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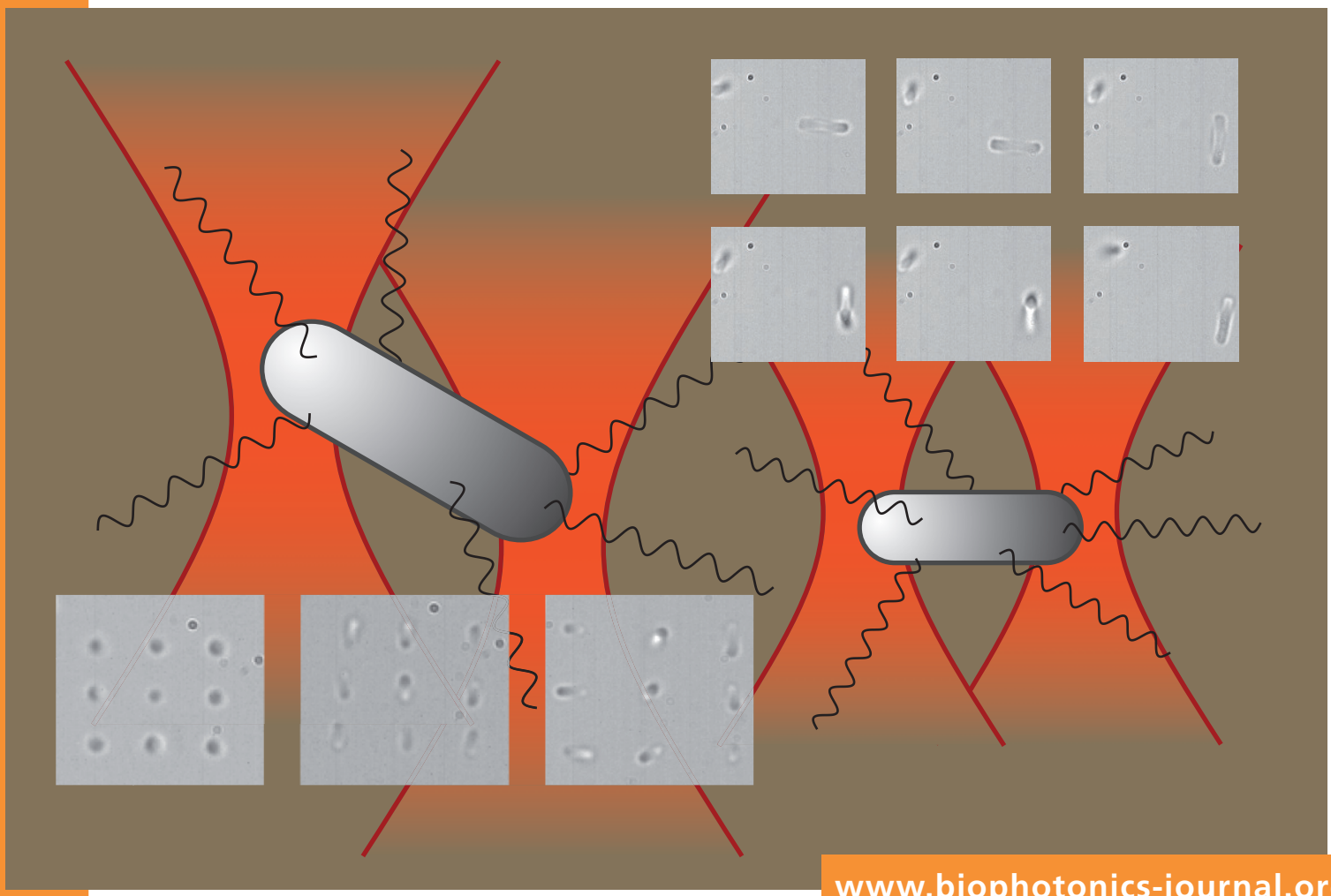

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FULL ARTICLE

Full 3D translational and rotational optical control of multiple rod-shaped bacteria

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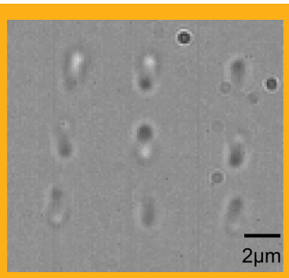
The class of rod-shaped bacteria is an important example of non-spherical objects where defined alignment is desired for the observation of intracellular processes or studies of the flagella. However, all available methods for orientational control of rod-shaped bacteria are either limited with respect to the accessible rotational axes or feasible angles or restricted to one single bacterium. In this paper we demonstrate a scheme to orientate rod-shaped bacteria with holographic optical tweezers (HOT) in any direction. While these bacteria have a strong preference to align along the direction of the incident laser beam, our scheme provides for the first time full rotational control of multiple bacteria with respect to any arbitrary axis. In combination with the translational control HOT inherently provide, this enables full control of all three translational and the two important rotational degrees of freedom of multiple rod-shaped bacteria and allows one to arrange them in any desired configuration.



Array of 3 × 3 bacteria of type *Bacillus subtilis* trapped in lateral orientation.

1. Introduction

Bacteria are highly interesting in biotechnology not only due to their capacity to synthesize materials [1], but also as part of bio-hybrid systems [2] for Lab-on-a-Chip applications. Nano-motors for example are needed for micro-fluidic applications like mixing processes [3] or material transport on nano scales. Thus especially flagellated bacteria that achieve motility due to rotational motion of their flagella are of large interest due to their well suited adaptation to constraints of movements at low Reynolds number [4]. That means they could work as a feasible model for future micro-machines.

For some questions it is sufficient to work with grown carpets of bacteria in which the bacteria are self-assembled [5]. However, investigations on bacterial movement in fluids [6, 7], the cell's reaction on changes of the environment [8], or the formation behaviour of biofilms [9] require detailed knowledge about single cell behaviour. Thus the capability to create defined orientations of bacteria and to manipulate on a single cell level is very important. Highly flexible tools to achieve this goal are optical laser

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traps, demonstrated first by Ashkin, who used two counter-propagating laser beams to stably trap particles of micrometre size [10]. It was also shown successfully with a single beam gradient laser trap (*optical tweezers*), one of the most commonly used trapping approaches nowadays, that manipulation of particles down to the nanometre scale is possible [11]. An important step for today's microbiological investigations was the demonstration of optical trapping not only of inanimate particles but also for living bacteria [12, 13].

While the investigation of a single particle gives insight into many of its properties and features, particle-particle interactions and the need for parallel processing of large amounts of particles in e.g. micro-fluidic applications required an expansion of optical tweezers to control multiple particles independently. This led to the development of dual trap tweezers [14] and finally multiple optical traps generated via phase-contrast methods [15, 16], time-sharing methods [17, 18] or holographic optical tweezers (HOT) [19–23] based on computer generated holograms. HOT are the most suitable technique if independent control of the axial (z) position for many particles is required. In contrast to the other methods, no additional components in the experimental setup are needed, but the axial control is achieved by simply updating the computer generated hologram.

Due to this flexibility and the precise control of a large number of micrometre-sized particles in parallel, optical tweezers are qualified for a wide range of studies in biology, like studies on gene expression of living cells [24], and in micro-fluidic applications. For example optically driven pumps, realized by rotating non-spherical particles, seem to be a promising development [25, 26]. Rotation of these pumps can be realized by transferring optical angular momentum [27, 28] or exploiting birefringence [29, 30].

For all these applications the orientation of particles is irrelevant, the particles find their designated position automatically, or the orientation control is limited to birefringent particles. However, the orientation of asymmetric particles is of high importance if chemically reactive areas or otherwise localised features, e.g. molecular motors, of these particles are studied or have to encounter each other. A defined control of orientation allows for example detailed studies on bacterial motility [31]. All available methods of orientational control of non-spherical particles in general and rod-shaped bacteria in particular can be classified by their degree of control. The most fundamental class of control is two-dimensional control of position and orientation. This can be realized for example by pushing a particle against a surface, thus trapping it laterally in an x-y-plane (perpendicular to the incident laser beam) directly above that surface and inducing a rotation within this plane [32, 33]. More sophisticated control techniques allow three-dimensional translational control but are usually still limited to orientational control in an x-y-plane. Important examples to realize this are traps with higher Hermite-Gaussian modes [34], linetraps [35, 36], or multiple beam traps [31, 37, 38]. Achieving full 3d position control and at the same time control over two or even all three rotational degrees of freedom represents the final level of particle manipulation. For this degree of control, usually two or three single traps are utilized, which can be steered individually to some extent [39–41].

Up to now all demonstrated schemes for complete control of rod-shaped bacteria are restricted to one single bacterium or a very low quantity. The main reason is that these schemes have strong requirements with respect to the mechanical or optical system. Fundamental limitations include timing of mechanically operating components [40, 41] or a direct correlation between quantity of desired traps and complexity of the setup [31, 40]. In this paper we demonstrate that HOT are an optimal tool to achieve full translational and rotational control of an enhanced number of rod-shaped bacteria. It is not limited to a single bacterium or a few bacteria, but enables simultaneous control of tens to hundreds of bacteria.

2. Materials and methods

As a model for rotational micro-machines we chose *Bacillus subtilis* because its genome is available [42] and the flagellar system is controllable both at the genetic level and through pharmacological treatment [8], which offers a good basis to utilise the motor of a bacterium for micro-fluidic applications and to create well-designed interaction scenarios.

2.1 Bacterial strains and media

B. subtilis is a Gram-positive, rod-shaped bacterium with a diameter of about 1 μm and a length of approximately 2.5 to 3 μm. During their replicating process individuals can exceed this length and still appear as one cell although they should be considered more precisely as chains of bacteria. *B. subtilis* is peritrichously flagellated [43, 44] and flagella rotation powers swimming through aqueous environment. For our demonstration of full 3d control, wild type strain BD 630 was defrosted, centrifuged at 9000 rpm for 2–3 minutes and resuspended at room temperature in chemotaxis buffer providing the apropriate conditions for smooth swimming [45]. Motility of the majority of these bacteria was visually confirmed.

2.2 Optical setup

For the observation of the samples we use the commercially available inverted fluorescence microscope Eclipse Ti from Nikon. A 100× oil-immersion objective with a numerical aperture of NA = 1.49 allows high resolution imaging and is also an important component of the optical tweezers setup. This setup (see Figure 1) consists of a 2.5 W $Nd:YVO_4$ laser operating at a wavelength of $\lambda = 1064$ nm allowing manipulation of biological samples with low photodamage [46] and a phase-only spatial light modulator (SLM) (Holoeye, Pluto) with a resolution of 1920 × 1080 pixels. The SLM, working in reflection geometry, is imaged onto the back aperture of the microscope's objective via a telescope, formed by lenses L2 and L3. A dichroic mirror (DM) is used to couple the laser beam into the microscope and together with a filter (stopband at $\lambda = 1064$ nm) it separates the laser beam path from the observation path. The SLM displays a sequence of holograms whose Fourier transform represents the desired trapping geometry. For hologram calculation we use a LabView program [47] which is based on a lenses and gratings algorithm [48]. This system allows independent control of multiple traps interactively. A half wave plate in combination with a polarizing beam splitter (PBS) is used for adjustment of intensity of the beam within our setup in order to minimise photodamage [49].

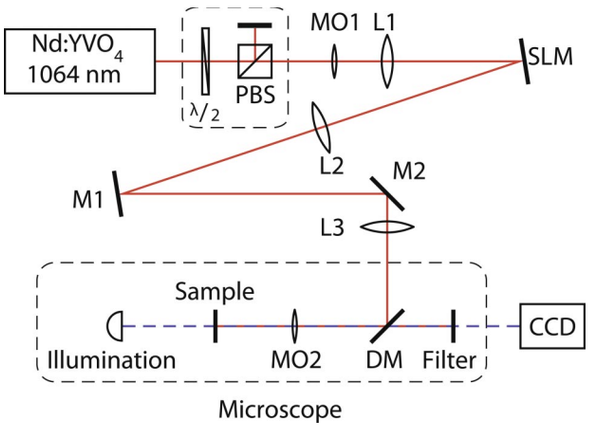

Figure 1 (online color at: www.biophotonics-journal.org) Schematic drawing of the experimental setup for holographic optical tweezers: a half wave plate and a polarizing beam splitter (PBS) are installed for laser power adjustment; a beam expander (microscope objective MO1 and lens L1) illuminates a spatial light modulator (SLM); L2 and L3 form a telescope to couple the beam into a commercial microscope and to image the SLM plane onto the MO2's back aperture; a dichroic mirror (DM) and a filter separate observation and laser path.

Images are acquired by a Photonfocus high-speed camera (MV2-D1280-640-CL) with up to 490 fps at full resolution (1280 × 1024 pixels). This camera is positioned, such that the plane of optical trapping and the observation plane coincide.

3. Results and discussion

In literature there are many examples of three dimensional translational control of rod-shaped bacteria with (holographic) optical tweezers. However, in these studies bacteria are almost always orientated parallel to the laser beam [24, 26, 49, 50]. This axial orientation prohibits the observation of the cell's interior, the bacterium's flow characteristics or movement behaviour within a fluid. We first demonstrate that two adjacent optical traps, generated with HOT, are capable of trapping bacteria in lateral orientation. In contrast to previous studies [31] both traps can be controlled by a driving computer and additional traps can be added easily, without any changes in the mechanical or optical setup. Independent axial control of these traps allows direct access to the orientation of the bacterium with respect to the *x*-*y*-plane [39]. Finally, it is shown that the advanced control through HOT by no means is limited to a single bacterium, but applicable to large numbers of individually controlled bacteria.

3.1 Full 3D and rotational control of a single B. subtilis bacterium

The most common usage of optical tweezers in biological studies is a single trap. A rod-shaped bacterium automatically aligns its major axis parallel to the laser beam as soon as it is trapped (cf. Figure 2g). While the bacterium is trapped in a single trap, its apparent shape as observed with the microscope is that of a circle (Figure 2a). This trapping position is very stable, although very motile bacteria are sometimes able to escape if the laser power is not high enough to compensate for their locomotive force. Rotating the bacterium out of this natural axial position requires a continuously applied torque. We create two spots very close (<0.5 μm) to each other in different *x*-*y*-planes in order to change the orientation of the rod-shaped bacterium. These two traps effectively hold the bacterium at two different points and thus enable to exert torque. When one of the traps is moved slightly, it causes a change of the bacterium's orientation (Figure 2h). This change in orientation is observed as a change of the apparent shape of the bacterium to an ellipse (Figure 2b). With

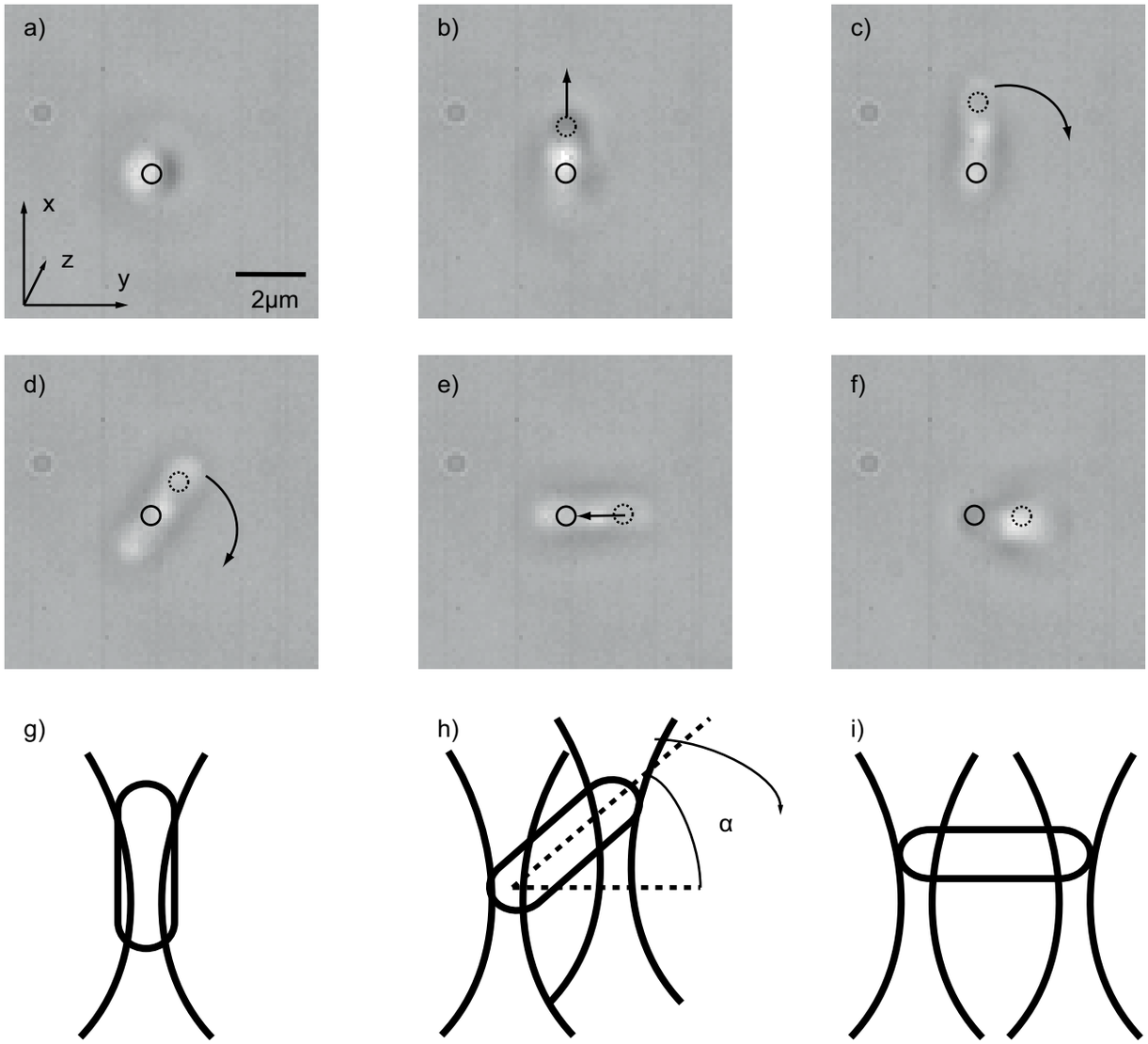

Figure 2 Demonstration of trapping and rotating a single rod-shaped bacterium. Circles indicate the approximate position of the optical traps, the dashed circle is the only one moved; (**a**) Bacterium in axial orientation, trapped by a single trap, (**b**) a second trap is generated near the first one in a different x-y-plane to achieve an angle $-90° < \alpha < 90°$ between the bacterium's major axis and the image plane, (**c**)–(**e**) bacterium is trapped and rotated within the image plane, (**f**) the bacterium is rotated out of the image plane again, (**g**)–(**i**) sketch of the bacterium's position within the two traps, corresponding to (**a**)–(**c**) as a sideview.

the appropriate relative position of both traps, any arbitrary angle α between $-90°$ and $+90°$ with respect to the x-y-plane can be achieved. Figure 2i shows the particular case of a fully horizontally trapped bacterium ($\alpha = 0°$). Therefore, one trap is left fixed and the second trap is moved to the same plane as the first one. This is done by alternately changing its axial position and then adjusting the lateral position, such that the distance between both traps remains roughly constant. The effect of any change in the positions of the traps can be observed in real-time. Very motile bacteria are able to escape from the traps if the laser power is not chosen to compensate for their locomotive forces. Most likely the flagella themselves and their dynamics also influence the optical traps to some extend. Regardless of these effects the horizontal position (Figure 2c) enables a stable side view of the bacterium.

With the axial control of the positions of both traps, any angle α with respect to an x-y-plane can be set. Of course it is also possible to rotate the bacterium in a given x-y-plane. Figures 2d and 2e de-

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monstrate this rotation around the z-axis. Therefore, one trap is kept fixed while the other is translated on a circular trajectory in the x-y-plane until an angle of $\theta = 90°$ with respect to the x-axis is reached. For the intermediate state shown in Figure 2f the trap is moved towards the fixed one resulting in a rotation of the trapped bacterium of $\alpha \approx 45°$ out of the x-y-plane. If one of the traps finally is removed, the bacterium flips back into the remaining trap and takes its initial axial position.

All of these positioning steps can be combined with a three dimensional translation of the particle. By this means, control of all three translational degrees of freedom and the two important rotational degrees of freedom is achieved. The bacterium is free to rotate around its major axis, which allows for example analysing the natural rotation induced by the bacterial flagella motor [31].

In the process of duplication bacteria become longer and form bacterial chains, i.e. they have not yet separated. These chains exceed the usual length of *B. subtilis* of 3 μm and orientational control with only two spots becomes difficult due to the chains dimensions. Although this is not reported in literature, we assume that other schemes for rotational control of rod-shaped bacteria share these difficulties if they are also based on a discrete trap approach [31]. However, with HOT it is very simple to add more traps to effectively stabilise position and orientation [37] of the bacterium. Figure 3 illustrates, that there are no restrictions in applying the suggested technique for longer bacterial chains to reach the translational control together with the control over its orientation. In this example we used four traps which are equally spaced. By moving the group of these 4 involved traps simultaneously and maintaining their relative positions even bacterial chains of such dimensions can be controlled in all three dimensions. The bacterial chain is translated (Figure 3a, 3b and 3f), rotated around both axes (Figure 3b–3e) and remains stably trapped for at least many minutes.

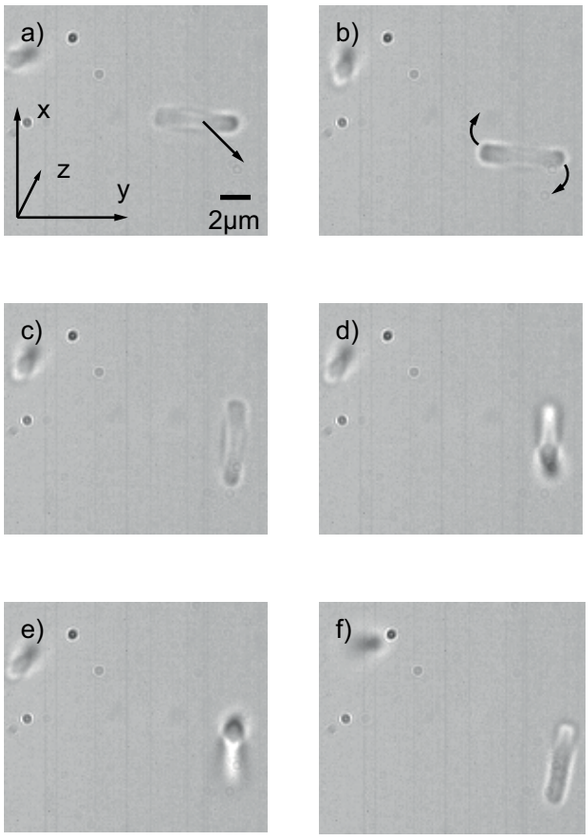

Figure 3 Demonstration of control over orientation and position of a duplicating bacterium (length of approximately 5.6 μm); because of its length, 4 traps are used to show an example of defined motion of bacteria with large lengths; (**a**)–(**c**) orientation of the bacterium in an x-y-plane, (**d**) angle α between image plane and bacterium $35° \pm 5°$ (**e**) angle $\alpha = 27° \pm 5°$, (**f**) demonstration of translation in z-direction, (**a**)–(**f**) bacterium in the upper left corner is adhered to the coverslip; it indicates that the manipulation of the trapped bacterium is done above the coverslip at a certain distance from any boundary.

3.2 Defined arrangements of multiple B. subtilis

Many rod-like bacteria accumulate motility-associated organelles at their cell poles, including formations of flagella bundles or pili [51]. The relative orientation of these bacteria is highly important, e.g. to achieve or control specific chemical reactions or cooperative phenomena. Although it is known that large numbers of bacteria can be trapped and positioned with HOT [23], up to now no scheme has been proposed that enables rotational control of multiple bacteria. With HOT, in contrast to other methods, it is easy to create multiple traps in different x-y-planes and to control them independently. If N optical traps are required to fully control the position and rotational state of a single bacterium, $M \times N$ independent traps enable control of M individual bacteria. As demonstrated in the previous section, typical bacteria can be fully controlled with $N = 2$ single traps. Thus, for full translational and rotational control of M bacteria only 2M traps are required.

A simple example of multiple trapped bacteria is shown in figure 4a. Nine bacteria are arranged in a 3×3 array pattern by means of nine single traps. The bacteria are aligned with their major axis parallel to the optical axis. This is clearly indicated by the apparent circular shape of the rod-shaped bacteria.

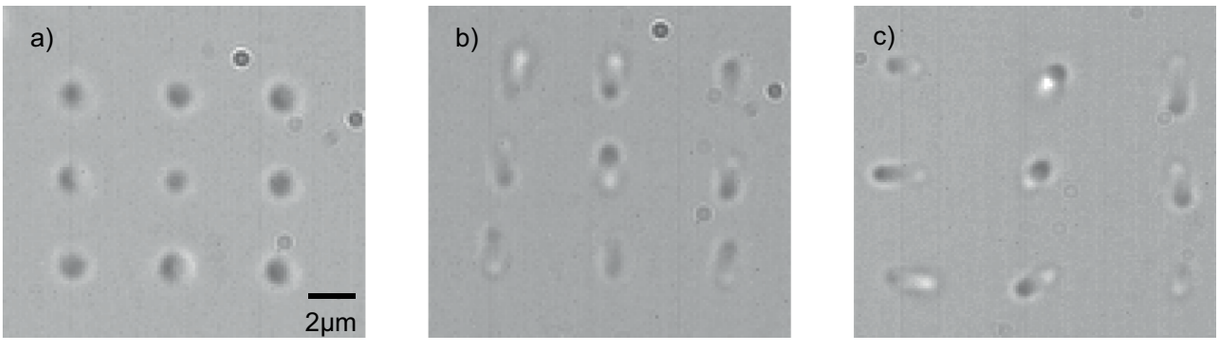

Figure 4 Demonstration of the orientational control of a larger array of *Bacillus subtilis* (**a**) a 3×3 array of bacteria each trapped with a single spot and each in axial orientation, (**b**) according to the case with one bacterium, for each trap another one was generated to rotate all bacteria into the image plane, (**c**) independent control over the bacteria is demonstrated.

In order to demonstrate rotational control with respect to the *x*-*y*-plane, all bacteria are rotated by 90° to the $\alpha = 0°$ orientation (Figure 4b). This is done by adding a second trap close to each of the nine initial traps, resulting in 18 individual traps with approximately 10–15 mW per trap in the sample plane. The additional traps are manually positioned to bring all bacteria into the horizontal orientation. This configuration is well suited to study, for example, the flagella's rotation [30] of multiple bacteria simultaneously or interactions between bacteria.

Figure 4c demonstrates that every bacterium can be controlled individually and shows different orientations simultaneously. The bacteria in the first column are horizontally orientated ($\alpha = 0°$). In the mid column, the bacteria are rotated with an angle $\alpha \approx 45°$ to the image plane. The bacteria in the last column are trapped in horizontal orientation ($\alpha = 0°$) but rotated by $\theta = 90°$ in the *x*-*y*-plane with respect to the bacteria in the first column.

Of course this technique is not limited to simple arrays. Depending on the experimental requirements, almost any configuration with tens of bacteria in arbitrary positions and with arbitrary orientation is possible. Figure 5 demonstrates another example of such an arrangement. Six individual bacteria are trapped on a circle with a diameter of a few bacterial lengths. In Figure 5a three bacteria are fixated laterally; the other three are only slightly rotated out of their initial position. By rotating all bacteria into a lateral orientation, a ring within the image plane can be realized (Figure 5b). This particular arrangement could be useful for example to create confined flow scenarios or complex scattering scenarios which allow detailed bottom-up studies of the formation of structures within bacterial suspensions [52, 53].

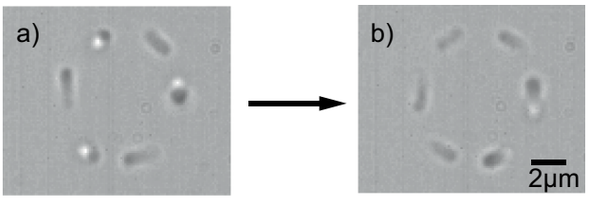

Figure 5 Six bacteria trapped in a circular configuration; (**a**) Three bacteria are orientated in a lateral position, the other three bacteria are rotated some degrees out of their initial axial position; (**b**) all bacteria are arranged within the object plane.

4. Conclusion

Control of position and orientation of rod-shaped bacteria is of topical interest for sophisticated studies on the bacterial motility and the statistics of run-tumble-processes of bacteria [30], their interaction with the environment [5] and for possible applications. Although translational control of single bacteria has been possible for more than 20 years and although there are many schemes available that allow for rotational control, all common approaches have significant limitations. Either they are limited with respect to the accessible rotational or translational degrees of freedom or they are restricted to a few bacteria or even a single bacterium.

By creating two or more optical traps next to each other and being able to tune their relative position precisely with HOT we successfully demonstrated manipulation of the position and orientation of *B. subtilis*. Even more important, the ability of HOT to easily generate large numbers of optical traps provides combined position and orientation control of many bacteria simultaneously. The number of bacteria that can be controlled is limited only by the laser power available at the sample plane

which means structures of up to 40–50 individually controllable bacteria with the present optical setup. In particular the orientation control of multiple bacteria could be of highest interest since it enables for example advanced fabrication of micro-sized machines, parallel observation of multiple bacteria at the same time for investigations where statistical analysis is required or studies on orientation dependent particle-particle interactions.

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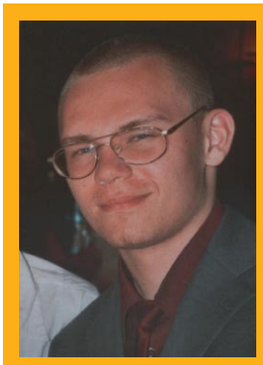

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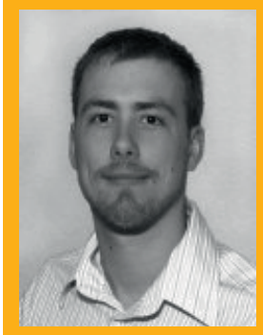
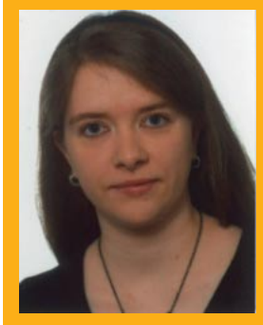


Mike Woerdemann received his Diplom degree in the field of physics in 2007 from the University of Münster, Germany. Currently he is working in the research group "Nonlinear Photonics" of Cornelia Denz. His major research interests are structured light fields and their application for optical trapping.



Stephanie Müller received her diploma in Biology in 2008 and is currently a Ph.D. student in the „Single-molecule-biophysics" research group of Berenike Maier at the LMU München, Germany. Her major research interests are the mechanisms of bacterial motors detected in laser-trap experiments.

Berenike Maier studied physics and received her Ph.D. in 2001 at the TU München, Germany. After three years research in cell biology at Columbia University she started an Emmy Noether group at the LMU München. Since 2004 she is associate professor for biophysics and single molecule analysis. Her research interests are the physics of bacterial interactions focussing on bacterial motors and their regulation through genetic networks.

Cornelia Denz is head of the Institute of Applied Physics and the Center for Nonlinear Science at University of Münster, Germany. In 1992 she received the Lise Meitner-Award for her work on optical neural networks and in 1999 the Adolf Messer-Award for the development of a nonlinear optical motion detection microscope, respectively. Her main research interests are on nonlinear optics and photonics, emphasizing basic nonlinear phenomena in optics as well as applications in information technology and life sciences.

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