Optofluidics, Microfluidics and Nanofluidics
Research Article

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Mike Woerdemann, Florian Hörner, and Cornelia Denz

Structured attachment of bacterial molecular motors for defined microflow induction

Abstract: Bacterial rotational motor complexes that propel flagellated bacteria possess unique properties like their size of a few nanometres and the ability of self-reproduction that have led to various exciting applications including biohybrid nano-machines. One mandatory prerequisite to utilize bacterial nano motors in fluid applications is the ability to transfer force and torque to the fluid, which usually can be achieved by attachment of the bacterial cell to adequate surfaces. Additionally, for optimal transfer of force or torque, precise control of the position down to the single cell level is of utmost importance. Based on a PIV (particle image velocimetry) evaluation of the induced flow of single bacteria, we propose and demonstrate attachment of arbitrary patterns of motile bacterial cells in a fast light-based two-step process for the first time to our knowledge. First, these cells are pre-structured by holographic optical tweezers and then attached to a homogeneous, polystyrene-coated surface. In contrast to the few approaches that have been implemented up to now and which rely on pre-structured surfaces, our scheme allows for precise control on a single bacterium level, is versatile, interactive and has low requirements with respect to the surface preparation.

Keywords: holographic optical tweezers, micromanipulation, Bacillus subtilis, rod-shaped bacteria, microfluidics

DOI 10.2478/optof-2014-0001
Received January 29, 2014; accepted February 23, 2014.

1 Introduction

Microscopic arrangement, patterning, and organization of selected bacterial cells is believed to be the basis for novel perspectives in microbiology and biomolecular nanotechnology [1, 2] including nano- and microfluidic applications. From the microbiologist's point of view, the microtechnological control of single and multiple bacterial cells enables opportunities to understand the structure and intracellular organization of bacteria, phenotypic behaviour, cell culture, the biology of a single cell and to collect quantitative microbiological data [1, 3, 4]. Beyond that, the unique properties of bacteria open the door for novel, dynamic hybrid nano- and micro-devices [2] like propellers or induction of fluid dynamics at low Reynolds number.

Most motile bacteria are propelled by helical flagellar filaments that are rotated by a molecular motor complex [5]. The molecular motor is driven by an inward-directed proton flux, powered by a protonmotive force and is a most promising candidate as a nano scale bio motor for nano- and micro devices due to its ability to efficiently convert chemical energy into mechanical output on a nanoscopic scale. It even can be powered directly by external electric fields thus enabling the construction of functional nanoelectromechanical systems (NEMS) [5]. The force a single bacterial flagellar motor can exert is in the order of a few hundred piconewtons [6], and although a bacterium may contain many flagella distributed around its body, only the cooperative action of many individual bacteria can achieve significantly higher forces to move fluid and thus nutrition over larger scales, being independent of diffusion [3, 7].

A prerequisite for reasonable utilization of bacterial motors in general and efficient cooperative behaviour in particular is a method to attach, i.e. adhere or tether a single or a multitude of individual bacterial cells to a surface in a defined way, that gives the best possible control over the location and state of every single cell. With this demand in mind, a couple of techniques has been developed, mainly relying on pre-structuring a surface by various micro-patterning schemes such that areas, which allow bacteria to attach, alternate with areas, that prevent bacterial attachment as far as possible [1, 8, 9]. Many materials and coatings with well-known properties are available for both areas, as investigation of bacterial adherence to surfaces has a long tradition [10, 11], however the process of micro-patterning requires a relatively high effort, suitable material know-how and equipment. Moreover,

Mike Woerdemann, Florian Hörner, Cornelia Denz: Institute of Applied Physics, University of Münster, Corrensstraße 2/4, 48149 Münster, Germany

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fine control over individual bacteria is very limited and the approach lacks flexibility and reconfigurability with respect to new arrangements, a matching surface structure has to be developed and produced.

A few publications made up to now use of an optical tweezers based approach for studies on the single cell level [10, 12], though. Following a similar approach with respect to the optical tweezers tool, we extended it with respect to the capabilities of holographic optical tweezers to work with multiple objects simultaneously, thus increasing the throughput as well as allowing to freely orient trapped objects in order to potentially make use of site-specific interaction regions. Therefore, in this paper, we demonstrate and elaborate a generalized scheme to create structures of a single up to a few hundred individually selected and controlled motile bacteria on a functionalized, homogenous surface without the need of pre-structuring. It is a flexible, reconfigurable method, which can be easily realized by surface preparation and can be applied to a range of available materials and coatings. Our scheme relies on holographic optical tweezers (HOT) [13, 14] that are used to trap and confine single and multiple bacterial cells and move as well as attach them in a controlled way to a surface that is optimized to enable the bacteria to adhere easily. To get an estimate of the size on which such patterns should be structured we analysed the flow of fluids in close proximity to attached bacteria.

2 Experimental Procedures

2.1 Holographic Optical Tweezers System

The optical system used for observation as well as for handling and attachment of the bacteria consists of a microscope workstation that combines a commercially available inverted Nikon Ti Eclipse fluorescence microscope with integrated, in-house developed and implemented holographic optical tweezers (HOT) [14, 15]. The sample is positioned with a precise piezo-motor-driven x-y-z translation stage. For highest-resolution observation a microscope objective with a numerical aperture of NA = 1.49 (100× magnification) is used. The focal plane of this objective is imaged onto a Photonfocus CMOS high-speed camera with a resolution of 1280 × 1024 px and a frame rate of up to 490 Hz at full resolution with a field of view of 153.6 × 122.88 μm^2 being observed. HOT are integrated by means of a 2.5 W Nd:YVO_4 laser emitting at a wavelength of λ = 1064 nm (chosen to reduce photodamage [16]) which illuminates a Holoeye Photonics Pluto phase-only spatial light modulator (SLM) with a resolution of 1920 × 1080 px.

A LabView program [17] is used to calculate holograms of the desired trap geometries, which are displayed on the SLM. The SLM plane is imaged onto the back aperture of the microscope objective. Its Fourier transform, which is generated by focusing through the microscope objective into the observation plane, represents the distribution of the optical traps.

2.2 Bacterial Cell Preparation

Bacillus subtilis of strain BD 630 was kept at −80°C (10 % glycerol was added to the bacterial culture before freezing) for storage. For our experiments, a small amount was unfrozen and centrifuged at 9000 rpm for 5 minutes to separate *B. subtilis* from the used frost protection agent. The bacteria were resuspended in a chemotaxis buffer (0.14 mM $CaCl_2$, 0.3 mM $(NH_4)_2SO_4$, 0.1 mM EDTA, 5 mM K-Lactat, 0.05% Glycerol, and 0.01 mM potassium phosphate, ph = 7[18]) to achieve bacterial motility.

2.3 Bacterial Adherent Surface Preparation

Different surfaces were investigated. i) Standard microscopy cover glasses were used as supplied. ii) Special petri dishes with a cover glass equivalent plastic bottom with a physical surface treatment for cell adherence ("ibiTreat", Ibidi, Martinsried, Germany) were used as supplied. iii) Similar petri dishes from Ibidi with Poly-L-Lysine coating and iv) Collagen IV coating were used. v) Standard cover glasses were coated with polystyrene. For this purpose, after washing the cover glasses 10 times with purified milli-Q water (Merck Millipore, Billerica, MA, USA) they were put into 2 % Hellmanex solution (Hellma Analytics, Hellma GmbH & Co. KG, Müllheim, Germany) for about 2 hours. Then they were dried with a dry airflow of N_2. A constant stream of polystyrene, dissolved in toluene, was sprayed manually with a pipette onto the spinning cover glass. In a final step the cover glasses are baked out at a temperature of 120°C for 3 hours.

2.4 Scheme for Structured Bacterial Adherence

Holographic optical tweezers (HOT) are known to be able to trap hundreds of bacteria simultaneously with full control on any individual bacterium [14, 19, 20]. However, the

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imposed pattern of bacteria is only maintained as long as the laser is interacting with the bacteria. For the vision of a self-reliant bacterial driven hybrid micro-machine [21], fixation of the bacterial structure without requirement for continuous laser exposure is mandatory because continuous exposure, depending on the wavelength of the utilized trapping laser and the laser power, may result in photo damage [16, 22], and thus reduction of the bacterial motor capability. Only a few schemes have been demonstrated that allow for fixation of bacterial cells after prestructuring with HOT without preventing their viability, and all of them rely on embedding in gel-like supports [19, 20] that necessarily also immobilize the bacterial flagella, thereby stalling any motion of the bacterial motor. In contrast, our proposed scheme does neither require a specifically patterned surface nor a fixation material therefore allowing utilizing bacterial motion.

We use HOT to trap a multitude of bacteria, prestructure them and attach the whole structure to a surface. This process can be repeated to create patterns of almost arbitrary extent and complexity, which is shown exemplarily with structures of regular arrays. 1-6 holographically generated optical tweezers were created to trap a corresponding number of *B. subtilis* in a user-defined pattern. These traps were placed a few micrometres above the cover glass to prevent immediate adherence of bacteria. Laser power was adjusted to decrease the strengths of the traps such that every trap was able to trap exactly one single bacterium. In order to finally attach the defined structure of *B. subtilis* cells to the cover glass, the traps were moved in axial direction to the immediate vicinity of the surface for a few seconds, where they adhere and remain in the predefined structure when the laser is disabled (cf. Figure 1). Although rod-shaped bacteria like *B. subtilis* preferentially align along the axis of the incident beam, whereby the described technique is per se especially suitable in combination with bacteria having a single polar flagellum, the described procedure is due to the use of HOT not limited to such species or such an orientation [14]. The typical laser exposure time is in the order of ten seconds, minimizing the effects of photo damage [16].

2.5 Quality of Surface Adherence

Different bacteria utilize different mechanisms of surface adherence [11]. While some species are known to adhere end-on [10], the employed *B. subtilis* strain usually adheres by means of their flagella. In the common case that exactly a single flagellum adheres, the bacterial body is free to rotate, only tethered by the flagellum [23]. Tethered

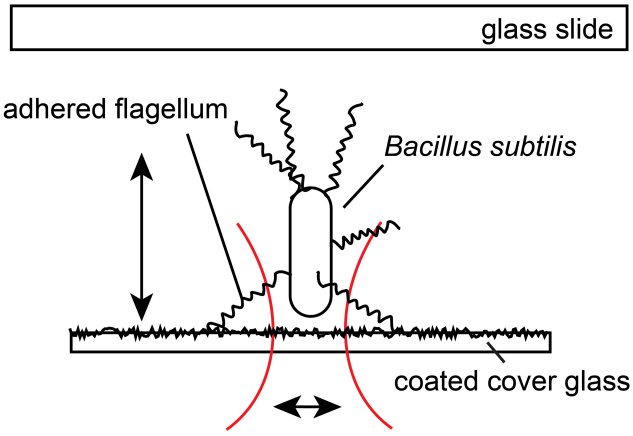

Fig. 1. Bacterial cells are approached to an appropriately prepared surface by means of optical tweezers and adhere, dependent on the surface coating, with their poles, flagella or the whole cell body.

bacteria that do not exhibit rotation in our study are either attached by more then one flagellum, by other adherence mechanisms, or they are non motile or dead. Conversely, an actively rotating tethered bacterium unambiguously is motile.

Bacterial adhesion depends strongly on the ionic strength and pH of the used buffer as well as cell surface characteristics like its hydrophobicity or its charge [24, 25]. Therefore, to obtain the desired strength of adhesion, all these parameters need to be thoroughly adjusted, according to the used type [25] as well as strain of bacteria [24].

2.6 Analysis of Bacteria-induced Flow

Flow analysis was performed by tracing fluorescent beads sufficiently small to reveal details of the flow in a submicrometer regime. However, in order to avoid undesired interference of beads with the manipulation and adherence of the bacteria first *B. subtilis* was manipulated within the pure chemotaxis buffer. Several bacteria were trapped and attached to the polystyrene-coated glass surface following the procedure mentioned above and shown in Figure 1 with the exception that no glass slide was used. In a subsequent step a small amount of fluid containing the fluorescent beads (Thermo Scientific, Fremont, CA, USA; R600, diameter of 0.6 µm, absorbing/emitting at λ = 542/612 nm) was added with a pipet. Movement of these beads was captured over a period of 4 seconds at a frame rate of 50 fps. After this, a PIV (particle image velocimetry) algorithm [26, 27] was applied to analyse the fluid flow

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within the field of view. For the analysis interrogation windows of 20×20 px and an overlap of 0.75 have been chosen.

3 Results

3.1 Optimization of adherence conditions

In order to find optimal working conditions, we characterized different potential substrates for their adherence properties with the employed *B. subtilis* strain. For this purpose, we analysed polystyrene coated glass, untreated glass, Poly-L-Lysine, Collagen IV, and "IbiTreat" surfaces (see above). For each material, 5 bacteria were trapped simultaneously, moved to the surface as described above and kept there for 20 s in order to let them interact. This procedure was repeated 10 times. The results are summarized in Figure 2a). Glass and polystyrene provide the best probabilities for adhering bacteria; roughly 50 % of the bacteria adhere. The bacterial adhesion is for example decreased due to strong motility, leading to a reduced interaction time with the substrate. In order to exclude time-dependent influences, we always tested glass as a reference (with 6 sets of 5 bacteria) before and after testing a new surface. However, since all experiments on glass result in a similar probability, we conclude that there are no significant time-dependent influences, at least during a time interval of 6 hours.

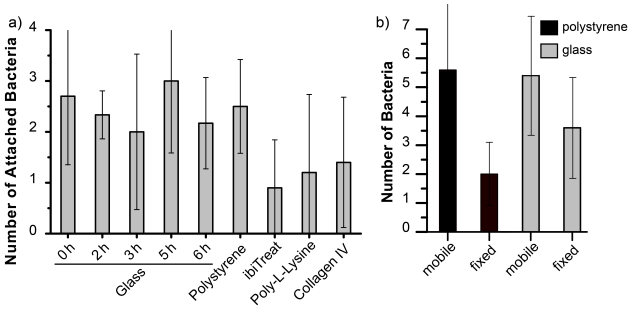

Fig. 2. a) Attachment of sets of 5 bacteria, moved to different surfaces. The non-glass surfaces were examined chronologically from left to right. Examination of glass was repeated between two different surfaces to ensure no time dependent effect (life cycle dependent adherence), therefore examination of glass and non-glass surfaces was alternating. For all examinations 10 sets of 5 bacteria were analysed, except for glass at 2-6 h, for which 6 sets were analysed. Overall glass as well as polystyrene showed the best probability of attachment for further experiments. b) Number of bacteria in a given field of view that were attached to the surface either entirely ("fixed") or in a way that they were free to rotate ("mobile"); number of fields of view tested is n = 5 for both surfaces.

Adherent bacteria might either be completely immobilised ("fixed") or still able to rotate ("mobile"). We examined the mobility properties of bacteria attached to the different substrates, in order to account for possible differences in the quality of adherence (see above). For this, we brought a drop (20 µl) of bacteria suspended in chemotaxis buffer in contact with the surface and counted the number of self-adhered bacteria within the total field of view after 2 minutes. Over the following 3 minutes the evaluation was repeated in 4 additional areas of the surface. We considered a bacterium as "mobile" if it showed at least passive (rotational) movement, either due to energized motors (motile bacteria) or only due to Brownian motion, and "fixed" otherwise. We restricted the investigation to the two substrates with the highest probability of adherence – glass and polystyrene. As can be seen in Figure 2b), we found a certain tendency of polystyrene to have a higher chance to enable mobile adhered bacteria. For this reason and because the prepared polystyrene-coated surfaces are better defined (cf. preparation procedure described above) than the untreated glass substrates, all following experiments were carried out on polystyrene surfaces.

3.2 Creation of extended arrays of attached bacteria

Figure 3 shows an array pattern of 5×5 bacterial cells attached to a polystyrene-coated cover glass that we created as an example of a wide variety of possible patterns. Approximately 50 % of the bacteria adhere to the surface within a contact time of roughly 2 seconds. Defects in the pattern can easily be filled with additional bacteria. Many of the bacteria are rotating around a rotation axis in close proximity to the cell body as indicated by their relative positions in the image sequence. These bacteria are not only obviously motile, but following a simple mechanical model are tethered to the surface with exactly one flagellum [16, 19]. Attachment with more than one flagellum would prevent any continuous rotation. The majority of the bacteria, however, are immobilized. As there was no pre-selection of the bacteria before the structuring process, a certain amount of the non-rotating bacteria might be dead or in a non-motile state. Another reason for absence of rotational motion, however, is the unspecific nature of the mainly hydrophobic [28] or van-der-Waals interaction of the bacterial cell with the polystyrene surface [11] that enables adherence of poles, cell body and flagella. Polystyrene coated surfaces were chosen to demonstrate that even very cheap and easily produced materials enable stable structuring of bacterial cells and due to the well-

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known property of polystyrene not to affect the viability of adhered bacteria [28].

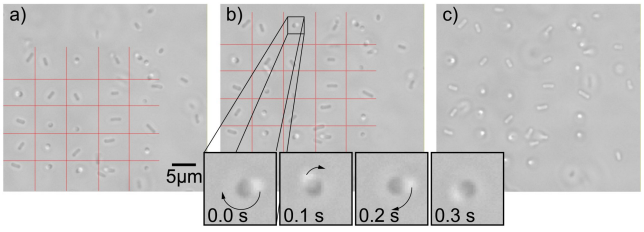

Fig. 3. Array pattern of 5×5 *B. subtilis*, attached to a polystyrene-coated microscope cover glass. (a,b) Red grid illustrates the regular pattern of the attached bacteria. The sample is translated transversally (b) and axially (c) to demonstrate that all bacteria are attached to the surface. Insets show rotation of one bacterium.

However, our approach is general, and any sufficiently transparent surface modification with more specific or more efficient binding properties can be used with our scheme, like e.g. suitable antibodies, proteins, or polysaccharides [11], in order to attach defined regions of the cellular body. The essential difference for patterned bacterial attachment being that the surface does not need to be prestructured, almost any modification can be prepared in a very simple, i.e. homogeneous way with established techniques like spin-coating or vaporising.

3.3 Analysis of flow during bacterial rotation

To get an estimate of the structure size needed for arrays to achieve interactions between bacteria, we analysed the impact by single rotating bacteria on the surrounding medium. With the above-described procedure we attached bacteria to a coated glass surface and subsequently added fluorescent polystyrene beads as standard tracer particles. We observed the action of two exemplarity long-time attached bacteria (Figure 4, black arrows) on the fluid by analysing the motion of the tracer particles by classical particle image velocimetry [26, 27].

We would like to remark that although neither fluorescence illumination nor tracer beads on their own had any impact on the rotation of the attached bacteria, that is bacteria were in a continuous rotation state over an observation period of 10 s, in combination they may lead to an inhibition of rotation within a few seconds (cf. Table 1, these conditions were checked in several fields of view independently). We assume the Stokes shift of the fluorescent beads in combination with strong fluorescence illumination, which needs to be maximized in order to observe

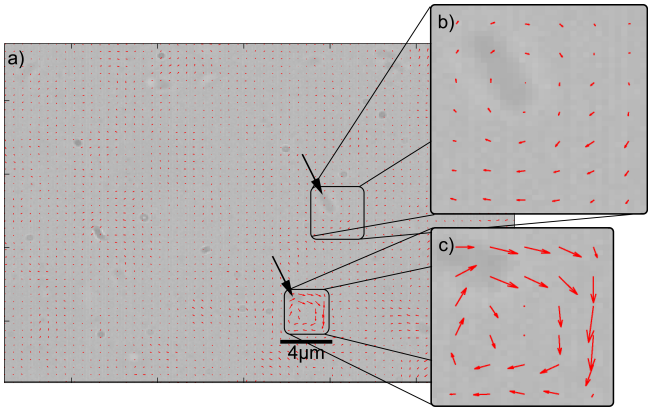

Fig. 4. Analysis of the flow induced by two attached bacteria (black arrows). Fluorescent tracer particles were analysed performing PIV over a sequence of 200 images. a) 30 × 36 µm^2 region of interest with the overlaid evaluated vector field (red arrows); b) + c) Enlargements of the region around the attached bacteria demonstrating circular flow in close proximity to them.

the tracer particles with a frame rate of 50 fps, lead to a heat induced effect on the bacteria.

Table 1. Influence of factors on the rotation of adhered bacteria. Rotation of adhered *B. subtilis* was categorized 'continuous' if the bacteria were performing persistent rotation during the observation period.

		Tracer particles	
		absent	present
Fluorescence illumination	absent	continuous	continuous
	present	continuous	< 5 s

Therefore, the analysis of the bacteria-induced flow has been limited to a period of 4 s. The evaluation was performed using a PIV algorithm [27] and averaging over the full observation length of 200 images. We found that the bacteria depicted in Figure 4b) and c) induced a circular flow of noticeable though different strength (indicated by different length of red arrows, Figure 4 b) and c). This difference can be explained simply by the natural variation in the strength of rotation (rotation frequency varies between individual bacteria as well as for the same bacterium over time, see for example supplementary movie 1 and [29]). We detect an influence of the bacteria rotation on the surrounding fluid. Our method gives a range of about 2 µm around the rotational centre, displaying a reasonable and reproducible limit for the distance of bacteria within larger structures to create strong impact on the fluid flow. For detailed studies on the torque bacteria can generate, see e.g. [30, 31].

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3.4 Extended arrays of rotating bacteria

Finally, we combined our previous findings to demonstrate a pattern of fully rotating bacteria. To ensure best rotation properties, we preselected all bacteria individually with respect to their motility as indicated by their rotational motion frequency in the optical trap. Only bacteria that have visually been confirmed to exhibit a significant motion in the optical traps clearly different from standard Brownian motion are attached to the surface in a pattern of 2×4 bacterial cells (cf. Figure 5). All bacteria are rotating continuously with a maximal frequency of $v = 9\ \text{Hz} \pm 1.4\ \text{Hz}$ for at least a couple of minutes, changing their rotation direction occasionally and abruptly from counter-clockwise to clockwise or vice versa. Interestingly, it is sometimes possible to stop the rotation of a selected bacterium and completely immobilize it by means of re-exposure to the laser trap, even though power level and exposure time are much too short for serious photo damage [16]. We believe that the optical trap increases the cross section of surface interaction by imposing geometrical constraints to the position of the bacterial cell with respect to the surface and hence favours adherence of additional flagella or extracellular components.

The initial screening process, ensuring the bacteria's desired qualities, is much easier to realize if performed before attachment, underscoring the advantage of using optical traps for the described selective attachment. This screening process allowed the assembly of a fully functional array (in terms of the capability to rotate) of bacteria.

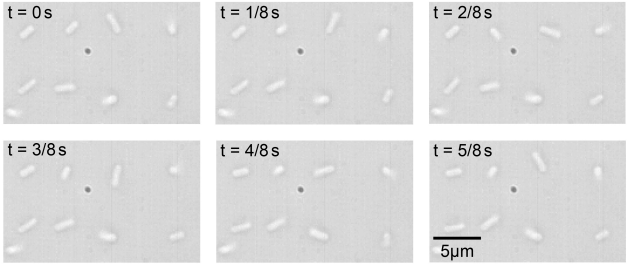

Fig. 5. Array pattern of 2×4 vivid *B. subtilis*. Bacteria are rotating with different frequencies as indicated by the image sequence. See also Supplementary Movie 1.

4 Discussion

In conclusion, we have suggested and demonstrated an innovative scheme for HOT-induced attachment of arbitrary patterns of living bacteria to a surface. The fundamental difference in contrast to established techniques of bacterial surface attachment is that our technique allows pre-structuring of bacteria with very high precision and flexibility by holographic optical tweezers. In a subsequent, application-oriented step, bacteria can be deposited on a homogenous surface. Following this protocol, not a specifically structured but rather homogenous and therefore easy to manufacture surface can be chosen. Thus, numerous configurations of self-propelled bacterial rotational motors can be arranged on surfaces in such a way that they may act as mixers, guide motors for particles, or induce dynamic fluid motion. Choosing specific bacterial strains with desired rotational [32–35] or adherence properties [24, 25] will further fine tune these applications.

To summarize, we demonstrated successfully the controlled adherence of patterns of motile bacteria to a simple and inexpensive polystyrene surface and showed the impact of these arrays on the fluid motion. Single bacteria can be selected individually for desired properties like their motility. The low requirement of a homogeneous surface allows implementing an easy way to prepare surface modifications that enable more specific binding to e.g. the poles of bacterial cells and thus even higher organized assemblies.

So far, studies have been performed on either a single bacterium or on large amounts or baths of bacteria. With the presented technique, we bridge the gap between these two extreme cases. On the one hand, this technique opens the door to previously inaccessible experiments on interactions of bacteria being able to specifically design scenarios to investigate basic scientific questions. An example is e.g. hydrodynamically-mediated synchronization, or chemical interactions between bacteria. We recently could demonstrate higher-order synchronization of two bacteria attached in such a way on a polystyrene surface.

Moreover, combining the presented approach with nano-containers [36] to biohybrid nanorobots capable of holding drugs or biological material like RNA or proteins, novel methods for self-propelled drug delivery can be envisaged and created. Recently, some of us could demonstrate the facilitation of these bacterial nanorobotic structures. These novel biohybrid systems pave the way to novel biomedical approaches in healing: for guiding these nanorobots, the ability of sensing their environment

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would make bacteria a well-suited choice to transport a drug towards a location of interest, like an inflammatory site, within a body.

Therefore, due to its simplicity, precision and versatility, we believe that our original approach is ideally suited for prototyping of biohybrid micro- and nano-machines as well as for fundamental studies on hydrodynamic interactions between individual bacteria [37] or lab-on-a-chip applications [38].

Acknowledgement: The authors thank B. Maier and J. Ribbe, both University of Cologne, for kindly supplying *B. subtilis* samples and for their help in preparing the polystyrene coated cover glasses and C. Alpmann, University of Münster, for helpful discussions. The authors would like to thank the Deutsche Forschungsgemeinschaft for the financial support of this project in the frame of the German-Chinese transregional research project TRR61.

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Supplemental Material

Supplemental Movie 1
This movie shows the rotating array pattern of 2×4 bacteria of Figure 5 in real time.

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