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Optical tweezer micromanipulation of filamentous fungi

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Abstract

Optical tweezers have been little used in experimental studies on filamentous fungi. We have built a simple, compact, easy-to-use, safe and robust optical tweezer system that can be used with brightfield, phase contrast, differential interference contrast and fluorescence optics on a standard research grade light microscope. We have used this optical tweezer system in a range of cell biology applications to trap and micromanipulate whole fungal cells, organelles within cells, and beads. We have demonstrated how optical tweezers can be used to: unambiguously determine whether hyphae are actively homing towards each other; move the Spitzenkörper and change the pattern of hyphal morphogenesis; make piconewton force measurements; mechanically stimulate hyphal tips; and deliver chemicals to localized regions of hyphae. Significant novel experimental findings from our study were that germ tubes generated significantly smaller growth forces than leading hyphae, and that both hyphal types exhibited growth responses to mechanical stimulation with optically trapped polystyrene beads. Germinated spores that had been optically trapped for 25 min exhibited no deleterious effects with regard to conidial anastomosis tube growth, homing or fusion.

Index Descriptors and Abbreviations: Germ tube; Hyphal tip growth; Micromanipulation; *Neurospora crassa*; Optical tweezers; Organelle; Spitzenkörper

1. Introduction

Optical tweezers, also known as optical traps or laser tweezers, were first described in the 1980s by Ashkin and colleagues (Ashkin et al., 1986). They permit the non-invasive micromanipulation of inert and biological objects by means of optical radiation alone.

Optical tweezers use the forces generated when light interacts with matter to trap objects near the point of focus of an objective lens. To understand the origin of trapping forces for objects interacting with a laser beam, one needs to appreciate that when photons are absorbed, reflected or refracted by an object, the momentum they possess is changed, i.e. the object exerts a force on the photons. Newton's third law states that for every action force there is a corresponding reaction force that is equal in magnitude and opposite in direction. The object exerting the force on the photons will therefore experience a reaction force (Fig. 1A). Optical tweezers require an intense laser light source, tightly focused by an objective lens of high numerical aperture. They produce forces in the piconewton range that are sufficient to trap microscopic particles and move them relative to their surroundings (Fig. 1A and B). For a particle to be trapped efficiently it must have a refractive index that is sufficiently higher than its surroundings. The trapped object can be moved either by moving the microscope stage or by moving the trap position within the field of view (e.g. with galvanometric mirrors). The sizes of particles that can be trapped range from large objects such as whole cells down to particles of a few tens of nanometer in size. The trapping force can be adjusted by changing the laser power. Most recently, multiple traps with positions

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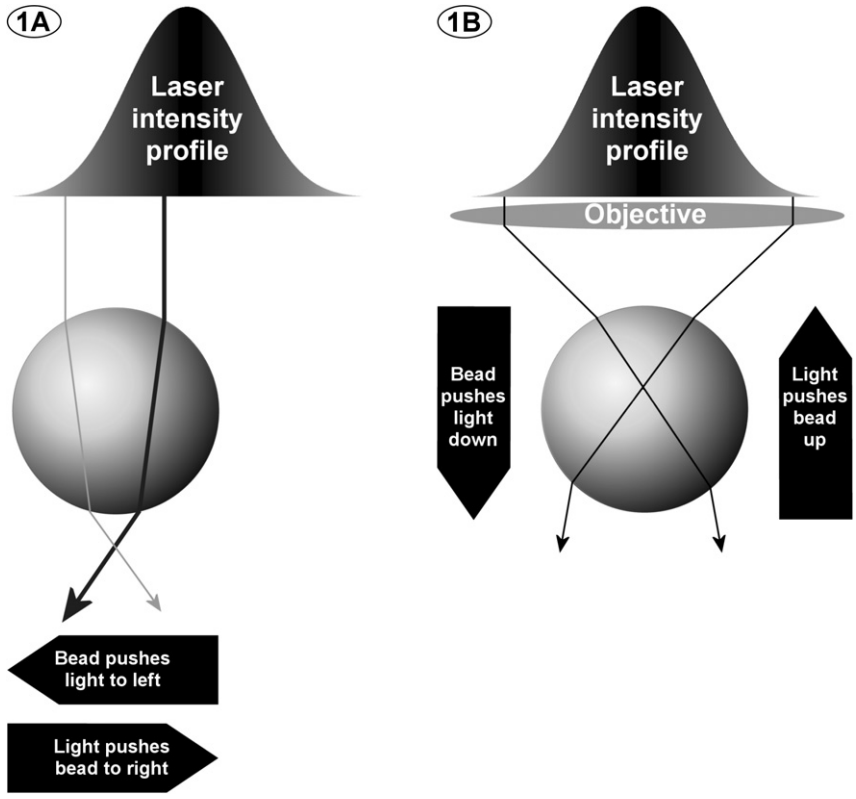

Fig. 1. Ray diagrams showing the interaction of light with a transparent sphere (e.g. a polystyrene bead). The sphere causes a change in direction of the light (an action force) and results in an equal and opposite reaction force on the sphere in accordance with Newton's third law. (A) As the centre of a laser beam with a Gaussian profile is more intense, the ray from this region generates a greater force than the ray from the periphery of the beam. Therefore the net reaction force on the sphere pushes the bead to the right. Upon reaching the centre of the beam the sphere would be trapped in x and y directions but not z. (B) To also trap in the z direction the light is focused by an objective lens. In both cases the object is attracted to and trapped in the region of highest light intensity, which is at the point of focus (figure adapted with permission from Dholakia et al., 2002).

controllable in real time have been implemented (Grier and Roichman, 2006). A number of general reviews on optical tweezers are available: Block (1990, 1992), Kuo and Sheetz (1992), Ashkin (1997), Sheetz (1998), Greulich (1999), Molloy and Padgett (2002) and Neuman and Block (2006).

Optical tweezer micromanipulation of living cells provides a powerful method for investigating many aspects of cell biology. Optical tweezers can be used to manipulate whole living cells or organelles directly, deliver molecules in microspheres to localized cell regions, or mechanically stimulate cells by pushing trapped objects against them. Using light to manipulate cells has the following advantages over the more traditional mechanical methods of micromanipulation: it is much less invasive and can inflict negligible damage and stress to cells; it allows objects within living cells to be trapped; it offers very precise control of the position of the object being manipulated; and it allows instant trapping or release of objects (Wright et al., 1990; Block, 1990; Berns et al., 1991; Kuo and Sheetz, 1992; Weber and Greulich, 1992; Svoboda and Block, 1994; Sheetz, 1998; Molloy and Padgett, 2002). Optical tweezers have been used in a wide range of biological applications which include: micromanipulating cells to redirect their growth (Leitz et al., 1995; Bracker et al., 1997; Erlicher et al., 2002); micromanipulating organelles within cells (Berns et al., 1992; Leitz et al., 1995; Tolić-Norrelykke et al., 2005); isolating individual cells, organelles and chromosomes (Leitz et al., 2003; Liu et al., 2004); measuring the forces produced by motor proteins and RNA polymerases (Block et al., 1989; Finer et al., 1994; Greulich, 2005; Kuo and Sheetz, 1993) measuring the biophysical properties of DNA (Cluzel et al., 1996; Wang et al., 1997; Greulich and Pilarczyk, 1998; Baumann et al., 2000; Greulich et al., 2000; Lang et al., 2003); fusing cells (Block, 1992); providing localized mechanostimulation of a cell (Wang et al., 2005); and automating cell sorting (Kuo and Sheetz, 1992).

Whole cells and organelles of budding yeast (*Saccharomyces cerevisiae*) have been micromanipulated in a range of studies to evaluate different optical tweezer systems (Ashkin, 1991; Grimbergen et al., 1993; Daria et al., 2004; Goksor et al., 2004; Sacconi et al., 2005; Lafong et al., 2006). Optical tweezers have also been used more experimentally with yeast cells to: investigate the viscoelastic properties of the cytoplasm (Tolić-Norrelykke et al., 2004); demonstrate the specification of the division plane by the nucleus in the fission yeast *Schizosaccharomyces pombe* (Tolić-Norrelykke et al., 2005); and examine the inhibition of growth of *Hanseniaspora uvarum* cells by confining them with optically trapped cells of *Sacch. cerevisiae* (Arneborg et al., 2005).

Filamentous fungi are also well suited to being micromanipulated by optical tweezers, particularly because they can be analyzed as monolayers of germinating spores or growing hyphae, can exhibit rapid growth, can respond quickly to stimuli, and often possess large hyphae or cells. Berns et al. (1992) were the first to use optical tweezers with filamentous fungi, and showed that organelles could be trapped and moved within hyphae without obvious damage. They found that the order of responsiveness of organelles to trapping was: lipid bodies > nucleoli > mitochondria > chromosomes. Bracker et al. (1997) later showed how optical tweezers could be used to move the Spitzenkörper within a hyphal tip, change the direction of hyphal growth, redistribute secretory vesicles in the cytoplasm, and induce branch formation. Their study provided compelling evidence that the Spitzenkörper is responsible for hyphal growth directionality (also see Fig. 3 in Bartnicki-Garcia, 2002). Of particular interest was their observation that rather than being trapped, the Spitzenkörper was repelled by the laser, as was later shown by Wright et al. (2005). The Spitzenkörper is a predominately phase dark structure when observed by phase contrast light microscopy (Lopez-Franco and Bracker, 1996) indicating that, overall, it has a higher refractive index than the surrounding cytoplasm. It had therefore been expected that the Spitzenkörper should be trapped rather than repelled by optical tweezers (Bracker et al., 1997).

Roca et al. (2005) used optical tweezers in a novel assay to assess unambiguously whether conidial anastomosis tubes (CATs) of *Neurospora crassa* home towards each other or not. This technique allows a conidium or conidial germling to be optically trapped and to have its position relative to another changed. Using this method, Roca et al. (2005) showed that CAT tips were both the sites of chemoattractant secretion and reception. Furthermore, by using mutants defective in signalling, it was shown that cAMP is not the CAT chemoattractant (Roca et al., 2005) and that the mutant *soft* forms CATs that are unable to home towards or fuse with other CATs (Fleißner et al., 2005).

The first attempts to use optical tweezers to measure the growth forces generated by leading hyphae of *N. crassa* were made by Wright et al. (2005) by placing beads trapped with known forces in their paths. It was concluded that the growth forces of the hyphae analyzed were greater than the piconewton forces which optical tweezers can be used to readily measure. This supported a previous study (Money et al., 2004) in which it was estimated from measurements using a miniaturized strain gauge that vegetative hyphae generate growth forces which are several orders greater than can be measured with optical tweezers.

Our aim in this paper is to evaluate the use of optical tweezers as experimental tools in a range of cell biology applications on filamentous fungi. We designed and built a simple, compact, easy-to-use, safe and robust optical tweezer system that was mounted on a research grade inverted microscope. This system was used to trap and move whole cells, organelles and beads in combination with differential interference contrast (DIC), phase contrast and widefield fluorescence imaging.

2. Materials and methods

2.1. Fungal strains and culture conditions

The *N. crassa* wild-type strain 74-OR23-1VA (# 2489, FGSC, Kansas City, KS, USA) was used. It was grown and maintained on solid Vogel's minimal medium (Davis, 2000) in continuous light at 25 °C.

2.2. Sample preparation

Macroconidia were harvested from Petri dish cultures of *N. crassa* by pipetting liquid Vogel's medium onto the colony, swirling, and then removing the resulting spore suspension before adjusting its concentration to 1×10^6 macroconidia ml^{-1} using a haemocytometer. A 200 µl droplet of this macroconidial suspension was transferred either into an eight well slide culture chamber (Nalge Nunc International, www.nalgenunc.com) or onto coverslips pre-coated with poly (vinyl) alcohol (PVA) which prevents the macroconidia from adhering to the glass (Krylov and Dovichi, 2000).

To image, stain and manipulate hyphal tips, the inverted agar block culture method was used (Hickey et al., 2005). The hyphal vacuolar system was stained with 20 µM Oregon Green 488 (DFFDA) (Invitrogen, Molecular Probes, www.probes.invitrogen.com) made up in liquid Vogel's medium. The inverted agar block method was adapted when trapping polystyrene beads which were 4 µm in diameter (Interfacial Dynamics Corp., www.idclatex.com). For this purpose the agar block was supported on a coverslip with adhesive electrician's insulation tape. This provided more space for the beads in the liquid layer between the coverslip and agar, and it prevented the beads from adhering to the agar. Beads were also coated with bovine serum albumin (BSA) to prevent them from adhering to the fungal cells. For this purpose, the beads were suspended in a 1% aqueous solution of BSA (Sigma – www.sigmaaldrich.com) for 10 min before centrifugation and resuspension in Vogel's liquid medium. 1 µm porous silica beads (a kind gift from C.G. Hunt, USDA Forest Products Laboratory, Madison, WI, USA) were used for the localized delivery of a 50 µM latrunculin-B (Calbiochem – Merck Biosciences, www.merckbiosciences.co.uk) solution made up in Vogel's liquid medium.

2.3. Microscopy

The optical tweezer system was mounted on a Nikon Eclipse TE2000-U inverted microscope. A Nikon plan apo 100×, 1.4 N.A. DIC H oil immersion objective was used for DIC and fluorescence microscopy, and a Nikon plan fluor 100×, 1.3 N.A. Ph 3 DLL oil immersion objective was used for phase contrast microscopy. To increase the camera's

field of view, a 0.7× demagnifying lens was used when necessary. For widefield fluorescence microscopy a mercury vapour lamp with a Nikon B-2A filter cube (containing a 450–490 nm excitation filter, 500 nm long pass dichroic mirror, and 515 nm long pass emission filter) was used.

2.4. Optical tweezer set-up

The custom built optical tweezer system (Fig. 2) contains a near-infrared ($\lambda = 785$ nm) diode laser, the output power of which is adjustable up to 70 mW (VPSL-0785-070-x-5-A; Blue Sky Research, www.blueskyresearch.com). The laser beam is directed first towards two galvanometric beam steering mirrors and then into the rear of the microscope (Fig. 2A and C). A slight modification made to the microscope, which involved lifting the nosepiece, allows the optical tweezers to be combined with fluorescence imaging. A dichroic mirror reflects the beam into the objective lens. The beam is expanded to fill the back aperture of the objective to offer maximal trapping efficiency. A computer program written in LabVIEW 6.1 (National Instruments, www.ni.com) allows the user to move the trap in x and y directions across the field of view by controlling the galvanometric mirrors at the click of the mouse. The computer takes the images generated by the CCD camera (Applied Vision Technologies, Dolphin 145-F, from FirstSight Vision, www.firstsightvision.co.uk), and moves the trap in a straight line at constant speed to any position in the field of view. The speed of trap movement can be preset in $\mu m\,s^{-1}$ in the tweezer control program. A double click releases the trapped object. Movement in the z axis is made by adjusting the focus of the microscope. It is important to note here that the laser power at the specimen is approximately 50% of the laser output power due to attenuation through the microscope, particularly the objective lens (Svoboda and Block, 1994; Neuman et al., 1999). In this paper we refer to laser power in terms of output power.

2.5. Digital image processing and animation

Images were captured either singly through the control software directly from the camera or as a time course using screen capture software (HyperCam, www.hyperionics.com). Further processing was carried out with Paintshop Pro (v. 7; JASC Software, www.jasc.com). Time courses of

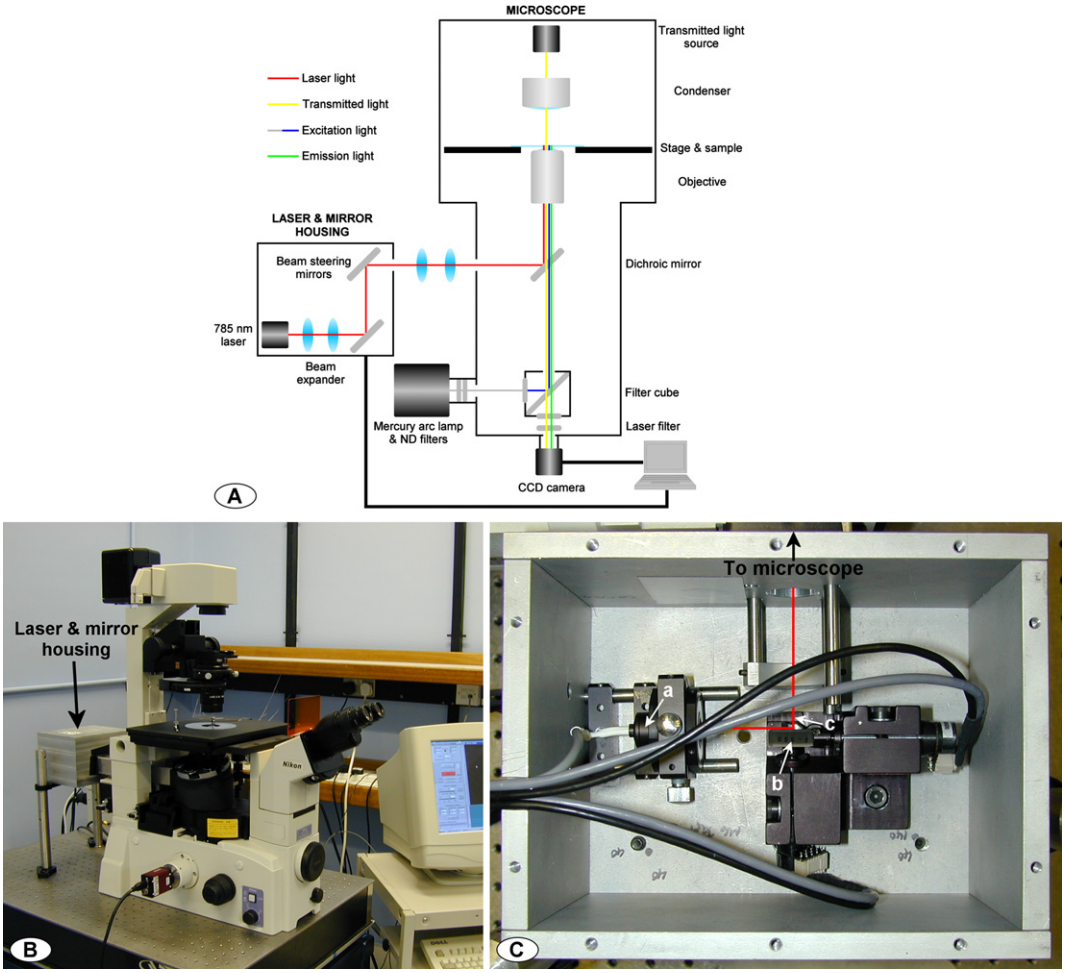

Fig. 2. (A) Schematic diagram of the optical tweezer system used. (B) The tweezer system mounted on the microscope. (C) The interior of the laser and mirror housing in which (a) is the 785 nm diode laser, (b) are the galvanometric mirrors, and (c) is the path taken by the laser beam. The optical elements required for phase contrast, DIC and fluorescence can be incorporated into the microscope whilst maintaining the full functionality of the optical tweezers.

images were edited and built up into animation movies (.avi and mpeg files) using Animation Shop (v. 3; JASC Software, www.jasc.com), Premier Pro (v. 1.5; Adobe, *www.adobe.com*), and ImageJ (freeware; http://rsb.info.nih.gov/ij/).

3. Results

3.1. Manipulation of whole cells

Ungerminated macroconidia (Fig. 3) and germinated macroconidia (Fig. 4) were trapped and moved around relative to their surroundings. The semi-spherical, ungerminated macroconidia were easily moved within the field of view in x and y directions with the computer-controlled steerable trap controlled by the galvanometric mirrors (Supplementary movie 1 at www.fungalcell.org/tweezermovies.htm). Macroconidia could be moved over greater distances by trapping them in a fixed position and then carefully moving the microscope stage. Using a laser output power of 40 mW it was possible to move a macroconidium at a speed of up to 15 $\mu m\,s^{-1}$ in x and y directions, without risking losing the macroconidium from the trap. The speed of movement achievable varied depending on the size and optical properties of a given macroconidium, the laser power used, and the viscosity of the medium. With time, macroconidia tended to adhere to the coverslip, after which they could not be detached from the glass surface even when using the highest output laser power (70 mW). To overcome this problem the coverslips were coated with PVA, which prevented spore adhesion to the glass without perturbing germ tube growth. Germ tubes up to 100 μm long could be trapped at different positions along their lengths, thereby allowing control of their orientation (Fig. 4; Supplementary movie 2 at www.fungalcell.org/tweezermovies.htm).

Recently a new type of hypha, the conidial anastomosis tube (CAT), has been described (Roca et al., 2005). CATs arise from conidia or germ tubes and home towards and fuse with each other. Optical tweezers have been used to trap and move one CAT relative to another to unambiguously determine whether the two CATs are homing towards each other or not. This technique is being used to

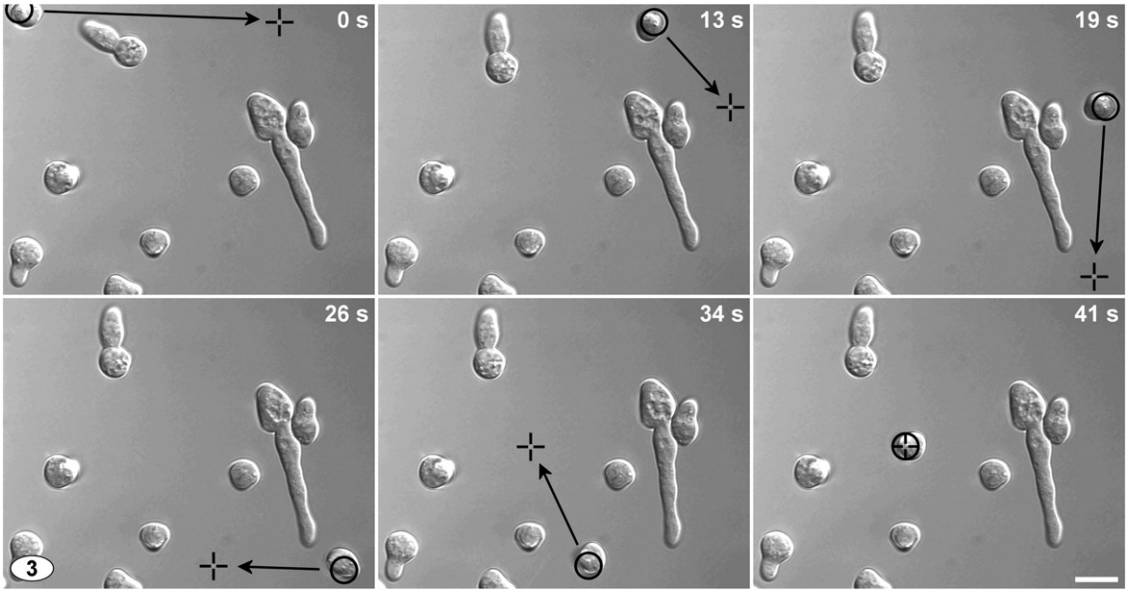

Fig. 3. Micromanipulation of an ungerminated macroconidium of *Neurospora crassa*. The circle represents the current position of the laser whilst the cross-hair shows the position to which the trap is about to be moved. Bar = 10 μm. (See Supplementary movie 1 at www.fungalcell.org/tweezermovies.htm.)

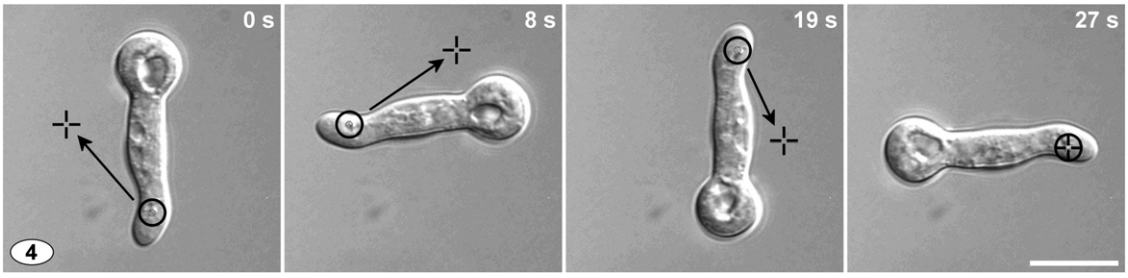

Fig. 4. Rotation of a macroconidial germling of *N. crassa*. The circle represents the current position of the laser whilst the cross-hair shows the position to which the trap is about to be moved. Note that as the germling was manipulated it rotated around its centre point and the field of view remained constant throughout the time course. Bar = 10 μm. (See Supplementary movie 2 at www.fungalcell.org/tweezermovies.htm.)

study the signalling pathways involved in CAT homing, particularly by using mutants (Fleißner et al., 2005; Roca et al., 2005). In order to conduct this assay, conidia or germlings were previously only trapped for up to 1 min. Here we found that we could extend the trapping time to 25 min and found that CATs continued to home towards and fuse with each other, without any apparent ill effects (Fig. 5).

3.2. Manipulation of organelles

Organelles of high refractive index (e.g. Woronin bodies) visualized by differential interference contrast (DIC) or phase contrast optics were readily trapped within living cells (Fig. 6; Supplementary movie 3 at www.fungalcell.org/tweezermovies.htm). For some organelles it was necessary to hold the organelle in the trap for some time, during which period it seemed that the organelle ceased to be attached to the cytoskeleton, before it became possible to move the organelle around within the cytoplasm. Some Woronin bodies were found to be strongly tethered to the lateral plasma membrane adjacent to septa such that the tweezer trapping forces were insufficient to move them. In order to move organelles within the viscous hyphal cytoplasm it was necessary to move the trap slowly (~1–2 $\mu m\, s^{-1}$) so as not to lose the organelle from the trap. Upon the release of an organelle from the trap it was common to observe the organelle 'float' around in the cytoplasm or move with the bulk cytoplasmic flow (Lew, 2005). Some unidentified, refractile organelles, similar to those described as 'vesicles' by Riquelme et al. (2002), were observed moving tens of micrometers in straight lines within hyphae. These organelles were probably tethered by motor proteins to microtubules. It was often possible to optically trap these organelles but it was apparent that their movement was force-driven. As a result, they were sometimes lost from the trap, particularly when using the trapping laser with low power (data not shown). Moving trapped organelles in a direction that was more-or-less at right angles to the direction in which they were moving along putative microtubules/microfilaments commonly resulted in the organelles being lost from the trap and 'springing back' to the putative cytoskeletal element.

Organelles of lower refractive index than their surroundings were repelled by the trap. Fig. 7A–C shows vacuoles, which are of lower refractive index than the cytoplasm, being moved around within hyphae whilst being observed with phase contrast, DIC or fluorescence microscopy. To move the vacuoles, a technique of 'chasing' was employed, much akin to repelling magnets, in which the vacuoles were pushed away from the trap when it got close to them (Fig. 7A; Supplementary movie 4 at www.fungalcell.org/tweezermovies.htm). Applying the trap directly to a vacuole resulted in it 'popping' out of the trap (Fig. 7B; Supplementary movie 5 at www.fungalcell.org/tweezermovies.htm). If vacuoles were pressed against the plasma membrane, it was possible to split them in two (Fig. 7C; Supplementary movie 6 at www.fungalcell.org/tweezermovies.htm).

Using what appeared superficially to be the same 'organelle chasing' technique, it was possible to influence the position of the Spitzenkörper within the growing tip, as

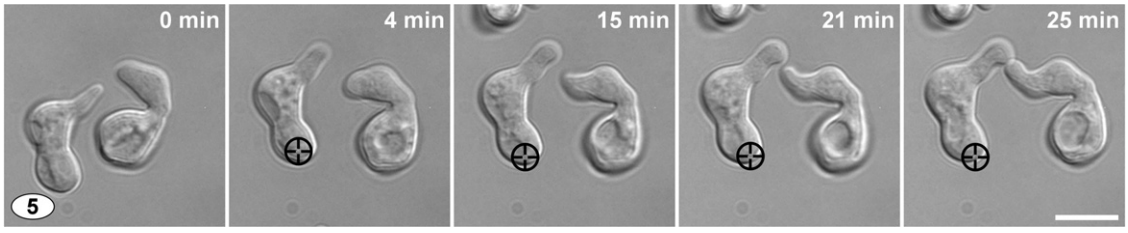

Fig. 5. An example of a conidial anastomosis tube (CAT) homing assay. The two conidia had germinated and their CATs were homing towards each other (0 min). The left hand germling was repositioned (here shown 4 min after repositioning). The CAT tips then changed their orientation to home back towards each other (15 and 21 min) before making contact (25 min) and subsequently fusing (not shown). The left hand conidium remained trapped throughout the entire 25 min period without apparent inhibition of CAT growth, homing or fusion. The position of the trap is represented by the cross-hair in the circle. Bar = 10 μm.

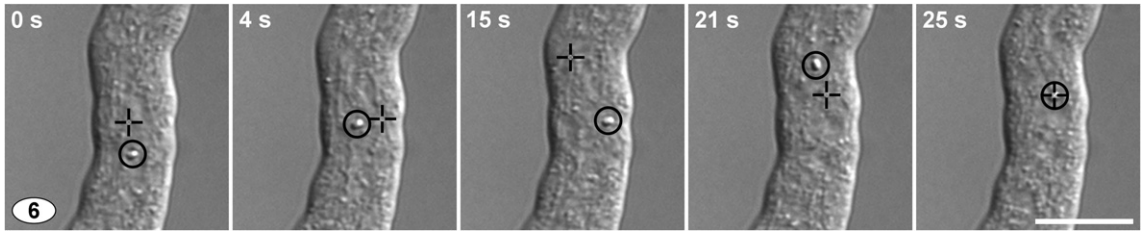

Fig. 6. Trapping and moving a Woronin body within a hypha of *Neurospora crassa.* The hyphal region shown was just behind the hyphal tip, and is where Woronin bodies can be found floating free in the cytoplasm and not, as is more usual, associated with the hyphal cell cortex or septum (Markham and Collinge, 1987; Jedd and Chua, 2000). The Woronin body could be moved in the direction of the prevailing bulk cytoplasmic flow (towards the hyphal tip), laterally across the hypha and against the flow. The circle represents the current position of the laser whilst the cross-hair shows the position to which the trap is being moved to. Bar = 10 μm. (See Supplementary movie 3 at www.fungalcell.org/tweezermovies.htm.)

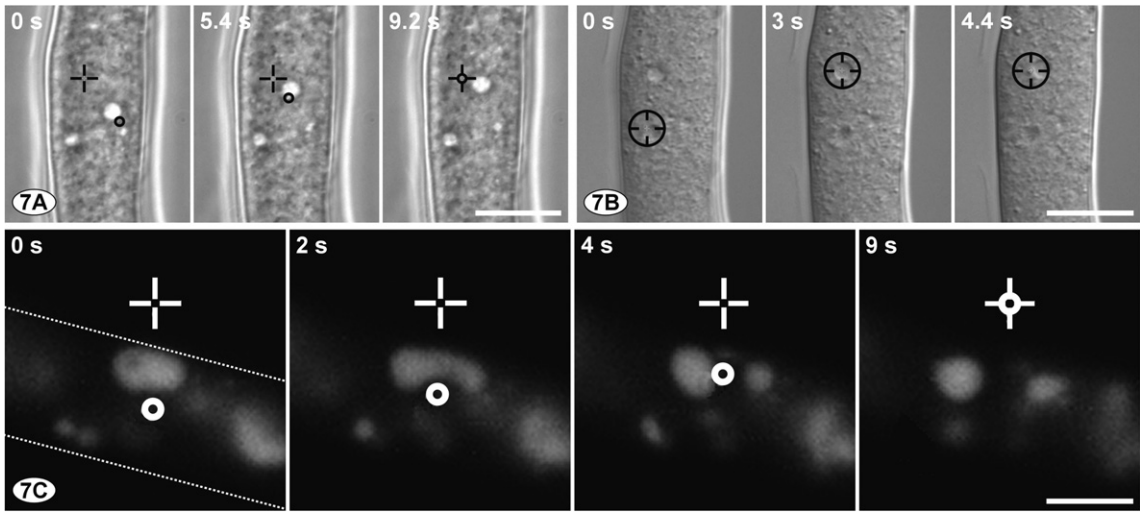

Fig. 7. Repulsion of vacuoles which have a lower refractive index than their surrounding cytoplasm in *Neurospora crassa*. (A) Vacuoles are visualized as bright objects (phase light) with phase contrast optics, (B) as depressed 'hollows' with DIC optics, or (C), as brightly fluorescent objects when stained with Oregon Green 488 (DFFDA) and imaged with widefield fluorescence microscopy. If the laser was applied directly to the vacuole they 'popped out' of the trap (B, compare the 3 and 4.4 s time points). As a result of being repelled, the vacuole could be pushed around the cytoplasm (A). If the vacuoles were pressed against the plasma membrane, it was possible to split them into two (C). The outline of the hypha in (C) is indicated by dotted lines. Bar = 10 μm (A and B). Bar = 5 μm (C). (See Supplementary movie 4–6 at www.fungalcell.org/tweezermovies.htm.)

previously described (Bracker et al., 1997; Wright et al., 2005). Placing the trap to the side of the Spitzenkörper resulted in the redirection of growth on the side of the hyphal tip away from the trap (Fig. 8; Supplementary movie 7 at www.fungalcell.org/tweezermovies.htm).

3.3. Manipulation of microspheres

Polystyrene beads and other types of transparent microspheres can usually be efficiently trapped, and can provide powerful experimental tools in live-cell studies. Here we have explored their uses in making growth force measurements, generating mechanical stimuli, and providing localized extracellular sources of molecules.

A bead situated at the centre of a laser trap experiences no force. As it is displaced from the trap centre, a restoring force develops that is initially proportional to the displacement, i.e. the trap acts as an ideal ('Hooke's law') spring. Eventually, however, the trap 'softens' and the force decreases to zero: the bead escapes the trap beyond a maximum displacement. Precise measurement of the position of the trapped bead allows the determination of the force exerted on the bead (Sheetz, 1998). On the other hand, an estimate of the forces involved can conveniently be obtained by measuring the maximum trapping force, which increases linearly with the incident laser power (Wright et al., 2005). This is achieved by trapping a bead at a given laser power and then dragging it through a liquid of known viscosity at increasing velocity until it escapes from the trap (Supplementary movie 11 at www.fungalcell.org/tweezermovies.htm). Applying Stoke's law to calculate the drag at the 'escape speed' allows us to calculate the maximum trapping force at that laser power. As shown in Fig. 9, and as has been previously reported by Wright et al. (2005), the growth force of a leading hypha of *N. crassa* is sufficient to push a 4 μm polystyrene bead out of a trap even when using the highest laser power (Fig. 9; Supplementary movie 8 at www.fungalcell.org/tweezermovies.htm). Performing the same experiment using a germ tube yielded very different results (Fig. 10). The tip of a germ tube, which grows much

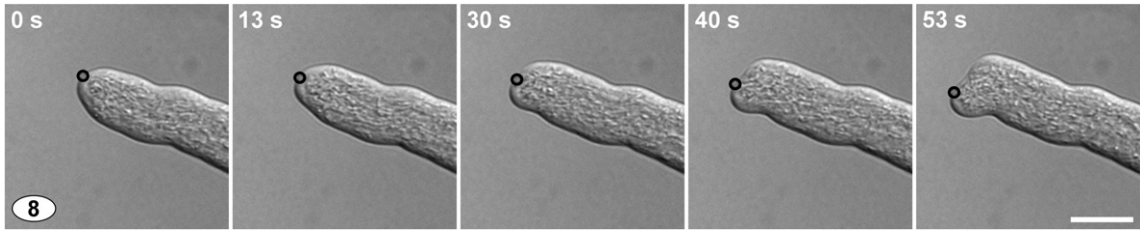

Fig. 8. Redirection of growth by manipulating the Spitzenkörper. The Spitzenkörper is repelled by the laser. When the laser was located to the side of the Spitzenkörper, this resulted in the redirection of hyphal tip growth away from the trap. In this experiment, the trap was repositioned gradually to maintain its position just to the side of the Spitzenkörper as growth progressed. The position of the trap is represented by the circle. Bar = 10 μm. (See Supplementary movie 7 at www.fungalcell.org/tweezermovies.htm.)

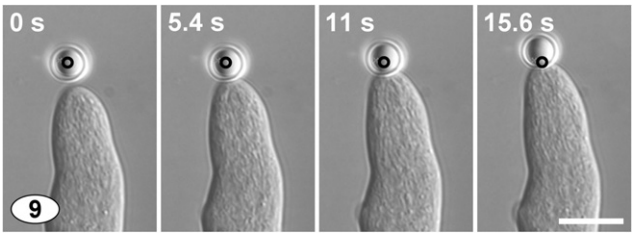

Fig. 9. Inability to halt the growth of a leading hypha with an optically trapped bead. Using an optically trapped 4 μm polystyrene bead as an obstacle, the tip of this leading hyphae was able to push the bead out of the trap. The highest output laser power (70 mW) was used in this experiment, which equates to a trapping force of 19 pN (Wright et al., 2005). The circle represents the position of the laser. Bar = 10 μm. (See Supplementary movie 8 at www.fungalcell.org/tweezermovies.htm.)

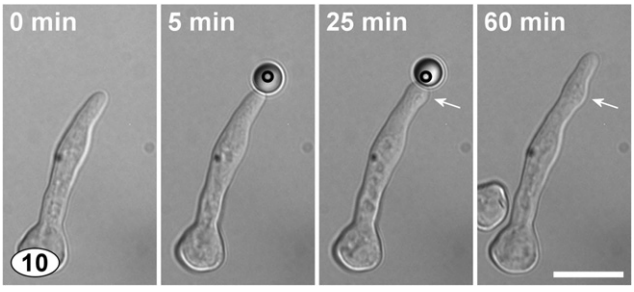

Fig. 10. Inhibition of germ growth with an optically trapped 4 μm bead used as an obstacle in front of a germ tube. Note that upon first making contact with the bead the germ tube tip begins to push the bead slightly forward (at 5 min), but is unable to push it out of the trap. The tip then proceeds to swell (arrow at 25 min). When the obstacle was removed growth resumed at the germ tube tip, leaving a subapical swelling (arrow at 60 min). The highest output laser power (70 mW) was used in this experiment, which equates to a trapping force of 19 pN (Wright et al., 2005). Bar = 10 μm. (See Supplementary movie 9 at www.fungalcell.org/tweezermovies.htm.)

slower than leading hyphae, became swollen upon contact with a bead trapped at full laser power, and then stopped growing. This suggests that germ tubes produce a smaller growth force than leading hyphae. Upon removal of the obstructing bead, growth resumed at the germ tube tip, leaving a subapical swelling (Fig. 10; Supplementary movie 9 at www.fungalcell.org/tweezermovies.htm).

Another method of applying a mechanostimulus to growing hyphal tips was attempted. This involved using a 10 μm bead to repeatedly hit a growing tip of a leading hypha by moving the trapped bead at high speeds (up to 40 μm s^{-1}) back and forth against the hyphal tip. The hyphae treated in this way continued to maintain a uniform linear rate of extension but some slight redirection of growth was sometimes observed following the time point at which the stimulus was applied (Fig. 11; Supplementary movie 10 at www.fungalcell.org/tweezermovies.htm).

Optical tweezers were used to deliver chemicals to localized cellular regions. In Fig. 12A latrunculin-B, a drug inhibiting actin polymerization (Spector et al., 1983), was delivered selectively in a group of three porous silica beads to a hyphal tip. The extension rate of this hypha was dramatically reduced and as it continued to grow slowly it underwent significant swelling. The growth of neighbouring hyphae up to ~300 μm away continued to grow normally during the 30 min period of this experiment (Fig. 12B). The porous beads, having been soaked in latrunculin-B, were added to the edge of the agar block then trapped and taken to the hyphal tip through the liquid growth medium between the agar block and the coverslip.

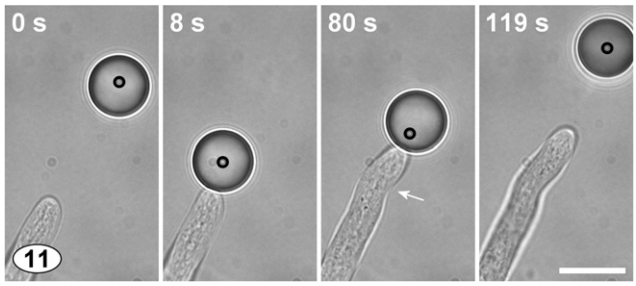

Fig. 11. Effect of repeatedly hitting a tip of a growing vegetative hypha with a 10 μm polystyrene bead. Note that the hypha continued to grow (with an extension rate of 7.7 μm min^{-1}) throughout the period of mechanostimulation but there was a slight redirection of growth during the time (arrow at 80 s) that the hypha was stimulated. Bar = 10 μm. (See Supplementary movie 10 at www.fungalcell.org/tweezermovies.htm.)

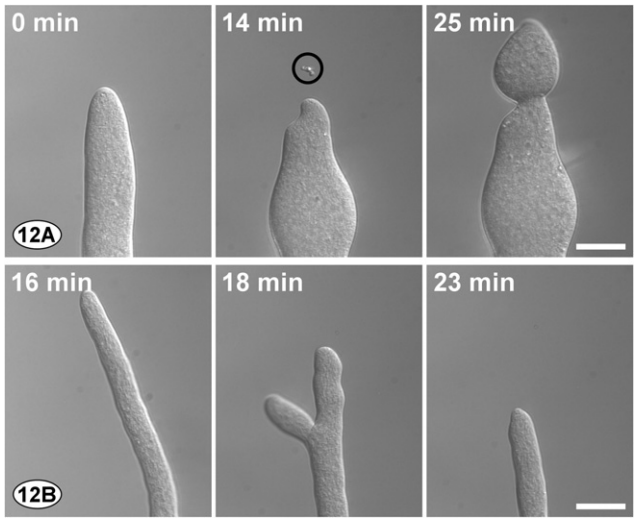

Fig. 12. Using trapped beads to deliver chemicals to a localized region of a cell. (A) Delivery of a localized dose of latrunculin-B to a hyphal tip from a group of three porous silica beads. The drug, which disrupts actin polymerization, has caused hyphal tip growth to be significantly inhibited and to be accompanied by a gross swelling of the hyphal tip region. (B) A neighbouring hypha, which was ~300 μm away from the treated hyphae, was unaffected during the period of this experiment, and is shown branching at time 18 min. In both (A) and (B) the field of view has been moved in order to keep the growing hyphal tips in the field of view. Bar = 10 μm.

3.4. Does the laser adversely affect trapped cells?

A spore germination assay to assess possible damage caused to cells by the trapping laser was performed. This involved analyzing the germination of a population of macroconidia in which 50 spores were individually exposed to

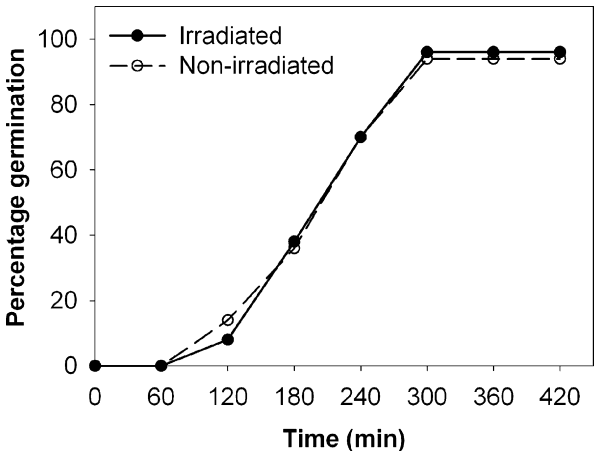

Fig. 13. Macroconidial germination was not significantly affected in spores optically trapped compared with those which were not. Fifty freshly inoculated ungerminated spores were each trapped for 30 s and their germination followed for 7 h. Similar results were obtained when this experiment was repeated three times.

the trapping laser for 30 s. Their rate of germination was compared to that in a non-irradiated population of macroconidia as a control (Fig. 13). No significant difference in the timing of the onset of germination, the rate of germination or final percentage of germinated spores was found.

4. Discussion

This study has demonstrated that optical tweezers are a powerful tool for fungal cell biology research. We have shown that optical tweezers give us the ability: to manipulate whole cells, altering their position relative to one another to study the interaction between them; to manipulate organelles within cells; and apply localized forces, mechanical stimuli or doses of chemicals to cells. All this can be achieved without significant damage to the cells being micromanipulated. The potential opportunities for novel experimentation with optical tweezers when combined with live-cell imaging are vast.

Both ungerminated macroconidia and macroconidial germlings with germ tubes up to 100 μm long have been trapped and moved in a very controlled manner. In these cases, a region of the germ tube of high refractive index is trapped sufficiently strongly to allow the whole germ tube to be moved. Moving one CAT relative to another using optical tweezers has formed the basis of a novel CAT homing assay developed to investigate cell signalling between CATs (Fleißner et al., 2005; Roca et al., 2005). In our previous studies, conidial germlings were only trapped for up to 1 min because of concern that longer periods of irradiation might be deleterious to the cells. However, a big problem we have encountered is that untrapped CATs tend to drift and rotate and their relative positions to each other can change independently of tweezer micromanipulation (Fleißner et al., 2005). Here, we have shown here that it is possible to continuously trap conidia for periods of at least 25 min without any discernible effects on CAT growth or homing. Long term trapping prevents the problem of the drifting of individual CATs. Furthermore, we have recently developed a two-trap optical tweezer system which allows us to prevent the drifting and rotation of both CATs in a homing assay (unpublished results).

The 785 nm laser used in this study did not cause any discernible damage to fungal cells even when used at its full output power (70 mW). One previous study highlighted two wavelengths ranges, 800–850 nm and 950–990 nm, which were the least harmful to the cloning efficiency of Chinese Hamster Ovary (CHO) cells. The same study reported that 740–760 nm and ~900 nm were the most harmful. The duration of exposure, irrespective of wavelength, was also shown to be a significant factor in a cell's ability to survive (Liang et al., 1996). The results from two further studies were consistent with these findings. Photodamage to bacterial cells of *Escherichia coli* was found to be minimal at 830 nm and 970 nm and maximal at 870 nm and 930 nm (Neuman et al., 1999). Cells of the fission yeast *Schizosacch. pombe* were found to exhibit visible photodamage at 880 nm but not 830 nm. Despite this, trapping lipid granules in these yeast cells with 830 nm light at high laser power (5–6 times that used in our study) delayed cell division (Sacconi et al., 2005). In the latter study, a temperature-sensitive process, mitotic spindle elongation, was used to demonstrate that the yeast cells were not substantially heated by the 830 nm trapping laser. Photodamage can potentially be caused by the photochemical generation of reactive oxygen species, two-photon absorption (even from a continuous wave laser of the type used in this study), or transient local heating (Neuman et al., 1999; Sacconi et al., 2005). Reducing photodamage to a minimum requires one to judiciously select an optimal combination of laser wavelength, laser power, and the duration of exposure to the laser beam, as well as taking into account the type of biological sample being irradiated (Liang et al., 1996).

Fungal organelles of higher refractive index than their surrounding cytoplasm (e.g. Woronin bodies) are usually trapped whilst organelles of lower refractive index (e.g. vacuoles) are repelled by the trapping laser. Essentially organelles that are visible when viewed microscopically, due to their refractive index being different from that of the surrounding cytoplasm, can be manipulated by optical tweezers unless they are tethered to other cell components. Trapped organelles with high refractive indices can be moved through the naturally viscous cytoplasm at slow speeds. Organelles with lower refractive indices are repelled by the trap and can be pushed or chased around in the cytoplasm. Organelles or particles that are below the resolution of what can be discerned with a light microscope can also be optically trapped if they can exert a force on the photons of the laser beam by absorbing, reflecting or refracting these photons. In this respect, Bracker et al. (1997) used optical tweezers to move locally high concentrations of secretory vesicles, which individually were not resolvable with the light microscope, to new locations

within hyphal tips and, as a result, induced the formation of new branches at those sites. Use of the repulsion method to move organelles of low refractive index is more difficult and less precise to control but still can be a useful technique for organelle micromanipulation. This method also enabled larger spherical vacuoles to be split in two when they were pushed against the plasma membrane.

One apparent exception to the ability of high refractive index organelles being trapped related to the micromanipulation of the Spitzenkörper. As previously reported, the Spitzenkörper in many fungal species is predominantly of high refractive index and is visually phase dark (Girbardt, 1957; Lopez-Franco and Bracker, 1996; Harris et al., 2005). However, it has been previously reported that the Spitzenkörper is repelled by the trapping laser (Bracker et al., 1997; Wright et al., 2005). Closer analysis of the Spitzenkörper of *N. crassa* reveals it to possess a more complex composite structure containing regions of both high and low refractive index (Lopez-Franco and Bracker, 1996; Harris et al., 2005). This may go someway to explaining why the Spitzenkörper is repelled. Another possible cause of the negative phototropic response of hyphal tips may be because it is photoreceptor-mediated, and involves a photoreceptor in the plasma membrane that is linked via a signal transduction pathway to the Spitzenkörper. Two red/far red light phytochrome-like photoreceptor proteins have been identified as being encoded in the *N. crassa* genome (Galagan et al., 2003; Froehlich et al., 2005).

We found that it was not always possible to move organelles within hyphae with optical tweezers. Some organelles were clearly tethered to other cell components (e.g. cytoskeletal elements) or the plasma membrane. Indeed, cellular organelles are commonly transported along microtubules and actin microfilaments by means of motor proteins (Steinberg, 2000; Lee and Plamann, 2001; Xiang and Plamann, 2003). The tethering of Woronin bodies was demonstrated in *Nectria haematococca* by Berns et al. (1992). They described the trapping and moving of Woronin bodies up to a distance of 2 μm from a septum before the Woronin bodies were lost from the trap and sprung back to the septum. They inferred that Woronin bodies were tethered close to septa by an unidentified elastic filament which was not visible in the light microscope. The Woronin bodies manipulated in the present study were located close to the hyphal tip in the apical hyphal compartment and were not associated with septa or cell cortex. These Woronin bodies were mostly untethered and could be readily moved within the hyphal cytoplasm.

Optical tweezers are frequently used to manipulate transparent beads or microspheres of different sizes, chemical composition or physical properties (Block, 1990; Greulich, 1999; Sheetz, 1998) In this study we used them to: make preliminary measurements of the 'growth forces' generated by the growing tips of germ tubes; mechanically stimulate hyphal tips; and deliver chemicals to localized regions of hyphae.

We previously found that the trapping forces which can be generated by our laser tweezer system with 4 μm beads are in the range of 1–19 pN. We showed here and previously (Wright et al., 2005), that the growth force of a leading hypha of *N. crassa* is sufficient to push a 4 μm polystyrene bead out of a trap even when using the highest laser power (equivalent to a trapping force of 19 pN). However, the extension of germ tubes was inhibited by a bead of similar size trapped with a similar force. This indicates that the growth force generated by a germ tube is less than that of a leading hypha. This is consistent with results obtained by Money et al. (2004) who estimated from measurements using a miniaturized strain gauge that vegetative hyphae generate growth forces which are several orders greater than those that can be measured with optical tweezers. The reason why we used 4 μm beads rather than smaller ones (e.g. 1 μm beads) was to avoid the problem of directly irradiating and potentially influencing the behaviour of the Spitzenkörper with the laser trap. In future studies it will be necessary to determine the force per unit area experienced by the germ tube tip. This will require high resolution imaging of the contact area (e.g. by using low-temperature scanning electron microscopy, Read, 1991).

When germ tubes made contact with a trapped bead they exhibited a swelling growth response. Fungal hyphae have been previously found to show a range of responses to physical mechanostimuli (Read et al., 1992). These include asymmetrical cell growth or spore germination in response to contact with a surface (Kwon et al., 1991; Read et al., 1992; Kuo and Hoch, 1996), directional growth and infection structure differentiation in response to microtopographical signals (Hoch et al., 1987; Read et al., 1992; Gow, 1994; Gow et al., 1994; Read et al., 1997), multicellular infection plaque differentiation in response to compression (Lucas, 2004), and intracellular calcium changes in response to shaking (Nelson et al., 2004; Bencina et al., 2005). How germ tubes sense and respond to trapped beads is not known. However, we have recently found that mechanical perturbation (by shaking in liquid culture) of germ tubes often causes them to exhibit a swelling response and this is preceded by a transient increase in cytosolic free calcium (Marris, P.I., Hickey, P.C., Read, N.D., unpublished results). This suggests that the mechanostimulus may be transduced intracellularly by calcium signalling.

The localized delivery of controlled doses of specific chemicals to different cellular regions is another way to use optically trapped beads. In this study we demonstrated how this could be performed by using porous silica beads soaked in the actin polymerization inhibitor, latrunculin-B (Spector et al., 1983). As a result of this treatment, the extension rate of hyphae was inhibited and they underwent significant swelling in contrast to hyphae that were ~300 μm distant that continued to grow normally. In the future, this could be a very powerful method to deliver chemicals to localized regions of fungal cells when used in combination with live-cell imaging. Porous beads soaked in pharmacological inhibitors and agonists, caged compounds

or fluorescent dyes could be used for this purpose. In our study this technique was used rather crudely and needs some improvement and refinement. It would he highly desirable, for instance, for the beads/microspheres/nanocapsules to retain the chemical until it needs to be released, and then to have a method which actively releases the chemical at the appropriate point in time. If an impermeant microsphere/nanocapsule is used to house the chemical, then it is possible to selectively burst it by laser photolysis (Sun and Chiu, 2004). A third method that has been used to locally apply chemicals is to coat beads with chemicals such as pheromones, and to press the beads up against cells in order to activate ligand–receptor responses (Wei et al., 1999).

In this study we have described an optical tweezer system with a single trap that can be readily use as an experimental tool in combination with live-cell imaging. We have recently developed a two-trap system which we are also using successfully to manipulate filamentous fungi (unpublished). In addition, we have also developed an entirely different tweezer system using holographical optical trapping (Lafong et al., 2006) that involves using computer-generated holograms to create three-dimensional configurations of single-beam optical traps (Grier and Roichman, 2006). Using this approach we have been able to trap and move <6 yeast cells simultaneously (Lafong et al., 2006).

It is clear from the present study, from other biological applications of optical tweezers that have been reported, and from recent developments in tweezer technology, that optical tweezers will become increasingly important in live-cell studies to address novel questions in fungal biology.

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