The Mycelium as a Network

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SUMMARY 250 WORDS

The characteristic growth pattern of fungal mycelia as an interconnected network has a major impact on how cellular events operating on a micron scale affect colony behaviour at an ecological scale. Network structure is intimately linked to flows of resources across the network, which in turn modify the network architecture itself. This complex interplay shapes the incredibly plastic behaviour of fungi, and allows them to cope with patchy, ephemeral resources, competition, damage and predation in a manner completely different to multi-cellular plants or animals. Here we try to link network structure with impact on resource movement at different scales of organisation, to understand the benefits and challenges of organisms that grow as connected networks. This inevitably involves an interdisciplinary approach whereby mathematical modelling helps to provide a bridge between information gleaned by traditional cell and molecular techniques or biophysical approaches at a hyphal level, with observations of colony dynamics and behaviour at an ecological level.

INTRODUCTION

Growth as an interconnected mycelial network is characteristic of filamentous fungi and has been subject to scientific investigation since the seminal works of Buller at the start of the 20th century (1-3). We have increasingly detailed understanding of the fundamental cellular processes needed to form a network, such as hyphal tip growth (4), septation (5, 6), hyphal orientation (7), branching (8) and fusion (9-13) (Fig. 1A,C). In contrast, we know far less about the molecular events at the next physical scale that leads to hyphal aggregation and hyphal differentiation, and how these impact on physiological processes such as long-distance resource distribution and biomass recycling. For example, whilst direct uptake and intra-hyphal nutrient diffusion may be sufficient to sustain short-range local growth when resources are abundant (14), long-distance translocation is required to deliver nutrients at a sufficient rate to growing tips, particularly in fungi that form large networks on the forest floor that are too large to distribute nutrients through diffusion alone. We know little about the quantitative contribution of different potential transport pathways, such as cytoplasmic streaming, vesicle transport, growth-induced mass flow or evaporative mass-flow, to net fluxes and overall nutrient dynamics, and how they might vary between species and developmental stage (15-17). Nevertheless, the behaviour of the growing mycelial network emerges from the interaction of many such processes and requires an integrated view to understand the overall impact on fungal behaviour (18-20). Our understanding is further constrained by inferences drawn from a limited number of genetically-tractable model filamentous species grown under lab conditions (abundant, evenly dispersed, low molecular weight resources, high relative humidity, constant light and temperature) compared to real world conditions (patchy, recalcitrant, ephemeral resources, fluctuating temperature, light and relative humidity).

At the whole colony level, there is a wealth of observational data describing fungal behaviour (usually of non-model, but ecologically relevant species) in qualitative terms, and some progress has been made in quantitative measurements of network architecture and dynamics, in parallel with development of predictive models for network function (21-33). The challenge is to construct a framework that can integrate information across scales into a coherent model to explain fungal behaviour. We start this construction process by considering the biophysical properties of the mycelial network at each scale, with particular emphasis on the interplay between the network architecture and transport of resources through the network. Most of our knowledge on network structure and dynamics comes from non-resource-unit-restricted (i.e. fungi that can extend as mycelia from their food source) wood decay basidiomycetes that forage on the forest floor for resources distributed heterogeneously in space and time (34-36). We focus on these for discussion here for this reason, and because mycorrhizal networks have been comprehensively reviewed recently (37, 38). We also examine advances in image analysis techniques to extract the network architecture, empirically-based modelling and simulation techniques to predict network behaviour, and experimental approaches to map the actual functional flows within the network. We finally explore how network concepts may help to define a new set of fitness traits that can be experimentally determined (39).

THE BUILDING BLOCKS FOR NETWORK CONSTRUCTION

Growth at the hyphal tip

The mycelial network comprises individual hyphae ranging from about 2-20 μm in diameter that grow by tip expansion following highly localized polar secretion of wall materials

(4)(Fig. 1A). Secretory vesicles are transported to the apical cell wall using a combination of microtubule and microfilaments, powered by molecular motors such as kinesin, dynein and myosin (4, 40-42). Excess membrane is then recycled locally by endocytosis or sorted more extensively by motile early endosomes distal from the tip (40, 41, 43). Early endosomes may also play a role as a general transport system for other cellular components, such as ribosomes and mRNA (40, 44).

Motor-driven transport of vesicles and organelles concomitantly generates cytoplasmic streaming that serves to increase the effective transport rate for solutes and other cytoplasmic components (40). This is important as synthesis of sufficient new wall material for maximal growth requires a minimum volume of cytoplasm distal to the tip (see section: Modelling tip growth and branching) which has to be supplied by nutrient uptake and transport, often remote from the tip (42). Synthesis starts with accumulation of external ions, sugars and amino acids present in the soil or following breakdown of organic substrates, using proton-coupled co-transporters or in response to the membrane potential via ion channels (Fig. 1A). The low intracellular water potential from solute accumulation leads to water uptake, which is required for the increase in hyphal volume during growth, and also leads to an increase in turgor pressure. Turgor is sensed by the conserved HOG (high-osmotic glycerol) MAP kinase cascade and regulates plasma-membrane ion transport (Fig. 1A). In addition, osmolytes such as glycerol, arabitol, other polyols, proline or trehalose may be synthesized internally to maintain turgor (45, 46). Stretch-activated plasma-membrane channels, such as Mid1, may respond directly to membrane stretch and also regulate solute uptake (Fig. 1A). For most fungi, some minimum turgor pressure is required for tip extension, but turgor pressure *per se* does not appear to be rate limiting for normal growth, as hyphae experiencing different turgor pressures can grow at the same rate. Indeed, some fungus-like oomycetes and wall-less

fungal mutants can grow in the absence of turgor pressure, when the cytoskeleton alone may be sufficient to drive the tip of the hyphae forward (41, 47-50).

Transport at the tip

Several parallel systems contribute to solute and organelle movement within the apical and sub-apical compartments. In all systems, solutes will move by diffusion through the cytosol (51, 52) or the dynamic tubular vacuolar system (53-55), and this may be sufficient to allow bi-directional source-sink nutrient movements in slow growing species (56), particularly as diffusion is constrained effectively to one dimension within the hyphae (52, 53), or 'non-translocating' species that do not show protoplasmic streaming (57). For example, the time (t_D) for a species j to diffuse length l scales as l^2/D_j. Thus, ions and small molecules with a diffusion coefficient (D_j) of 1-2 x 10^{-9} m^2 s^{-1} would transit a typical apical compartment in *Phanerochaete velutina* (400 µm) in ~2 min, whilst proteins, with a diffusion coefficient of $2x10^{-11}$ would take ~2 h. Transport over a small colony (10 mm) would take 20 h for small molecules and 7 weeks for proteins, and over a 25 cm microcosm 1.5 and 100 years, respectively. In practice, macromolecules are unlikely to have to move across the whole colony diameter, as transcription, translation and localisation in the case of proteins, or synthesis, packaging and vesicle delivery in the case of wall components, are spatially localised on a much shorter length scale. Nevertheless, there is some evidence for long-distance protein translocation during fruiting in *Agaricus* (58).

By comparison to diffusive movement, motor-driven organelle transport can provide constant velocity transport in both directions over medium length scales (mm-cm) both for the cargo transported, and also the bulk cytoplasm that becomes entrained (40). Fungi have some of the fastest microtubule motors known (1-4 µm s-1, 59), whilst myosin V in yeast transports

secretory vesicles at ~3 μm s^{-1} (44, 60). The maximum speed that motors can move at also places an upper bound on the velocity that can be achieved by motor-driven movement to around 4 μm s^{-1} (14.4 mm hr-1, 61). Actin-myosin based cytoplasmic streaming in other systems, such as pollen tubes, can reach higher velocities, typically 40-60 μm s^{-1}, and reaching 100 μm s^{-1} with myosin XI in characean algae (62), but such high speed motors do not seem to operate in fungi. The energetic costs of motor-driven movement would be expected to scale with the length of the transport pathway, particularly the cost of assembling the microfilament and/or microtubule tracks, the size of vesicle or organelles transported, the speed of movement and the cytoplasmic viscosity (62). There are no quantitative estimates in fungi to our knowledge, but it is likely that the energetic costs are excessive, particularly in comparison to mass-flow (see below). Nevertheless, this rate of movement may well be sufficient for species that do not forage far beyond their food resource, termed resource-restricted or non-translocators (57, 63), or for mycorrhizal fungi that have access to an abundant and consistent supply of carbon/energy from the host plant.

In addition to motor-driven transport, passive movement of solutes and organelles, such as vacuoles and nuclei towards the tip, can occur through mass flow of cytoplasm arising from a combination of expansion of the apical cell wall to create new volume, and sub-apical uptake of water and solutes (32, 33, 64-68), termed growth-induced mass flow (see Fig. 1B, Fig. 2B and Fig. 3, 33). Quantitative microscopic measurements have been made on *Neurospora* hyphae, which have a relatively large diameter and grow at a maximum rate of 1 μm s^{-1} equivalent to 3.6 mm h^{-1} (69), thus capturing the fastest local transport events required for tip growth in any species. Injection of oil droplets, which cannot interact directly with motors and the cytoskeletal system, provides direct evidence for growth-induced mass flow, as the rate of movement (about 5 μm s^{-1}) exceeds the rate of tip extension (0.2-0.5 μm s^{-1}) in these

experiments (66). The actual velocity expected for mass-flow depends on the number of downstream tips (Fig. 2B, see Section: The impact of branching on transport). Likewise, nuclei, vacuoles and mitochondria in apical compartments all migrate towards the growing tips at the same or greater rate than the tips extend (64, 66-68). Such movement would be consistent with mass flow driven by the continuous sub-apical water influx required to sustain volume increases at the tip during growth.

The time (t_A) taken for solutes or organelles to advect a distance l at velocity v, is l/v, whilst the relative importance of advection compared to diffusion is given by the Péclet number, $t_A/t_D = vl/D_j$ (62). Advection dominates if this ratio is greater than 1, whilst diffusion dominates if less than 1. Thus the Péclet number for the apical compartment of *Neurospora* growing at 1 μm s^{-1} is around 0.3 for solutes and 20 for proteins. An alternative measure considers the length scale for localisation of macromolecules, such as proteins and mRNA, depending on diffusion, advection and their lifetime (τ) before degradation (65). Thus, localisation scales as $\sqrt{D_j\tau}$ if diffusion and degradation dominate, $v\tau$ if flow and degradation dominate. The ratio $C = \sqrt{D_j/v^2\tau}$ of these two distances is a dimensionless number. If $C > 1$ then most transport occurs by diffusion, and if $C < 1$, then most transport is by flow (65).

Water permeability and water uptake

The extent to which longitudinal mass flow occurs is critically dependent on precisely where water influx occurs in the colony. In principle water could be taken up anywhere along the hyphae, but the actual site of uptake has profound implications for the magnitude and direction of internal mass flows. A second critical observation is that regardless of intra-hyphal concentration gradients and turgor pressure resulting from solute accumulation, mass flow only takes place when water is able to exit the translocation pathway. A range of

possible routes exist through either localized exudation (70-73), hydraulic redistribution to soil with lower water potential (74), evaporation during fruit body formation (e.g. Phycomyces sporangia 75), coupling to the plant evapotranspiration stream in mycorrhizas (76), or by moving into a region of new growth (33, 66).

Fungi have a particular challenge to maintain a balanced water economy to allow water uptake for growth, while preventing excess evaporation from their extensive surface area. Equally, controlled evaporation may drive solute movement into aerial hyphae (75) and evaporative cooling may be required to aid spore dispersal by establishing convection currents (77), or through formation of 'Buller's drop' by condensation (78). The plasma-membrane provides some impedance to water movement, with permeability dependent on the lipid composition and temperature (79). Thus the osmotic permeability (P_f) of the plasma-membrane in yeast mutants lacking aquaporins (AQPs) increases from 0.34 µm s^{-1} at 7°C to 2 µm s^{-1} at 23°C (80), equivalent to a plasma membrane water hydraulic conductivity coefficient ($L_w^{pm} = P_f \bar{V}_w / RT$) of ~2.5-15 x 10^{-15} m s^{-1} Pa^{-1}. It is worth noting that these values are 1-2 orders of magnitude lower than plant and mammalian cell membranes at the same temperature. Indeed, the presence of ergosterol in fungi, rather than cholesterol in other organisms, may be linked to greater resistance to desiccation (81, 82).

The presence of aquaporins (AQPs, Fig. 1B) also significantly increases water permeability in parallel to the lipid bilayer. For example, over-expression of AQPs in yeast increased P_f by 9 fold at 7°C. Likewise, expression of AQPs from the filamentous mycorrhizal fungus *Laccaria bicolor* in *Xenopus* oocytes increased the membrane permeability to up to 147 µm s^{-1} (83). Whilst the precise role of AQPs in water uptake have not resolved, AQPs do appear

to be critical in hydraulic coupling during fruiting, and between mycorrhizal fungi and the host plant (84-86).

In both plant and animal systems there is also evidence for movement of water against the free energy gradient by chloride/cation co-transporters (CCC, 87, 88). CCC sequences are present in fungal genomes, but their role in fungal water economy has not been investigated to date. Nevertheless, this presents the interesting possibility that the low intrinsic plasma membrane water permeability in fungi, particularly at temperatures typically encountered in soils, might allow much greater regulated spatial control of the water economy through the localisation and activation of aquaporins and CCC water pumps.

Hydrophobins and control of water loss

To restrict water loss, many mycelia produce small (15 kD) secreted cysteine-rich hydrophobin proteins that self-assemble to form a rodlet layer, particularly on aerial hyphae (Fig. 1B, Fig. 2A), and increase the hydrophobicity of the surface, thus restricting water movement (89-91). One benefit of using hydrophobins to reduce evaporation is that their intrinsic organisation varies with water availability, with no additional sensing or control other than their synthesis and secretion, allowing them to function autonomously outside the cell. Nevertheless, hydrophobins represent an expensive investment of scarce nitrogen resources, particularly as the cost of forming a functional barrier scales with the surface area of the hyphae covered. Thus, in more mature systems and multi-hyphal aggregates, hyphal walls tend to be impregnated with other compounds, such as melanin and phenolics, to reduce water evaporation and act as protection against damage and fungivores (92). Species vary in the extent of hyphal hydrophobicity, and this characteristic can be used to group mycorrhizal species as hydrophobic or hydrophilic (73).

In addition to chemical impregnation, there are biophysical constraints on water evaporation through the wall interstices depending on the diameter of pores in the cell walls generating a matric potential. There are few measurements of pore size in fungi, but in the water mould *Achlya* a value of 2 nm was determined experimentally (93). If terrestrial fungi have similar values, this would restrict evaporation, even at low external relative humidity, depending on the radius of the pores and wettability of the walls (79). Whilst the role of matric potentials is well understood for the soil-plant-atmosphere continuum (79), we are only at the beginning of understanding water fluxes through the more complex soil-mycorrhiza-plant-atmosphere or soil-saprotroph-atmosphere systems (76, 94).

Insulating the hyphal wall leads to secondary problems on how the fungus can then sense the external chemical environment, how enzymes needed to decompose organic resources can be delivered, and how the products are then absorbed. Interestingly, there is evidence that enzyme delivery may be mediated by unconventional secretion through extracellular vesicles (EVs, exosomes), possibly derived from multi-vesicular bodies, that cross the wall (Fig.1A) by an unknown mechanism (43, 95).

The impact of septation on transport

In Dikarya, after a period of growth, the apical cell divides to establish a series of septal compartments that retain cytoplasmic continuity through the septal pores (Fig. 1C). Septa exhibit a variety of pore structures from narrow plasmodesmatal connections or simple uniperforate pores, to pores with associated Woronin bodies or other pore occluding structures in the Ascomycota, and the more complex septal pore caps in the Basidiomycota (Fig. 1C, 96). The pore size ranges from 10-70 nm in the case of plasmodesmata, to 50-500

nm for most other pore types, but is often partially occluded by electron-dense material (6, 96). Septal pores allow passage of nutrients, macromolecules and organelles between septal compartments. In some species nuclei can also migrate through the septal pore (67, 69), whilst in the basidiomycetes, nuclear movement in heterokaryons is strictly controlled through formation of clamp connections adjacent to the septum. The pore may be blocked reversibly or irreversibly by Woronin bodies (WB) and septal pore associated (SPA) proteins in ascomycetes, or the septal pore cap (SPC) and its various elaborations in basidiomycetes (6, 97). Interestingly, the precise positioning and tethering of WBs has recently diverged in the Pezizomycotina, with more distal anchorage in rapidly-growing species such as *Neurospora* (Fig. 1C), regarded as an adaptation to prevent accidental pore occlusion as high flow velocities sweep the WBs into the pore (98). Whilst pore occlusion has a role in preventing loss of cytoplasmic contents after damage (6), there is increasing evidence that pore closure (99) or opening (100) can occur as part of a developmental sequence, and closure may be reversible depending on environmental conditions (101).

Even when open, the septal pore places an interesting constriction on fluid flow between septal compartments (Fig. 1D). The velocity of flow through septal pores (v_p) increases in proportion to the relative area of the pore to the cross-sectional area of the hypha (r_h^2/r_p^2), with experimentally-measured velocities in excess of 200 μm s^{-1} in *Neurospora* (69), sufficient to restrict backward diffusion against the flow (65). Various organelles also accumulate in local eddies adjacent to the septa and, in the case of nuclei, appear to alter their protein complement as a result (69). Indeed, accumulation of secretory vesicles at septa may also be part of the trigger initiating branch formation (102, 103)

Fluid flows induce wall shear stresses, and in the case of laminar flow the wall shear stress τ produced can be estimated using the formula $\tau = 4\eta v/r$, where η is the dynamic viscosity of the fluid, v is the mean velocity of fluid flow and r is the radius of the vessel (104). In the typical case where the fungal cytoplasm has dynamic viscosity of 2 $gs^{-1}m^{-1}$ and the hypha has a radius of 6 μm, the wall sheer stress is $\tau = v \times 10^{-3}$, where τ is measured in Pascals or Nm^{-2} and v is measured in μm s^{-1}. By way of comparison, the wall shear stresses in mammalian arterial systems are in the range 0.2–2 Nm^{-2}, and it is known that stresses of that scale induce changes in gene expression (105). Hence it is plausible that a 6 μm radius hyphae could detect and respond to internal velocities of the order of 1 mm s^{-1}, but along the length of most hyphae the wall shear stress will be negligible. However, because the mean velocity of fluid flow is inversely proportional to cross-sectional area, and wall shear stress is proportional to the velocity gradient at the wall, wall shear stress at the septal pore will be approximately r_h^3/r_p^3 greater than the wall shear stress elsewhere (where r_h and r_p, are the radii of the hyphae and pore, respectively). This means that even the modest flows that have been measured in fungi can be expected to produce physiologically significant mechanical forces at the septal pore. Indeed, the scale of those forces can be estimated by measuring the elasticity of the cell wall and the amount of deflection that occurs during transient pore plugging, and in *N. crassa* it has been calculated that the deflection associated pressure difference is as large as 2 bar (69). Also note that the above equations somewhat underestimate wall shear stress, as the velocity profile deviates from the parabolic profile expected from Poiseuille flow due to the high density of organelles moving within the fluid (68). This analysis suggests that septal dissolution, branching and other aspects of hyphal developmental may be directly influenced by the relative scale of flows within the hyphae (33), as mechanical forces are large enough to degrade septa (69), and it is also plausible that fluid flows could be detected by proteins located at the septal pore, leading to flow dependent

changes in the pattern of gene expression.

Modelling tip growth and branching

The number of growing tips at the colony margin increases by apical or sub-apical branching and daughter hyphae typically show avoidance behaviour (negative autotropism, Fig. 2A) regulated by the Cdc42 signalling pathway (7, 106) to increase exploration of the substrate (Fig. 2A). The fact that fungi grow by apical extension, although intercalary growth has been observed (107, 108), implies that there is a fundamental relationship between the overall colony growth rate and the branching rate. This relationship can be clarified by letting A denote the mean cross-sectional area of individual hyphae, $N(t)$ denotes the number of hyphal tips at time t, $g(t)$ denotes the specific growth rate of the colony (that is, the rate of change of volume per unit volume of fungus), and $v(t)$ denotes the mean velocity of hyphal tips. If all growth is due to apical extension, it follows that the mean volumetric growth rate of an individual tip is $Av(t)$, and the total volumetric rate of growth $\frac{dV}{dt} = V(t)g(t) = AN(t)v(t)$. It follows that:

$$N(t) = \frac{V(t)g(t)}{Av(t)} \quad (1)$$

When the specific growth rate g and the mean velocity of hyphal tips v are constant, the rate of change of the number of tips is:

$$\frac{dN}{dt} = \frac{\frac{dV}{dt}g}{Av} \quad (2)$$

Equations (1) and (2) imply that when the specific growth rate g and velocity of hyphal tips v are constant, we have:

$$\frac{\frac{dN}{dT}}{N} = \frac{\frac{dV}{dt}}{V} = g \quad (3)$$

Note that the number of tips is increased by each branching event and decreased by each fusion (anastomosis) event, so we cannot conclude that the branching rate is simply equal to the specific growth rate g. Nevertheless, it is reasonable to assume that the branching rate is only slightly larger than the specific growth rate g, which is a claim that has empirical support (102, 109). If the branching rate is equal to the specific growth rate, there must be a constant relationship between the volume of hyphae and the number of hyphal tips. In 1959 Plomley (110) observed that exponential colony growth requires continual branching, and he speculated that when a species is growing exponentially, there is a constant volume of fungus per hyphal tip termed the "hyphal growth unit". This is the key concept behind the mathematical models of hyphal growth developed in the 1970's (102, 109, 111-113), which recapitulate the experimental observation that the velocity of hyphal tips is initially proportional to the volume per hyphal tip (e.g. 69). Thus the rate of germ tube extension is initially exponential as the increasing volume of cytoplasm provides more vesicles per tip. As the colony grows, the rate of vesicle delivery reaches a maximum and the mean tip velocity approaches a constant as a result, obtaining a value for the hyphal growth unit that is characteristic of the species in the given environment and manifest at a colony level as the width of the peripheral growth zone (111).

Linking growth and branching to nutrient status

Like all organisms, fungi require a source of energy and nutrients to sustain growth, and so the specific growth rate and branching rate of fungi must be smaller for fungi that grow on substrates that are recalcitrant or nutrient poor (114, 115). This kind of energetic constraint on fungal growth has implications for the optimal allocation of resources towards vegetative growth, the production of digestive enzymes, and the production of spores (31). Any successful model of hyphal growth must recapitulate the fact that nutrient availability enables

growth, and rapid growth is associated with rapid branching. However, due to the difficulty of observing fungal growth and a lack of understanding of the control of branching, there are few statistics concerning the location of branching events in a growing fungus (116). On a macro scale, models of the branching process generally make the reasonable assumption that branching is more likely to occur in regions with a higher density of tip-growth vesicles (29, 102), which may occur when vesicles are too far downstream of the apex to be able to contribute to growth of the apex itself and are therefore diverted to form a new growing tip (102, 117). On a micro scale, it has been suggested that branching is associated with septa because vesicles may accumulate around septal pores (102, 103, 109, 117). However, it is not known whether all else being equal, younger hyphae are more likely to branch, or whether hyphae that carry relatively large mass flows are more likely to branch.

Hyphae can also form in older parts of the colony by secondary or lateral branching, to an extent dependent on the species, the medium of growth, and other environmental factors (8, 118, 119), but there are significant differences in the cellular mechanisms involved in apical and secondary tip formation (120). Watters *et al.* (121) demonstrated that the distribution of distances between adjacent branch points is independent of tip extension rate in *N. crassa* at range of temperatures. However, following a sudden increase in temperature (and metabolic rate), there is a transient increase in branching rate, while following a sudden decrease in temperature hyphae produce an unusually long branch interval (122). These observations are consistent with the following four assumptions:

1. The rate of tip extension is proportional to the rate of vesicle deposition.
2. The rate of branching is proportional to the number of vesicles in the colony.
3. The rate of vesicle production is proportional to the metabolic rate of the colony, and this rate changes relatively rapidly when the metabolic rate is altered by changing temperature.

4. The rate of vesicle deposition at the tips is proportional to the metabolic rate of the colony, and this rate changes relatively slowly when the metabolic rate is altered by changing temperature.

If these plausible assumptions are correct, a sudden increase in temperate will increase the rate of vesicle production more quickly than it increases the rate of vesicle deposition, creating a transient increase in the density of vesicles within the colony, which produces an increase in the branching rate. Also note that if the velocity of hyphal tips and the specific growth rate of a fungus remain fairly constant, the diameter of the fungal colonies will grow in a linear manner, while the volume of that same colony will grow exponentially. This implies that the density of the fungal growth will increase, though at some point population effects will start to inhibit growth, as in the dense core of the colony the concentration of inhibitory waste products will be relatively high, and there will be relatively few nutrients or space to enable further growth.

The impact of branching on transport

Branching reduces the radial distance that can be supplied by diffusion alone (53), but has no effect on the velocity of motor-driven transport, although the total flux is halved at each branch point. The extent that branching affects organelle and resource distribution by mass-flow depends on the site of water uptake (16, 32, 33). At one extreme, if water uptake only occurs at the initial inoculum, volumetric mass flow scales with the number of downstream tips that are growing (Fig. 2B). If water uptake takes place equally across the network and the cross-sectional area is constant, the velocity in each hypha matches the rate of growth, whilst if all uptake takes place at the tips, there is no mass flow in the rest of the colony. In wild-type or the *so* mutant of *Neurospora*, which is unable to fuse and thus only grows as a

branching tree (123), nuclear movement occurs predominantly by mass flow and the rate scales with the number of downstream tips, reaching values of 5-20 μm s^{-1} (67, 69), consistent with water uptake at the centre of the colony or germinating conidium. Thus, in *Neurospora*, even though water is freely available in the substrate, uptake is highly spatially regulated to ensure high rates of longitudinal flow through the colony.

The impact of hyphal fusion on transport

Fusion may take place early in colony formation *via* conidial anastomosis tubes (CATs) that potentially link multiple conidia into a supra-cellular network (11-13). Secondary branch hyphae also develop in more mature regions when daughter hyphae typically show positive autotropism and fuse with neighbours to create a micro-hydraulic network with loops (7, 9, 11-13, 49). Fusion appears to be required for high rates of long-distance nutrient translocation, as the *so* mutant in *Neurospora* shows reduced radiolabelled amino-acid movement (124). Nevertheless, loops are probably more critical in providing multiple pathways to improve network resilience and to allow rapid re-allocation of resources in response to local demands. For example, lateral movement of radiotracers at the colony margin (125) probably occurs as a result of anastomosis.

Hyphal differentiation and the formation of multi-hyphal aggregates

In many species, there is some differentiation into leader or trunk hyphae and aerial hyphae at the colony margin (Fig. 2C). However, formation of linear hyphal aggregates, such as cords and rhizomorphs, only occurs to a lesser extent on agar, so there is comparatively little information on the development and internal structure of these organs from laboratory studies.

Cords form behind the front of apically extending hyphae, with the degree of hyphal aggregation within the growing front varying between species (126, 127). Close to the growing front, both large and smaller diameter hyphae are normally present with many branches and anastomoses interconnecting both hyphal types. In corded systems, large diameter hyphae are initially cytoplasmic, with progressive cellular disorganisation and appearance of empty vessel hyphae with distance from the front (17, 128). In *Serpula lacrymans* narrow tendril hyphae emerge from the clamp connections and then branch and grow both acropetally and basipetally around neighbouring hyphae (129). In more developed cords, cross sections at the light or EM level show further differentiation of thick-walled fibre hyphae and vessel hyphae, with diameters around 10-15 µm, that are likely to improve flow (Fig. 2C). During early development, movement of radiotracers and fluorescent dyes appears to be restricted to particular routes that probably correspond to developing cords (125, 130-132), although tracking the internal connectivity has been rarely attempted. The size of the vessel hyphae can have an impact on the flow rate, which scales as r^4, but flow will be more critically dependent on the size of the septal pores and the extent of septal dissolution associated with cord formation (126, 127). Nevertheless, ectomycorrhizal cords of *Paxillus* have swollen clamp connections at the nodes that provide continuity with the branches, but only limited septal dissolution in the vessel hyphae (133).

Rhizomorphs are more highly differentiated root-like structures that extend from a meristematic-like region at the tip (Fig. 2C). They consist of a central lacuna surrounded by an inner medulla with large thin-walled vessel hyphae that form high-conductivity channels, an outer medulla containing loosely packed hyphae which are only about 2 µm wide, and a thick melanised, hydrophobic rind that insulates them from the environment. The longitudinal hydraulic conductivity ($L_w^{\parallel}$) through the cords varies was 1.5-3 x 10^2 cm^2 bar^{-1} s^{-1} (134). The

hydraulic conductivity perpendicular to the hyphal axis ($L_w^{\perp}$) for such cords has not been measured, but is likely to be low.

Pressure-driven mass-flow on a macroscopic scale

The velocity of water movement in corded systems or rhizomorphs is vastly in excess of values that could be achieved by motor-driven movement, and initially led to the idea of pressure-driven mass-flow as the major transport mechanism in foraging fungi (15, 135). Thus estimates of water transport in *Suillus bovinus* were 27 cm h^{-1} (136), whilst measurements in *Serpula lacrymans* have a mean value of 148 cm h^{-1} (70). Likewise, a range of different solutes are transported in *Armillaria* at velocities in excess of 20 mm h^{-1} (137), in *Serpula* ranging from 10-50 mm h^{-1} (138) to 20-30 cm h^{-1} (135, 139), or 20-100 mm h^{-1} in *Phanerochaete velutina* depending on the extent of cord formation (Fricker et al., unpub., 32, 33, 131, 132). Similarly, even movement of larger organelles, such as nuclei, by mass flow can reach velocities of up to 4 mm h^{-1}, although there is wide variation in velocity between different hyphae in the network (67).

The magnitude of growth-induced mass-flows has been empirically-determined measurements of network growth at the level of individual cords (Fig. 3A, B, 33), and input into an advection-diffusion-delivery (ADD) model to predict the pattern of resource translocation (Fig. 3C, 32). The actual pattern of nutrient movement was then determined experimentally using non-metabolised radio-tracers and photon-counting scintillation imaging (PCSI, Fig. 3D). The ADD model of growth-induced mass flow is surprisingly effective at predicting the distribution of the amino acid analogue ^{14}C-amino iso-butyrate (^{14}C-AIB) in a complex network of fungal cords (compare Fig. 3C with Fig. 3D). This is

remarkable considering the conceptual simplicity of the biophysical model, and that there is essentially only one free parameter – the fraction of the cord that carries nutrient flow.

The magnitude and direction of mass flows can be altered by experimental manipulation of the external water potential, but it is also clear that under natural conditions, the external water potential can have an impact on transport through the network. Thus, substantial water flow (18 cm h^{-1}) can be driven by hydraulic redistribution from moist to dry soil (74), or through hydraulic coupling to the host plant (27 cm h^{-1}) in the case of mycorrhizas (136, 140).

Biomass recycling

As some hyphae expand and mature, other regions of the mycelium regress and their contents are recycled. In particular, there is extensive remodelling of redundant parts of the mycelium in larger mycelia grown from woody resources (See Section: Colony dynamics and behaviour), presumably to re-deploy resources to the growing margin to forage more efficiently in highly heterogeneous environments (14, 31, 56, 141, 142). Interestingly, in other systems such as blood vascular networks and slime molds, recycling and network re-modelling occurs in response to flow rate (143), and there is evidence that a similar relationship between high flow and cord thickening, versus low flow and cord thinning holds for fungi (33, 144).

At a molecular level, recycling probably involves autophagy and controlled apoptotic-like mechanisms, rather than necrosis (145, 146). Nevertheless, how decisions are made to trigger autophagy in particular hyphae are not clear, nor is it clear how the cytoplasm and wall materials are broken down and transferred to the growing front.

Bidirectional movement and oscillations

There is plenty of evidence for nutrient translocation from resources to the growing margin where they are needed for growth. However, in several systems, radiotracer measurements indicate that nutrients can move both acropetally and basipetally in the same colony or following colony fusion (125, 137, 138, 147-152). Bi-directional movement is likely to be critical to achieve mixing of spatially distributed food resources between constantly changing sources and sinks and therefore promote maximum colony growth in a heterogeneous environment, and may also contribute to colony-wide control of behaviour (56). Over short length scales, diffusion and motor-driven transport may achieve transport at a sufficient rate, but responsive resource allocation in larger colonies requires mass-flow. However, simultaneous bi-directional movement is difficult to explain based solely on pressure-driven mass flows, which would be expected to operate in one direction at a time, so models have invoked distinct pathways that involve acropetal mass flows in vessel hyphae and basipetal streaming in adjacent cytoplasmic hyphae (Fig. 2D, 17). In addition, some species, such as *Phanerochaete velutina* (131, 132, 153), but not others, such as *Serpula lacrymans* (138) or *Neurospora crassa* (124), show marked oscillations superimposed on the net transport. The origins of the oscillations are not known, but may represent a reciprocating transfer of material, rather than the continuous circulatory system shown in Fig. 2D. This is reminiscent, albeit at a much longer time scale, of the shuttle streaming in *Physarum polycephalum* that leads to efficient mixing within the plasmodium (154).

COLONY DYNAMICS AND BEHAVIOUR

In discrete organic resources saprotrophic mycelial networks typically operate on a mm to cm scale, depending on the size of the resource and size of the genetic individuals. However,

large individuals occupying extensive decay columns in tree trunks and branches operate over a larger scale. Further, those fungi which are non-resource-unit-restricted extend from their main food base into the surrounding environment in search of new resources (Figs. 4-7), and often operate on the scale of metres. Thus, saprotrophic systems of mycelia cords and rhizomorphs cover several square metres to many hectares (Fig. 4, 135, 155, 156, 157). Currently, the largest recorded genet is of *Armillaria ostoyae* spanning 965 hectares, with a maximum separation of extending fronts of 3810 m (157), though the extent of internal connectivity is unknown.

There is little information on mycelia networks within organic resources (see Section: Extension of network analysis to 3-D), except for the boundaries of territory occupied by individual mycelia, indicated by interaction zone lines (36). Likewise, little is known of the structure of networks of non-resource-unit-restricted fungi foraging in relatively homogenous resources, e.g. fairy ring formers and colonisers of leaf litter patches (36). The latter are particularly interesting as they extend through the leaf litter layer as an ever increasing annulus of mycelium, about 30 - 40 cm wide in the case of *Clitocybe nebularis*, differentiated into several distinct zones (158).

A wide variety of patterns of mycelial outgrowth from organic resources has evolved ranging between slowly extending search fronts densely populated by hyphae, which are unlikely to miss new resources, e.g. *Hypholoma fasciculare*, to more rapidly extending but sparser systems, e.g. *Phanerochaete velutina*, and at the furthest extreme *Armillaria* species. The slow-dense foragers are often considered short range foragers, and have sometimes been termed ‘phalanx or phalangeal foragers’ (34, 35, 159), by analogy with foraging of clonal plants that grow relatively slowly and have tight aggregated rosettes (160-163). The fast-

effuse foraging, on the other hand, is often considered long-range foraging, and has been likened to ‘guerrilla’ foraging of plants (clones of which have fast growing branches that are loosely aggregated). Similar concepts have been developed for mycorrhizal networks that include short-range ‘contact exploration types’ and ‘long-distance exploration types’, where the mycelium is highly differentiated into strands (164). Other similar, but not identical, ideas have been put forward by plant ecologists, such as ‘clump’ plants characterized by frequent branching at large angles and with short spacers, which exploit resources at a particular site, and ‘runners’ with infrequent branching and long spacers, which explore more widely for resources (165, 166). Parallels have also been drawn between fungal and animal foraging (163, 167).

Perhaps the most dramatic alterations in mycelial network structure occur when new resources are discovered, either by a foraging mycelial front, or when resources land on an established system (Fig. 5). When mycelium encounters a new resource, parts of the network connecting the original with the new resource aggregates to form mycelial cords, whilst non-connecting mycelium dies back (168). The rapidity and extent to which this occurs depends, amongst others, on the foraging strategy of the fungus, the relative size/quality of the original and new resources, and the presence/absence of invertebrate grazers (34, 35, 169, 170). Short-range foragers re-allocate their biomass rapidly and extensively, with often only the connective cord and outward growth from the newly colonised resource remaining after a few months. In contrast, little reallocation of mycelia biomass occurs in long-range foragers unless the newly encountered resource is considerably more substantial than the original resource. The process is speeded up if grazing invertebrates are present; fine mycelium disappears more rapidly than if they are absent leaving only larger mycelial cords (Fig. 5). These processes occur repeatedly on the forest floor producing large, open networks of cords interconnecting discrete organic resources ranging from small twigs etc. to large tree trunks

(Fig. 4). How the decision is made to strengthen or recycle a particular cord is not clear, nor is the mechanism that serves to retrieve useful breakdown products and re-direct them to regions of new growth.

Interestingly, strands *Pleurotus ostreatus* and rhizomorphs *Armillaria gallica* (formerly *bulbosa*) both show spontaneous action potentials between 0.5-5 Hz and the frequency increases in response to addition of nutrients (171, 172). Furthermore, the signal propagated across the colony at a speed of 0.5 mm s^{-1} over distances of a few cm when nutrients were added remotely. Similar potentials have been reported in a number of other species, and it has been proposed that they may play a role in coordinating growth and differentiation by preventing hyphae from undergoing apoptosis and strengthening cord formation (172).

QUANTITATIVE ANALYSIS OF NETWORK ARCHITECTURE

Constraining the problem to 2-D

Our understanding of the co-ordinated growth and behaviour of fungal networks has been limited by the immense practical difficulties associated with measuring organisms that exhibit indeterminate, highly plastic growth. Whilst fungi would normally grow as three-dimensional networks through opaque media such as soil, wood, plant or animal tissue, most morphological studies have constrained growth to two spatial dimensions to simplify image collection and analysis. Furthermore, the substrate is usually limited to transparent media such as agar plates (e.g. 173), often overlaid with cellophane membranes (174, 175), or as dispersed mycelia in submerged culture (176-178). There is a challenge to achieve both sufficient magnification to resolve individual hyphae and sufficient scale to map the entire network. Magnifications typically range from 10-100x using microscopes (179, 180), but

photographic enlargers (174, 175, 181) or flatbed scanners can also be used (173). Colonies can also be grown on cellulose nitrate membranes on agar (182) or soil (183), and visualised following fixation, staining and rendering the membrane translucent with immersion oil to enhance contrast (182). Alternatively, colonies grown on soil can be repeatedly sampled and growth quantified using an immunoblotting technique (184).

Network organisation is relatively easy to observe at the growing margin of the colony on transparent media. However, sub-marginal growth is denser and includes aerial hyphae, which make it difficult to track individual hyphae and discern the pattern of branching and anastomosis. Thus most network analysis is limited to a short period (<72 h) of surface growth over short distances (mm-cm) (e.g. 173), although confocal imaging can be used to analyse 3-D distribution of aerial and penetrative hyphae labelled with Congo Red (185, 186). More recently, confocal fluorescence imaging following wall labelling with Calcofluor White has allowed quantitative measurements of early colony formation in 3-D over an extended period (17 days) and represents the first time the dynamics of critical parameters such as branching length and angle have been determined throughout a colony (116). At a more macroscopic scale (cm-m), saprotrophic fungi have been studied the most as they are straightforward to culture from woody resources as a planar 2-D system on non-sterile compressed soil or sand that can be readily imaged (Figs. 4-7).

Fractal measures and hyphal coverage

The simplest quantitative measure of colony behaviour is surface area coverage from the number of pixels that make up a mycelia image. However, like many natural structures, mycelia are approximately fractal, and fractal geometry maybe more appropriate to describe the systems (173, 187, 188). The fractal dimension quantifies the extent to which mycelia fill

space relative to the size of the mycelial system. In 2-dimensions, e.g. the surface of soil, its value ranges between 1 (a line) and 2 (a filled surface). In 3-dimensions it could take an upper value of 3, though 3-dimensional mycelial systems have rarely been investigated. Two types of fractal dimension have been used to provide different measurements of the branching/space-filling of mycelia - the mass fractal dimension (D_M) and the surface (or border) fractal dimension (D_S, a subset of D_M), which only describes the edge of the mycelial system. Both are important because they allow a distinction to be made between systems where there are gaps inside (i.e. hyphae do not entirely fill the space), and those systems that have plane-filled interiors and are only fractal at their edges/borders.

Several methods are available for calculating fractal dimension, the most commonly used for mycelia in natural and semi-natural (i.e. not agar) situations, e.g. mycelia growing across the surface of soil, is the box-counting technique (188). Effectively, a series of grids of square boxes of different sizes (i.e. different numbers of pixels in an image; 3-63 being appropriate for mycelia in small - 24 x 24 cm - soil microcosms) is overlaid onto an image of the mycelium. The number of boxes intersecting pixels of the mycelium is recorded. The box-count fractal dimension is given by D in Equation (4):

$$N(s) \approx cs^{-D} \tag{4}$$

where $N(s)$ is the total number of boxes having side length s, that intersects the mycelia image; c is a constant. There are two types of boxes: interior boxes (*i.e.* those that contain only pixels of the mycelium) and border boxes (i.e. those that contain at least one mycelial pixel and which contain or adjoin at least one non-mycelial, e.g. soil, pixel). So the total number of boxes, $N(s)$, is given by Equation 5:

$$N(s) = N_{border}(s) + N_{interior}(s) \tag{5}$$

Regression of the linear part of a plot of log $N_{border}(s)$ against log s provides an estimate of D_S(Equation 6):

$$\log N_{border\,(s)} = \log c - D_s \log s \qquad (6)$$

Likewise, regression of the linear part of a plot of $N_{interior}(s)$ against log *s* provides an estimate of D_M. D can be estimated for entire mycelia, or for distinct areas of local interest using software such as FracLab for MatLab (https://project.inria.fr/fraclab/) or FracLac for ImageJ (189)

Fractal dimension and hyphal coverage per unit area vary considerably between species, during mycelial development, depending on abiotic conditions, and are altered by interspecific interactions with other organisms (188). D_M of mycelia in small (24 x 24 cm) microcosms of compressed soil (non-sterile, i.e. similar to the natural environment) ranges between 1, for the linear rhizomorphs of fungi such as *Armillaria* spp. and *Megacollybia platyphylla*, to close to 2, for the densely packed mycelia of *Hypholoma fasciculare* and *Stropharia caerulea* (Fig. 6). However, even for the latter two species, D_M decreases with time and increasing size of the system, with networks becoming sparser as mycelial cords form and interstitial hyphae die back. These mycelia with, at least, initially high D_M have lower D_S, indicating that they are surface/border fractal systems, i.e. only the edge of the system is fractal. Others have similar D_M and D_S, indicating that the whole system is fractal. Those fungi that are mass fractal tend to extend more rapidly than those that are border fractal, implying that there is a balance between space-filling and extension rate. Trade-offs when deploying mycelial biomass in search of new resources, and for other attributes such as resilience, are considered in more detail below.

Fractal geometry is affected by the quantity and quality of the resources available to the mycelium, including: size of resource, number of resources, addition or encounter with new resources, and the nutrient status of soil through which the mycelium is growing (35, 187, 188). For example, D_{BM} and D_{BS} of *Hypholoma fasciculare*, *Phanerochaete velutina* and *Resinicium bicolor*, but not *Stropharia caerulea*, increased with increasing inoculum size (from 0.5 to 4 cm^3). *S. caerulea* systems growing from well decayed (84 d old) inocula took 10 days longer to achieve the same D_{BM} and D_{BS} values as when mycelia were growing from relatively undecayed (22 d old) inocula. D_{BM} and D_{BS} was often lower for mycelia growing across soils with relatively low phosphorous and nitrogen content, short range foragers often being more responsive to nutrient addition than the longer range foragers. Non-nutrient environment factors, including water potential, temperature and pH, also sometimes affect *D* (188). For example, D_{BS} of *S. caerulea* decreased with increasing water potential (i.e. getting wetter) from -0.02 MPa to -0.002 MPa, whereas with *P. velutina* there was little effect. D_{BM} and D_{BS} of *P. velutina* was significantly less at 5ºC than at 10 - 25ºC for several weeks, though effects on *S. caerulea* were variable. Interactions with other fungi can also dramatically influence mycelia characteristics including hyphal coverage and fractal dimension, which varies between combinations of species, and alters locally reflecting attack or defensive responses (190).

Graph-theoretic network representations

Fractal measures capture some aspects of network organisation, but advances in image analysis now mean that an explicit representation of the network structure is possible. Early image analysis routines (reviewed by 176, 177) used simple grey-scale thresholding (116, 180) or Sobel or Canny edge detectors followed by image opening and hole filling (191) to

segment the hyphae. The resultant binary image is typically thinned to a single pixel wide skeleton and can be converted to a graph representation, where each junction is classed as a node and the intervening segment a link. These approaches have the advantage that they can be readily implemented in open-source software packages, such as imageJ (e.g. 182, 192).

More recent approaches use additional ridge enhancement algorithms, adaptive thresholding and pruning steps to aid segmentation (173, 193, 194). Most current algorithms for network extraction were originally developed for blood vascular systems or neurons, and typically involve enhancement of the 'ridge' nature of the hypha or hyphal aggregate. The most common approaches use second-order derivatives of a Gaussian over a range of angles and scales (e.g. 195) , although pattern matching templates can also be used (196). Anisotropic kernels are more powerful as they emphasise the elongated shape of network segments and have recently been developed specifically to analyse mycelial networks (197). The maximum response of the filter provides information on the edge strength and its orientation, whilst additional measures, such as the anisotropy of the first three eigenvalues, gives a measure of how strongly the edge corresponds to a (blood) vessel ('vesselness', 195). An alternative approach uses local phase-congruency to give an intensity-independent approach to edge enhancement. This provides better segmentation for larger microcosms with cords, when the edges vary in intensity as well as scale and orientation (Fig. 7A, B), but at the expense of greater computational cost. The phase-congruency measure can also be combined with tensor-based measurements to give the phase-congruency equivalent of 'vesselness' (193, 198). The enhancement step is followed by segmentation using non-maximal suppression and hysteresis thresholding (197), watershed segmentation and edge pruning (193), or a reverse diffusion-based algorithm (196) to give a single pixel-wide skeleton.

A complete description of the mycelial network also requires an assumption of the hyphal width (e.g. 173), or experimental estimation from the network image (32, 33, 144, 169, 175, 193). This is still a technically challenging area with a number of methods available that use slightly different assumptions. For example, a granulometry approach can be used whereby the intensity image is subject to a series of image openings (erosion followed by dilation) that successively remove structures as the size of the opening kernel exceeds the underlying object (193, 198). The intensity of each pixel initially decreases slowly as the kernel samples more of the object, but then reduces dramatically once the boundary of the object is reached, and the kernel only samples the background. The transition point for any pixel is determined from the maximum (negative) gradient of the granulometry curve. However, this approach constrains the radius to integer pixels values, and also suffers from digital approximation of small kernels to a true disk shaped kernel. Thus, rather than extract a specific size threshold, the integrated intensity under the granulometry curve can be calculated to provide a more nuanced interrogation of the local image intensity profile. The integrated intensity cannot be directly related to the physical width without additional assumptions about the relationship between intensity and sampled volume through calibrated microscopic measurements. Nevertheless, this approach does help with estimation of relative hyphal widths, even if they are sub-resolution objects, provided it is assumed that the intensity scales with the width of the hyphae (Fig. 7B). Including estimates of the hyphal width gives the volume of the mycelium at any point and, if time series are collected, how the volume changes with growth or recycling.

Conversion to a graph representation

The graph representation is based on translation of the pixel skeleton to a planar, weighted, undirected graph (Fig. 7C), with nodes located at hyphal tips, branches and anastomoses, and

edges representing hyphae or cords, with weights based on the Euclidean length (L) and radius (r) of each cord, combined either as the cylindrical volume ($V = \pi r^2 L$), to represent the material cost of the cord, or the predicted conductance ($G = r^2/L$), assuming cords are bundles of equally sized vessels rather than a single vessel with increasing radius (144, 173, 175, 182, 199-201). The network cannot be resolved within the resource, so it is represented as a single node connected to all the edges incident on the resource boundary (Fig. 7C). The graph conversion step also makes additional assumptions about the functional connectivity of the network as every junction or crossing-point is automatically defined as a fully connected node in 2-D planar systems irrespective of whether there is an actual anastomosis present, although 3-D imaging can help to separate fusions from overlaps (116). In addition, the inferred conductivity assumes all the septal pores are open and hyphae behave as simple pipes.

Summary statistics, such as the number of tips, junctions and edges, the total hyphal length, area and volume, or the distribution of branching angles and internodal lengths can be readily extracted from the pixel skeleton or graph representation of the fungal network (116, 144, 173, 175, 199-202). Measures can also be referenced to 2-D space to give tip densities and fractal dimensions (173, 175). Topological network measures, such as node degree, α-index or shortest path metrics, such as betweenness centrality (Fig. 7D), can also be readily calculated, and show species-dependent developmental changes over time (144, 173, 199-203).

In more realistic microcosms with patchy resources, in competition with other species, or in the presence of fungivores, there is considerably more variation in spatial network architecture than are captured in a single summary network statistic. Such heterogeneity may

be identified by algorithms that identify regions of the network with a higher local density of connections than other regions of the network than would be expected by chance and are termed ‘communities’ in network parlance. Communities are initially identified by progressively partitioning the network into smaller and smaller units by breaking links depending on the strength of their interaction by tuning a resolution parameter. There is considerable flexibility in the choice of the interaction term that forms the basis of the analysis, which can be based on measures such as edge volume, conductance, or resilience (204, 205). The profile of key summary statistics, such as the number of communities, or the energy and entropy of the system, as the resolution parameter is changed, are used to define a set of mesoscopic response functions (MRFs) for each network. Networks are then clustered based on an estimate of the distance between the MRFs to give a dendrogram (Fig. 8). This provides a biologically-sensible clustering of networks from different species, and also of developmental stages for a particular species, or exposure to different experimental conditions (204, 205). These approaches allow objective groupings of networks across species, treatments and laboratories. Nevertheless, these methods are in their infancy and the challenge now is to understand whether the groupings can be interpreted from a biological perspective to yield additional insight that cannot be captured from qualitative description of each network alone.

INTEGRATING STRUCTURE AND FLOWS USING MODELLING

Constructing models that meaningfully represent processes ranging from the micron scale in individual hyphae to hyphal networks operating at an ecologically relevant scale is extremely challenging. At the microscopic scale, sophisticated biophysical models have been developed to explain the growth of individual hyphal tips, including vesicle delivery and viscoelastic

wall deformation (42, 206-211), followed by wall aging through cross-linking of wall polymers (4, 212, 213). Whilst, at a macroscopic scale, the growth of fungi in the environment can be modelled by using differential equations to represent changes in the density of fungal mycelia and spore production, along with changing resource density. Such an approach can be effective for modelling the spread of fungal crop pathogens (214), or modelling carbon cycling in the environment (215), but these kinds of model are essentially blind to the fact that fungal biomass grows as an interconnected network. Here we consider an intermediate scale, namely the growth of individual mycelial networks.

Continuum models at the colony level

One strategy for modelling the interaction between tips, hyphae, growth limiting nutrients and inhibitory waste products is to represent the mycelium as a continuum, and use differential equations to model processes such as the uptake of nutrients by hyphae, the extension of tips to form hyphae, the formation of new tips through branching, and the loss of tips due to anastomosis. Such models have their roots in the work of Edelstein and co-workers (216), and they have been reasonably successful in explaining the gross morphology of colonies with dense mycelia, which can be observed growing on agar, plant surfaces or building materials. For example, when *Aspergillus orzae* grows on agar, the morphological of the colony depends on the concentrations of nutrient and agar (217, 218). At low nutrient concentrations, colonies are uniform with a smooth, circular growth front provided that it is difficult for nutrients and waste products to diffuse through the agar. If the agar concentration is decreased, the effective diffusion coefficient of waste products and nutrients is increased, and colonies condense into branched forms, with a tortuous, irregular growth front. This morphological instability can be explained by constructing a reaction-diffusion type model that takes into account the build-up of waste inhibitors and the provision of growth-limiting

nutrients. Essentially, if the nutrients needed for growth can only be obtained by growing into new territory, a low density colony will gradually fill the dish, maintaining circular symmetry. If nutrients can diffuse into already colonised regions, which will enable denser growth. However, in that case the build-up of inhibitory molecules can prevent some regions from growing out of the dense core, and any point on the growth front that happens to be further away from the inhibitory core will be more likely to grow, producing positive feedback and an unstable, irregular growth front.

Including biomass recycling in continuum models

Another feature of fungal physiology that can be incorporated into continuum models is the distinction between mobile and immobile fungal biomass. Metabolically active hyphal tips mature into relatively inert hyphae, or fully vacuolated vessels that are not metabolically active. Uptake of nutrients is generally believed to be greatly reduced behind the hyphal tips, and in some species hyphae develop into cords or metabolically inert transport vessels that are highly insulated from the environment (17, 35, 219). Although some biomass is static, the cytoplasmic contents of the fungal colony is free to move by pressure driven mass flows, diffusion, or various mechanisms of active transport. Furthermore, some internal resources may be remobilised, as parts of the fungal colony are broken down and recycled to fuel further growth (See Section: Biomass recycling). Energetic arguments suggest that the process of recycling is of much greater benefit to organisms that live on recalcitrant or nutrient poor substrates (31), and modelling suggests that the rate of recycling may also have a significant impact on colony morphology (142). There are three extreme morphologies: (i) `fairy-rings', with an annulus of high density growth surrounding a region with low or zero density; (ii) colonies of relatively constant density; or (iii) rings that alternate between higher and lower densities (220, see Section: Colony dynamics and behaviour). The distribution of

densities of fungal growth depend on both the nutrient environment and the species in question, and models indicate the importance of nutrient transport and biomass recycling in determining such morphologies (24, 26, 142). The fact that fungal growth is space filling is critically important, as the capacity to take up nutrients, water or pollutants from the environment is proportional to the surface area. There is no reason to suppose that the total surface area of a fungal colony is a simple function of its diameter, which suggests that fungal growth and its environmental consequences may not be well represented by assuming some density of growth. One approach is to represent the space filling capacity of fungi in terms of fractal dimension (188, 221-224) as the dimension of a space will influence the dynamics of any exchange process (225) (see Section: Fractal measures and hyphal coverage).

Spatially explicit network models

A promising but computationally intensive approach is to adopt a spatially explicit representation of all the hyphae in a network (26, 29, 226). Each hyphal tip can be associated with a location and a direction, and the movement of those tips can be modelled so that tip velocity and changes in tip direction recapitulate empirical observations. These agent-based models can either be constrained to a lattice, or at the expense of additional computational cost, the orientation of hyphal tips can be lattice-free (28, 30). In all such `tip-and-trail' models, hyphae are present wherever the moving tips have passed, and those locations can be given traits such as internal and external concentration of growth limiting resource. This approach enables the modelling of hyphal fusion, which is assumed to occur whenever a tip grows into a location that is already occupied by hyphae or tips.

Branching angle can also be modelled to recapitulate empirical observation, but there are fundamental difficulties in modelling branching frequency. One approach is to simply impose the distribution between branch points that has been observed experimentally (226). This may be appropriate when branching rate is constant and all branching events are apical or subapical, as in that case it is relatively easy to measure branching frequency, and the location of branching events is relatively well determined. Such a model can be used to determine the relationship between branching rate, branching angle, and the expected distribution of fungal density for colonies of various size (226), but in cases where the environment is heterogeneous and branching rate is not constant, it is not clear which branching rate should be imposed. An alternate approach is to suppose that tip growth and branching rate are a function of the internal concentration of some growth limiting resource (29, 227). Such models effectively reveal the importance of nutrient transport, but the internal concentration of nutrients will depend on the scale and mechanisms of nutrient uptake and translocation of which we still have little quantitative information (16, 56, 147, 228). Thus it is not clear whether these models represent the mechanisms of nutrient transport with sufficient accuracy, nor is it clear whether they capture the actual relationship between branching rates and local conditions. Crucially, the connected nature of mycelial networks means that local behaviour can be affected by conditions in remote parts of the colony, especially by the translocation of nutrients (32, 219, 229, 230).

Furthermore, because the flow of cytoplasmic contents is relatively unimpeded, turgor pressure will tend to equalise rapidly across the colony. Hence turgor pressure and the rate of water uptake necessarily reflect the osmotic potential of the entire colony and its environment, not just the local conditions (49, 231). This implies that the behaviour of the colony is dependent on the interaction between the genotype and a complex spatial

convolution of environmental conditions (26, 142, 232, 233). It should also be noted that not all cords are equally important when a dynamic process occurs on a network. In a heterogeneous environment some cords will carry more resource than other, nearby edges (28), and empirical observations confirm that the distribution of velocities of fluid flow within a fungal network are highly heterogeneous, with some cords carrying much larger mass flows than others (32, 33, 67). This heterogeneity is presumably related to the fact that some hyphae branch and form hyphal aggregates or cords, while other hyphae are degraded and recycled. There is some evidence that cords which carry greater currents are more likely to thicken (33), but there are is a substantial body of open questions concerning the developmental logic of fungal networks.

Comparison with other types of transportation network

Given the importance of nutrient transport, it is worth considering how fungal networks compare to other transportation networks (32, 144, 219). In particular, modelling may be able to shed some light on the trade-offs involved in forming cross links, as it is notable that some fungal networks contain numerous loops, while other fungi essentially grow as branching trees (16, 199, 201). There is a considerable literature on the optimal design of distribution networks, and fractal branching trees are known to be optimal for the case where a single source is connected to multiple sinks, (234-236). Furthermore, when a metabolism limiting resource is distributed through a fractal branching tree, we should expect to see scaling effects such that larger organisms have a lower metabolic rate. As theory predicts, there is evidence in the animal kingdom that metabolic rate is proportional to $M^{3/4}$, where M represents the mass of the animal (234, 235). However, while mammals use a cardio-vascular system to distribute oxygen, glucose and other metabolism limiting substances throughout the body from a single heart, fungal colonies generally cannot be represented as a

single source system. Nutrients may be gathered from across the colony, and such organisms may thrive by foraging phosphate from one location and nitrogen from another, and distributing both resources throughout the mycelium (147, 219). Hence in the case of fungi, the distance that determines the scale of the transport challenge is not the radius or the mass of the colony, but the typical distance between the site of nutrient uptake, and the place where those nutrients are consumed.

Whatever the spatial distribution of sources and sinks, any efficient flow pattern must be such that at every point, the flow moves materials away from the source and towards the sink. In other words, although there may be loops in the network, optimally efficient flows always transport materials in a directed manner. This feature of efficient material flows is common to many definitions of `efficient', and it is inevitable when the flows are driven by differences in potential. That is because at every point in any given network, material moves from regions of high potential to low potential. Hence material can never backtrack, as that would require movement against the potential gradient. However, although it is most efficient for particles to move in a directed manner, there are circumstances in which the most efficient network is one that contains loops. In particular, if a network has to function in the face of random damage, or a network needs to connect a fluctuating distribution of sources and sinks, the most efficient choice of network may be one that contains loops (237). Further modelling may help to elucidate the trade-offs that arise between the conflicting demands of efficient directed transport and efficient mixing. In many cases the primary interest is in the functional consequences of fungal growth, such as the production rate of desirable compounds, the rate of nutrient or pollutant uptake, rates of soil acidification, wood degradation and so on (227). Some of these macroscopic rates of interest have been connected to microscopic fungal parameters, such as branching rate or fungal density, but more effective predictive models

may require a deeper understanding of how fungal development shapes and responds to the environment in which growth occurs. The increasingly quantitative nature of experimental data concerning tip growth, branching, nutrient transport and cytoplasmic flow should provide valuable material for future modelling efforts (116), and advances in imaging technology will surely enable a more detailed analysis of the architecture and functional significance of developing fungal networks.

NETWORK ROBUSTNESS AND RESILIENCE

In natural environments mycelial networks are subject to physical damage and attack by fungivores. The intrinsic robustness of the network at a particular time-point is determined by the network architecture, including thickness and toughness of hyphae and mycelia cords, palatability to grazers, and extent of interconnectedness. In particular, networks with loops formed by anastomosis are more robust, as they already maintain alternative routes for nutrient translocation to circumvent damaged regions. Fungi can also respond to damage or attack by network remodelling leading to greater resilience. Experiments can be conducted with real networks in the presence of fungivores, or using an *in silico* approach based on network analysis in other domains, whereby a property of the network, such as the largest connected component or the predicted transport efficiency, is calculated as cords are removed according to some rule. Thus, long thin cords might be removed first to simulate grazing by Collembola with small mouth parts, or a local region of the network might be deleted as a chunk to simulate grazing by wood lice.

Experiments in soil microcosms

The effects of invertebrates grazing on mycelia of cord-forming wood decay basidiomycetes has been studied extensively in microcosms (24 x 24 cm or 50 x 50 cm) of compressed non-

sterile woodland soil (238-240). Intense grazing can completely destroy mycelia and prevent them from growing out of organic resources, but less intense grazing can dramatically alter mycelial morphology and physiology, in different ways depending on the grazing species, the fungus and the grazing intensity. Mycelia are grazed in different regions and to different extents depending on fungal species: collembola grazing on the relatively unpalatable *H. fasciculare* tends to be at hyphal tips; in contrast, *R. bicolor* extending from beech wood is indiscriminately grazed, and with *P. velutina* fine hyphae within the colony and hyphal tips are grazed (241). While microfauna and mesofauna often graze on fine hyphae, macrofauna such as woodlice (*Oniscus asellus*) graze in large swathes including severing thick cords (242).

Susceptibility to grazing also depends on the age and stage of development of the mycelium, and the resources to which it has access. Mycelia comprising individual hyphae or thin cords, at early stages of development or supported by a dearth of resources, are more prone to grazing than mycelia that have formed highly sclerotised cords interconnecting between woody resources (170, 239). Mycelial systems occupying large (50 x 50 cm) trays of soil had similar hyphal coverage after several months irrespective of whether or not they had been grazed by collembola (170), apparently because collembola grazed on the fine hyphae that would have died anyway as the system became more open. Greater mycelial interconnectedness, i.e. more cross-links forming tangential connections, also limits the effects of grazing and other damage (Fig. 5 and Fig. 9, 169, 243). Connectedness is greater in some species than others, e.g. greater in *Phallus impudicus* than *P. velutina* (see above), in mycelia developing from larger resources and in more mature systems (144, 169, 243, 244).

Effects of grazing depend not only on the intrinsic robustness but also on how mycelia respond to damage or grazing, i.e. resilience. Though extension rate often decreases as a result of invertebrate grazing (239), it sometimes increases, switching from slow, exploitative to fast, explorative growth (242). These changes sometimes occur at or close to the site of grazing, further away, and in all directions around a mycelial margin. The latter, is invertebrate-density dependent (239), and has been interpreted as compensatory growth, similar to that which occurs in plants during herbivory (238). During interactions between mycelia of *P. velutina* and *H. fasciculare* growing across soil, grazing by collembola curtailed growth of the former except in the region of the opponent, where rate of overgrowth rapidly increased (243). Remarkably, though collembola grazing of *P. velutina* tends to reduce interstitial hyphal biomass overall, some of these fine hyphae survive, and thicken, increasing the local level of cross-connectivity, and hence increasing robustness (169).

Network risks: control of systemic infection

Network organization may be useful for long-distance transport and co-ordinated colony behaviour, but also poses a risk of rapid systemic infection. Indeed, approximately 30-80% of fungi are thought to harbour persistent systemic mycovirus infections that spread horizontally via hyphal anastomosis between compatible fungal hosts, or vertically through spore formation, with no extracellular mode of infection (245, 246).

FUTURE PROSPECTS

Extension of network analysis to 3-D

It is already possible to collect sets of tiled 3-D confocal images of fungi growing on agar with sufficient resolution to identify individual hyphae, and sufficient spatial and temporal

scales to map colony development over a period of days, which gives a much richer source of information for modelling (116). Recent progress has also been made on 3-D imaging of fungi in wood or soil with sub-micron resolution using X-ray computed tomography (247-250). However, manual or automated segmentation and analysis routines are still challenging, as the invasive hyphae can be very narrow and it is currently difficult to achieve sufficient contrast to segment fungal hyphae from the woody tissue (247, 248, 250). Imaging mycelial networks in soil has used osmium vapour staining and use of X-ray translucent polystyrene substrate to create sufficient contrast (249). Nevertheless, at this point, it is not possible to extract a connected 3-D network from either substrate, but it is anticipated that further methodological improvements will overcome some of the current technological challenges.

Inter-cellular and intra-cellular hyphal networks formed during host-mycorrhizal interactions have been successfully imaged at cellular resolution using confocal microscopy after labelling with various fluorophores (133, 251-253), using label-free imaging (254), by in situ hybridization (255), or following combined KOH maceration, resin embedding, physical sectioning and confocal imaging of fluorescently labelled WGA lectin (256, 257). Quantitative measurements of volumes and surface areas are possible, even from dense arbuscules (251). Techniques to visualise arbuscular mycorrhizal networks by sandwiching between Millipore filters gives impressive images of the mycelial network and has allowed quantitation of hyphal lengths and number of anastomoses (258, 259), and could easily be subject to network analysis. Likewise, cord forming ectomycorrhizal networks can be imaged and analysed using the same approaches developed for saprotrophic systems (Fig. 10), although it is more complex to segment the fungal mycelium from the root as they span different scales and the colour discrimination from each other and the soil is difficult.

In a similar manner, pathogens invading their host can be labelled using non-specific stains, fluorochromes or following expression of fluorescent proteins (260-262). Lesion development can be followed by automated segmentation and analysis using the HyphArea program (191), which has the potential to provide a complete, quantitative representation of the invasive hyphal network. The length scale that most pathogen infections operate over is quite limited (mm-cm), which suggests that growth and internal nutrient movement can be accomplished easily by diffusion and cytoplasmic streaming rather than mass-flow.

Network analysis and fitness traits

The network formed by different species is recognisable at a macroscopic level and tools to quantify network architecture and dynamics are emerging. At this point, we do not know which metrics may best describe network organisation and how these relate to fitness (263), but it is not unreasonable to propose that each species operates a slightly different set of local rules to balance growth, transport efficiency, recycling, resilience and reproduction that collectively maximize the long-term global success of the organism (31, 264). Thus network analysis may help to provide quantitative measures of traits that fulfil the criteria of ecological versatility, wide scope throughout the fungal kingdom and measurability set out by Aguilar-Trigeros *et al.* (39).

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FIGURE LEGENDS

Figure 1: Mycelial network formation – Tip growth and fluid flows

A – Hyphae grow by extension at the tip through polarised secretion of wall materials from macrovesicles and chitosomes at the apex, choreographed by the Spitzenkörper. Membrane is recycled distal to the tip into early endosomes by endocytosis. Endosomes may also form multi-vesicular bodies (MVB) that may be involved in unconventional secretion of exosomes. Transport of secretory vesicles, endosomes and other organelles along microtubule and microfilament networks also generates cytoplasmic streaming within the apical compartment. Although wall plasticity controls hyphal extension, the driving force involves maintenance of sufficient turgor pressure by water uptake through osmosis in response to accumulation of solutes. Turgor pressure may be sensed and regulated by a MAP Kinase cascade or stretch-activated channels, such as mid1. If the site of water uptake required for the volume increase during growth is distal to the tip, growth-induced mass-flows will also help to move organelles and solutes towards the tip. Based on (4, 43, 49, 64).

B – Water uptake into the hypha occurs to allow tip expansion and is driven by the transverse hydrostatic pressure in response to the difference in concentration of osmotically active solutes between the medium and the hypha. The transverse hydraulic conductivity depends on the permeability of the plasma membrane and aquaporins (AQPs) in parallel, and the wall and other surface layers, such as hydrophobins, in series. Longitudinal flow in the lumen of the hypha is lamina and follows Poiseuille flow. Based on (32, 33, 49, 79).

C – Variation in septal pore structure in different fungal taxa. Redrawn from (96) with permission.

D – Impact of the septa and septal pores on fluid flows. The change in cross-sectional area causes an increase in velocity by several orders of magnitude and also increases the wall

shear stress within the pore. Flow may deviate from parabolic profile expected from the Hagen-Poiseuille equation due to the density of organelles in the cytoplasm. In addition, there may be eddy currents near the pore opening that trap nuclei, vacuoles and other organelles. Based on (32, 65, 68, 69).

Figure 2: Mycelial network formation - Branching, fusion and multi-hyphal aggregate formation. A – Hyphae may branch sub-apically or by tip splitting to explore the substrate or to form aerial hyphae that are often insulated by hydrophobins. At the colony margin tips avoid each other, whilst secondary branch hyphae in the colony interior can show positive auto-tropism and fuse by anastomosis.

B – The velocity of mass-flow within the network depends critically on the site of water uptake for growth. If all water uptake is distal from the tips, the velocity scales in proportion to the number of downstream tips. If uptake is equal everywhere, the flow rate is constant at the speed of hyphal extension, whilst if uptake is solely at the tips, there is no long-distance movement. Based on (32, 33).

C – Schematic representation of the formation of strands, cords and rhizomorphs, showing progressive differentiation of vessel hyphae as potential conduits for long-distance transport. Scale bars are approximate. Based on (127, 129, 134, 228, 265).

D – Schematic representation of how circulating fluid flows might operate within a hyphal cord. Acropetal mass flows would take place in the vessel hyphae in response to growth, evaporation or exudation at the tips, whilst basipetal flows take place simultaneously through cytoplasmic hyphae by cytoplasmic streaming. Redrawn from (17) with permission.

Figure 3. Growth-induced mass flows explain long-distance nutrient movement in *Phanerochaete velutina.* A – Structure of the mycelial network after 21 d growing from a

wood resource across compressed black sand. B – The network architecture is extracted using intensity-independent, phase-congruency tensors and watershed segmentation from experimental time-series. The output is a set of weighted adjacency matrices of the length, width and volume of each cord. In this image the cord width is pseudo-colour coded from 50 µm (blue) to 500 µm (red). C – The network structure and growth are input into a biophysical advection/diffusion/delivery (ADD) model, using growth-induced mass flow to predict the pattern of resource translocation. The predicted amount of radiolabel is colour-coded on a rainbow scale from blue (zero) to red (maximum). D – The actual pattern of nutrient movement is then determined experimentally using the non-metabolised amino acid analogue ^{14}C-amino iso-butyrate (^{14}C-AIB) and photon-counting scintillation imaging (PCSI). The amount of ^{14}C-AIB is colour-coded on a rainbow scale from blue (low) to red (high) . The ADD model of growth-induced mass flow predicts the distribution of radiotracer in a complex network of fungal cords with a Pearson correlation coefficient 0.56. From Heaton *et al.* (32) with permission.

Figure 4. Mycelial networks in woodland. A – Map of a mycelial cord network of *Phanerochaete velutina* revealed by carefully removing surface litter layer, recovering and then re-revealing 13 months later. From Thompson and Rayner (155) with permission. B – Network of *Megacollybia platyphylla* on the floor of a mixed deciduous woodland, following removal of surface litter.

Figure 5. Effects of resource addition and grazing on mycelial networks. Mycelial cord systems (99 days old) of *Phanerochaete velutina* growing from centrally positioned beech wood inocula across the surface of compressed non-sterile soil in 50 x 50 cm trays. A – With four additional beech wood blocks added behind the mycelia margin 36 d after central

inoculation. B – With no additional resources added. C – As (A) but with 250 lab-reared collembolan, *Folsomia candida*, added 49 d after the central inoculums. D – As (C) but with no additional resources. From Wood *et al.* (170), with permission.

Figure 6. **Species variation in fractal dimensions.** Mycelia of six wood decay fungi extending, for 28 d, from centrally positioned beech wood inocula across the surface of compressed non-sterile soil in 24 x 24 cm trays. White circles are inert plastic discs. Note the different surface (D_{BS}) and mass (D_{BM}) fractal dimensions of the mycelia of different species. Photographs courtesy of Damian P. Donnelly.

Figure 7: Macroscopic network analysis of *Phallus impudicus* growing on compressed soil. A – original image of the mycelial network after 21 days. B – network architecture automatically extracted using phase-congruency edge enhancement, watershed segmentation and link pruning to give a single-pixel wide skeleton pseudo-colour coded by the cord width. C - Conversion to a graph representation whereby each node (junction or tip) is connected to by edges that are pseudo-colour coded by the average width of the cord segment from 50 µm (blue) to 500 µm (red). The structure of the network within the wood resource cannot be defined so any cord incident on the boundary is connected to a central node with a link set to the maximum width. D – Characterisation of the cord betweenness centrality as a measure of how important each cord is to transport through the network from the resource to every other node, with the colour-code ranging from blue (low importance) to red (high importance).

Figure 8: Network taxonomy. Taxonomies of 270 fungal (and slime-mould) networks based on community structure using modularity optimisation with path-score (PS) values as the edge weights (205). The dendrogram was produced from the mesoscopic structure of each

network as an indication of how similar different networks are to each other. The species abbreviations are coded as follows: Species: Pp, *Physarum polycephalum*; Pv, *Phanerochaete velutina*; Ag, *Agrocybe gibberosa*; Pi, *Phallus impudicus*; Rb, *Resinicium bicolor*; Sc, *Strophularia caerulea*. The of resources, and grazing are colour-coded from low (blue) to high (red). Substrate is coded as blue for Agar; white for sand, and red for compressed, non-sterile soil. Interactions are coded as blue for no interaction, or red grown in competition with *Hypholoma fasciculare*. At the bottom of the figure the logarithms of number of nodes *N*, number of edges *M*, and the edge density $\rho = 2M/N(N-1)$ are also shown. From (205) with permission.

Figure 9. *In silico* evaluation of network robustness. Networks of *Phanerochaete velutina* (red) and *Phallus impudicus* (green) were attacked *in silico* by progressively removing links in the network at random, and calculating the number of paths in the network that still remain. In each case 5 examples are shown. Note that the *P. velutina* network breaks down more rapidly than the *P. impudicus*.

Figure 10: Network analysis of the ecto-mycorrhizal fungus *Paxillus* on pine. A - *Pinus* seedling infected with *Paxillus*. The dotted white circle marks the loading site for radioactive ^{14}C-AIB. The dotted red lines indicate the routes for preferential transport of isotope to the roots from (C). B - Automated extraction of both the plant root and extra-radical mycorrhizal network using phase-congruency enhancement, watershed segmentation and cord width measurement. C - Scintillation image of nutrient movement along selected pathways from the fungus to the plant root. (R. Tajuddin, D. Johnson and M.D. Fricker, unpub.)

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