REVERSE FOUNTAIN CYTOPLASMIC STREAMING

IN *RHIZOPUS ORYZAE*

By

Phakade Mdima Shange

A Thesis Presented in Partial Fulfillment

Of the Requirements for the Degree

Master of Science

Approved April 2020 by the

Graduate Supervisory Committee:

Robert Roberson, Chair

Page Baluch

Gillian Gile

ARIZONA STATE UNIVERSITY

May 2020

ABSTRACT

The intracellular motility seen in the cytoplasm of angiosperm plant pollen tubes is known as reverse fountain cytoplasmic streaming (i.e., cyclosis). This effect occurs when organelles move anterograde along the cortex of the cell and retrograde down the center of the cell. The result is a displacement of cytoplasmic volume causing a cyclic motion of organelles and bulk liquid. Visually, the organelles appear to be traveling in a backwards fountain hence the name. The use of light microscopy bioimaging in this study has documented reverse fountain cytoplasmic streaming for the first time in fungal hyphae of *Rhizopus oryzae* and other members in the order Mucorales (Mucoromycota). This is a unique characteristic of the mucoralean fungi, with other fungal phyla (e.g., Ascomycota, Basidiomycota) exhibiting unidirectional cytoplasmic behavior that lacks rhythmic streaming (i.e., sleeve-like streaming). The mechanism of reverse fountain cytoplasmic streaming in filamentous fungi is currently unknown. However, in angiosperm plant pollen tubes it's correlated with the arrangement and activity of the actin cytoskeleton. Thus, the current work assumes that filamentous actin and associated proteins are directly involved with the cytoplasmic behavior in Mucorales hyphae. From an evolutionary perspective, fungi in the Mucorales may have developed reverse fountain cytoplasmic streaming as a method to transport various organelles over long and short distances. In addition, the mechanism is likely to facilitate driving of polarized hyphal growth.

.

.

ACKNOWLEDGEMENTS

I would like to thank my mentor and advisor Dr. Robert Roberson for his patience, guidance and knowledge he has shared about filamentous fungi and microscopy. I would also like to thank my committee members Dr. Baluch, Dr. Gile, and Dr. Wilson-Rawls for their advice and feedback. Finally, I would like to thank our collaborators Dr. Mourino Perez and Dr. Diego Delgado at CICESE.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Figure Page

Introduction

I.a. The Fungi

Mycology is the study of fungi, though etymologically it refers strictly to the study of mushrooms (Greek: myces = mushroom; logos – discourse). Indeed, mushrooms and other larger fungal fruiting bodies have been the major emphasis of mycologists for hundreds of years because these are the most visible parts of the fungal body. Initially these organisms were believed to be phylogenetically closely related to plants (Kingdom = Plantae) because of some morphological similarities. However, unlike plants fungi do not contain roots, leaves, stems, a vascular system, nor photosynthetic capabilities. In fact, from an evolutionary standpoint, fungi are more closely related to humans than plants (Baldauf and Palmer, 1993). Defining the fungal kingdom (*i.e.*, Mycota) is difficult because of their diverse morphology and behavioral characteristics. One of the most succinct definitions for the Mycota comes for Alexopoulous *et al.* (1996): 'Fungi are eukaryotic, spore-producing, achlorophyllous organisms with absorptive nutrition that generally reproduce both sexually and asexually, and whose usually filamentous, multicellular branched somatic cells (*i.e.*, hyphae) or unicellular yeast cells are surrounded by a chitin-rich cell wall during all or part of life cycle.' Fungi are most familiar to the public as yeast, molds, and mushrooms. They are eukaryotic organisms that inhabit diverse ecological niches around the world and are critical to the health of ecosystems performing roles in decomposition of organic matter and nutrient recycling (Heath, 1990). Fungi are heterotrophs, unlike plants, and comprise the most successful and diverse phyla of eukaryotic microbes, interacting with all other forms of life in associations that range from beneficial (*e.g.*, mycorrhizae) to antagonistic (*e.g.*, pathogens). Some fungi can be parasitic or pathogenic on plants (*e.g.*, *Cryphonectria parasitica*, *Magnaporthe grisea*), insects (*e.g.*, *Beauveria bassiana*, *Cordyceps* sp.), invertebrates (*e.g.*, *Drechslerella anchonia),* vertebrates (*e.g.*, *Coccidioides immitis*, *Candia albicans*) and other fungi (*e.g.*, *Trichoderma viride*, *Ampelomyces quisqualis*). However, most fungi are saprophytes, obtaining

nutrition through the break down of non-living organic matter. Fungi are important in the food and pharmaceutical industries, as well as studies in molecular and cellular biology. In the food industry, fruiting bodies of fungi in the Basidiomycota (*e.g.*, mushrooms) are farmed at an industrial level and play a direct role in the production of consumable products for the domestic and international business markets. Some of the common fungi used in mushroom production include *Agaricus bisporus* (*i.e.*, the button or portobello mushroom), *Lentinus edodes (i.e*., the shiitake mushroom) and *Pleurotus ostreatus* (*i.e.*, the oyster mushroom). Fungi also have an indirect role in the food industry via fermented products. Some fermented products include tempeh (soybean fermented by *Rhizopus oryzae*), tofu (Chinese cheese, soybean fermented by *Mucor* sp.), and alcoholic products (plant products fermented by *Saccharomyces cerevisiae)*. The contributions that fungi have made in the pharmaceutical industry are just as significant. The famous antibiotic penicillin was first derived from the fungus *Penicillium notatumn* and later produced on a commercial scale by *Penicillium chrysogenum*. A second notable antibiotic is griseofulvin produced by *P. griseofulvin*, which has proven to be a useful cytoskeletal inhibitor for the disruption of microtubules. *Claviceps purpurea* the notoriously known ergot of rye fungus contains medicinal alkaloids such as ergometrine used during childbirth if uterine contractions are irregular and weak (Carlile *et al*., 1997).Also, they are also administered post childbirth if there is excessive bleeding (Carlile *et al*., 1997).

In addition, to the food and pharmaceutical industry, fungi are utilized for model organisms in molecular and cellular biology. The in depth understanding of many fundamental concepts are derived from *Saccharomyces cerevisiae* and *Neurospora crassa*. Yeast is formally known as *Saccharomyces cerevisiae* and is one of the oldest eukaryotic model organisms. In fact, yeast has been employed in studies of cell cycle regulation and events of vesicle trafficking and secretion. Another model organism is *Neurospora crassa,* used in the development of the single

gene/single protein concept and many basic aspects of genetics (Beadle and Tatum,1941). The Mycota kingdom is composed of seven phyla. The majority of these phyla are strictly terrestrial

(*e.g*., *Ascomycota, Basidiomycota, Mucoromycota*, *Zoopagomycota*). Fungi reproduce sexually and asexually primarily by spores; fragmentation is also a common means of asexual reproduction. Fungi belonging to the Ascomycota produce sexual spores called ascospores. These spores contain a single haploid nucleus, a result of one meiotic division, and are formed within a specialized cell called an ascus. In addition, asexual spores (conidia) are produced on specialized aerial hyphae known as conidiophores. The Basidiomycota produce haploid sexual basidiospores on a structure known as the basidium. Within the basidium two haploid nuclei of compatible mating types undergo karyogamy and meiosis giving rise to haploid nuclei that migrate into developing basidiospores (Carlile *et al*., 1997). The Blastocladiomycota and Chytridiomycota are aquatic fungi. During the life cycle of aquatic fungi, motile sexual and asexual zoospores are produced and characteristically contain with a single posterior whiplash flagellum. The fungi that are of interest in this study are members of Mucoromycota and Zoopagomycota; previously known as Zygomycota. These fungi are of particular interest due to the paucity of basic research and their apparent polyphyletic nature. The research performed here has been focused primarily on the selected members of the Mucoromycota within the order Mucorales. The focus was *Rhizopus oryzae* (Mucoraceae), with additional experiments done with *Cunninghamella echinulate* (Cunninghamellaceae), *Gilbertella persicaria* (Choanephoraceae) and *Neurospora crassa* (Ascomycota, Sordariales, Sordariaceae). All members of the Mucorales examined thus far display an atypical cytoplasmic behavior to fungi known as reverse fountain cytoplasmic streaming (RFCS). Reverse fountain cytoplasmic streaming is caused by a combination of cytoskeletal action and bulk cytoplasmic flow. The motion can be described as an anterograde movement along the cell cortex, followed by a retrograde movement down the cell center. This phenomenon has been well documented in angiosperm plant pollen tubes (Chebli *et al.,* 2013). However, little is known about RFCS in filamentous fungi.

I.b. The Mucorales (Mucoromycota)

I.b.i. *Rhizopus* (Mucoroaceae). The genus *Rhizopus* is a member of the order Mucorales, the subphylum Mucoromycotina, and the phylum Mucoromycota (Spatafora *et al*., 2017). The Mucorales are mostly saprotrophs and are commonly found in soil and the droppings herbivores and rodents (Carlie *et al*.,1997; Souza *et al*., 2017). A few members are weak parasites of crop plants (*e.g.*, *Gilbertella*, *Choanephora*) and others are animal pathogens (*e.g.*, Mucor). The cell wall composition of Mucoromycota is chemically complex. While chitin microfibrils are existent, chitosan may be the most copious component. Chitosan is derived from chitin enzymatically by removing most of the acetyl groups which yields the polymer β (1-4) glucosamine (Adriana *et al*., 2019). Other cell wall polymers include polyglucuronic acid and mannoproteins. In addition to proteins, lipids and various ions are also present. A unique characteristic to the Mucorales is the ability to form a reproductive structure known as a sporangium. The Mucorales sporangium produces thousands of asexual spores (sporangiospores). The spores are non-motile and are dispersed passively in the environment by wind, rain splash, insects or other animals. The spores can contain one or several haploid nuclei. Several members of the Mucorales are heterothallic. This means, they are self-sterile and need 2 mating types (+/-) to sexually reproduce via zygospores. However, there are homothallic species such as *Rhizopus sexualis, Syzygites megalocarpus*, *Zygorhynchus molleri* and *Absidia spinose* (Webster,1980).

I.b.ii. *Mucor* (Mucoroaceae). This is the largest genus in the Mucorales order is found in similar environmental niches as *Rhizopus*. Species of *Mucor* are differentiated from species of *Rhizopus* by the lack of rhizoids and branched sporangiophores (McDonald, 2018). Some *Mucor* species form chlamydospores, which are another type of asexual spore with a thick wall that is generated within the hyphae. This can be a helpful medical diagnostic characteristic since some *Mucor* sp. are opportunistic pathogens of humans. *Mucor* maybe dimorphic in anaerobic environments, changing from a filamentous growth form to a yeast growth form.

I.b.iii. *Cunninghamella* (Cunninghamellaceae). Members of this genus differ from many in the Mucorales by asexual reproduction. This genus solely reproduces by spores called conidia and does not form sporangia/sporangiola. The asexual conidia are formed naked on globose vesicles that can be attached to branched or unbranched conidiophores. In some species such as C. *elegans* and *C. echinulate* the conidia are spiny yet in others they are smooth.

III.b.ii. *Gilbertellla* (Choanephoraceae). In this family both sporangia and sporangiola are present. The sporangia often hang downwards are typically columellate. The sporangia contain dark brown sporangiospores with bristle like appendages and a striate epispore. The function of the appendages is unknown. However, it is hypothesized that they are needed for dispersal of 'conidia' by insects. The genus *Gilbertella* is unique since it does not contain sporangioles but rather releases striate appendages spores via sporangia split. *Gilbertella persicaria* is a known parasite of tomatoes and peaches.

I.c. The Sordariales (Ascomycota)

I.c.i. *Neurospora* (Sordariaceae). The genus *Neurospora* belongs to the order Sordariales and the subphylum Pezizomycotina. *Neurospora crassa* is the most well-known species of the *Neurospora* genus. The hyphae of this species grow rapidly 0.4 µm/sec (Riquelme *et al.*, 2002) and possess hyphae with diameters of up to 8-10 µm in diameter. The sexual spores are produced in an ascus, which has eight haploid ascospores, half of one mating type (+) and half of another mating type (-). *Neurospora crassa* is a model organism it studies of biochemical and genetic research. There are several reasons for their popularity and wide-spread use amongst molecular and cellular biologists. First, *Neurospora crassa* wild type has basic nutritional requirements (simple mineral salts, one vitamin and a carbohydrate source). Second, mutations can be easily induced by irradiation of conidia. The third is its rapid growth, ease in laboratory manipulation, and cell cycles that include asexual and sexual reproduction. Asexual reproduction occurs with both macroconidia and microconidia. Macroconidia have an ellipsoidal morphology

and contain several nuclei. Conidia are produced on conidiophores that rises above the substrate (*i.e.*, arial conidiophores). The presence of carotenoids in the macroconidia make them pink in

color. These spores are produced in enormous quantities, are easily detached and dispersed by air currents, and readily germinate upon landing on a suitable substrate. Microconidia are uninucleate and do not germinate as readily as macroconidia. In tropical regions, burnt vegetation is often decorated with the pink macroconidia and mycelium of *Neurospora*. *Neurospora* is also found in other warm and humid places such as wood-drying kilns and bakeries.

II. Sub-cellular Structural Characteristics of the Fungi

Understanding cellular and sub-cellular structures is one way by which the fungi are classified into their proper phylogenetic niches. Therefore, understanding growth patterns and sub-cellular structural characteristics is imperative. The following section will discuss various sub-cellular characteristics such as Spitzenkörper (Spk), apical vesicular crescent (AVC), septations, and spindle pole bodies.

II.a. Apical Organization of Secretory Vesicles

II.a.i. The Spitzenkörper

The Spitzenkörper (Spk) is one of several hallmark characters of dikaryotic fungi (*i.e.*, Ascomycota, Basidiomycota). Interestingly, Spk are also present in only few fungi other than the dikarya (*e.g.*, *Allomyces macrogynus. (*Blastocladiomycota*)*, *Conidiobolus cornatus (*Zoopagomycota*)*, *Basidiobolus sp. (*Zoopagomycota*)*. The Spk is present only at the tips of actively growing hyphae and is a non-membrane-bound spherical inclusion composed mostly of Golgi-derived secretory vesicles, cytoskeletal elements, signaling proteins, and ribosomes (only in the Ascomycota), (Roberson *et al*., 2010). It is clear that the Spk is primarily involved the final stages of exocytosis of secretory vesicles and its position within the apical dome dictates growth direction (Girbardt, 1957; Reynaga-Peña *et al*.,1997; Riquelme *et al*.,1998). There are a few different models that explain how the Spk facilitates apical growth. One of the most popular models is known as the “vesicle supply center” (Bartnicki-Garcìa *et al*.,1989; Riquelme *et al*.,1998). This model explains hyphal tip expansion via delivery of exocytic vesicles to the

growing apical region. The model does assume that radial distribution of vesicles is significant enough to form the shape of the tip. Another model for apical cell wall growth is the unitary model. This model introduces the idea that cell wall construction during hyphal tip extension requires a delicate balance of lytic and secreted synthesis enzymes (Adriana *et al*., 2019). Finally, the integrated model has a hypothesis of chitinases and glucanases facilitating hydrolysis of chitin and glucan; respectively. Once hydrolyzed, the polysaccharides possess a free terminus which functions as a substrate for cross-linking enzymes (Adriana *et al*., 2019). This cross-linking is ultimately needed to harden the cell wall in order form the mature cell wall. The main difference between the unitary model and integrated model is the location of rigidification and maturing of the cell wall. In the integrated model crosslinking that enables rigidification occurs behind the extension zone, which is contrary to the unitary model that has crosslinking and rigidification at the apex. The Spk can vary in size, shape, position and behavior even within the same genus. Transmission electron microscopy (TEM) reveals the Spk is composed of two classes of secretory vesicles: microvesicles (30 to 40 nm in diameter) and macrovesicles (70 to 100 nm in diameter). The macrovesicles are typically found on the periphery of the Spk while microvesicles are found within the central region *(i.e*., core) of the Spk. In fact, most of the core contains significantly less vesicles and thereby yielding a lower refractive index making it appear brighter than the periphery when viewed with phase-contrast light microscopy (Roberson *et al*., 2010) (Fig. 1).

II.a.ii. The Apical Vesicle Crescent

Structural characteristics are dramatically different in most members of the Mucoromycota and other early fungal lineages studied to date. One of the defining structural characteristics of this phylum is an apical vesicle crescent (AVC) (Fig. 1). The AVC is characterized as a thin band of closely packed secretory vesicles in the shape of a crescent. It's located just underneath the plasma membrane of growing hypha and is believed to be functionally analogous to the Spk. Although, the AVC is not as structurally complex as the Spk, it is required for optimal growth. In addition, the AVC is dynamic and frequently shifts positions as the hypha changes directions and

expands. Secretory vesicles are also divided into two groups depending on their diameter: macrovesicles 130 nm and smaller microvesicles 65 nm (Fisher and Roberson, 2016)

II.b. Septations and Spindle Pole Bodies

II.b.i. Septa (*i.e.*, cellular cross walls) divide hyphae into separate cellular compartments and regularly occur in the Ascomycota and Basidiomycota. These cross walls contain a central pore that vary in structural complexity and are used as a phylogenetic character. The Ascomycota contain a simple septal pore, while the Basidiomycota have more complex septal pores called dolipores. Ultimately, septal pores have a dual responsible of compartmentalization of cytoplasmic materials between cells as well as cytoplasmic continuity between. The number of nuclei in each compartment may vary depending on the type of fungus. Basidiomycota typically contain two nuclei of different mating types (*i.e.*, '+'/'- ', n+n, the dikaryotic state). Compared to the Ascomycota, which can have multiple nuclei (*i.e.*, coenocytic) in one compartment all of the same mating type, though an individual mycelium my contain all '+' mating types while another mycelium may have the '-' mating type (*i.e.*, heterokaryotic). This is due to the simple septal pore enabling nuclei to move freely. However, sometimes septal pores can become plugged with Woronin bodies. Woronin bodies are electron dense organelles that have a range of 150 to 500nm. The shape can vary from oblong, rectangular, spherical or even hexagonal. The Mucoromycota have large, coenocytic cells and rarely produce septa.

II.b.ii. A forth sub-cellular character is the spindle pole body (*i.e.*, microtubular organizing center). Spindle pole bodies of terrestrial fungi are typically globular in nature. In contrast, aquatic fungi have centriole-like spindle pole bodies. Interestingly, some Zoopagomycota species which are terrestrial fungi, have centriole-like spindle pole bodies. The presence of the centriole-like spindle pole bodies in terrestrial fungi suggest a phylogenetic linage to aquatic organisms. Hence, the hypothesis that this phylum was one of the first to transition from aquatic to terrestrial life-style.

III. Polarized Growth in Fungal Hyphae

Polarized growth in fungi is a complex process involving cell wall and plasma membrane biosynthesis, polarized vesicle transport, constitutive exocytosis and endocytosis, turgor pressure, organelle motility and positioning, and cytoplasmic migration. These actions give rise to a tubular morphology of the cell. Three aspects critical to sustain polarized growth of hyphae are the secretory pathway, the cytoskeleton, and cell wall synthesis; all are briefly addressed below.

III.a. The Secretory Pathway

At the heart of hyphal tip growth is the outward secretory pathway that starts with the synthesis of proteins at the rough endoplasmic reticulum (ER) and from there they travel to the Golgi equivalent and finally to the cell surface via the transport of vesicles. Vesicles are small, spherical cytoplasmic inclusions bound by a single unit membrane with a cargo of soluble and transmembrane proteins. Vesicles are delivered to the apical surface via intracellular transport along microtubules where they join at the Spk. From there, these vesicles are delivered to the plasma membrane via actin microfilaments where they dock and fuse (*i.e.*, exocytosis). Exocytosis is regulated by the Arp2/3 complex and an exocyst complex (Machesky *et al.,*1999). An additional group of apical proteins called the polarisome, contains the essential Cdc42 and Rho GTPase that are responsible for recruitment of actin and other components required for polarized cell growth. This, process involves the secretory pathway, cytoskeleton function, and the activities of multimeric protein complexes that establish and maintain polarity and providing the needed biomolecules for plasma membrane extension and cell wall synthesis for sustained growth.

III.b. The Cytoskeleton

Cells regulate their morphology, organize and support cytoplasmic components, interact with the environment, and control their movements and intracellular transport. These functions are carried out through coordinated actions of the cytoskeleton. The fungal cytoskeleton is composed primarily of two structural elements, the microtubules (MTs) and actin microfilaments (MFs). Cytoplasmic intermediate filaments, common in animal cells, are scarce in the fungi. Ultimately, these elements extend throughout the cytoplasm and make connections with themselves and other cellular elements to form a continuous interconnected cytoplasmic system. Each cytoskeletal element has discrete mechanical and dynamic characteristics and performs specific and solitary or shared duties. Cytoskeletal function and behavior are direct results of the inherent characteristics of their proteins and the actions of hundreds of associated proteins. These proteins interact with the cytoskeleton and cytoplasmic components in highly regulated and precise ways.

III.b.i. Actin

Actin is highly conserved amongst plants, mammals, insects, and fungi. In most eukaryotic cells, actin is the most abundant cytoplasmic protein and is present in all cell types, muscular and non-muscular cells. Actin is referred to globular (G) actin or filamentous (F) acitn. Globular actin is the free monomeric subunit that has a with a molecular weight of 43KDa. These subunits, like α/β tubulin heterodimers, can undergo regulated polymerization into filament polymers (i.e., F-actin). Filamentous actin is composed of two protofilaments for a helical arrangement. Filamentous actin can be of variable lengths, though generally smaller than MTs, with a diameter of 7 nm.

In fungi, actin plays an important role endocytosis, exocytosis, and septum formation. These cellular processes are carried out by three higher order actin configurations (*i.e.*, rings, patches, cables; Berepiki *et al*., 2011). Every configuration has different localization patterns, mechanisms of assembly and cellular functions. Actin rings, also known as contractile actomyosin rings (CAR), are vital components for cytokinetic machinery in filamentous fungi. These rings are

composed of actin, myosin 2 and other affiliated proteins (Berepiki *et al*., 2011). Contractile actomyosin rings connects to the plasma membrane and generates contractile force via myosin II used during septal formation. Actin patches mediate endocytosis and are associated in a complex with over 60 proteins (Berepiki *et al*., 2011). Actin patches and associated proteins coat newly formed endocytic vesicles and are involved in their formation. Actin cables linked by fimbrin/tropomyosin function as transportation tracks in fungi. The cargo that is transported among actin cables varies from peroxisomes, mitochondria and secretory vesicles. Traditionally, actin cable dynamics are hard to visualize. However, recent advances with molecular markers has made visualization of actin cables possible (Heath 1990: Mouriño-Pérez and Roberson, 2015).

III.b.ii. Microtubules

In filamentous fungi, microtubules (MTs) are organized in axial arrays and are most abundant in the cortical region of the cytoplasm. Microtubules are composed of α/β tubulin heterodimer subunits that polymerize to generate 13 protofilaments arranged in a parallel bundle that organize into tube-shaped structure of variable lengths and with a diameter of 25 nm. These macromolecular structures play several roles and facilitate essential functions such as intracellular transportation, sorting of organelles as well as spatial organization in the cytoplasm. According to Horio (2007)**,** MTs are necessary for rapid polarized growth by their long-distance transport of secretory vesicles to the hyphal apex.

III.c. The Cell Wall

The cell walls of fungi are not static structures; indeed, they are dynamic and essential for cell viability, morphogenesis, and pathogenesis. Some refer to the cell wall as a dynamic ‘organelle’ with a makeup that is regulated in response to environmental conditions and cellular stresses (Hopke *et al.,* 2018). Fungal walls are architecturally similar: fibrous and gel-like carbohydrate polymers form a tensile and robust core upon which various other proteins and components are

added that together make strong, but flexible, and chemically diverse cell walls. Cell walls are mostly composed of two cell wall layers: an inner layer and outer layer. The inner wall of most

fungi is made up of a core of covalently attached branched β-(1,3) glucan and chitin that together form intra-chain hydrogen bonds and can assemble into fibrous microfibrils. These microfibrils form a basket-like scaffold around the cell (Souza *et al*.,2017). This wall component represents the load bearing, structural component that resists the substantial internal hydrostatic pressure exerted on the wall by the cytoplasm and plasma membrane. Relatively, the inner layer has more conserved structural components. In contrast, to the outer layer which is more heterogeneous in composition and constancy. In filamentous fungi, chitin synthase and glucan synthase are critically important cell wall synthesizing integral membrane proteins that are delivered to the apical plasma membrane via secretory pathways.

III.c.i. Chitin Synthase

In *Neurospora crassa* chitin synthase 3 (CHS-3) and chitin synthase 6 (CHS-6) were found two have different distributions and morphology. Hyphae were labeled with CHS-3-GFP and CHS-6-GFP. However, there was some very interesting similarities in distributions of the two chitin synthases. Fluorescence microscopy reveals that both CHS-3 and CHS-6 form a round aggregate near the hyphal apex (Riquelme et al., 2007). Phase-contrast microscopy reveals that this location corresponds to the Spk. When the hyphae were labeled with FM4-64 the round apical structure appeared larger (Riquelme *et al.,* 2007). This suggest that CHS-3/CHS-6 is incorporated into the internal core of the Spk. In addition, both chitin synthases were visualized in the formation of the septum. However, CHS-6 did not persist longer than 20 minutes post septum formation (Riquelme *et al.,* 2007). The role of chitin synthase enzymes can be different in the Mucoromycota. In fact, it was found that this phylum has four of the seven chitin synthases observed outside of dikarya.

.

III.c.ii. Glucan Synthase

In order to achieve polarized growth, it's mandatory that filamentous fungi have continuous synthesis of cell wall polymers at their apical domain. A major polysaccharide of the fungal cell wall is β-1-3-glucan. In *Neurospora crassa*, β-1-3-glucan comprises 80% of the fungal cell wall mass (Sánchez-Leon and Riquelme, 2015). Biosynthesis of β-1-3-glucan is generated at a complex known as the β-1-3-glucan synthase complex. This complex includes one or more Fks catalytic subunits and at least one Rho regulatory subunit. An additional requirement for β-1-3-glucan synthesis is a protein called GS-1. How GS-1 works in conjunction with β-1-3-glucan synthase complex is not well understood. A recent discovery in *Rhizopus oryzae* was a high proportion of is β-1-3 linked to polysaccharides containing fructose (Sánchez-Leon and Riquelme, 2015). This finding correlates to a gene affiliated with fructose metabolism in the Mucoromycota.

IV. Polarized Cell Growth in Plant Pollen Tubes

There are several examples of polarized growth in plants cells. Polarized growth is well documented in plant root hairs, algal rhizoids, and pollen tubes. This uniaxial expansion enables root hairs and algal rhizoids cells to explore its local environment for nutrients and water. However, pollen tubes differ because their sole function is reproduction. Plant pollen tubes, like fungal hyphae, contain a cell wall and must be synthesized at the apex to sustain polarized growth (Hepler *et al.,* 2001). The cell wall must be pliable enough to enable cell growth yet rigid enough to sustain internal turgor pressure (Hepler *et al.,* 2001). As in fungal hyphae, microtubules and actin compose the cytoskeletal array that is needed for long-distance and short distance intracellular transport. All organelles are transported with these arrays but at various velocities (Chebli *et al.,* 2013). In plant pollen tubes the actin cytoskeletal system is organized into cortical and central bundles (Chebli *et al.,* 2013). These bundles are oriented parallel to a dense cortical network in the subapical region. In angiosperm plant pollen tubes, organelles move retrograde on this centrally positioned array. Contrary, to the cortical array which moves organelles

anterograde. It is a combination of all these movements which ultimately create RFCS (Chebli *et al.,* 2013). This type of streaming is the opposite of fountain cytoplasmic streaming which typically seen in gymnosperm plant pollen tubes. Fountain cytoplasmic streaming can be described as organelles moving towards the apical dome via central array and away from the apex along cell cortex (de Win *et al.,*1996). The difference in cytoplasmic streaming is believed to be due to the variance in cytoskeletal organization (Chebli *et al*.,2013). The actin polarities and orientation are believed to be barbed end facing toward apical dome (central array) and barbed end facing subapical region (cortical array). These actin orientations are the opposite in angiosperm plant pollen tubes.

V. Overlap Between Plants and Fungi

Both plant pollen tubes and fungal hyphae illustrate a high degree of polarity zonation. For example, there is a clear zone at the extreme apex. This means that large organelles are positioned several microns from the apex. This cytoplasmic organization is linked to a cyclic motion of organelles and cytoplasmic bulk flow. Both pollen tubes and Mucorales hyphae studied to date exhibit cytoplasmic regions that move organelles anterograde along the cortex of the cell. Simultaneously, there is retrograde cytoplasmic flow that moves organelles down the center of the cell. An important note is that these streaming 'lanes' do not stretch to the extreme apex of the cell but reverse within the clear zone.

The tip growth of plant pollen tubes is comparable to fungal hyphae. Both have highly restricted growth (predominately within 5 µm of the apex) and contain separate but complementary processes. These processes entail: 1) vectoral transport of vesicles mostly generated from the Golgi apparatus, 2) localized vesicle exocytosis in appropriate gradient, 3) movement of the cytoplasm, corresponding to the lateral walls of the hyphae thereby leaving sub-apical regions mostly composed of vacuoles, 4) migration of organelles such that they maintain characteristic distribution with respect to the advancing tip during and after their replication (Heath, 1990). All these processes are synchronized together, however cytoplasmic migration and organelle

movement can be independent to some extent. This enables, vacuole distribution and abnormal organelles can be elicited via disruption of microtubules.

VII. Hypothesis, Goals & Objectives

The hypothesis of this research is that cellular structure and behavior are robust characters in elucidating fungal phylogeny and evolution. Many fungi belonging to the Mucoromycota, specifically within the Mucorales, possess a unique cytoplasmic behavior known as RFCS, a behavior previously only documented in angiosperm plant pollen tubes. Fungi within the phyla of the Basidiomycota and Ascomycota exhibit bulk flow and saltatory cytoplasmic behavior. Current experimental data illustrates RFCS in several of the Mucorales species. These fungi have an aggregation of secretory vesicles at the extreme apex of growing hyphal cells termed the AVC. In contrast, a vesicle aggregation known as the Spk is unique to higher order fungi (*i.e*., Ascomycota and Basidiomycota). Therefore, it is a strong possibility that the AVC is linked in unresolved ways to RFCS. However, this claim is not supported in angiosperm pollen tubes that exhibit RFCS but have a cone-shaped vesicle accumulation at the apical region and not an AVC (Lancelle and Hepler, 1992; Bove et al., 2008). The goals of this research are to describe RFCS in selected members of the Mucorales to better understand the significance of RFCS (*i.e*., do fungi with AVC have an evolutionary advantage over fungi with a Spk or vise-versa?).

II. Materials and Method

I. Organisms

Rhizopus oryzae (NRRL 43880), *Gilbertella persicaria (*NRRL 2700), and *Cunninghamella echinulate* (NRRL 1382) were maintained on potato dextrose agar (PDA, Sigma-Aldrich, St. Louis, MO) at room temperature (25 Cº).

II. Light Microscopy

For low magnification imaging of mycelia, Petri dishes containing 24-hour old cultures of *Rhizopus oryzae* were visualized using a Nikon SMZ-2T stereo scope (Nikon Instruments Inc., Melville, New York) coupled to an Olympus DP72 camera and imaged with Olympus CellSens software (Olympus, Tokyo, Japan).

Culture slides were prepared by applying either a thin layer of potato dextrose broth and 15% gelatin or VMM broth and 15% gelatin over one surface of sterilized microscope slides (FIG. 2). Hyphae were inoculated onto the surface of the coated slides and the samples grown for 24 h in a moist chamber at 23 C. After placing coverslips (No. 1.5) over the colony's leading edge, hyphae were imaged using an Axioskop light microscope (Carl Zeiss Inc., Thornwood, New Jersey) equipped with differential interference contrast (DIC) and phase contrast (PC) optics using plan-neofluar 100x/1.3 NA (oil immersion) objective and 0.9 NA or 1.4 NA (oil immersion) condenser lens. The microscope was coupled to a Hamamatsu Orca Flash 4.0 digital camera (Hamamatsu Photonics, Hamamatsu City, Japan) and images captured using HCImage Live (Hamamatsu Photonics, Japan).

Hyphae of all organisms were also imaged using wide field and confocal fluorescence optics. Hyphae growing on culture slides were stained with N-(3-triethylammoniumpropyl)-4-(6-(4-(diethyl amino) phenyl) hexatrienyl) pyridinium dibromide (FM4-64; Thermo Fisher Scientific, Waltham, MA). Cells were stained in 50 µM FM4-64 dissolved in PD broth for a period of 40 to 60 minutes

before imaging. This rather long period of dye incubation led to FM4-64 being incorporate into most membranes throughout the cell providing a means to visualize organelle dynamics, particularly mitochondria and Golgi equivalents. Cells were imaged in wide-field fluorescent mode using an Axioskop compound light microscope (Carl Zeiss Inc.) equipped with a mercury vapor bulb (50 watts) as the source of FM4-64 excitation using appropriate excitation and emission filters and dichromatic mirror. Stained cells were also imaged using a Nikon Ti E scanning disk confocal microscope (Nikon Inc., Melville, NY)

III. Data Processing and Analysis

All data were processed and analyzed using ImageJ (NIH, Bethesda, Maryland) and Adobe Photoshop 8.0 (Adobe Systems Inc., San Jose, California).

III. Results

Hyphae of fungi within the Mucorales examined here have a relatively simple cytoplasmic organization composed to two regions (Fig. 1). Region 1 is defined as the apical dome composed primarily of the AVC. This region begins at the tip of the cells and typically extends back two to three micrometers. This limited area is structurally simple. However, it is the highly active site of processes that are fundamental to hyphal growth and morphogenesis (*e.g.*, vesicle motility, exocytosis, cytoskeletal dynamics, cell wall biosynthesis) and exhibits dynamic flow of organelles characteristic of RFCS. Below the apical dome is region II composed of cytoplasmic components common eukaryotic inclusions and organelles including nuclei, cisternae and vacuoles, rough and smooth ER, mitochondria, cytoskeletal elements, vesicles, ribosomes, and Golgi equivalents. Region II appears to be uniformly organized for a long distance, an increase of vacuoles occurs the most distal regions only. Members of the Mucorales are coenocytic and mostly aseptate (*i.e.*, lacking regular occurring cellular cross walls), which may explain the extended length of region II. Members of the Ascomycota and Basidiomycota have regularly occurring septations with cellular compartments containing either two nuclei of the opposite mating type (*i.e.*, heterokaryotic, Basidiomycota) or multiple nuclei of the same mating type (*i.e*., homokaryotic, Ascomycota) (data not shown). The cytoplasm of these hyphae (Fig. 1 illustrating Ascomycota hyphae) are divided into three regions: region I containing Spk with no other organelles present except mitochondria. These mitochondria either remain stationary, just posterior to the Spk, or briefly move anteriorly into the region I and then back out into the subtending region. Region II is the subapical zone subtending the apical dome and in optimally growing hyphae can extend between 15 to 25 micrometers subapically. Region III extends subapically behind region II until the first septum appears. The primary feature that differentiates regions II from III in septate and aseptate fungi is the presence of nuclei in region III.

Mature hyphae of *R. oryzae.* were approximately 7 µm (N = **, SD = 0.** µm) in diameter, with no hyphal septations, and exhibited a growth rate of .33µm/min (N = 25, SD = .12 µm/min) (Figs. 3, 4). Growing hyphae maintained an AVC within the apical dome subtending the plasma membrane that was clearly visible with phase contrast microscopy (Fig. 4). *Rhizopus oryzae* control growth under the light microscope yielded consistent patterns of RFCS identified as an anterograde cytosolic/organelle movement along the cortex of the hyphal cell into hyphal region I, and then reversing direction to travel in a retrograde direction down the center of the cell through hyphal region II (Figs. 5-7). Discrete mitochondria and other organelles (*e.g.,* multivesicular bodies) provided useful points of motility references (Figs. 5-7). The currents of cytoplasmic flow were also observed with the orientation of organelles, primarily mitochondria, around the apex. For example, in figure 7, a mitochondrion is shown with one end near the apex and undergoing a morphological change such as bending subapically in the direction of retrograde flow. The velocity of these organelles was variable depending on size. Interestingly, the anterograde and retrograde mitochondrial velocities were variable. Anterograde velocity was 0.8 µm/s (N = 50, SD = .66 µm), retrograde motility was slower averaging 0.6 µm/s (N = 50, SD = .61µm).

FM4-64 is an amphipathic molecule with a polar head and hydrophobic tail. This dye binds within the hydrophobic regions of the plasma membrane which enables the fluorescent characteristic. It is used primarily as a fluorescent marker for live cell studies of endocytic pathways. The application of FM4-64 in this study was to produce general florescent staining of organelle outer membranes. This was accomplished by ‘over staining’ cells with higher concentrations of the dye than typically used and for longer incubation periods. This resulted in FM4-64 diffusing through the plasma membrane and into the cytosol where it labeled most organelle membranes. Interestingly, the nuclear and endoplasmic reticulum membranes were not labeled. The higher doses and longer incubation times of FM4-64 used in this study showed no adverse effect on living fungal cell growth and cytoplasmic behavior. The use of FM4-64 in

both wide-field and confocal fluorescence microscopy, demonstrated the confocal to be superior method for visualization of RFCS the Mucorales fungal hyphae (Figs. 8-12). As with phase contrast microscopy, a variety of organelles were clearly noted traveling anterograde along the cortex until they reach the apical region and then turn around and travel retrograde down the center array of the cell. Mitochondria are the most common and easily recognized organelles for illustrating (Fig. 8). However, other organelles such as multivesicular bodies (Fig. 9) and Golgi equivalents (Fig.10) and were also observed undergoing RFSC. Images recorded with the wide-field fluorescent microscope were performed before phototoxic effects were detectable.

I also studied two additional additional members of the Mucorales after staining with FM4-64, *Cunninghamella echinulata* (Fig. 11) and *Gilbertella persicaria* (Fig. 12). Both mucoralean fungi were imaged using spinning disc confocal microscopy. The primary advantage of this imaging modality is reduced phototoxicity, allowing longer time periods of imaging. The disadvantage of the confocal microcoscope, is seen in (Fig.12) where a mitochondrion undergoing a 'tumbling' behavior resulted in its appearance as a spherical organelle and not the true thread-like structure. Both *C. echinulata* and *G. persicaria* clearly exhibited RFCS. It is notable that one of the most important advantages of using FM4-64 was its ability to stain, and thus allowing detection of structures that lacked the ability to create adequate phase shifts of light required for visualization with phase contrast light microscopy (*e.g.*, Golgi equivalents).

IV. Discussion

The present study has shown that fungi within the order Mucorales exhibit a form of hyphal cytoplasmic dynamics that, up to this time, has never been observed in fungal hyphae. This cytoplasmic behavior is referred to as reverse fountain cytoplasmic streaming (RFCS). The RFCS form of cytoplasmic dynamics has previously been observed only in growing angiosperm pollen tubes (Lancelle and Hepler, 1992; Bove et al., 2008; Chebli *et al.,* 2013). Thus, RFCS represents a new characteristic for phylogenetic consideration of fungi. Traditionally, fungal phylogenies were performed by chemical, morphological, and anatomical characteristics (Zhao *et al.,* 2015). In the 1960's biochemical pathways, amino acid biosynthesis, and cell wall chemistry were used to clarify the evolutionary relationships of fungi. Over more recent decades additional modalities of studying fungal phylogenies have been developed including, genetic sequencing and advanced bioimaging (McLaughlin et al., 2015; Spatafora et al., 2017). Much of our knowledge of subcellular hyphal structure has come from advances in cell fixation for TEM using cryomethods (Howard and Aist, 1979; Hoch and Howard, 1980; Howard, 1981; Roberson and Fuller, 1988; Vargas et al., 1993; Roberson and Chandler, 1998) and coupling this with the application of electron tomography for high-resolution, three-dimensional analysis of cytoplasmic structure and organization (Müller et al., 2000; Hohmann-Marriott et al., 2006). By the mid-1980s, the advancement in ultrastructural studies was supported greatly by the development of new tools for light microscopy and live-cell imaging. Using high-resolution cameras, computer technology, and fluorescently conjugated molecular probes, the potential for studying many structural components in space and time became possible (Freitag et al., 2004; Mouriño-Pérez et al., 2006; Uchida et al., 2008; Mouriño-Pérez and Roberson, 2015).

Live cell light microscopy imaging using traditional phase contrast and fluorescence microscopy (i.e., wide-field, spinning disc confocal) were deemed the most suitable methods for achieving the

objective of this study. Of these two methods, fluorescence microscopy observations using the FM4-64 fluorophore to label cytoplasmic organelles was clearly superior to phase contrast optics.

FM4-64 is an amphiphilic dye used to investigate endocytosis and vesicle trafficking in living eukaryotic cells. It is thought the dye inserts into the outer leaflet of the plasma membrane and via endocytosis, is passed on to intracellular membrane compartments. It is important to note, however, that the molecular interactions of FM4–64 with phospholipid membrane leaflets are not well understood (Rigal *et al*., 2015; van Gisbergen *et al*., 2008). For the purpose of this study cells were overloaded with FM4-64 by using higher than suggested concentrations of the dye and by increased incubation time. This resulted in the labelling of many endomembranes, including organelle membranes, via the typical endocytosis at the plasma membrane and through the diffusion of the dye through the plasma membrane and into the cytosol. The organelles that were labelled included mitochondria, Golgi equivalents, and endosomal membranes (i.e., multivesicular bodies). It is important to note that that the over staining of FM4-64 did not manifest as evidence of cell stress. Use of phase contrast images was primarily useful for the visualization of mitochondrial movements, which directly corresponded to the FM4-64 images. The combination of both phase contrast and fluorescence microscopy where invaluable in illustrating the myriad of cytoplasmic dynamics in living fungal cells. Cytoplasmic streaming, or cyclosis, represents vigorous intracellular organelle and cytosolic movement and is characteristic of many plant pollen tubes. This cytoplasmic behavior is caused by a combination of cytoskeletal action (i.e., actin/myosin) and bulk cytoplasmic flow. (Kroeger and Geitmann, 2011; Chebli *et al.,* 2013). Cyclosis allows for the (1) transport of nutrients and metabolites between different locations of the cell, (2) the positioning of organelles during steady state growth and in response environmental conditions that effect growth, and (3) the generation of continuous cytoplasmic mixing (i.e., convention), which enhances the occurrences of chemical and enzymatic reactions (Chebli *et al.,* 2013). There are two forms cyclosis observed in pollen tubes. One is RFCS that is characteristic of angiosperm pollen tubes. Here, organelles and cytosolic materials move in an anterograde fashion along the cellular cortex and retrograde through the cell's central region. The second is referred to as the fountain cytoplasmic streaming (FCS) found in gymnosperm pollen tubes in which a fountain-like streaming pattern is observed by the anterograde movement along the central axis and a retrograde movement down the cell's cortex (Lazzaro, 1996; Fernando *et al*.,

2005). As mentioned above, like angiosperm pollen tubes, fungal hyphae within the Mucorales exhibit RFCS. Interestingly, members of the Ascomycota, Basidiomycota, Blastocladiomycota, and some members of the Zoopagomycota that have been examined do not exhibit this streaming behavior (Girbardt, 1957; Roberson *et al*., 1989; Vargas et al., 1993; Lopez-Franco and Bracker, 1996; McDaniel and Roberson, 2000; Riquelme *et al.,* 2002; Ramos-Garcia *et al.,* 2009; Roberson et al., 2011; Fisher *et al.,* 2018; Roberson unpublished observations). Rather, fungal hyphae in these phyla (*e.g*., *Neurospora crassa* (Ascomycota, Shange, unpublished observations; *Conidiobous coronatus* (Entomophthorales; Fisher et al., 2018) display cytoplasmic behavior as unidirectional bulk flow that lack rhythmic reversals. Live cell imaging of mitochondrial and nuclear motility show that these organelles often move via bulk flow (i.e., moving at the same rate as the expand tip) (Ramos-Garcia *et al*., 2009; Fisher *et al*., 2018). This suggests that these organelles are tethered by cytoskeletal elements and are moving via bulk flow. However, this is not to say that individual organelles and vesicles exhibit periodic intracellular saltatory anterograde and retrograde motility independent of and faster than bulk flow. Indeed, saltatory motility is a common feature in hyphae (McKerracher and Heath, 1987) and in eukaryotic cells. These rapid movements suggest that organelles are independent of bulk flow and are propelled by molecular motors moving on cytoskeletal elements (i.e., actin microfilaments, microtubules).

Cytoplasmic order is also different in those fungi that exhibit RFCS verses unidirectional bulk flow. One of the differences in cytoplasmic order is the compartmentalization of hyphae via septal cross walls. Dikaryotic fungi have regularly occurring septal cross walls. In comparison to the Mucorales, hyphal septations are scant. The biggest difference in cytoplasmic order is seen at the extreme hyphal apex and the nearby subapical regions. In dikaryotic fungi and limited members of Zoopagomycota (*i.e., C. coronatus*), can be partitioned into several regions based on cytoplasmic order. The apical dome, or region I (McDaniel and Roberson, 2000; Fisher *et al*., 2018) begins at the tip of the cell and typically extends back two to three micrometers. This limited area is structurally simple; however, it is the highly active site of processes that are

fundamental to hyphal growth and morphogenesis (*e.g.,* vesicle motility, exocytosis, cytoskeletal dynamics, cell wall biosynthesis). Mitochondria, ribosomes, Worrin bodies (Ascomycota) and

occasionally smooth endoplasmic reticulum are all found in region I. As mentioned above, the primary structure at the apex of growing hyphae in the Ascomycota, Basidiomycota is the Spk. A structural equivalent of the Spk is not common or has not been observed in hyphae of the zygosporic fungi (e.g., Mucoromycota), or the other relatively early diverging lineages. As a notable exception to this statement, Spk have been identified in hyphae of *Allomyces macrogynus* (Blastocladiomycota) (Vargas et al., 1993), *C. coronatus* (Zoopagomycota)(Fisher *et al.*, 2018) and *Basidiobolus* sp. (Zoopagomycota)(Roberson *et al*., 2011). Below the apical dome, the hyphal cytoplasm contains common eukaryotic inclusions and organelles including nuclei, cisternae and vacuoles, rough and smooth ER, mitochondria, cytoskeletal elements, vesicles, ribosomes, and Golgi equivalents. In the septate fungi, typically two subapical regions are recognized, regions II and III. Region II is the subapical zone just behind the apical dome and in optimally growing hyphae, it can extend between 15 to 25 micrometers (Riquelme et al., 2002). In hyphae growing at less than optimal rates the size of region II is reduced. Region III extends subapically behind region II until the first septum appears. The primary feature that differentiates regions II from III in both septate and aseptate fungi is the presence of nuclei, region II being devoid of nuclei. In the Mucoromycota, the hyphal cytoplasm is can be divided into two regions. Like the dikarya, region I begins at the tip of the cells and typically extends back two to three micrometers. However, instead of a Spk, these fungi contain an AVC - a thin crescent-shaped band of closely packed vesicles is present just beneath the apical plasma membrane. Region I, exhibits dynamic flow of organelles characteristic of RFCS. Region II(below region I) contains a heterogeneous composition of organelles and continues subapically becoming highly vacuolate in older regions of the cell.

Determining evolutionary advantages between RFCS /unidirectional bulk flow, the presence of an AVC/ Spk, and two hyphal regions versus three hyphal regions is difficult. However, there are clear correlations such as hyphae with an AVC exhibit RFCS, contain two cytoplasmic zones, and are typically aseptate. Those containing a Spk exhibit unidirectional bulk flow, contain three

cytoplasmic zones, and are generally regularly septated. Is the presence of the Spk and unidirectional bulk flow superior to the AVC and RFCS? It seems not, as all of these fungi are

ecologically successful, their hyphal growth rates are comparable. In addition, all have members that are obligate or facultative saprophytes, endophytes, or are obligate or facultative parasites, to plants and animals. Clearly, the Spk is not unique to the dikarya with fungi such as *Allomyces* (Vargas *et at.,* 1993)*, Basidiobolus* (Roberson *et al.,* 2011) and *Conidiobolus* (Fisher et al., 2017) all possessing an Spk. Although, they are structurally and at least in the case of *Allomyces;* functionally diverse. Did these organisms evolve a single form of cytoplasmic behavior and a primitive form of the Spk that diversified over the millennia? Or did the Spk, the AVC, and cytoplasmic behaviors evolve independently throughout the history of fungal evolution? One thing that is clear, the Spk is more widespread among the Mycota than the AVC. This suggests that the Spk has an evolutionary advantage over the AVC. However, the AVC is less complex than the Spk and contains fewer secretory vesicles than the Spk. This would indicate that hyphae with AVC would be less competitive than those with Spk. However, this does not seem to be the case. It is possible that the evolutionary coupling of RFCS with the presence of an AVC provided a synergistic advantage for the mucoralean fungi.

References

Alexopoulos C, Mims C, Blackwell M. 1996. *Introductory mycology*. New York: Wiley. pp:7-45

Baldauf S, Palmer J. 1993. Animals and fungi are each other's closest relatives: congruent evidence from multiple proteins. Proc.Natl. Acad. Sci.USA **90**: 11558-11562

Bartnicki-Garcia S, Hergert F, Gierz G. 1989. Computer simulation of fungal morphogenesis and the mathematical basis for hyphal (tip) growth. Protoplasma. **153**: 46-57

Beadle G, Tatum E.1941. Genetic Control of Biochemical Reactions in Neurospora. Proc. Natl. Acad. Sci. USA. **27**: 499-506

Berepiki A, Lichius A, Read N. 2011. Actin organization and dynamics in filamentous fungi. Nature reviews microbiology.**9**:876-887

Bove J, Vaillancourt B, Kroeger J, Hepler P, Wiseman W, Geitmann A. 2008. Magnitude and direction of vesicle dynamics in growing pollen tubes using spatiotemporal image correlation spectroscopy and fluorescence recovery after photobleaching. Plant Physiology. **147**, 1646–1658.

Carlile M, Watkinson S, Gooday G. 1994. The Fungi. Academic Press. pp: 38-79

Chebli Y, Kroeger J, Geitmann A. 2015. Transport Logistics in Pollen Tubes. Molecular Plant. **6**:1037-1052

Cheung A, Wu H.2008. Structural and Signaling Networks for the Polar Cell Growth Machinery in Pollen Tubes. Annul Review of Plant Biology. **59**: 547-572

Fisher K, Romberger I, Lowry D, Shange P, Roberson RW. 2018. Subcellular characters of the zygomycetous fungus, Conidiobolus coronatus (Entomophthorales). Mycologia.**110**: 31-3877

Fragoso de Souza C, Lima D, Gurgel L, Santiago A. 2017. Coprophilous Mucorales (ex Zygomycota) from three areas in the semi-arid of Pernambuco. Brazilian J Microbiology. **48**:79-86

Fuchs F, Westermann B.2004.Role of Unc104/KIF1-related Motor Proteins in Mitochondrial Transport in *Neurospora crassa*. Molecular Biology of the cell.**16**:153-161

Fuchs F, Prokisch H, Neupert W, Westermann B.2002.Interaction of mitochondria with microtubules in the filamentous fungus Neurospora crassa. J Cell Sci 2002.**115**:1931-1937.

Girbardt M.1957. Über die Substruktur von Polystictus versicolor L. Archives of Microbiology. **28**: 255-269

Girbardt, M. 1957. Der Spitzenkörper von *Polystictus versicolor*. Planta **50**:47-59.

Heath I.1990. Tip Growth in Plant and Fungal Cells. Academic Press. pp: 119-171

Hepler P, Vidali L, Cheung Y. 1970. Polarized cell growth in higher plants. Ann. Rev. Cell and Developmental Biology. **17**:159-187

Hopke A, Brown A, Hall R, Wheeler R.2018.Dynamic fungal cell wall architecture in stress adaptation and immune evasion. Trends Microbiology.**26**(4): 284–295.

Horio T. 2007. Role of microtubules in tip growth of fungi. Journal of plant research. **120**: 53-60

Jaspersen S, Winey M. 2004. The budding yeast spindle pole body: Structure, duplication, and function. Ann.Rev.Cell and Developmental Biology. **20**:1-28

Lancelle S, Hepler P**.** 1992. Ultrastructure of freeze substituted pollen tubes of *Lilium longiflorum*. Protoplasma.**167**: 215–230

López-Franco R, Bracker C. 1996. Diversity and dynamics of the Spitzenkörper in growing hyphal tips of higher fungi*.* Protoplasma. **195**:90-111.

Machesky L, Gould K. 1999. The Arp2/3 complex: a multifunctional actin organizer. Current opinion in cell biology. **11**: 117-121

McDaniel D, Roberson R. 2000. Intracellular motility and mechanisms of control during hyphal tip growth in *Allomyces*. Fungal Genetics and Biology. **31**:223-234

McKerracher L, Heath I.1987. Cytoplasmic migration and intracellular organelle movements during tip growth of fungal hyphae. Experimental Mycology. **11** :79-100

McLaughlin D, Healy R, Celio G, Roberson R, Kumar A. 2015. Evolution of zygomycetous spindle pole bodies: evidence from *Coemansia reversa* mitosis. American Journal of Botany **102**:1-11

Mouriño-Pérez RR, Roberson R. 2015. Applications of confocal laser scanning microscopy in filamentous fungi. In: TES Dahms, K Czymmek (eds), Advanced Microscopy in Mycology. Springer International Publishing, Switzerland

Ramos-García S, Roberson R, Freitag M, Bartnicki-García S, Mouriño-Pérez R. 2009. Role of cytoplasmic bulk flow and microtubule-related motor proteins in nuclear displacement in growing hyphae of *Neurospora crassa*. Eucaryotic Cell. **8**:1880-1890.

Reynaga-Peña C, Gierz G, Bartnicki-Garcia S. 1997. Analysis of the role of the Spitzenkörper in fungal morphogenesis by computer simulation of apical branching in Aspergillus niger. Proc.Natl. Acad. Sci. USA. **94**: 9096-9101

Riquelme M, Roberson R, Sánchez-León E. 2016. 3 Hyphal Tip Growth in Filamentous Fungi. In Growth, Differentiation and Sexuality. The Mycota (A Comprehensive Treatise on Fungi as Experimental Systems for Basic and Applied Research). Wendland J. Springer. pp:47-66

Riquelme M, Reynaga-Peña C, Gierz G, Bartnicki-García S. 1998. What Determines Growth Direction in Fungal Hyphae? Fungal genetics and biology. **24**: 101-109

Riquelme M, Bartnicki-García S, González-Prieto J, Sánchez-León E, Verdín-Ramos J, Beltrán-Aguilar A, Freitag M. 2007.Spitzenkörper Localization and Intracellular Traffic of Green Fluorescent Protein-Labeled CHS-3 and CHS-6 Chitin synthases in Living Hyphae of *Neurospora crassa*. Eukaryotic Cell. **6**: 1853-1864

Riquelme M, McDaniel D, Roberson R, Bartnicki-García S, 2002. The effect of ropy-mutation on cytoplasmic organization in mature hyphae of *Neurospora crassa*. Fungal Genetics and Biology **37**:171-179.

Roberson R, Fuller M, Grebski C. 1989. Effects of the sterol biosynthesis inhibitor cyproconazole on hyphal tip cells of *Sclerotium rolfsii*. I A light microscopic study. Pesticide Biochemistry and Physiology **34**:130-142.

Roberson R, Abril M, Blackwell M, Letcher P, McLaughlin D, Mouriño-Pérez R, Riquelme M, Uchida M. 2010. Hyphal Structure, Chapter 2. In: *Cellular* and *Molecular Biology of Filamentous Fungi*. Borkovich K, Ebbole D (eds). ASM Press, Washington, D.C. pp. 8 - 27

Roberson R, Saucedo B, MacLean D, Propster J, Unger B, Oneil TA, Parvanehgohar K, Cavanaugh C, Lowry D. 2011. The hyphal tip structure of Basidiobolus sp.: a zygomycete fungus of uncertain phylogeny. Fungal Biology **115**: 485-492. Sánchez-León E, Riquelme M (2015) Live Imaging of β-1,3-Glucan Synthase FKS-1 in Neurospora Crassa Hyphae. Fungal Genetics and Biology. **82**: 104-107

Roberson R, Chandler D. 1998. Rapid freezing and deep etching of cells and molecules. In: *Cell Biology: A Laboratory Handbook*. Spector D, Goldman R, Leinwand,L (eds). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. (pp.129.1-129.23)

Spatafora J, Chang Y, Benny G, Lazarus K, Smith M, Berbee M, Bonito G, Corradi N, Grigoriev I, Gryganskyi A, James T, O'Donell K, Roberson R, Taylor T, Vigalys R, White M, Stajich J. 2017. A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. Mycologia. **108**:1028-1046

Spatafora J , Aime C,Igor V, Grigorie V, Martin F, Stajich J, Blackwell M. 2017. The Fungal Tree of Life: from Molecular Systematics to Genome-Scale Phylogenies. Microbiol Spectrum 5:FUNK-0053-2016

Vargas M, Aronson J, Roberson R. 1993. Cytological organization of hyphal tip cells of *Allomyces macrogynus*. Protoplasma **176**:43-52.

Verchot-Lubicz J, Goldstein R. 2009. Cytoplasmic streaming enables the distribution of molecules

and vesicles in large plant cells. Protoplasma. **240**:99–107

Verdín J, Sánchez-León E, Rico-Ramírez A, Martínez-Núñez L, Fajardo-Somera R, Riquelme M. 2019. Off the wall: The rhyme and reason of *Neurospora crassa* hyphal morphogenesis. The Cell Surface. **5**: 10020

Webster J.1970. Introduction to Fungi. Syndics of the Cambridge University Press. pp: 105-154

Zhao X, Zhang L, Zhao Z, Wang W, Leavitt S, Lumbsch H.2015. A Molecular Phylogeny of the Lichen Genus *Lecidella* Focusing on Species from Mainland China. PLos One **10**(9): e0139405.

Appendix A

Reverse fountain cytoplasmic streaminging

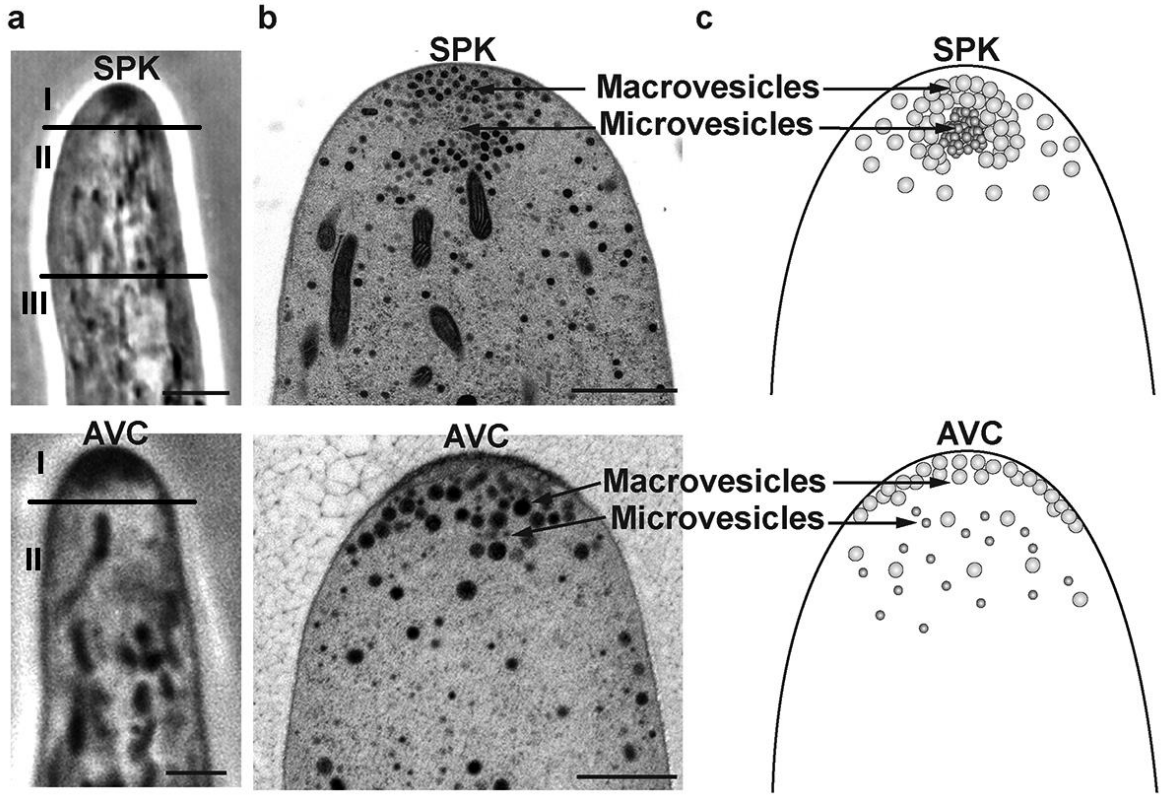

Figure 1. Hyphal dome organization in *Neurospora crassa* (Ascomycota, top panels) and *Mucor indicus* (Mucoromycota, bottom panels) illustrating typical hyphal regions (I, II, III). (A) Phase-contrast microscopy; Scale Bar: (top) 3µm; (bottom) 1 µm. (B) Transmission electron microscopy; scale bars: 1 µm. (C) apical vesicular organization models of fungal hyphae: the Spitzenkörper (Spk) and the apical vesicle crescent (AVC). (Modified from: Riquelme *et al*. 2016)

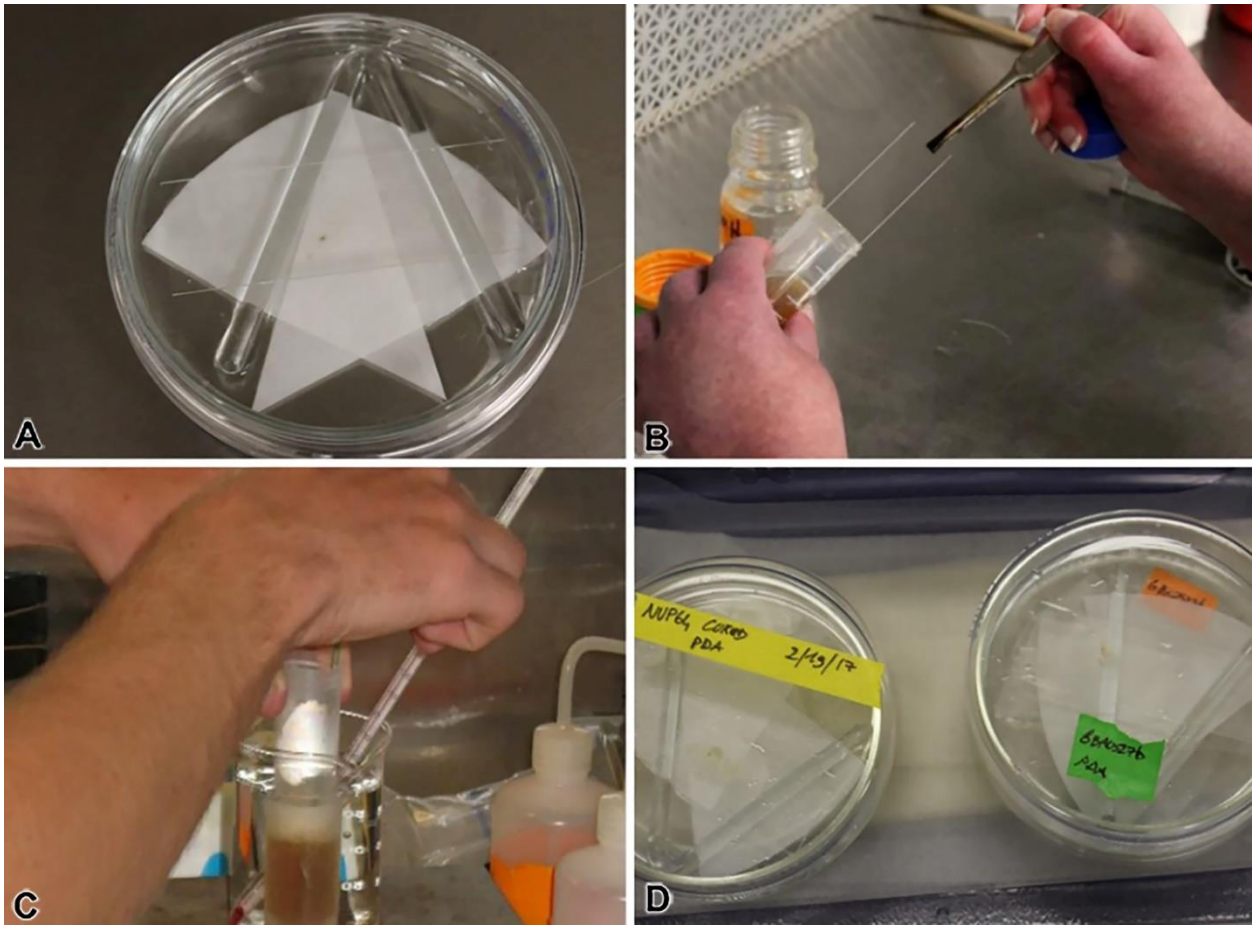

Figure 2. Preparation of culture slides live cell imaging of hyphal growth. (A) Sterile glass Petri dish containing moistened filter paper, support glass rod bend in shape of arrowhead, and glass slide. (B) Glass slide is dipped into molten growth media containing 15% gelatin. (C) Media maintained at ~48 C. (D) After media has cool, surface is inoculated with organism and allowed ~ 24 h of growth in moist chamber before imaging with light microscopy.

Figure 3. The leading edge of a hyphal colony *Rhizopus oryzae* growing on semi-solid agar. Bar: 50 µm.

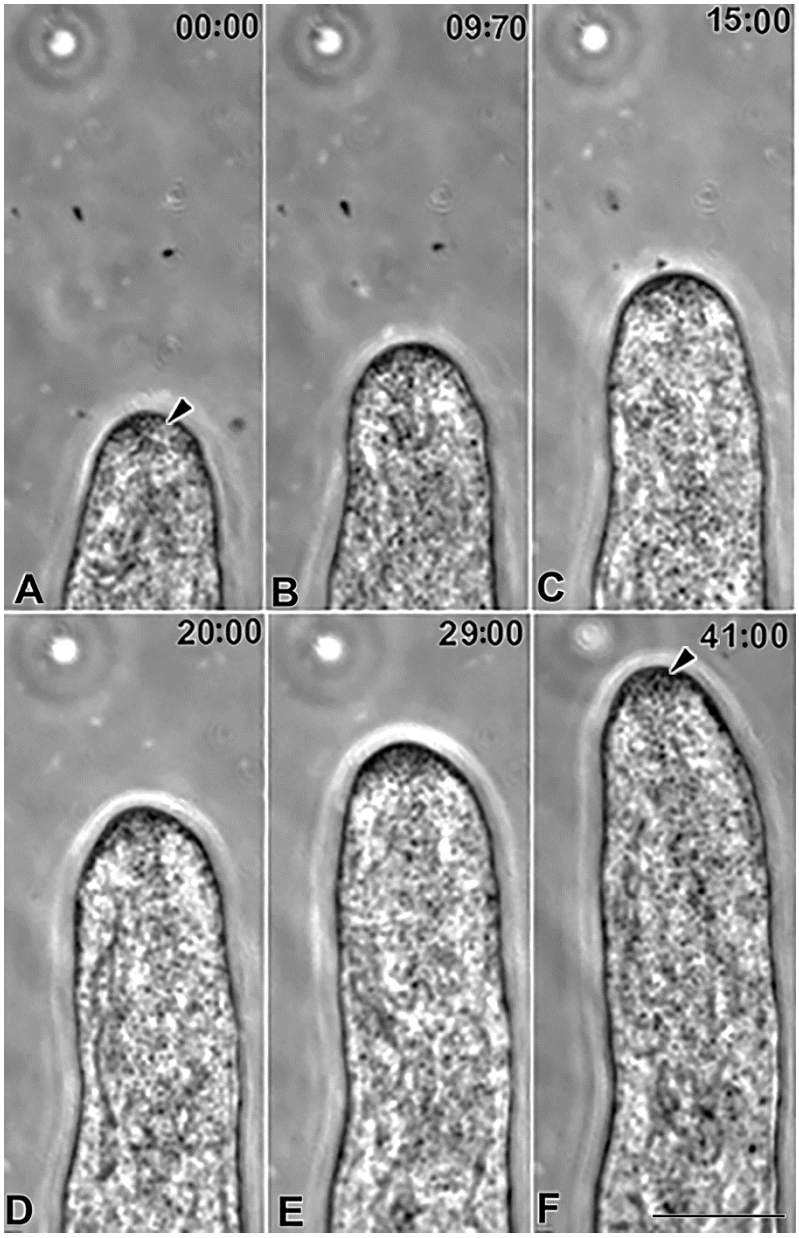

Figure 4. Phase contrast light microscopy of growing *Rhizopus oryzae* hypha. (A, F) An accumulation of secretory vesicles (AVC) was detected within the hyphae apex (arrowhead). Elapsed time (second: millisecond) is represented in the upper right-hand corners of each image. Scale bar: 5.0 µm.

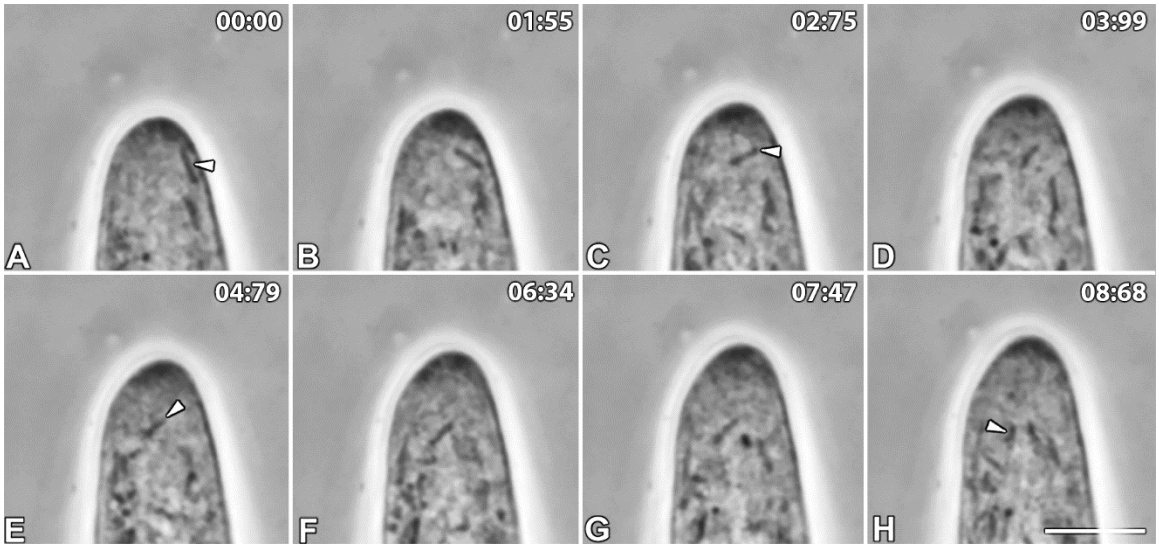

Figure 5. Apical growth of *Rhizopus oryzae* hypha illustrating RFCS observed using phase contrast optics. A mitochondrion (arrowhead) is moving anterograde (A-D) and (E-H). Elapsed time (second: millisecond) is represented in the upper right-hand corners of each image. Scale bar: 5.0 µm.

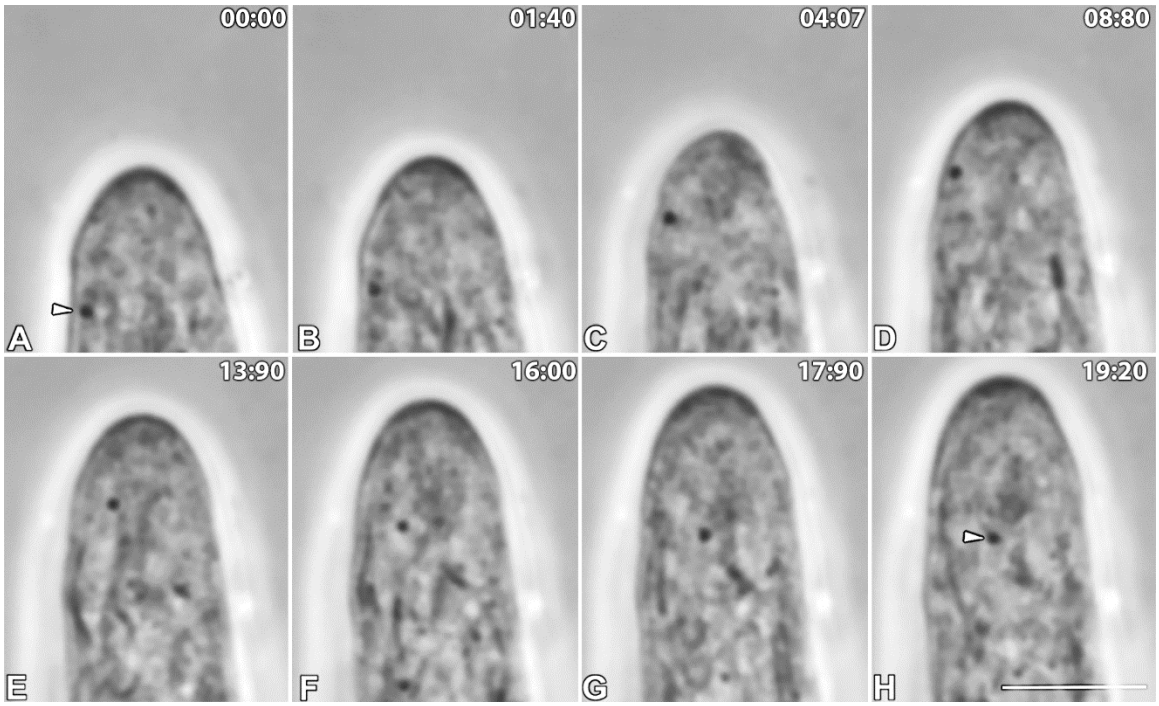

Figure 6. A multivesicular body (arrowhead) demonstrating RFCS. Apical growth of *Rhizopus oryzae* hypha illustrating RFCS observed using phase contrast optics Elapsed time (second: millisecond) is represented in the upper right-hand corners of each image. Scale bar: 5.0 µm.

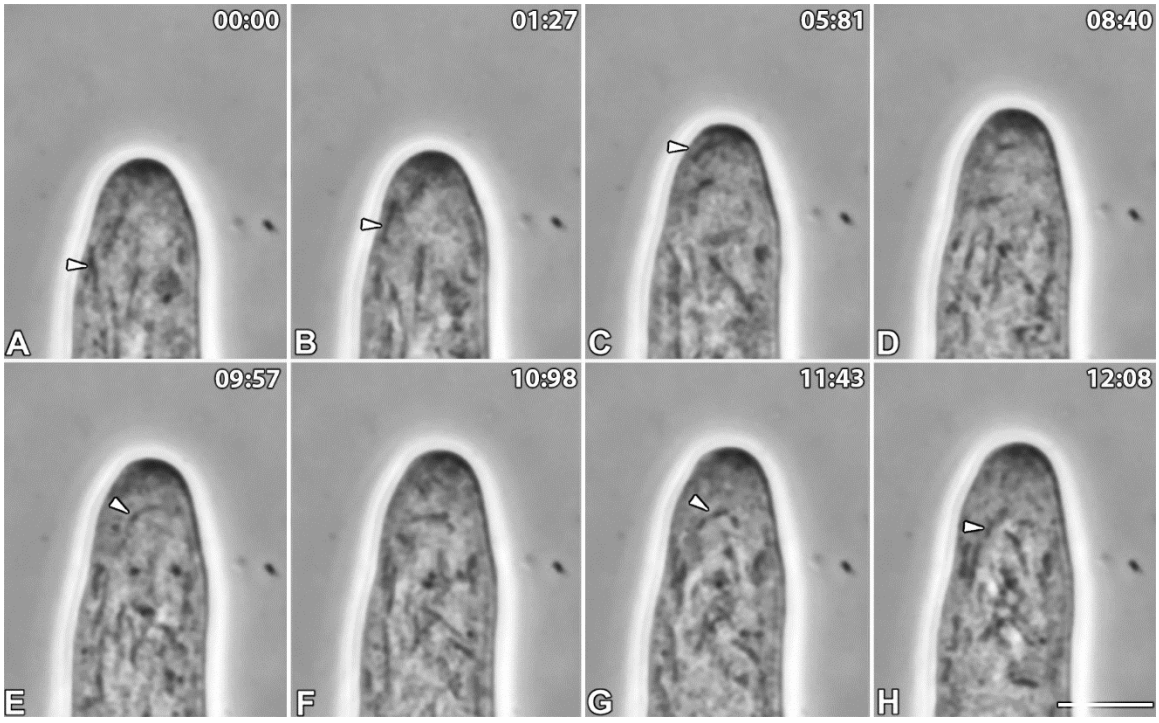

Figure 7. Demonstration of reverse fountain cytoplasmic streaming in *Rhizopus oryzae* with mitochondrion (arrowhead) undergoing a morphological change such as bending subapically in the direction of retrograde flow. Elapsed time (second: millisecond) is represented in the upper right-hand corners of each image. Scale bar: 5.0 µm.

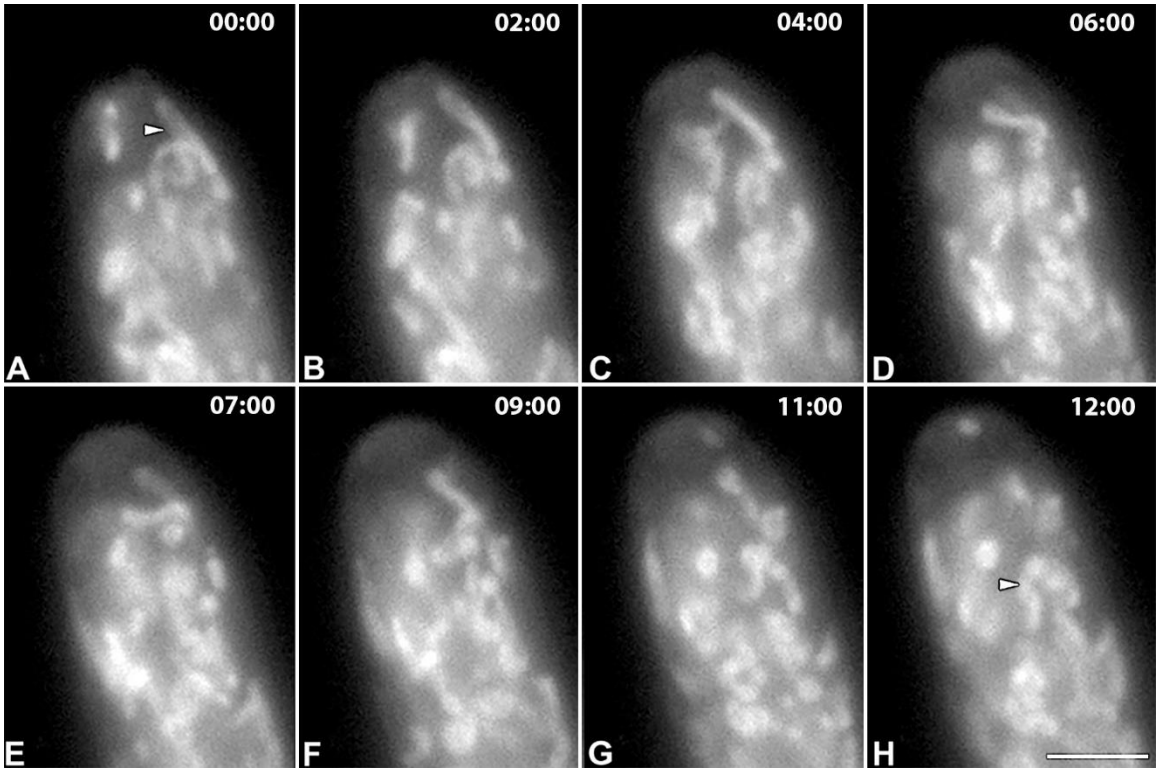

Figure 8. *Rhizopus oryzae* labelled with FM4-64 imaged with wide-field epifluorescence microscopy. (A-C) A mitochondrion (arrowhead) moves anterograde, (D, E) bends, and begins motility in a retrograde fashion (F-H) through the center of the hypha. Elapsed time (second: millisecond) is represented in the upper right-hand corners of each image. Scale bar: 5.0 µm.

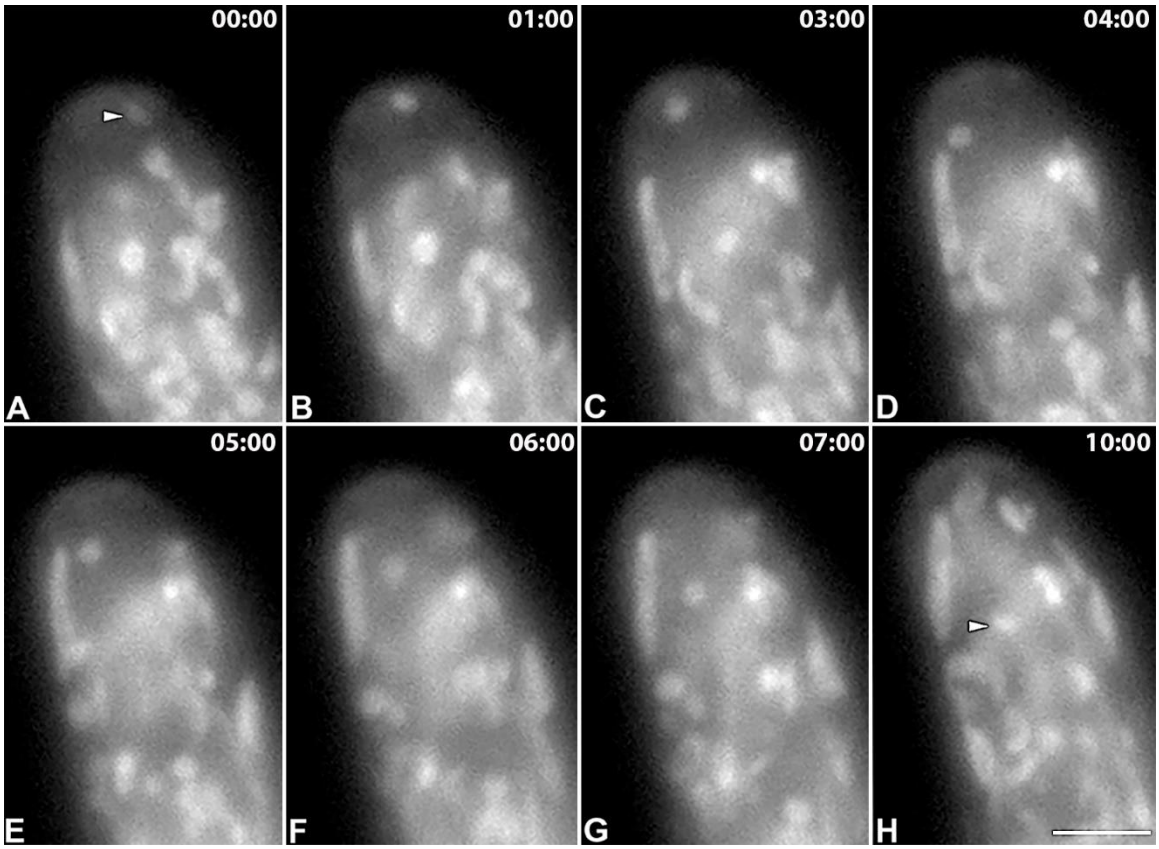

Figure 9. Reverse fountain cytoplasmic streaming in *Rhizopus oryzae* labelled with FM4-64 and imaged with wide-field epifluorescence microscopy. (A, B) A small organelle (arrowhead), identified as a multivesicular body, moves anterograde into region I of the hypha, (C) stops and then moves in a retrograde fashion down the central cytoplasmic area (D-H). Elapsed time (second: millisecond) is represented in the upper right-hand corners of each image. Scale bar: 5.0 µm.

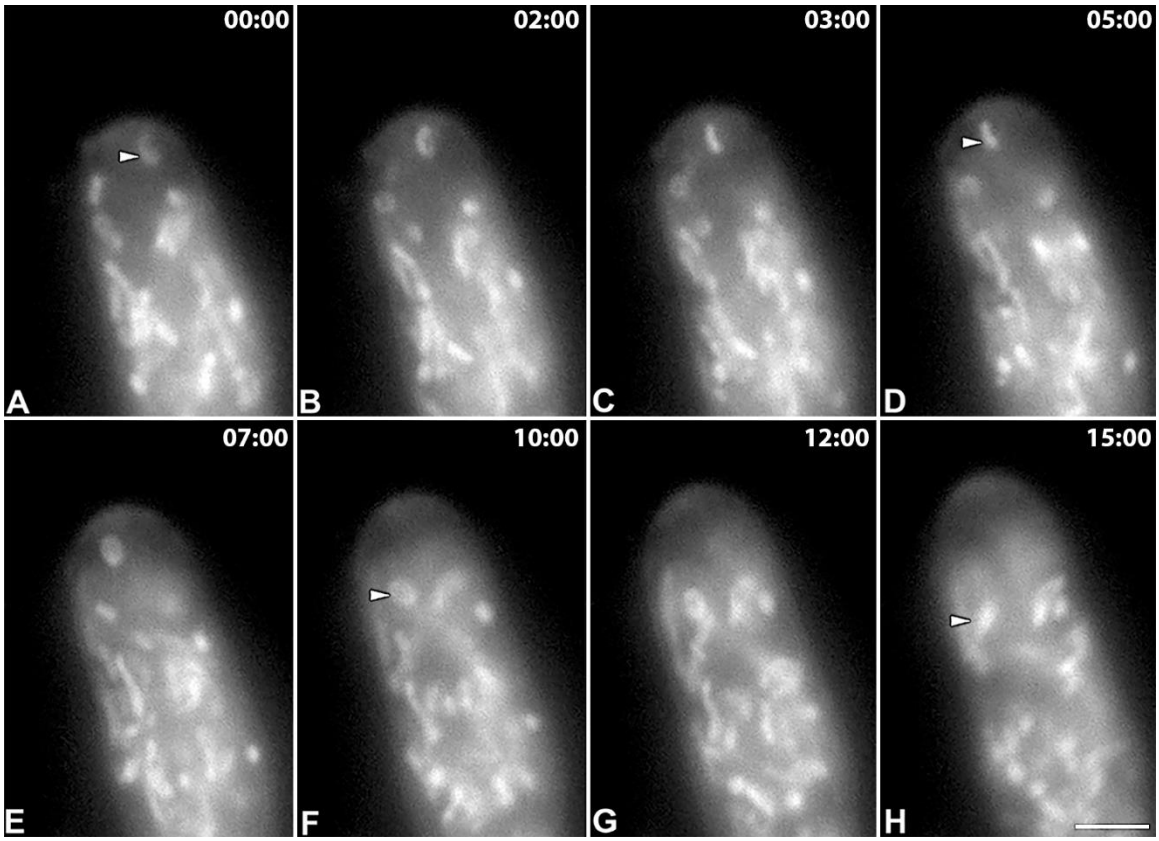

Figure 10. Wide-field fluorescence microscopy of *Rhizopus oryzae* hypha labeled with FM4-64. (A-D) The organelle indicated at the arrowhead (A) represents a Golgi equivalent. After entering the apical dome, the Golgi equivalent moves down the center of the hyphal cytoplasm. Elapsed time (second: millisecond) is represented in the upper right-hand corners of each image. Scale bar: 5.0 µm.

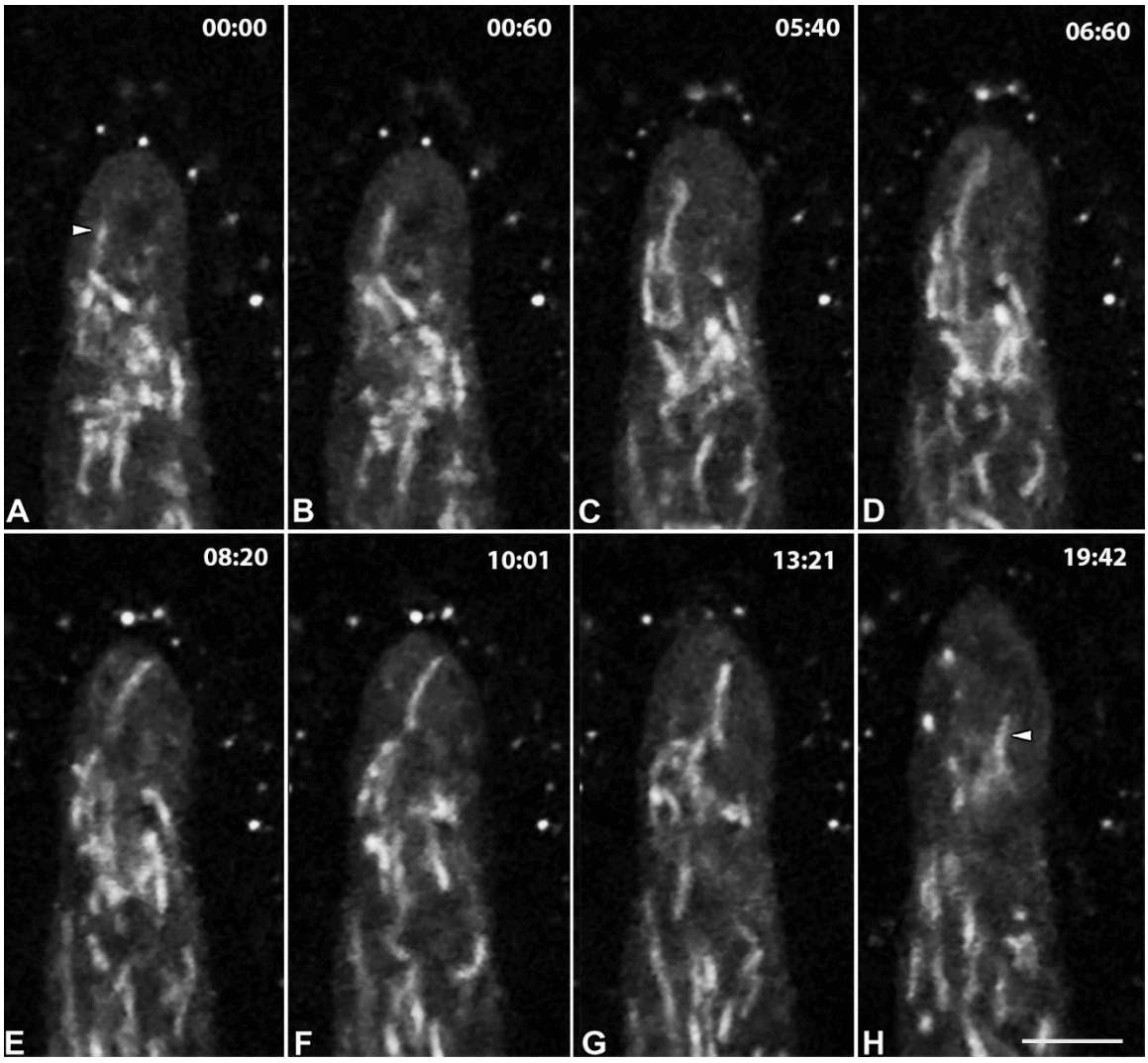

Figure 11. Hypha of *Cunninghamella echinulate* labelled with FM4-64 and imaged with spinning disc confocal microscopy. (A-E) A mitochondrion (arrowhead) moves anterograde along the cell cortex and (G, H) without exhibiting a morphological change moves in a retrograde fashion down the central cytoplasmic region of the cell. Elapsed time (second: millisecond) is represented in the upper right-hand corners of each image. Scale bar: 5 µm.

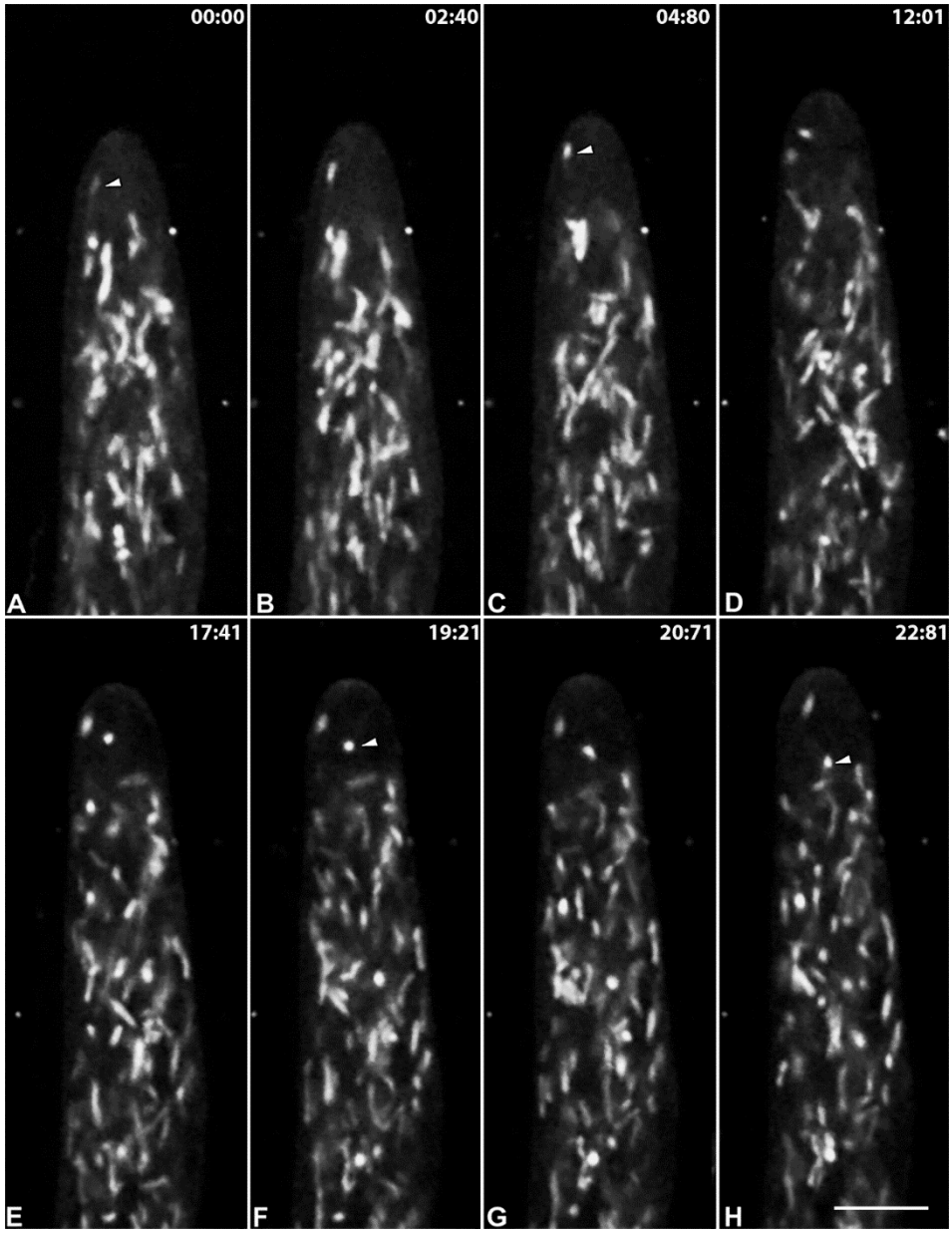

Figure 12. Hypha of *Gilbertella persicaria* labelled with FM4-64 and imaged with spinning disc confocal microscopy. (A-D) A mitochondrion (arrowhead) moves anterograde along a cortical path into region I and retreats subapically through the center of the cell (E-H). In this case, the mitochondrion appeared to undergo tumbling behavior and, in many images (E, F, H), appeared spherical in nature due to the confocal imaging. Elapsed time (second: millisecond) is represented in the upper right-hand corners of each image. Scale bar: 5 µm