative endosomes</td>
</tr>
<tr>
<td>1.5-3 min</td>
<td>Apical vesicle cluster</td>
</tr>
<tr>
<td>10 min</td>
<td>Subapical spherical vacuoles</td>
</tr>
<tr>
<td>15 min</td>
<td>Putative tubular vacuoles</td>
</tr>
<tr>
<td>~ 40 min</td>
<td>Mitochondria</td>
</tr>
</tbody>
</table>
Fig. 3.2 *Neurospora crassa*. Sub-apical region of hypha. Time-course following addition of FM4-64. Note the strong staining of the plasma membrane and appearance of bright spots (putative endosomes) after 2 min. After 10 min membranes around the large spherical vacuoles became clearly stained. Bar = 10 μm.
Fig. 3.3 Sclerotinia sclerotiorum stained with FM4-64. Time-course following addition of FM4-64. Bar = 10 μm.

3.2.2 Staining of the apical vesicle cluster

FM4-64 strongly stained the AVC within the main Spitzenkörper of growing hyphal tips (Fig. 3.1c, Fig 3.3a,b). Comparison of the properties of FM1-43 and FM4-64 (Fig. 3.4; Table 3.1) showed that FM4-64 is best suited for this purpose (Fischer-Parton et al. 2000). Time-lapse sequences highlight the variable staining pattern achieved with FM4-64 (Movies 3.4, 3.5) and FM1-43 (Movies 3.6, 3.7).
Fig 3.4. *Neurospora crassa* hyphae stained with (a) FM4-64 and (b) FM1-43. Images taken at 30 s intervals. The growth rates were: 12.0 μm min⁻¹ (a), 15.3 μm min⁻¹ (b). Note the AVC stained with FM1-43 photobleaches and stains up tubular mitochondria more noticeably. Bar = 10 μm. Published in Fischer-Parton et al. (2000).

Table 3.2 Comparison of the fluorescent dyes FM1-43 and FM4-64. Published in Read and Hickey (2001).

<table>
<thead>
<tr>
<th>Property</th>
<th>FM4-64</th>
<th>FM1-43</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Excitation wavelengths</em></td>
<td>488 or 514 nm</td>
<td>488 or 514 nm</td>
</tr>
<tr>
<td><em>Emission wavelengths</em></td>
<td>&gt; 550 nm</td>
<td>&gt; 550 nm</td>
</tr>
<tr>
<td><em>Staining of Spitzenkörper</em></td>
<td>Very good</td>
<td>Poor</td>
</tr>
<tr>
<td><em>Staining of mitochondria</em></td>
<td>Slowly</td>
<td>Quickly</td>
</tr>
<tr>
<td>(typically &gt; 1 h)</td>
<td>(typically &lt; 30 min)</td>
<td></td>
</tr>
<tr>
<td><em>Photobleaching</em></td>
<td>Reasonably resistant</td>
<td>Reasonably resistant except Spitzenkörper which photobleaches very rapidly</td>
</tr>
<tr>
<td><em>Phototoxicity</em></td>
<td>Not a major problem</td>
<td>Not a major problem</td>
</tr>
</tbody>
</table>
3.2.3 Inhibition of FM4-64 uptake by sodium azide

Dye internalisation was found to be an active process because it is energy-dependent. Loading hyphae with FM4-64 in the presence of the metabolic inhibitor sodium azide or at 4 °C (Fischer Parton et al. 2000) only resulted in staining of the plasma membrane whilst inhibiting the uptake of the dye into the cytoplasm and staining of organelles. These findings are consistent with dye internalization being by endocytosis and rules out the possibility that the dye could be taken up by passive diffusion.

Fig. 3.5 Aspergillus nidulans. Hyphal tip stained with FM4-64 for 30 min in the presence of 10mM sodium azide. Note staining of the plasma membrane, but lack of dye internalisation within the cytoplasm. Bar = 5 μm.

Fig. 3.6 Neurospora crassa hyphae stained with FM4-64 for 30 min in the presence of 10 mM sodium azide. (a) Hyphal tip (b) subapical region showing septum. Note staining of the plasma membrane, but lack of dye internalisation within the cytoplasm. Bar = 10 μm. Published in Fischer-Parton et al. (2000)
3.2.4 Oregon Green 10 kDa dextran is not taken up by fluid-phase endocytosis

In order to seek further evidence for the occurrence of endocytosis in *N. crassa*, hyphae were treated with the membrane-impermeant, fluid-phase endocytosis marker Oregon Green dextran. This involved growing hyphae on agarose containing 40 μM Oregon Green 488 conjugated to a 10 KDa molecular weight dextran. After 4 h, the cells remained negatively stained, suggesting that molecules of this size are not taken up by fluid-phase endocytosis (Fig. 3.7).

![Image](image_url)

**Fig. 3.7** *Neurospora crassa* grown for 4 h in the presence of 40 μM Oregon Green 488 10 kDa dextran conjugated dye. (a) Transmitted light image of hyphal tip; (b) confocal image of same hyphal tip as in (a); (c) transmitted light image of subapical hyphal region; (d) confocal image of the same subapical region as in (c). Bar = 10 μm.
3.2.5 Hyphal tip organisation in different species: the apical vesicle cluster stained with FM4-64

Different species stained with FM4-64 were observed and time courses of hyphal growth revealed the movement and dynamic behaviour of the stained AVC within the Spitzenkörper (Fischer-Parton et al. 2000). Table 3.3 lists the different species observed. High-resolution confocal images of hyphal tips stained with FM4-64 were captured in four ascomycetes (Fig. 3.8), five basidiomycetes (Fig. 3.9), four deuteromycetes (Fig. 3.10), and two zygomycetes (Fig. 3.11). Time-lapse sequences showed the diversity of hyphal tip organization between several different species: *Aspergillus fumigatus* (Movies 3.8, 3.9); *Basidiobolus ranarum* (Movie 3.10); *Sclerotinia sclerotiorum* (Movies 3.11, 3.12); and *Phycomyces blakesleeanus* (Movie 3.13). The AVC in ascomycete hyphae was generally donut-shaped, often possessing a less-stained core region, especially in those species with wide hyphae (e.g. *Neurospora crassa*) (Figs. 3.8, 3.12). Long strand-like structures were sometimes observed in hyphal tips (Figs. 3.8c, 3.10c). These possibly represented lines of vesicles moving along microtubules. Hyphae of basidiomycetes were generally smaller than those of ascomycetes with more compact AVCs (Fig. 3.9). However, the large hyphae of *Rhizoctonia solani* possessed a core region (Fig. 3.9e).

FM4-64 stained aggregations of apical vesicles were observed in hyphal tips of zygomycetes (Fig. 3.11). However, the staining pattern in these fungi was unlike that of the AVCs observed in higher fungi. In *Phycomyces*, stained vesicular structures were diffusely packed within the apical dome (Fig. 3.11a), whereas in *Basidiobolus*, the stained vesicles were organised in a spherical aggregation (3.11b) resembling the AVC within the Spitzenkörper of higher fungi. Hyphae of *Basidiobolus ranarum* are extremely large in diameter (~20 μM). Staining of *Basidiobolus ranarum* hyphae was relatively dim compared to other species. Increased dye concentration (50 μM) and a laser power of 3% were required for reasonable quality confocal images of the AVC to be obtained. Hyphae appeared to continue growing normally, despite the increased dye concentration and laser power that they were exposed to. Vacuolar membranes also became stained with FM4-64 and the single giant nucleus remained unstained.
Table 3.3 Different species stained with FM4-64.

<table>
<thead>
<tr>
<th>Ascomycotina</th>
<th>Basidiomycotina</th>
<th>Deuteromycotina</th>
<th>Zygomycotina</th>
</tr>
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<tbody>
<tr>
<td><em>Morchella esculenta</em></td>
<td><em>Coprinus cinereus</em></td>
<td><em>Aspergillus nidulans</em></td>
<td><em>Basidiobolus ranarum</em></td>
</tr>
<tr>
<td><em>Neurospora crassa</em></td>
<td><em>Ganoderma lucidum</em></td>
<td><em>Aspergillus fumigatus</em></td>
<td><em>Phycomyces blakesleeanus</em></td>
</tr>
<tr>
<td><em>Sclerotinia sclerotiorum</em></td>
<td><em>Lentinulus edodes</em></td>
<td><em>Botrytis cinerea</em></td>
<td></td>
</tr>
<tr>
<td><em>Sordaria macrospora</em></td>
<td><em>Mycena citricolor</em></td>
<td><em>Penicillium claviforme</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Rhizoctonia solani</em></td>
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Fig. 3.8 Ascomycete hyphae stained with FM4-64. (a) *Morchella esculenta*; (b) *Neurospora crassa*; (c) *Sclerotinia sclerotiorum*. Note the strand-like structures (arrows). (d) *Sordaria macrospora*. Note the presence of a less-stained region of the AVC, which is a differentiated “core” region within the AVC of each species. Bar = 10 μm.
Fig. 3.9 Basidiomycete hyphae stained with FM4-64. (a) Coprinus cinereus; (b) Ganoderma lucidum; (c) Lentinulus edodes; (d) Mycena citricolor; (e) Rhizoctonia solani. Note the presence of a differentiated “core” region in the AVC in (e). Bar = 10 μm.
Fig. 3.10 Deuteromycete hyphae stained with FM4-64. (a) *Aspergillus fumigatus*; (b) *Aspergillus nidulans*; (c) *Botrytis cinerea*. Note the strand-like structures (arrows) (d) *Penicillium claviforme*. Bar = 10 μm.

Fig. 3.11 Zygomyces hyphae stained with FM4-64. (a) *Phycomyces blakesleeanus*. Note the diffuse cloud of stained vesicles within the apex. (b) *Basidiobolus ranarum*. Note the near-spherical AVC. Bar = 10 μm.
3.2.6 The Spitzenkörper core

A wide variation in the staining and morphology of the AVC was observed in *N. crassa* hyphae stained with FM4-64 (Fig. 3.12). The AVC was highly mobile within the tip, and often moved in and out of the plane of focus. The AVC sometimes appeared as a "donut" or "cup" shaped ring, and the core region remained unstained or less stained than the rest of the AVC.

![Figure 3.12](image)

*Neurospora crassa.* Different hyphal tips stained with FM4-64. Note the variation in AVC and Spitzenkörper core morphology. Bar = 5 μm.

3.2.7 Satellite Spitzenkörper

Although satellite Spitzenkörper (López Franco *et al.* 1994, 1995) were observed in hyphae of a range of different species, they were most frequently observed in hyphae of *Botrytis cinerea* stained with FM4-64 (Fig. 3.13; Movie 3.14). In this sequence, the satellite formed just below the main Spitzenkörper, migrated towards the tip and then within 15 sec of being formed, it fused with the main Spitzenkörper.
Fig 3.13 *Botrytis cinerea* hyphal tip. Note the satellite Spitzenkörper that migrates towards the tip and eventually fuses with the main Spitzenkörper. Bar = 10 μm. Published in Fischer-Parton *et al.* (2000).
3.2.8 Retraction of Spitzenkörper

In FM4-64 stained hyphae of *Sclerotinia sclerotiorum* and *Morchella esculenta*, retraction of the AVC was frequently observed in response to laser light stress. Combined factors that appeared to induce retraction were high laser power (3%), slow scanning speed (F1) and image collection at rapid intervals (every 3 s). In Fig. 3.14, the Spitzenkörper of *Sclerotinia sclerotiorum* hyphae initially retracted 1-2 μm from the tip then suddenly pulled back and apparently disappeared. Usually the tips recovered within 1-2 min, and a new Spitzenkörper was formed (Movies 3.15, 3.16). In *Morchella esculenta* hyphae stained with FM4-64, repeated laser scanning at a high zoom factor (4) caused retraction of the Spitzenkörper, accompanied by cessation of growth (Fig. 3.15; Movie 3.17). Fluorescent fibrous projections were often observed extending back from the Spitzenkörper, as it retracted (Movie 3.16).

![Fig. 3.14 Sclerotinia sclerotiorum. Hyphal tip stained with FM4-64. Note retraction of the Spitzenkörper. Bar = 10 μm.](image-url)
Fig. 3.15 *Morchella esculenta*. Hyphal tip stained with FM4-64. Note retraction of the Spitzenkörper and formation of a new one after 24 s. Bar = 10μm.
3.2.9 Hyphal Branching

Time-lapse sequences showed the development of subapical branches from hyphae (Movie 3.22). High-resolution images revealed branch formation close to the tip of a *N. crassa* hypha (Fig. 3.16; Movie 3.18). Note the appearance of a stained vesicle cluster immediately adjacent to the plasma membrane, which developed into a bulge, and eventually a discrete Spitzenkörper was observed. In a time-lapse sequence of *Sclerotinia sclerotiorum* shown in Fig. 3.17 and Movie 3.19, a branch was initiated, but then aborted and a new branch was formed ~1 min later. These branches occurred closer to the hyphal tip than was typical for *N. crassa* (Fig. 3.16), and possibly originated from a satellite Spitzenkörper. Normally hyphal branches form subapically (Figs. 3.16, 3.17, 3.20 and 3.21). However, Fig. 3.18 and Movie 3.20 show what appeared to be an example of dichotomous apical branching. In this unusual case, the main hypha stopped growing and 2 branches were initiated. The emergence of the two branches was preceded by what appeared to be the formation of two new AVCs. This branching was probably a result of stress on the microscope stage. In hyphae of the zygomycete *Phycomyces blakesleeanus* stained with FM4-64, a fluorescent staining plaque appeared at the site of branch initiation (Figs. 3.19 a, b) and bright fluorescence was observed in the tip of a developing branch (Fig. 3.19 c).

3.2.10 Septum formation

Septum formation was imaged in 2 different species: *N. crassa* and *A. fumigatus*. The sequence of septum formation was observed in growing colonies of *A. fumigatus* (Fig. 20; Movie 3.21) and *N. crassa* (Fig. 3.21; Movie 3.22). In *N. crassa*, the septa were formed sequentially with the newest septa appearing closer to the tips (Fig. 3.22b). Surprisingly, in *A. fumigatus*, the sequence of septum formation was more random (Fig. 3.22a). High-resolution images showed the growth of septa resulting from the in growth of stained plasma membrane and septal wall material, and time-lapse movies showed movement of cytoplasm through a septal pore (Fig. 3.23; Movie 3.23, 3.24).
Fig. 3.16 *Neurospora crassa* hypha stained with FM4-64. Note the patch of fluorescence (arrow) on the plasma membrane that appeared just before the branch emerged. The AVC was observed before, during and after branch formation. Bar = 10 μm.
Fig. 3.17 Sclerotinia sclerotiorum stained with FM4-64. Note the appearance of an AVC (0 s), close to a bulge in the hypha. This AVC subsequently disappeared from view. After 105 s, another AVC is observed which gives rise to a branch. Bar = 10 μm.
Fig. 3.18 Sclerotinia sclerotiorum hyphae stained with FM4-64 showing apical branching. Bar = 10 μm.
Fig. 3.19  *Phycomyces blakesleeanus* stained with FM4-64. (a) Branch initiation. Note fluorescence at site of branch formation; (b) Extending branch that grew at an angle of approximately 90° to its parent hypha. Bar = 10 μm.
Fig. 3.20 *Aspergillus fumigatus* hyphae in the peripheral region of the colony stained with FM4-64. (a) Time = 0 min. (b) Time = 83 min. Bar = 100 μm.
Fig. 3.21 Neurospora crassa hyphae in the peripheral region of the colony stained with FM1-43. (a) Time = 0 min. (b) Time = 28 min. Bar = 50 μm.
Fig. 3.22 Contrasting sequences of septum formation in two species. (a) *Aspergillus fumigatus* stained with FM4-64, negative image. Bar = 100 μm. (b) *Neurospora crassa* stained with FM1-43, negative image. The numbers in each image refer to the order in which the septa were formed. Bar = 50 μm.
Fig. 3.23 Neurospora crassa stained with FM4-64. (a) Mid-power magnification of septum formation. (b) High-power magnification of septum formation. Note in growth of the septa from the lateral walls of the hyphae. In each case only the stained plasma membrane associated with the septum and lateral cell wall is fluorescing. Bar = 10 μm.
3.2.11 Remote sensing and redirection of hyphal growth

Hyphae of *Neurospora crassa* display both positive and negative autotropisms. An example of hyphal avoidance (negative autotropism) is shown in Fig. 3.24 and Movie 3.25. Two hyphae that were growing together bent and grew away from the other hypha. Growing tips appeared to sense the presence of nearby hyphae in advance, and responded by changing their direction of growth. Positive autotropism occurs during "hyphal homing" leading up to hyphal fusion (anastomosis) (Figs. 3.25 - 3.26).

![Image](image-url)

Fig. 3.24 *Neurospora crassa* stained with FM4-64. Note the two hyphae (which appear to be stuck together) grow away from the hypha on the left. Bar = 20 μm.
3.2.12 Hyphal fusion and cytoplasmic flow

In mature regions of a *Neurospora* colony, hyphal fusion occurs (see review by Glass *et al.* 2000). Fusions are rarely observed in the outer periphery of the colony. They mostly occur in more central regions. The process of hyphal fusion in growing colonies of *N. crassa* was studied in time-lapse movies using confocal microscopy. The fluorescent dyes FM4-64 and FM1-43 enabled us to visualize the plasma membrane, Spitzenkörper, mitochondria, vacuolar network, and nuclei during compatible fusions. Fusion-competent hyphae were generally morphologically distinct and often showed remote sensing, resulting in branch initiation and redirection of growth to facilitate contact. Time-lapse sequences of hyphae in the fusion zone showed that hyphae remotely sense each other, and sometimes a branch was initiated in response to the presence of an adjacent growing tip. Overall, the time taken for anastomosis varied between 7 and 18 min, from the point of cell wall contact to cytoplasmic mixing. The time taken for the complete fusion process, from the point of contact to the moment of cytoplasmic transfer, varies from 8-20 min. A wide variety of morphologically different types of fusions were observed (Fig. 3.25). After staining with FM4-64, intense membrane activity and a "bright spot" was seen where fusion-competent hyphae met (Figs. 3.25 - 3.27; Movies 3.26, 3.27). Time-lapse sequences of differentially stained mycelium revealed dramatic post-fusion changes in organelle movement and cytoplasmic flow (Figs. 3.28 - 3.29; Movies 3.28, 3.29). In the fusion sequence shown in Fig. 3.30, it appears that the turgor-pressure of the two hyphae is similar because a "membrane bubble" is observed before cytoplasmic transfer occurs.

![Image of hyphal fusion](image)

**Fig. 3.25** *Neurospora crassa* hyphae stained with FM4-64. Different examples of hyphal fusions. Note the "bright spots" which are usually on both sides at the fusion point. Bar = 10 μm.
Fig. 3.26 *Neurospora crassa* hyphae undergoing homing and fusion after staining with FM4-64. The complete homing and fusion sequence shows the polarised growth of two branches towards each other, contact, and formation of pore between the fused branches. Note that the branches on the right are also growing towards each other. Bar = 10 μm.
Fig. 3.27 *Neurospora crassa* hyphae undergoing fusion after staining with FM4-64. Note the "bright spot" (arrow) is persistent throughout the fusion process. Bar = 10 μm.
Fig. 3.28 *Neurospora crassa* hyphae from two separate mycelia showing fusion and cytoplasmic flow after differential staining with FM1-43. Note that the hyphal tips superficially resemble swollen appressoria, and that most of the staining is of brightly stained mitochondria. Bar = 10 μm.
Fig. 3.29 *Neurospora crassa* hyphae from two different mycelia, differentially stained with FM1-43, undergoing fusion (transmitted light bright field image at the top). Note that the contents of the upper unstained hypha flow into the stained hypha following fusion. Bar = 10 μm.
Fig. 3.30 *Neurospora crassa* hyphae fusing after staining with FM1-43. In this unusual case, a bubble-like segment of membrane (arrow) extends through the fusion pore and eventually bursts. Bar = 10 μm.
3.2.13 Asexual reproduction

The reproductive stage of *Aspergillus fumigatus* involves the production of conidia which develop from a bulbous vesicle that is located at the end of the conidiophore. The process of conidiophore development was followed using time lapse and confocal microscopy (Figs. 3.31, 3.32; Movie 3.30). The optical sectioning capabilities of confocal microscopy have enabled 3-D reconstruction of developing conidiophores in this medically important fungus (Figs. 3.33, 3.34; Movies 3.31, 3.32). An AVC was observed during conidiophore growth, but disappeared after the swollen vesicle started to form (Figs. 3.32, 3.34). A 3-D reconstruction of germinating spores from a detached conidiophore is presented in Movie 3.33.

![Image of A. fumigatus conidiophores](image1)

**Fig. 3.31** *Aspergillus fumigatus* stained with FM4-64. Developing conidiophores in a colony. Note the presence of an AVC (arrows) in developing conidiophores. Bar = 10 μm.

![Image of A. fumigatus conidiophores](image2)

**Fig. 3.32** *Aspergillus fumigatus* stained with FM4-64. Different stages of conidiophore and conidium development shown in four different conidiophores. Note the presence of an AVC which disappears after the conidiophore swells. Bar = 10 μm.
Fig. 3.33 *Aspergillus fumigatus* stained with FM4-64. Different stages of conidiophore and conidium development. (a) Single optical section; (b) 3-D projection of 50 x 0.5 µm optical sections. Bar = 20 µm.
Fig. 3.34 *Aspergillus fumigatus* stained with FM4-64. Different stages of conidiophore development. (a) Single optical section; (b) 3-D projection of 35 x 0.5 μm optical sections. Note the presence of AVCs (arrows) in the extending conidiophores but their absence after the swollen vesicle has developed. Bar = 10 μm.
3.2.14 Protoperithecia and trichogyne development

The early stages of sexual reproduction in Neurospora crassa were examined using confocal microscopy. To induce formation of protoperithecia, Neurospora colonies were grown for 7-14 days on tap water agar. The nutrient poor media promoted formation of protoperithecia and reduced aerial mycelium and conidia. The young protoperithecium develops from a hyphal branch, which appears to have grown around itself forming a coiled complex (Fig. 3.35; Movie 3.34). The mature protoperithecium consists of a complex aggregation of hyphae, from which emerges growing trichogynes (Fig. 3.36; Movie 3.35). The trichogynes grew slowly (~1.0 μm min⁻¹) and trichogynes stained with FM4-64 had faintly stained fluorescent spots at their tips, which resembled stained AVCs (not shown).

Fig. 3.35 Neurospora crassa stained with FM4-64. Early stage of protoperithecium development. (a) 3-D projection; (b) 3-D stereogram. Bar = 10 μm.
Fig. 3.36 *Neurospora crassa* stained with FM4-64. Late stage of protoperitheciun development. Note the long slender trichogynes emerging from the near spherical protoperitheciun. (a) 3-D projection of optical sections; (b) 3-D stereogram. Bar = 20 μm.
3.2.15 Clamp development in basidiomycetes

Clamp connections are the structures formed by many basidiomycetes during dikaryon formation. The early stages of clamp connection formation were followed in the basidiomycete *Ganoderma lucidum* using time-lapse confocal microscopy (Fig. 3.37; Movie 3.36). A bright spot resembling a stained AVC was observed asymmetrically located within the emerging clamp. Its location in the growing clamp was consistent with it supplying the wall-building vesicles contributing to asymmetric growth pattern of this structure.

![Fig. 3.37 Ganoderma lucidum stained with FM4-64. Early stages of clamp development. Note the presence of a "bright spot" resembling a stained AVC within the developing clamp. The main hyphal tip of the hypha from which the clamp has emerged is to the right of these images. Bar = 10 µm.](image-url)
3.3 Discussion

3.3.1 Endocytosis and the vesicle trafficking network

Little is known about endocytosis in filamentous fungi and until recently, neither physiological nor ultrastructural studies had provided good evidence that it even occurs (Ashford 1998). However, recently compelling evidence that endocytosis occurs in filamentous fungi has come from reports of the uptake of the endocytosis marker dye FM4-64 by fungal hyphae (Hoffmann and Mendgen 1998; Read et al. 1998, Read and Hickey 2001).

From the data presented in this chapter, time- and energy-dependent staining of hyphae supports the hypothesis that FM4-64 enters cells by endocytosis. It should be emphasized that the visualization of individual vesicles is beyond the resolution of the imaging techniques employed here. Evidence against FM4-64 entering budding yeast cells by unfacilitated diffusion was obtained by inhibiting dye internalization in the presence of sodium azide, or by applying dye at low temperature (Vida and Emr, 1995). My experiments showed that dye internalization in N. crassa is an active process, because it is inhibited by the metabolic inhibitor sodium azide. Sodium azide has also been shown to inhibit FM4-64 internalization by yeast cells (Vida and Emr, 1995) and pollen tubes (Parton et al. 2001). Similar inhibition of FM4-64 uptake was obtained by Fischer-Parton (1999) when N. crassa hyphae were stained with FM4-64 at 4°C; when the temperature was subsequently increased to 25°C normal dye uptake was observed. Time-dependent staining of pollen tubes with FM4-64 was overcome by injecting them with aqueous FM4-64. This resulted in uniform staining of the pollen tubes (Parton et al. 2001). Equivalent results were obtained when protoplasts of the N. crassa mutant slime were lysed with FM4-64 present in the surrounding medium (see sections 5.2.4 and Fig. 5.15). Strong evidence that FM4-64 is transported to the vacuole via an endocytic pathway comes from studies on mutants of budding yeast defective in vesicle trafficking at different steps in this pathway. In these mutants dye transport to the vacuole was inhibited (Vida and Emr, 1995).

The time-dependent staining of hyphae shown here is consistent with the model of vesicle trafficking proposed by Fischer-Parton et al. (2000) and Read and Hickey (2001) (see section 1.2.4). The results described in this chapter show that FM4-64
was internalized by hyphae of 15 different species representing the zygomycetes, ascomycetes, basidiomycetes and deuteromycetes. Interestingly, in contrast to our observations, Cole et al. (1997) did not observe uptake of FM4-64 by fungal hyphae of the basidiomycete *Pisolithus tinctoris*. The reason for this difference is not clear. It may be a species-specific phenomenon. In this respect, I did find that hyphae of *Mucor mucedo* failed to exhibit intracellular staining with FM4-64 and were only weakly fluorescent when stained with 10 times the normal concentration of dye used in my studies (data not shown). There is also a possibility that FM4-64 may become immobilized and prevented from entering hyphae of *M. mucedo* or *P. tinctorius*. Dye molecules might be unable to pass through the cell wall or they may become trapped and retained by the extracellular matrix. Cole et al. (1997) also found that membrane impermeant probes such as Lucifer Yellow carboxyhydrazide, which is used as an indicator of fluid phase endocytosis in budding yeast (Dulic et al. 1991), were not taken up into *Pisolithus tinctoris* hyphae. Although FM4-64 was rapidly internalized, the fluid phase marker Oregon Green 488 10 kDa dextran apparently was not taken up into *Neurospora crassa* hyphae, even after long periods of immersion in the dye (Fig. 3.7). Again, in contrast, it has recently been shown that both the fluid phase markers Lucifer Yellow carboxyhydrazide and FITC-dextran (10 kDa) are internalised by germinating conidia of *Magnaporthe grisea* (Atkinson, 2000). The significance of this disparity in results between different species is unclear and the occurrence of fluid-phase endocytosis in filamentous fungi needs future research.

### 3.3.2 Hyphal tip organization

The staining patterns of FM4-64 and FM1-43 stained hyphae were quite different. Differences in the chemical structures of these dyes may affect their fluorescent properties and how they become distributed amongst different membranes. FM4-64 and FM1-43 molecules are composed of three elements (Fig 1.2): a hydrophobic tail (which promotes partitioning into membranes), a dicationic head (which prevents passage across membranes) and a body or nucleus (which determines the spectral properties of the dye). The apical vesicle cluster of hyphae stained more brightly with FM4-64 and was more resistant to photobleaching than in hyphae stained with the same concentration of FM1-43.

In hyphae stained with FM4-64 for >1 h, many different organelles became stained, including mitochondria and vacuoles. However, in all the species examined the
nuclear envelope remained unstained, even after prolonged exposure to the dye (>6 h). One possibility resulting in different patterns of staining by the two dyes may be that they act as fluorescent lipid analogues. FM4-64 may become distributed amongst membranes by a lipid sorting mechanism. Besides the time-dependent staining of organelle membranes by FM4-64 (which we interpret as primarily involving the transport of the dye between organelles via the vesicle trafficking network), we suggest that a process of ‘lipid sorting’ may also play a role in the differential staining of organelle membranes. It has recently been found that fluorescent lipid analogues undergo sorting to different organelles in animal cells solely on the basis of the chemistry of their hydrophobic tails. This is believed to be the mechanism by which lipids normally become distributed between different membranes of the endomembrane system (Mukherjee et al. 1999). With FM4-64 we find that some membranes (e.g. the plasma membrane) stain strongly, some membranes stain at a reasonable level (most organelles) whilst others (e.g. the nuclear membrane) do not appear to stain at all, even though we would predict that the latter should stain up through connection with other components of the endomembrane system. With other styryl dyes which are internalised by endocytosis and which possess different hydrophobic tails (e.g. FM1-43) the pattern of membrane staining is different to that of FM4-64 (see Table 3.2) (Fischer-Parton et al. 2000; Read and Hickey 2001). It is conceivable that flippases could provide an alternative mechanism for the internalization of FM4-64 and FM1-43. Once dye has been translocated to the inner leaflet of the plasma membrane by flippase activity, lipid transfer proteins may then transport dye molecules to other organelles (Fischer-Parton et al. 2000). The ability of these dye molecules to flip across membranes and the different compositions of membranes may explain the staining of other organelles (e.g. mitochondria). A further possibility to be considered in relation to the staining of mitochondria with FM4-64 and FM1-43 is that mitochondria exhibit membrane continuity with other components of the endomembrane system. Evidence of this has previously been shown in hyphae (Bracker and Grove 1971; Franke and Kartenbeck 1971) at the ultrastructural level, albeit in chemically fixed material.

3.3.3 The apical vesicle cluster

FM4-64 proved to be an excellent fluorescent stain for imaging the AVC within the Spitzenkörper of a wide range of species spanning the ascomycota, deuteromycota,
basidiomycota and zygomycota. In general time-lapse confocal microscopy of FM4-64 stained hyphae showed that the Spitzenkörper was mobile and moved around the apex of growing tip, although it usually remained close to the plasma membrane at the apical pole. Movement of the Spitzenkörper often corresponded to changes in direction of growth on the same side that the Spitzenkörper had moved to. This supports the view that the Spitzenkörper supplies wall-building, secretory vesicles for hyphal growth (Grove and Bracker, 1970; Howard, 1981; Roberson and Fuller, 1988).

Hyphae stained with FM4-64 often possessed a region exhibiting reduced staining that correlated with the differentiated 'core' region observed in unstained hyphal tips at the light microscope level (e.g. López Franco and Bracker, 1996), and at the electron microscope level (e.g. Bourett and Howard, 1991). These unstained core regions were observed in many different species, including Botrytis cinerea, Sclerotinia sclerotiorum, Morchella esculenta, Neurospora crassa and Rhizoctonia solani. The core was more obvious during the early stages of staining (2-30 min) following application of the dye, and usually in larger diameter hyphae >10 μm. In most species, the core is largely devoid of vesicles (e.g. see Bourett and Howard, 1991). However, in some species (e.g. A. niger), the core contains microvesicles, which are surrounded by larger apical vesicles (e.g. Grove and Bracker, 1970).

FM4-64 stained the AVC of the lower fungi Phycomyces blakesleeanus and Basidiobolus ranarum (Fig. 3.11). Phycomyces blakesleeanus belongs to the zygomycota, and was reported to possess a crescent-shaped band of apical vesicles instead of a Spitzenkörper, lacking a core region (Grove and Bracker, 1970; López Franco et al. 1995).

3.3.4 Satellite Spitzenkörper

Small satellite Spitzenkörper were shown to arise in a location immediately below the plasma membrane several micrometers back from the main Spitzenkörper. Normally they migrate within 15 s towards the main Spitzenkörper and fuse with it (López Franco et al. 1994, 1995; Fischer-Parton et al. 2000). If the satellite Spitzenkörper do not immediately migrate towards the main one, then a bulge in the hypha typically appears adjacent to them indicating that they are delivering wall-building vesicles to the region of the plasma membrane which they are in close
proximity with (López Franco et al. 1994). It is possible that a satellite Spitzenkörper may be involved in the initiation of a branch when the branch forms very close to the hyphal tip (see Fig. 3.17).

3.3.5 Spitzenkörper retraction

Retraction is a stress response where the apical vesicle cluster loses its apparent tethering to the tip, retracts and usually breaks down. This phenomenon is commonly observed under conditions of laser light stress although may also be induced by osmotic stress (López-Franco and Bracker 1996). Prolonged exposure to the laser beam caused the Spitzenkörper to lose its apparent tethering to the tip, and stained apical vesicles disappeared into the cytoplasm.

3.3.6 Hyphal branching

FM4-64 provided a powerful tool for observing branch formation in living hyphae. Before a branch emerged, an aggregation of what seemed to be brightly stained vesicles was observed adjacent to the plasma membrane (Fig. 3.16). In wild type Aspergillus fumigatus branches usually occur >50 μm from the tip. The hyper-branching mutant of A. niger, ramosa-1, displays near apical branching at the restrictive temperature and video-enhanced phase contrast microscopy of this mutant showed formation of new Spitzenkörper de novo in branching tips (Reynaga-Peña and Bartnicki-Garcia 1997; Reynaga-Peña et al. 1997). In this study, FM4-64 was used as an alternative method for tracking the apical vesicle cluster and has revealed new aspects of Spitzenkörper behaviour during branch formation. Branches that were observed close to the apex in many cases may have been induced in response to environmental stress because they were not common in unperturbed cultures (data not shown). Several possible explanations are proposed for the unusual example of apical / near apical branching observed in Sclerotinia sclerotiorum (Fig. 3.19): (1) the Spitzenkörper disappears and two new Spitzenkörper are formed independently; (2) the Spitzenkörper moves to one side and a new Spitzenkörper is formed adjacent to it; (3) the main Spitzenkörper splits into two daughter Spitzenkörper. However, from the images shown in Fig. 3.19, it is not possible to determine which one of these explanations is correct because only a single thin optical section through the hypha was imaged, and one does not know what other things might be going on in different focal planes.
3.3.7 Septum formation

Septa are the cross walls which form within fungal hyphae and physically separate adjacent hyphal compartments although septa often possess open pores. Cytoplasm and organelles (including nuclei) are able to pass through the septal pores of unplugged septa (Markham 1995). When an ascomycete or deuteromycete hypha becomes damaged, septal pores are rapidly plugged by a structure termed the Woronin body. Septa of ascomycetes are often different in structure from those of basidiomycetes and the zygomycetes lack septa completely (Gull 1978). FM4-64 staining of the plasma membrane associated with septa allowed detailed observations on the development of septa with time. An unexpected result was a distinct difference in the timing of septum formation in peripheral regions of Aspergillus and Neurospora colonies. Septa were formed sequentially in N. crassa, with newest septa developing towards the hyphal tip, whereas unexpectedly in A. fumigatus, septa appeared to develop randomly (see section 3.9). The significance of these differences is not known.

3.3.8 Hyphal fusion

Fungal hyphae are clearly capable of remote sensing, although we know very little about the signaling mechanisms involved. Possible signaling molecules in the external environment surrounding hyphae might include pheromones or metabolites (e.g. sugars). A wide range of morphologically different fusion types were observed. The commonly described types are “tip-tip”, “tip-peg” and “peg-peg” fusions (Fig. 3.31) (Buller 1933; Glass et al. 2000). Another type of fusions observed were “tip-trunk” fusions, which often bore some superficial similarity to swollen appressoria (e.g. Fig. 3.28). The bright spot (Figs. 3.25 - 3.27) which stained with FM4-64, was possibly an aggregation of vesicles delivering compounds to (1) agglutinate cell walls at the point of contact and (2) to soften cell walls at the fusion pore. Differential staining of mycelia and time-lapse sequences allowed the observation of the moment of fusion when cytoplasmic transfer occurs. The direction of flow appears to be governed, at least in part, by the differential turgor pressures of participating hyphae, although this was not measured in my studies. In other examples, it appeared that the fusing hyphae maintained similar pressure and a “membrane bubble” was observed (see Fig. 3.29). The direction of cytoplasmic flow
often changed (Fig. 3.26), presumably because of fusion events upstream and downstream in that region of mycelium. Cytoplasmic flow, and particularly the movement of organelles will also be regulated by the cytoskeleton (McKerracher and Heath, 1987). Based on our observations, discrete physiological states of the participating hyphae can be divided into 3 stages: pre-contact, post-contact, and post-fusion (Fig. 3.32).

Fig. 3.31 Diagram representing the different types of hyphal fusions observed. (a) tip-tip. (b) tip-trunk. (c) tip-peg. (d) peg-peg.

Fig. 3.32 Diagram representing the observed stages of the fusion process. (a) Remote sensing, (b) hyphal contact, (c) agglutination, (d) fusion and (e) fusion pore enlargement.

3.3.9 Conidiophore and conidium development

FM4-64 proved to be a very useful stain for imaging the living reproductive structures of fungi, especially for 3-D reconstruction. The asexual reproductive structures of Aspergillus are comprised of upright conidiophores, which become
swollen at their apices to form so-called vesicles. From these vesicles, chains of conidia are formed from phialides (Cole and Samson, 1979). The hyphae that developed into conidiophores were generally larger in diameter (>10 μm) than vegetative hyphae (~8 μm) from which they were derived in mature regions of the colony. The early stages of conidiophore development were examined in *Aspergillus fumigatus* colonies, which had been stained with FM4-64. The swollen, growing hyphal tips of developing conidiophores contained a brightly stained region that resembled a giant AVC. Staining intensity became reduced as the vesicle grew to ~20 μm in diameter and the developing conidia started to bud from the surface. Individual AVCs were not observed in budding conidia, although this detail may have been beyond the resolution that could be obtained in the present study. Many nuclei were observed within the cytoplasm of FM4-64 stained conidiophores; these appeared as dark spherical regions ~3 μm in diameter.

### 3.3.10 Sexual Reproduction

The protoperithecium starts as a branch that coils around itself and develops into a mass of interwoven, enveloping hyphae (Read 1983, 1994). FM4-64 was used to image the early stages of protoperithecium development and serial sections revealed the complex 3D structure of protoperithecia. Trichogynes are the specialized hyphae that emerge from the protoperithecium (e.g. see Broxholme *et al.* 1991) and were observed growing for the first time with the confocal microscope.

Clamp connections are found exclusively in many basidiomycetes and play an important role in the separation of nuclei during sexual reproduction (Girbardt, 1957). A clamp connection is formed when a branch coils back on itself, forming a loop that fuses with the hypha from which it emerged. FM4-64 stained the AVC that appeared to be asymmetrically localized on one side of the branch as it curled. This is consistent with the asymmetrically positioned AVC supplying wall-building vesicles for the polarised growth of the clamp connection.
Chapter 4: Imaging organelle organisation and dynamics in living hyphae

4.1 Introduction

Relatively little is known about the dynamic morphology and organization of organelles in living hyphae of filamentous fungi. Most of the research on analysing organelle organisation and morphology has involved studies on fixed specimens, especially at the ultrastructural level, which cannot show the dynamics of organelle organisation in vivo. Video-enhanced microscopy of living hyphae (e.g. López-Franco et al. 1994, 1995; López-Franco and Bracker, 1996) allows visualization of many different organelles (e.g. mitochondria and nuclei) and organelle complexes (e.g. the Spitzenkörper), but does not provide selective staining of specific organelles, which can be obtained using fluorescent dyes and recombinant fluorescent probes (especially GFP). Confocal microscopy combined with the use of vital dyes or GFP probes allow us to non-invasively interrogate living hyphae, and obtain time-lapse sequences of images at high spatial and temporal resolution. The work in this chapter has involved the development and use of new techniques which complement previous microscopical studies, adding a new perspective to our understanding of organelle dynamics in living hyphae. The overall aim of this chapter was to examine the dynamic morphology and organisation of organelles in living hyphae using GFP and vital fluorescent stains at a higher spatial resolution that has previously been achieved. The following organelles were imaged:

- **Vacuolar network** using GFP fused to the CPYA vacuolar protein or the vacuolar selective dye Oregon Green 488 DFFDA.
- **Mitochondria** using GFP fused to the SUAPRGA1 mitochondrial protein or mitochondrion-selective dyes Rhodamine-123, FM1-43, DASPMI and Mitotracker Green. The potentiometric dye, Rhodamine-123 was also used to image and measure mitochondrial activity.
- **Nuclei** using GFP targeted to the GAL4 DNA binding domain or the nucleus-selective dyes SYTO-11 and SYTO-13. Spindle pole bodies and nucleoli were also imaged using GFP fused to the BIMG PP1 phosphatase.
- **Endoplasmic reticulum (ER)** using GFP fused to an ER retention signal and chitinase export signal.
- **Golgi** using GFP fused to the COP1 α-coatamer protein.
4.2 Results

4.2.1 Vacuolar network

The vacuolar network was imaged in hyphae of *A. nidulans* expressing GFP fused to the CPYA vacuolar protein or stained with the vacuolar selective dye Oregon Green 488 carboxylic acid diacetate (carboxy-DFFDA). In general, carboxy-DFFDA was found to be brighter but more susceptible to photobleaching, and more phototoxic than the GFP targeted to the vacuole. The strains were grown on ethanol induction medium because the fusion protein was under the control of the ethanol inducible promoter, AlcA. Bright fluorescence from the GFP expressing strain was observed in colonies viewed with a fluorescence stereomicroscope (Fig. 4.1). Confocal microscopy revealed a gradient in different vacuolar morphology, although considerable morphological variation was observed from hypha to hypha. Two extremes of the variation are shown in Fig. 4.2, which compares the morphology of the vacuolar network with GFP targeted to it and after staining with carboxy-DFFDA. Confocal microscopy of hyphal regions 0-50 μm from their tips revealed mobile spherical/near-spherical fluorescent structures of varying shape from 1-3 μm in size (Fig. 4.3a,b; Movie 4.1). 50-200 μm from hyphal tips, tubular vacuoles and small spherical/near-spherical vacuole compartments were observed (Fig. 4.3c,d; Movie 4.2). In subapical regions, >200 μm from hyphal tips, large spherical/ovoid vacuole compartments were observed, and these varied in fluorescence intensity (Fig. 4.3e,f; Movie 4.3). Often different vacuolar compartments split apart and fused with each other. Movie 4.3 shows the fusion of 2 spherical vacuoles exhibiting different fluorescence intensities. Time-lapse sequences, revealed the highly mobile behaviour of the vacuoles and their dynamic pleiomorphic nature. In subapical regions separated by septa there appeared to be a gradient of vacuolar staining within each individual compartment, with brightly fluorescent tubular vacuoles adjacent to the septum towards the tip, and less intensely fluorescent, large spherical/near-spherical vacuoles towards subapical regions (data not shown). The overall staining was similar although GFP appeared to be localized to some of the finer tubular structures that could not be as readily resolved with carboxy-DFFDA. Thus, the GFP probe revealed vacuolar compartments in more detail than could be resolved with the carboxy-DFFDA dye (Fig. 4.3c,d).
Fig. 4.1 Aspergillus nidulans expressing GFP targeted to vacuolar network viewed using a fluorescence stereomicroscope. (a) Low power image. Bar = 1 mm. (b) High power image. Bar = 100 μm.
To compare the vacuolar morphology of *A. nidulans* with that of *N. crassa*, hyphae were stained with the vacuolar selective stain carboxy-DFFDA and the general membrane-selective dye FM4-64. Staining with carboxy-DFFDA showed that hyphae were largely devoid of the vacuolar network in the hyphal region 30-50 μm back from hyphal tips of *N. crassa* (Fig. 4.4a, 4.5; Movies 4.4, 4.5). Within the first 0-50 μm of hyphal tips, small mobile spherical/near spherical, stained components of the vacuolar network were observed (Fig. 4.5a). 50-200 μm from the hyphal tips, the cytoplasm was predominated by an extensive tubular network of "spaghetti-like" vacuolar compartments (Fig. 4.5b). Membranes of tubular vacuoles could be readily visualised after prolonged staining with FM4-64 (Fig. 4.4b; Movie 4.7). In regions >200 μm from hyphal tips, mainly large spherical vacuoles were stained with carboxy-DFFDA (Fig. 4.6a) and this corresponded with vacuolar membrane staining by FM4-64 (Fig. 4.6b). Hyphae of *Phycomyces blakesleeanus* stained with carboxy-DFFDA contained an extensive tubular vacuolar network that extended to the hyphal tip (Fig. 4.7). These results showed that the vacuolar morphology is different in different regions of hyphae, and that considerable variation in their morphology exists between species.

Hyphae were sensitive to the phototoxic effects of the carboxy-DFFDA. An example of this is shown in hyphae of *Lentinus edodes*, which were very sensitive to phototoxic effects of this dye at 50 μM concentration (Fig. 4.8; Movie 4.6). Initially the vacuoles appeared as tubular elements extending throughout the apical hyphal compartment. However, after several scans of the laser beam, the vacuoles changed shape dramatically, fused and rounded up into spherical fluorescent structures, with negatively stained inclusions.

![Fig. 4.2](a) *A. nidulans* grown on ethanol induction medium and expressing GFP targeted to the vacuolar network; (b) *A. nidulans* grown on ethanol induction medium stained with carboxy-DFFDA. Bar = 20 μm.
Fig. 4.3 Aspergillus nidulans grown on ethanol induction medium. (a) GFP targeted to vacuolar network, 0-30 μm from the hyphal tip (arrow); (b) stained with carboxy-DFFDA, 0-30 μm from the hyphal tip (arrow); (c) GFP targeted to vacuolar network, ~100 μm from the hyphal tip; (d) stained with carboxy-DFFDA, ~100 μm from the hyphal tip; (e) GFP targeted to vacuolar network, ~200 μm from the hyphal tip; (f) stained with carboxy-DFFDA, ~200 μm from the hyphal tip. Bar = 5 μm.
Fig. 4.4 *Neurospora crassa* vacuolar network. (a) Hyphae stained with carboxy-DFFDA. Note that the hyphae are largely devoid of the vacuolar network 30-50 μm from hyphal tips. Bar = 50 μm. (b) Hyphae stained with FM4-64 for 3 h. Note the long "spaghetti-like" tubular vacuoles predominant in the subapical regions of the hyphal compartment. Bar = 10 μm.
Fig. 4.5 *Neurospora crassa* stained with carboxy-DFFDA. Note that the region occupied by the Spitzenkörper remains less stained. Bar = 10 μm.
Fig. 4.6 *Neurospora crassa*. (a) Sub-apical region of a hypha stained with carboxy-DFFDA. (b) Sub-apical region of a hypha stained with FM4-64. Note staining of the membrane of a large spherical vacuole adjacent to septum. Note the internal staining of the large spherical vacuole adjacent to a septum (indicated by arrows). Also note staining of much smaller near spherical vacuolar compartments of variable size. Bar = 10 μm.

Fig. 4.7 *Phycomyces blakesleeanus* stained with carboxy-DFFDA. Note the "spaghetti-like" tubular vacuoles that extend close to the tip (arrow). Bar = 10 μm.
Fig. 4.8 *Lentinus edodes* stained with carboxy-DFFDA. Note the phototoxic effects: the hyphal tip stopped growing and the vacuolar compartments fused with each other. Bar = 10 μm.
4.2.2 Mitochondria

A comparison of five different fluorescent probes selective for mitochondria that were used to stain *A. nidulans* hyphae is presented in Fig. 4.9. Plasma membrane staining was particularly apparent in Rhodamine-123, DASPMI and FM1-43 stained hyphae. The fluorescence of GFP was uniform in mitochondria throughout individual hyphae (Fig. 4.10; Movie 4.8) and no plasma membrane fluorescence was observed. GFP fluorescence varied between individual hyphae (Fig. 4.11). The division of a single mitochondrion is shown in Fig. 4.12. Initially, a narrowing region was observed in the mitochondrion, and within 10 s, the mitochondrion split into 2 daughter mitochondria that rapidly moved apart. The mitochondria in hyphal tips stained with the potentiometric dye Rhodamine-123 were brighter in fluorescence towards hyphal tips (Figs 4.9, 4.10, 4.13). Graphs of fluorescence intensity within hyphae are presented in Fig. 4.14. Fluorescence of GFP was uniform within hyphae (Fig. 4.14a) whereas fluorescence of Rhodamine-123 decreased sharply over the 40 µm region from hyphal tips (Fig. 4.14b). A similar fluorescence gradient was observed in hyphae of *N. crassa* stained with Rhodamine-123 (Fig. 4.13b).

DASPMI stained mitochondria far more selectively in hyphal tips of *N. crassa* compared with that in *A. nidulans* (Figs. 4.9 and 4.15). In contrast to mitochondria in hyphal tips of *A. nidulans* (Figs. 4.9, 4.10, 4.13a, 4.14), mitochondria in hyphae of *N. crassa* and *Sclerotinia sclerotiorum* tended to extend further towards the apical pole and were packed more tightly within the hyphal tips, although they rarely penetrated the region occupied by the AVC (Figs. 4.15, 4.16; Movies 4.9-4.13). Fig. 4.17 shows that mitochondria in hyphae of *Phycomyces blakesleeanus* were much shorter (5-7 µm) than observed in other species (7-12 µm). Both Rhodamine-123 and DASPMI exhibited phototoxic effects at dye concentrations >100 µM (normal concentration 10-65 µM) and during prolonged imaging. Phototoxic effects were also particularly noticeable in hyphae of *A. nidulans* stained with a high concentration of DASPMI (100 µM). After successive laser scanning at slow speed, the mitochondria rounded up, and appeared to vanish, whilst nuclei were weakly fluorescent (Fig. 4.18; Movie 4). *Basidiobolus ranarum* hyphae stained with dihydro-Rhodamine-123 exhibited weak mitochondrial fluorescence. However, an unexpected apparent photoactivation of dye was observed at the hyphal apex (Fig. 4.19; Movie 4.14), although it is not clear what cellular components were stained up.
Fig. 4.9 *Aspergillus nidulans* stained with different probes targeted to mitochondria. (a) GFP; (b) DASPMI; (c) Rhodamine-123; (d) FM1-43; (e) Mitotracker green FM. Bar = 5 μm.
Fig. 4.10 *Aspergillus nidulans*. (a) Mitochondria stained with Rhodamine-123. (b) GFP targeted to mitochondria. Note the gradient in mitochondrial fluorescence observed towards hyphal tips in (a), whilst in (b) the mitochondrial staining is more or less uniform. Bar = 10 μm.
Fig. 4.11 Aspergillus nidulans expressing GFP targeted to mitochondria. Note that the lower hypha is not as fluorescent as the upper branched hypha. Bar = 10 μm.

Fig. 4.12 Aspergillus nidulans expressing GFP targeted to mitochondria. Note the division of a single mitochondrion (pseudo coloured green). Bar = 5 μm.
Fig. 4.13  (a) *Aspergillus nidulans* stained with rhodamine-123. (b) *Neurospora crassa* stained with Rhodamine-123. Note that the fluorescence is higher in the hyphal tip regions where the mitochondria are most active. Bar = 20 μm.
Fig. 4.14 *Aspergillus nidulans*. Graphs showing fluorescence intensity along a median transect down the lengths of growing hyphae. (a) GFP targeted to mitochondria (b) Stained with Rhodamine-123. Note the relatively uniform fluorescence in (a) compared to gradient observed in (b). Bar = 5 μm.
Fig. 4.15  *Neurospora crassa*: (a) Hyphal tip stained with FM1-43 (b) Hyphal tip stained with DASPMI. Bar = 10 μm.

Fig. 4.16  *Sclerotinia sclerotiorum* stained with DASPMI. Bar = 10 μm.

Fig. 4.17  *Phycomyces blakesleeanus* stained with Rhodamine-123. Bar = 10 μm.
Fig. 4.18 Aspergillus nidulans stained with 100 μm DASPMI. Note the phototoxic effects of laser scanning, which resulted in the rounding up and sudden de-staining of mitochondria. Bar = 5 μm.
Fig. 4.19 Basidiobolus ranarum stained with dihydro-Rhodamine-123. Note the appearance of bright fluorescence at the hyphal tip after successive scans. Bar = 10 μm.
4.2.3 Nuclei

Aspergillus nidulans expressing GFP targeted to a GAL4 DNA binding domain proved to be an excellent system in which to study nuclear division in fungal hyphae (Figs. 4.20-4.23; Movies 4.15-4.17). Time-lapse images revealed a wave of nuclear division within single hyphae followed by a period in which the daughter nuclei moved away from each other (Fig. 4.22). The waves of nuclear division were in either direction or bi-directional and the speed at which they travelled varied. In larger diameter tips (>5 μm), cytoplasmic streaming appeared to be faster and the waves were less obvious, because of the greater number of nuclei not arranged in a uniseriate row (Fig. 4.21; Movie 4.16). Waves of division often propagated for long distances along hyphae (>500 μm) and in sub-apical regions could be followed over the course of several minutes (Fig. 4.22; Movie 4.17). The average rate at which waves propagated through apical regions (within 100 μm of hyphal tips) was ~25 μm min⁻¹ (n = 3) and in sub-apical regions (100 - 500 μm from hyphal tips) was ~11 μm min⁻¹ (n = 3) (Table 4.1).

Aspergillus nidulans expressing GFP fused to BIMG PP1 phosphatase exhibited staining of nuclei and bright staining of the spindle pole bodies (SPBs) (Figs. 4.24, 4.25). GFP in these transformants was also associated with the plasma membrane within the apical dome of growing hyphal tips (Fig 4.24, 4.25b), branches (Fig. 4.25a) and developing septa (Fig. 4.25b). Waves of nuclear division were also observed in this transformant (Movie 4.18). The SPBs often appeared to be transporting nuclei along tracks, which are presumably microtubules.

A range of nuclear stains (DAPI, Propidium Iodide, SYTO11, SYTO13, SYTO14, SYTO15, SYTO16), were tested in living hyphae. None of these dyes proved to be useful stains for nuclei in living, unperturbed hyphae. Only the SYTO dyes were readily taken up into living hyphae. However, all the SYTO dyes resulted in much apparent non-specific staining and were extremely phototoxic (Fig. 4.26; Movie 4.19). Optical sections of Basidiobolus ranarum hyphae stained with SYTO11 allowed the 3-D structure of the single giant nucleus with a stained nucleolus and unstained nuclear vacuole to be examined in detail (Fig. 4.27; Movie 4.20) although the hyphae did not grow during the period of observation.
Fig. 4.20 *Aspergillus nidulans* expressing GFP targeted to nuclei, and grown on ethanol induction medium. This hypha is relatively narrow because it was grown on minimal medium. Note that nuclear division has been undergone between successive images in hyphae labelled A and B. Bar = 20 μm.
Fig. 4.21 *Aspergillus nidulans* expressing GFP targeted to nuclei, and grown on ethanol induction medium. Although a wave of nuclear division moving away from the tip is occurring, this is not very clearly seen because of the large number of nuclei which are not arranged in uniseriate manner in this hypha. Bar = 10 μm.
Fig. 4.22 Aspergillus nidulans expressing GFP targeted to nuclei, and grown on ethanol induction medium. Sequence of nuclear division in sub-apical region of a hypha. Note the sequential division (tip is towards the right). Bar = 10 µm.
Fig. 4.23 *Aspergillus nidulans* expressing GFP targeted to nuclei, and grown on ethanol induction medium. These nuclei are located in sub-apical compartments 300-400 µm from the hyphal tip. Sequences of nuclear division in sub-apical regions of 3 different hyphae (a-c). The images on the right show the same hyphae after the nuclei have divided (tip is towards the right). The numbers indicate parent nuclei (e.g. 1) and their daughter nuclei (e.g. 1a and 1b). The times between division of nuclei in (a), (b) and (c) are detailed in Table 4.1. Bar = 10 µm.

Table 4.1. Times between the division of successive nuclei and rate of movement of waves of nuclear division, in sub-apical regions of the hyphae shown in Fig. 4.23.

<table>
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<tr>
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<th>Nuclei numbered</th>
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<tr>
<td></td>
<td>1 and 2</td>
<td>2 and 3</td>
<td>3 and 4</td>
<td>Average</td>
</tr>
<tr>
<td>(a)</td>
<td>Time</td>
<td>70 s</td>
<td>65 s</td>
<td>55 s</td>
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<tr>
<td></td>
<td>Speed</td>
<td>12.8 µm min⁻¹</td>
<td>11.0 µm min⁻¹</td>
<td>5.45 µm min⁻¹</td>
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<tr>
<td>(b)</td>
<td>Time</td>
<td>90 s</td>
<td>70 s</td>
<td>100 s</td>
</tr>
<tr>
<td></td>
<td>Speed</td>
<td>13.3 µm min⁻¹</td>
<td>10.2 µm min⁻¹</td>
<td>9.0 µm min⁻¹</td>
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<td>(c)</td>
<td>Time</td>
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<td>Speed</td>
<td>13.2 µm min⁻¹</td>
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Fig. 4.24 Aspergillus nidulans expressing GFP targeted to BIMG PP1 phosphatase, grown on malt extract agar medium. (a) Medium power image. (b) High power image. Note the brightly stained SPBs (arrows), staining of the nuclei and staining associated with the apical dome of the hypha. The region occupied by the Spitzenkörper is less stained (asterisk). Bars = 10 μm.
Fig. 4.25 *Aspergillus nidulans* expressing GFP targeted to BIMG PP1 phosphatase, grown on malt extract agar medium. (a) GFP-BIMG localised at the area from which a branch emerges (arrow) and subsequently associated with the plasma membrane in the apical dome of the branch tip. (b) GFP localization in a developing septum (arrows) and associated with the plasma membrane in the apical domes of two growing hyphal tips. Bar = 10 µm.
Fig. 4.26 *Neurospora crassa* stained with SYTO 13. Note the apparent staining of mitochondria, which swelled and possibly burst within the hypha after laser irradiation. The nuclei (arrows) become increasingly stained, whilst the tubular mitochondria become decreasingly stained with time. Bar = 10 μm.

Fig. 4.27 *Basidiobolus ranarum* stained with SYTO 11. (a) Transmitted light image showing nucleus (n), nucleolus (nu) and nuclear vacuole (nv); (b) confocal image; (c) high magnification confocal image. Note the large brightly stained nucleolus and unstained nuclear vacuole in (b) and (c). Bars = 10 μm.
4.2.4 Endoplasmic reticulum (ER)

The ER was imaged in *A. nidulans* expressing GFP fused to an chitinase export signal and ER retention signal peptide, under control of the AlcA promoter. Fluorescence was observed throughout hyphae and was brighter towards tips of growing hyphae (Fig. 4.28a; Movies 4.21, 4.22). High-resolution images revealed the extensive, reticulate network throughout the cytoplasm and which surrounded nuclei (Fig. 4.28b; Movie 4). Brightly stained regions were also frequently observed (Fig.4.28b), and possibly represented over expressed GFP sequestered within organelles. Hyphal tips became narrow after prolonged laser irradiation, indicating phototoxic effects resulting from the presence of GFP.

**Fig. 4.28** *A. nidulans* expressing GFP localized to the ER. (a) Low magnification. (b) High magnification: tip region. (c) High magnification: sub-apical region. Note the reticulate network extends through the cytoplasm, more fluorescent towards the hyphal tip (arrow). Also note the presence of brightly stained regions (B) in (b). Negatively stained nuclei are indicated by asterisks. Bars = 10 μm.
4.2.5 Golgi

*Aspergillus nidulans* expressing a GFP fused to the α-coatamer of the COP1 protein revealed the structure and behaviour of Golgi cisternae in living hyphae. At low magnification, fluorescence from Golgi was brighter towards the tips of growing hyphae (Figs. 4.29a,b; Movie 4.23) mainly because the tip regions were more densely populated with Golgi. High power images showed that some Golgi formed donut-shaped structures 1-2 μm in diameter (Fig. 4.29c). Golgi were extremely mobile within the hyphae (Movies 4.23, 4.24), and often appeared to fuse together and break apart. A 3-D reconstruction that showed the distribution of Golgi within a hypha can be seen in Movie 4.25.

![Fig. 4.29](image)

**Fig. 4.29** *Aspergillus nidulans* expressing GFP targeted to Golgi. (a) Transmitted light image of a germinating conidium. (b) Golgi localisation in the same germinating conidium as in (a). Bar = 10 μm. Note the increased amount of Golgi towards the hyphal tips. (c) High magnification image of Golgi in a growing hyphal tip. Bar = 10 μm. (d) Digitally enhanced images showing donut-shaped Golgi cisternae. Bar = 1 μm.
4.3 Discussion

4.3.1 The vacuolar network

Using vacuole-targeted GFP and the fluorescent dyes, carboxy-DFFDA and FM4-64, the vacuolar network was imaged in living hyphae of *A. nidulans*. These probes indicated that distinct morphological types of vacuoles exist in different hyphal regions. Interestingly, the intensity of GFP fluorescence was found to vary within hyphae. Furthermore, the smaller spherical and tubular vacuoles were more brightly stained, whereas the larger spherical vacuoles in sub apical regions were less intensely fluorescent. It is unclear why this variation in fluorescence occurred. It may be related to different concentrations of soluble GFP in different vacuolar compartments, or variations in vacuolar pH (Kneen et al. 1998). The fusion of two differently stained vacuoles was observed, and it appeared that the fluorescence became equilibrated in the resultant vacuolar compartment (Movie 4.3). In this study, the tubular and spherical vacuoles observed in *A. nidulans* bear similarities to those observed in *Pisolithus tinctorius* hyphae stained with carboxy-DFFDA (Cole et al. 2000). However, considerable variation in vacuolar morphology exists between different species, particularly in those with large diameter hyphae (e.g. *N. crassa* and *Phycomyces blakesleeanus*).

4.3.2 Mitochondria

In the apical hyphal compartment mitochondria are typically tubular organelles, resembling bacteria, and are responsible for ATP production within hyphae (Markham, 1995). In subapical regions of *N. crassa*, mitochondria tended to be shorter and more compact (Fischer-Parton et al. 2000). Hyphal tips of filamentous fungi are relatively tightly packed with active mitochondria, which are obviously an important requirement for fast tip growth. Mitochondria were mainly longitudinally arranged within tips of hyphae, although they often moved in different directions, both longitudinal and lateral directions. The staining pattern of mitochondria was similar using fluorescent dyes and mitochondrion-targeted GFP. Variations in mitochondrial size and number were observed between different species.

Rhodamine-123 is a potentiometric dye, which accumulates in mitochondria, and its fluorescence is dependent on membrane potential. Increasing dye activity indicates increasing mitochondrial membrane potential (Goldstein and Korczack 1981). My
results demonstrate that the mitochondrial membrane potential is greatest towards growing hyphal tips and this indicates that mitochondria are most active in this region. This is not surprising since hyphal tip growth requires considerable amounts of energy.

In a time-lapse sequence, the division of a single mitochondrion was observed for the first time in a living hypha. The confocal section through a hypha was focused close to the glass coverslip, as there appeared to be more longitudinally arranged mitochondria adjacent to the plasma membrane. The sequence showed pinching in the middle of a mitochondrion, which then broke apart and the two daughter mitochondria moved rapidly away from each other. This binary fission of mitochondria resembles binary fission in bacteria (Alberts et al. 1994).

4.3.3 Nuclei

GFP has proved to be an excellent probe for monitoring nuclei within living fungal hyphae with minimal perturbatory effects. Extensive time-lapse sequences were obtained using confocal microscopy, allowing detailed observation of mitosis in living hyphae. So far, I have not been able to find any fluorescent dyes that permit observations of nuclei in living hyphae without cytotoxic effects. The waves of nuclear division, which have been previously reported by Aist and Morris (1999), were particularly striking and were shown to move in either direction along a hypha, and occasionally in both directions from a central region of a hypha (data not shown). The nature of the signal that induces nuclear division is unclear, but calcium waves are a possibility (Jaffe and Creton, 1998). The SPB is a small fibrillogranular organelle, attached to the outside of the nuclear envelope that separates and divides in early prophase. The roles of SPBs and microtubules in independent nuclear motility were reviewed recently by Aist (1995). In the confocal sequences obtained in this study, it is apparent that the SPBs play a role in the movement and positioning of nuclei within fungal hyphae.
4.3.4 Endoplasmic reticulum

In this study, the ER was imaged in *A. nidulans* using GFP fused to a chitinase export signal and an ER retention signal peptide. It formed a complicated reticulate network through the cytoplasm and around nuclei, and extended right up to the hyphal tip. Cole *et al.* (2000) used ER tracker and BIODIPY-Brefeldin A to differentiate the ER and Golgi cisternae from the tubular vacuolar system in *Pisolithus tinctorius*. The images of *Pisolithus tinctorius* stained with ER-Tracker published by Cole *et al.* (2000) superficially resembled that of GFP localisation in *A. nidulans*. However, the confocal images obtained in this study were of higher resolution and showed the ER in far more detail. In the GFP-ER strain analysed here, brightly stained structures were observed throughout hyphae, particularly at their tips. It is unclear whether these structures were an artifact resulting from accumulation of GFP in organelles (e.g. vacuoles), or actually sheet-like components of the ER. My preliminary observations of *N. crassa* hyphae stained with the uv-excited dye ER-tracker blue white, and imaged using 2-photon microscopy, revealed similar fluorescent staining to that of GFP targeted to the ER in *A. nidulans* (data not shown).

4.3.5 Golgi

An interesting feature of Golgi cisternae in filamentous fungi is that they do not typically form stacks, as is characteristic of plant and animal cells (Howard 1981). Here, GFP targeted to the Golgi apparatus has been imaged in filamentous fungi for the first time. Golgi moved extensively throughout the hyphae. The resolution of confocal microscopy was not sufficient to resolve the cisternal structures in detail. However, enhanced images clearly showed the donut-shapes that individual Golgi cisternae often had. Similar structures have been observed in freeze-substituted hyphae of *A. nidulans* observed at the ultrastructural level (R. Roberson, personal communication). Brefeldin-A is an inhibitor known to disrupt transport from the ER to the Golgi. Surprisingly, no noticeable effects were observed following addition of Brefeldin-A to hyphae, even at high (100 μM) concentrations (data not shown). From this unexpected result, it could be concluded that Brefeldin-A was either not taken up into hyphae, or the strain of *A. nidulans* might be resistant to Brefeldin-A. Anomalous effects of Brefeldin A have been previously reported in hyphae of *Magnaporthe grisea* (Bourett and Howard 1991).
Chapter 5: Analysis of mutants

5.1 Introduction

*Neurospora crassa* provides an excellent system for the study of gene function. An extensive range of morphological mutants have been described (Perkins *et al.* 2000). Many of the mutant genes have been cloned and analysed at the molecular level. However, there is a distinct lack of detailed analyses of the phenotypes of living mutant cells. Fluorescent staining, time-lapse imaging and 3-D imaging of living hyphae are powerful tools that can reveal the complex characteristics of mutants. The study of living mutant cells should significantly increase our understanding of how fungal genes function *in vivo.*

Four morphological mutants of *N. crassa* were studied using FM4-64 and other vital fluorescent dyes:

1. **cot-I**: a temperature-sensitive hyperbranching mutant. The *cot-1* gene has been shown to encode the COT1 protein, a serine-threonine specific protein kinase (Yarden *et al.* 1992). After shifting to the restrictive temperature (>32°C) hyphal extension is inhibited and there is a massive induction of hyphal branching (Collinge and Trinci 1978).

2. **spray**: a hyperbranching mutant which displays near apical branching. The *spray* gene has been cloned and found to encode a membrane protein thought to be involved in Ca\(^2+\) regulation (Bok *et al.* 2001).

3. **Nkin**: a null mutant that lacks the conventional kinesin motor protein. Seiler *et al.* (1996) reported that hyphae of this mutant lacked a discrete Spitzenkörper and exhibited abnormal morphology but showed virtually no defects in organelle movement.

4. **slime**: a combination of three mutations: *fz* (fuzzy), *sg* (spontaneous germination), and *os-I* (osmotic), which results in cells being unable to produce a functional cell wall. Mutant cells thus exist as protoplasts (Emerson 1963). Studies by Steinberg and Schliwa (1993) showed that organelle movement in *slime* protoplasts are mediated by microtubules.

The primary aim of this chapter was to analyse the phenotypes of these mutants, in significantly more detail than has been previously achieved, using the live-cell imaging techniques described in chapters 3 and 4. These studies were carried out...
in order to gain a greater insight into the functional roles of the various genes mutated.

5.2 Results

5.2.1 *cot-1*: colonial temperature-sensitive hyperbranching mutant

Hyphae of *cot-1* grown at the permissive temperature of 25°C appeared identical to wild type hyphae (Fig. 5.1). Colonies of *cot-1* were grown at 37°C and stained with FM4-64. The stained hyphae were then imaged by confocal microscopy on a temperature-controlled stage at 37°C. After 3 h at 37°C, hyphae underwent increased septation, with individual compartments becoming very swollen. Many of these swollen hyphal compartments produced short branches that narrowed towards their tips, which were very strongly fluorescent (Fig. 5.2; Movie 5.1). The apical hyphal compartment frequently underwent apical or near apical branching (Fig. 5.2a). Accumulation of strongly stained intracellular inclusions were observed throughout hyphae and the large spherical vacuoles, commonly observed adjacent to septa in wild type hyphae, were rarely seen (Fig. 5.2). In order to observe these changes in more detail, samples were incubated at 37°C, to induce the mutant phenotype, and examined at hourly intervals. However, in these experiments the fungi were imaged at room temperature (~25°C). Although hyphae were imaged within 1 min of being transferred from the restrictive to the permissive temperature, surprisingly, the wild type phenotype had already started to recover (Figs. 5.2a, 5.3a, 5.4a). The recovery of hyphae was manifested by slight swelling of the branch tips. Nevertheless, it was still possible to identify changes that had occurred at hourly intervals after the upshift from 25°C to 37°C in these images (Fig. 5.2a, 5.3a, 5.4a). After 1 h at the restrictive temperature, hyphae had stopped growing and narrowed towards the tips. Multiple septa appeared within 100 μm of hyphal tips and accumulation of stained material was observed towards the tips (Fig. 5.3). After 2 h at 37°C more septa and branches had formed and the hyphal compartments of the main hyphae had started to become swollen (Fig. 5.4b). After 3 h the mutant phenotype had become further accentuated (Figs. 5.2 and 5.5a).

Following a shift down to 25°C the hyphae recovered rapidly and resumed normal growth (Figs. 5.3 - 5.5). The narrow tips (2-3 μm) became bulbous, and increased in diameter up to a width of 5-10 μm, and eventually, within ~30 min, resumed the appearance of normal growing hyphae. AVCs were observed in the tips of
recovered hyphae within ~15 min of the shift to 25°C. 4D (x,y,z and time) rendering provided a new method to observe the recovery of hyphae (Fig. 5.6; Movie 5.2). After 24 h at the restrictive temperature the phenotype was even more dramatic (Fig. 5.7). Hyphae of cot-1 grown at 37°C, stained with Nile Red, contained what appeared to be large central accumulations of lipid material (Fig. 5.8).

**Fig. 5.1** Neurospora crassa stained with FM4-64. (a) Wild type grown at 37°C; (b) cot-1 grown at 25°C. Bar = 10 µm.

**Fig. 5.2** Neurospora crassa cot-1 grown at 37°C for 3 h and stained with FM4-64 for 30 min. (a) Apical region; (b) 3D stereogram; (c) sub-apical region (d) 3D stereogram. Note the brightly stained material within hyphae and at the tips of branches. Bars = 10 µm.
Fig. 5.3 Neurospora crassa cot-1 stained with FM4-64, grown at 37°C for 1 h and shifted back to 25°C. Each image represents a projection of 30 x 1.0 μm optical sections through the hypha. The times represent the periods after which the hyphae were shifted to 25°C. Note the recovery of branched hyphae which become wider. Bar = 20 μm.
Fig. 5.4 *Neurospora crassa* cot-1 stained with FM4-64 grown at 37°C for 2 h and shifted back to 25°C. Each image represents a projection of 30 x 1.0 μm optical sections through the hypha. The times represent the periods after which the hyphae were shifted to 25°C. Note the swelling of hyphal compartments of the main hyphae and presence of multiple septa. Bar = 20 μm.
Fig. 5.5  *Neurospora crassa* cot-1 stained with FM4-64 grown at 37°C for 3 h and shifted back to 25°C. Each image represents a projection of 30 x 1.0 μm optical sections through the hypha. The times represent the periods after which the hyphae were shifted to 25°C. Note the bulbous compartments of the main hypha and the finely tapered branches which become wider during recovery. Bar = 20 μm.
Fig. 5.6 Neurospora crassa cot-1 stained with FM4-64, grown at 37°C for 3 h and then shifted to 25°C. Each image represents a projection of 30 x 1.0 μm optical sections through the hypha. The times represent the periods after which the hyphae were shifted to 25°C. Green = growth at 37°C; red = growth at 25°C. Bar = 20 μm.
Fig. 5.7  *Neurospora crassa* cot-1 stained with FM4-64 and grown at 37°C for 24 h. (a) Projection of 40 x 2.5 μm optical sections; (b) 3D stereogram. Bar = 20 μm.
Fig. 5.8 *Neurospora crassa* cot-1 grown at 37°C for 3 h and stained with Nile Red. (a) Transmitted light, bright field image; (b) Confocal fluorescence image indicates that the hyphal compartments probably contain large accumulations of lipid material. Bar = 20 μm.
5.2.2 spray: hyper-branching mutant

Hyphae of spray grew slowly and branched to form a dense mycelial network. Hyphae stained with FM4-64 exhibited bright staining towards their tips, and small AVCs were observed in all growing tips (Fig. 5.9; Movies 5.3, 5.4). Septa appeared to form much closer towards the hyphal tips compared with that in wild type and as a result, hyphal compartments were much shorter (compare Fig. 5.9 with Fig. 3.21). A comparison of FM4-64 and FM1-43 stained colonies is shown in Fig. 5.10. FM4-64 staining revealed the AVCs to be very mobile, within the hyphal apices, and this accounted for the very irregular growth pattern of individual hyphae. The AVC was much smaller in spray compared with that of wild type hyphae (Figs. 5.11a,b; Movie 5.4). Hyphae of spray stained with FM1-43 showed mitochondria, which were brighter within tips (Figs. 5.10b, 5.11d). This degree of variation in fluorescence was not as distinct in the wild type (Fig. 5.11c). Negatively stained nuclei were often observed ~10 μm from hyphal tips, whereas in wild type hyphae nuclei rarely were observed less than 20 μm from the tip (Figs. 5.11a,b). Overall in spray, the internal organization seems to be more compact and hyphal compartments shorter than in the wild type.

Abnormal hyperbranching was often observed in stressed slide cultures of wild type Neurospora crassa that had become dehydrated and anoxic (Fig. 5.13).

Fig. 5.9 Neurospora crassa mutant spray stained with FM4-64. Note the bright staining towards hyphal tips, and multiple septa. Bar = 50 μm.
Fig. 5.10 Neurospora crassa mutant spray. (a) Stained with FM4-64. Note the stained AVCs, negatively stained nuclei (n), and the gradient in membrane staining towards hyphal tips. (b) Stained with FM1-43. Note the brightly stained mitochondria at tips. Bar = 10 μm.
Fig. 5.11 *Neurospora crassa* wild type and mutant *spray*. (a) Wild type stained with FM4-64; (b) *spray* stained with FM4-64; (c) wild type stained with FM1-43; (d) *spray* stained with FM1-43. Note that the negatively stained nuclei (n) appear closer to the hyphal tip in *spray* (b) than in wild type (a). Bar = 10 μm.

Fig. 5.12 *Neurospora crassa* wild type hyphae under stressful conditions and stained with FM4-64. Note multiple branching, irregular hyphae and presence of septa relatively close to hyphal tips. Bar = 20 μm.
5.2.3  *Nkin*: kinesin deficient mutant

A kinesin deficient mutant (*Nkin*) of *Neurospora crassa* was generated by Seiler et al. (1996). The authors reported that hyphae exhibited abnormal growth and lacked a discrete Spitzenkörper, but otherwise the movement and organisation of organelles were normal. In my study, fluorescent dyes were used to compare differences in organelle organisation (AVC, mitochondria and the vacuolar network) between *Nkin* and wild type hyphae. Figure 5.13 shows low power confocal images of *Nkin* hyphae stained with FM4-64 (for the AVC), DASPMI (for mitochondria) and carboxy-DFFDA (for vacuoles), respectively.

Hyphae of *Nkin* stained with FM4-64 showed bright fluorescent staining towards their tips, and grew much slower than wild type hyphae. The AVCs were much smaller than in wild type hyphae (Figs 5.13 a, 5.14b). The AVCs were very mobile and moved around within the apical region, causing very irregular growth pattern (Movies 5.5, 5.6). In contrast to wild type hyphae (section 3.2.12), hyphal fusions occurred in regions around the leading edge of the colony and within 200 μm the of tips of leading hyphae (data not shown). Mitochondria stained with DASPMI were shorter in length in *Nkin* than in wild type hyphae. In most of the *Nkin* hyphae observed, mitochondria were mostly absent from the first 10 μm of hyphal tips (Figs. 5.13b, 5.14d; Movie 5.7). However, single mitochondria could often be observed moving in and out of the tip region (Movie 5.7). Carboxy-DFFDA staining revealed the tubular vacuolar network in *Nkin* hyphae, which was much more compact and globular than the spaghetti-like vacuoles observed in wild type hyphae (compare Fig. 5.13c with Fig. 4.5). Similarly, the tubular vacuolar network stained with FM4-64 exhibited less dynamic movement compared with that in wild type hyphae (Movie 5.6).
Fig. 5.13 Neurospora crassa kinesin deficient mutant (Nkin). (a) Hyphae stained with FM4-64 to show AVCs within hyphal tips; (b) hyphae stained with DASPMI to show mitochondria (arrow = hyphal tip); (c) hyphae stained with carboxy-DFFDA to show the vacuolar network. Bar = 20 μm.
Fig. 5.14 *Neurospora crassa* wild type and kinesin deficient mutant (*Nkin*). (a) Wild type AVC stained with FM4-64; (b) *Nkin* AVC stained with FM4-64; (c) wild type mitochondria stained with DASPMI (arrow = hyphal tip); (d) *Nkin* mitochondria stained with DASPMI (arrow = hyphal tip). Note that the mitochondria are mostly absent close to the hyphal tips of *Nkin* but not the wild type, and that the AVC in *Nkin* is much smaller than in the wild type. Bar = 5 μm.

### 5.2.4 *slime*: wall-less mutant

The pattern of FM4-64 staining in *slime* protoplasts was similar to that of wild type hyphae: the plasma membrane became immediately stained, followed by the staining of small fluorescent cytoplasmic inclusions (putative endosomes) (Fig. 5.16a) and finally staining of vacuolar membranes after 20 min (Fig. 5.16b). An AVC was not observed. *Slime* cells in the presence of FM4-64 exhibited rapid staining of their cell contents when they burst (Fig. 5.16c; Movies 5.8, 5.9).
Fig. 5.15 *Neurospora crassa* slime mutant after staining with FM4-64, which is continuously present in the external medium. (a) Stained for 10 min (left - transmitted light, bright field image; right - fluorescence image); (b) stained for 20 min (left - transmitted light, bright field image; right - fluorescence image); (c) The same cell as in (b), which has burst. Bar = 10 μm.
5.3 Discussion

5.3.1 cot-1

In *N. crassa*, COT-1 kinase was shown to be essential, as insertional inactivation of the *cot-1* gene was lethal (Yarden *et al.* 1992). Gorovitz *et al.* (1999) identified 2 COT-1 isoforms, and co-immunoprecipitation studies revealed that the isoform of lower molecular weight (67 kDa) was greatly reduced at the restrictive temperature. Because of the apparent structural and functional similarities of COT-1 kinase to mammalian dystrophia myotonica (DM) kinases (Mahadevan *et al.* 1993), *N. crassa* may provide an excellent model system in which to understand DM kinase function. In the present study it was found that the mutant phenotype of *cot-1* became noticeable within 1 h of shifting from the permissive temperature (25°C) to the restrictive temperature (37°C). The main hyphae grew very slowly, underwent multiple septation and the hyphal compartments became swollen. The hyphal branches induced at the restrictive temperature were typically thinner in diameter than that of the wild type, and unlike the wild type were markedly narrower towards their tips. The branches were packed with brightly stained material (after staining with FM4-64) and their branches grew extremely slowly. The fast recovery of hyphae and their branches following a shift back down to the permissive temperature was revealed by time-lapse sequences.

Large central regions of *cot-1* at 37°C that did not stain with FM4-64 were probably lipid reserves, because they stained up with Nile Red (Greenspan *et al.* 1985). The large spherical vacuoles that are often adjacent to septa in wild type hyphae were rarely observed in *cot-1* hyphae that had been grown at 37°C. Overall, the present study has shown that a number of dramatic changes occurred within hyphae during the expression of the mutant phenotype over a period of several hours, but that the shift back to the permissive temperature resulted in a rapid recovery of hyphae within minutes. The molecular mechanism of these changes is not known, but may be related to the disappearance of the lower molecular weight COT-1 isoform at the restrictive temperature (Gorovits *et al.* 1999). The results here provide further evidence that the COT-1 kinase is required for hyphal tip elongation and involved in septation and hyphal branching (Collinge *et al.* 1978; Yarden *et al.* 1992). The results reported here also provide a much more detailed insights into the complex pleiotropic effects of the *cot-1* mutation on the *cot-1* mutant phenotype.
5.3.2 spray

The spray gene has been cloned by Griffiths et al. (2001) and has been proposed to encode a protein involved in calcium regulation. The hyphae of spray were thinner in diameter and slower growing than wild type hyphae. Stained with FM4-64, the AVC appeared to be smaller and more mobile than wild type hyphae, which was reflected in the more irregular growth pattern of individual hyphae. In FM4-64 stained hyphae, unstained nuclei were closer (<10 μm) to the tips of spray hyphae compared to wild type hyphae (>30 μm). FM1-43 provided an excellent mitochondrial marker to compare the organisation and distribution of mitochondria in wild type and spray mycelia. Mitochondria in spray appeared to be relatively normal in morphology, although smaller and more tightly packed within the tips of hyphae compared to that observed in wild type hyphae.

5.3.3 Nkin

The previous studies on Nkin lacked detailed observations on organelle dynamics in growing hyphae (Seiler et al. 1996). The data presented in this chapter showed that a lack of the conventional kinesin motor protein had several effects on the cytoplasmic organisation within hyphae and together these defects had apparently resulted in the abnormal hyphal morphologies observed. FM4-64 provided an excellent marker for following hyphal tip growth and revealed the AVCs within hyphal tips, which often appeared as small clouds of fluorescence, rather than discrete, strongly fluorescent structures as observed in wild type hyphae. The growing hyphal tips grew much slower than wild type (Seiler et al., 1996) and exhibited a more irregular and branched growth pattern.

Previously, it was also reported that the Nkin mutant lacked a clearly observable Spitzenkörper (Seiler et al. 1997). In the present study, and also based on the findings of Fischer-Parton and Read (unpublished), it is clear that this is not the case. However, it is clear that the Spitzenkörper of Nkin is abnormal, being smaller than the wild type, often not as discrete, and much more mobile. Conventional kinesin may therefore have a role in regulating the mobile behaviour of the Spitzenkörper and in concentrating the secretory vesicles in a discrete AVC. Seiler et al. (1997) also reported that conventional kinesin may play a role in transporting wall-building secretory vesicles to the hyphal tip. This may be true but because an
AVC was still present and mutant hyphae still continued to grow, albeit more slowly than wild type, it seems that other mechanisms for transporting secretory vesicles to the hyphal tips may also exist.

The results reported here also indicate that conventional kinesin plays a role in positioning mitochondria within the hyphal tip, because, in contrast to wild type, a 10-15 μm region largely devoid of mitochondria was observed in the Nkin mutant. A similar observation was made in Nectria haematococca in which the conventional kinesin gene had also been mutated (Wu et al. 1998). Conventional kinesin may also be important in regulating mitochondrial size because in Nkin hyphae they were much shorter than in the wild type. Staining of the vacuolar network using carboxy-DFFDA showed that tubular vacuoles occupied a smaller region (50-100 μm) than that in wild type hyphae, in which they typically extend for 200-500 μm (see Fig. 4.4). The pattern of vacuole staining in Nkin was also found to be abnormal, indicating that kinesin plays an important role in regulating vacuolar organisation.

Overall, these results with the Nkin mutant suggest that conventional kinesin is an important motor protein in N. crassa and may be involved in the delivery of secretory vesicles to the tip, the behaviour of the AVC, the positioning and size of mitochondria, and in the organisation of the tubular vacuolar network.

5.3.4 slime

Slime was originally described by Emerson (1963) as a mutant of Neurospora, lacking functional cell walls, that exists as protoplasts. The cells are prone to osmotic stress, and require careful handling. Although slime cells were described as protoplasts, it is important to remember they are defective in 3 different genes and therefore fundamentally different from protoplasts that are routinely prepared by enzymatic digestion of the cell walls. The pattern of FM4-64 staining was similar to that of wild type hyphae (section 3.2.1), and is believed to be indicative of the endocytic pathway via endosomes to the vacuole. When cells surrounded by the dye burst, the membranous contents became stained immediately. This observation supports the finding that FM4-64 is not internalised by diffusion (see section 3.2.1) and provides further evidence for the occurrence of endocytosis in fungal hyphae. FM4-64 staining of an AVC was not observed in slime cells, as expected from their non-polar morphology.
Chapter 6:

Summary and future work

Chapter 3 was initially focused on the endocytic internalisation of the dye FM4-64 into living hyphae. The experiments showed that dye uptake is time and energy dependent in Aspergillus nidulans and Neurospora crassa. Previously, there has been little evidence for the occurrence of endocytosis in filamentous fungi. The results indicated that endocytosis is an important and dynamic process, which takes place in both apical and sub-apical regions of hyphae. FM4-64 was further exploited as a general membrane-selective stain to generate a detailed study of several key processes in living fungal hyphae including tip growth, branching, septum formation and sporulation. The results obtained using FM4-64 indicated that it is currently the best vital dye for live cell imaging of filamentous fungi.

Chapter 4 used the techniques developed in Chapter 3, which were applied to imaging organelle dynamics using vital fluorescent dyes and GFP. The comparison of GFP with fluorescent dyes revealed important differences in staining patterns, and indicated that GFP is a more specific probe for targeting organelles. In addition, GFP was shown to be less susceptible to phototoxicity and photobleaching.

In order to further correlate the localisation and organisation of organelles in living cells, it would be interesting to compare organelle morphology and organisation of fluorescently stained hyphae with that observed in unstained hyphae observed by video enhanced microscopy (López-Franco et al, 1995). At the ultrastructural level, freeze-substituted hyphae could be observed with the transmission electron microscope using anti-GFP immunolabelling to localise the GFP. Further work involving Nipkov spinning disc confocal microscopy would permit imaging organelle dynamics with a higher temporal resolution. Two-photon microscopy has been reported to result in reduced phototoxicity and photobleaching (Cox and Sheppard, 1999). Two-photon microscopy could also be used to image organelle dynamics, with the possibility of uv-excited organelle dyes (e.g. ER-tracker).

Recently, a wide range of recombinant fluorescent proteins have been developed which exhibit different spectral properties (e.g. cyan fluorescent protein [CFP], yellow fluorescent protein [YFP] and red fluorescent protein [dsRFP]). These new
probes may be used to image multiple organelles in the same hypha at the same time. Experiments should be carried out to examine the effects of various pharmacological agents (e.g. Latrunculin A, benomyl), which perturb the cytoskeleton, in combination with vital dyes and recombinant probes. This additional data should provide a greater understanding of the role of the cytoskeleton in regulating the dynamics and organisation of individual organelles.

Chapter 5 provided a study of four morphological mutants of *Neurospora crassa* and using the fluorescent dyes and imaging techniques developed in the previous chapters. Four different mutants were selected based on their phenotypes. The temperature sensitive mutant *cot-1*, produces a highly branched phenotype which is difficult to image using conventional light microscopy. The use of optical sectioning and 3-D reconstruction allowed me to resolve the structure of mutant hyphae in detail. A mutant lacking the conventional kinesin motor protein was showed to display an abnormal growth pattern and imaging using the mitochondrial dye DASPMI demonstrated that mitochondria in this mutant are mostly absent from the tip region. The hyperbranching mutant *spray*, about which relatively little is known, was imaged using time-lapse techniques and revealed the dynamic branching pattern. Important differences in organelle distribution were also observed. The wall less mutant *slime* exists as protoplasts and the experiments provided further evidence that FM4-64 is actively internalised by endocytosis. Live-cell imaging will provide a greater understanding as to how mutations affect the physiology of living cells and could be used to analyse a wider range of mutants (e.g. strains compromised in endocytosis, secretion, tip growth).

Overall, this thesis has resulted in the development of many new techniques for live-cell imaging in hyphae and has provided a significantly novel insight into the biology of living fungal cells. These techniques will be useful to other researchers and help them to produce novel and high quality data from living fungi using confocal microscopy. The time lapse and 3-D movies will also provide valuable educational resources, and will hopefully encourage future research on fungal cell biology, especially involving the analysis of living cells.
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Chapter 8: Publications


Appendix: Movies

Contents

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Chapter 3: Analysis of the vesicle trafficking network

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Chapter 5: Analysis of mutants

Viewing instructions

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Chapter 2: Materials and Methods

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Neurospora crassa

06

Nkin

FM4-64

Hyphal growth (High Power)

07

Neurospora crassa

Nkin

DASPMI

Mitochondria

08

Neurospora crassa

slime

FM4-64

protoplast lysis

09

Neurospora crassa

slime

FM4-64

protoplast lysis

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Confocal microscopy of FM4-64 as a tool for analysing endocytosis and vesicle trafficking in living fungal hyphae

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Key words. Confocal microscopy, endocytosis, FM1-43, FM4-64, fungal hyphae, Spitzenkörper, tip growth, TMA-DPH, vesicle trafficking.

Summary
Confocal microscopy of amphiphilic styryl dyes has been used to investigate endocytosis and vesicle trafficking in living fungal hyphae. Hyphae were treated with FM4-64, FM1-43 or TMA-DPH, three of the most commonly used membrane-selective dyes reported as markers of endocytosis. All three dyes were rapidly internalized within hyphae. FM4-64 was found best for imaging the dynamic changes in size, morphology and position of the apical vesicle cluster within growing hyphal tips because of its staining pattern, greater photostability and low cytotoxicity. FM4-64 was taken up into both the apical and subapical compartments of living hyphae in a time-dependent manner. The pattern of stain distribution was broadly similar in a range of fungal species tested (Aspergillus nidulans, Botrytis cinerea, Magnaporthe grisea, Neurospora crassa, Phycomyces blakesleeanus, Puccinia graminis, Rhizoctonia solani, Sclerotinia sclerotiorum and Trichoderma viride). With time, FM4-64 was internalized from the plasma membrane appearing in structures corresponding to putative endosomes, the apical vesicle cluster, the vacuolar membrane and mitochondria. These observations are consistent with dye internalization by endocytosis. A speculative model of the vesicle trafficking network within growing hyphae is presented.

Introduction
Vesicle trafficking is fundamental to numerous activities in eukaryotic organisms, and underlies many of the basic processes involved in cell growth and differentiation. The vesicle trafficking network includes exocytosis and endocytosis (Gruenberg & Clague, 1992; Rothman, 1994). In filamentous fungi a reasonable amount is understood about exocytosis, whereas very little is known about endocytosis.

Most of our current understanding of vesicle trafficking in filamentous fungi is concerned with tip growth and is based upon ultrastructural studies (e.g. Grove & Bracker, 1970; Howard, 1981), pharmacological treatments (e.g. Howard & Aist, 1980), analyses of mutants (Wu et al., 1998; Seiler et al., 1999) and mathematical modelling of vesicle trafficking in relation to tip growth (e.g. Bartnicki-Garcia et al., 1989). During tip growth, extension of the hypha is confined to a region occupying only a few micrometres at the hyphal apex and involves highly polarized exocytosis. Secretory vesicles deliver membrane, cell wall precursors and wall-building enzymes to the hyphal tip, and many extracellular enzymes released into the surrounding medium are also believed to be secreted from this region (reviewed by Wessels, 1993).

In higher fungi (i.e. members of the Ascomycota, Basidiomycota and Deuteromycota) vesicle trafficking to the apex during tip growth is highly organized and involves the activity of a specific, multicomponent organelle complex which, in most cases, is called the Spitzenkörper (= 'apical body'). This structure is predominated by secretory vesicles which make up what is commonly described as an 'apical vesicle cluster' (Grove & Bracker, 1970; López-Franco & Bracker, 1996). The dynamic behaviour of the Spitzenkörper has indicated that it is intimately associated with the precise growth pattern of the hyphal apex (Girbardt, 1957; Bartnicki-Garcia et al., 1995; López-Franco & Bracker, 1996).

From studies of tip-growing plant cells (Steer & Steer, 1989; Miller et al., 1997) it has been suggested that membrane recycling via endocytosis is a critical part of the process of apical extension. However, reports of the existence of endocytosis in filamentous fungi have been conflicting. Work by Caesar-Ton That et al. (1987) identified a fraction from hyphae of Neurospora crassa that was rich in coated vesicles and possessed a major polypeptide with a molecular weight similar to that of the heavy chain of
clathrin, the major coat protein of endocytic vesicles in animal and plant cells (Hawes et al., 1995; Mellman, 1996). However, convincing ultrastructural evidence for clathrin-coated vesicles or pits in fungal hyphae is lacking. Evidence against the occurrence of endocytosis in filamentous fungi was obtained by Cole et al. (1997), who were unable to observe uptake of membrane-impermeable fluorescent probes by fluid-phase endocytosis into hyphae of the basidiomycete *Pisolithus tinctorius*. Recently, uptake of the membrane-selective endocytosis marker FM4-64 by germ tubes of *Uromyces fabae* (Hoffmann & Mendgen, 1998) and hyphae of *N. crassa* and *Trichoderma viride* (Read et al., 1998) has been taken as positive evidence for membrane internalization by endocytosis.

Amphiphilic styryl dyes, such as FM4-64, insert into the outer leaflet of the plasma membrane and are believed not to directly enter intact cells by unfacilitated diffusion (Illinger & Kuhry, 1994; Betz et al., 1996). They have, therefore, been widely used as fluorescent reporters of endocytosis and other components of the vesicle trafficking network in animal cells (e.g. Betz et al., 1996) and the budding yeast (Vida & Emr, 1995; Rieder et al., 1996).

In this paper we have used confocal imaging of amphiphilic styryl dyes to study endocytosis and vesicle trafficking in living fungal hyphae. Although we tested three of these dyes (FM4-64, FM1-43 and TMA-DPH), most of our work has concentrated on FM4-64 because of its superior properties. Time courses of FM4-64 staining have been performed and considerable emphasis placed on imaging the stained apical vesicle cluster within growing hyphal tips. Possible pathways of dye internalization and distribution have been assessed. However, our analysis supports the view that endocytosis and vesicle trafficking are probably the predominant pathways by which FM4-64 is internalized and distributed between organelles within hyphae. Based on the interpretation of our results in the context of current knowledge in other cell types, we present a speculative model of the vesicle trafficking network within growing hyphae.

**Materials and methods**

**Dyes and other chemicals**

The dyes FM4-64, FM1-43 and TMA-DPH were obtained from Molecular Probes Inc. (Eugene, OR, U.S.A.). All other chemicals were supplied by Sigma (Poole, U.K.).

**Fungal material**

The following fungi were used: *Aspergillus nidulans* (R153 from Fungal Genetics Stock Center [FGSC], Arcata, CA, U.S.A.); *Botrytis cinerea* (from the Plant Disease Control Group, DuPont Co., Wilmington, DE, U.S.A.); *Magnaporthe grisea* (strain 0-42 from B. Valent, DuPont Co.); *Neurospora crassa* 74 A (strain 262 from FGSC); *Phycomyces blakesleeanus* (isolated from soil by P. Hickey, Edinburgh, U.K.); *Puccinia graminis* f. sp. *tritici* (isolate 84 from the National Institute for Agricultural Botany, Cambridge, U.K.); *Rhizoctonia solani* (No. 283 from E. E. Butler, University of California, Davis, U.S.A.); *Sclerotinia sclerotiorum* (from T. S. Abney, Purdue University, IN, U.S.A.); and *Trichoderma viride* (No. 2011 from J.E. Tuite, Purdue University).

**Growth conditions and dye loading**

*Aspergillus nidulans*, *B. cinerea* and *P. blakesleeanus* were grown on 2% w/v malt extract solidified with 2% w/v agar. Twenty-four-hour-old cultures of *P. blakesleeanus* and 48-h-old cultures of *A. nidulans* and *B. cinerea* were used for experiments. Agar bearing the leading edge of the colony was cut out and carefully placed, hypha side down, in liquid malt extract medium containing 25 μM FM4-64 on a glass coverslip.

*Magnaporthe grisea* was grown on oatmeal agar at 24 °C in continuous light (as described in Jelitto et al., 1994). Conidia were harvested from 10-day-old cultures, inoculated onto glass coverslips in distilled water and incubated in darkened humid chambers. The resultant germlings were stained with 7.5 μM FM4-64.

*Neurospora crassa*, *R. solani*, *S. sclerotiorum* and *T. viride* were grown on Vogel's medium N (Vogel, 1956) plus 2% w/v sucrose (VMS medium) and prepared for microscopic observation on coverslips, as described by Parton et al. (1997). After 10 min of loading with 6.4 μM FM4-64, the medium containing dye was replaced with fresh medium lacking dye. *Neurospora* hyphae were similarly stained with 6.4 μM FM1-43 or 10 μM TMA-DPH. Additionally, *N. crassa* was grown on a thin layer of VMS medium solidified with 2% w/v agarose evenly spread on a glass coverslip (Parton et al., 1997). Shortly before imaging, the mycelium was covered with 100 μL liquid VMS medium containing 6.4 μM FM4-64. For double staining of *N. crassa* hyphae with Rhodamine 123 and FM4-64, the stains were added sequentially. Firstly, 10 μM Rhodamine 123 was applied in 100 μL liquid VMS medium for at least 30 min then a further 100 μL medium containing 6.4 μM FM 4-64 was added.

*Puccinia graminis* ureudiospores (obtained from uredia on wheat as described by Read et al., 1997) were inoculated onto glass coverslips and subsequently incubated in a humid chamber for 3–5 h to allow germ tubes to form. Germlings were loaded with dye by placing 20 mm HEPEs-buffer (pH 7.2) containing 3.2 μM FM4-64 over them, followed by a coverslip supported by strips of lithographer's tape (No. 616, Scotch Brand).

For time course experiments, osmotic shock during dye...
application was avoided by acclimatizing hyphae to standard liquid medium before experimentation.

Confocal microscopy

For routine confocal microscopy we employed a Bio-Rad MRC 600 confocal laser scanning microscope fitted with a 25 mW argon laser and connected to a Nikon Diaphot TMD inverted microscope with epifluorescence equipment (all supplied by Bio-Rad Microscience, Hemel Hempstead, U.K.). The laser power used was 1 or 3% of full intensity. Excitation was at 514 nm, and fluorescence was detected at >550 nm. Simultaneous confocal fluorescence images and corresponding brightfield images were collected. A ×40 dry plan apo (NA 0.95) and a ×60 oil immersion plan apo (NA 1.4) objective were used.

UV confocal microscopy of TMA-DPH was performed using a Leica TCS NT confocal microscope (Leica Microsystems Heidelberg GmbH, Germany) fitted with a 2 W UV argon laser. The Leica system was also equipped with a 100 mW argon ion laser, which allowed simultaneous imaging of Rhodamine 123 and FM4-64 without ‘bleed through’ of signal between the two channels used (488 nm excitation; Rhodamine 123 fluorescence detected at 530/30 nm, FM4-64 fluorescence detected at >640 nm). A ×63 water immersion plan apo (NA 1.2) objective was used.

Results

Application and imaging styryl dyes in hyphae

Amongst the most commonly used fluorescent dyes for imaging endocytosis in living cells are the styryl-based dyes FM4-64, FM1-43 and TMA-DPH (Figs 1A, 2A and 3A, Illinger & Kuhry, 1994; Betz et al., 1996; Haugland, 1996). These compounds were tested in N. crassa. All three dyes were found to be taken up by both apical and subapical hyphal compartments in a time-dependent manner: immediate staining of the plasma membrane was followed by dye internalization and staining of organelles. All dyes stained the apical vesicle cluster within the Spitzenkörper (Figs 1B, 2B and 3B), although considerable differences were observed in the patterns of organelle staining and in the photosensitivity and phototoxicity of each dye.

The staining pattern with each dye was examined over the apical 50 μm of hyphae, 40 min after dye application (Figs 1B, 2B and 3B). At that time FM4-64 had clearly stained the plasma membrane and Spitzenkörper region, with a more diffuse background staining of the cytoplasm. Staining of organelles was also evident but not marked. There was little staining of mitochondria (Fig. 1B). In addition, FM4-64 caused little disturbance to apical extension, even with repeated laser scanning at 15 s intervals over periods of 10 min when imaged with a ×40 dry plan apo (NA 0.95) objective. The extension rate of dye-loaded hyphae imaged in this way was 16.7 ± 1.1 μm min⁻¹ (SE) (n = 11), whilst the extension rate of control hyphae lacking dye was 17.2 ± 0.3 μm min⁻¹ (SE) (n = 10). FM1-43 staining of the Spitzenkörper was similar except that it photobleached much more rapidly, making it more difficult to follow with time. In addition, the elongated mitochondria were more intensely stained with FM1-43 (cf. Figs 2B and 1B) unless the period of FM4-64 staining was significantly extended (Fig. 4). As with FM4-64, little significant effect on growth was observed (data not shown). TMA-DPH stained the plasma membrane and Spitzenkörper but the latter was less clearly stained than with FM4-64 (cf. Figs 3B and 1B). The cytoplasm was diffusely stained with TMA-DPH but pronounced staining of organelles was lacking (Fig. 3B). Repeated scanning with the UV laser led to drastic photobleaching of TMA-DPH and cessation of apical extension within three or four scans. These limitations render TMA-DPH useless for the type of prolonged examination required to image the dynamic behaviour of the Spitzenkörper or other aspects of vesicle trafficking.

Characteristics of FM4-64 internalization

Examination of early dye uptake within hyphal tips revealed that after immediate plasma membrane staining, signs of internalized dye could first be discerned as early as 30 s following dye application, and more clearly after 60 s (Fig. 5). Initial dye internalization was observed by contrast adjusting images and could be seen as a slight staining of the apical cytoplasm, most obviously in a 10–15 μm long region commonly 8–10 μm from the apical pole. Discrete, roughly spherical fluorescent organelles, ~0.75 μm in diameter, which corresponded in size and in their time of appearance to the putative endosomes visualized by FM4-64 staining in budding yeast cells (Vida & Emr, 1995), were first discernible 110 s after dye application. These organelles tended to be more numerous or obvious in a particular region behind the extreme tip although the precise distance of this zone from the apical pole varied between hyphae (data not shown). Spitzenkörper staining was first evident after 180 s (Fig. 5). Subsequently, the Spitzenkörper region and the small, roughly spherical organelles became brighter whilst numerous other small organelles also became stained. In hyphae stained for longer than ~15 min, numerous roughly circular regions of dye exclusion, ~2–3 μm in diameter, could be seen up to within ~20 μm of the tip (Figs 1B and 4). These regions of dye exclusion correspond to the size and location of nuclei (Zalokar, 1959). Staining of nuclear membranes was never observed.

The subapical compartments we investigated after staining with FM4-64 were within the peripheral growth zone of the mycelium (i.e. the mycelial region needed to maintain the maximum extension rate of the colony’s leading hyphae) and possessed unplugged septa. These hyphal
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Fig. 1. (A) Molecular structure of endocytosis marker dye FM4-64. (B) Confocal image of growing hyphal tip of *Neurospora crassa* stained for 40 min with FM4-64; images 30 s apart; hyphal extension rate = 12.0 μm min⁻¹.

Fig. 2. (A) Molecular structure of endocytosis marker dye FM1-43. (B) Confocal image of growing hyphal tip of *Neurospora crassa* stained for 40 min with FM1-43; images 30 s apart; hyphal extension rate = 15.3 μm min⁻¹.

Fig. 3. (A) Molecular structure of endocytosis marker dye TMA-DPH. (B) Confocal image of growing hyphal tip of *Neurospora crassa* stained for 40 min with TMA-DPH; images 1 s apart; growth rate not determined.

Note the staining of elongated mitochondria in Fig. 2(B) but not in Figs 1(B) and 3(B). Bar = 10 μm.

Compartments are metabolically very active and contribute to tip growth by the vectorial transport of cytoplasm and organelles towards growing hyphal apices (Trinci, 1971). In these compartments (Fig. 6) the immediate staining of the plasma membrane after dye application was followed within 1–2 min by the appearance of faintly stained roughly spherical organelles, ~0.75 μm in diameter, which were similar in appearance to the previously described organelles in the apical hyphal compartments. These organelles were distinct from the similarly sized, more-or-less spherical mitochondria found in subapical regions. This was shown by double staining with FM4-64 and the mitochondrion-selective stain, Rhodamine 123, which did not colocalize (Fig. 7). With time, FM4-64 staining increased in the small spherical organelles which were not mitochondria, and also in the surrounding cytoplasm and other organelles, until the small spherical organelles could no longer be clearly distinguished (3–9 min in Fig. 6). The large spherical vacuole, normally found lying adjacent to the septum of subapical hyphal compartments, was originally visible as a zone of dye exclusion. Initial staining of the vacuole membrane was observed after 9 min and the degree of staining increased thereafter (Fig. 6). Strong staining of the septum, lined on each
Fig. 4. Confocal image of a growing hyphal tip of *Neurospora crassa* stained for 105 min; hyphal extension rate = 11.5 μm min⁻¹. Note pronounced staining of elongated mitochondria (cf. Fig. 2B) and cytoplasmic region of dye exclusion (asterisk) which probably represents an unstained nucleus. Bar = 10 μm.

The intense dye fluorescence associated with the plasma membrane remained relatively constant during experiments in which dye was continuously present in the extracellular medium. Application of FM4-64 in the presence of the metabolic inhibitor sodium azide inhibited dye internalization but still allowed staining of the plasma membrane of apical and subapical hyphal compartments (Fig. 8).

FM4-64 uptake into the apical hyphal region of different fungal species (Fig. 9) was broadly similar to that recorded for *N. crassa* (Fig. 5). However, there were clear differences between species in the rate and extent to which FM4-64 was taken up into organelles, most obviously the elongated mitochondria within hyphal tips. *Aspergillus nidulans*, in particular, accumulated FM4-64 more rapidly (i.e. within 30 min) within these mitochondria (Fig. 9D).

**Staining of Spitzenkörper in different species**

In higher fungi (Ascomycota, Deuteromycota and Basidiomycota) the Spitzenkörper is a highly dynamic and pleomorphic multicomponent structure believed to contain the secretory vesicles responsible for tip growth (Grove & Bracker, 1970; López-Franco & Bracker, 1996). López-Franco & Bracker (1996) investigated Spitzenkörper morphology in the unstained hyphal tips of 32 species using computer-enhanced phase-contrast microscopy. In the present study, we have compared FM4-64 staining of the apical vesicle cluster within the Spitzenkörper of seven higher fungal species: *N. crassa* (Fig. 9A), *S. sclerotiorum*.

**Fig. 5.** Time course of FM4-64 internalization within the hyphal tip of *Neurospora crassa*. Numbers indicate time(s) after dye application (continuous loading). Confocal fluorescence images (median optical sections) are displayed alongside the same images contrast adjusted and shown in ‘negative’, in which dye-stained structures appear dark. Early uptake of dye is more easily seen in the contrast-adjusted negative images. The earliest signs of dye internalization can be seen after 30 s, most prominently within a (10–15 μm) region 8–10 μm from the apical pole. Small, roughly spherical organelles (arrowheads) are first evident between 110 s and 180 s. Staining of the Spitzenkörper region (asterisk) is first seen 180 s after dye application. Hyphal extension continued at an average rate of 21 μm min⁻¹ throughout the time course. Bar = 10 μm.

Fig. 6. Time course of FM4-64 internalization with continuous loading of a subapical hyphal region of Neurospora crassa. Numbers indicate time (min) after dye application. Small, roughly spherical structures (arrowheads) are clearly visible after 2 min. Bar = 10 μm.
Spitzenkörper). FM4-64 was found to clearly stain satellite Spitzenkörper in *S. sclerotiorum, T. viride* (Fig. 9E) and *B. cinerea* (Fig. 10). Such satellites were most frequently observed in *B. cinerea*. Satellite Spitzenkörper generally emerged adjacent to the plasma membrane a few micrometres behind the main Spitzenkörper, migrated to the apex and finally merged with the main Spitzenkörper within 15 s (Fig. 10).

Finally, we also imaged the FM4-64 stained apical vesicle cluster of the lower fungus *P. blakesleeanus* (Fig. 9H). This fungus belongs to the Zygomycota, which have been reported to possess a crescent-shaped band of apical vesicles instead of a Spitzenkörper (Grove & Bracker, 1970). FM4-64 staining revealed a crescent-shaped zone of bright fluorescence in a zone corresponding to the expected location of these vesicles.

**Discussion**

In the present paper we have demonstrated FM4-64 staining of hyphae in a range of fungal species, spanning the Ascomycota, Deuteromycota, Basidiomycota and Zygomycota. We have shown that FM4-64 is clearly an excellent stain for tracking the apical vesicle cluster or main Spitzenkörper and satellite Spitzenkörper. FM4-64 also has significant potential for the study of endocytosis and other aspects of vesicle trafficking in living fungal hyphae. Below, we discuss how, with the aid of this dye, we can gain a better understanding of endocytosis and the vesicle trafficking network of hyphae.

**Does endocytosis occur in filamentous fungi?**

Little is known about endocytosis in filamentous fungi and neither physiological nor ultrastructural analyses have provided concrete evidence that it even occurs (Ashford, 1998). The best evidence for its occurrence so far available are reports of the uptake of FM4-64 by fungal hyphae (Hoffmann & Mendgen, 1998; Read *et al.*, 1998). Our current results further substantiate this with observations of FM4-64 uptake by nine different fungal species. In contrast to this, it is interesting to note that Cole *et al.* (1998) did not observe uptake of FM4-64 by healthy hyphae of *Pisolithus tinctorius* (Basidiomycota). The reason for this difference in results is unclear. Cole *et al.* (1997) also found that membrane-impermeant fluorescent probes, such as Lucifer Yellow carbohydrazide (LYCH), which is used as an indicator of fluid-phase endocytosis in budding yeast (Dulic *et al.*, 1991), were not taken up into hyphae of *P. blakesleeanus*.
P. tinctorius (fluid-phase endocytosis involves the uptake of molecules in the lumen of endocytic vesicles). We found that although FM4-64 was rapidly internalized by *N. crassa*, *LYNH* and *Oregon Green* 10 kDa dextran did not appear to be taken up into hyphae of this fungus even after long periods of immersion in the dye (unpublished results).

In the light of such seemingly contradictory evidence, questions still need to be raised as to the occurrence of endocytosis in filamentous fungi and whether the styryl dyes do in fact reliably report endocytosis in filamentous fungi. In the absence of good evidence from other experimental techniques, the burden of proof presently lies heavily upon the FM4-64 data and is critically dependent upon the assumption that FM4-64 is internalized by endocytosis (Fig. 11A). This is certainly believed to be the case where it has been used with animal and yeast cells (Cochilla et al., 1999).

**Evidence supporting endocytic uptake of FM4-64**

The FM4-64 molecule is composed of three elements (Fig. 1A): a hydrophobic tail (which promotes partitioning into membranes), a dicationic head (which prevents passage across membranes), and a body or nucleus (which determines the spectral properties of the dye). The structure of this dye therefore places a significant energetic barrier to direct passage across the plasma membrane (Betz et al., 1996). Evidence against FM4-64 entering budding yeast cells by unfacilitated diffusion was obtained by inhibiting dye internalization in the presence of the metabolic inhibitor sodium azide, or by applying dye at low temperature (Vida & Fair, 1995). This was confirmed for fungal hyphae by our experimental observations with azide reported here. Similar inhibition was also obtained by Fischer-Parton (1999) when *Neurospora* hyphae were loaded with dye at 4 °C; when the temperature was subsequently increased to 25 °C normal dye uptake occurred.

Whilst unfacilitated diffusion of styryl dyes across membranes is unlikely, it is known that membrane phospholipids, which would similarly be expected to be resistant to passage from one side of membranes to the other, are able to cross over with the aid of flippases (Menon, 1995). Flippases are enzymes that facilitate the movement of specific phospholipids from one leaflet of the lipid bilayer of membranes to the other. Their activity is an essential requirement for membrane biosynthesis. In mammalian
Fig. 10. Confocal images of an FM4-64-stained satellite Spitzenkörper (asterisk) of Botrylis cinerea showing a time course (in s) of different stages in its formation, migration and fusion with the main Spitzenkörper. Bar = 5 μm.

cells they have been identified in both the plasma membrane and endoplasmic reticulum (ER) (Menon, 1995) but they have not, to our knowledge, been described in fungi. It is conceivable that the action of flippases could provide an alternative mechanism for the internalization of FM4-64, FM1-43 and TMA-DPH (Fig. 11B). Once dye has been translocated to the inner leaflet of the plasma membrane by flippase activity, lipid transfer proteins may then transport dye molecules to the cytosolic face of the membranes of other organelles (Fig. 11B). Lipid transfer proteins have been identified in numerous eukaryotic cells, including filamentous fungi (Record et al., 1998). Alternatively, owing to the water solubility of these dyes and reversible incorporation into many membranes, they could enter the cytosol from the cytoplasmic face of the plasma membrane and then label the external leaflet of organelle membranes (Fig. 11B). Lipid transfer proteins have been identified in numerous eukaryotic cells, including filamentous fungi (Record et al., 1998).

The case for endocytosis in fungal hyphae

Although inconclusive, it is clear that uptake of FM4-64, FM1-43 and TMA-DPH provide the best evidence to date for endocytosis in filamentous fungi. The case for endocytosis in fungal hyphae is further supported by the wealth of knowledge available from research with the budding yeast fungus. Endocytosis and components of the endocytic pathway have been well characterized in this organism at the genetic, biochemical and ultrastructural levels (Pelham, 1997; Geli & Riezman, 1998; Prescianotto-Baschong & Riezman, 1998). There is an array of physiological functions carried out in animal, plant and yeast cells which are mediated by endocytosis (Dulic et al., 1991; Hawes et al., 1995; Mellman, 1996; Geli & Riezman, 1998). Evidence that such activities are also carried out in filamentous fungi strengthens the case for endocytosis. Our data are consistent with the interpretation that endocytosis occurs in both apical and subapical hyphal compartments. At present we do not know exactly what roles endocytosis might serve in these different compartments. Nevertheless, based on what is known about the biology of hyphae and about endocytosis in other organisms we can speculate generally as to possible roles that endocytosis may serve in filamentous fungi.

Removal of excess plasma membrane. It has been suggested that, in pollen tubes, insertion of membrane by the fusion of vesicles delivering cell wall components to the apex exceeds the membrane necessary for tip extension (Picton & Steer, 1983). The most likely retrieval mechanism for superfluous membrane is endocytosis (Steer, 1988). This is supported by Derksen et al. (1995), who identified a zone 6–15 μm behind the tip with a concentration of clathrin-coated pits implicating it as a major site of endocytosis. In rust germ tubes, Hoffman & Mendgen (1998) observed initial FM4-64 uptake within a region 5–20 μm away from the apical pole, indicating active endocytosis in that zone. This broadly matches our observations of early uptake in N. crassa. Calculations of the amount to the vacuole was inhibited (Vida & Emr, 1995). Indeed, in budding yeast FM4-64 has been successfully used in a fluorescence assisted cell sorter to screen for proteins involved in endocytosis (Gaynor et al., 1998).

Our observations of the time-dependent sequence of FM4-64 internalization, with staining appearing in a defined sequence of organelles, is consistent with uptake by endocytosis as described for styryl dyes applied to animal and yeast cells (reviewed in Cochilla et al., 1999). In these latter systems, the first obvious stained organelles have been identified as, or proposed to be, early endosomes (Vida & Emr, 1995; Cochilla et al., 1999). The roughly spherical ~0.75 μm organelles observed here correspond well to this and thus we now refer to them as putative endosomes. However, it must be emphasized that endosomes have not yet been identified at the ultrastructural level in filamentous fungi.
of apical vesicle membrane relative to the volume of these vesicles in fungal hyphae have indicated that a significant excess of membrane relative to wall material is probably added during hyphal tip growth (C. E. Bracker, personal communication, 1998). In subapical compartments endocytosis may be important for retrieving excess membrane delivered by secretory vesicles during septum formation.

Recycling of membrane proteins. Endocytosis in apical hyphal compartments may function in tip growth by providing a means for retrieving displaced membrane proteins (e.g. ion channels and cell wall-building enzymes) and returning them to the tip for re-use. In budding yeast, there is strong evidence that two of the three chitin synthases in the plasma membrane are recycled back to the plasma membrane via endocytosis (Chuang & Schekman, 1996; Ziman et al., 1996, 1998; Holthuis et al., 1998). It makes economic sense for a hyphal to recycle and reuse some of the proteins involved in tip growth rather than to synthesize all of these proteins de novo. As discussed in the following section, rapid recycling of membrane proteins in hyphae may occur via endosomes and/or satellite Spitzenkörper.

Transport of membrane proteins and lipids to the vacuole for degradation. Our findings showed that spherical vacuoles in

N. crassa became stained with FM4-64 in a manner similar to that reported for the vacuole of budding yeast, which functions as the site of degradation of plasma membrane proteins (Vida & Emr, 1995; Wendland et al., 1998).

Uptake of molecules in the fluid phase of endocytic vesicles. This may be important for the uptake of certain nutrients (Dulic et al., 1991).

Receptor-mediated uptake of ligands. It has been shown that, in budding yeast, the mating pheromone α-factor and its plasma membrane receptor are internalized by receptor-mediated endocytosis (Wendland et al., 1998). Although filamentous fungi produce pheromones (Bölker & Kahmann, 1993), their mechanism of internalization has not been studied.

Tracking the vesicle trafficking network in fungal hyphae. FM4-64 was found to be of low toxicity and relatively resistant to photobleaching during repeated imaging. This makes it an excellent dye for following dynamic processes in living cells over time without perturbing those processes. Confocal imaging has allowed us to visualize putative endosomes and other organelles (e.g. mitochondria and vacuoles). However, it should be emphasized that visualization of individual vesicles is beyond

the limits of the imaging techniques employed here. This is because the vesicles are very small (typically < 100 nm in diameter) and are present at a high density within the hyphal cytoplasm. The diffraction limitation of fluorescence imaging means that such small, closely spaced fluorescing vesicles cannot be individually identified because they appear blurred together (Betz & Angleson, 1997).

On the basis of our observations with FM4-64 uptake we have proposed a speculative model (Fig. 12) which interprets our observations in the context of current knowledge of the vesicle trafficking pathways in yeast and animal cells (e.g. Mellman, 1996; Petham, 1997; Geli & Riezman, 1996).

Our observation of diffuse fluorescence within the cytoplasm within 30 s of adding FM4-64 to hyphae may be interpreted as primarily representing a cloud of stained endocytic vesicles. Although endocytic vesicles have not been identified at the ultrastructural level, possible candidates are filasomes (Howard, 1981). These are vesicles possessing a fibrillar coating that contains actin (Bourett & Howard, 1991; Roberson, 1992). These coated vesicles were found to be concentrated principally in the first 12 μm of growing hyphal tips of the basidiomycete Sclerotium rolfsii (Roberson, 1992). This is consistent with our observations of initial dye internalization in a localized region behind the apical dome. The first FM4-64-stained organelles that we could visualize clearly in hyphae were small and roughly spherical and, as previously discussed, are interpreted as putative endosomes. In other cell types endosomes function as sorting compartments for proteins and lipids, and are classified into two functional types: ‘early’ and ‘late’ endosomes (Ashford, 1998; Presciannoto-Baschong & Riezman, 1998; Mukherjee et al., 1999).

In subapical compartments, the next obviously stained organelle was the large spherical vacuole. This pattern of staining was similar to that in budding yeast, in which staining of yeast vacuolar membranes followed that of putative endosomes (Vida & Emr, 1995). In addition to the large spherical vacuoles, the vacuolar system in hyphae also consists of an extensive tubular network in both subapical and apical compartments (Ashford, 1998). We have also found that the membranes of these tubular vacuoles in apical hyphal compartments become clearly stained after prolonged immersion in FM4-64 (unpublished results).

In addition to vacuolar membranes we would also expect that both the Golgi and ER of fungal hyphae would become stained with FM4-64 via retrograde pathways which connect the endosomal system, Golgi and ER, as occurs in budding yeast (Pelham, 1997; Fig. 12). Staining by FM4-64 of these membranes in fungal hyphae would provide an explanation for the observed increase in staining of other organelles in the cytoplasm with time. However, it should also be stressed that the staining of the small, roughly spherical organelles proposed earlier to be endosomes could conceivably be Golgi instead.
The traditional view of hyphal tip growth has been that the wall-building, secretory vesicles which reside in the Spitzenkörper prior to exocytosis are generated exclusively by Golgi cisternae (e.g. Grove & Bracker, 1970; Howard, 1981). Recently, evidence has been obtained that indicates that satellite Spitzenkörper also supply wall-building vesicles to the growing hyphal tip (LópeFranco et al., 1994, 1995). Satellite Spitzenkörper arise immediately beneath the plasma membrane a few micrometres behind the apical pole, and then migrate towards and merge with the main Spitzenkörper. These fusion events were correlated with a transient increase in the hyphal extension rate and are believed to be responsible for the pulsed growth behaviour of fungal hyphae resulting from the pulsed delivery of wall-building secretory vesicles (LópeFranco et al., 1994, 1995). However, as yet, the source(s) of the vesicles within satellites is/are unknown. These vesicles may be derived from the Golgi and/or endocytic vesicles internalized from the plasma membrane.

We suggest that secretory vesicles may be additionally derived from endosomes. This is consistent with our observations that Spitzenkörper stained shortly after the putative endosomes. In the previous section we proposed that a likely function of endocytosis in hyphae is the recycling of proteins involved in tip growth back to the apical plasma membrane. Satellite Spitzenkörper may also have a role in this process. However, the longer route for recycling these proteins from endosomes to the Spitzenkörper via the Golgi cannot be discounted (Fig. 12).

Mitochondria were always stained by FM4-64 with longer incubation times than necessary to stain Spitzenkörper or putative endosomes, and these times varied between species. A possible explanation of this phenomenon, still consistent with FM4-64 internalization by endocytosis, is that FM4-64 stains mitochondria through direct contact with the ER (Fig. 12). Continuity between the ER and the outer mitochondrial membrane has been shown at the ultrastructural level in various cells including hyphae (Bracker & Grove, 1971; Franke & Kartenbeck, 1971). We found that FM4-64 and FM1-43 stained mitochondria at different rates in any one species and the rates of staining varied between species. An explanation for this may be found in the recent finding that fluorescent lipid analogues undergo endocytic sorting to different organelles in animal cells solely on the basis of differences in the chemistry of their hydrophobic tails (Mukherjee et al., 1999). Variations between species (e.g. N. crassa and A. nidulans) in the composition of their organelle membranes may also explain the differential rates at which their mitochondria become stained by FM4-64.

The hypothetical model of vesicle trafficking presented in Fig. 12 now needs to be rigorously tested and, in particular, endocytic intermediates and components of the secretory pathways need to be identified and characterized. Towards this aim, we are currently performing double labelling experiments in living hyphae by following the time course of FM4-64 staining in which the green fluorescent protein has been targeted to specific organelles (e.g. Fernández-Ábalos et al., 1998). This will need to be correlated with the localization of FM4-64 at the ultrastructural level as has been done for FM1-43 in nerve cells (Henkel et al., 1996). The model in Fig. 12 is also being subjected to genetical analysis and in this respect our unpublished results have shown that FM4-64 is proving to be a very powerful tool for analysing growing hyphae of mutants compromised in vesicle trafficking.

Acknowledgements

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mutants provide novel insights into the roles of vesicle trafficking during cell morphogenesis in *Neurospora*. *Curr. Biol.* 9, 779–785.


The Vesicle Trafficking Network and Tip Growth in Fungal Hyphae

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Abstract. Experimental methods are described for analysing vesicle trafficking in living fungal hyphae involving confocal microscopy of fluorescent dyes. Results from the use of one of these dyes, FM4-64, are summarised. Based on this work, a speculative model of the vesicle trafficking network is presented. The likely multifunctional roles for endocytosis in hyphal tip growth are discussed. Finally, a new model (the Exocytosis-Endocytosis Equilibrium [or ‘Triple E’ model]) of how vesicle trafficking may regulate hyphal tip morphogenesis is proposed.

1. Introduction

Vesicle trafficking underpins hyphal tip growth. Although the significance of polarised secretion has been long appreciated as central to tip growth, only recently has it emerged that endocytosis may also play important roles in this process.

The growing tips of fungal hyphae characteristically possess a concentration of vesicles (the ‘apical vesicle cluster’). In higher fungi (Ascomycota, Deuteromycota and Basidiomycota), these vesicles are the predominant components of a multi-component complex, which is usually called the Spitzenkörper (= ‘apical body’) [1-3]. The dynamic behaviour of this remarkable structure, and also smaller versions of it (so-called satellite Spitzenkörper [4-6]), have been shown to be intimately associated with the precise and often subtle growth pattern of the hyphal tip in ways which are consistent with Spitzenkörper delivering wall-building vesicles to local domains of the apical plasma membrane.

Increasing evidence for endocytosis in growing hyphal tips is coming from studies using the amphiphilic styryl dye, FM4-64 [6-8], which is commonly used as an endocytosis marker in budding yeast [9] and animal cells [10].

Vesicle trafficking is very dynamic and extensive within growing hyphal tips. A common rate of hyphal extension for Neurospora crassa growing in open culture in a Petri dish is ~ 36 μm min⁻¹. In order for a 10 μm wide hypha of Neurospora to supply sufficient plasma membrane to the hyphal tip to maintain this growth rate it has been estimated that ~ 600 secretory vesicles per second would have to fuse with the apical plasma membrane [11]. However, even this magnitude of membrane transport must be a significant underestimate of the amount of vesicle trafficking occurring within a growing hyphal tip because it does not take account of vesicles moving between organelles or the activity of endocytic vesicles.

The aims of this paper are to: (a) describe dye labelling approaches for the experimental analysis of vesicle trafficking in growing hyphal tips imaged with confocal microscopy; (b) summarise results that we have obtained in analysing vesicle trafficking...
using the dye FM4-64; (c) based on this data, provide a speculative model of the vesicle trafficking network in growing hyphal tips; (d) consider the likely multi-functional roles of endocytosis in hyphal tip growth; and finally (e) use our current knowledge of the vesicle trafficking network in growing hyphal tips to propose the Endocytosis-Exocytosis Equilibrium (or 'Triple E') model of hyphal morphogenesis.

2. Approaches for Imaging Vesicle Trafficking in Living Fungal Hyphae

Considerable emphasis is currently being placed on analysing the cell biology of individual living hyphae. Much of this has been due to important advances in imaging and dye technologies [12]. In this respect, we have developed techniques for monitoring vesicle trafficking by using confocal laser scanning microscopy of living hyphae loaded with the fluorescent dye FM4-64 [6].

Confocal microscopy has been used to image living hyphae at high spatial resolution without perturbing their growth. Dye-loaded hyphae of different species have been found to vary in their sensitivity to being scanned with the confocal microscope laser. However, repeated laser scanning at 3 sec intervals for periods greater than 10 min with a x60 (1.4 N.A.) objective are often possible without inhibiting the rate of hyphal extension, and using this approach animated time courses of growing hyphae are routinely obtained in our laboratory.

We have used three different styryl dyes (FM4-64, FM1-43 and TMA-DPH) commonly employed as indicators of endocytosis in other systems [e.g. 9, 10, 13], and assessed their suitability for monitoring vesicle trafficking in fungal hyphae. Comparison of the properties of these dyes (Table 1) showed that FM4-64 is best suited for this purpose [6].

Our published and unpublished findings support the endocytic uptake of dye shown in Fig. 1 as the predominant mechanism by which FM4-64 is internalised within hyphae [6].

<table>
<thead>
<tr>
<th>Property</th>
<th>FM4-64</th>
<th>FM1-43</th>
<th>TMA-DPH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excitation wavelengths</td>
<td>488 or 514 nm</td>
<td>488 or 514 nm</td>
<td>351 and 364 nm</td>
</tr>
<tr>
<td>Emission wavelengths</td>
<td>&gt; 550 nm</td>
<td>&gt; 550 nm</td>
<td>&gt; 400 nm</td>
</tr>
<tr>
<td>Staining of Spitzenkörper</td>
<td>Very good</td>
<td>Poor</td>
<td>Poor</td>
</tr>
<tr>
<td>Staining of mitochondria</td>
<td>Slowly (typically &gt; 1 h)</td>
<td>Quickly (typically &lt; 30 min)</td>
<td>None</td>
</tr>
<tr>
<td>Photobleaching</td>
<td>Reasonably resistant</td>
<td>Reasonably resistant</td>
<td>Poor</td>
</tr>
<tr>
<td>Phototoxicity</td>
<td>Not a major problem</td>
<td>Not a major problem</td>
<td>Very bad</td>
</tr>
</tbody>
</table>
3. Experimental Analysis of Vesicle Trafficking in Fungal Hyphae Using FM4-64

Below is a summary of some of the main features of FM4-64 staining of hyphae:

(a) The sequence of organelle staining is time-dependent. The time course and pattern of hyphal staining with FM4-64 is broadly similar in the > 15 species representing the Zygomyccota, Ascomycota, Basidiomycota, and Deuteromycota [6, 14] we have examined. Initial staining of the plasma membrane is followed by staining in the cytoplasm (Fig. 2A), and then successive staining of putative endosomes (Fig. 2B), other membranous organelles, the apical vesicle cluster (Fig. 2C), vacuolar membranes, and mitochondria. The time course of staining of hyphae of N. crassa is summarised in Table 2. This time-dependent staining of different organelles is consistent with dye internalisation by endocytosis and subsequent staining of different organelles by the distribution of dye via the vesicle trafficking network.

(b) Dye internalisation involves an active process which is energy dependent. Loading hyphae with FM4-64 in the presence of the metabolic inhibitor sodium azide or at 4 °C allows staining of the plasma membrane whilst inhibiting uptake of the dye into the cytoplasm and staining of organelles [6, 7, 15]. This indicates that FM4-64 uptake into hyphae is an active, energy-dependent process (consistent with it being mediated by endocytosis) and does not involve unfacilitated diffusion.

Figure 2. Confocal images of hyphal tips of N. crassa showing the time course of FM4-64 staining. 25 μM FM4-64 was continuously present in the external medium. (A) Pronounced staining of the plasma membrane (30 sec after adding dye). (B) Staining of putative endosomes (1 min after adding dye). (C) Staining of the main Spitzenkörper (30 min after adding dye). Bar = 10 μm.
Table 2. Summary of the time course of staining of a hypha of *N. crassa* continuously loaded with FM4-64.

<table>
<thead>
<tr>
<th>Time after adding FM4-64</th>
<th>Cell component stained</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1 sec</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>&lt; 10 sec</td>
<td>Putative endocytic vesicles</td>
</tr>
<tr>
<td>30 sec</td>
<td>Putative endosomes</td>
</tr>
<tr>
<td>1.5-3 min</td>
<td>Apical vesicle cluster</td>
</tr>
<tr>
<td>10 min</td>
<td>Subapical spherical vacuoles</td>
</tr>
<tr>
<td>15 min</td>
<td>Putative tubular vacuoles</td>
</tr>
<tr>
<td>~ 40 min</td>
<td>Mitochondria</td>
</tr>
</tbody>
</table>

(c) **Vesicles within Spitzenkörper become stained.** FM4-64 strongly stains the apical vesicle cluster within the main Spitzenkörper of growing hyphal tips (Figs. 2C, 3A and 3B; Table 2). Because it is assumed that the apical vesicle cluster is composed primarily of secretory vesicles, this observation indicates that the endocytic and secretory pathways within the vesicle trafficking network are integrated [6]. There is good agreement between the morphology and behaviour of stained Spitzenkörper [6] and unstained Spitzenkörper previously analysed by computer-enhanced, phase-contrast microscopy [3, 16]. Stained Spitzenkörper commonly possess a region exhibiting reduced staining [6]. This correlates with a differentiated ‘core’ region observed in unstained hyphal tips [3] and at the ultrastructural level [e.g. 1, 2, 17]. This region commonly possesses few vesicles although in some genera it contains a concentration of microvesicles [1]. Small satellite Spitzenkörper also arise in a location immediately below the plasma membrane several micrometers back from the main Spitzenkörper. Normally they migrate within 15 sec towards the main Spitzenkörper and fuse with it [5, 6]. If these satellite Spitzenkörper do not immediately migrate towards the main one then a bulge in the hypha typically appears adjacent to them indicating that they are delivering wall-building vesicles to the region of the plasma membrane which they are in close proximity with [5].

(d) **Fluorescence recovery after photobleaching (FRAP) indicates vectorial transport of secretory vesicles to the main Spitzenkörper.** In these experiments, Spitzenkörper of growing hyphae of *Rhizoctonia solani* which had been pulsed-stained with FM4-64 were photobleached with the laser of the confocal microscope without inhibiting tip growth. Fluorescence within the Spitzenkörper subsequently recovered within 10 min whilst the hyphae continued to grow. FRAP within the apical vesicle cluster is interpreted as involving the addition of new unbleached, stained wall-building secretory vesicles to the Spitzenkörper following their formation in a subapical hyphal region [18].

(e) **Internalised dye is recycled back to the plasma membrane.** FM4-64 only fluoresces within a hydrophobic environment (e.g. when inserted within a membrane [19]). Thus when hyphae are immersed in dye, there is no background staining within the surrounding medium (Figs. 2 and 3). When hyphae are ‘continuously loaded’ with dye in this way the plasma membrane typically exhibits consistently pronounced staining. However, if a hypha is ‘pulse-loaded’ with dye (i.e. the dye is washed out from the growth medium after a period of staining), the plasma membrane remains stained even for periods of 2 h or more (cf. Figs 3A and 3B). This indicates that dye internalised within hyphae by endocytosis is
recycled back to the plasma membrane via exocytic vesicles (Fig. 1). Furthermore, these results indicate that significant amounts of FM4-64 are not released into the lumen of exocytic vesicles and lost during secretion as occurs dramatically in nerve cells loaded with the dye FM1-43 [20, 21].

**Figure 3.** Confocal image of a hyphal tip of *N. crassa* stained with 25 μM FM4-64 and imaged 2 h after initially applying dye. (A) Pulse-loaded hyphal tip (hyphae were incubated with FM4-64 for 30 sec before washing the dye out of the surrounding medium). Note that the plasma membrane has remained stained. (B) Continuously stained hyphal tip (the dye was left in the surrounding medium). Bar = 10 μm.

*(f) FM4-64 may become distributed amongst membranes by a lipid sorting mechanism.* Besides the time-dependent staining of organelle membranes by FM4-64 (which we interpret as primarily involving the transport of the dye between organelles via the vesicle trafficking network), we suggest that a process of ‘lipid sorting’ may also play a role in the differential staining of organelle membranes. It has recently been found that fluorescent lipid analogues undergo sorting to different organelles in animal cells solely on the basis of the chemistry of their hydrophobic tails. This is believed to be a mechanism by which lipids normally become distributed between different membranes of the endomembrane system [22]. With FM4-64 we find that some membranes (e.g. the plasma membrane) stain strongly, some membranes stain at a reasonable level (most organelles) whilst others (e.g. the nuclear membrane) do not appear to stain at all even though we would predict that the latter should stain up through connection with other components of the endomembrane system. With other styryl dyes which are internalised by endocytosis and which possess different hydrophobic tails (e.g. FM1-43) the pattern of membrane staining is different to that of FM4-64 (Table 1) [6].

### 4. Model of the Vesicle Trafficking Network in Hyphal Tips

In Fig. 4 we provide a diagrammatic representation of our current working model of the vesicle trafficking network within growing hyphal tips. Below is a summary of the evidence for each of the component pathways which have been numbered in the diagram.

[1] **Plasma membrane → endocytic vesicles → endosomes → vacuole.** Dye fluorescence is initially detected within the cytoplasm within 10 sec of adding FM4-64 to hyphae and this is interpreted as representing stained endocytic vesicles which have been budded off from the plasma membrane. Although biochemical evidence for the presence of clathrin, the major coat protein of endocytic vesicles in animal and plant cells, has been found in *N. crassa* [23], convincing ultrastructural evidence for the existence of clathrin-coated vesicles or pits is lacking in filamentous fungi. The best candidates for endocytic vesicles in hyphae are filasomes [2, 6]. These are vesicles found throughout hyphae and which each possess a fibrillar coating rich in actin [17, 24]. The next small organelles to stain within the hyphal cytoplasm after endocytic vesicles (Fig. 2B) are believed to be endosomes. In animal and
yeast cells endosomes act as the cell compartments responsible for molecular sorting, and are of two types 'early' and 'late' endosomes. Endosomes have not yet been identified at the ultrastructural level in fungal hyphae. However, one candidate for endosomes in hyphae are multivesicular bodies [e.g. see 2, 25] for which a function has not been established. Ten to fifteen minutes after adding FM4-64 to hyphae of *N. crassa* the spherical and tubular vacuolar elements become stained (Table 2).

**Figure 4.** Working model of the vesicle trafficking network within the growing hyphal tip (adapted from [6]). Each of the numbered vesicle trafficking pathways are described in the accompanying text.

[2] **Endoplasmic reticulum (ER) → Golgi → main Spitzenkörper → apical plasma membrane.** The traditional view of the secretory process involved in tip growth is that proteins synthesized on the ER are transported via vesicles to the Golgi within which they are processed and transported in secretory vesicles to the apical vesicle cluster within the main Spitzenkörper [2]. These secretory vesicles are then directed to the apical plasma membrane with which they fuse. An interesting feature of Golgi cisternae in filamentous fungi is that they do not typically form stacks as is characteristic of plant and animal cells [2].

[3] **ER → Golgi → subapical plasma membrane.** Secretion also occurs from subapical regions of fungal hyphae. This is particularly important during the delivery of wall-building vesicles to new sites of branch formation (Fig. 6) [26]. Other extracellular enzymes are possibly also secreted from subapical locations.

[4] **ER → Golgi → satellite Spitzenkörper → main Spitzenkörper → apical plasma membrane.** Satellite Spitzenkörper also appear to contain wall-building vesicles as is indicated by the observation that a bulge in a hypha often appears adjacent to these multicomponent structures [5]. However, it is not clear exactly where these vesicles are generated. One possibility is that at least some are derived from Golgi cisternae. The repeated delivery of wall-building vesicles by the fusion of satellite Spitzenkörper with the main Spitzenkörper is believed to result in the pulsed growth pattern of hyphae [4, 5].
[5] Plasma membrane → endocytic vesicles → satellite Spitzenkörper → main Spitzenkörper → apical plasma membrane. It is possible that satellite Spitzenkörper may also contain endocytic vesicles derived from the plasma membrane below which these Spitzenkörper characteristically arise. These endocytic vesicles could play a role in recycling proteins and lipids back to the hyphal tip (see Section 5).

[6] Endocytic vesicles → endosomes → main Spitzenkörper → apical plasma membrane. A second pathway which may be important for recycling membrane proteins and lipids back to the growing hyphal tip is via endosomes. This would have the added advantage that the endosomes could provide a ‘molecular sorting’ function to select those proteins and lipids to be returned to the hyphal tip for reuse (see Section 5). A third possible pathway for recycling membrane proteins and lipids may be from endosomes to the main Spitzenkörper via the Golgi.

[7] ER → Golgi → endosomes → vacuoles. Proteins (e.g. lytic enzymes) within vacuoles are ultimately derived from ER and then transported via the Golgi and endosomes in which they are respectively processed and sorted [27].

[8] Retrograde vesicle trafficking. Retrograde pathways of vesicle trafficking must occur to maintain the correct balance of membrane between different organelles and also to allow the recycling of specific molecules [27].

5. Importance of Endocytosis for Hyphal Tip Growth

We believe that endocytosis may play critical roles in hyphal tip growth. Important functions may include:

(a) Removal of excess plasma membrane. Calculations have indicated that in the tips of hyphae and pollen tubes, insertion of new membrane by secretory vesicle fusion results in an excess in apical plasma membrane relative to the amount of cell wall components necessary to maintain tip extension [28-31]. Endocytosis could provide a mechanism to retrieve this membrane. Studies in which the time course of FM4-64 uptake into hyphae has been followed indicate that initial internalisation of the dye is concentrated in a region 2-20 μm back from the apical pole (Fig. 2A) [6, 7, 14]. Interestingly, it has been found that filasomes, which have been suggested as candidates for endocytic vesicles [6], are concentrated in this zone [24]. Furthermore, clathrin-coated endocytic vesicles have also been shown to be concentrated in a similar region in pollen tubes [31]. In chemically fixed hyphae, membranous aggregates associated with the plasma membrane and termed plasmalemmasomes or lomasomes [e.g. see 32] often develop. These structures which, in most cases are artefacts generated during the slow process of chemical fixation, may represent the buildup of excess membrane which failed to be retrieved normally by endocytosis in the dying hyphae.

(b) Generation of vacuolar system. If the assertion is correct that an excess of membrane inserted by secretory vesicles into the apical plasma membrane has to be internalized, it may be that much of this membrane plays an important role in generating vacuolar membranes of the vacuolar system, especially in the apical hyphal compartment. When the vacuolar system in the apical compartments of N. crassa is stained using a vacuole-selective dye such as Oregon Green 488 carboxylic acid [33], we find that tubular and small, near spherical vacuolar compartments start to appear 10-30 μm back from the apical pole of the hyphal tip. Behind this region the tubular elements get progressively longer. The much larger spherical vacuolar compartments, characteristic of subapical hyphal compartments [6, 27], do not appear until the first septum back from the tip.
Figure 5. Vacuolar network behind the growing hyphal tips of a main hypha and branch of \textit{N. crassa} stained for 20 minutes with 50 \mu M of the vacuole-selective dye Oregon Green 488 carboxylic acid diacetate. Note the tubular and small, near spherical vacuolar compartments. Bar = 10 \mu m.

(c) \textbf{Recycling of membrane proteins.} Endocytosis could provide a mechanism for recycling enzymes and lipids involved in tip growth (e.g. cell wall synthases, ion transporters and possibly membrane lipids involved in signalling). This would maintain a concentration of these molecules in the tip region where they are active and where these molecules once recycled can be reused. Besides providing one of probably several mechanisms for polarising the distribution of important proteins and lipids in the tip region, recycling these molecules makes economic sense for a hypha rather than having to synthesise all of these molecules \textit{de novo} which would require a much greater energy expenditure. In the budding yeast there are numerous examples of proteins with a polarised distribution concentrated at the site growth (i.e. buds). Furthermore, two of the three chitin synthases in budding yeast are recycled via endocytosis [34-37]. Endocytic recycling of membrane proteins has also been shown to occur in animal cells (e.g. in neurones [20]).

(d) \textbf{Transport of membrane proteins and lipids to the vacuole for degradation.} Besides endocytosis possibly supplying vacuolar membrane, many membrane proteins and lipids may also be transported to vacuoles for degradation. Fungal vacuoles are well established as the main lytic compartments in hyphae [27].

(e) \textbf{Uptake of molecules in fluid-phase of endocytic vesicles.} Small molecules may be taken up by endocytosis within the lumen of endocytic vesicles. This has been suggested as a possible mechanism for the uptake of certain small nutrients in budding yeast [38].

(f) \textbf{Uptake of signalling molecules involving receptor-mediated internalisation of ligands.} Pheromones and other signalling molecules may be internalised by receptor-mediated endocytosis in the hyphal tip region. In budding yeast, for example, this is the way in which \alpha-factor is internalised [39] and results in yeast cells exhibiting polarized growth in the direction of the highest concentration of the pheromone [40]. There are a number of examples in different filamentous fungi where hyphae (e.g. trichogynes in \textit{N. crassa}) exhibit positive chemotropisms towards pheromones. Endocytosis may also be important for the uptake of host signal molecules by hyphae in host-pathogen interactions.
6. The Endocytosis-Exocytosis Equilibrium Model of Hyphal Morphogenesis

We have formulated what we term the *Endocytosis-Exocytosis Equilibrium* (or *'Triple E'*) model of *hyphal morphogenesis*. The background to this model is based on the following key attributes of fungal hyphae:

(a) They exhibit tip growth (i.e. hyphal extension is limited to a region occupying a few micrometers at the hyphal tip) and this involves polarized secretion and localised cell wall synthesis [41].

(b) The major cell wall synthesizing enzymes (chitin and glucan synthases) involved in hyphal tip growth are integral membrane proteins and thus firmly anchored within the plasma membrane [42].

(c) The cell wall in the tip region of a growing hypha is plastic but becomes progressively more rigid behind the tip; changes in hyphal morphology are only possible where the cell wall is plastic [26, 43].

In this paper and elsewhere [6, 7], it has been argued that extensive endocytosis occurs in the hyphal tip region.

Our Triple E model proposes that the amount of cell wall synthesizing plasma membrane encased by plastic cell wall in the hyphal tip region is regulated by the *dynamic equilibrium* between exocytosis and endocytosis. The predicted effects of the steady state of this equilibrium on hyphal morphogenesis are shown diagrammatically in Fig. 7. We further propose that the dynamic equilibrium in exocytosis and endocytosis will vary in different parts of the apical dome of a hypha because of gradients in each of these processes, and this may also influence the precise pattern of hyphal tip morphogenesis.

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**Figure 6.** A diagrammatic summary of the Endocytosis-Exocytosis Equilibrium ("Triple E") model of hyphal morphogenesis showing the effects of three different steady state equilibria between exocytosis and endocytosis in the hyphal tip region. If the concentration of active, integral cell wall synthesizing enzymes per unit area of plasma membrane is uniform for these three different equilibria then the correct balance between the amount of plasma membrane present and the amount of cell wall synthesized should be maintained. This simplified diagram does not take account of likely gradients in the exocytosis-endocytosis equilibrium, or how gradients in cell wall plasticity, or in the activity of cell wall synthesising enzymes, within the hyphal tip region might influence hyphal tip morphogenesis.
Finally, we propose that the capacity for the endocytosis-exocytosis equilibrium to influence hyphal morphogenesis will be further modulated by the local plasticity of the encasing cell wall which again we would expect to vary in different regions of the hyphal dome.

In relation to possible gradients in exocytosis and endocytosis, it is well established that exocytosis is highly polarized in the growing hyphal tip [44] and various mechanisms have been proposed to explain this phenomenon (e.g. a mobile vesicle supply centre, calcium promoting localized vesicle fusion, and targeted transport along cytoskeletal elements to marked sites on the apical plasma membrane). Indeed, a range of physiological devices may be employed by hyphae to generate gradients in exocytosis [45]. Our preliminary evidence suggests that gradients in endocytosis also exist in the hyphal tip region with a concentration of endocytic activity just behind the main Spitzenkörper [14].

If the Triple E model proves to have credence in explaining important aspects of hyphal tip morphogenesis then it is clear that in order for a hyphal tip to maintain the normal hyphoid shape of an actively growing hypha [44] then exocytosis and endocytosis will have to be exquisitely regulated in a coordinated manner. A major challenge in the future will be to experimentally test the Triple E model by using pharmacological and genetic tools which interfere with these regulatory mechanisms, and to analyse the effects of these treatments in growing hyphae using methods similar to that described in this paper. It will also be interesting to determine whether a similar mechanism plays a role in the morphogenesis of tip-growing cells (e.g. pollen tubes and root hairs) of the plant kingdom where the mechanism of tip growth may have evolved independently of that in the fungal kingdom.

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