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Chapter 13

Using Optical Tweezers Combined with Total Internal Reflection Microscopy to Study Interactions Between the ER and Golgi in Plant Cells

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Abstract

Optical tweezers have been used to trap and micromanipulate several biological specimens ranging from DNA, macromolecules, organelles to single celled organisms. Using a combination of the refraction and scattering of laser light from a focused laser beam, refractile objects are physically captured and can be moved within the surrounding media. The technique is routinely used to determine biophysical properties such as the forces exerted by motor proteins. Here, we describe how optical tweezers combined with total internal reflection fluorescence (TIRF) microscopy can be used to assess physical interactions between organelles, more specifically the ER and Golgi bodies in plant cells.

Key words Optical trap, ER, Golgi, Tweezers, GFP

1 Introduction

Trapping and micromanipulation for biological samples using optical tweezers has a wide range of applications from DNA, macromolecules, organelles to single celled organisms (reviewed in [1]). The basic premise of optical traps is that only microscopic particles that display a significantly different refractive index to the surrounding medium will be trapped in the focused infrared laser beam (Fig. 1). Infrared laser beams are routinely used as they generally result in the lowest sample absorbance and therefore cause the least photon damage. Optical traps can either be fixed in position, and therefore require lateral stage movement to move the sample relative to the trapped object, or the system can use mirrors, acousto-optic deflectors, or spatial light modulation to move the position of the trap and therefore the object [2]. Here, we present our system which utilizes a single beam gradient trap that is fixed in position combined with TIRF microscopy. Readers
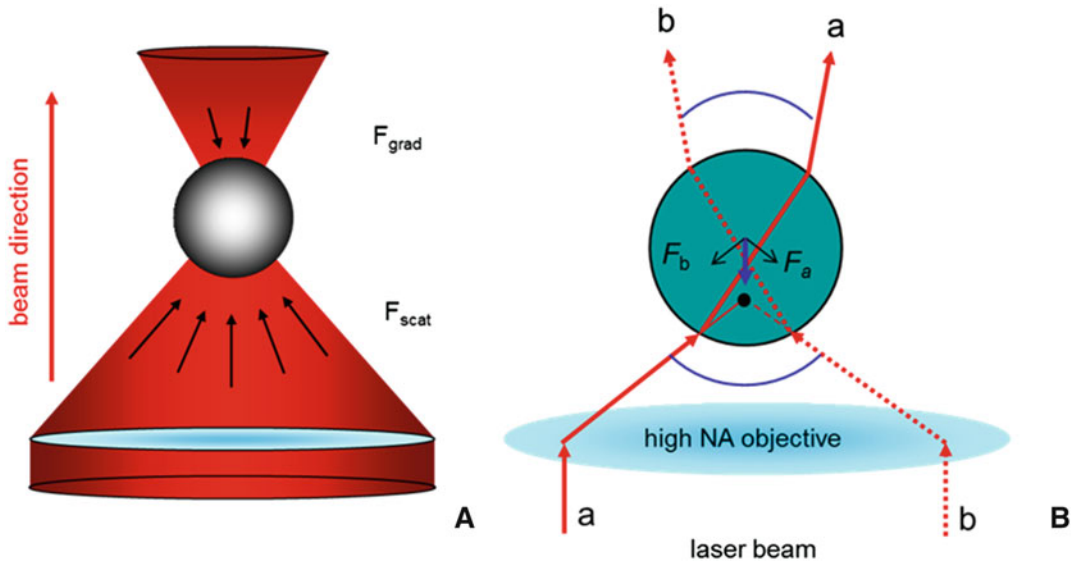
Fig. 1 Mechanism of action of a gradient optical trap. (**A**) Scattering and gradient forces are balanced in a tightly focused laser beam using a high numerical aperture objective lens to form a stable three-dimensional trap. (**B**) Gradient restoring forces (F_a, F_b) result from the refraction of the laser beam as it passes through the trapped object, with rays of light (*a* and *b*) depicting the paths taken by the edges of the beam

are directed to Ketelaar et al. [3] for discussion of integrating optical traps with confocal microscope systems.

Optical trapping has been used to probe several aspects of subcellular architecture in plant cells (reviewed in [3]). For example, the effects of nuclear positioning on root hair growth [4], interactions between several organelle pairings including peroxisome-chloroplasts [5], ER-Golgi [6] and ER-chloroplasts [7], and cytoskeletal organization [8]. We have used optical tweezers to characterize interactions between several organelles in plants (ER-Golgi and peroxisome-chloroplast), where the measurement of such interactions is only possible if one of the organelles is preferentially trapped. The efficiency with which organelles are trapped is related to a number of factors including refractive index, size, shape, and laser wavelength. For example, unlike the endoplasmic reticulum (ER), Golgi bodies in leaf epidermal cells are amenable to being trapped and moved within the confines of the cell. Since the ER and Golgi are functionally linked through the secretory pathway, this raised the question as to whether they were also physically linked. Using optical tweezers, we were able to show that lateral movement of trapped Golgi bodies resulted in the remodeling of the ER indicative of a physical association between the two organelles [6]. These studies provided a qualitative analysis of the interaction between the two structures. Here, we provide an overview of our current optical trapping platform which enables the user to quantify interactions between organelles. We have used this

system successfully to characterize the physical interaction between peroxisomes and chloroplasts, which are functionally linked through the photorespiratory pathway [5]. We will discuss important considerations relating to system calibration, sample generation, developing a quantifiable platform for measuring interactions and post-acquisition analysis. We also include caveats to force measurement calculations in plant cells. Of course, variations will apply subject to the optical trap system available to the user, but the general premise and pitfalls herein are universal.

2 Materials

2.1 System Setup and LabVIEW Interface

An optical trap with a two-channel TIRF microscope (TIRF-M) was constructed around a Nikon Ti-U inverted microscope. The near infrared (NIR) trapping laser at 1090 nm was delivered to the trapping objective (100×, oil immersion, NA 1.49 with both temperature and cover glass correction ring) using a commercial Elliot Scientific optical trapping platform. Fluorescence excitation for the TIRF-M imaging was obtained from an Omicron laser hub fiber coupled to a manual Nikon TIRF-M unit. For GFP and RFP chromophores fused to the proteins of interest, 488 and 561 nm were used, respectively. Fluorescence emissions were detected using two electron multiplying charge-coupled device (EMCCD, iXon, Andor) cameras. These are connected via a twin-cam (Cairn) unit (Fig. 2). This allowed the flexibility of full field of view per imaging channel as well as the ability to further magnify the image without restricting the observation area. A single camera may be used for a dual channel imaging (requiring technologies such as the Cairn OptoSplit II). This leads to restricted field of view but has the advantage of reduced cost and no need for synchronization of two separate cameras.

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2.2 Microscopy Consumables

1. Standard glass slides (76 mm × 26 mm, 1 mm thick) to fit the stage.
2. Borosilicate No. 1 coverslips (*see* **Note 1**).
3. Electrical tape.
4. Polystyrene beads (1 μm diameter) are commercially available.

3 Methods

Carry out all procedures at room temperature unless otherwise stated.

3.1 Calibrating the Optical Trap Against Known Standards

The trap position, optical trapping force, and laser power transmission at the microscope objective are measured daily and compared for consistency to ensure the laser trapping setup is performing to
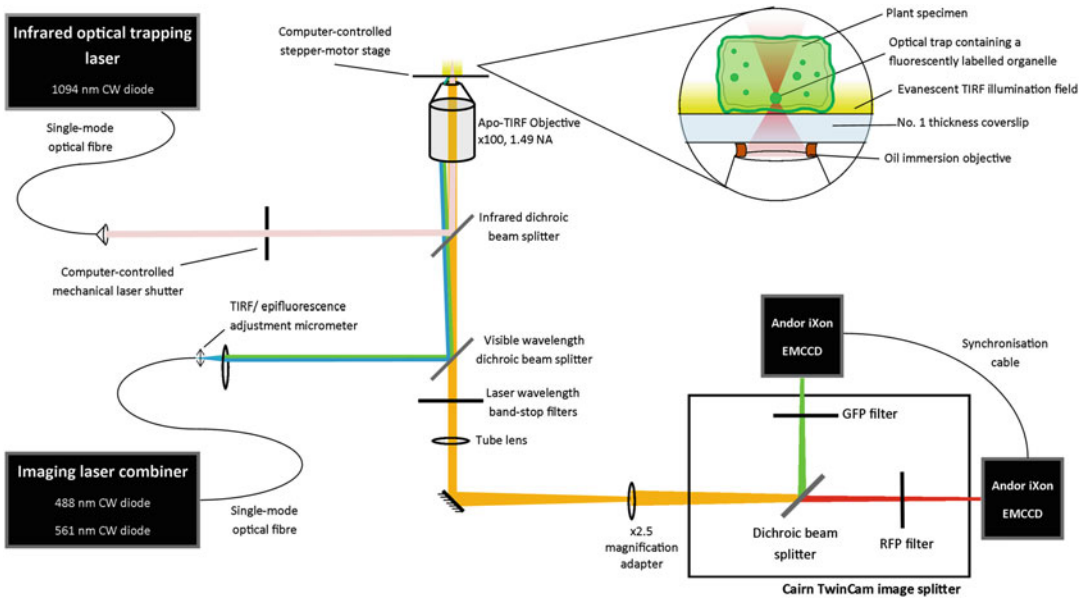
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Fig. 2 Schematic of the microscope and optical trap system setup. The infrared trapping laser and visible-wavelength imaging lasers are fiber-coupled to independent microscope ports. The TIRF angle is tuned by adjusting the position of the fiber aperture in relation to the collimating lens using a micrometer. A computer-controlled mechanical shutter is placed in the trapping laser beam path which provides fast shuttering of the optical trap. The sample is mounted on a computer-controlled variable speed (Märzhäuser) stepper motor stage. The fluorescence is imaged through a (Nikon) ×100 1.49 NA objective and ×2.5 magnification adapter on two (Andor) EMCCD cameras, filtered for GFP and RFP fluorescent dyes, respectively. The associated computer-controlled hardware is interfaced using National Instruments LabVIEW which provides full automation for each trapping routine

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specification. You are referred to **Note 2** for a critical evaluation of objective lenses for tweezers and TIRF microscopy.

3.2 Nominal Laser Power Transmission at the Sample

Using a power meter set to the wavelength of the trapping laser (1090 nm), a range of power readings of the optical trap laser transmission (0–130 mW) at the objective are measured on a daily basis. Large fluctuations of these readings (i.e., >5% change) between daily experimental runs could be indicative of changes in laser performance and stability or changes in laser alignment affecting the percentage transmission at the objective.

3.3 Trap Position and Force

Trap position is determined by trapping a 1 μm diameter polystyrene bead dispersed in water and imaging the position under bright field illumination or directly observing a significantly attenuated laser using the EMCCDs with the infrared filter removed.

To assess the trap strength, the escape force of spherical polystyrene beads is calculated. This is a relationship between the power of the optical trap that is required to maintain beads in the trap at a given stage speed, and is carried out as follows:

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1. Place a drop of polystyrene beads on a coverslip and leave to settle for ~30 min.

2. Trap a polystyrene bead at the upper end of the optical trap power spectrum (*see* **Note 3**).

3. Move the stage back and forth with a preset velocity of 100 μm/s.

4. Repeat **step 3** after decreasing the power incrementally.

5. Note down the trap laser power at which the bead escapes the trap at 100 μm/s stage oscillation.

6. Repeat **steps 2–5** using 200, 300, 400, and 500 μm/s stage speeds.

7. Using the equation for Stokes viscous drag, calculate the escape force at which the bead escapes the trap (i.e., is no longer trapped) for each of the five different stage speeds.

$$F_{drag} = 6\pi\mu r v$$

where F_{drag} is the viscous drag force acting on the bead, μ is the fluid viscosity (water, 0.00089 Pa s), r is the object radius (bead), and v is the velocity measured.

8. Plot laser power against escape force for each stage speed (i.e., calculate the F_{drag} for the given velocity at which the bead escapes the trap). For beads, the relationship is linear as there are no constraints on moving the bead other than viscosity (Fig. 3).

Comparison between escape force profiles between different experimental runs enables the user to determine whether the trap "strength" has altered, i.e., is it "easier" or "harder" to maintain beads in the trap at a given stage speed. Similar bead
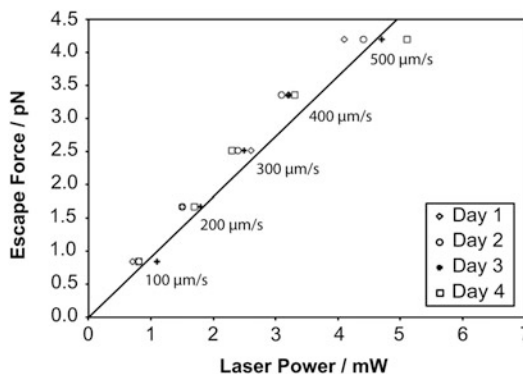

Fig. 3 Calculation of the optical trap escape force of a 1 μm polystyrene bead. Example plot of laser power (mW) against bead escape force (pN) for a range of indicated stage speeds (100–500 μm/s). Data displayed was collected on 4 consecutive days

trapping profiles ensures that the system is behaving in a consistent manner and therefore allows results from biological samples to be directly compared.

3.4 Sample Preparation

To be able to specifically trap Golgi bodies, fluorophores need to be introduced which are specifically targeted to, and highlight the Golgi. Similarly, fluorophores for ER, or other organelles are required if monitoring the effects of interaction between two organelles. Genetically encoded fusions to targeting sequences, or functional proteins can be introduced into the plant material through agrobacterium-mediated transformation. Integration and expression can either be stable or transient. Readers are referred to standard protocols for these transformation procedures [9, 10]. Note, a bank of fluorescent organelle markers generated by the Nebenführ lab [11] are available either as plasmids or stable transgenic Arabidopsis lines through public repositories (ABRC or NASC).

Plant leaf material expressing the relevant fluorescent markers to be subjected to optical trapping is prepared in the following manner:

1. Pieces of tissue (~5 mm^2) are cut from the plant leaves and immersed in latrunculin b (25 μM for 1 h) to depolymerize actin and prevent organelle movement (*see* below).

2. The leaf samples are carefully mounted on the microscope slide and a No. 1 coverslip secured over the top using thin strips of electrical tape at either end of the slide.

3. Slides are mounted coverslip down onto the microscope stage and secured with spring clips. We are aiming to minimize the working distance between the coverslip and leaf to enable efficient trapping and uniform TIRF illumination. If the samples are incorrectly mounted, then trapping efficiency and imaging can be compromised.

4. To this end, a metal plate with a hole to allow imaging of the sample is placed under the slide and the spring clips of the slide holder placed over the top. The metal plate allows equal pressure to be applied evenly over the slide to help minimize the distance between sample and coverslip (i.e., plane of optimal trap force). Note, the level of pressure does not affect cell viability as evidenced through cytoplasmic streaming in samples which have not been treated with latrunculin b.

5. Regions of the sample are imaged ensuring the level of excitation lasers for the fluorophores is minimal so as to reduce any effects from fluorophore bleaching.

We have been able to trap Golgi bodies in both *Arabidopsis thaliana* [6] and *Nicotiana tabacum* leaf epidermal cells (Figs. 4 and 5). Both ER and Golgi are highly motile and so to be able to
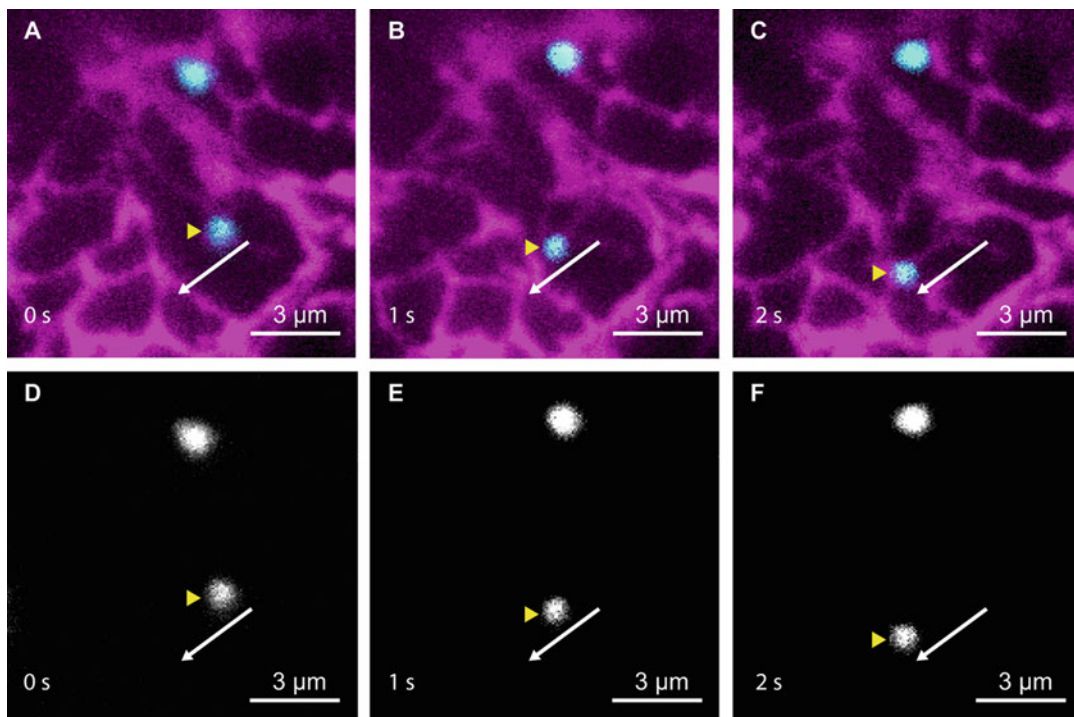
Fig. 4 Optical trapping and movement of an ER-associated Golgi body in tobacco leaf epidermal cells. Micrographs showing sequential frames of a movie of a Golgi (**d**–**f**) that is trapped under the force of the optical trap as the microscope stage is moved a distance of 6 μm at 6 μm/s. This movement is referred to as the stage translation. Panels (**a**)–(**c**) show the same frames of the trapped Golgi (*cyan*) in relation to the position of the surrounding ER (*magenta*). The ER is observed to be attached to and to move with (behind) the trapped Golgi. The *yellow* arrow indicates the position of the trapped Golgi and the *white arrow* indicates the direction of movement of the stage. Scale bar, 3 μm

carefully dissect interaction the movement of both organelles needs to be inhibited [6]. Any subsequent motion is therefore due to the trapping and micromanipulation of the trapped object. Figure 4 highlights trapping of Golgi in a motile system. Comparisons between image frames highlight dynamic rearrangement of the ER making it difficult to observe the resulting changes in the ER as a result of trapping and moving a Golgi body which is physically associated with the ER. Figure 5 clearly shows trapping and movement of Golgi which are not attached to the ER.

3.5 Developing an Automated Trapping Routine

Qualitative analysis of interaction between ER and Golgi can be carried out by trapping and manually moving the Golgi and observing the effects on the ER [6]. To quantify interactions, the relationship between trap strength and organelle trapping efficiency and behavior is required (*see* **Note 3**).

1. Prior to data acquisition, a live image is displayed on the computer monitor and a potential organelle of interest is
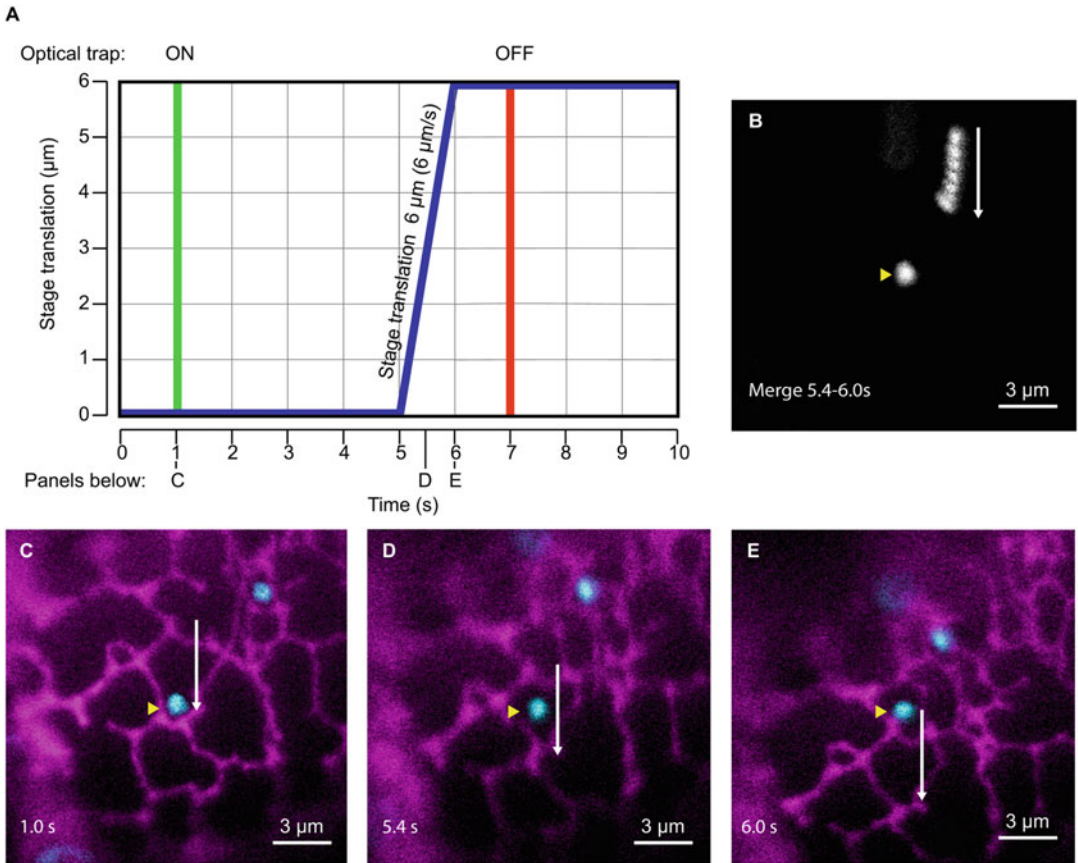
Fig. 5 Automated trapping routine applied to a Golgi body in tobacco leaf epidermal cells. Schematic representation of the position of the optical trap as considered radially from the starting point of the trapping routine (**a**). The TIRF image is recorded from 0 s, the trap is turned on after 1 s, the stage movement (translation) of 6 μm at 6 μm/s begins at 5 s and ends at 6 s, the trap is turned off after 7 s, and the image recording ends at 10 s. Images taken from a representative movie where the trapping routine (**a**) was used to trap a Golgi (*cyan*) and record the event in relation to the surrounding ER (*magenta*) are depicted; the moment the trap is turned on (**c**), the midpoint of the translation (**d**), and the end of the translation before the trap is turned off (**e**). The *yellow arrow* indicates the position of the trapped Golgi and the *white arrow* indicates the direction of movement of the stage. In this case, the Golgi is not attached to the ER and as it is moved the ER does not follow (**d**). Merged frames of the Golgi signal that correspond to 5.4–6.0 s of the routine are provided (**b**). The trapped Golgi (*yellow arrow*) does not change position relative to the field of view as the stage moves relative to the trapped object. Merged image therefore shows the trapped organelle as a single point (*yellow arrow*), whereas an organelle which was not trapped appears to be moving in the merged composite (*white arrow*) as its position changes relative to the field of view as the stage moves. Scale bar, 3 μm

located manually using a joystick and aligned with the optical trapping position.

2. The organelle then undergoes a recorded trapping routine such as that highlighted in Fig. 5. Here, the trap is turned on, stage moved laterally at a fixed speed over a fixed distance (referred to as stage translation), trap turned off, and the movement of the organelle post trapping monitored.

3. Organelles are scored as either being stably trapped (i.e., remaining in the trap during stage translation), or escaping the trap during the stage translation, or are not trapped during the procedure.

4. Distribution of trapping outcomes among these three categories can serve to determine the relative differences in trapping efficiency between different conditions, and can be compared when optical trapping power is changed [5]; tethered organelles will tend to require greater force to trap and move them a fixed distance than organelles which are not tethered. This procedure was followed for characterizing interactions between peroxisomes and chloroplasts [5].

5. The major considerations are the laser power used to trap the organelle, and the distance and speed at which to move the surrounding environment relative to the trapped organelle. If the trapping force is too strong, it will simultaneously trap several refractile objects, and if it is too weak, then the object will not be trapped. Similarly, if the object is moved too quickly, or too far (i.e., beyond the cell boundary), it will escape the trap.

Through development of the experiment we have built a custom system to allow the interface between imaging and optical trapping to be integrated. The major benefit of an integrated system is that customized scripts can be written in LabVIEW® to control the stage lateral motion (i.e., speed and distances traversed), the timing of trap activation, and the subsequent data capture [5]. The essential components are therefore:

1. An optical trap.
2. A means of turning the trap on and off (we use a shutter that can be automated).
3. A microscope stage that can be moved at the sub-micron resolution scale at controlled velocity.
4. A means of imaging the organelles of interest in a real-time (TIRF).

3.6 Setting the Optical Trap Laser Power

If the optical trap is too strong, it will likely trap the majority of organelles it is centered over (which includes organelles which do not contain fluorophores). If the goal is to determine whether trapping efficiency is affected by molecular perturbation, then it is important that not all organelles can be trapped under control conditions. Therefore, trapping 20 organelles and assessment of whether they remain in the trap during the micromanipulation procedure (*see* above) is deemed necessary. In practice, we classify organelles that have undergone the trapping routine into three categories: stably trapped (i.e., stay in the trap over the entire

movement), escape the trap (i.e., "fall out" of the trap during the stage movement), or are not trapped at all. Once this distribution has been ascertained, a full characterization can be carried out with at least five samples from each condition where at least ten organelles are trapped per sample.

3.7 Optimizing Distance and Speed at Which to Move the Trapped Organelle

Determining the distance to move the trapped organelle is an important consideration, too far and organelles may escape the trap due to opposing force components encountered (e.g., moving it into cytoplasmic streams if working in a motile system or encountering other subcellular structures), too short and it will be difficult to assess whether the trapped organelle is still attached to the ER.

3.8 Post-Acquisition Data Analysis

Analysis of data generated from the integrated TIRF-M optical tweezer setup requires certain META data to be assigned to each data set. The META data enables the user to determine the coordinates of the optical trap, to overlay trails of trapping events for ease of initial analysis and indicates the crucial time points during the trapping routine (e.g., trap on/off).

3.9 META Data Output for Post-Acquisition Analysis

For each recorded trapping event, five data files are generated based on post-acquisition analysis requirements:

1 and 2. Raw images acquired from the synchronized EMCCDs as 16-bit TIFF stacks, one for each fluorescent channel.

3. An 8-bit false-color (RGB) TIFF stack containing overlaid channels for quick reference.

4. A txt file containing the lateral (x/y) trap (shutter) position at the start of the routine and any additional META data (e.g., optional notes).

5. A csv file containing lateral (x/y) positions as measured in μm relative to the top-left stage limit. These positions are measured at the end of each frame but could be measured more often if required.

3.10 Data Analysis

Data can be analyzed based on the distribution of organelles into the three categories (stable, escaped, not trapped classification), and how this changes with optical trap power or under molecular perturbation. In addition, the distance and rate at which stably trapped organelles move back towards their original pre-trap position after the trap has been turned off can be measured. These measurements enable the user to understand the nature of physical interaction or the surrounding environment of the organelle. Assuming constant viscosity, differences in recoil rate can be used to model the physical tethering process [5]. Statistical analysis undertaken is dependent on the hypothesis under test and the nature of the material. Keeping sample sizes constant over experimental runs allows for less complicated downstream statistical analysis. Commercial or free software such as ImageJ can be used to

analyze the TIFF stacks post acquisition, and using python or similar programming language the lateral stage positions can be used to map and subtract the movement of the stage for automatic/batch analysis of the relative movement (e.g., speed, distance, recoil) of the trapped organelle. Determination of precise force components in vivo is difficult owing to variability in the biological specimen; slight variations in organelle size and morphology, changes in microenvironments within the cell such as viscosity, cytoplasmic streaming events and potential shear forces from boundary layers generated in the constrained cytoplasmic environment. Due to these inherent complications generating variation of trapping efficiencies across the cell environment, a large number of interaction events need to be probed to obtain statistically significant data; we routinely trap at least 50 organelles in total taken from several samples from at least three independent plants from independent transformations.

4   Notes

1. Some batches or individual coverslips can have aberrations in the glass making trapping difficult. A manifestation of these issues is a significant change in Z focus upon trapping. If this occurs, move to another area on the slide or make a fresh sample.

2. Using a high numerical aperture TIRF objective lens to create a single beam gradient trap (laser tweezers) requires some care. The TIRF objectives with oil immersion have aberration when used in aqueous environments such as plant cells which reduces the effective numerical aperture for trapping from 1.49 to a value between 1.0 and 1.1. This both reduces the efficiency of trapping from a focusing perspective but also means that not all the laser power entering the objective is used for trapping. While we have used the TIRF objective in a conventional manner by slightly overfilling the input pupil, other researchers have found more efficient trapping by underfilling the objective. The transmission of laser light at 1090 nm of our TIRF objective was determined to be 70% using the double objective method suggested by Mahamdeh et al. [12].

3. Beads which have settled and adhered to the coverslip cannot be trapped and moved laterally. Beads which are "free floating" can undergo the trapping routine.

Acknowledgements

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