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A Double Tweezers and Laser Ablation (Scissors) Microscope for Biological Studies

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The thesis of Shahab Parsa is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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For the memory of my father, Mahmud, and the love of my mother, Soheila, my sister, Sara, and my brother, Arash.

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Abbreviations and Symbols

Abbreviations

CW – Continuous Wave

NA – Numerical Aperture

FSM – Fast-Scanning Mirror

HWP – Half-Wave Plate

PBS – Polarizing Beam Splitter

CCD – Charge Coupled Device

MT – Microtubule

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ABSTRACT OF THE THESIS

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Optical tweezers and scissors are two common applications of laser micro-beams in biological research. Previously in our lab, there have been experiments that utilized both optical trapping and cutting, but in these experiments, optical trapping and cutting were performed at different time-points as the sample would have to be transferred from an optical cutting system to an optical trapping system or vice versa. In this project, we designed and built a combined laser scissors and tweezers microscope that (1) has two trapping beams and (2) uses a short pulsed tunable 200 fs 76 MHz 710-990 nm Ti:Sapphire laser for laser microsurgery. Both the position and power of all three beams can be independently controlled. Moreover, experiments

were performed to test the ability of the system to trap and move whole chromosome or chromosome fragments inside living PTK-2 cells as well as chromosomes in suspension. The optical scissors laser was used at 730 nm to successfully cut chromosomes inside live cells. The optical traps were able to move whole chromosomes or chromosome fragments (after cutting) inside live Nocodazole-treated cells and also chromosomes isolated in suspension. The optical traps were not able to move intact or cut chromosomes inside un-treated cells, cold-treated cells or cells where the kinetochore or microtubule spindle was damaged using the optical scissors. Our experiments show that chromosome's geometry and index of refraction allow for easy trapping. However, when inside cells, the microenvironment around them, especially the microtubule spindle cage, can prevent any trapping movement.

Chapter 1 - Background and Objective

1.1 - Theory background on optical trapping and optical cutting

1.1.1 - Optical Trapping Theory

Arthur Ashkin was the first person to describe optical forces on micro-particles in 1970 [1] and observe the resulting trapping forces on such micro-particles [2]. Since then, optical trapping has been explored for many years as a means for manipulating micro and nano sized particles in biological or non-biological experiments. In this section, I will describe the theory behind optical trapping and how lasers can be used to create stable optical traps.

A beam of light consists of photons. Theoretically, photons at rest have no mass; however, photons at rest do not exist, and they travel at speed of light c (300,000 m/s in vacuum). Therefore, to calculate a travelling photon's mass (and its momentum), the energy of the photon calculated by the special theory of relativity ($E = mc^2$) is set equal to the photon's energy calculated by quantum theory ($E = \frac{hc}{\lambda}$). In this manner, it can be shown that a travelling photon has mass ($m = \left(\frac{h}{\lambda}\right)\left(\frac{1}{c}\right)$) and thus momentum ($p = m.c = \left(\frac{h}{\lambda}\right)$). When a photon enters a transparent particle, its speed and thus its momentum will be changed. This change of momentum is transferred to the particle as a force in the opposite direction to the change of momentum.

These forces can be significant relative to other forces if the object's size is small (nano to micro-meter range). For these particle sizes, there are three different

regimes when analyzing the trapping forces on a particle: Rayleigh, intermediate, and Mie. In the Rayleigh regime where the particle's diameter is much smaller than the laser wavelength, particles are treated as induced dipoles, and there are two major force components: the scattering force which pushes the object in the direction of light propagation and only acts in the z-direction (laser beam axis), and the gradient force which pulls the object towards the highest beam intensity, acting in the x, y, and z directions. Particles can be stably trapped when these two force components equal each other in the z-direction. The translational position of the trapped particle is determined by the gradient forces only [3] and for symmetrical beams pulls the particle towards the center of the beam. This induced dipole analysis fails as particles become larger compared to the wavelength [4]. As particles get larger, the analysis enters the intermediate regime, where rigorous wave analysis is required to define the forces present, and simple force equations cannot be derived [5-9]. Nevertheless, particles do trap in this regime, and other methods can be used for measuring the force exerted by the laser beam. In the Mie regime, the laser beam is treated as an infinite sum of rays of light. In this region, ray optics can be used to trace the path of each ray and the force it exerts on the particle. The results are then summed together to derive equations for the present forces. When the index of refraction of the particle is larger than the index of refraction of its surrounding medium, this net gradient force pulls the object towards the source of light. Stable trapping is achieved when this gradient force is equal to the scattering force (light pressure in the direction of light propagation). For

the gradient force in this region to be significant enough to equal the scattering force, the laser beam must be highly focused using a high numerical aperture objective [1-2].

Chromosomes generally have a diameter of approximately 1 μm and are also not spherical. Therefore, they fall in the intermediate regime and equations for the forces exerted on them cannot be simply derived. Although other methods can be used to calculate the forces exerted on the chromosomes which usually lie in the pico-Newton range, these were not measured during these experiments. The power of the laser at the focus plane, however, is reported as a reference.

1.1.2 - Optical cutting background

Different materials absorb different wavelengths of light. This phenomenon is due to the fact that energy is absorbed in a quantized manner, meaning that the material receiving the energy has to be able to absorb the specific quanta that the incident wave contains. This is dependent on the specific amounts of energy that the materials dipole can absorb to be excited to a higher energy state. The absorbed energy can then be reradiated or converted to other forms of energy such as thermal, or photochemical bond energy.

When a laser beam is focused by an objective, the light absorbed at the focused spot can be used to create sub-micron lesions whose size is determined by the numerical aperture of the objective and the wavelength of the laser beam, given by $1.22\lambda/NA$ [10]. Moreover, depending on the incident object's optical properties, the wavelength of light, the pulse duration, and its frequency, the mechanism of the damage can be different. For example, for various laser systems used for studying

DNA damage and repair, the mechanisms for damage were identified as single-photon absorption, multi-photon absorption and plasmid formation [11].

1.2 - Project Objective

Optical trapping and cutting have been utilized for many years as useful tools to non-invasively manipulate the environment inside the cell or the cell itself. Optical trapping has been used for exerting forces on cells or cellular organelles either directly or indirectly by using micro-beads [12].while optical cutting has been used to induce damage on or inside the cell in a repetitive and controllable manner [13].

The objectives of this project are to 1) design and build a system that allows for independent and simultaneous control of power and position for two optical tweezers (traps) and one optical scissor (cutter) and 2) show that such system can be useful for various biological studies, specifically for studying forces on chromosomes during mitosis (cell division).

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Chapter 2 - System Design and Construction

2.1 - Optical layout and configuration of the trapping and cutting beams

When designing the optical layout of a system, many parameters must be considered. First a choice must be made on the type of microscope objective that will be used. A higher numerical aperture objective is preferred as it will create a smaller focal spot of the laser and thus a higher gradient force, resulting in better capabilities. Moreover, slightly over-filling the back aperture of the objective with the trapping laser beam optimizes the trapping stiffness at a given input, and since different objectives have different back-aperture dimensions, for each objective a different set of lenses must be used to adjust the beam size. In this project, two different objectives were used at different points in time (Zeiss Plan-Aprochromat 63x 1.4 Oil Ph3 and Zeiss EC Plan-Neofluar 40x 1.3 Oil Ph3) on an inverted Zeiss microscope (Zeiss Axio Observer.A) that allows both phase and fluorescent microscopy. The advantages and disadvantages of using each setup will be discussed in section 2.4. Once the desired beam size at the back-aperture is known, various optical elements as described below are used to produce an ideally collimated beam of the right diameter at the back-aperture.

The complete optical hardware includes the inverted microscope, two different lasers and various optical elements. The lasers are a 1064nm CW Nd:YVO4 laser, model Millennia by Spectraphysics, Newport Co. (Newport Beach, CA) for optical trapping and a wavelength-tunable 710-990nm 76MHz 200fs Ti:Sapphire laser, model Maitai by Spectraphysics, Newport Co. (Newport Beach, CA) for optical cutting. A

detailed diagram of the final optical setup is presented in Figure 2.1, and a detailed discussion will follow. Working to from the objective towards the laser, the function of the various lenses inside the inverted microscope and the "scan lens" are to make the surfaces of the back-aperture of the objective and the external dichroic high-pass filter #1 mirror images of each other. It is important that the surfaces of the Fast-Scanning Mirrors (FSM-300-M-04, protected gold mirror, Newport Co.) are mirror images of the back-aperture of the objective. This is achieved by using beam relays that are two lenses of equal focal lengths, f, placed so that the two surfaces A and B are mirror images of each other. This means that the distance between A and B should be 4f, and the distance between each surface A or B and the two lenses should be 1f and 3f. Ensuring that the FSM plane and the back-aperture of the objective are mirror images means that ideally when the FSM is tilted resulting in movement of the focused beam spot, the beam does not move at the back-aperture. The advantage is that the power measured at the back-aperture when the beam is going straight into the adapter would ideally be the same as when the beam is tilted while moving the focused spot. If these two surfaces were not mirror images, when the trap is moved by the FSM, the beam would move slightly out of the back-aperture of the objective and thus less power would enter the objective. This would make accurate dosimetry very difficult.

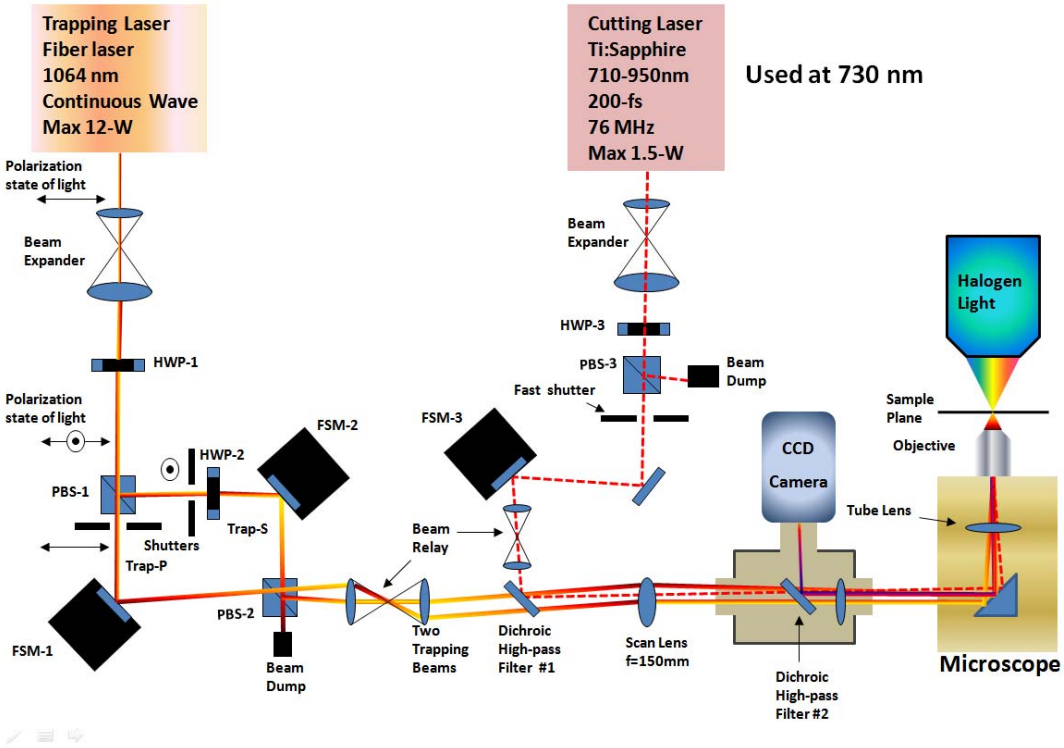

Figure 2.1 - A detailed diagram of the optical setup of the system. The diagram is explained in the text in detail.

To control the power in each optical trap and the optical cutter, a combination of a half-wave plate (05RP02-34, Zero-Order Quartz Wave Plate, Newport Co., Newport Beach, CA) and polarizing beam splitter (10BC16PC.9, Polarizing Cube Beamsplitter, Newport Co., Newport Beach, CA) is used in that order. The laser beam coming out of the trapping laser is fully polarized in one direction. A polarizing beam splitter (PBS) divides the laser beam into two beams with perpendicular polarizations as shown in figure 2.2 and also has the capability to join two beams that have perpendicular polarizations in one direction. A half-wave plate (HWP) has the capability to rotate the polarization of the laser beam. The amount of rotation depends

on the angle between the polarization direction of the incoming beam and the primary axis of the HWP crystal. Thus, each HWP is placed inside a motorized rotational stage (PR50PP, Newport Co.). The rotational stages can be controlled by the computer through a DAC board and a controller. Rotating the HWP using the motorized rotational stage changes the polarization direction of the laser beam and dictates what percentage of it will pass through the PBS and what percentage will be reflected by it. It is important to note that an HWP can only rotate the beam but cannot polarize an unpolarized beam. Therefore, if the initial beam is randomly polarized in all directions (for example from a fiber laser), the HWP will have no effect on it, and the PBS will simply split the beam into two beams of roughly equal power. As shown in figure 2.1, HWP-1 and PBS-1 together control the power in trap-P, while HWP-2 and PBS-2 together control the power in trap-S, and finally HWP-3 and PBS-3 together control the cutting beam power. This allows independent control of the power for all three beams. The positions of the three beams (trap-P, trap-S, and the cutting beam) are also controlled by FSM-1, FSM-2, and FSM-3 respectively. Therefore, this setup allows for independent control of power and position of two optical traps and one optical cutter.

It should be noted that the inverted microscope is equipped with an x-y stage micro-manipulator that can be controlled by the software in addition to a manual joystick. However, the z-direction's adjustment can only be done manually. Thus, the depth of the imaging must be adjusted manually while the translational position of the sample and the traps can be controlled by the software or the manual joystick. It is

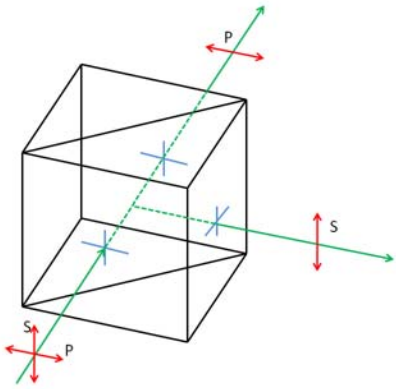

Figure 2.2 - A schematic diagram illustrating how a polarizing beam splitter (PBS) splits an incoming light beam into two outgoing light beams.

noted that for a single trap setup, an x-y stage micro-manipulator can essentially perform the same functions as an FSM, since moving the stage while the laser beam is stationary changes the location of the beam compared to other objects in the sample. However, when there are two different trapping beams present, moving the stage moves the elements inside the sampled dish relative to both beams by the same amount. Therefore, to achieve independent and easier control of both traps, having two separate FSMs is preferred.

The femto-second Near InfraRed (NIR) laser used for optical cutting is shared with another system. The most commonly used wavelengths for the laser microsurgery are 730nm or 780nm, and throughout the experiments described here the laser was used at 730nm. The beam directed to this system is expanded to fit the back aperture of the objective. As mentioned previously, the position and power of the beam can be controlled independently using an FSM and a pair of HWP and PBS. All three beams use milli-second shutters (uniblitz LS6ZM2, Rochester, NY) for turning the beam on

and off. However, it is important that the shutter for the cutting beam is a fast shutter because too much exposure to this beam can result in a catastrophic cell destruction. Therefore, when cutting a "line," multiple dots with adjustable distances are ablated. This is a way to minimize the amount of energy added to the cell. The fs laser beam joins with the two trapping beams by reflecting off a dichroic beam splitter while the trapping beams pass through the beam splitter. The three laser beams are focused by a 150mm scan lens to ensure the scissor and tweezers can be manipulated across the whole field of view of a Charge-Coupled Device (CCD) camera. A 1,344 X 1,024 pixel 12-bit digital CCD camera (ORCA R2, Hamamatsu) is used for fluorescence or phase contrast imaging at up to 28 frames per second without binning. For real time imaging, the camera and the laser beams share the same port from the microscope (left imaging port) using another dichroic beam splitter which reflects visible light to the camera while allowing the laser beams to pass.

2.2 - The software used for controlling trapping and cutting

The computer software portion of the system is needed for controlling various hardware functions that can be broken up into two general functions. The first function is acquiring images from the samples (phase or fluorescent) through the images received from the CCD camera and controlling various microscope shutters. The second function is to control the laser beams using the various hardware components of the optical layout such as the FSMs, rotational stages, and the translational stage on the microscope. The hardware control was achieved using self-designed 'RoboLase' software based on a Labview (National Instruments) environment. This software

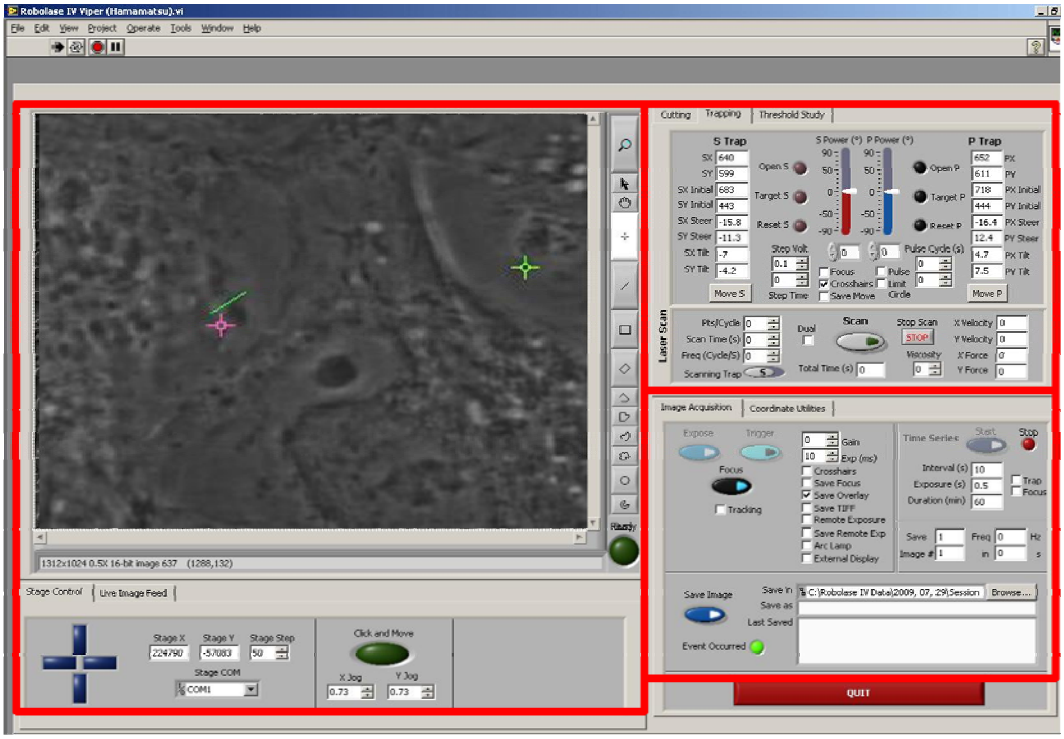

Figure 2.3 - The software interface used for controlling the hardware. This software was developed by our lab members, Dr. Linda Shi and Stevie Harsono using LabView.

provides the ability to control laser power in both laser systems, to maneuver all laser beams independently, to provide complete control of the microscope stage in the x and y directions, and to allow microscope focus and image acquisition (Figure 2.3) [1]. In addition, the system is designed as an open platform which allows users to operate the system through the internet [2].

2.3 - The dual-objective method used for determining power loss in objectives

To measure the transmission properties of the two different objectives used, the standard dual-objective method was used. Figure 2.4 represents a schematic of the setup used. The various laser beams as they would normally enter the inverted

microscope and reach the objective were used. The power of the beam before it enters the first objective is measured by removing the objective and measuring the power at the back-aperture plane of the objective using a power meter (1830-C, Newport Co., Newport Beach, CA). This is the initial power or P_i. Next, the first objective is positioned properly followed by a layer of oil, a glass slide, a second layer of oil, and finally the second objective positioned upside down and held in place by an xyz micro-manipulator (Figure 2.4). Finally the power meter is placed at the back-aperture plane of the second objective to measure the beam's power after passing through both objectives. Then, the micro-manipulator is used to move the second objective in 3-

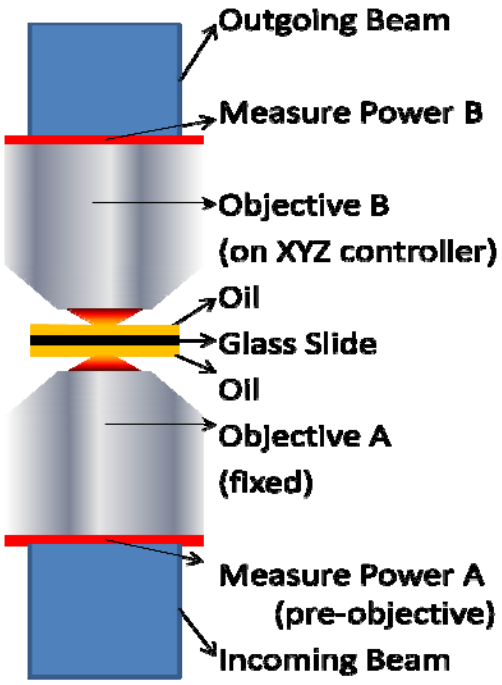

Figure 2.4 - A schematic illustrating the double-objective method for determining transmittance of objectives.

directions until the maximum read-out power is achieved. At this point, the two objectives are aligned with each other in the vertical direction as long as the incoming beam is vertical, which is tested for before this procedure. The final power measured, P_f, is related to the initial power, P_i, by the transmission coefficients of the two objectives, T_1 and T_2, through the following equation: $P_f = T_1 T_2 P_i (eq. 2.1)$ where the transmissions are expressed as decimals.

As can be seen from eq. 2.1, in one experiment, there are two measurements (P_i and P_i) made and there are two unknowns T_1 and T_2. Therefore, one experiment with two different objectives cannot completely determine the transmission of the two objectives. However, there are multiple solutions to this problem. First, it is possible that the transmission of one of the two objectives for the desired wavelength is known from other experiments or from the manufacturer's specification sheets. Here, the known transmission allows us to measure the transmission of the second objective by performing one experiment. A second option is to assume that both objectives have equal transmissions, which is a reasonable assumption if the two objectives are identical and brand new. However, as objectives are used they are damaged differently, and their transmissions are likely to be different. The final option is the only one that can be used with a combination of different objectives when none of the transmission coefficients are known. However, it requires that there are three objectives present. The three objectives can be used in three different pairs of experiments, resulting in three different equations: $T_1 T_2 = A$, $T_1 T_3 = B$, and $T_2 T_3 = C$, where A, B, and C are measured constants (P_f / P_i). These three equations can then

be solved for the three unknowns by simple substitutions. In this way, the transmission of all three objectives is determined without any prior knowledge or assumptions.

It should be noted that it is necessary that the upper objective has an NA equal to or larger than the lower objective's NA to be able to capture all the light that has exited the lower objective. Therefore, if a 40x 1.3NA objective and a 63x 1.4NA objective are to be used in one experiment, the 63 x 1.4NA objective must be the upper objective (in figure 2.4). Also, to make sure that the measured power before the first objective is precise an adapter that has a hole with the same size as the back-aperture of the objective being used is necessary. If not present, a pinhole adapter that will let only a small portion of light pass through may be used at a small distance before the first objective during both measurements in each experiment. This is necessary to make sure all the beam power that is measured actually goes through the objectives.

2.4 - The optical differences between the 63x 1.4 NA and the 40X 1.3NA setups

During the experiments performed with the system, two different objectives were used: one with 63x magnification and 1.4 NA (Zeiss Plan-Aprochromat, Oil Ph3) and one with 40x magnification and 1.3NA (Zeiss EC Plan-Neofluar, Oil Ph3). Table 2.1 compares aspects of these two setups. As can be seen in table 2.1, the sizes of the back-apertures of the two different objectives as well as each objective's transmittance of the trapping wavelength (1064nm) are different, resulting in different trapping power at the focal plane using all the power in one trap. However, these numbers must

Table 2.1 - A comparison between two different conditions of the system with a 63x 1.4NA objective and a 40x 1.3NA objective.

	Setup 1	Setup 2
Objective	63x	40x
Numerical Aperture	1.4	1.3
Objective Transmittance for 1064 nm	~26%	~46%
Back Aperture Diameter	7.9 mm	10.9mm
Measured Power Before Objective (using laser at 10W max, the 63x adapter and all power in one trap)	~1356 mW	~1750 mW
Calculated Power Post-objective	~352 mW	~800 mW

be viewed with caution as the 63x objective has a higher numerical aperture and thus would be able to create a higher gradient force (see section 1.1.1) than would the 40x objective at the same laser power.

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Chapter 3 - Biological Experiments

3.1 - Background on biological trapping experiments and cutting experiments

3.1.1- Trapping background

Since optical traps were described in 1986 [1], the optical trap has been employed as a useful tool in many different types of biological experiments: for manipulating yeast cells, blood cells, protozoa and various algae and plant cells [2], for trapping of viruses and bacteria [3], for measuring the compliance of bacterial flagella [4], for internal cell surgery [5], for manipulating chromosomes [6], for trapping and force measurements on sperm cells [7-8], for measuring the forces exerted by the motor molecules kinesin and dynein along microtubules [9-10], and for cell sorting with optical recognition [11]. Biological and medical applications of optical traps include probing the viscoelastic properties of single biopolymers such as DNA, probing cell membranes and aggregated protein fibers such as actin, and characterizing cells with different size and refractive indices [12-16].

3.1.2 - Cutting Background

Laser scissors (cutting) have been used as a method for creating sub-micron lesions over the past 40 years. In 1969, Berns et al. used a focused laser beam to generate sub-micron lesions in living cells [17]. This localized irradiation can be used to selectively deactivate the nucleolus genes and to create subsequent cellular lines [18]. In addition to chromosomes, other cellular structures such as the mitotic spindle

can be manipulated by laser microdissection [19]. Studies also indicate that multi-photon effects from short pulsed lasers can be utilized in subcellular surgery [20-21].

3.2 - Biological experiments

The initial objective in the biological experiments performed was to carry out a combination of optical trapping and cutting experiments on live cells concurrently with the goals of examining the forces on chromosomes during mitosis and intentionally creating cells with polyploidy or monoploidy. It was initially hypothesized that optical trapping could be used to hold chromosomes in place while a cell undergoes mitotic division, and the minimum trapping power required to perform this task could be used to approximate the forces exerted on chromosomes by the microtubule spindle. Furthermore, it was hypothesized that it may be possible to use optical tweezers to direct whole or fragments (after cutting a chromosome by optical scissors) of chromosomes away from the daughter cell where they belong and into the other daughter cell. Such an experiment would produce daughter cells with too much and too little genetic information, and the fate of such daughter cells would be interesting to follow. Moreover, it was hypothesized that splitting the power of one strong optical trap into two weaker optical traps could result in the same amount of chromosome movement but would decrease the heating damage as the energy would be deposited over a more diffuse area.

All biological experiments on live cells were performed on *Potorous tridactylus* (Rat kangaroo) kidney epithelial cells (PTK2, originally obtained from American Type Culture Collection, ATCC CCL-56) or one of its stably transfected

lines graciously provided by Professor Jagesh Shah at Harvard Medical School, Boston, MA(CFP tagged tubulin (P133) or YFP tagged kinetochores (F105)). PTK-2 cells have been a stable cell line since 1962 and can be stably transfected. They have 11 distinguishable chromosomes and unlike most other animal cells, stay flat during mitosis. These properties make them an ideal cell line for experiments involving mitosis and manipulation of condensed chromosomes during mitosis. Cells were grown in modified Eagle's medium supplemented with 10% fetal bovine serum. The cells were incubated at 37°C with 5% CO2 and then were seeded into Rose chambers at least 48 hours before scissor/tweezers experiment as previously described [22].

3.2.1 - Experiments on live cells

3.2.1.1 - Trapping experiments on untreated cells

The first set of experiments involved trying to move chromosomes of parental cells in different stages of mitosis. Multiple cells in pro-metaphase, metaphase and anaphase were experimented on. The two optical traps, each containing approximately 165 mW at the focus plane, were placed approximately a micron away from the cut pieces of chromosomes. No movement was observed that could be attributed to action of the optical trap. Even if the traps were positioned on top of the chromosome fragments and slowly moved in any direction, no noticeable movement of the chromosome pieces was observed. The existence and location of the optical trap was confirmed by trapping of debris of similar size (1-2 μm) outside the cell but in an area that was devoid of cells. It was often attempted to move the debris transversely inside the cell when both the cell and the debris were in focus. In each case, the debris would

get stuck at the cell membrane and would not pass into or over the cell. These experiments confirm that the optical trap is focused inside the cell and is at the same depth as the microscope's focus. The depth of the traps were often tested using 1 and 5 μm beads by trapping the beads and reassuring that they are trapped in the imaging plane.

While performing these experiments, optical cutting of chromosomes was often performed with the expectation of creating smaller chromosome fragments whose movement due to trapping would theoretically be easier to detect. Successful cutting of chromosomes was often possible and reproducible using irradiances ranging from 7-10 * 10^{11} W/cm^2. Figure 3.1 shows a successful cutting attempt on a dividing cell during metaphase. As can be seen in (a), the cell is in the metaphase stage of

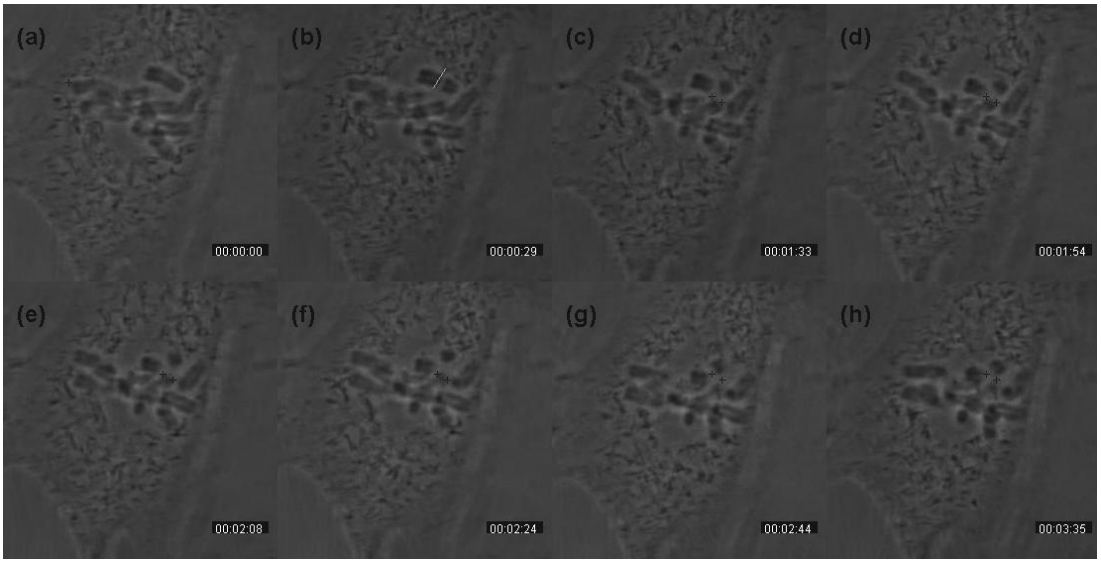

Figure 3.1 - (a) shows a mitotic cell at metaphase. One chromosome has not reached the metaphase plate yet. (b) shows the line that was used for cutting this chromosome while (c-h) show the movement of the two cut pieces with respected to each other over time. The two (+) signs in (c-h) also denote the position of the two optical traps but as can be seen no movement was observed that could certainly be attributed to the action of these optical traps.

division with all but one of the chromosomes pairs aligned at the metaphase plate. In (b), a line was drawn and optical cutting was performed using 10^{11} W/cm^2 irradiance. As can be seen in (c-h), the two pieces become separated and change their orientation compared to each other which suggests that the two chromosomes were completely severed from each other. Moreover, the two black (+) signs in (c-h) denote the position of the two optical traps, each with approximately 220mW of power in the focal spot. However, the movements of the chromosomes were not consistent with the position of the traps and could not be attributed to their presence.

One interesting result encountered in this series of experiments is depicted in figure 3.2. As can be seen in (a), the cell is in the prophase stage of mitosis. During an attempt to cut a chromosome, not depicted in the figure, a microplasma was formed which created a whole in the cell membrane and allowed the surrounding media to enter the cell as can be seen by comparing the position of the cell membrane (b-h). The arrow points at the position of the trap represented by a black cross-hair in some portions of the figure. By moving the trap, the chromosome is toggled back and forth multiple times between (b) and (h). The chromosome would simply pop into the trap once the trap was targeted to be approximately 1 micron away from the chromosome and then turned on. It was also possible to simply bend the chromosome back and forth by dragging it from one position to the other by the trap. However, it was not possible to pull the chromosome completely out of its position.

This experiment along with other experiments demonstrating the ease with which trapping could be performed outside cells on objects of similar size to

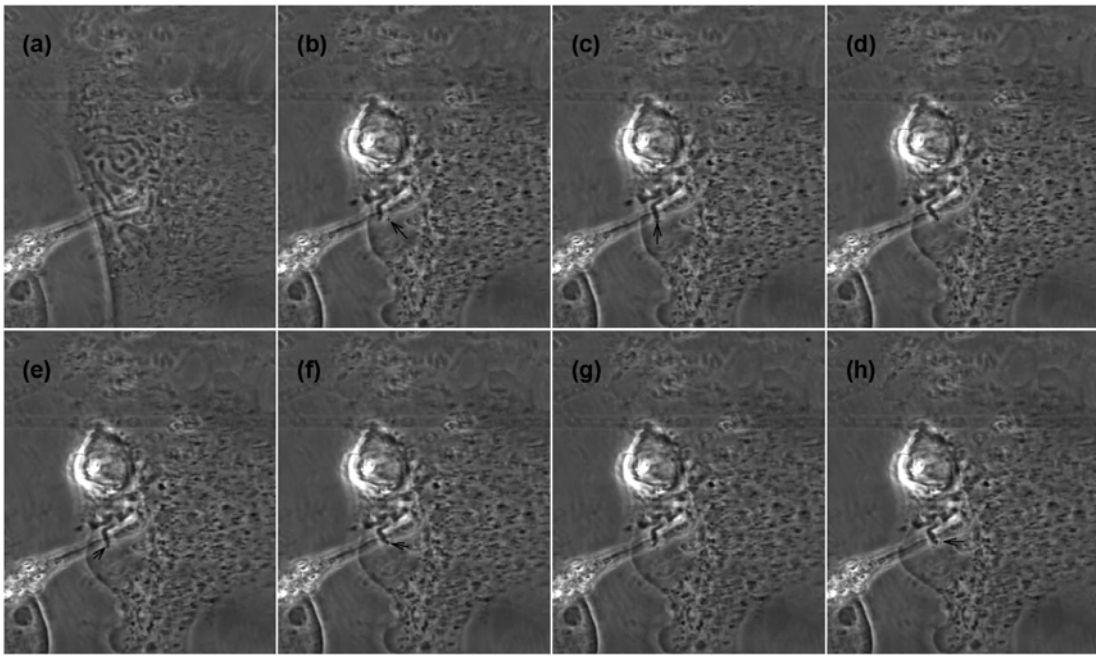

Figure 3.2 - (a) shows a mitotic cell at metaphase. One chromosome has not reached the metaphase plate yet. (b) shows the line that was used for cutting this chromosome while (c-h) show the movement of the two cut pieces with respected to each other over time. The two (+) signs in (c-h) also denote the position of the two optical traps but as can be seen no movement was observed that could certainly be attributed to the action of these optical traps.

chromosomes (floating debris or microbeads) suggested that it was the micro-environment around the chromosomes inside the cells that did not permit trap-induced movement to occur inside parental PTK-2 Cells. It is important to note that whether or not the chromosome resides at the trap's focal spot can only be verified if some movement of the chromosome is observed when the trap is turned on or moved. Therefore, if the chromosome resides at the focal point of the trap but the trap cannot move the chromosome to a nearby location, no movement will be observed and thus the chromosome is said not to be trapped. Therefore, it was hypothesized that the micro-environment around the chromosomes inside a living cell did not allow for movement of the chromosomes even if they were trapped.

When one looks at the intracellular environment, it can be seen that the cytoskeleton consisting of various filaments (actin, microtubule, etc.) along with many other intracellular organs can provide a high resistance to the chromosome movement. It is important to note that during mitosis, the microtubule spindle consists of numerous microtubules that control the movement of chromosomes to either end of the cell by direct attachment to their kinetochores. It was hypothesized that it was mainly the presence of this microtubule cage that was responsible for the inability to observe chromosome movements inside a living cell when the laser trap was applied.

3.2.1.2 - Experiments on drug-treated cells

To test the theory that the microtubule spindle was creating the resistance to the chromosome movement by the trap, we hypothesized that the use of a well known microtubule inhibitor, such as Nocodazole, to dissolve the microtubule spindle would result in release of the spindle inhibition such that trapping and movement of chromosomes would occur. In this set of experiments, cells that had been incubated in Nocodazole (1μg/ml for periods of 4-24 hours) were used. The trapping of chromosomes and chromosome fragments was successful in multiple cells. These cells were mostly arrested in pro-metaphase or metaphase due to Nocodazole's action. An example of chromosome trapping is illustrated in figure 3.3. In (a), the two traps (165 mW at the focal plane) are located close to, but not directly on the chromosome. After turning the trap on, the chromosome quickly tilted towards the traps (b) and then moved completely into the two traps (c). When the traps were moved to a new location, the chromosome moved to the new location with the traps (d). Even one

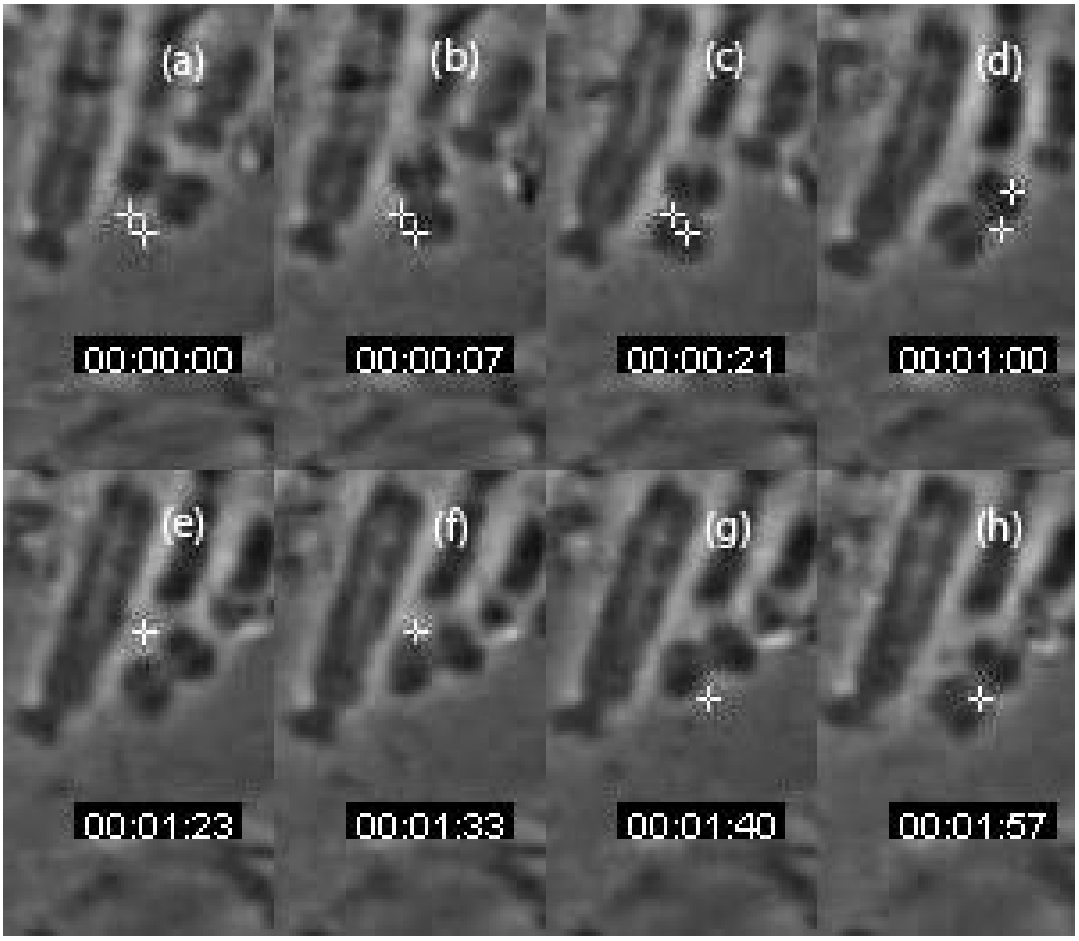

Figure 3.3 - Optical trapping of a chromosome in a Nocodazole treated cell. Two optical traps, each with 165 mW are used to move and rotate a piece of chromosome. The two traps are off in (a), (e) and (g) but on in (b), (c), (d), (f) and (h). Images (b), (c) and (d) show the response of the chromosome to the positioning of the two traps. In (f) and (h), one trap (165 mW) is used to move the chromosome back and forth after first positioning the trap while it was off (in (e) and (g)).

trap with 165 mW was sufficient to move the chromosome back and forth (e-h). Other chromosomes were also movable inside the same cell and in other cells.

In Nocodazole-treated cells, chromosome fragments were also moved after the chromosome had been cut using the 730-nm 200-fs beam as described in figure 3.4. The pre-cut chromosome can be seen in figure 3.4 with the line drawn on the

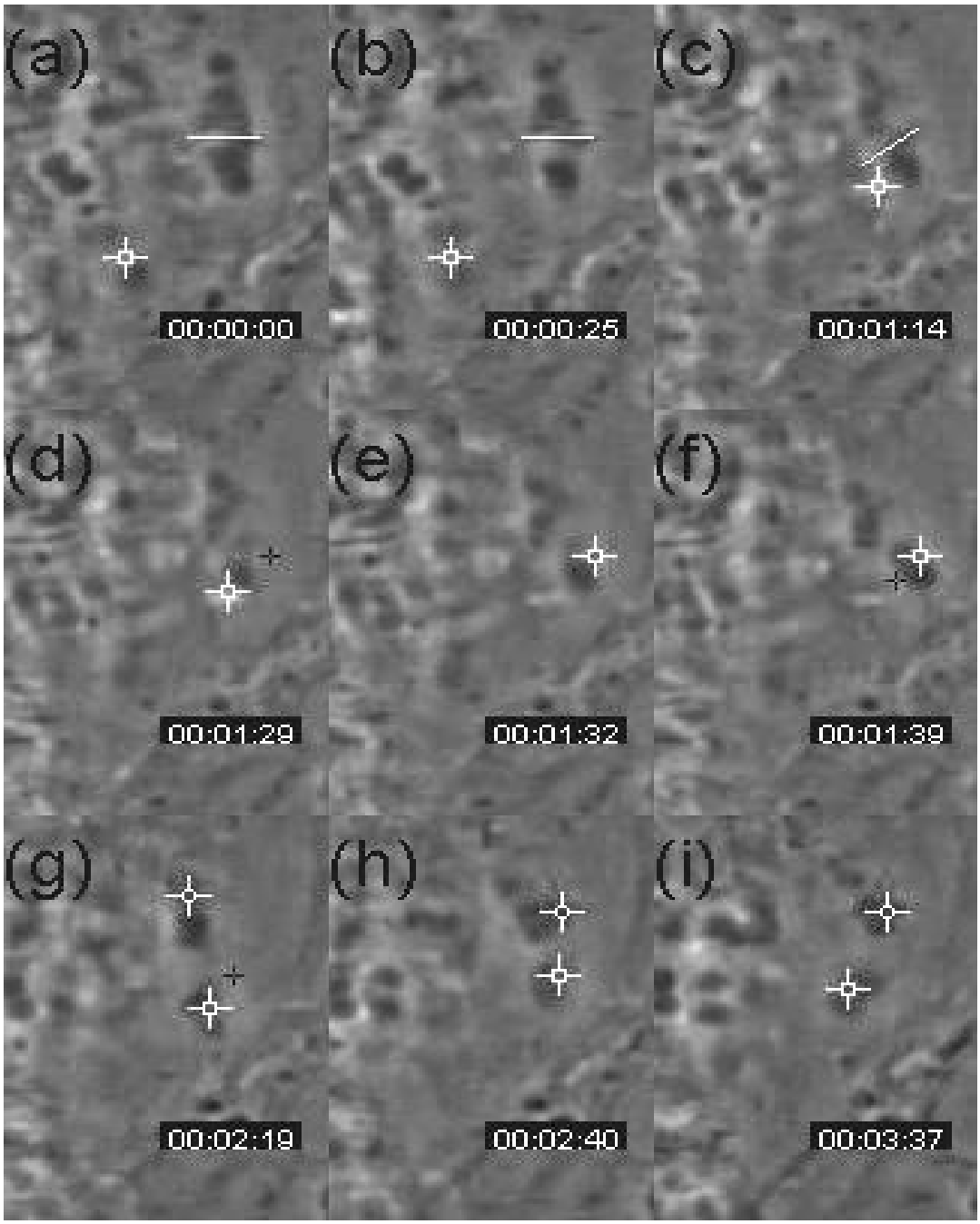

Figure 3.4 - Laser microsurgery of a chromosome on a Nocodazole treated cell. The selected chromosome is cut using a 200-fs, 76 MHz, 730nm beam once in (a) and again in (c). (b) shows the chromosome after the first cut and shows small connections between the two fragments. (d) shows the fragments after the second cut, while in (e) and (f) the lower fragment is moved by use of an optical trap with 165 mW. In (g-i), a second trap of 165 mW is positioned on the upper chromosome fragment and used to move the upper fragment to the right. The lower chromosome fragment is moved up from (g) to (h) and then back down to (i) from (h) by use of the optical trap.

computer indicating the region that was subsequently cut. In (b), the cut immediately following laser exposure is shown. To ensure that the two pieces were severed from each other, another cut was made along the line depicted in (c). In (d), the position of the trap after the cut is shown. In (d-f), the lower chromosome fragment is moved by the optical trap to a new location. In (g), the second trap is positioned on the second chromosome fragment. This chromosome fragment is then moved to a new location as shown in figures 4(h-i). Simultaneously, the lower chromosome fragment is moved to a new location as illustrated in (g-h). It is then moved back to its original position (i).

Different chromosome behaviors observed under optical trapping force in Nocodazole treated and untreated mitotic cells suggest that microtubules play an important role in holding chromosomes in place, and in particular, in resisting externally applied forces to whole chromosomes and their fragments. Figure 3.5 shows two images of microtubule immunofluorescence staining for untreated and Nocodazole treated cells. In the treated cell, the microtubule network has disappeared. This may explain why the cytoplasmic resistance is so much less in the Nocodazole treated cells. On the other hand, not all the chromosomes in Nocodazole treated mitotic cells could be moved with the optical trap. This indicates that there might be other cytoskeleton components (such as actin) creating resistance to pulling the chromosome during mitosis. This appears to be substantiated by actin immuno-staining (data not presented). It was also noticed that the chromosomes of prophase cells are easier to move with the laser tweezers.

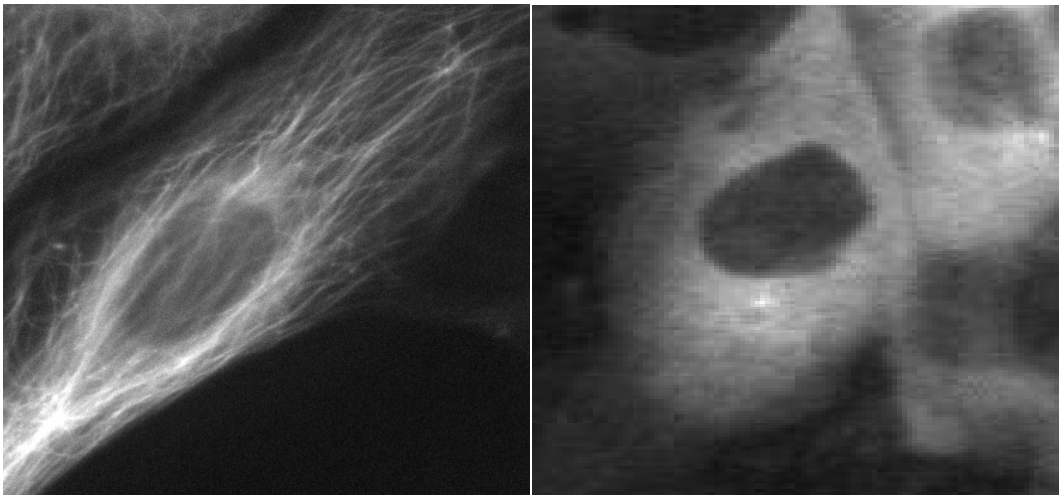

Figure 3.5 - The effect of Nocodazole treatment on microtubule filaments as shown by fluorescently tagged microtubules. On the left, a normal cell where clear filaments are visible. On the right, a cell treated with Nocodazole (usually 0.1-1 ug/mL), where microtubules are visible as a fuzzy cloud. Picture Courtesy of Nicole Wikada.

3.2.1.3 - Experiments on cold-treated cells

In addition to drugs, treating live cells with cold is known to affect microtubule (and other cytoskeleton elements) dynamics and shift the equilibrium towards dissolved microtubules from microtubule filaments [23]. Therefore, as another attempt to verify that the cytoskeleton and mainly the microtubules are responsible for our inability to move chromosomes in non-treated cells, we performed other experiments on live PTK-2 cells stably transfected with a CFP-microtubule (P133). In these experiments, the cells were put inside the refrigerator (4^{o}C) for periods of 30 minutes to 2 hours while their temperature was monitored using an infrared temperature-recording device (Raytek MX – RAYMX4PCFU, Santa Cruz, CA). After being removed from the refrigerator and being placed on the microscope, icepacks were placed on top of the chambers to keep the cells cold for a longer period of time. When

removed from the refrigerator, the chambers had a temperature of 4^{o}C and gradually increased in temperature to the room temperature within approximately 1 hour if the icepack was not replaced. Fluorescent pictures were taken of cells before and after being treated with cold to evaluate the dissolving of microtubules. Figure 3.6 shows a sample fluorescent picture of the same cell before and after being treated with cold. As can be seen in the pictures, some microtubule filaments can be distinguished as lines both before and after cold-treatment, but in general, cells under both conditions have a diffuse cloud of fluorescent material. Therefore, it was not possible to confirm whether the microtubule filaments had fully dissolved as a result of the cold-treatment.

It was attempted to trap the chromosomes or chromosome fragments in these cold-treated cells using the same parameters as before (~165 mW in each trap at focus or ~350 mW in one trap). However, no movement due to trapping could be observed in any of the n = 15 cells. The reason for this could be either that the microtubules were not dissolved properly or the micro-environment changes as a result of the cold

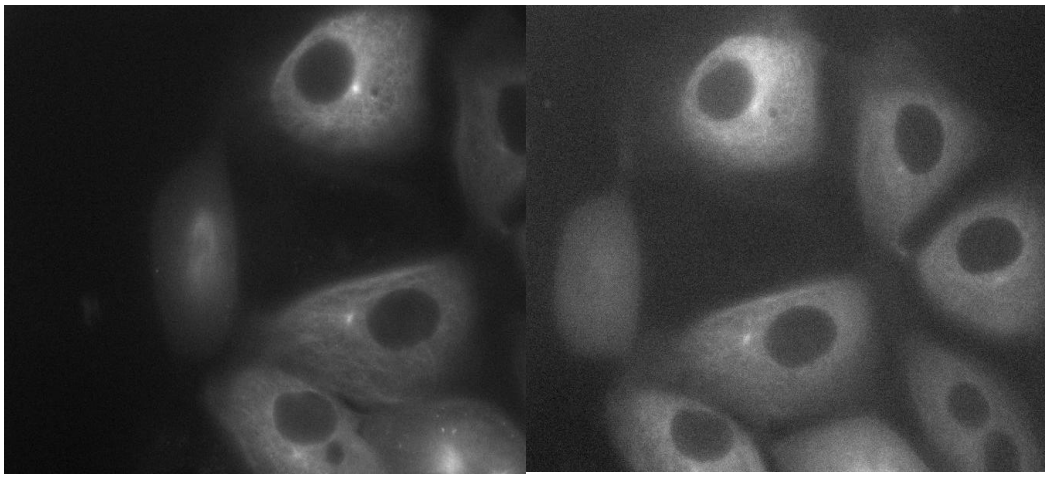

Figure 3.6 - The effect of cold-treatment on microtubule filaments as depicted by PTK-2 cells with CFP-tagged microtubules. On the left, normal cells at room temperature. On the right, cells after being at 4 ^{o}C for approximately one hour.

prevented movement of chromosomes. Theoretically, the lowered temperature can also make the environment inside the cell more viscous as well as create micro-crystals that would inhibit chromosome movement.

3.2.1.4 - Experiments on non-drug-treated cells with cut kinetochore/MT

Another set of experiments aimed at determining the effects of microtubules on the ability to move chromosomes with traps was also performed. In these experiments, the cutting laser system was used to ablate the YFP-fluorescent kinetochores of dividing cells PTK-2 cells in order to detach the chromosome from the microtubule spindle. Although the disappearance of the fluorescence cannot be considered as proof of detachment of the chromosome from the microtubules, an observed "sling-shot" effect where the chromosome quickly moves to a side of the cell after ablation indicates that one of the chromosome kinetochores is destroyed. Both kinetochores of one chromosome were ablated in some instances to theoretically completely detach the chromosome from the microtubule spindle. This should have a similar effect as the previous experiments where chromosome fragments were cut away from the remaining part of the chromosome that was attached to the kinetochore. Figure 3.7 shows fluorescent and phase images of a cell before and after the kinetochore ablation followed by an attempted chromosome trapping. In these experiments, no difference was noticed from the un-treated experiments. In both series of experiments, the chromosomes are theoretically detached from the microtubule spindle but are still inside an organized network of microtubules which we have theorized are blocking the chromosome movement when the trap is applied.

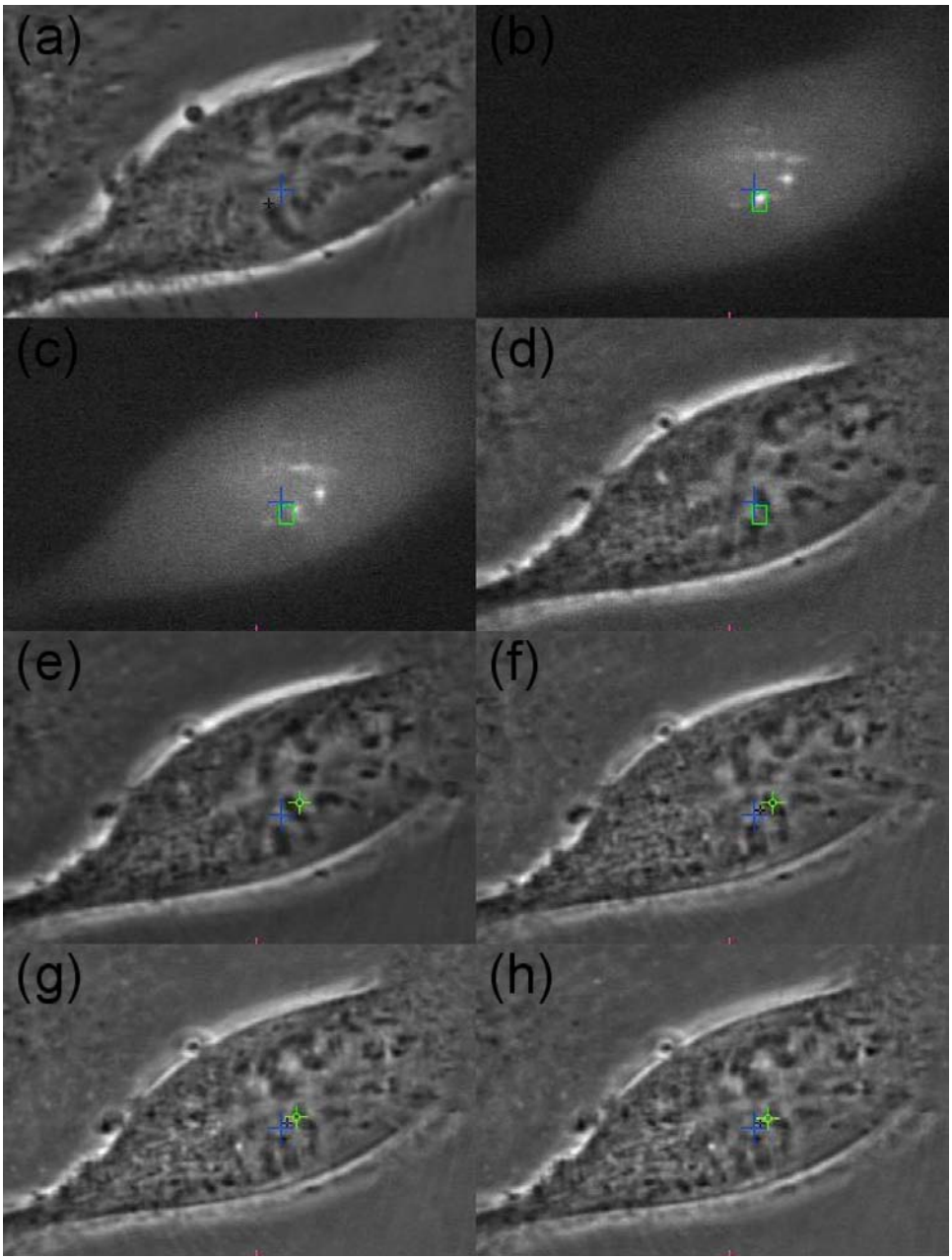

Figure 3.7 - Kinetechore ablation of YFP- tagged PTK-2 cells. The blue cross-hair in all pictures shows only the center of the computer screen. (a) shows the cell before any ablation. The green box in (b) is where a kinetechore exists and is ablated between (b) and (c). No fluorescence is detectable in (c) after the kinetechore ablation. (d) shows a phase image of the cell after the ablation. The green cross-hair in (e-h) is the position of the trap which was on throughout. Although the trap is moved back and forth between (e) and (h) no movement of chromosome is observed that can be attributed to the movement of the optical trap. ~150 mW at focal spot of the optical trap.

3.2.2 – Experiments performed on isolated chromosome extracts

To achieve a better understanding of the system's chromosome trapping capabilities, it was decided to perform trapping experiments on different samples of chromosome suspensions outside living cells. The system is able to trap 5 and 10 micron beads with great ease in a suspension although 1 micron beads can either be stably trapped or be pushed by the trapping force. In both cases, it is obvious that the trap can exert a force on the 1 micron bead, which is similar in diameter to the thickness of PTK-2 chromosomes, although the chromosomes can be much longer. Therefore, it was theorized that it should be possible to trap and move a chromosome in suspension. Three different sources of chromosome suspensions acquired from different types of cells were used as described below.

3.2.2.1 - Chromosome isolations made from primary cultures

Initially help was sought from the researchers and scientists at San Diego Zoo's Institute for Conservation Research. As the holder of the largest bank of animal karyotypes, this institute routinely extracts chromosomes and prepares suspensions from primary culture cells. The extraction protocol is detailed in part A of the appendix and was generously provided by Marlys Houck, a genetics researcher at this institute. Various primary cultures were used in these experiments including Antelope cells treated with Colcemid and fixed with methanol/acetic acid solution, and Deer mouse cells treated with and without Colcemid.

In addition to the hypotonic medium's effect on cell lysing, the last step is to release a drop of the solution on a slide and the drop contact would help with the

lysing process. However, we could not use this method as we needed the chromosomes to be in suspension and not attached to the glass slide as would happen as a result of the drop. It is important to note that after the chromosomes attached to the glass slide, it was not possible to move them using the optical trap, suggesting that the attachment force between the chromosomes and the glass slide was higher than that of the trapping force. To overcome this problem, we tried using a 23G syringe to aspirate the solution in and out of the samples dish in attempt to more fully lyse the cells. However, since the chromosomes in these samples were not marked with any fluorescent tags, it was not possible to distinguish between the floating chromosomes and other floating debris even if there were successful lysis. However, the floating pieces whether small or large were trappable and could easily be moved in the solution with similar trapping power as before (~150 mW at focus point) and only using one trap. Therefore, to make sure that we can distinguish between the floating chromosomes and floating debris, we decided to make use of two other sources of isolated chromosome suspensions: HeLa (human) chromosomes with YFP tagged histones and Chinese Hamster Overy (CHO) cells which have much larger chromosomes and are easily distinguishable as chromosomes when in suspension. CHO chromosomes are also similar in size to PtK2 chromosomes.

3.2.2.2 - Experiments on isolated HeLa chromosomes

These chromosomes were provided as a courtesy from Dr. Don Cleveland's lab at UCSD. The protocol for purification of these chromosomes from HeLa cells is provided in part B of the appendix as performed by Dr. Anita Kulukian. This protocol

provides a much more purified and concentrated solution of chromosomes with minimal debris. In addition to this, the fact that the histone molecules on the chromosomes are YFP tagged make distinguishing the chromosomes much easier. The chromosomes were stored in a liquid nitrogen tank and re-suspended in a PIPES buffer solution after thawing. These floating chromosomes were easily trappable and moveable using the trapping system with low powers. Initially powers of up **to** 150 mW (at focus point) in one trap were used to trap and move the chromosome pieces. However, this power could be lowered and the chromosomes were still trappable and moveable at powers as low as 10 mW (at focus point) and finally most chromosomes were released from the trap at extremely low powers around 3-4 mW (at focus point). Figure 3.8 shows a sample of how trapping was performed and how fluorescence was used to confirm that in fact chromosomes were being trapped. The floating chromosome is trapped in (a) and (b) and is moved between these two pictures. In (c) and (d) two fluorescent images are taken of the chromosome once the trap was released (fluorescence could not be used at the same time as trapping due to the fact that two different dichroic mirrors and entering ports were used). In (e) and (f) two more phase images that also suggest the shape of a chromosome are shown. However, these phase images alone would not be a definite confirmation that the piece is a chromosome and not a floating piece of debris. Please note that since the fluid has a very low viscosity, the chromosome moves around when not trapped due to normal fluid disturbances. The green arrow is the position of the trap that is on in (a-b) and off in (c-f).

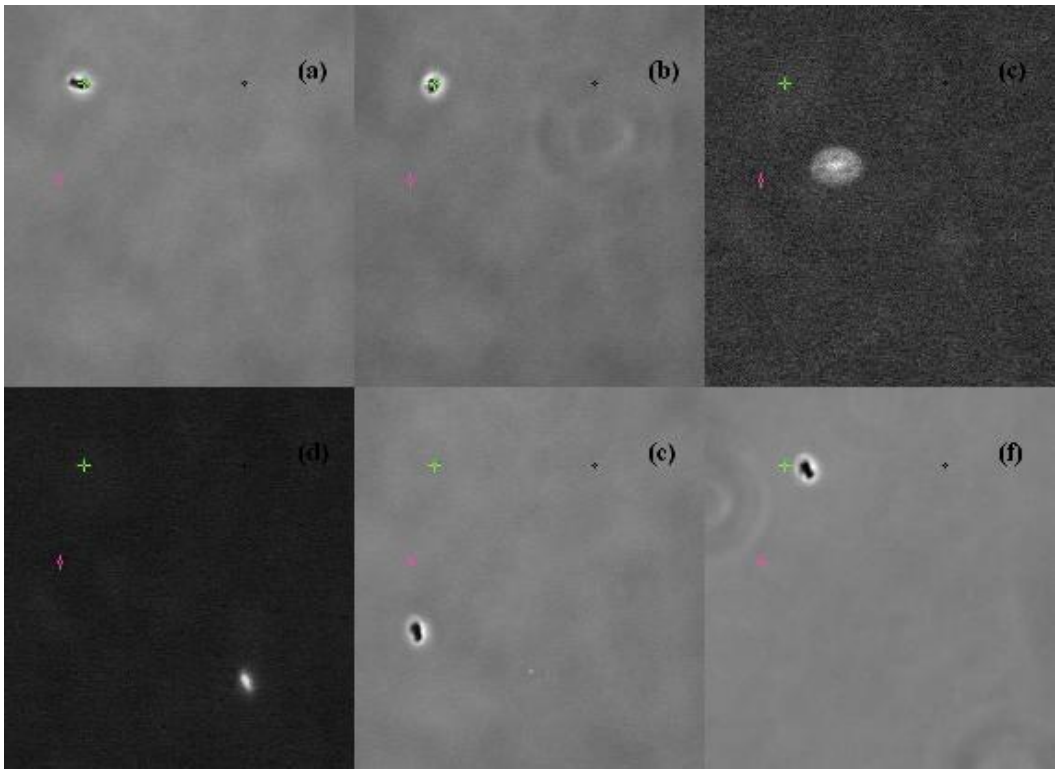

Figure 3.8 - A sample trapping experiment on HeLa chromosomes in suspension. (a-b) show the chromosome trapped and moved using the trap between (a) and (b). (c-d) show fluorescent pictures of the floating chromosome confirming that it is a chromosome. (e-f) show two more phase images of the chromosome when not trapped that suggest the shape of the chromosome. The green arrow is the position of the trap that is on in (a-b) and off in (c-f).

3.2.2.3 - Experiments with larger Chinese hamster ovary (CHO) chromosome extracts in various viscosity solutions

Trapping experiments with HeLa chromosomes were successful. However, HeLa chromosomes, which are human chromosomes, are much smaller than PTK-2 chromosomes in length and thickness. Therefore, since a purified sample of PTK-2 chromosomes were not available (these cells are difficult to synchronize and thus a purification process yields low amounts of chromosomes, if any), purified chromosome suspensions of CHO cells, which are much larger chromosomes than

HeLa chromosomes and comparable in size to PTK-2 cells were used. Also, in addition to the regular buffer solution used to re-suspend the chromosomes, two more buffer solutions created by using the same buffer medium with various concentrations of methylcellulose were used. The use of the methylcellulose permitted the production of solutions of different viscosities so that chromosome movements could be assessed under different viscous conditions.

The methylcellulose powders used for creating buffer solutions with higher viscosity were purchased from Sigma Aldrich (St. Louis, MO). The two different powders (with different chain lengths and thus different viscosities after dissolved in water) had the following part numbers: M6385 (viscosity 25 cP, 2 % in H2O(20 °C)) and M0262 (viscosity 400 cP, 2 % in H2O(20 °C)). The product information sheets provided by Sigma Aldrich for these products provide a detailed protocol for preparing the solution with the difference that we used a buffer solution as opposed to simple deionized water. Briefly, 1/3 of the final desired volume of solution is heated to about 80^{o}C and then the powder is added with continued agitation. The remainder of the solution is added as a cold solution and agitation follows for another 30 minutes.

Trapping and moving the chromosomes was attempted in the regular buffer solution (with viscosity close to that of water's, around 1 cP), the 25 cP solution and the 400 cP solution. In all solutions the CHO chromosomes could be trapped and moved with ease although the movement was slower in the 400 cP solution and required higher laser trapping power. In the 25 cP solution, approximately 6 mW of trapping power at the focus plane was enough to visibly trap and slowly move the

chromosome. In the 400 cP solution, approximately 30 mW of trapping power at the focus plane was enough to visibly trap and slowly move the chromosome. Figure 3.9 shows trapping of floating chromosomes in the 25 cP solution. The shape of the chromosome was used to visibly detect chromosome pieces.

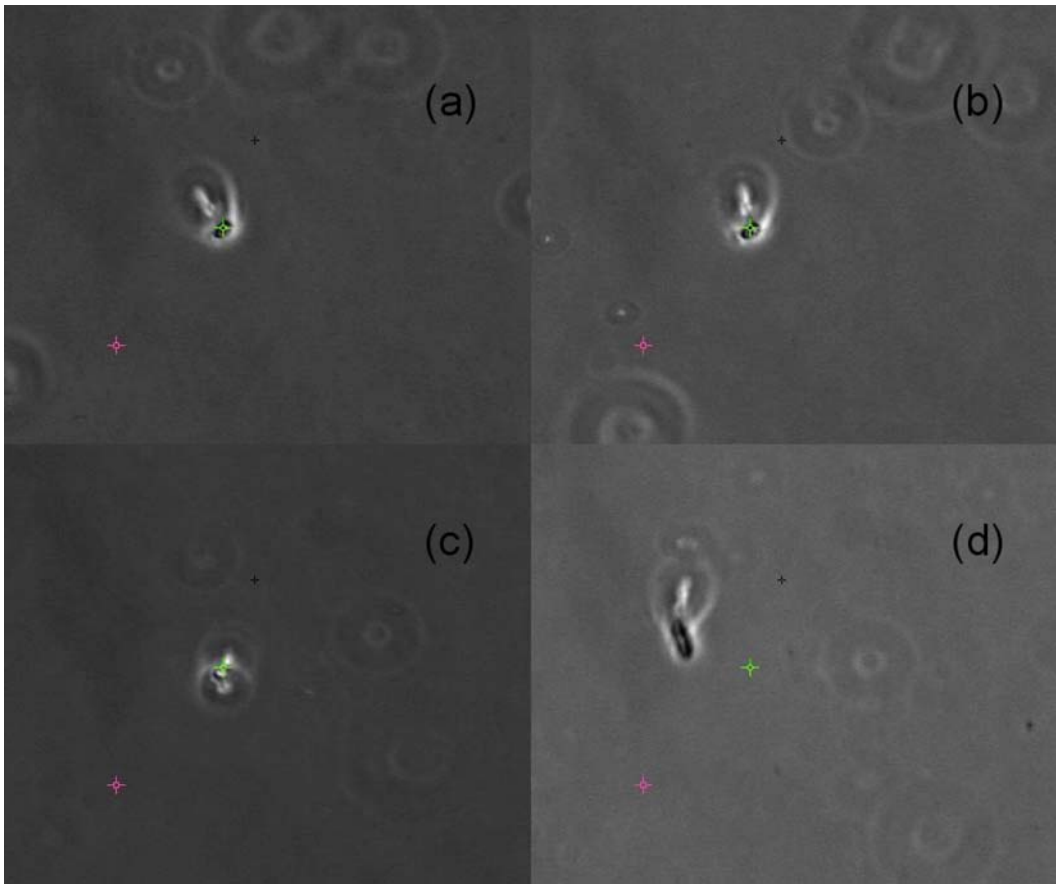

Figure 3.9 - Trapping of a CHO chromosome in a 25 cP buffer solution. The green cross-hair depicts the position of the optical trap which was on in (a-c) and off in (d).The chromosome is trapped and moved in (a-c) while in (d) a better shape of the chromosome can be seen to confirm that it is a chromosome.

3.3 - Discussion

During the biological experiments, it was possible to show that the system was capable of trapping chromosomes in suspension and in some Nocodazole-treated live cells. The system is also capable of cutting chromosomes in live cells at various stages of mitosis. However, the system was not able to successfully trap and move intact chromosomes or chromosome fragments (after optical cutting) inside un-treated live cells, cold-treated cells or cells where the kinetochore or microtubules were ablated using optical cutting. Moreover, isolated chromosomes of HeLa and CHO cells were easily trapped in suspension. Even at high viscosity solutions, the optical traps were capable of moving the CHO chromosomes although slower. It should be noted that the cytosol of various cells has been approximated to have a viscosity of 1.5 cP. Therefore, it was concluded that the inability to move chromosomes inside a living cell is not due to a high-viscosity of the cytosol solution itself but to a resistance to movement of chromosomes by the intracellular organelles, mainly the microtubule spindle during mitosis.

Acknowledgements

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Chapter 4 - Conclusion and Future Directions

4.1 – Conclusion

In conclusion, an optical system was built that combined two lasers, a 1064 nm ND:YVO4 laser with maximum 12 W of power for trapping and a wavelength tunable Ti:Sapphire 200fs 76 MHz laser with maximum power of 1.5 W used at 730 nm for cutting, into a system that allowed for control of two optical trapping beams and one cutting beam. Both the position and power of all three beams can be controlled independently of each other which allows for flexibility in performing various experiments. The system can be used both with a 40x 1.3 NA objective and a 63x 1.4 NA objective although recalibration of position and depth of the traps is required every time a change in objective is made.

During the biological experiments, optical cutting of chromosomes inside live PTK-2 cells was successful and reproducible. Typically a power around 7-10 * 10^{11} W/cm^2 was used for making successful cuts. Lower powers with multiple cuts could also be used to make a successful cut. In the trapping experiments, successful trapping and moving of chromosomes was not possible in un-treated (non-Nocodazole), cold-treated, kinetochore-ablated, or microtubule spindle-ablated cells. However, optical trapping and moving of chromosomes was observable in Nocodazole-treated cells. Optical trapping of isolated chromosomes in suspension was easily achieved. Chromosome trapping and moving was achievable with HeLa and CHO chromosome isolations and in high viscosity (400 cP) solutions containing CHO chromosomes. The fact that we can easily trap chromosomes suspended in a buffer solution along with the

experiments where trapping of chromosomes in live Nocodazole-treated cells were possible, suggest that it is the caging microenvironment of the microtubule spindle around the chromosomes that hinders the ability to move them. Also, the viscosity of the cytosol itself is estimated at 1.5 cP as it is mostly water, which also suggests that the intracellular organelles surrounding the chromosomes (mainly microtubules during mitosis) is prohibiting the movement of the chromosomes by the trap. The maximum trapping power of the system was approximately 150 mW for each trap at the focal point if the power is split between two traps, and approximately 350 mW if all of the laser power was directed to one trap using the 63x objective. This power should be at least able to move the chromosomes slowly inside the cell if the only inhibitor of movement was the viscosity of the cytosol.

4.1 – Future Directions

To further our understanding of the forces needed to move chromosomes inside cells and the effects of the microtubule spindle and other intracellular organelles on our trapping and movement capabilities, additional experiments can be pursued. One idea is the concept that liquid flow in small confined areas is much more affected by boundary flow and conditions as opposed to flow in larger containers. A small object like the chromosomes "sees" a much higher resistance due to viscosity and the boundary layer in a small container like the cell with a few microns diameter compared to a sample dish with 1-2 mL of volume. Therefore, if we consider a cell that only contains chromosomes and buffer solution, the trapping force required to move this chromosome inside the cell is higher than what we measured for the

chromosome outside the cell in suspension due to the boundary layer effects. An experiment to examine this is possible. Micron-size liposomes can be created using various techniques and they can be filled with a suspension of free-floating chromosomes. In this way, the environment inside the cell can be more accurately modeled and the trapping forces required to move the chromosomes inside such a liposome would more closely reflect the forces required to trap and move a chromosome inside a cell whose microtubule spindle has been dissolved using the methods described in this thesis. The experiments that were performed where the chromosomes are trapped and moved in suspension do not give an accurate estimate as to the amount of force required to move the same chromosomes inside live cells but merely confirm that the geometry and index of refraction of the chromosomes allow for trapping with our system.

Another possible continuation project is to use Xenopus (clawed toad) extracts that can form microtubule spindles in a suspension outside of a cell along with chromosomes. Observing the trapping force required for moving the chromosomes attached to the spindle outside of the cell, if possible, would provide a much better idea as to the amount of force required for moving the chromosomes inside an intact live cell. Finally, it should be noted that the inside environment of different cells can be vastly different between plant and animal cells and even within different animal cells. Therefore, the inside environment of a specific cell line may provide a specific "pathway" that allows for chromosome trapping and movement without any treatments while another may not. An example of such is the newt lung cells that have

an intermediate filament cage much larger than the microtubule spindle cage and therefore would provide an area through which movement of chromosomes with optical traps is possible [1].

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Appendix

Part A - Primary Culture Chromosome Extraction Protocol Provided and Performed by Marlys Houck

1. Check secondary cultures for optimal growth of cells. This usually occurs approximately 24 hours after a 1:2 passage. Evidence of mitotic doublets and rounded-up cells should be evident and abundant.

2. To each T75 flask add 50 µl colcemid for 15 min- 1 hr.

3. Transfer culture fluid to a 15-ml centrifuge tube. Add 1-2 ml EBSS to the flask to rinse all medium out. Pour this rinse into your centrifuge tube. Add 1-2 ml ATV to the flask and make sure all cells get coated. Pour ATV into centrifuge tube, leaving a tiny amount still in flask. Wait a few minutes. Rap sides of flask gently to detach cells. Check under microscope to make sure all cells are removed. Add 3 ml EBSS and rinse all cells out.

4. Centrifuge 10 min at 1000 -1200 RPM. Remove supernatant, leaving approximately 0.5 cc. Resuspend pellet gently.

5. Add 7 ml 0.075 M KCl warmed to 37° C. Resuspend gently. Incubate in 37° C H2O bath for 10-30 min. Incubation time varies for different cultures; you will have to find the best times by trial and error. Meanwhile, make fixative.

6. Add 3 drops of cold fix to the hypotonic solution while gently agitating the tube. Resuspend the solution.

7. Centrifuge at 1000 RPM for 10 min.

8. Remove all but 1 cc supernatant. Resuspend well. Carefully pull up the suspension into the Pasteur pipet. Add approx. 6 cc fixative to the tube. Place the pipet with your cells into the fixative and carefully release the suspension in a slow steady stream. Resuspend thoroughly with pipet. Refrigerate at least 30 minutes (optional).

9. Spin down and wash with fixative at least two more times.

10. After last rinse, depending on size of pellet, leave approximately 0.5-1 cc of fixative to make a cloudy suspension. You are now ready to make slides.

Part B – HeLa Chromosome Extraction Protocol Provided and Performed by Dr. Anita Kulukian

- Eat large breakfast. Turn on music.
- Collect mitotic HeLa cells arrested with 10ug/ml Colcemid (1:200, 150ul per T150 flask containing 25 ml media; 50ng/ml final.) for 16 hrs. 40 flasks at 90%-100% confluency works best. It is better to wait one day than to use less confluent cells.
- Count the number of mitotic cells using hemacytometer.
- Note total volume of cells.
- Spin at 1500 rpm for 5min to collect cells in 250ml conical tube.
- Resuspend cell pellet into 5ml media and transfer to 15ml tube. Pool together.
- Spin down cells for 1500rpm in clinical for 5min. Remove sup.
- Note the size of the pellet.

- Resuspend and swell cells in a total of 50ml 1X MPME (pre-warmed) by incubating at 37C for 5 min (pool into 50ml conical tube).

- Pellet swollen cells by spinning at 1500rpm for 5 min.

- Remove supernatant.

- Pipet 10 ml of ice cold Lysis buffer onto cell pellet with protease and phosphatase inhibitors. Within 20sec must start douncing. Note: You don't want to exceed the volume of the dounce, but adjust as necessary.

- Pipet up and down and transfer to metal dounce. Dounce cells with 10 strokes with metal dounce. Don't let it "pop."

- Transfer lysate to 50 ml conical on ice.

* Save a .25ml aliquot (5 x 50ul) as "input".

- Spin down at 900rpm for 1 min to pellet nuclei and other debris.

- Remove and keep supernatant on ice.

- Resuspend the pellet in another 6ml lysis buffer, redounce, and repeat above steps.

- Repeat potentially a third time if "debris" pellet is large.

- Note: total volume of lysate should not exceed 26ml, which is the max you can layer above the sucrose gradient in the Corex tube.

- Add 5M NaCl to bring salt concentration up to 100mM NaCl (18ul per ml of lysate). Spin quickly in lysate and depress the pipette slowly.

* Save a .25ml aliquot (5 x 50ul) as "lysate".

- Prepare 2 sucrose gradient tubes in 30 ml Corex tube by adding drop wise 4ml of each sucrose step. Best NOT to prepare the gradient in advance.

- Layer 13 ml supernatant over each sucrose gradient.

- Spin at 5500rpm in HB4 rotor for 20 min at 4C with brake off.

- Collect flocculent white layer at the 40-50 and 50-60 interphase. Do not collect pellet at the bottom of the 60% layer, which contains nuclei.

- Save aliquot as "sucrose purified chromosomes", in (5 x 50ul) aliquots. Snap freeze.

- Resuspend chromosomes into total of 30ml of hsMPME wash buffer.

- Pour chromosome mix into 40ml dounce and homogenize with 5-10 gentle strokes.

- Place into one Corex centrifuge tube and spin in clinical centrifuge at 4C for 15min at 2900xg (3700rpm).

- Carefully aspirate away supernatant, leaving behind a ring pellet.

- Dislodge pellet and resuspend each pellet into 10ml hsMPME wash buffer.

- Transfer to 15ml dounce. Homogenize with 5-10 gentle strokes.

- Prepare 1 sucrose gradient tube in 30 ml Corex tube by adding drop wise 4ml of each sucrose step.

- Layer chromosome solution over sucrose gradient.

- Spin at 5500g for 20 min at 4C with brake off.

- Collect flocculent white layer at the 40-50 and 50-60 interphase. Do not collect pellet at the bottom of the 60% layer, which contains nuclei.

- Resuspend chromosomes into 30ml 1x hsMPME wash buffer.

- Pour chromosome mix into 40ml dounce and homogenize with 5-10 gentle strokes.

- Place into one Corex centrifuge tubes and spin in clinical centrifuge at 4C for 15min at 2900xg (3700rpm).

- Carefully aspirate away supernatant, leaving behind a ring pellet. Dislodge pellet and resuspend into 300-800ul chromosome storage buffer. Adjust volume as necessary. This is an arbitrary step and depending on amount of recovered chromosomes.

- Carefully transfer to 2ml dounce.

- Wash Corex tube with an additional 200ul or so of buffer to recover remaining chromsomes. Transfer to dounce

- Gently dounce chromosomes with 10 strokes until homogenized.

- Aliquot into 25ul and snap freeze. Store at -80C.

Additional Notes:

-Volumes of lysis buffer, sucrose gradient, wash buffers, etc., can be adjusted as necessary. If you have a lot of chromosomes, you can add additional tubes of sucrose gradients or washes, using either 15ml or 30ml Corex tubes.