Mass flow and velocity profiles in *Neurospora* hyphae: partial plug flow dominates intra-hyphal transport

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INTRODUCTION

Fungal hyphae grow into new territories while forming an interconnected mycelium behind the colony edge where nutrients are actively absorbed to fuel continued growth. Intracellular hydrostatic pressure is the major driving force for cellular expansion of hyphae at the edge of the fungal colony (Lew, 2011). Behind the colony edge, nutrients are transported throughout the interconnected mycelium. The transport of nutrients can be measured with radioactive tracers and has velocities in the range of 3–70 μm s\(^{-1}\) (Jennings, 1987). These velocities would result in translocation that is further than could be expected for diffusion alone. In 60 s, a protein with a diffusion coefficient of 7 × 10\(^{-11}\) m\(^2\) s\(^{-1}\) would travel a mean (bidirectional) distance of ~35 μm compared to 180–4200 μm for unidirectional nutrient translocation. With recent advances in imaging techniques, it is now clear that there is a highly complex network of translocation (Fricker et al., 2008) that adapts dynamically (Bebber et al., 2007). Some of the genes that affect translocation have been identified in *Neurospora crassa* (Simonin et al., 2012); the gene products function in hyphal fusions that create a cytoplasmic continuum. At a localized scale, cytoplasmic movement translocates cellular components acropetally from vegetative hyphae to the growing edge of the colony (Riquelme et al., 2002) and to developing aerial hyphae in conidia formation (Bleichrodt et al., 2003).

The driving force for translocation could be molecular motors or a trans-hyphal pressure gradient, or both (Lew, 2011). When silicon oil was injected into hyphae, it moved through the hyphae similarly to vacuoles. Since the silicon oil should not interact with molecular motors, the likely cause of movement was trans-hyphal pressure gradients (Lew, 2005). Taking advantage of the ability to express green fluorescent protein (GFP) in *N. crassa* (Freitag et al., 2004), Ramos-García et al. (2009) monitored the movement of nuclei labelled with GFP-tagged histone. Movement of nuclei towards the growing edge of the colony was still observed in strains with mutations in microtubule-related motors (dyenin and kinesin) and after treatment of WT strains with disruptors of the cytoskeleton, corroborating the idea that bulk flow is an important determinant of organelle movement. Genetic intermixing – in which mass flow plays a primary role – has been directly imaged using nuclei labelled with DsRed or GFP (Roper et al., 2013). In Basidiomycetes, at least, an alternative transport mode relies upon movement through the vacuole system (Darrah et al., 2006).

In this paper, we explore the nature of mass flow in hyphae in greater detail. We use dual-imaging of mitochondria and either nuclei or vacuoles to correlate their vectorial movement within hyphae. Profiles of velocity versus distance from the hyphal wall were constructed to test for known models of bulk flow at a low Reynolds number (Cox & Mason, 1971). Experimental manipulations of external osmolarity were used to directly modify the trans-hyphal pressure gradients required to drive mass flow through the hyphal network. The results indicate that mass flow dominates cytoplasm movement. Due to the high density of organelles, the movement deviates from Hagen–Poiseuille flow and is better described as partial plug flow.

Abbreviation: GFP, green fluorescent protein.
METHODS

Strain preparation and media. A GFP-tagged histone strain (rid Pccg-1-hH1::sgfp+, FGSC 10174) was obtained from the Fungal Genetics Stock Center (School of Biological Sciences, University of Missouri, Kansas City, MO, USA) (McCluskey et al., 2010) and maintained on slants of Vogel’s minimal medium (Vogel, 1956) plus 1.5 % (w/v) sucrose and 2.0 % (w/v) agar. The GFP-tagged histone strain was used to visualize the fluorescently labelled nuclei. For visualizing vacuoles, a his-3”::Pccg-1::nca-2”::sgfp+ (FGSC 10160) strain was used. This strain has a GFP-tagged NCA-2. NCA-2 is a calcium transporter that is found in both vacuoles and a tubular internal membrane network (Bowman et al., 2009). Movement of the internal network was difficult to track with GFP-NCA-2 because of the small size of the fluorescent structures (movement could be observed qualitatively), but large vacuoles could be readily tracked.

For the dual imaging experiments – in which both mitochondria and nuclei (or vacuoles) velocities were measured – the mitochondria were labelled with MitoTracker Red FM (Invitrogen Molecular Probes, Catalogue Number M22425). Conidia from the GFP-tagged strains were spread in a 2 cm streak along the edge of a 55 mm Petri dish containing organic medium (OM) plus 1 % agar. Organic medium contains: 1%, w/v, glucose; 0.1%, w/v, peptone; 0.01%, w/v, yeast extract; 0.1%, w/v, KH2PO4 and 0.03%, w/v, MgSO4·7H2O. The peptone and yeast extract were obtained from Difco. The cultures were grown overnight at 28 °C. To label mitochondria, 2.5 ml of OM containing MitoTracker Red FM (final concentration 12 μM from a 2 mM stock in methanol) was pipetted into the Petri plates on top of the mycelium after overnight growth so that the fungi continued to grow submerged in the medium. To maximize loading with MitoTracker, the plates were incubated for another 5–6 h at 28 °C in the dark (less loading was observed when the Petri plates were incubated at room temperature). The MitoTracker fluorescence intensity was strongest at the hyphal tips, presumably because of the highly polarized membrane potential of tip-localized mitochondria (Levina & Lew, 2006), but could be readily visualized in hyphal trunks behind the colony edge for tracking movement of the mitochondria.

Dual fluorescent imaging using a confocal microscope. To image the hyphae in situ, the culture plates were mounted directly on the microscope stage and a cover-slip gently placed over the colony edge. Hyphal trunks were selected on the basis of noticeable cytoplasm movement and strong fluorescence intensity of the mitochondria. Fluorescence scanning was performed using an EC Plan-Neofluar ×100 oil immersion objective (N.A. 1.3) on a Fluoview 300 confocal system (Olympus). The nuclei and vacuoles (GFP labelled) were imaged with an excitation wavelength of 488 nm. The mitochondria (MitoTracker Red labelled) were imaged with an excitation wavelength of 579 nm. For both, the emission wavelength was 622 nm. Only a region of interest of the selected hypha was imaged to minimize the time required for scanning. Time series of 120 images were acquired at 1 s intervals with Kalman filtering. The image stacks were analysed using ImageJ (Rasband, 2013).

Quantifying organelle flow. For nuclei and vacuoles, it was relatively easy to track the movements of the organelles using ImageJ. Mitochondrial fluorescence was not as discretely located, due to the pleiomorphic structure of the mitochondria (tubular to filamentous; Luck, 1963). In all cases, the fluorescence images were digitally enhanced with linear contrast stretch and Gaussian filtering (1.5 pixel radius) in ImageJ to make organelle tracking easier (Fig. 1). For any given experiment, a sample of 5–7 mitochondria and nuclei or vacuoles were selected in each digital image. Their movement along the hyphal trunk was tracked for three sequential images and their displacement (x-y coordinates) over the two 1 s intervals was recorded (Fig. 1). The x-y coordinates for the two 1 s intervals were converted to vectors of mean velocity (μm s−1) and direction (as an angle) using MATLAB. We did not document the zero velocities at the wall boundary because we expected that cytoskeleton effects would be more pronounced at the immobile wall and to avoid the complexities of boundary effects.

To analyse the correlations between velocity and direction for the mitochondria and nuclei or mitochondria and vacuoles, the means of the vectors at any specified time were calculated (Fig. 2). For analysis of velocity profiles, the individual vectors were mapped along the width of the hyphae. Since velocity and hyphal diameter varied from one experiment to another, both velocity and location along the hyphal width were normalized (to maximal velocity and the hyphal radius, respectively). Best fits of the velocity profiles were to a parabolic model (a simplified version of the Hagen–Poiseuille equation: v(τ) = a(1 − r²), where v(τ) is the velocity at normalized

Fig. 1. Image enhancement and organelle tracking. (a) Examples of (from left to right) fluorescence images of mitochondria, nuclei and vacuoles (arrow). Upper panels are the original images; lower panels are processed with Gaussian filtering and linear contrast stretch using ImageJ. In all cases, clearly delineated organelles were selected for tracking. (b) Example of tracking a nucleus. The x-y coordinates of the nucleus (red dots) are tracked for three sequential images (overlaid in a single image). (c) The x-y coordinates for the three sequential images (overlaid in a single image) were transformed into a mean velocity and direction for the two 1 s intervals. The mean velocity (v, in μm s−1) was calculated as

\[ v = \sqrt{v_x^2 + v_y^2} \]

where

\[ v_x = \frac{(x_3 - x_1) + (x_2 - x_1)}{2} \]

and

\[ v_y = \frac{(y_3 - y_1) + (y_2 - y_1)}{2} \]

The direction (angle) of growth was calculated as the mean of tan⁻¹ \( \frac{y_3 - y_1}{x_3 - x_1} \) and tan⁻¹ \( \frac{y_2 - y_1}{x_2 - x_1} \).

Individual flow vectors for organelles moving through the region of the hyphae that was being imaged are shown in Fig. 2. Bars, 10 μm.
Modulating organelle flow. To assess as directly as possible the role of pressure gradients in driving organelle movement through the hyphae, the mycelia were placed in a chamber that allowed the extracellular osmoticum to be varied at will in separate compartments. The GFP-tagged histone strain was grown between two layers of dialysis membrane (molecular mass cut-off of 12000–14000) overnight at 28 °C in the dark. A section of the mycelium that included the growing edge of the colony was cut with a razor blade and carefully lifted and placed in a chamber with a cover-slip window to allow imaging (a schematic of the set-up is shown in Fig. 6a). Two beads of petroleum gel were applied above the upper dialysis membrane and a 22 × 50 mm cover-slip overlaid on the petroleum gel so that three water impermeable zones were created. Movement of nuclei was imaged in the central zone with a ×10 objective on a Zeiss Axioskop fluorescent microscope. Fluorescent imaging used filter set 15 (excitation at 546 nm; longpass emission at 590 nm) and a Hamamatsu Orca C4742-95 camera controlled by Openlab software (Improvision); images were collected at 1 s intervals. For these experiments, we selected larger trunk hypha within the mycelial network for which movement of nuclei was towards the colony edge.

The nuclei were tracked for four sequential images and mean velocities were calculated from the three 1 s intervals. The two outer zones were filled with a buffered solution (BS) that contained: 10 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 133 mM sucrose and 10 mM MES; pH adjusted to 5.8 with KOH. To modify the trans-hyphal pressure gradient, BS plus 500 mM sucrose was added to one of the outer compartments. The osmolarities of the solutions were 205 mOsmol for BS and 970 mOsmol for BS plus 500 mM sucrose, measured with an osmometer (5005 Osmette II, Precision Systems). The absolute magnitude of the pressure gradient between the two outer compartments was calculated from the Van’t Hoff relation \[ \Delta P = R \frac{\Delta c}{c} \] where \( \Delta P \) is the pressure difference, \( R \) is the gas constant (8.314 l kPa K⁻¹ mol⁻¹), \( T \) is the temperature (K) and \( c \) is the osmolarity and the distance between the two compartments (1.5 cm), yielding the result \(~1200 \text{ kPa cm}^{-1}\). Because the dialysis membrane slows diffusive equilibration at the hyphal layer, the actual gradients will be much lower. Although we are unable to image growth at the colony edge simultaneously, we expect that the treatments would inhibit growth for at least a brief period of time based on general observations when measuring colony growth.

Statistics. Results are shown as mean ± 1 S.D. (sample size). Statistics and linear regressions were calculated in Excel (Microsoft). Non-linear regressions were performed in Kaleidagraph (Synergy Software).
RESULTS

To study the nature of flow in *N. crassa*, we mapped the velocity vectors of organelles using confocal microscopy. The GFP-tagged histone (for imaging nuclei) and GFP-tagged calcium transporter NCA-2 (for imaging vacuoles) provided us with *in situ* tools for mapping organelle movement. The MitoTracker allowed us to label mitochondria so that imaging of two different organelles could be done simultaneously. The vector maps of flow through a region of the hypha (see Fig. 2 for an example) revealed that the movement of the uniformly distributed nuclei (or vacuoles) and mitochondria was consistently unidirectional and normally acropetal (towards the growing edge of the colony). This well-defined directionality was very clear a small distance behind the colony edge (within 1 cm). Further back – where anastomoses would be expected to create a more complex network – acropetal movement was less clear (especially in smaller hyphae). Even here, unidirectional movement was consistently observed for any given hypha. For correlative analysis, the mean velocity vectors were calculated for each specific time interval. A total of 11 hyphae were used in the analysis of mitochondria and nuclei, and five hyphae for mitochondria and vacuoles. The direction of flow deviated very little from parallel movement within the hyphae (Fig. 3), and plots of angles for mitochondria and nuclei (Fig. 3a) or vacuoles (Fig. 3b) were strongly clustered at 0° as expected for unidirectional flow parallel to the hyphal walls.

![Flow vectors for nuclei and mitochondria (a) and vacuoles and mitochondria (b). Each point in the plot represents the correlation between the velocity of the nuclei and mitochondria (or vacuole and mitochondria) in the same hyphal region and time-frame of 2 s. The angles and velocities are plotted in the upper graphs, and correlations of angles in the lower graphs. There is a close correspondence of direction for the three organelles.](http://mic.sgmjournals.org)
Organelle velocities were strongly correlated (Fig. 4). The regression slope of the velocities of nuclei and mitochondria was nearly 1 (Fig. 4a). Comparing vacuole and mitochondria velocities (Fig. 4b), vacuoles appeared to move slower than mitochondria, but even here the regression slope was close to 1. It is possible that the slightly slower velocity of the vacuoles may be due to their larger size, and thus more likely to be affected by immobile elements in the cytoplasm (such as the cytoskeleton).

A hallmark of low Reynolds number flow in hyphae (Lew, 2005) is the expectation that velocity profiles of the cytoplasmic fluid will be parabolic: maximal at the centre of the hypha and decreasing to zero at the hyphal walls. This relation of velocity as a function of radial distance \( v(r) \) is predicted from the Hagen–Poiseuille equation:

\[
v(r) = \left( \frac{\Delta p}{l} \right) \left( \frac{1}{4\eta} \right) (R^2 - r^2)
\]

where \( \Delta p/l \) is the pressure gradient, \( \eta \) is the viscosity, \( R \) is the hyphal radius and \( r \) is the radial location. Velocity profiles were constructed for movement of nuclei and mitochondria; vacuole movements were not examined due to the lower sample size (and the large size of the vacuoles). In order to fit velocity profiles, the Hagen–Poiseuille equation was simplified to the form \( v(r) = a(1 - r^2) \), where \( a = [\Delta p/(l/4\eta)] \) and \( r \) (radial location) is normalized so that \( R \) is equal to 1. The predicted parabolas fit the data very poorly (Fig. 5). Instead, there was a flat profile of velocity, independent of radial distance. This is probably due to the presence of high concentrations of organelles in the cytosol, resulting in ‘partial plug flow’ (Karnis et al., 1966; see Discussion).

The close correspondence of velocities for the three organelles is strong indirect evidence of the dominant role of pressure-mediated organelle movement in the hyphae. It is unlikely that molecular motors could transport disparate organelles with such consistent velocity. Direct evidence for pressure mediation was obtained by modifying the extracellular osmoticum on either side of petroleum gel barriers. The basic set-up is shown in Fig. 6(a). Hyphae were constrained to two dimensions by being grown between two layers of dialysis membrane. Petroleum gel beads overlaying the upper dialysis membrane created three compartments. The two end compartments were filled with BS. After imaging for 40 s to establish a baseline of nuclear velocities, the solution in one of the compartments was changed to BS plus 500 mM sucrose. The effect of the addition depended on whether the solution change was made behind the colony edge (basal) or at the colony edge (apical). If the addition was basal, velocities decreased or even reversed (Fig. 6b); if apical, velocities increased (Fig. 6c). For all experiments, the change in velocity was \( 2.3 \pm 1.6 \mu \text{m sec}^{-1} (n=12) \). The velocity change was reversed by a return to BS after treatment with BS supplemented with sucrose (Fig. 6d). It is not possible to
determine the actual magnitude of the trans-hyphal pressure gradient accurately because the high osmolarity solution would take time to diffuse through the dialysis membrane to the hyphae. Therefore, the overall gradient will be significantly lower than that calculated from the Van’t Hoff relations for the two compartments (see Methods). In other experiments measuring turgor, injection of a large bolus of silicon oil into a hypha causes fast cytoplasmic flow (R. R. Lew, unpublished) even though the change to the trans-hyphal pressure gradient is low relative to the high hydrostatic pressure of the hyphae (~400–500 kPa; Lew & Nasserifar, 2009). Although we are unable to quantify the relation between the pressure gradient and flow velocity, the effect of modifying the trans-hyphal pressure gradient on cytoplasmic flow clearly indicates the role of pressure-driven flow directly.

**DISCUSSION**

In order to study the nature of mass flow in fungal hyphae, we tracked the movements of two different organelles simultaneously (nuclei and mitochondria, or vacuoles and mitochondria) using dual fluorescent imaging on a confocal microscope. Correlated organelle movements – both velocity and direction – provide evidentiary support for the primary role of mass flow in movements of cytoplasm in the trunk hyphae. It is important to note that movement of nuclei in a direction opposite (basipetal) to that of the normal tip-directed movement was occasionally observed near the hyphal wall, consistent with some contribution by a cytoskeleton/motor system (cf. Ramos-Garcia et al., 2009). But basipetal movement of individual nuclei at the wall was rare. Certainly, molecular motors have been implicated in numerous aspects of fungal growth (Steinberg, 2007), but flow can be independent of the activity of molecular motors (Lew, 2011). This idea arose from a previous report in which silicon oil was injected into the hyphae, and moved similarly to vacuoles (Lew, 2005) and warranted the more detailed exploration described here.

Using fluorescent imaging, it was possible to map the velocity profiles of nuclei and mitochondria. This allowed a direct test of the mechanism of flow. At low Reynolds number, flow through a tube is expected to be laminar, with a continuous change in shear from the immobile cell wall to the centre of the hyphal tube. This will cause a gradient of velocities – highest in the centre of the tube – that has a parabolic shape (the Hagen–Poiseuille equation). When data from multiple experiments are collected and normalized to maximal velocity and hyphal width, there is considerable scatter, but the data do not fit the parabolic shape predicted for laminar flow. Instead, velocity is nearly the same at the centre of the hyphae and close to the cell wall. This velocity profile is consistent with low Reynolds number flow of particle suspensions (reviewed by Cox & Mason, 1971). Karnis et al. (1966) measured particle movements in tubes at low Reynolds number (<10−3) similar to the Reynolds number of hyphal flow, ~10−4 (Lew, 2005). They observed a transition from a parabolic velocity profile to a ‘flat-top’ velocity profile when the volume fraction of particles was increased to values above ~0.18, and described the velocity profile as ‘partial plug flow’. It was observed with both spherical and disc-shaped particles.

**Fig. 5.** Velocity profiles for mitochondria (a) and nuclei (b). The individual velocities of all measurements are shown (mitochondria, n=3784; nuclei, n=4007). They were normalized to the maximal velocity for each hypha. Radial distance is normalized to each hyphal radius. The mean hyphal diameter was 18.4 ± 1.9 µm (n=11) (range, 15.0–20.8 µm). Open circles are the mean velocities for binned normalized radial distances of 0.1 (except mitochondria at the walls of the hyphae, which were binned from ± 0.8 to ± 1 to increase the sample size near the cell walls). Best fits are to a Hagen–Poiseuille model (a), which predicts a parabolic profile with maximal velocity at the centre of the hyphae, and an arbitrary catenary function (b) that emphasizes that the velocity profile is much flatter than that predicted for Hagen–Poiseuille mass flow.
particles, and was more pronounced with larger particles. In hyphae, the volume fraction of organelles flowing in the hyphae is considerably higher than 0.18 (cf. Fig. 1), and the velocity profile we observe is consistent with ‘partial plug flow’. An intuitive explanation of the partial plug flow is that the organelles themselves affect laminar flow as they disrupt shearing of the fluid from the immobile cells walls to the hyphal centre causing a flat velocity profile. This would maximize the integrity of the cytoplasm continuum since all the cytoplasmic components would move in tandem.

It could be argued that the coordinated movement of very different organelles is a consequence of molecular motors and their transported cargo. As the cargo moves through the cytoplasm, it could ‘entrain’ the surrounding fluid, resulting in mass flow. Such a process has been invoked in cytoplasm streaming of the giant cells of the green alga Chara (Verchot-Lubicz & Goldstein, 2010). Indeed, silicon oil droplets injected into the cytoplasm of Chara move at the same rate as visible cargo, regardless of the oil droplet size (from 30 to 300 μm diameters) (K. Cross & R. R. Lew, unpublished). Even in small plant cells, it has been suggested that entrained mass flow occurs (Esseling-Ozdoba et al., 2008). The movement of free GFP in cytoplasmic strands was measured using fluorescence bleaching recovery and found to be affected by treatments with a myosin inhibitor, and correlated with the movement...
of organelles, providing support for cytosol entrainment. The situation for plant cells is different from that of fungal hyphae, which are cytoplasm-rich and do not exhibit the relatively narrow trans-vacuolar cytoplasmic strands common to plant cells. The existence of cytosol entrainment in plants would impact on one proposed function for cytoplasmic streaming in plants: higher fluxes (either uptake or secretion) to and from the streaming organelles (Pickard, 2006) because of a thinner diffusion zone in the absence of cytosol entrainment. This would allow the moving organelles to interact with a larger volume of the surrounding cytosol. If entrained cytosol moved with the organelle, it would create a thicker diffusion zone and limit fluxes. In fungi, the typically acropetal movement of cytoplasm apparently fulfils a different role of supplying cytoplasm to the ever-expanding hyphal tips.

Different lines of evidence suggest that cytosol entrainment by the activity of molecular motors is unlikely in fungal hyphae. Nuclei move towards the growing edge of the fungal colony even in strains with defective molecular motors (Ramos-García et al., 2009). By directly modifying the trans-hyphal pressure gradient and showing it has rapid effects on nuclei movement, we provide direct evidence for the alternative explanation – pressure-mediated flow. Addition of external osmoticum basal to the colony edge causes water flow out of nearby hyphae, creating a basopetal pressure gradient in the hyphal tubes. This caused the velocity of nuclei movement towards the colony edge to slow down or even reverse. Addition of external osmoticum at the colony edge causes water flow out of the tips, creating an acropetal pressure gradient in the hyphal tubes. This caused an increase in the velocity of nuclei movement towards the colony edge. Both effects can be attributed to changes in the pressure gradients within the hyphal network of the colony. The nature of the mass flow in the hyphal tubes is in some ways analogous to mass flow in phloem of higher plants, although the physical mechanisms causing mass flow in phloem are more complex than pressure-driven flow alone (Knoblauch & Peters, 2010), similar to the role of the vacuolar network in nutrient translocation in the more complex architecture of the Basidiomycetes (Darrah et al., 2006). Some aspects of the physical mechanisms of osmotic-pressure-driven translocation (and flow of particle suspensions) may be best addressed in micro-fluidic model systems (Jensen et al., 2009).

In summary, pressure-driven mass flow dominates organelle movements in N. crassa hyphae. Because of the high concentrations of organelles in the cytosol, the movement is best described as ‘partial plug flow’. The long-distance mode of pressure-driven translocation should complement the short distance transport mediated by the cytoskeleton.

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