Optical trapping inside living organisms
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ABSTRACT
We use optical tweezers to investigate processes happening inside living cells. In a previous study,\textsuperscript{1} we trapped naturally occurring lipid granules inside living yeast cells, and used them to probe the viscoelastic properties of the cytoplasm. However, we prefer to use probes which can be specifically attached to various organelles within the living cells in order to optically quantify the forces acting on these organelles. Therefore, we have chosen to use nanometer sized gold beads as probes. These gold beads can be conjugated and attached chemically to the organelles of interest. Only Rayleigh metallic particles can be optically trapped\textsuperscript{2} and for these it is the case that the larger the beads, the larger the forces which can be exerted and thus measured using optical tweezers.\textsuperscript{3} The gold nanoparticles are injected into the cytoplasm using micropipettes. The very rigid cell wall of the \textit{S. pombe} yeast cells poses a serious obstacle to this injection. In order to be able to punch a hole in the cell, first, the cells have to be turned into protoplasts, where only a lipid bilayer separates the cytoplasm from the surrounding media. We show how to perform micropipette delivery into the protoplasts and also how the protoplasts can be ablated using the trapping laserlight. Finally, we demonstrate that we can transform the protoplasts back to normal yeast cells.

Keywords: optical tweezers, in vivo, \textit{Schizosaccharomyces} pombe, cell mechanics, gold nanoparticles, protoplasts, micropipettes, ablation

1. INTRODUCTION
Optical tweezers are one of the few nano-tools which are capable of reaching inside living cells without punching a hole through the membrane. Therefore, this technique has superior potential for probing processes happening inside living cells. In our study we use \textit{Schizosaccharomyces pombe} yeast cells as model organisms to study the nano-mechanics inside living cells. In order to use the optical trap inside living cells it is crucial that there exists a handle for the technique, i.e. a dielectric object with an index of refraction larger than the surrounding media which has a size that makes it appropriate for trapping. Naturally occurring lipid granules in the cytoplasm possess these properties, but they cannot be made to adhere to the intracellular organelles. These granules are ideal for studying the viscoelastic properties of the cytoplasm\textsuperscript{1} or they can be used to push around e.g. the nucleus inside the cell.\textsuperscript{4} However, for quantitative measurements of the forces acting on a particular organelle the handle needs to be firmly attached to the organelle and its size has to be characterized to sufficient precision to allow for quantitative determinations of forces. Therefore, we insert well characterized gold beads into the living organism, and by functionalization of the particles with anti-GFP (green fluorescent protein) these beads can specifically bind to organelles expressing GFP. In practice, we have a number of yeast strains with different organelles expressing green fluorescent protein (GFP). In general, \textit{in vivo} studies suffer from being very complex, but still they are receiving an increasing amount of interest and the living cell has the potential of becoming the test-tube of the 21st century.

\textit{S. pombe} yeast cells work as excellent model organisms for human cells. E.g. the cell division process of \textit{S. pombe} to a large extent resembles that of human cells. Compared to human cells, the \textit{S. pombe} yeast cell has the advantage that is has a very reproducible behavior, it is easy to work with, its genetics is well determined, and its cytoskeleton is fairly sparse. Especially the simplicity of the cytoskeleton is an advantage for our experiments as we in the future wish to study e.g. the forces pulling on the nucleus during cell division and the polymerizing forces of microtubulus \textit{in vivo}.

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Figure 1. *S. pombe* yeast cell, picture taken with DIC microscopy. The lipid granules naturally occurring inside the cell are clearly visible. The width of the cell is approximately 4 µm and the length ∼ 12 µm.

One disadvantage of the *S. pombe* yeast cell is the extremely stiff outer cell wall and the inability of the cell to engulf foreign objects. Other cell types, as e.g. fibroblasts readily engulf foreign objects as polystyrene microspheres, and this cell type has been used to study intracellular transport. However, due to the above mentioned advantages of *S. pombe* it is our choice as model system.

The lipid granules that can be optically trapped and which we used to probe the viscoelastic properties of the living cell cytoplasm are easily observable in a microscope. Figure 1 shows a picture of such a yeast cell taken with differential interference contrast (DIC) microscopy. The lipid granules are visible, however, the nucleus and the other organelles are not. These structures need to be tagged e.g. by fluorophores in order to become clearly visible.

Therefore, our library of strains have different organelles expressing green fluorescent protein. Figure 2 shows four such cells which have respectively the membrane systems and microtubules fluorescently marked with GFP. The cells are in interphase except the lower right which is in late M phase and has completed division of the nucleus into two daughter nuclei.

Monitoring the motion of such lipid granules over 5 decades of time using optical tweezers and multiple particle tracking showed that different types of motion occur inside living cells; the types can be distinguished by the exponent $\alpha$ in the power law behavior of the mean square displacement $\langle |\Delta \vec{r}(t)|^2 \rangle \propto t^{\alpha}$. On short timescales only subdiffusive motion is present ($\alpha = 0.75$). However, other types of motion are also present as e.g. confined motion ($\alpha = 0$), normal Brownian motion ($\alpha=1$), and superdiffusive motion ($\alpha > 1$). Each of these classes of motion is the sign of a particular biological process taking place inside the living cell.

The long term goal of our efforts is to quantitatively measure forces inside living cells. There are several obstacles on the route to this goal, some of these are dealt with in the present paper. The outline of the following is that first we present the arguments for a qualified choice of which sizes of gold beads to use for this study. This, of course, relates to the optical trapping properties of gold nanoparticles. Then we show how to get gold nanoparticles inside the yeast cells; this step involves enzymatically transforming the yeast cells into protoplasts and insertion of the beads by micropipetting. Also, we show how the trapping laser light can be used for ablation of the protoplasts. A very crucial step is the revival of healthy *S. pombe* yeast cells from protoplasts, and we describe this process in detail.

2. METHODS

2.1. Optical trap

We use a single beam optical trap based on a 1064 nm Nd:YVO$_4$ laser and implemented in an inverted Leica DMIRE2 microscope. The sample is mounted on an XYZ piezo stage (PI 731.20) with capacitative feedback and nano-meter resolution. The detection system includes a Si-PIN quadrant photodiode and a CCD camera (Sony XC-E150, 25 Hz). The optical trap and the detection schemes are as described earlier and have a spatial resolution of about 2 nm in all dimensions and a sampling frequency up to MHz using the quadrant photodiode.
Figure 2. Gallery of *S. pombe* yeast cells. The cells are in interface except the lower right which is in mitosis and have respectively the membrane systems and microtubules fluorescently marked.

2.2. Preparation of yeast cells

The type of fission yeast strain used in this study was SPK10(h−). Cells were transformed into protoplasts and regenerated by slightly modified standard procedures. Cells were transformed into protoplasts and regenerated by slightly modified standard procedures. Cultivation of the cells was performed at 30°C on agarplates containing rich media (glucose, nitrogen, sulfate and aminoacids). The cells were transferred to YPD broth (yeast extract 1%, peptone 2%, dextrose 2%) 15 hours before removal of the cell wall. Cells were washed once in millipore water and resuspended in E-buffer containing 50 mM sodium citrate and 100 mM sodium phosphate (pH = 5.7) containing 1.2 M sorbitol as osmotic stabilizer. Enzymatic treatment was performed by adding 5mg/mL enzymes extracted from Trichoderma Harzianum (Sigma-Aldrich) and incubating for 2 hours at 30°C in a rotary shaker. The transformation frequency was 100%. The protoplasts were harvested by centrifugation at 270 g and washed once in E-buffer containing 1.2 M sorbitol and once in E-buffer containing 0.6 M sorbitol. Finally, the protoplasts were inoculated into regeneration medium (YPD medium containing 0.9 M sorbitol). In order to monitor the regeneration process in the microscope the protoplasts were incubated in a temperature controlled chamber (chamber: RC-30, controller: TC-324B/344B Warner Instruments) at 30°C ± 1°C for 20 hours. The regeneration frequency was close to 100%. One picture was taken every 15 seconds to monitor the whole process. The protoplasts used for injection experiments were transferred into E-buffer containing 1.2 M sorbitol.

2.3. Sample preparation for microinjection

The yeast cells were flown into a perfusion chamber. This chamber consists of two sides of glass slides, each with a thickness of 0.15 mm, separated by double sticky tape. Before use, the glass slides were cleaned by ultrasonication in ethanol. The micropipettes were pulled from glass rods with inner diameter 0.58 mm and outer diameter 1.0 mm. The suction pipette was firmly attached to the chamber. In the experiments where injection was performed no lid was used on the chamber. The injection micropipette was translated using a
3. OPTICAL TRAPPING OF GOLD NANOPARTICLES

The choice of which gold nanoparticles to use for insertion into living cells was based on knowledge of which sizes can actually be optically trapped and which forces one can exert on a given gold nanoparticle size. We were able to trap gold nanoparticles with diameters from 18 nm to 254 nm for a time long enough to perform a quantitative measurement of the trapping strength. We were not able to trap a 12 nm gold nanoparticle. Due to lack of availability of larger gold nanoparticles we were not able to put an upper bound on the sizes of gold nanoparticles which could be optically trapped, but according to literature, Mie metallic particles with diameters larger than 500 nm cannot be optically trapped in 3 dimensions.

The optical trap exerts a harmonic potential on the trapped bead, this potential being characterized by the spring constant $\kappa$. Thus, the force exerted by the optical trap on the particle is in one dimension given by $F_{\text{trap}} = \kappa x$, where $x$ is the deviation from the equilibrium position and $\kappa$ is the trap stiffness in the $x$-direction. By performing a power spectrum analysis on the Brownian fluctuations of a gold particle of a given size in the optical trap, we were able to determine the normalized trapping strength ($\kappa$/laserpower) as a function of bead size. The results from this study was that the trapping strength scaled with the volume of the particle for the smallest particles ($d < 100$ nm) while for larger particles ($d > 100$ nm) the trapping strength increased more slowly. This conclusion is valid both in the direction orthogonal to and parallel to the propagating laserlight.

Another consideration regards the fact that the gold nanoparticles need to have a diameter which is smaller than the inner diameter of the micropipette used for injection. The final choice of particle size used for injection into the protoplasts was $d = 100$ nm.

As the trapping potential is harmonic, the histogram of the positions visited by the bead is a Gaussian distribution. Such a histogram is shown in Figure 3. During the trapping of a single gold nanoparticle, it could happen that one or more beads would diffuse into the trap. When more scatterers are present in the trap, the position histogram still appears Gaussian, however, with a larger standard deviation, $\sigma$. Therefore, $\sigma$ can be used as a measure of the number of beads in the optical trap.

When the particles are successfully inserted in the living cells and attached to the organelles of interest, we would probably not be able to control the number of particles actually present in the trap. But we expect that from estimations of $\sigma$ we might be able to deduce the number of particles. The more particles in the trap, the larger the total force as the contributions from the individual beads add up. Another issue which has to be considered is that of calibration of the force: Due to the complexity of the cytoplasm the calibration procedures for a gold nanoparticle inside a cell will be different from those of gold nanoparticles just in a simple liquid.

4. REVIVAL OF YEAST CELL Spheroblasts

The stiff cell wall of S. pombe yeast possesses a major obstacle for injection of gold nanoparticles. Large molecules and particles cannot pass through the dense cell wall. Therefore, it is a major challenge to get the gold nanoparticles across the cell wall and into the cytoplasm. Our first strategy was to use a 'gene-gun' to shoot gold beads into the yeast cells. However, a major drawback of this technique is that only a few cells out of a large number of bombarded cells will be penetrated by a particle. Hence, cells with embedded beads are difficult to find in a microscope. Therefore, we turned to a single cell technique where we use enzymes to degrade the cell wall (as described in the Methods section). This degradation removes the network of interlaced sugar molecules in the cell wall resulting in a protoplast consisting of the cell’s cytoplasm surrounded by a plasma membrane. If the protoplasts are placed in a hypotonic solution water will swell the protoplasts, resulting in crenation. On the other hand, if the solution is hypertonic the protoplasts will swell as a result of water intake and eventually burst (lysis). S. pombe cells are among the few yeasts which can be regenerated from protoplasts in liquid media. If the protoplasts are provided appropriate conditions (as described in the Methods section), they are able to re-build the outer cell wall, recover, and return to the state of a totally normal and healthy yeast cell within $\sim 20$ hours. A recovery from protoplast to dividing, healthy yeast cell is shown in the picture panels of Figure 4.
**Figure 3.** Histograms showing the positions (in volts) visited by gold nanoparticles, diameter $d = 40$ nm, optically trapped in water. The standard deviation of the histograms increases abruptly every time a particle drops into the trap. Standard deviations: $\sigma_1 = 13$ mV, $\sigma_2 = 18$ mV and $\sigma_3 = 27$ mV.

**Figure 4.** This series of pictures was taken over 20 hours. It shows how a protoplast is able to recover back into a healthy, dividing yeast cell.
Figure 5. These pictures show the laser ablation of a protoplast held in a micropipette. The laser is focused on the membrane at the top of the protoplast (in the figure). After $\sim 30$ s of exposure ($1.5$ W) the laser had burned a whole in the membrane and the cytoplasm (white material) leaked out into the surrounding solution.

5. LASER ABLATION OF PROTOPLASTS

By focusing the trapping laser light on the bilipid membrane of a protoplast, we were able to burn a hole in the membrane. The laser power used for this experiment was $\sim 1.5$ W measured at the output of the objective. Figure 5 shows such a laser ablation experiment where the protoplast bursts after $\sim 30$ s of exposure. During the ablation experiment the protoplast was held taut in a micropipette while the trapping laser light was focused on the membrane at the top on the protoplast.

As visible from Figure 5 the cytoplasm leaks out into the surrounding media when there is a hole in the membrane. For this particular cell, a recovery is not possible. If laser ablation should be used e.g. to cut a hole into the membrane through which gold nanoparticles could be inserted, at first one would have to overcome the problems of leakage of the cytoplasm during ablation. Two possible ways to control leakage would be: 1) Better adjustment of the osmotic pressure in the surrounding liquid. 2) Applying a gentle suction by the micropipette holding the protoplast because suction is well-known to create further tension in the bilipid layer.

Pulsed lasers are typically used for transfection of single cells and to perform laser ablation experiments. Photoporation of single cells has been performed with a continuous violet diode laser with low intensity and short exposure times to avoid damage to the DNA. As the wavelength of our trapping laser, $1064$ nm, was chosen to minimize absorption by the biological specimen and also to avoid heating of the surrounding liquid (mainly water), the ablating effect of the laser occurs only at high laser powers and long exposure times $\sim 30$ s. Another way to ablate the protoplast membrane was to trap a $d = 200$ nm particle at fairly high powers outside the protoplast. Bringing the trapped particle in contact with the plasma membrane caused immediate rupture of the membrane. This effect can be explained by the fact that a trapped metallic particle absorbs light and emits heat. The heating of the trapped gold particles inside living cells can be minimized by choosing smaller particles and use lower powers to avoid thermal damage to the cytoplasm.

6. INSERTION OF GOLD BEADS INTO YEAST CELLS

Using a sharp micropipette with an outer diameter of approximately $0.5 \mu$m, it is possible to punch a hole in the membrane of a protoplast. Figure 6 shows such an injection.

Through this micropipette gold nanoparticles with diameters smaller than the inner diameter of the micropipette can be delivered into the protoplast at any wanted location. If the diameter of the gold nanoparticles is larger than $\sim 70$ nm the particles in water are visible by eye in bright field microscopy. In differential interference contrast (DIC) microscopy the particles produce a large contrast (see Figure 7) and should be distinguishable from the cytoplasm once inside the cells.
Figure 6. A micropipette (outer diameter $\sim 0.5 \, \mu m$) is used to make a hole in the bilipid membrane of a protoplast with the goal of injecting gold nanoparticles into the cytoplasm.

Figure 7. DIC image of a $d = 70$ nm gold particle adhered to the coverslip.
7. PERSPECTIVES
Once the gold particles are within the successfully recovered yeast cells, one of the remaining challenges is to optically trap the delivered gold nanoparticle and to devise a way to perform a calibration procedure inside the living cell. Conjugated gold nanoparticles will then be inserted and attached to the organelles expressing GFP, thus allowing for experiments where forces inside living cells can be quantitatively measured. A sketch of the proposed experiment is given in Figure 8.

8. CONCLUSIONS
We have used optical tweezers to investigate the properties of living \textit{S. pombe} yeast cells. The long term goal is to insert gold nanoparticles into the cells and use these as handles for the optical tweezers and hence quantitatively probe the dynamics inside living cells. The larger the gold nanoparticle, the larger the optical force exertable on them.\(^3\) In order to get the gold beads through the very rigid outer cell wall, the yeast cells must be enzymatically treated and transformed into protoplasts. Once they are protoplasts it is substantially easier to get the gold nanoparticles into the cytoplasm, e.g. by micropipetting through the lipid bilayer. The trapping laser light was used to ablate the bilipid membrane of the protoplasts, and the osmotic pressures must be carefully controlled to prevent the cytoplasm from leaking through the hole. After the enzymatic degradation of the cell wall, we have successfully transformed the protoplasts back into healthy \textit{S. pombe} yeast cells.

We can expect to exert forces of up to 30 pico-Newton\(^3\) using a single gold nanoparticle as handle inside a living cell. If more beads attach and are simultaneously trapped, even larger forces can be exerted. Therefore, this setup is ideal to study e.g. the motion of molecular motors carrying out their tasks inside living cells; the maximum force exertable by such a motor, the stall force, is e.g. around 6 pN for kinesin\(^{16}\) and \(\sim 25\) pN for the RNA-polymerase.\(^{17}\) Polymerizing microtubulus are expected to exert forces up to \(\sim 50\) pN\(^{18}\) and therefore, measuring the action of \textit{in vivo} polymerizing microtubulus should also be within reach using the model system and procedures presented here.

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