CHAPTER 10

Optical Trapping and Laser Ablation of Microtubules in Fission Yeast

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Abstract

Manipulation has been used as a powerful investigation technique since the early history of biology. Every technical advance resulted in more refined instruments that led to the discovery of new phenomena and to the solution of old problems. The invention of laser in 1960 gave birth to what is now called optical manipulation: the use of light to interact with matter. Since then, the tremendous progress of laser technology made optical manipulation not only an affordable, reliable alternative to

METHODS IN CELL BIOLOGY, VOL. 97
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978-0-12-381349-7
DOI: 10.1016/S0091-679X(10)97010-6

traditional manipulation techniques but disclosed also new, intriguing applications that were previously impossible, such as contact-free manipulation. Currently, optical manipulation is used in many fields, yet has the potential of becoming an everyday technique in a broader variety of contexts. Here, we focus on two main optical manipulation techniques: optical trapping and laser ablation. We illustrate with selected applications in fission yeast how *in vivo* optical manipulation can be used to study organelle positioning and the force balance in the microtubule cytoskeleton.

I. Introduction

Manipulation is an ideal tool to investigate the complex system of mechanical interactions taking place inside a living cell. The idea underlying all the manipulation techniques is to perform controlled modifications of a selected structure and to observe the reaction to those alterations. With the help of manipulation it is therefore possible to study a specific interaction by selectively perturbing only the involved players. The very early examples of manipulation consisted generally in removing a single cell to study, e.g., its role during embryonic development. These manipulation experiments were usually performed by physically destroying the cell by means of a glass capillary or a needle. In the last two decades, optical micromanipulation became the most prominent manipulation technique. Thanks to the rapid advances in laser technology, developing an optical manipulation setup has become relatively uncomplicated and more and more research projects exploit optical manipulation to study a great variety of phenomena. The advantages of optical manipulation over other, nonoptical techniques are manifold: it is easily integrable with many microscopy setups, including confocal or multiphoton microscopes, and it allows for higher spatial and temporal resolution. Moreover, by using optical manipulation it is possible to minimize the interaction with the sample: in contrast to other manipulation techniques, optical manipulation is in fact contact free, the interaction being mediated only by photons.

II. Optical Manipulation

A. *In Vivo* Optical Manipulation

Optical manipulation uses light as a means of interaction with the sample. Light carries momentum and energy, and both can be used to modify the sample structure. The two most widely used optical manipulation techniques are optical tweezers and laser ablation.

1. Optical Tweezers

Optical tweezers rely on the momentum exchange between the photons of a laser beam and the sample to apply forces (Ashkin, 1992, 1998; Ashkin and Dziedzic,

1987; Ashkin *et al.*, 1987). Usually, an optical tweezers setup consists of an infrared, continuous wave (CW) laser focused using a high numerical aperture (N.A.) objective. The high N.A. is required to maximize the optical forces that can be exerted on the sample at a given laser power. When working *in vitro*, micrometer-sized homogeneous microspheres can be trapped with forces ranging from a few pN to several tens of pN, depending on the optical properties of the microspheres and of the medium. Moreover, it is possible to track the position of the trapped particle with sub-nanometer accuracy at high (several MHz) repetition rates (Simmons *et al.*, 1996). When working *in vivo* several difficulties arise: beside the risk of inducing optical damage when focusing the trapping laser beam, the cytoplasm is not an optically homogeneous medium. Moreover, it may be difficult to microinject a cell with particles with known optical properties. These factors prevent a reliable measurement of the applied optical forces. Nevertheless, it is still possible to exploit optical forces to trap either single cells or particles having suitable mean optical properties (e.g., lipid granules) that are naturally present in the cytoplasm (Maghelli and Tolic-Norrelykke, 2008; Sacconi *et al.*, 2005b; Tolic-Norrelykke *et al.*, 2004a, 2005). The applied forces, tough difficult to measure, are sufficiently large to study biologically relevant phenomena (Ashkin and Dziedzic, 1987; Ashkin *et al.*, 1987; Simmons *et al.*, 1996).

2. Laser Ablation

Laser ablation exploits the confined deposition of energy induced by a highly focused laser beam to locally modify the sample. Depending on the characteristics of the laser (wavelength, power, pulse duration), on the exposure time and on the exposed area, the physical mechanisms underlying the ablation process might vary (Heisterkamp *et al.*, 2005; Vogel and Venugopalan, 2003; Vogel *et al.*, 2005). To perform laser ablation with high spatial resolution, it is necessary to carefully control the beam shape and to use highly corrected optics to focus the beam to the smallest possible area.

B. Integration with Microscopy Setups

Combining optical tweezers and laser ablation with microscopy setups requires basic knowledge of optics. For optical tweezers, the key aspects to consider are the beam quality, the quality of optical components and their correction (particularly for what concerns spherical aberration), and the power control method. For laser ablation, it is desirable to have in addition an accurate control of the exposure time. Working with fluorescently labeled samples can greatly enhance the potential applications, especially when working *in vivo*. However, one should consider the additional optical components necessary for visualizing the fluorescence signal when designing the setup.

III. Optical Tweezing in Fission Yeast

A. Background

Optical forces can be exploited to displace the nucleus in fission yeast (Maghelli and Tolic-Norrelykke, 2008; Sacconi *et al.*, 2005b; Tolic-Norrelykke *et al.*, 2005). Since the refractive indexes of the cytoplasm and of the nucleoplasm do not differ significantly, it is not possible to directly apply optical forces to the nucleus. Therefore, it is necessary to trap a suitable particle and use it as a handle to apply forces on the nucleus. Due to the rigid cell wall of fungi, microinjecting particles that can be easily trapped using optical tweezers is cumbersome (Riveline and Nurse, 2009). However, it is possible to apply optical forces to the lipid granules naturally present in the cytoplasm (Maghelli and Tolic-Norrelykke, 2008; Sacconi *et al.*, 2005b; Tolic-Norrelykke *et al.*, 2005). The trapped granule can then be moved inside the cell and used to displace the nucleus.

B. Experiment

To demonstrate how optical tweezers can be used to perturb the intracellular arrangement, we set up an experiment using a *Schizosaccharomyces pombe* strain in which the nuclear envelope and the spindle pole body, a centrosome analogue in fission yeast, were labeled with GFP. The cells, fixed to the glass bottom of a Petri dish, were treated with a microtubule poison drug (MBC) to depolymerize the microtubules. By switching on the optical trap, and focusing inside the cell, it was possible to trap lipid granules in the cytoplasm (Fig. 1A and B). The trapped granule was successively moved against the nucleus (Fig. 1C). The manipulation was performed while simultaneously acquiring two-photon images of the cell. During the interaction, the nuclear envelope was deformed, clearly showing an indentation corresponding to the point where the trapped particle pressed against it (Fig. 1C, the trapped particle is marked by white arrows). After switching off the trap, the nuclear membrane relaxed into its original shape (Fig. 1C and D).

We next repeatedly applied forces on the nuclear envelope using a trapped lipid granule over a time interval of several minutes. As a result, the whole nucleus was displaced (Fig. 2A). After switching off the optical trap, and washing out the microtubule-depolymerizing drug, we imaged the manipulated cell, tracking the position of the nucleus (Fig. 2B).

C. Discussion

We used optical tweezers to deform and displace the nucleus in a living cell. Although displacing the nucleus in fission yeast has been achieved by other methods, such as centrifugation, working at a single cell level has several advantages as follows:

1. Allows for following of the displacement and relaxation processes
2. Permits a comparison of the same cell before and after the manipulation

3. Makes it possible to change the position of the nucleus only, without affecting other organelles or molecular gradients

When used *in vivo*, care must be taken to avoid inducing damage while trapping the lipid granules. We choose to perform trapping using a CW, near-infrared laser emitting at 970 nm. According to different studies (Ashkin and Dziedzic, 1987; Liang *et al.*,

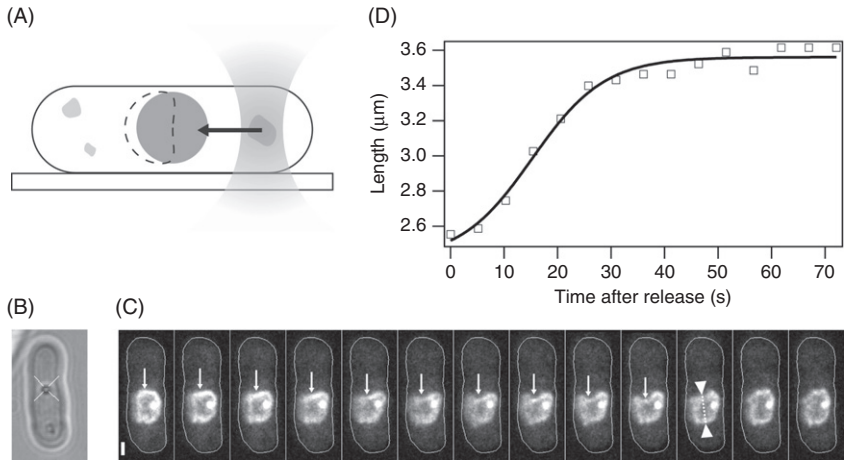

Fig. 1 (A) Schematics of the trapping experiment (side view). By focusing the optical trap inside the cell, it is possible to trap a lipid granule, naturally present in the cytoplasm. The trapped granule can then be used to apply forces on the nucleus. (B) Transmitted light image of a cell in which a lipid granule is trapped (white cross, the trapping laser is perpendicular to the image plane). In the same cell, the lipid granule was used to apply forces on the nuclear membrane: the time series (C) shows two-photon fluorescence images of the nucleus (labeled with Cut11-GFP) during the interaction with the trapped granule (white arrows). The optical trap is switched off (third last image) and the relaxation of the nuclear membrane, measured between the white triangles, is plotted as a function of time in (D). Scale bar is 1 µm, time between images in (C) is 25 s.

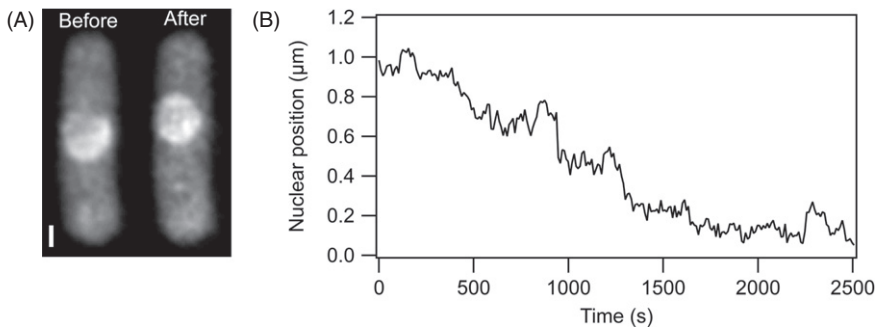

Fig. 2 (A) Displacement of the nucleus achieved by optical manipulation. Scale bar is 1 µm. After allowing for microtubule repolymerization by washing out MBC, the nucleus relaxes back to its original position in around 20 min (B).

1996; Liu *et al.*, 1995; Neuman *et al.*, 1999), wavelengths in the 950–1000 nm range appear to minimize the damage induced to the sample during the trapping process. In our experiments, we could not notice any changes in the two-photon signal from the nucleus during the manipulation. Moreover, after being deformed the nucleus always relaxed back to its original shape. Taken together, these results suggest that manipulating fission yeast cells with optical tweezers as described does not induce significant damage.

Analyzing how the nucleus of living cells reacts to such manipulation can provide information about its mechanical properties. In Fig. 1C it is possible to follow the length of the deformed nucleus, taken along the dotted line, after the optical trap has been switched off. The nucleus relaxes back to its original shape: fitting the data with a Sigmoid function $f(t) = y_0 + y_{\max}/(1 + \exp((t_0 - t)/\tau))$, solid line in Fig. 1D) gives a value of $\sim$7 s for the characteristic rate τ. This characteristic time is determined by the mechanical properties of the nucleus and of the cytoplasm.

Optical forces can be used to displace the whole nucleus away from its natural position (Fig. 2A). We first depolymerized the microtubules using MBC and then employed the trapped granule to alter the geometrical arrangement of the cell. After washing out MBC, we were able to follow the position of the nucleus in time (Fig. 2B). We noticed that the nucleus came back to its original position, implying the existence of centering forces that actively keep the nucleus around the geometrical center of the cell. Previous studies (Sacconi *et al.*, 2005b; Tolic-Norrelykke *et al.*, 2005) have shown that the centering forces depend on microtubules, which microtubules exerting pushing forces on the nucleus (Daga *et al.*, 2006; Tolic-Norrelykke, 2008, 2010; Tolic-Norrelykke *et al.*, 2005; Tran *et al.*, 2001). Therefore, analyzing the time evolution of the nuclear position can yield information about the pushing forces generated by the microtubules.

IV. Laser Ablation of Microtubules

A. Background

Similarly to optical tweezers, laser ablation may be used to perturb the internal force balance of a cell. Laser ablation does not require any particle to be present inside a cell but can directly target cytoskeletal elements (Colombelli *et al.*, 2005; Khodjakov *et al.*, 2004; Maghelli and Tolic-Norrelykke, 2008; Raabe *et al.*, 2009; Sacconi *et al.*, 2005a; Tolic-Norrelykke *et al.*, 2004b; Vogel *et al.*, 2009) or organelles (Amy and Storb, 1965; Berns *et al.*, 1977; Sacconi *et al.*, 2007; Stiess *et al.*, 2010). It is possible to use laser ablation to investigate, e.g., the specific function of an organelle in a cell, or to study how a cell or an organism reacts to a modification of its structure. Laser ablation experiments can hence provide information about cellular processes that are complementary to the data that can be collected using genetic approaches. Technically, laser ablation can be implemented in any optical microscopy setup: by carefully designing the optical path, laser ablation can be used to manipulate the sample at a higher spatial and temporal resolution in comparison with optical tweezers. To perform laser ablation

experiments *in vivo*, it is necessary to minimize any unspecific damage that could result from the ablation process. By controlling the laser power at the sample and the exposure time during ablation, it is possible to find the optimal parameter set that guarantees high ablation efficiency while minimizing the unspecific damage (Maghelli and Tolic-Norrelykke, 2008; Raabe *et al.*, 2009; Sacconi *et al.*, 2005a).

B. Experiment

To illustrate how laser ablation can contribute to the understanding of different biological processes, we ablated GFP-tagged microtubules in fission yeast during different phases of the cell cycle. The experiments were performed on a custom-built two-photon setup, using a pulsed femtosecond laser both for imaging and ablation (Maghelli and Tolic-Norrelykke, 2008). We started by searching for the ablation parameters maximizing the ablation efficiency while keeping the unspecific damage as low as possible. By keeping the power at the sample (100 mW) and the wavelength (895 nm) constant, we tried to ablate interphase microtubules using different exposure times [Fig. 3A, data taken from Maghelli and Tolic-Norrelykke (2008)]. We concluded that the optimal exposure time is between 20 and 30 ms. Using exposures in this range, the ablation efficiency was above 50% while no cell died as a consequence of the ablation.

We next used these settings to ablate interphase microtubules. During interphase, fission yeast microtubules are organized into antiparallel bundles. In each bundle, the plus ends point toward the cell periphery (scheme in Fig. 3B I). The ablation (Fig. 3B II, ablation marked by a white arrow) cuts the bundle creating a new plus and a new minus end. The newly created minus end is unstable; as a consequence, the severed fragment depolymerized mainly by shrinking from its minus end. As a control, we observed that the other, nonablated microtubules were not affected (Fig. 3B II–IV).

In our third experiment, we ablated the mitotic spindle. Fission yeast has a closed mitosis, i.e., the nuclear envelope does not break down. During mitosis, the two spindle

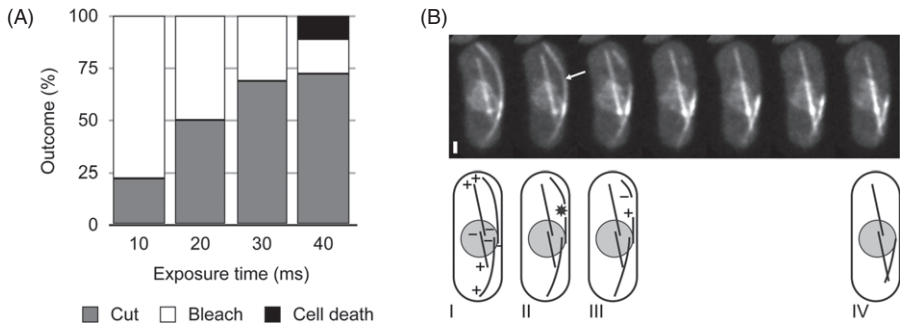

Fig. 3 Outcome of ablations performed on interphase microtubules using different exposure times (A) [data taken from Maghelli and Tolic-Norrelykke (2008)]. The optimal exposure time is between 20 and 30 ms. (B) By ablating interphase microtubules a free, unstable minus-end is created. As a result, the fragment depolymerizes mainly by shrinking from its minus end. Scale bar is 1 μm, time between frames is 5 s.

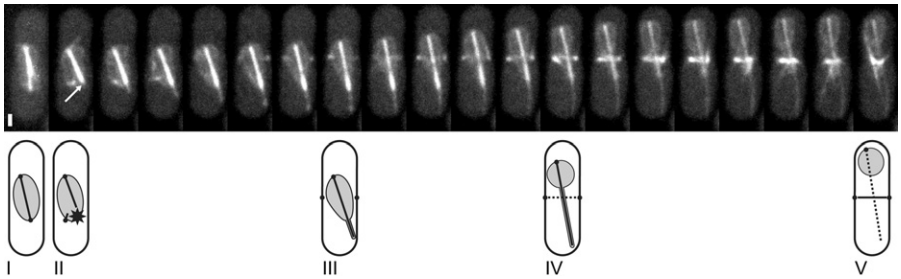

Fig. 4 Ablating the mitotic spindle near one spindle pole body induces asymmetric division. The ablated spindle continues to grow, deforming the nuclear envelope (ablation marked by the white arrow). The polymerization forces displace the nucleus, eventually leading to an abnormal mitosis, in which one daughter cell inherits all the nuclear material. Scale bar is 1 μm, time between frames is 100 s.

poles remain embedded in the nuclear membrane (Fig. 4 I). We performed the ablation near a spindle pole (Fig. 4 II, ablation spot marked by a white arrow). The ablation cut the spindle asymmetrically: one spindle pole remained embedded in the nuclear membrane (upper pole in Fig. 4 III), while near the other pole the microtubules forming the spindle deformed the nuclear membrane creating a protrusion (lower side in Fig. 4 III). As the spindle elongated, the outgrowing protrusion contacted the cell cortex: further polymerization of the spindle displaced the nucleus upward (Fig. 4 IV). As the cell started to divide, the nucleus had already been pushed across the septum. As a result, the cell segregated the nuclear material asymmetrically: one sibling retained the whole nucleus (the upper cell), while the other one was deprived of the nucleus (the lower cell in Fig. 4 V) (Raabe *et al.*, 2009).

We next used laser ablation to study the force balance during fission yeast meiosis. During meiosis, two cells fuse forming a zygote and their nuclei merge. In fission yeast, the meiotic prophase is accompanied by prominent oscillations of the fused nuclei, termed horsetail nuclear movement (HNM) (Chikashige *et al.*, 1994). By using laser ablation, it has been demonstrated that dynein generates the nuclear movement by pulling on cytoplasmic microtubules (Vogel *et al.*, 2009; Yamamoto *et al.*, 1999). To further investigate the mechanism underlying the nuclear oscillations, we cut a microtubule bundle during the HNM in front of the moving spindle pole (Fig. 5 II, ablation spot marked by a white arrow). The ablation disconnected a microtubule bundle from the moving nucleus, creating a free fragment. We observed that the severed microtubule bundle continued its movement along the cell cortex (Fig. 5 III–V).

C. Discussion

In our experiments, we used laser ablation to manipulate the cytoskeleton during different phases of the cell cycle. The ablations perturbed the mechanical equilibrium or the geometrical arrangement of the cytoskeleton. We then inferred information about the forces acting in the cell by observing how the cell reacted to the modifications. When performing ablation in living specimens, the major pitfall is to

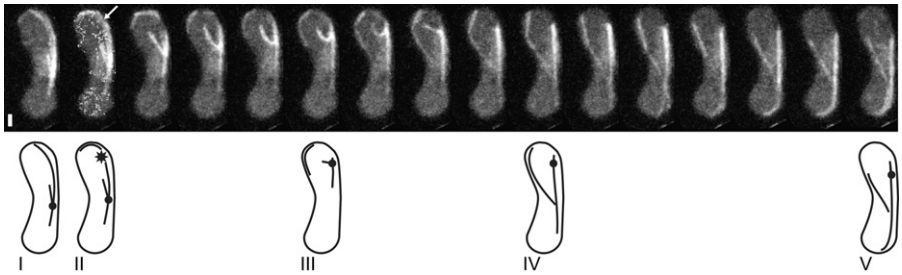

Fig. 5 Ablation of microtubules during meiosis. A microtubule bundle is cut (ablation marked by the white arrow) and a fragment detaches from the spindle pole body (black circle in the scheme). The fragment moves along the cell cortex, independently from the spindle pole body. Scale bar is 1 μm, time between frames is 12 s.

mistake an ablation-induced artifact for a real, physiological reaction of the cell. It is therefore necessary to perform controls to rule out any spurious effect. We therefore started our experiments by calibrating the ablation efficiency, trying to find the parameter set allowing us to perform ablations without inducing significant damage (Fig. 3). Using these parameters, we then performed ablations to perturb the force balance during mitosis (Fig. 4), exploiting the cell internal forces to asymmetrically segregate the nuclear material in a dividing cell. Compared with creating enucleated cells by centrifugation (Carazo-Salas and Nurse, 2006), the ablation-based method described here has several advantages: in the first place it permits one to follow the displacement process, allowing for insight into the intracellular forces leading to the asymmetric segregation. In the second place, since no external forces are used, the spatial arrangement of other organelles is not perturbed.

In our next experiment, we used laser ablation to study the force generators responsible for the nuclear movement during meiosis (Fig. 5). Previous work has shown that the nuclear movement is driven by dynein (Yamamoto *et al.*, 1999). A recent study employed laser ablation to dissect the force balance during the nuclear movement and put forward a model in which the force generators self-organize (Vogel *et al.*, 2009). Here, we observed independent movement of the microtubule bundle, disconnected from the moving spindle pole body (Fig. 5). The observed movement fits well with the model proposed in Vogel *et al.* (2009). The movement is most likely a consequence of the pulling forces exerted by dynein motors anchored at the cell cortex, which pull independently on the fragment and on the microtubules connected to the spindle pole body (Fig. 5 III–V).

V. Methods

A. Cell Culture and Sample Preparation

Fission yeast cells were grown in liquid yeast extract medium at 25° C. During imaging and manipulation, the cells were attached to the glass bottom of a Petri dish using ~2 μl of

2 mg/ml lectin. The Petri dish was filled with ~3 ml of liquid minimal medium. On the microscope stage, the sample was kept at 25° C. When needed, carbendazim (MBC) at a concentration of 25 μ g/ml was used to depolymerize microtubules.

B. Microscopy

Laser ablation and optical trapping were performed using a custom-built two-photon setup (Maghelli and Tolic-Norrelykke, 2008). For ablation, the pulsed laser of the microscope was tuned to a wavelength of 895 nm. The power of the laser at the sample plane was ~5 mW during imaging and ~100 mW during ablation. Optical trapping was achieved using a near-infrared (970 nm) CW laser. To perform manipulation using the optical trap, the focused beam was either kept fixed with respect to the objective while moving the sample, or the sample was kept fixed with respect to the objective while steering the optical trap using a pair of computer-controlled galvanometer mirrors.

The objectives used were either a 63× 1.0 N.A. water dipping lens or a 100× 1.4 N.A. oil immersion objective.

The image acquisition and manipulations were performed using a custom-written software (LabView) controlling the setup. Image analysis was performed using ImageJ, data analysis using Igor Pro or Matlab.

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