Current Biology

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Review

Cellular anatomy of arbuscular mycorrhizal fungi

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https://doi.org/10.1016/j.cub.2025.03.053

SUMMARY

Arbuscular mycorrhizal (AM) fungi are ancient plant mutualists that are ubiquitous across terrestrial ecosystems. These fungi are unique among most eukaryotes because they form multinucleate, open-pipe mycelial networks, where nutrients, organelles, and chemical signals move bidirectionally across a continuous cytoplasm. AM fungi play a crucial role in ecosystem functioning by supporting plant growth, mediating ecosystem diversity, and contributing to carbon cycling. It is estimated that plant communities allocate ~3.93 Gt CO_2e to AM fungi every year, much of which is stored as lipids inside the fungal network. Despite their ecological significance, the cellular biology of AM fungi remains underexplored. Here, we synthesise the current knowledge on AM fungal cellular structure and organisation. We examine AM fungal development at different biological levels — the hypha and its content, hyphal networks and AM fungal spores — and explore key cellular dynamics. This includes cell wall composition, cytoplasmic contents, nuclear and lipid organisation and dynamics, network architecture, and connectivity. We highlight how their unique cellular arrangement enables complex cytoplasmic flow and nutrient exchange processes across their open-pipe mycelial networks. We discuss how both established and novel techniques, including microscopy, culturing, and high-throughput image analysis, are helping to resolve previously unknown aspects of AM fungal biology. By comparing these insights with established knowledge in other, well-studied filamentous fungi, we identify critical knowledge gaps and propose questions for future research to further our understanding of fundamental AM fungal cell biology and its contributions to ecosystem health.

Introduction

The arbuscular mycorrhizal (AM) symbiosis is one of the most important terrestrial symbioses on Earth. More than 70% of land plants form partnerships with AM fungi[1], covering over 55% of global vegetation[2,3]. AM fungi comprise the subphylum Glomeromycotina, which together with the closely related subphyla of Mucoromycotina and the Mortierellomycotina, form the early divergent fungal phylum Mucoromycota[4,5]. AM fungi are obligate biotrophs, as they rely on carbon from plant partners in the form of sugars and lipids. In return for carbon, they provide nutrients and water. More than 50% of a plant's phosphorus uptake[6] and up to 35% of the total water transpired by plants daily[7] come from AM fungi. The fungus develops extensive hyphal networks in soil (extraradical mycelium (ERM), outside the root), to collect nutrients inaccessible to plants, such as phosphorus and nitrogen. These nutrients are then transferred via the intraradical mycelium (IRM, inside the root), to specialised structures known as arbuscules formed in host plant cells (Figure 1), to trade for carbon. As a result, mycorrhizal networks represent a key entry point for the drawdown of carbon into soil[2]. Recent estimates suggest that, globally, approximately 3.93 Gt of CO_2e $year^{-1}$ are allocated belowground to AM hyphal networks[2]. This symbiosis enhances the diversity of plant communities[8,9] and provides multiple ecosystem functions, including soil formation and increased macroaggregate stability[10,11].

The cellular organisation of AM fungi shares some similarities with early-diverging fungal clades, exhibiting unique features that distinguish them from the cells of most eukaryotes, including plants and animals. AM fungal cells are large multinucleate eukaryotic cells that can extend for many centimetres[12–14], placing them among the largest single-celled organisms on Earth. Collectively, their networks in prairie soils have been shown to reach upper hyphal lengths of ~100 m/cm^3 [15]. The hyphal networks of AM fungi can be thought of as a network of open pipes, as they lack physical barriers, called septa. Aseptate, or coenocytic, hyphae are a common feature among early-diverging fungi. This morphology contrasts with later-diverging fungal lineages, in which living hyphae are compartmentalised by porous septa[16]. Consequently, in AM fungi, all cellular components are contained in — and must be regulated across — a continuous cytoplasmic fluid. While we have begun to understand some aspects of AM fungal biology, such as nuclear dynamics and cytoplasmic flows, we lack a comprehensive overview at the cellular level. This is due to challenges culturing these obligate biotrophs, the inability to genetically transform them with reporter genes and other tools, and difficulties in developing microscopy techniques to visualise living hyphae at high resolution to identify and characterise organelles and other cellular components.

This review synthesises the current understanding of AM fungal cellular structure and arrangement. We explore three levels of organisation of AM fungi: the hypha and its anatomy; the collective

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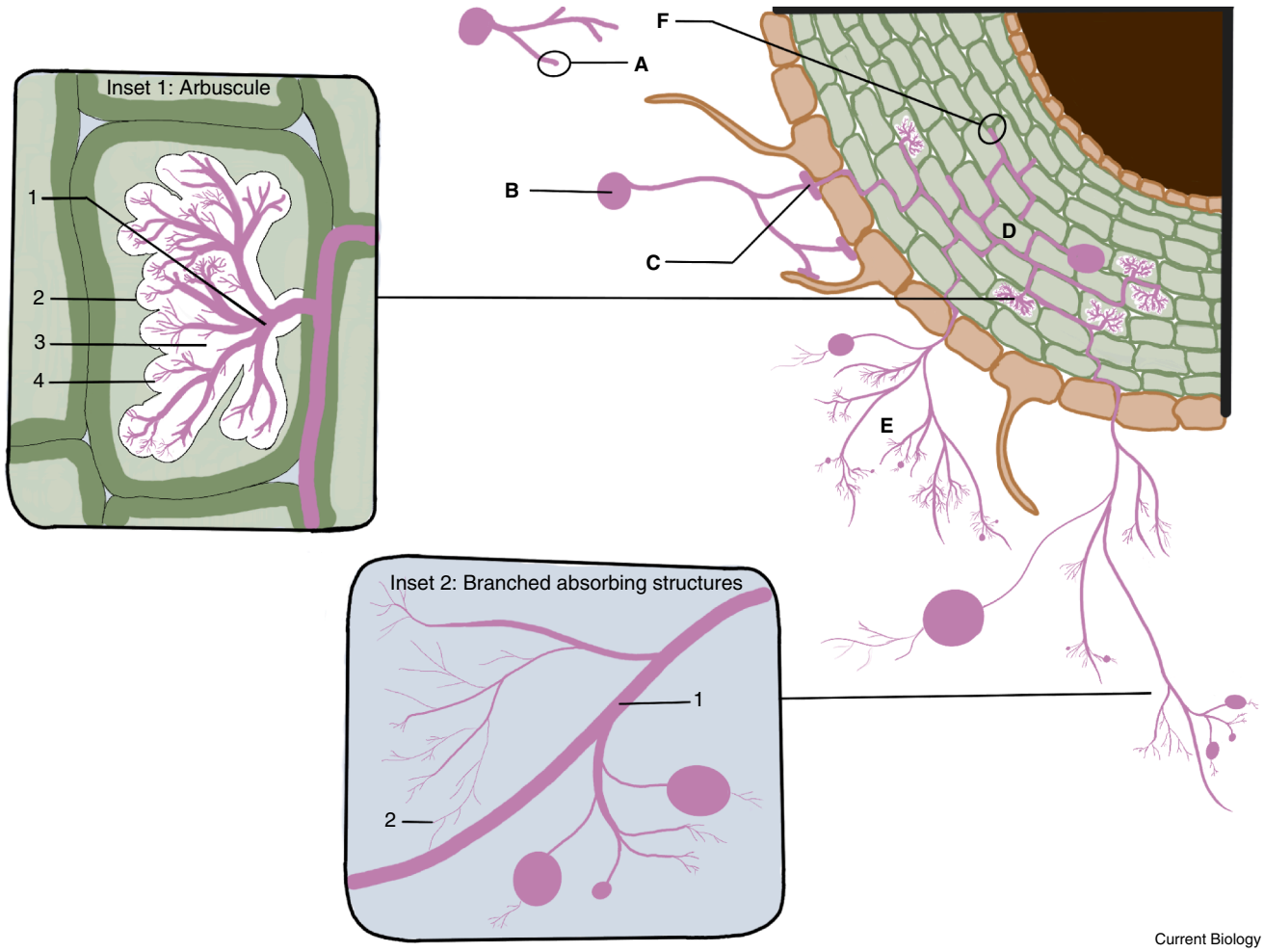


Figure 1. Morphological variation of the arbuscular mycorrhizal (AM) fungal hyphae.
Despite being a continuous single cell, AM fungal hyphae demonstrate remarkable morphological and functional diversity. Germ tubes (A) emerge from spores (B) and play a crucial role in initiating fungal growth, soil exploration and host colonisation. Hyphopodia are specialised, swollen structures that develop where fungal hyphae encounter the root epidermis, functioning as adhesion and entry points to plant roots[151] (C), specialised structures formed at the root surface, facilitate host root penetration. Part of the intraradical hyphal network (D), formed within the cortical root cells, arbuscules (Inset 1) develop as highly branched, intracellular structures optimised for nutrient exchange[152]. The arbuscule trunk (Inset 1.1) forms the base of the arbuscule and is enveloped by the periarbuscular membrane (PAM; Inset 1.2), an expansion of the plant plasma membrane[153]. The arbuscular trunk shares protein markers with the plasma membrane of the plant cell[153]. The periarbuscular space (PAS; Inset 1.3) comprises an apoplastic interface between the PAM and the fungal hyphae and facilitates nutrient and signal exchange[40]. The branch domain of the PAM envelops the fine hyphal branches (Inset 1.4) and contains specific phosphate transport proteins[154]. In the soil, branched absorbing structures (BAS; Inset 2) form sub-apically in the extraradical mycelium and are linked to either nutrient absorption, particularly phosphorus, or to spore formation (spore-BAS)[101]. Runner hyphae (Inset 2.1), which constitute the skeleton of the extraradical mycelium (ERM) (E), grow out into the soil in search of new hosts and act as conduits for nutrient translocation[155,156]. When considering the hyphal growth dynamics, hyphal tips can be found simultaneously intraradically in apical colonisation expansion zones (F) and in thin arbuscule branches (Inset 1.4), and extraradically in the BAS (Inset 2.2) and at the advancing front of the ERM (E). (Image courtesy of Sean C. Maston.)

hyphal network and network level cellular dynamics; and AM fungal spores. Understanding the biology of the AM fungal cell is important because it allows us to make predictions about how, why, and where symbiotic trade with plants takes place, and how these processes are coordinated across the network and under changing environmental conditions. We also know that AM fungal species differ greatly in ecologically relevant traits, such as growth, sporulation, and nutrient-exchange patterns. A better understanding of AM fungal cell biology would help us link these important traits to underlying cellular functions. We conclude by identifying key knowledge gaps and describe new technologies that can help explore these unknown areas.

Hyphal anatomy

Despite the unique morphologies and specialised functions of the various hyphal structures (Figure 1), the cellular organisation of AM fungal hyphae is composed of conserved components that exhibit spatial plasticity. From the exterior inward, these components include the cell wall, the plasma membrane, the cytoskeleton and the cytoplasm, which houses numerous organelles.

Cell wall

The fungal cell wall is a dynamic, complex, and mechanically robust extracellular matrix that is crucial for maintaining cellular integrity, turgor pressure and cell permeability, while also protecting the fungus[17]. This structurally complicated and

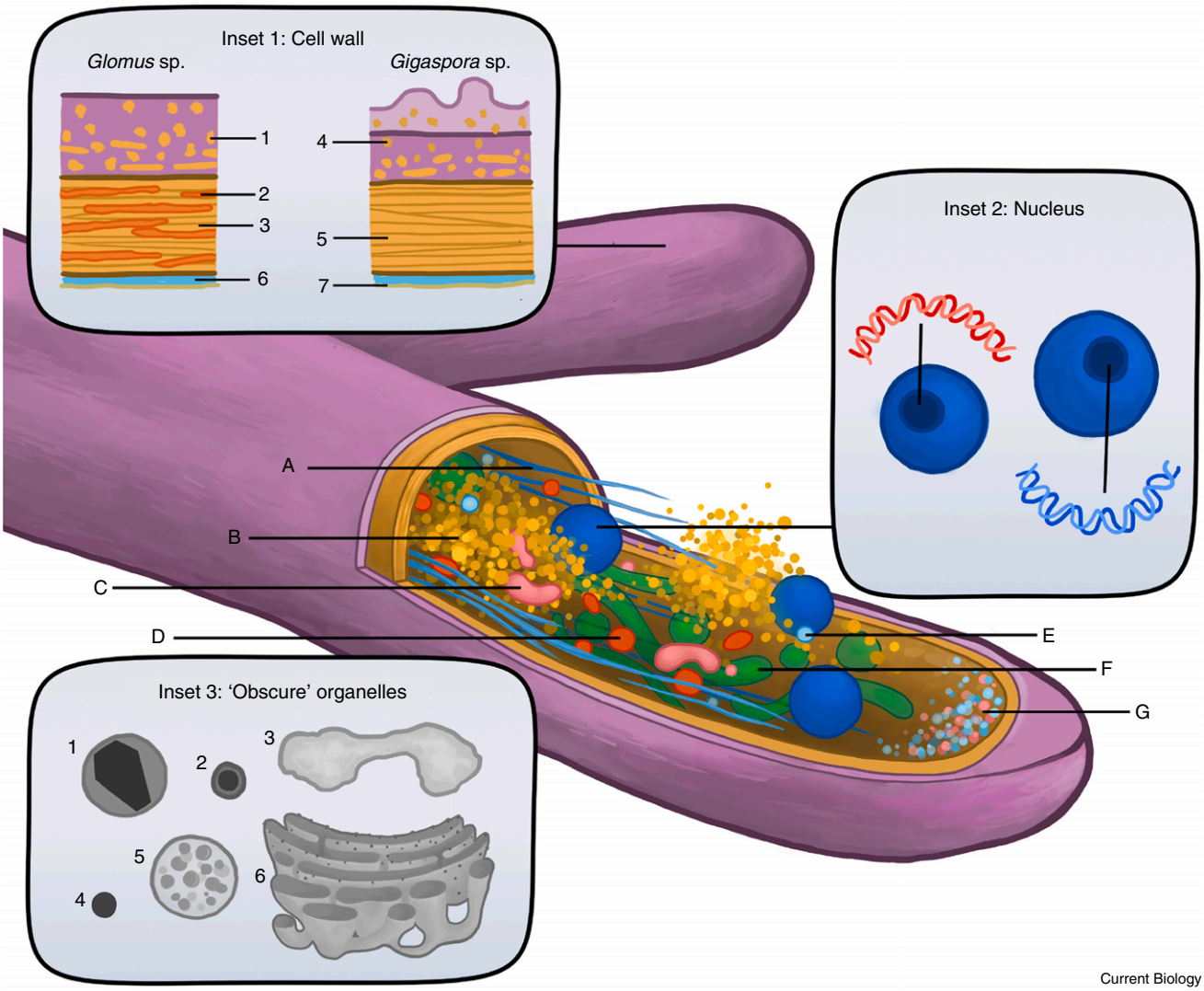


Figure 2. Illustration of characterised and obscure cellular components in AM fungal hyphae.
Schematic illustrations based on published literature showing organelle arrangement within AM fungal hyphae using transmission electron microscopy (TEM) and fluorescence microscopy. Main hypha: (A) microtubules (light blue), (B) lipid droplets (orange), (C) Golgi bodies (pink), (D) mitochondria (red), (E) vesicles (cyan), (F) vacuoles (green) and (G) the apical vesicle crescent (AVC, small light blue and pink spherical structures). Proposed differences in cell wall structure between Glomeraceae and Gigasporaceae species are shown in Inset 1, including differences in wall layering, outer wall structure and glucan content. (Inset 1.1) monomeric chitin, (Inset 1.2) β (1-3) glucans, (Inset 1.3) chitin polymer, (Inset 1.4) monomeric chitin, (Inset 1.5) chitin polymer, (Inset 1.6) extracytoplasmic space, and (Inset 1.7) cell membrane. Inset 2 displays nuclei (blue) and illustrates the homokaryotic and dikaryotic nature of the model AM fungus *R. irregularis*. In most *R. irregularis* strains analysed to date, all co-existing nuclei are genetically similar; however, some strains are known to be dikaryotic in which the co-existing nuclei comprise two genetically distinct nuclear populations[106]. The relative abundance of these genomes can be actively adjusted in response to biotic[106] and abiotic factors[157]. Nuclei are not present at the hyphal apex and are observed abundantly in the sub-apical zones of growing hyphae[26,69]. Inset 3 illustrates the 'obscure organelles' observed mostly in TEM photographs that are yet to be well characterised in AM fungi. (Inset 3.1) membrane-bound crystals, (Inset 3.2) osmophilic granules, (Inset 3.3) glycogen deposits, (Inset 3.4) polyphosphate-like granules, (Inset 3.5) polyvesicular bodies and (Inset 3.6) smooth and rough endoplasmic reticulum (for more details see Box 1). (Image courtesy of Mary-Jane Woodward, Vrije Universiteit, Amsterdam.)

difficult-to-penetrate wall[18] has made it extremely difficult to successfully implement certain techniques on AM fungi, such as protoplast formation, fungal transformation, and hybridization[19,20]. Resolving the cell wall structure is crucial for understanding signal exchange and acquisition of various components from the environment.

Generally, the fungal cell wall comprises multiple layers distinguishable by electron density or texture[21–24] (Figure 2). Similarly, in AM fungi, the hyphal cell wall is multilayered, with varying patterns and thicknesses depending on the AM fungal species identity, as well as the age and type of hyphae[25]. Generally, thinner walls appear at the tips of the germ tubes[26], and there is progressive wall thinning of the intraradical hyphae moving from the outer to the inner root cell layer, indicating structural remodelling during different developmental stages[25]. Overall, shared features have been deduced from ultrastructural analyses, including: layers with distinct electron densities; a stratified fibrillar layer comprising the inner wall; and an outer wall

Table 1. Organelle and cellular structure visualisation techniques and material that have been applied or can be applied in the future on arbuscular mycorrhizal fungi.

Dye/antibodies	Target	Live/fixed sample	Microscopy method	Used on AMF
Acetoxymethyl ester (BCECF-AM)	Cytoplasm (intracellular pH)	Live[80]	Epifluorescence	Yes
Anti-actin antibody (N.350)	β-actin	Fixed[59]	Epifluorescence	Yes
Anti-α-tubulin antibody	α-tubulin proteins for microtubules	Fixed[59,61]	Confocal laser scanning (CLSM) Epifluorescence	Yes
Anti-β-tubulin antibody	β-tubulin proteins for microtubules	Fixed[59]	Epifluorescence	Yes
BODIPY	Vesicles Lipids Membranes	Live/fixed[138,158]	Confocal laser scanning (CLSM) Epifluorescence	Yes
Calcofluor White	Chitin in cell walls	Live[31]	Epifluorescence	Yes
DAPI (4′,6-diamidino-2-phenylindole)	Adenine-thymine-rich regions — DNA	Live/fixed[67,70,106]	Confocal laser scanning (CLSM) Epifluorescence Two-photon laser scanning (TPM)	Yes
FM4-64	Lipid bilayers, i.e. membranes and organelles	Live[32]	Confocal laser scanning (CLSM) Epifluorescence	Yes
Hoechst 34580	Nucleolus/DNA	Live[75]	Confocal laser scanning (CLSM) Epifluorescence	No
INT/MTT	Cell viability (cellular dehydrogenases)	Live[129]	Spectrophotometry	Yes
Lucifer yellow carbohydrazide	Extracytoplasmic (space between cell wall and cell membrane)	Live[7]	Confocal laser scanning (CLSM) Epifluorescence	Yes
LysoTracker Red	Vesicles	Live[78]	Confocal laser scanning (CLSM)	Yes
MitoTracker	Active mitochondria	Live[76]	Confocal laser scanning (CLSM)	Yes
Nile Red	Neutral lipids	Live[78,137,138]	Confocal laser scanning (CLSM) Epifluorescence Two-photon laser scanning (TPM)	Yes
Oregon Green 488 carboxylic acid diacetate (carboxy-DFFDA)	Vacuoles (tubular)	Live[77,78]	Confocal laser scanning (CLSM)	Yes
Succinate dehydrogenase (SDH) activity	Histochemical vital stain	Live[112,127,128]	Light microscopy	Yes
SYTO	Nucleic acids	Live/fixed[71,127,158]	Confocal laser scanning (CLSM) Epifluorescence	Yes
Uvitex2B	Chitin in cell walls	Live/fixed[33]	Confocal laser scanning (CLSM)	Yes
WGA + fluorophore (e.g., FITC, rhodamine)	N-acetylglucosamine (chitooligomers)	Live/fixed[23,25,31]	Epifluorescence	Yes

formed by amorphous material[21–23,27]. However, describing the specific cell wall structure is challenging, as chemical fixation of cells can introduce artifacts[28]. To date, most evidence has been acquired for a small number of species by using light and electron microscopy, and by *in situ* approaches mainly focusing on the distribution of chitin and glucans.

Like other fungi, chitin is a principal cell wall component of AM fungi[25]. Labelling of AM fungal cell walls with wheat germ agglutinin — a lectin that binds to chitin — bound to colloidal gold complexes (Table 1) showed accumulation of chitin throughout the cell wall[25] or localised in the outer wall layers[23]. Chitin appears present at all stages of morphogenesis, including the extra- and intraradical hyphae and arbuscles[25,29], although the form of chitin may change across these structures[25,30]. The use of fluorescent dyes such as Calcofluor-white[31,32] and Uvitex2B[33] (both bind to β-1-4-polysaccharides, such as chitin) further demonstrate the occurrence of chitin across the hyphal network (Table 1).

A correlation between cell wall thickness and chitin distribution has been suggested[27], with intraradical hyphae exhibiting a simplified structure[25,34], thinner walls[25,34], and irregular chitin labelling[22,23]. Arbuscules exhibit further modifications, as their walls lack a fibrillar texture and are amorphous[25]. Despite the absence of fibrillar chitin, remnants of chitin have been detected in

arbuscule walls by wheat germ agglutinin labelling, suggesting that the building blocks of chitin (oligomers of N-acetylglucosamine) are present, even though polymerised chains are not[25,30].

Chitin appears to exhibit a change in its final integration within ERM cell walls, potentially becoming encased and protected in non-chitinous cell wall components[25]. This suggests that AM fungi have high resistance to chitinolytic enzymes and to the organisms producing chitinases. This could also explain why the use of these enzymes alone does not result in the production of protoplasts, since they cannot cleave the protected chitin. Three lines of evidence support this hypothesis: firstly, chitin in extraradical hyphae was impenetrable to wheat germ agglutinin-conjugated fluorescence labelling, yet chemical treatments (in *Glomus versiforme*; synonym, *Diversispora versiformis*) enhanced binding[25]; secondly, chitinase pretreatment of *Funneliformis mosseae* cells revealed only a slightly diminished detection of chitin in external hyphae[29]; and thirdly, chitinase application to the apex of growing *F. mosseae* hyphae resulted in lytic events and consequent septation[35]. However, application to the subapical zone showed no effect[35]. The latter suggests that as the tip extends, the wall is more penetrable before chitin becomes embedded into non-chitinous components. Nevertheless, divergent chitin distribution is evident when compared with other filamentous fungi, in which chitin is predominantly deposited in the inner layers of the wall[17].

The distribution of specific glucans has also been investigated in AM fungi[24,29]. Glucans are the most abundant polysaccharide of the fungal cell wall, and their primary role is preserving the structural integrity of the wall[36]. Indirect immunolabeling confirmed the presence of β-(1,3) glucans in the inner walls of external hyphae, germ tubes, and intercellular hyphae in several species, including *F. mosseae*, *R. irregularis* and *Acaulospora laevis*[24,29]. β-(1,3) glucans progressively decrease as the fungus colonises the root yet are absent from arbuscules[29]. In contrast, β-(1,3) glucan was undetectable in *Gigaspora* and *Scutellospora* species[24], indicating that the cell wall composition of Gigasporaceae has diverged from that of the Glomeraceae. More recently, sequencing of the *Gigaspora margarita* genome corroborated this finding as homologues of FSK, the β-(1,3) glucan synthase, were not detected among the protein-coding genes[37].

While microscopy observations and *in situ* approaches have provided insights into the essential elements, we lack detailed information on the specific composition of the AM fungal hyphal cell wall. Sequencing of the *Gi. margarita* genome identified numerous mannoprotein biosynthesis enzymes[37]; however, wall components such as α glucans, galactomannan, hydrophobin rodlets and glucuronoxylomannan that affect the cell morphology and function of other fungi have never been investigated for their presence/absence in AM fungi. Biochemical analyses, such as mass spectrometry-proteomics, are required to deduce the entire cell wall composition[38] and to examine how it varies across morphogenetic stages and the AM fungal phylogeny.

Cell membrane

Cell membranes protect cells and regulate the transport of metabolites and solutes. Understanding the AM fungal cell membrane can provide insights into how AM fungi absorb water and nutrients from the soil and mediate nutrient exchange with their plant hosts[39,40]. The majority of our cytological knowledge comes from membrane examination in the arbuscule interphase[40], and no information is available for the status of the membrane for the extraradical hyphae. It is important to note, however, that molecular features of the AM fungal membrane of extraradical hyphae are well-described, including the identification and regulation of transporters for phosphate[41,42], ammonium[43], and iron[44].

Insights from early electron microscopy studies provided evidence that the periarbuscular membrane (PAM; the membrane that envelops the developing arbuscules) (Figure 1) is of plant origin, and the development of the PAM requires large-scale remodelling of the plant plasma membrane[45,46]. Recent electron tomography studies revealed intricate details in plant roots colonised by *R. irregularis*, including an extensive elaboration of arbuscle membrane tubules (memtubs) attached to the fungal protoplast, and an interconnected system of vesicles and membrane-bound compartments in the periarbuscular space (the matrix between the PAM and fungal hyphal membrane)[40,47]. While the function of the above structures is largely unknown, memtubs are hypothesised to function in the release of molecules or in nutrient assimilation. Additionally, extracellular vesicles are suggested to be involved in inter-organismal communication, possibly as mediators of this crosstalk; however, the recipient of these organelles in the symbiosis is currently unknown[48].

Cytoskeleton

Filamentous hyphae grow by polarised tip elongation[49]. This process involves cell wall extension, membrane expansion, and the continuous delivery of secretory vesicles to the hyphal apex via the fungal cytoskeleton[49–51]. The fungal cytoskeleton, consisting of microtubules (tubulin polymers) and actin microfilaments (polymers formed by the assembly of globular actin (F-actin)), regulates numerous cellular functions, including endocytosis, exocytosis, and intracellular organelle transport[49,52]. These polymers are also important for the maintenance of cellular polarity[53–55] and could play a crucial role in the morphogenesis of the various AM fungal structures (Figure 1).

The fungal cytoskeleton has been well studied in several model filamentous fungal species[53,54,56–58], yet efforts to visualise these structures in AM fungi have been nearly absent. Indirect immunofluorescence microscopy, in which two antibodies are used with a fluorophore-conjugated secondary antibody, of microtubule and actin subunits has yielded some results in hyphae emanating from germinating spores of *F. mosseae*[59]. Microtubules were located at the outer edges and central regions of hyphae[59]. Microtubules have also been identified using electron microscopy, often localised in the vicinity of the plasma membrane and within the cytosol[21]. However, visualisation of actin filaments by indirect immunofluorescence microscopy was only successful in thin hyphae. Here, actin microfilaments were observed in the outer regions of the hypha, with no observations of microfilaments at the apex[59]. The repeated washing steps required for indirect immunofluorescence microscopy likely hampered visualisations of the microfilaments at the hyphal tips as they are sensitive to physical disturbance.

Nuclei fluorescence staining revealed alignment with microtubule tracks[59], suggesting a possible role of microtubules in nuclear distribution, though direct evidence is still lacking (Figure 2). Experiments utilising microtubule inhibitors in *Neurospora*

crassa demonstrated a role for microtubule tracks for nuclear distribution and retrograde movement within filamentous hyphae[60]. Interestingly, the transport of nuclei is determined by a combination of forces in *N. crassa*, with cytoplasmic bulk flow mediating passive nuclear streaming[60]. A similar phenomenon was observed in *Aspergillus gossypii* in which microtubules drive nuclear oscillations and rotations, while transport is predominately driven by cytoplasmic streaming[58].

To date, only one study has successfully recorded features of the AM fungal cytoskeleton in symbiotic hyphae associated with host roots[61]. Indirect immunofluorescence microscopy targeting fungal tubulins revealed longitudinal bundles of microtubules in extra- and intraradical hyphae in *R. irregularis*[61]. Bundles or cables of microtubules were also observed in the large trunk hyphae and the fine branches of arbuscules[61]. These visualisations are crucial because they may highlight important transport activities for both the extra- and intraradical hyphae, and translocation routes to and from the arbuscules[61].

Despite some visualisations of the AM fungal cytoskeleton, we still require a comprehensive view of the cytoskeletal structure at the network level, and the role microtubules play in organelle migrations. In addition, much less is known about actin dynamics in AM fungi and other cytoskeletal components such as septins. Work performed on free-living filamentous fungi has benefitted from live-cell imaging of actin using GFP constructs[56]. Such techniques are unsuitable for AM fungi, as they are considered recalcitrant to genetic transformation due to their coenocytic and constant multinucleate organisation throughout all life stages[19]. While indirect immunofluorescence microscopy has provided some insights, cytoskeletal components are notoriously difficult to preserve during fixation in cells with complex cell walls[61]. Resolving the cell wall chemistry could allow us to identify pretreatment enzymes that make the cell wall permeable, increasing the efficiency of mainstream microtubule dyes seen to previously work on fungi (e.g., SiR-actin/tubulin, Lifeact, etc.). Alternatively, applying pre-stabilisation treatments with microtubule preservative agents[62] or using mild fixation protocols[63] could help preserve tubulin and actin for simple or multiplexing immunofluorescence[64]. Regardless, live-cell imaging techniques, such as nuclear dyes and microtubule inhibitors, used on free-living fungi could be adapted to AM fungi to explore the role of cytoskeletal components for organelle migration, potentially providing functional information that complements data obtained from fixed-cell techniques.

Cytoplasm and organelles

Organelle movement and positioning dictate cell growth and function. Ultrastructural studies are commonly used to examine cell cytology, but for AM fungi they have mostly focused on symbiotic interfaces and germ tubes. Again, this is because of the difficulty in manipulating living hyphae and the ability of the organelles to flow freely, making organelle dynamics difficult to study. Nevertheless, some studies have reported the presence of several organelles within the cytoplasm, including nuclei, vacuoles, mitochondria, Golgi equivalents, and extraordinarily large lipid droplets in hyphae[21,26,65,66]. The presence and distribution of important non-membranous, such as ribosomes or the cytoskeleton, and membranous organelles, such as the endoplasmic reticulum, have been mostly neglected in AM fungal cytology (Figure 2).

Fluorescent probes provide an alternative method to identify specific organelles and potentially acquire live cell dynamics (Table 1, Figure 3 and Video S1). The nuclei of AM fungi are typically well-suited to such studies. In particular, the multinucleate nature of AM fungal hyphae has been well-documented by using the fluorescent DNA-specific probe DAPI (4′,6-diamidino-2-phenylindole)[67–70] or the SYTO fluorescent nucleic acid stains in live and fixed cells[71] across a range of AM fungal species (Table 1). Still, fine nuclear details such as the nucleolus, which is the ribosome biogenesis location, have been rarely examined. A few studies have revealed fine details of the nucleolus in fixed samples using electron microscopy, including granular and fibrillar components, the nucleolar organiser and the status of heterochromatin surrounding this element[66,72]. Focusing further on the nucleolus, and particularly on its deformities, could inform on autophagy events[73] and overall cell health[74]. Nucleolus examination will benefit from live imaging using fluorescence and/or confocal microscopy with DAPI in combination with specific dyes, such as Hoechst 34580, targeting this structure[75]. In a similar manner, mitochondria-specific dyes such as MitoTracker (Table 1) give insight into the spatial organisation and dynamics of these organelles in AM hyphae[76]. Timelapse imaging of a developing spore revealed mitochondria migrating inside a hypha into the incipient *Glomus diaphanum* spore[76], yet observations of this kind are rare[76].

Beyond nuclei and mitochondria, which are the most studied organelles, a combination of laser scanning confocal microscopy and ultrastructural analysis of rapidly frozen AM fungal samples has uncovered an extensive tubular vacuole system[77,78]. This consists of a network of membrane-bound compartments that play key roles in maintaining cellular homeostasis, storage, and degradation processes. The vacuole system was identified in germ tubes, extraradical hyphae and intercellular hyphae of *Gi. margarita* using the vacuolar tracer Oregon Green 488 carboxylic acid diacetate (carboxy-DFFDA) (Table 1)[77,78]. The vacuoles are highly pleiomorphic[79] and can range from spherical to tubular and have a mildly acidic pH[78], which differs from the cytosolic pH of *Gi. margarita* and *R. irregularis*[80]. Vacuole acidification is essential for diverse physiological functions and aids in nutrient transport into and out of the vacuole[81,82]. Furthermore, a combination of labelling methods (Table 1) has shown that vacuoles store phosphate compounds[77]. The tubular vacuoles, which form longitudinal arrays and bundles from tip to base, are associated with cytoplasmic streaming and polyP granule transfer, and are proposed to occupy most of the cell volume[77]. This implies a role in long-distance intracellular translocation. Such a role for tubular vacuoles has been demonstrated in the ectomycorrhizal fungus *Pisolithus tinctorius*[83]. Overall, the physiological properties of the AM fungal tubular vacuole system are mostly unknown.

Finally, the cytoplasmic organisation of fungal hyphal tips, either in germ tubes or in mature networks, is phylogenetically informative and plays a key role in processes related to polarised tip growth[26,84]. Distinct zones of *Gigaspora* spp. germ tubes have been described based on their cytoplasmic content, using a combination of light and electron microscopy[26]. The hyphal apex represents the first zone and contains numerous secretory vesicles organised into a crescent behind the apex, resembling the apical vesicle crescent observed in early-diverging fungi[84], which is an analogous structure to the Spitzenkörper. The

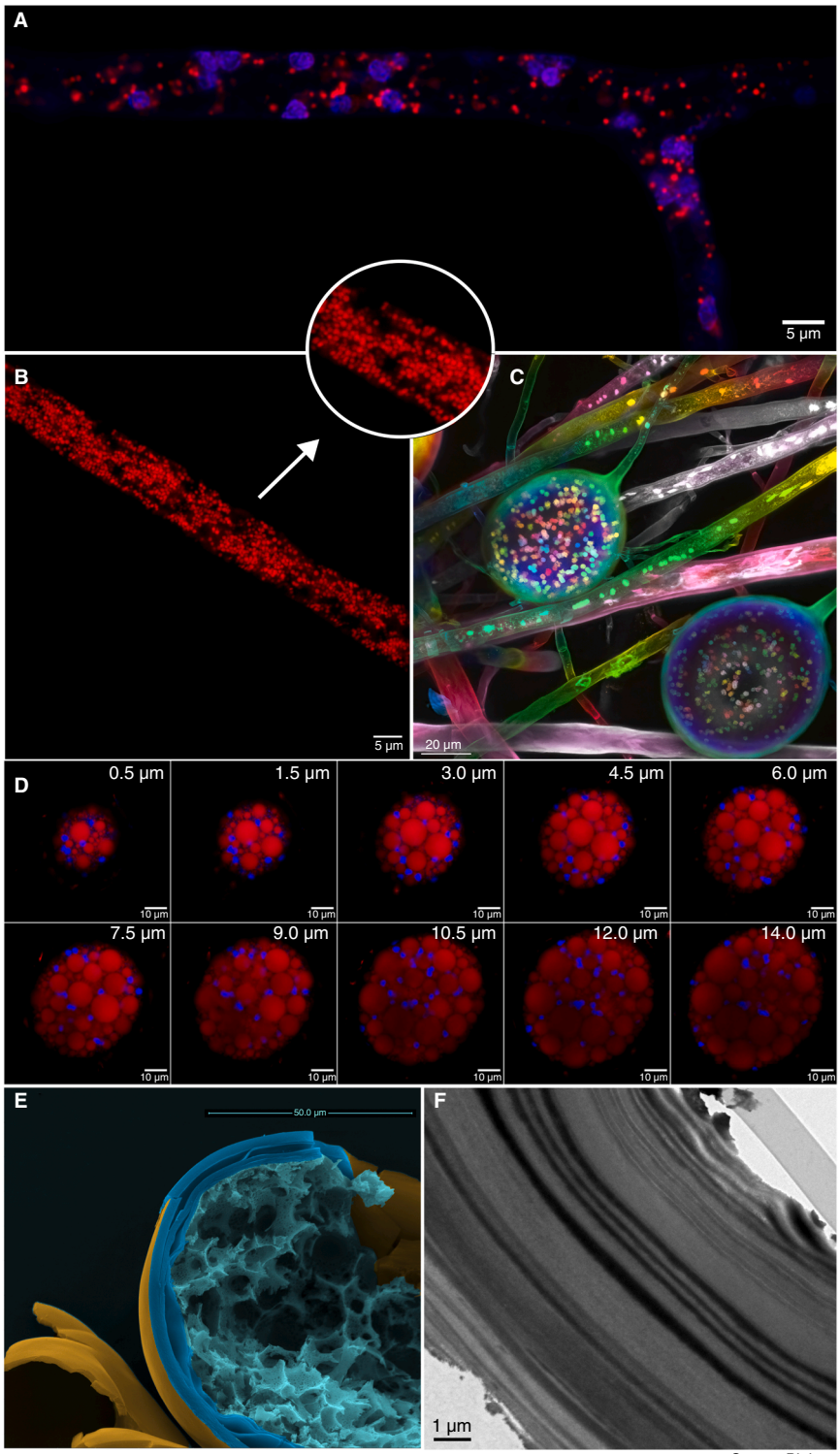


Figure 3. Localisation of nuclei and lipid droplets in hyphae and the multinucleate nature, lipid content, and ultrastructure of spores.

(A) Confocal microscopy of a branching hypha with nuclei stained with SYTO13 fluorescent nucleic acid dye (blue) and lipid droplets stained with Nile Red (red). (B) A hyphal section imaged with confocal microscopy showing lipid droplets stained with Nile Red and an insert highlighting accumulations of individual lipid droplets in the centre and near the periphery of the hypha. (C) Multinucleate spores stained with STYO13 and observed with a confocal microscope (ZEISS LSM 800). The image consists of multiple optical slices merged into a single 2D image. The final image was colour coded along the z-axis for depth recognition. (D) The multinucleate nature and lipid content of AM fungal spores. Shown are nuclei (blue) localised to the periphery of the spore and in between the lipid globules (red). Image data consist of multiple optical slices (97 z-stacks with 0.5 µm intervals). Selected slices are shown with the corresponding depth in µm through the spore. Z-stacks were acquired with a silicone immersion 100x lens. (E) Scanning electron microscopy (SEM) of a fractured spore, showing the spore interior and multi-layered wall. (F) TEM photograph of a spore showing a thick wall with laminations. Images in panels A, B, and D were acquired with a Nikon AXR confocal microscope (Amsterdam VUmc, Microscopy and Cytometry Core Facility). All images were acquired from *Rhizophagus irregularis* specimens.

Spitzenkörper is found across species of the Ascomycota and Basidiomycota Phyla and is a highly dynamic multicomponent complex that behaves as a vesicle organising centre and maintains hyphal growth and morphogenesis[84,85]. Interestingly, fungi in the Mucoromycotina and Mortierellomycotina lack a classical Spitzenkörper. Instead, they possess an apical vesicle crescent (Figure 2). Examination of germ tube tips of three AM fungal species (*Gi. gigantea*, *Gi. margarita* and *Gi. rosea*) supports the idea that AM fungi rely on an apical vesicle crescent and not on a Spitzenkörper[26]. Although electron microscopy helped to visualise the hyphal tip organisation of AM fungi, only germ tubes have been examined, leaving a gap in the study of vegetative hyphae. Challenges in collecting and fixing hyphae from the ERM raise the possibility that their tip organisation may differ.

Different microscopy methods have provided insights into AM fungal organelle content and organisation. Electron micrographs have confirmed the presence of common organelles and fluorescent probes have visualised nuclei, mitochondria and lipids in hyphae and an extensive tubular vacuole system. To build on previous research, a combination of fluorescent probes and novel antibody methods could be used to examine spatial distribution and relative organelle positioning. An important next step would be to target additional organelles to build a 3D model of relative organelle arrangement to better understand their spatiotemporal dynamics and infer functions. This would expand on transmission electron microscopy analysis by examining larger hyphal

Box 1. 'Obscure' organelles.

Multiple 'obscure' organelles and structures have been identified within the AM fungal hyphal cytoplasm. While the functions of these are largely unknown, they have been found in studies containing detailed independent electron micrographs or micrographs combined with energy dispersive X-ray analysis. Still, their function remains elusive (Figure 1). The list is long and includes, firstly, membrane-bound crystals, which possibly correspond to the hexagonal crystals described in other fungi such as *Neurospora crassa*. These organelles appear very electron-dense in electron micrographs and are bound by a single membrane[159]. Their function in septa-forming fungi seems to be related to that of the Woronin bodies, plugging septa to regulate traffic especially after wounding[160]. Future work should aim to link the localisation of these elusive organelles to cytoplasmic dynamics or hyphal disturbance. Secondly, osmophilic globules have unknown function but are suggested to play a role in cell wall formation or diverse enzymatic activities[101]. Thirdly, glycogen deposits, in which hexose is converted into glycogen, represent the predominant form of sugar transport to the ERM[161] and if further analysed for their spatiotemporal dynamics, could inform us about carbon translocation and storage dynamics across the network. Fourthly, polyphosphate-like granules (also referred to as dense granules) are only present in vacuolated hyphae and are entirely absent in collapsed arbuscules. These granules contain phosphorus and calcium and are hypothesised to be involved in phosphorus transfer to plants[162]. Interestingly, the calcium deposition observed in plant cells after arbuscule collapse supports this idea. Finally, polyvesicular bodies (syn. multivesicular bodies) are membrane bound cytoplasmic inclusions that hypothetically are involved in vesicle production[159]. Other important organelles such as lysosomes, which contain digestive enzymes, waste-related vacuoles and how they are disposed, smooth and rough endoplasmic reticulum, which are linked to creation/storage of lipids and steroids and protein synthesis, remain understudied in AM fungi.

segments with a focus on obscure organelles (Box 1 and Figure 2) that are rarely or never mentioned. In addition, custom-built microscopes and the use of microfluidics[86] that enable undisturbed sampling of the ERM could be used to capture live dynamics of multiple organelles.

The hyphal network

Network traits and morphogenesis

The living part of the hyphal network of AM fungi is a single interconnected massive cell linking colonised plant roots with the soil matrix. Although AM fungi are coenocytic, they are capable of forming septa under specific circumstances, such as to isolate dying regions of the ERM[70] or as a repair mechanism[87]. Septa also appear when germ tubes senesce in the absence of a host[69,88] (although multiple germination attempts are possible[89,90]) and upon arbuscle branch collapse in the IRM[91]. This process involves cytoplasmic retraction[88,92] and is described as a strategy to reallocate resources[86], to abandon suboptimal pathways[86], and enhance survivability. It is important to note here that, when assessing intraradical AM fungal colonisation with common histological techniques (e.g., McGonigle or Trouvelot method[93,94]), septated hyphae are dismissed as non-AM colonisation. Excluding possible AM fungal necromass can underestimate true colonisation values and ignore important colonisation dynamics. Future work should utilise monoxenic setups to explore the septation patterns of IRM and ERM in response to fungal/plant ontogeny and environmental variation.

At the initial stages of their formation, spores are also part of this interconnected open pipe system. AM fungal spores form terminally[95] or intercalary and singularly or in clusters[96] on sporogenic hyphae; both patterns have been observed on the same mycelium in root organ cultures with *R. irregularis*[97] and *F. mosseae*[98]. Upon maturation, the flow of cytoplasmic material ceases, and spores become distinct and independent units with the formation of septa. Variations in ontogeny lead to different spore types (e.g., glomoid, gigasporoid, etc.)[99] and there are differing germination modes among species, such as germination through the spore wall or with specialised structures such as germination shields[98,100] (for more details about spore structure and content see Box 2).

Research on the ERM residing in soil is of particular interest to our review. Previous studies on *R. irregularis* have provided insights into ERM architecture, defining key features such as runner hyphae, secondary and tertiary branching zones, and branching absorbing structures[13,101] (Figure 1). The formation of these structures seems under precise control: recent analysis of the *R. irregularis* transcriptome revealed structure-specific expression profiles for extraradical structures including germ tubes, immature and mature spores, runner hyphae, and branching absorbing structures[102]. The expression profiles demonstrated distinctive functions for nutrient absorption and metabolism of each fungal structure — for example, nitrate absorption in runner hyphae and fatty acid modification in mature spores — yet also revealed branching absorbing structure-specific functions, such as inorganic phosphate and ammonium absorption[102]. The analyses also identified putative genes associated with nutrient exchange expressed in the arbuscules. These data provide information on the dynamics of nutrient absorption and reveal key functional differences between branching absorbing structures and runner hyphae[102]. However, how the fungus fine-tunes expression across a widespread interconnected cell remains unknown. Techniques such as RNA/DNA fluorescence *in situ* hybridization (FISH)[103] have proved reliable for AM fungi[104,105] but could be exploited further. Single molecule FISH (smFISH), a technique able to quantify individual RNA molecules in fixed cells, can uncover fine-scale gene expression patterns but has never been applied to ERM so far. Combining DNA and RNA smFISH techniques on rare dikaryotic AM fungal strains[106,107] would help us understand how genes are expressed and regulated in these open pipe systems. This approach could reveal nucleotype-specific spatiotemporal expression patterns and their dynamics across the network,

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Box 2. Arbuscular mycorrhizal fungal spores.

Spores are AM fungal reproductive propagules. AM fungi produce some of the largest single-celled fungal spores on Earth, and their morphological traits differ among species[163]. The phenotypic characteristics and the structure of AM fungal spore walls have received much attention, especially from a taxonomic perspective[164,165]; comparative analyses of AM fungal spores have revealed interspecific differences in laminations, thickness, and texture[18,99]. Generally, the spore wall is a complex, multi-layered structure with chitinous microfibrils that arrange in a particular pattern[18,22,166]. Crystalline chitin fibrils are laid down in parallel planes and appear to form arcs due to a progressive rotation[18,30,166] . Helicoidal microstructures are not unique to AM fungal spores and are observed in the exoskeleton of arthropods and plant cell walls, with the latter formed by cellulose fibres[127,128]. These 3D structures provide tensile strength, yet the molecular control of the fibrillar organisation in AM fungi is currently unknown[30]. During maturation, the layering within the spore wall changes and the zonation of the spore wall becomes more distinctive[167] and the microfibrillar organisation is lost upon germination[30].

During sporulation, the juvenile spore is an extension of the coenocytic network and thus expected to share the cytoplasmic content. The lipid-rich content of AM fungal spores has been visualised using electron and fluorescence microscopy[137,167]. Initially, young spores are populated from the surrounding mycelium with small lipid droplets that coalesce into larger globules as the spore matures[65]. Fatty acid profiling showed that neutral lipids (NLFA) dominate the lipid fraction in ungerminated spores with a high TAG content[133,135]. However, TAG content varies among studies, with estimates ranging from 58% in *G. caledonium* to 80% in *D. versiformis*[133,134]. This variation may reflect spore maturity differences, diverse cultivation conditions[134] or simply phylogenetic-based differences. The large amounts of carbon stored in the spore are energy reservoirs that are rapidly depleted during spore germination (from 38 to 6.3 μg/100 μg wet cells)[133]. Many fungi produce multinucleate spores, yet AM fungi are distinct as they continuously carry hundreds to thousands of nuclei[71]. Various methods have been employed to estimate spore nuclear content, including confocal microscopy and modelling, with widely divergent results. (For a recent detailed review on spore nuclei see[71] and references therein). While the lipid and nuclear content of AM fungal spores has been extensively studied, much less is known about other organelles. Ultrastructural analyses have identified mitochondria and vacuoles[167], and some insights into mitochondrial dynamics during spore development have been gained using fluorescence staining[76]; however, such examples are limited.

New fluorescence protocols are now needed to visualise additional organelles in spores. While MitoTracker staining for mitochondria has been utilised[76], its application in AM fungi remains limited. Although the lipid-rich and multinucleate nature of AM fungal spores is well established (Figure 2), there is a lack of time-resolved data on the transport dynamics of these organelles. For example, it is currently unknown why nuclei exhibit bidirectional flow from spores[68] and data acquired on lipid expansion during development was extracted from individual spores of different ages[65,133], instead of single spores over time. Confocal microscopy has demonstrated that nuclei are positioned both at the periphery of the spore and amongst the lipid droplets (Figure 2). Timelapse studies on undisturbed hyphal networks using multiple fluorescent probes could help clarify the movement of lipids and nuclei into developing spores. Conducting such experiments at the network scale could provide valuable spatiotemporal data on organelle dynamics during spore development.

and help resolve debates on the function of the preserved meiotic machinery in the AM fungal genome[108] and the presence or absence of karyogamy (fusion of two genetically different nuclei), an indication of sexual reproduction in dikaryotic fungi[109].

In terms of network structure itself, the ERM of AM fungi exhibits a sigmoidal growth pattern typical of microbial growth, with distinguishable phases (lag, exponential, and stationary)[13,97,110]. Yet variation in the lag phase of mycelial growth and sporulation exists among *Rhizophagus* strains[110]. AM fungal species also differ in branching pattern, hyphal length and network density[111–113]. Such work has suggested that certain AM fungal species/isolates are faster colonisers, and differences in mycelial growth and colonisation rate can be attributed to propagule germination rates, the formation of primary infection units and branching morphology[110–112]. However, variations in ERM architecture have been shown under different experimental protocols, potentially imposing bias on architectural trait outcomes[114].

Another source of observed variation in network structure is hyphal self-fusion frequency[115]. Vegetative hyphal self-fusion, also known as anastomosis, facilitates interconnectedness between network regions of the same mycelium, potentially increasing nutrient acquisition and resource translocation across the fungal network[116]. Anastomoses create loops that facilitate damage recovery[87], and even possibly facilitate coordination, creating paths across large distances. The rate of anastomosis is thought to be related to fungal genotype[115], with Glomeraceae species showing higher self-fusion frequencies (sometimes exceeding 80% in *R. irregularis*[115,117]). However, anastomosis frequencies vary greatly between species and experimental contexts (Table 2 and Table S1). In contrast, Gigasporaceae species form very few fusions between hyphae of the same strain[87,115,117–119]. Analysis of *Gigaspora rosea* and *Scutellospora castanea* germlings revealed no fusions between hyphae belonging to the same germling or different germlings of the same strain[118]. This suggests that divergent strategies for fungal colony development and damage recovery mechanisms[87] have evolved between members of the Gigasporaceae and other AM fungal families. These differences might hold an important role in cytoplasm dynamics and nutrient trade between symbionts.

Experimental methods involving monoxenic cultures, network growth restriction to 2D and high-throughput image collection and analysis have enabled visualisations of network

Table 2. Hyphal anastomosis frequencies (AF) are self-fusion events related to mycelial interconnectedness (%) across the AM fungal phylogeny.

AM fungal species	AF within germlings	AF between germlings	AF within a mycelium	AF between mycelia
Acaulospora scrobiculata[118]	42.01 ± 6.38	72.86 ± 6.72		
Acaulospora spinosa[118]	32.81 ± 6.92	14.02 ± 7.36		
Ambispora leptoticha[119]		0		
Claroideoglomus etunicatum[118]	75.40 ± 3.57	63.79 ± 6.09		
Dentiscutata reticulata[117]			5.3	0
Funneliformis caledonium[115]	35.0–55.0	0–34.0		
Funneliformis coronatum[116]			1.2–7.7	
Funneliformis mosseae[115,116,127]	51.0–57.0	0–40.0	27.2–63.8	25.8–62.3
Gigaspora albida[118]	0	0		
Gigaspora gigantea[119]		0		
Gigaspora margarita[117]			4.5	0
Gigaspora rosea[115,118]	0	0		
Glomus formosanum[118]	79.84 ± 4.39	91.41 ± 3.92		
Glomus proliferum[117]			79.6	20.1
Paraglomus occultum[119]		0		
Rhizophagus clarus[118,119,168]	6.3–47.98	0–37.5	2.4–15.6	5.4–37.4
Rhizophagus irregularis[115–117,119,169,170]	45.8–69.0	0–90.0	13.9–87.5	12.5–49.5
Rhizophagus manihotis[118]	9.29 ± 3.01	24.29 ± 11.02		
Scutellospora calospora[118]	0	0		
Scutellospora castanea[115]	0	0		
Scutellospora heterogama[119]		0		

Range of values are shown per species. To view values per strain, see Table S1.

development over time, providing foundational information on ERM architecture and morphogenesis[13]. These techniques enable non-destructive observations that circumvent problems with extracting mycelium from field soil or pot cultures. A limitation of this approach is that only a few select species can be cultivated *in vitro*. Much of the information acquired about ERM growth dynamics has been extracted from *R. irregularis*, the model AM fungal species. To broaden understanding beyond model species, future research could utilise transparent, synthetic soils to grow plantlets with several AM fungal species[120]. This novel approach utilises a super absorbent polymer-based transparent soil in a two-compartment Petri dish system, allowing easy establishment of single spore cultures and the monitoring of fungal propagation and growth. While this technique would require optimisation for microscopy, it would enable non-sterile continuous microscopic observations and could expand knowledge of ERM architecture across the AM fungal phylogeny. Moreover, the necessity to develop a life-history trait database based on standardised protocols has been proposed[114]. Such a database would facilitate linkages between morphology and function, enhancing our ability to predict ERM traits and their influence on symbiotic performance, plant communities and AM fungal community dynamics[114].

A critical, but understudied, feature of the AM fungal hyphal network is the extracytoplasmic space, a region between the cell wall and the cell membrane that exists across the entire hyphal network, extraradically and intraradically. The extracytoplasmic space facilitates water movement along the hyphae and ultimately transfer to the host plant[7,121]. Direct observation of extracytoplasmic water transfer, which is driven by plant transpiration, was possible with the use of Lucifer yellow carbohydrazide (Table 1), a fluorescent dye that can penetrate the cell wall but not the cell membrane[7]. When combined with isotopic labelling of water, this method provided strong evidence that AM fungi can traverse air gaps in soil, enhancing plants' access to water[7]. Of interest for future work would be the use of water-soluble dyes with unique excitation/emission profiles to examine water relations between co-existing strains and interconnected hosts. Another unexplored aspect of this extracytoplasmic water flow is the potential role it could hold for the transfer of mycoviruses between compatible neighbouring AM fungal networks, and plant viruses between interconnected hosts via a single network[122,123]. This testable hypothesis could help explain the observed mycorrhiza-induced susceptibility of plants to viruses[124].

Finally, at the mycelial level, the use of dyes that rely on the membrane integrity (viable *versus* non-viable cell) can help us understand when different strains of AM fungi either fuse or compete. Fluorescein diacetate[125], propidium iodide[126], succinate dehydrogenase activity[127,128], INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-2H-tetrazolium chloride)[129], MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide)[129] and trypan blue[130] are commonly used dyes to assess AM fungal viability (Table 1). Fluorescein diacetate is non-fluorescent but, once inside living cells, esterases convert it to fluorescein, which

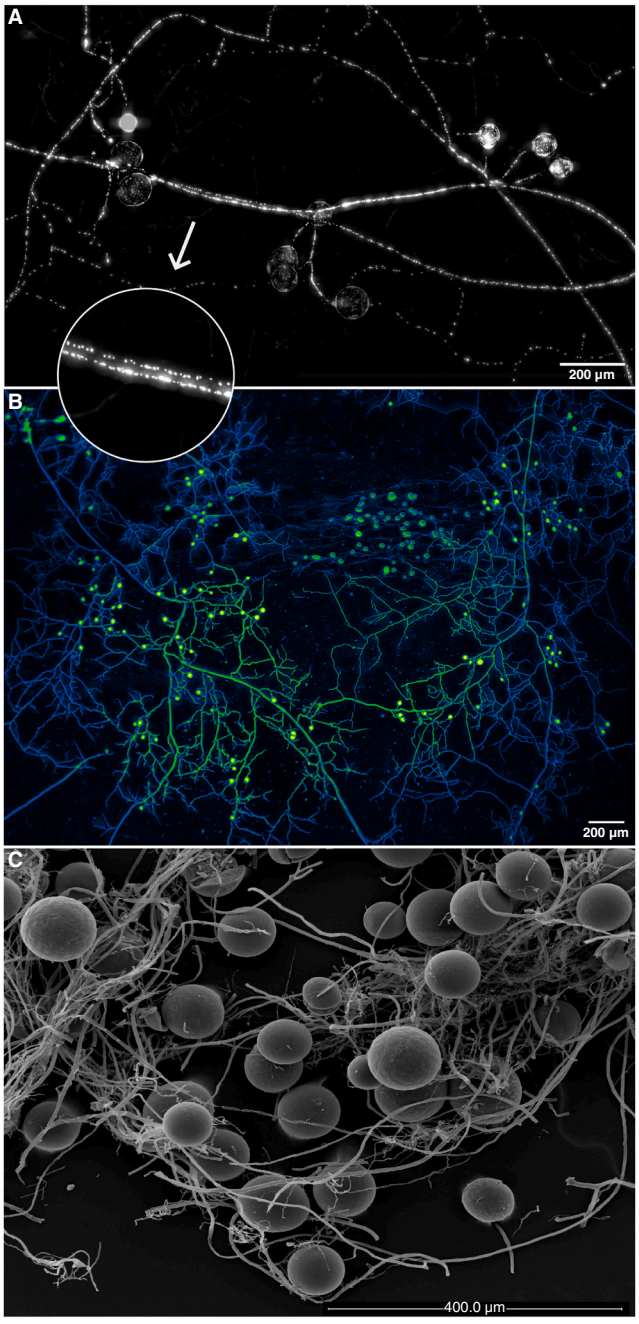


Figure 4. Techniques for AM fungal network imaging.
(A) AM fungal network grown asymbiotically in M-medium[171] with the addition of myristate (a fatty acid that sustains AM fungal growth in the absence of a host[158]) using a microfluidic chip system showing nuclear distribution in hyphae and spores, with an insert highlighting nuclear spacing in a hyphal section. Following fixation, the network was stained with DAPI (4′,6-diamidino-2-phenylindol) and imaged with a ZEISS Axio Zoom system (10x lens). The image was post-processed to remove uneven background using rolling ball background subtraction (rolling ball radius 50px) (image courtesy of Sander van Otterdijk, Vrije Universiteit, Amsterdam). (B) Image of ERM captured from *Glomus aggregatum* showing hyphae and spores. The network image was acquired using a 4x lens attached to a bespoke microscope designed to image live ERM without disturbance. False colour was applied in post-processing (image courtesy of Loreto Oyarte Gálvez, Vrije Universiteit and AMOLF, Amsterdam). (C) SEM photograph of intact spores attached to extraradical hyphae.

emits green fluorescence under UV or blue light, indicating metabolic activity[125]. In contrast, propidium iodide is membrane-impermeant and only enters cells with compromised membranes, binding to nucleic acids and emitting red fluorescence in non-viable cells[131]. Together, these dyes help differentiate between living and non-living fungal structures across diverse abiotic and biotic conditions. In the future, the use of such dyes may expand beyond individual strain samples to enable visualisations of interaction outcomes when different strains of AM fungi fuse to produce a shared mycelium. This method, in combination with high-throughput imaging, could elucidate whether regions of a shared network eventually septate to resolve competition, or whether the network remains viable, indicative of cooperation.

Organelle distribution

Examining the relative distribution of organelles at the network scale can help us infer information about the modes of transport of these organelles and resources (Figure 4 and Videos S1–S4). Lipid droplets have been observed with electron microscopy in all AM fungal structures[22,65]. It is well-established that carbon is predominately stored as triacylglycerol in lipid droplets at all stages of the fungal lifecycle[132–134], and lipid profiling studies have demonstrated that triacylglycerol can constitute up to an astonishing 90% of the total lipids in the ERM[132]. Calculations based on fatty acid profiling have suggested that 20% of the spore biomass is neutral lipid (*R. irregularis*), while other estimates suggest that total lipids account for 45–72% of the spore biomass, depending on growth stage[134,135]. Lipid storage patterns can also differ between species. Signature fatty acid analysis revealed that *R. irregularis* allocated more of its lipids to storage than *S. calospora* and positioned more to the IRM[136]. The latter result is expected, considering *S. calospora* does not form vesicles or intraradical spores[136].

Nile Red, a lipophilic neutral lipid-specific dye (Table 1), is a useful tool to track and quantify lipid content and therefore carbon flow in AM fungi (Videos S2 and S3). Nile Red staining of *R. irregularis* and *Gi. rosea* germ tubes revealed more lipid bodies near the germinating spore and less toward the germ-tube tip[137]. Similar ERM lipid distributions were found; lipid droplets accumulate closer to the root and were progressively fewer toward the growing colony front[137]. This suggests that lipid droplets are progressively consumed for hyphal elongation. While the direct transport pathway between plant host and fungus is unknown, it is well-established that the fungus receives fatty acids from the plant host and synthesises storage lipids in the IRM before exporting to the ERM[133,137,138]. Interestingly, the emergence of lipid droplets has been found to coincide with arbuscule collapse[138]. Future work should aim to quantify lipid flows and should aim to understand more about their physical properties.

Cytoplasm, cytosol, and cytoplasmic flows

Cytoplasm refers to the cell content enclosed by the cell membrane, while cytosol describes only the fluid portion of the cytoplasm, excluding the numerous organelles. Cytosolic free Ca^{2+} controls how fungi respond to environmental triggers and how they regulate nutrition[139]. Furthermore, changes in cytosolic pH (pH_c) can affect fungal behaviour and have been linked to hyphal chemotropism and host infection-related processes[140].

In line with what is seen in other fungal groups, cytosolic free Ca^{2+} in AM fungi also changes in response to environmental signals, including physical disturbance, temperature differences, and chemical cues[141]. Interestingly, it has also been hinted that cytosolic free Ca^{2+} plays an important role in fungal–endobacterial communication and in the AM fungal cellular metabolism by affecting mitochondrial function[142]. Furthermore, the use of the fluorescent (after it is hydrolysed) dye acetoxymethyl ester has allowed for fine pHc measurements for *Gi. margarita* and *R. irregularis* hyphae, from apical regions (approximately pH_c 6.7 with a sharp increase to pH_c 7 right beyond the apex) to subapical (stable pH_c 6.6)[80]. In the future, the use of Ca^{2+} microelectrodes in combination with Ca^{2+} fluorescent dyes and pH indicator dyes will be useful in revealing crucial information related to AM fungal cell coordination and behaviour, including how AM fungi control cytoplasmic flows.

Cytoplasmic flows, often referred to as cytoplasmic streaming, occur in diverse cell types and function as active transport pathways that overcome the slowness of diffusion[143]. These internal flows carry organelles and cellular components across the fungal colony, resulting in a constant motion of cellular contents in hyphae[144]. Organelles including nuclei and lipid droplets have been visualised in cytoplasmic flows in AM fungi[68,137], often travelling at velocities between 2 and 11 $\mu m \cdot s^{-1}$ [86,128,137]. In some cases, measurements from monoxenic cultures of *R. irregularis* detected cytoplasmic flow speeds exceeding 50 $\mu m \cdot s^{-1}$ [13,145]. Researchers have noted that the cytoplasmic flows of AM fungi are bidirectional and incredibly dynamic[86,145]. Such bidirectionality makes sense, because carbon from plant roots needs to travel to fungal tips and spores. Simultaneously, nutrients and water must flow in an anti-parallel direction to reach colonised plant cells. Bidirectional cytoplasmic streaming would also make sense in asymbiotically growing hyphae when considering energy being stored in the spore and minerals being taken up subapically. This bidirectionality results in fast and complex flows (Videos S2, S5 and S6). Lipid droplets, for example, can move independently of the flow of bulk cytoplasm or remain stationary while the cytoplasm is moving[86] (Video S3). Additionally, certain nuclei move in pulses, change directions, or remain anchored in specific zones[68]. This behaviour is postulated to favour nuclear mixing or to create discrete compartments for localised cellular functions in an otherwise continuous cytoplasm[68]. In the future, methods designed to analyse movement and dynamics of complex fluid systems such as particle image velocimetry[146] and kymograph analysis[13,147] could prove useful for describing the complex dynamics of the AM fungal cytoplasmic flows.

While a mechanistic understanding of fluid flows in AM fungi is lacking, evidence from other organisms suggest that motor-driven transport along the cytoskeleton plays a central role[144]. The molecular motor-mediated association of organelles to cytoskeletal components, such as microtubules, may enable anti-parallel trafficking and movement independent of the bulk flow[86,144,145]. Additional physical forces and material properties are postulated to control the movement of bulk cytoplasmic flow over long distances[144]. Evidence in *N. crassa* suggests that fluid flows exhibit a lack of shear due to the viscoelastic nature of the cytoplasm and behave like toothpaste in a tube rather than water in a pipe[144,148]. Moreover, pressure gradients are thought to modify bulk cytoplasmic flow, indicating that mycelia are micro-hydraulic networks[149]. Much of this evidence is obtained from non-AM fungi possessing septa and the closure of septa is thought to regulate flow dynamics[60,150]. Although similar mechanisms likely regulate cytoplasmic flows in AM fungi, we lack direct evidence.

Summary and future perspectives

Our understanding of the composition and dynamics of the AM fungal cell is still in its infancy. Resolving AM fungal cell anatomy requires addressing many remaining open questions, including: what is the specific protein composition and structure of the AM fungal cell wall, how does this vary across the AM phylogeny and developmental stages, and would it be possible that such structure facilitates endocytosis of large particles? How is the cytoskeleton structured at the network level, and what role do specific cytoskeletal components (i.e., actin and microtubles) play in nuclear and other organelle transport? Can additional fluorescence microscopy methods be employed to visualise unresolved organelles like the endoplasmic reticulum and Golgi apparatus, and what is their arrangement/localisation in the ERM? What drives cytoplasmic flows and how are they controlled in AM fungal networks? How is the growing hyphal tip organised in the ERM of AM fungi, and can we find evidence for an apical vesicle crescent-like germ tube? What are the physical properties of the lipid-droplet aggregates that underlie the unusual long-range flows of carbon through AM fungal networks? What are the identities and functions of the ‘obscure’ organelles?

The field will benefit from incorporating techniques like fluorescent probes for live and fixed cells, optimised xenic culturing, microfluidics, advanced microscopy, and image analysis in combination with machine learning approaches. Such innovations can deepen our understanding of cytoplasmic flow control, organelle arrangement and dynamics, network-scale morphogenesis within AM fungi, and ultimately, how it controls its nutrient-trade behaviours.

ACKNOWLEDGMENTS

This project was funded by the European Union (ERC, Nuclear Mix, 101076062). Views and opinions expressed are, however, those of the author(s) only and do not necessarily reflect those of the European Union or the European Research Council. Neither the European Union nor the granting authority can be held responsible for them. E.T.K. acknowledges support from NWO-VICI (202.012), NWO-Spinoza (SP!.2023.2), the Hefner Foundation, and an Ammodo Foundation grant. V.K. and E.T.K. acknowledge support from the NWO-Microp program (024.004.014). We thank Dr. Loreto Oyarte Gálvez and Sander van Otterdijk for generously providing microscopy images to be included in the figures. We also thank Mary-Jane Woodward for contributing the artistic representation of the AM fungal hyphae and its content, Sean C. Maston for contributing the artistic representation of the various morphologies of the AM hyphae and all the members of the Kokkoris group, Shimizu Group and Kiers Group for helpful and constructive discussions.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

Supplemental information including one table and six videos can be found with this article online at https://doi.org/10.1016/j.cub.2025.03.053.

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