Radial outward forces from centrosomes facilitate cell division

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Abstract

Forces play an important role in many cellular processes. Cell mechanics have been studied extensively, but information about the forces that occur during cell division is lacking. So far, experiments have shown that the cellular environment influences the mitosis by inducing forces on the cell from the outside. Measurements of the forces that the cell exerts during mitosis have not been done. In this report micropillars are used to measure the forces that the cell exerts during its division. With micropillars it is possible to measure forces of a single cell by looking at the deflections of each individual pillar. It is found that cells pull strongly on their surrounding just before their chromosomes start condensing in the prophase. This force is directed towards the center of the cell and is strongly polarized. From the direction of these forces it is possible to predict the orientation of the spindle and in which direction the cell divides. The cell stops pulling when it enters the prophase and it starts pushing radially outward. This force increases until the metaphase begins and then stays at a steady level until the cell enters the anaphase. The amount of force exerted depends on pillar stiffness. The total outward force at the beginning of the anaphase lies between 400 and 600 nN for high stiffness pillars and between 90 and 160 nN for low stiffness pillars. During the anaphase the pushing forces decrease. Microtubules emanate from the centrosomes to cell cortex. We hypothesize that polymerization on the plus end of the microtubules creates a pushing force on the spindle as well as the sides of the cell to align the spindle and enable chromosome segregration.

Contents

1 Introduction

A lot is known about how cells maintain their size and shape and how they function. Forces play an important role in cellular processes from the movement of molecules to cell division. Forces are exerted by different components in the cell. During division the cell goes through a multitude of processes subsequently involving the movement of chromosomes and organelles within the cell. These movements are excecuted in a precise way, where the position of the different components within the cell is key to the continuation of the process. The spatial organization is well documented, but how force exertion is executed is unknown [1].

It is difficult to measure forces inside cells, but the forces that act on the components inside the cell may be visible on the outside of the cell. In this report, micropillars are used to measure forces that a cell exerts on its environment during the different stages of the cell division. We propose that microtubules extent from the centrosomes and polymerization at the plus end generates a pushing force to aid in the alignment of the spindle.

1.1 Cell mechanics

The organization of all the molecules and organelles in the cell is determined by its cytoskeleton [2]. The cytoskeleton consists of three different kinds of filaments: the intermediate filaments, actin filaments and microtubules. Motor proteins exert forces on these filaments to either transport organelles or molecules or to maintain the shape of the cell.

Intermediate filaments provide structure to the cell. Their role is to hold components of the cell together and they do not participate in moving organelles. Intermediate filaments have a diameter of approximately 10 nm. To find out what forces a filament can bear, the persistence length is used. The persistence length is a measure of the flexibility and stiffness of a filament. For intermediate filaments this is 1 μm [3].

Actin filaments give the cell membrane strength and shape. Their persistence length is 17.7 μm [4]. Being close to the cellular membrane, actin filaments can form lamellipodia and filopodia. They are also important during division, where the contractile ring that divides the cell in two is made of actin.

The microtubules are used for maintaining the cells size and shape and for moving organelles within the cell. Microtubules are more rigid than the other components of the cytoskeleton and can physically bear both pushing and pulling forces. Their persistence length is 5200 μm, much higher than that of actin and intermediate filaments. These forces are generated by the growing or shrinking of one of the ends of the microtubule. When it changes from growing to shrinking it is called a catastrophy event and in the reverse case it is called a rescue event. Polymerization on one end creates a pushing force in that direction. When microtubules are attached to an organelle and depolymerize they create a pulling force. The pushing force is used for moving small distances while the pulling force can move organelles over large distances.

Another important function of the microtubules is that they position the nucleus and the mitotic spindle and they seperate the chromosomes during cell division. Kinetochores are assembled at the centromere of the sister chromatids and attach to the kinetochore microtubules to move the chromosomes.

Microtubules have a plus and a minus end. The minus end is generally located at the center and the plus end is located at the edge of the cell. At the plus end the depolymerization and polymerization is done quickly, while at the minus this is done slowly. Dynein and kinesin are the two types of motor proteins that can move along microtubules by ATP hydrolysis. Kinesin moves towards the plus end of a microtubule, and thus transports molecules and organelles from the centre of the cell to its edges. For dynein proteins, this is the other way around because they move towards the minus end. Dynein can move along microtubules to transport vesicles and proteins. They can also use microtubules to pull on large structures such as centrosomes and the mitotic spindle, so dynein is responsible for the movement of chromosomes during division [5]. Dynein can also be anchored to the cell cortex to exert forces on microtubules [6].

1.2 Cell division cycle

Multicellular organisms grow by increasing their number of cells [2]. New cells can only be made by dividing a cell that already exists. The process in which a cell divides in the two cells with the same genetic material is called mitosis. In order to achieve this, the DNA needs to be replicated before each cell division. Each of the cells then gets one set of DNA.

During division the cell goes through multiple phases, together called the cell division cycle. There are four of these phases: G_1, S, G_2 and M. During S-phase the chromosomes are duplicated so that there is one set of chromosomes for each daughter cell. The actual division of the cell happens in M-phase. Here the chromosomes are segregated and the nucleus divides. Also the whole cell divides in two, each with one nucleus. Between divisions, cells must grow in order to maintain their size and mass. The cell cycle contains two gap phases: G_1 and G_2. G_1 phase occurs between M-phase and S-phase, at the beginning of the cycle. G_2-phase occurs between S-phase and M-phase. During these gap phases the cell not only grows, but it also monitors its environment to check if the conditions are suitable to progress in the cell cycle. Especially in the G_1-phase this is important, because here the decision is made to start the cell cycle. The M-phase only takes up a small percentage of the total time of the division cycle. The M-phase is divided into 4 phases: Prophase, metaphase, anaphase and telophase.

At the beginning of the prophase, the cell has already doubled its number of chromosomes of which later half goes to each nucleus. The replicated chromosomes condense in this phase, they coil up around histones. Also, the mitotic spindle forms, which in a later phase will pull the two sets of DNA apart. This spindle is formed outside of the nucleus, controlled by the centrosomes. The spindle consists of microtubules.

At the transition between the prophase and metaphase, sometimes called prometa-

phase, the envelope around the nucleus breaks down, enabling the mitotic spindle to enter the nucleus. In this phase the spindle attaches itself to the two kinetochores which are located at the centromere of each chromosome.

In the metaphase the chromosomes are pushed towards the middle of the nucleus. The chromosomes are now aligned so that the spindle can attach to their centromeres. The sister chromatids are attached to the opposite poles of the mitotic spindle.

The sister chromatids are separated in the anaphase and become individual chromosomes. Each of the sister chromatids is pulled to an opposite spindle pole. This is done by shortening the kinetochore microtubules as well as the movement of the spindle poles.

The two sets of chromosomes arrive at the poles of the spindle in the telophase. Around each of the two sets, a nuclear envelope forms so that the cell has two nuclei. The chromosomes start to decondense at the end of the telophase. During the telophase the cytokinesis already starts. Cytokinesis divides the cell in two by forming a contractile ring in the middle of the cell. Each cell ends up with one nucleus and half of the cytoplasm. After a succesfull division the two daughter cells begin a new cycle, starting in the G1 phase.

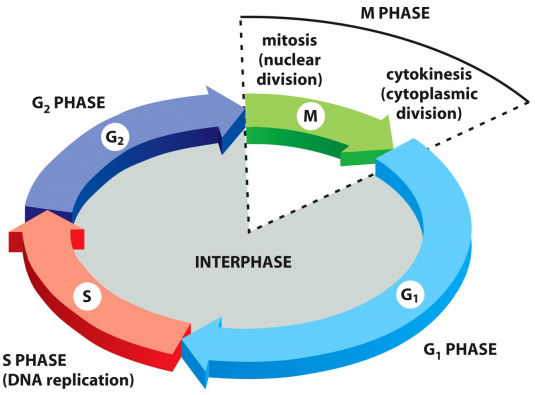


Figure 1: *Schematic of the different phases of the cell cycle and their relative duration. The interphase, consisting of the G_1, G_2 and S-phase takes up most of the cycle. The M-phase, where the nucleus and the whole cell actually divides takes up only a fraction of the cell cycle. For HeLa cells used in this experiment the whole cycle has a duration of 24 hours while the M-phase typicaly lasts 2 hours. Figure taken from Molecular Biology of the Cell [2].*

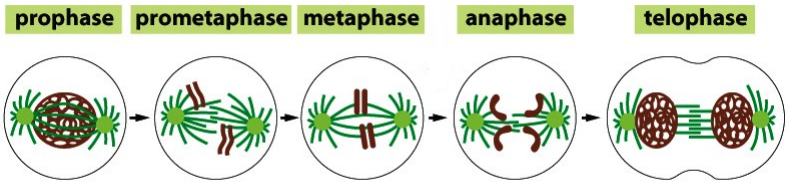


Figure 2: *Schematic of the different phases within the M-phase. The DNA is already replicated and the chromosomes will segregate in anaphase and form new nuclei in telophase. In telophase the cytokinesis already starts. Figure taken from Molecular Biology of the Cell [2].*

1.3 Cell mechanics during mitosis

Many experiments on cell mechanics have been performed, but during cell division information is lacking. The cell has to orient its spindle at the opposite poles of the cell and pull the chromosomes apart. The forces that govern these movements have not been studied extensively yet. Laser ablation experiments have shown that the orientation of the mitotic spindle depends forces exerted on the cell [7]. Cells were placed on substrates where they can only adhere on a cross shape. When the spindle had formed, the actin filaments in that direction were cut. The cells could not continue their division and had to reorient their spindle by 90 degrees. Up to now experiments have been done where forces are induced on the cell to see its response. Experiments where the cell is put on a stretchable substrate have shown that exernal forces influence the orientation of the nucleus and the mitotic spindle [7]. These experiments show that actin filaments are important during mitosis. Since actin filaments have a low persistence length and can only bear pulling forces, it is expected that mitosis is governed by pulling forces.

2 Materials and methods

2.1 Cell culture

We used HeLa cells for the experiments of this report. The HeLa cell line is the oldest and most commonly used cell line in research. The cell line is derived from cervical cancer cells that were taken on February 8 1951 of a woman named Henrietta Lacks. Telomerase prevents the telomers from shortening and this makes the HeLa cell line immortal. HeLa cells can divide indefinitely. They divide approximately once every 24 hours. Because they are so extremely proliferating they were found to be contaminating other cell lines in culture [8]. Their proliferation and immortality makes the HeLa cell line ideal to culture in the lab.

HeLa cells with two different kind of fluorescent labeling are used. One has a stable H2B-GFP transfection, making it possible to see the nucleus of the cell. During cell division this allows us to visualize the individual chromosomes. Also, unlabeled HeLa cells are transduced with a baculovirus. The baculovirus used here tranduces the cell so it transiently expresses tubulin-GFP. The virus is added 24 hours before seeding on pillars.

The cells are cultured at 37 degrees in 7% CO_2. The cells are seeded on a p60 dish in DMEM with 10% NBS (Newborn Bovine Serum) and passaged twice a week at a 1:10 dilution. The cells are trypsinized with 500 μl of 0.25% trypsin for 5 minutes and reseeded in a p60 dish. 16 hours prior to each experiment 100.000 cells are

seeded on the pillar array to ensure single cell density.

2.2 Micropillars

Forces that the cell exerts are measured by the deflection of micropillars. The micropillar arrays consist of a grid of pillars with 50 μm spacers on the sides. When doing experiments, the pillar arrays are placed upside down on a 100 μm coverslip.The spacers are approximately 50 μm so the pillars are within the working distance of the objective. The pillars have a diameter of 2 μm and a center-to-center spacing of 4 μm. so one cell can pull on many at a time. We use varying heights of 4.5μm and 6.9μm. Higher pillars have a lower stiffness. The stiffness of the pillars has been found to influence cellular differentation and migration and may also influence dvision [9, 10].

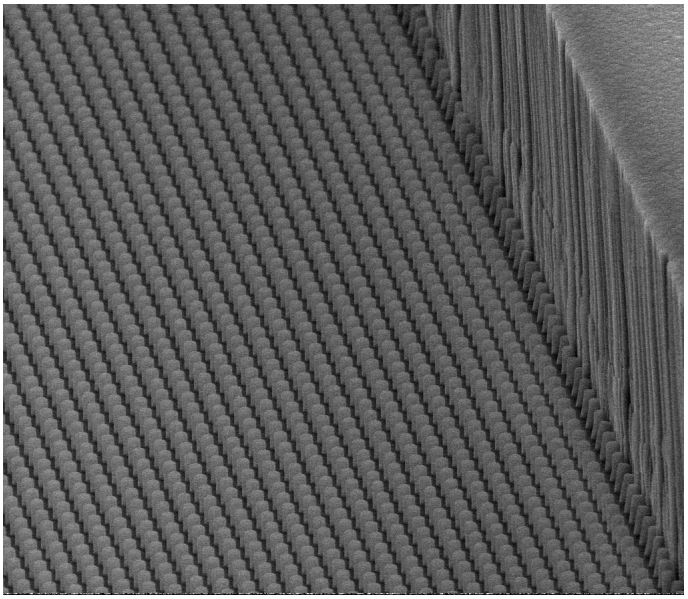


Figure 3: *Electron microscope image of micropillars with one of the spacers. The array of micropillars has an area of 1cm^2. The pillars are all equally spaced in a hexagonal grid and have a diameter of 2μm. The spacer is 50μm high.*

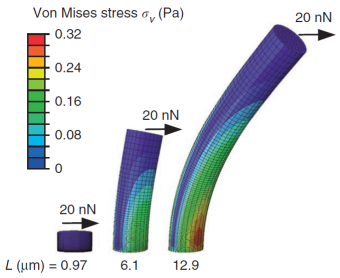


Figure 4: *Schematic of pillar deflection. Under the same force, a lower and stiffer pillar will show a lower deflection compared to a higher pillar.*

The pillars are made of Polydimethylsiloxane (PDMS, Sylgard 184) through replica-molding from a negative master silicon wafer. First base and curing agent are mixed with a ratio of 10 to 1 thoroughly for about 5 minutes [11]. The mixture is dessicated to release air bubbles and poured on a silicon wafer. The PDMS will fill the grid of holes of the wafer. The wafer is put in the oven to cure the PDMS for 20 hours at 110^oC. The pillar array is cut out and stamped with fibronectin. Pillars of different height are fabricated depending on the depth of the microholes in the wafer.

When the PDMS is cut out of the wafer, some of it stays behind between the pillars. These remnants are washed out with isopropanol after which they are blown dry with 100% nitrogen. After the wafer has been used twice it is silanized for one hour. With this process the surface of the wafer is covered with a layer of trichlorosilane [11]. This layer causes the PDMS to stick less to the wafer to ensure a precise replica of the negative mold.

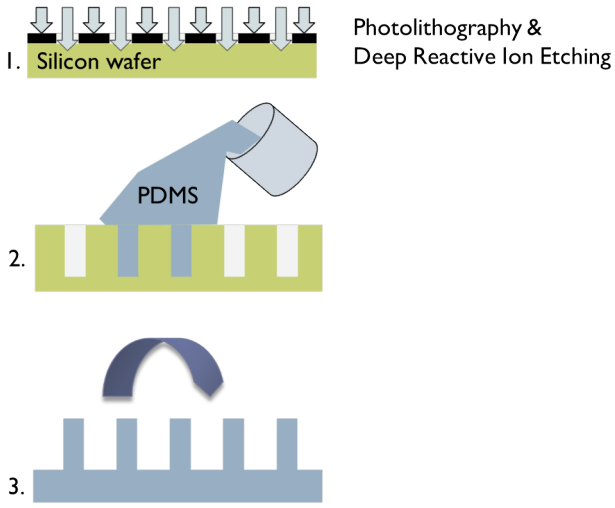


Figure 5: *Schematic of the fabrication of pillars. 1) Holes are etched in the wafer by photolithography and deep reactive ion etching with a constant distance between them so a grid is formed. This wafer can be used many times to create pillars. 2) PDMS is poured onto the silicon wafer so the holes are filled. These will form the pillars. 3) After curing the PDMS in the oven the PDMS is removed from the wafer and turned upside down so the pillars face up.*

2.3 Microcontact printing

The pillars are coated with fibronectin connected to fluorescent dye Alexa568. Fibronectin is a component of the extracellular matrix and the cells adhere to it [9, 12]. The labeling enables us to image the top of the pillars and the fibronectin provides sites for cells to attach. The fibronectin is applied with a technique called microcontact printing.

A PDMS stamp is prepared on a blank Si-wafer. The PDMS is mixed in the same way as done for the pillars but is cured at different conditions, for 16 hours in the oven at 65°C. 50 $\mu g/ml$ of fibronectin in PBS and 10 $\mu g/ml$ of fibronectin labeled with Alexa568 in PBS is mixed in mili-Q water and 40μl is applied per stamp. The stamp is then incubated in the dark for 60 minutes. The stamps are now cleaned

with water and dried. At the same time the pillars are treated in a Ultraviolet-Ozone cleaner so the PDMS becomes hydrophilic and it frees up OH-groups at the surface to bind protein. Afterwards the stamp is placed on top of the pillars to stick the Fibronectin onto the pillars. It is important that the stamp is flat so that the Fibronectin is evenly spread over all of the pillars. The stamps are removed again after 5 minutes. First the pillars are submersed in ethanol to minimize the damage done by peeling off the stamp. After the stamps are removed, 0.2% pluronic in PBS is added for one hour. This is neccesary because it will later prevent cells from adhering to the shafts of the pillars that are not stamped [13]. Next the pillars are washed 3 times with PBS. This ends the micro-contact printing of the pillars and they are now ready for seeding.

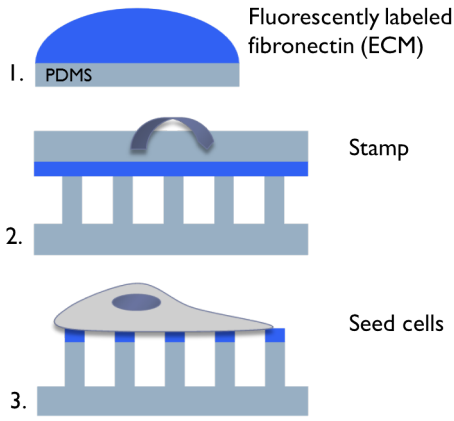


Figure 6: *Microcontact printing of pillars. 1) A flat piece of PDMS is coated with fluorescently labeled fibronectin. Fibronectin is part of the extracellular matrix and cells adhere to it. 2) The coated side is apllied to the top of the pillar array. The fibronectin will now attach to the top of the pillars. The labeled fibronectin can be imaged using a fluorescence microscope. 3) Cells can be seeded on the micro-contact printed pillars.*

2.4 Microscopy

A confocal spinning disk optical microscope is used for imaging. The Yokogawa confocal spinning disk unit (CSU) consists of two coaxially aligned disks that rotate with 5000 rpm. The first disk contains microlenses that focus the light onto pinholes in the second disk which gives an excitation pattern on the sample. The light emitted by the sample is collected by a CCD camera from Andor that has 512 by 512 pixels. A 100x oil objective with 1.4 NA from Zeiss is used. Lasers of different wavelengths are used to excite fluorescent molecules in the sample. A dichroic selects the specific wavelength and an Acousto-Optic Tunable Filter (AOTF) controls the laser intensity. Andor IQ software and homemade Labview (National Instruments) software is used to operate this system.

The samples are placed with the pillars facing downwards on a coverslip onto the microscope. In previous experiments, cell mechanical behaviour has been confirmed not to be affected by inversion of the sample [14]. During imaging the sample is kept at a constant temperature of 37°C and a CO_2 level of 5% by a Tokai Hit heat stage.

A joystick is used to move the field of view through the sample. Homebuilt labview software for this setup allows one to save different positions in the sample. This way the setup can automatically move the field of view to a certain position in the sample. A home-made automated focus system allows one to set the focal plane for each different position. The movement in the distance from the sample is done by a piezoelectric element (Physike Instrumente). The cells are observed by making a time lapse movie with a timelag of two minutes. In this time about ten locations are imaged. For each cell images are taken at four different distances from the sample around the focal plane. At each z-position one image is taken of the pillars at 561 nm and one image is taken of the cell at 488 nm. So with this setup it is possible to set all the positions in the sample that need to be imaged and then the imaging will be done automatically for a certain amount of time.

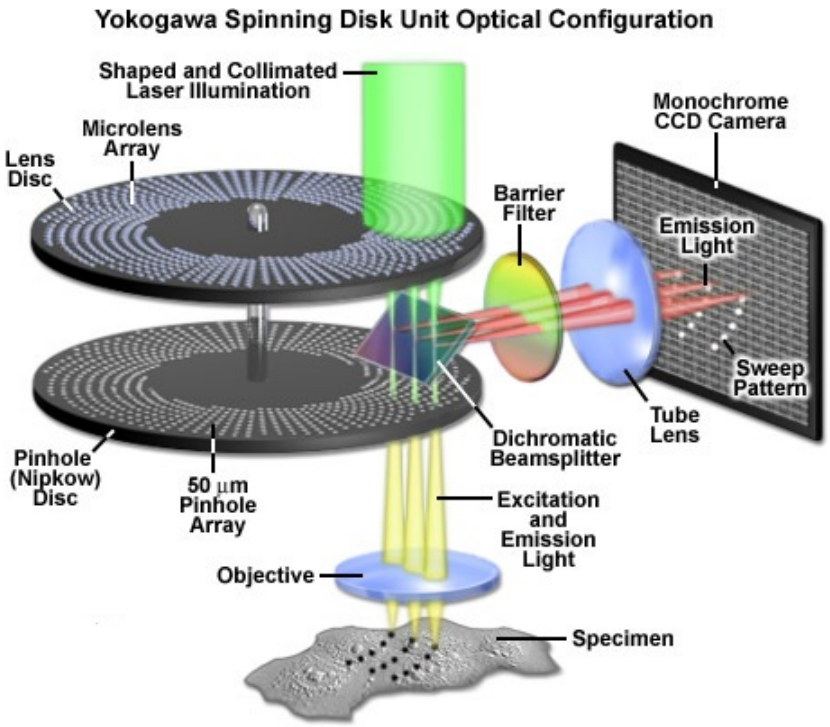


Figure 7: *Schematic of the Yokogawa Spinning Disk Unit. This setup is used for imaging in this report. Two disks rotating at 5000 rpm focus light on the sample to excite fluorescent molecules and the re-emitted light is collected by a CCD camera. Different lasers can be used to excite different fluorophores in the sample. Figure taken from http://zeiss-campus.magnet.fsu.edu/articles/spinningdisk/introduction.html.*

2.5 Data analysis

The data is analysed using Matlab (Mathworks). A projection is made of the 4 images that are subsequently taken of different z position of the cell. The images of the pillars are used to measure the deflection and calculate the forces that the cell exerts on the pillars. The images of the cell are used to determine the phase of the cell cycle. The forces exerted on the pillars is followed throughout the whole division cycle. Not only the absolute force is of importance here, but also the direction of the forces.

The cell cycle detection is done by making a black and white image of the GFP labeled histones. A threshold for the pixelvalue is set and every pixel that has a value higher than this is given a value one and every pixel that has a value lower than this is given a value zero. When the cell is in interphase, the black and white image has a round or elliptical shape and consists of one object. When the cell starts dividing, the condensing of the chromosomes creates holes in the black and white image. At the moment these holes start to appear we know that prophase has started. After the chromosomes are condensed, they are aligned at the center and they have a rod shape. The object is now compacted so there are no more holes visible. Metaphase is thus recognized by looking at eccentricity of the object in the image. When the nucleus starts dividing, this can be seen because the number of objects in the image increases to two. This way the anaphase is recognized. The telophase is recognized by an increase in the area of the nuclei because the chromosomes decondense.

First the position of the centers of the pillars is determined. This can be done using several methods, of which fitting a Gaussian to the correlation function of the pillar with a perfect circle gives the most accurate centre position and is therefor used. Assuming that the pillar array is nearly perfect, and that most of the pillars are not being pulled on, we can find the properties of the pillar grid. The average distance between the pillars is used to determine reference positions for all of the pillars. These reference positions are compared to the actual positions and the difference gives the deflection. The deflections are converted into forces by using the following formula.

$$F = k_1\delta(1 + k_2\delta^2) \tag{1}$$

Here k_1 and k_2 define the stiffness of the pillars and depend on their height and diameter. The bending constant is calculated using the following formula [15].

$$k = \frac{3\pi}{64}\frac{Ed^4}{h^3} \tag{2}$$

Here E is the Young's modulus, d is the pillar diameter, and h is the pillar height.

Height(μm)	k_1	k_2	Stiffness	Young's modulus (kPa)
4.5	65.9	0.04	high	2023
6.9	13.7	0.02	low	2314

The deflection analysis of the pillars gives us the absolute value of the force and its direction. The angle of this direction with respect to the nucleus is determined to check whether the force is outward or inward. The center position of the nucleus is determined from the black and white image of the cell. A line is drawn between the center and each pillar. The angle that this line makes with a horizontal line from the center is calculated. This angle is substracted from the angle of the direction of the pillar deflections to determine the direction of the deflections relative to the center.

The pillars on which the cell can exert forces are selected to be analysed further. From the black and white image the area of the nucleus is found. Since the cell

extends for a larger area than just the nucleus this area is increased by a certain amount of pixels. The pillars that are under the cell are selected. The radial component of the forces is now added up to get the total radial force. A positive value of the radial force is defined as an outward force and a negative value is defined as an inward force.

The center position of the pillars can be determined with a precision of 30 nm. This gives two different precisions in force for the two pillar stiffnesses. For high stiffness pillars this error becomes 2 nN and for low stiffness pillars this is 0.5 nN.

3 Results

3.1 Interphase force exertion

A cell which is not dividing also pulls on the pillars around it. The cell exerts forces in no particular direction. Following one particular pillar however does not give a static picture. Instead the force that the cell exerts on the pillar has fluctuations. On a timescale of 30 minutes the amount of force that the cell exerts on one high stiffness pillar can drop or rise by 10 nN. On high stiffness pillars the cell exerts forces directed outward and inward. On low stiffness pillar the majority of the forces is directed towards the center. During division the cell exerts a different pattern of forces at different phases.

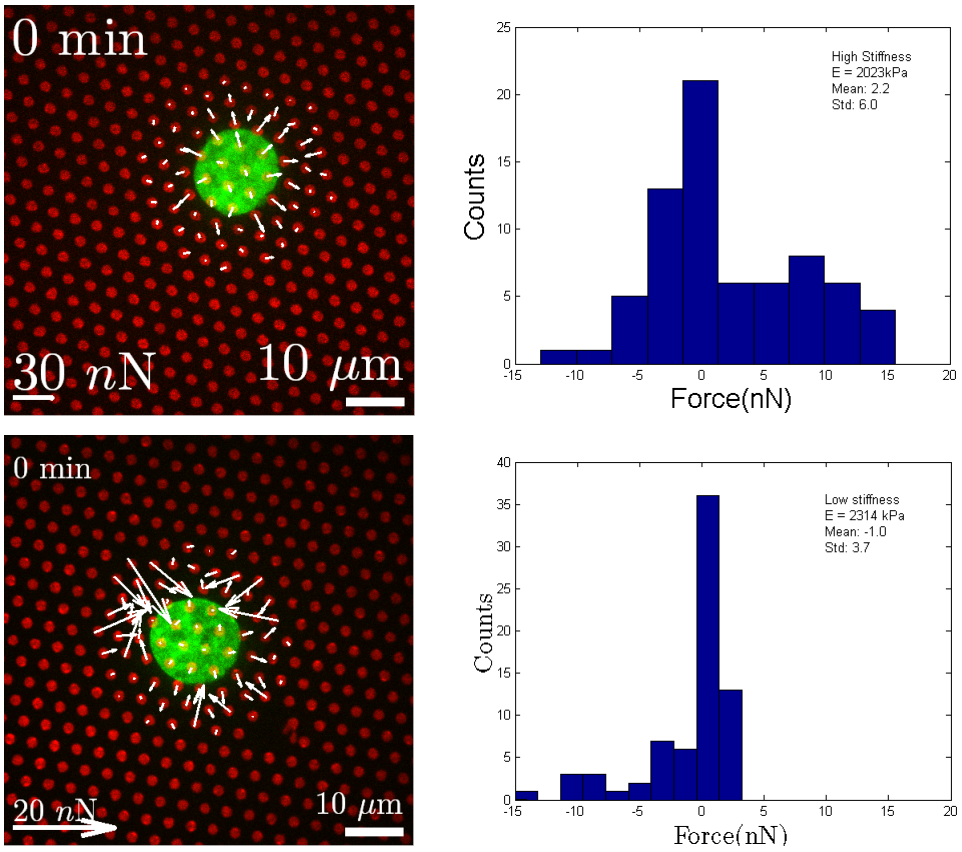


Figure 8: *Top left: A cell in interphase on high stiffness pillars. The green color shows the GFP molecule which is attached to the H2B histone. The red color shows the Alexa568 labeled fibronectin. The white arrows indicate the forces that the cell exerts on the pillars. Pillars at which the cell exerts forces are selected. The forces are directed both towards and from the nucleus. Top right: Histogram of the radial forces of the selected pillars. Inward forces are defined negative and outward forces are defined positive. The cell pulls strongly on some pillars and pushes on others. Most of the pillars however have small values that are within the measurement error. Bottom left: A cell in interphase on low stiffness pillars. The cell pulls heavily on most of the pillars, but the forces are not always directed exactly toward the center. Bottom right: Histogram of the radial forces of the selected pillars. Most of the forces are negative.*

3.2 Prophase

The prophase is recognized by the condensing of the chromosomes for the cells that express H2B-GFP. For the cells transduced with the baculovirus this phase is recognized because the cell starts rounding up and an increase in the amount of microtubules is visible just outside the nucleus. At the beginning of the prophase the cell strongly attaches itself to pillars at its edge and pulls them towards the center. From the direction of the forces in this phase, it can already be seen along which line the nucleus will orient and to what directions the spindle will pull the two new nuclei. The two new nuclei will separate along the direction of these forces. At the end of the prophase the pulling force towards the center dissappears. At the same time an outward force becomes visible around the nucleus. This force points radially outward from the center.

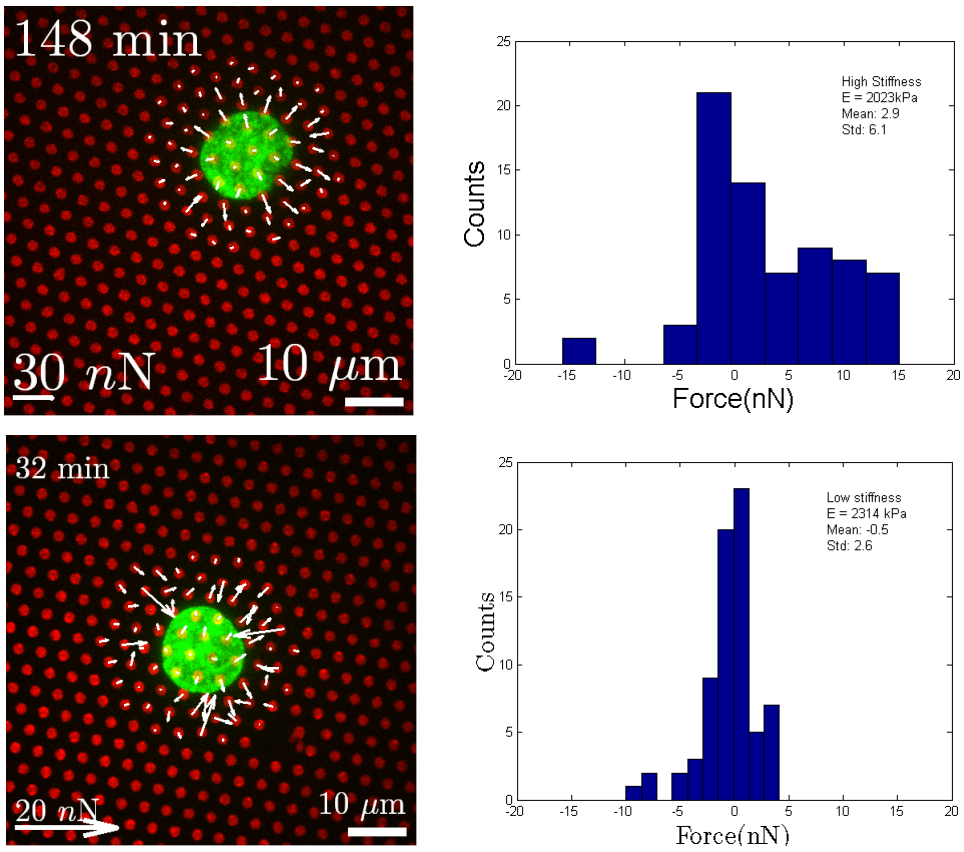


Figure 9: *Top left: A cell on high stiffness pillars at the beginning of the prophase. The chromosomes have just started condensing. From the arrows around the nucleus it can be seen that there is an outward directed force originating from the centre of the cell. Top right: Histogram of the radial forces of the selected pillars at the beginning of the prohase. Most of the forces are positive and thus directed outward. The cell pulls strongly inward on only 2 pillars. Bottom left: A dividing cell on low stiffness pillars at the beginning of the prophase. Bottom right: Histogram of the radial forces of the selected pillars at the beginning of the prohase. The cell pulls strongly on 8 pillars.*

3.3 Metaphase

In this phase the chromosomes align at the center of the nucleus. For the cells that express H2B-GFP, it can be seen that the chromosomes align in a bar shape in the center of the nucleus. For the cells with the baculovirus transduction, this phase is recognized by looking at the spindle, which attaches to the chromosomes in this phase. The total outward directed force increases during the first part of the metaphase and reaches its peak value at approximately 500 nN. At the end of the metaphase this force stops increasing and stays at a steady level.

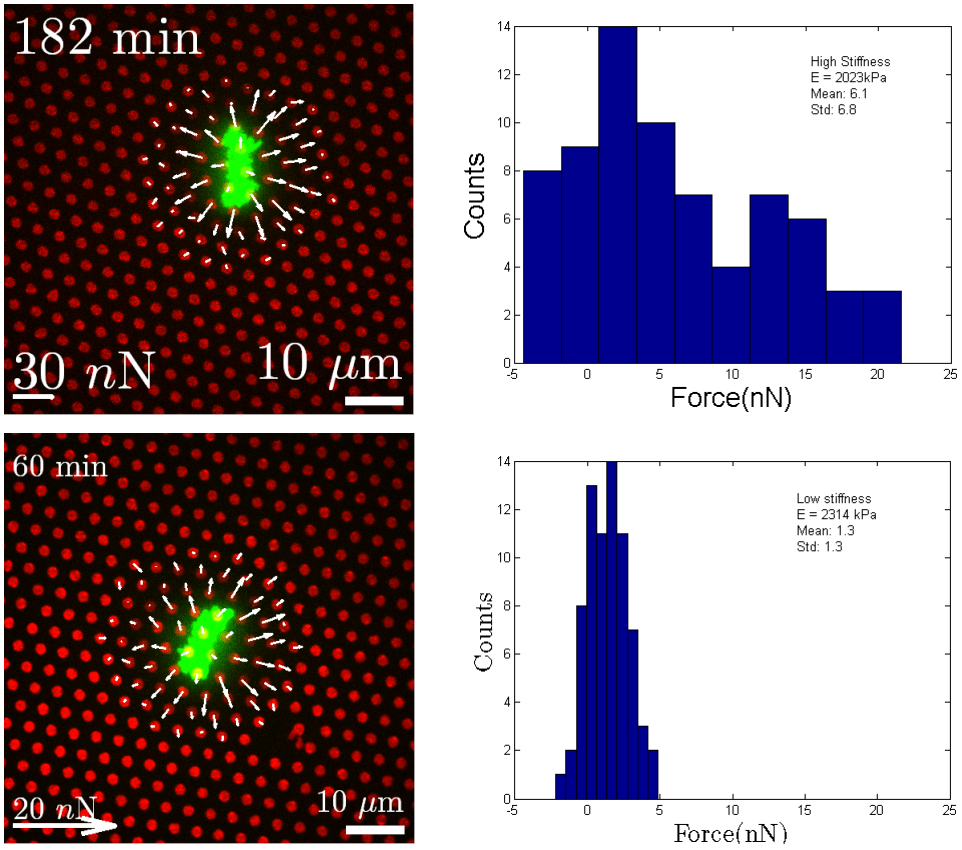


Figure 10: *Top left: A cell on high stiffness pillars at the beginning of the metaphase. The chromosomes have condensed and the mitotic spindle has formed. The chromosomes are aligned at the center of the nucleus. The outward forces have increased since the beginning of condensing. Top right: Histogram of the radial forces of the selected pillars at the beginning of the metaphase. Almost all forces are directed outward, and only a few have small positive values within the measurement error. Bottom left: A cell on low stiffness pillars at the beginning of the metaphase. Bottom right: Histogram of the radial forces of the selected pillars at the beginning of the metaphase. The cell pulls on 3 pillars. The other forces are negative within the measurement error or positive.*

3.4 Anaphase

In this phase the nucleus divides into two new daughter nuclei. The chromosomes can be seen to divide in two for the cells that express H2B-GFP. The images of the cells transduced with baculovirus show that the two poles of the spindle move apart. The cell itself does not yet divide in this phase. The forces still point outward just after the nucleus has divided. At the beginning of the anaphase the forces are still directed from one nucleus. Later in the anaphase the center from which the forces are directed is either one of the 2 centrosomes, depending on which of the centres of the nuclei is closer to the pillar. The total outward force decreases in this phase.

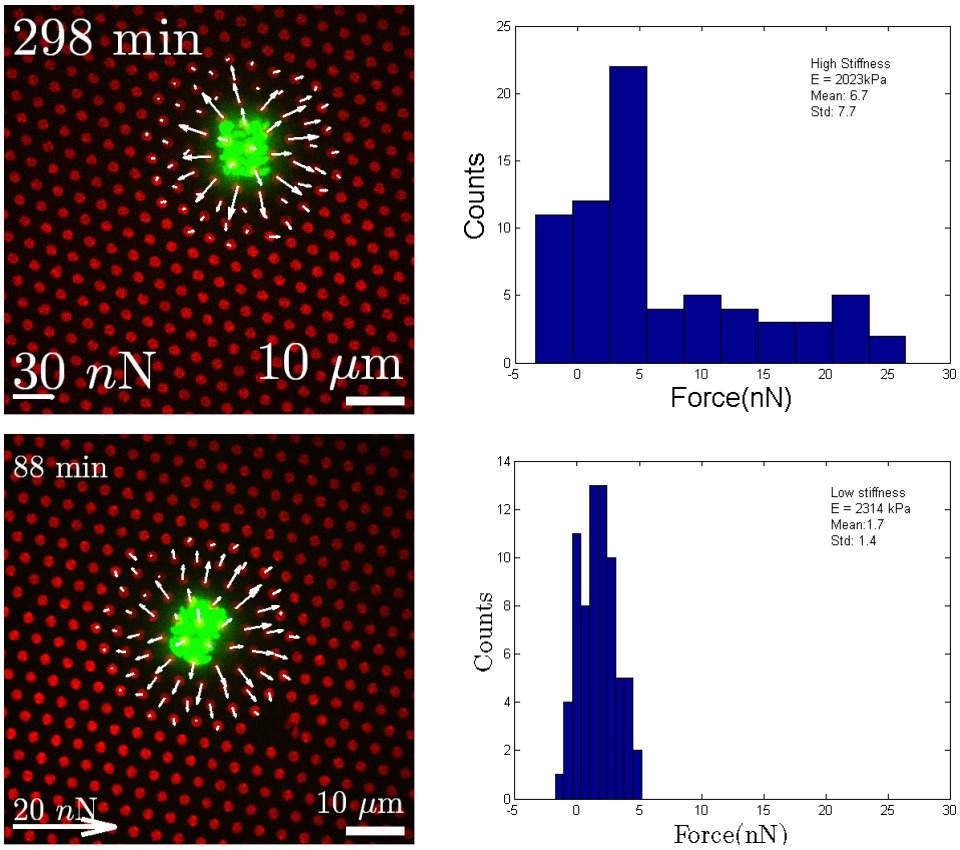


Figure 11: *Top left: A cell on high stiffness pillars at the beginning of the anaphase.The spindle pulls apart to two sets of chromosomes. The forces are directed outward, still originating from one center. Top right: Histogram of the radial forces of the selected pillars at the beginning of the anaphase. Still the majority of the forces are directed outward and higher forces than in metaphase are reached. Bottom left: A cell on low stiffness pillars at the beginning of the anaphase. Bottom right: Histogram of the radial forces of the selected pillars at the beginning of the anaphase. Most of the forces are directed outward.*

3.5 Telophase

In the telophase the nucleus has divided and the cytokinesis starts. This is visible in the cells that express H2B-GFP because the nuclei start to obtain a rounder shape because they decondense and move apart. Microtubules are spread throughout the whole cell, so they show its contour. For the cells that are transduced by the baculovirus the telophase is recognized because the whole cell splits in two. The outward force decreases to the level that it had during interphase. The cell starts to grab onto a few pillars at its edge to exert a pulling force and spread.

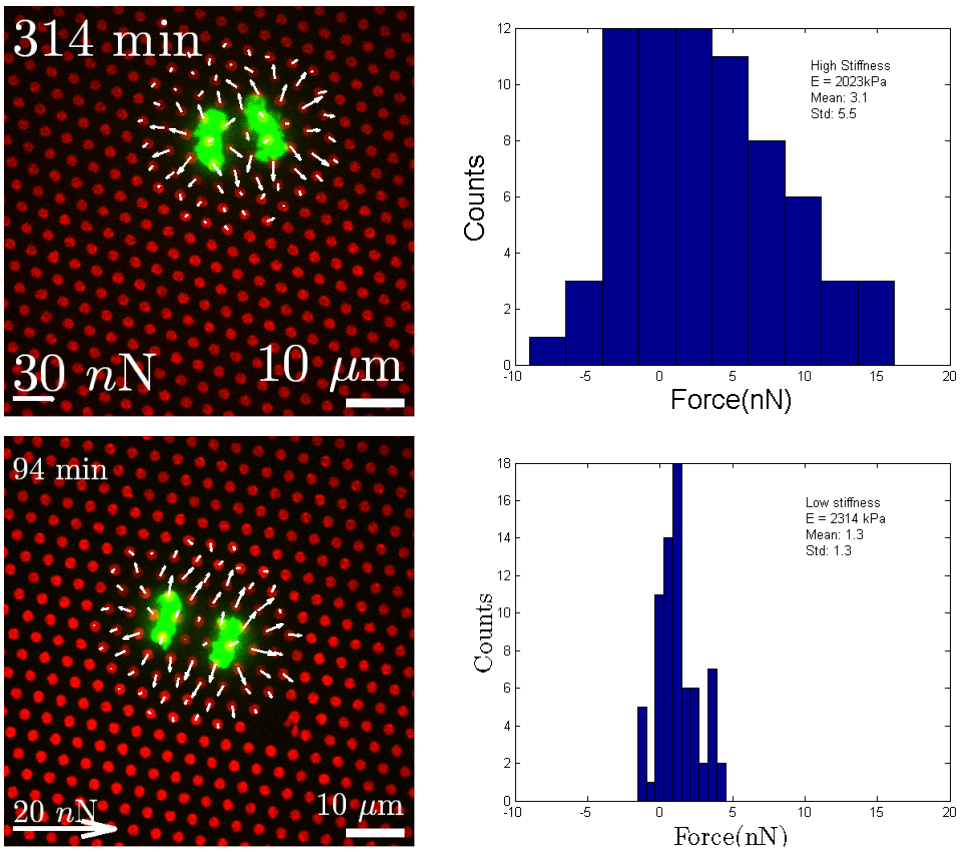


Figure 12: *Top left: A cell on high stiffness pillars at the beginning of the telophase. The two nuclei have arrived at the spindle poles. An envelope has formed around the two nuclei and the chromosomes start to decondense. The forces point outward from two nuclei and have decreased in value. Top right: Histogram of the radial forces of the selected pillars at the beginning of the telophase. The highest deflections have decreased in value. There are more inward directed forces than in anaphase. Bottom left: A cell on high stiffness pillars at the beginning of the telophase. Bottom right: Histogram of the radial forces of the selected pillars at the beginning of the telophase. The forces have decreased since anaphase.*

3.6 Outward directed forces

The outward directed force is exerted on many pillars. The total exerted force differs for every phase of the cell division and is lowest in the prophase. The highest outward force is reached at the beginning of the anaphase. At the beginning of the telophase the forces have decreased to nearly its value in interphase. The amount of force that is exerted depends not only on the phase but also on the stiffness of the pillars. The cells exert higher outward forces on stiffer pillars. During prophase the total outward force on high stiffness pillars is low and the values lie in a range between 0 and 200 nN. On low stiffness pillars some pillar exert inward forces so the radial force can be positive. It ranges from -20 to 30 nN. During metaphase the forces are high compared to those in prophase, ranging from 300 to 500 nN for high stiffness pillars and from 40 to 120 nN for low stiffness pillars. During anaphase the highest forces are exerted, from 400 nN to 600 nN for high stiffness pillars and 90 to 160 nN for low stiffness pillars. In the telophase the outward force has lowered to values between 150 and 250 nN for high stiffness pillars and 30 to 90 nN for low stiffness pillars.

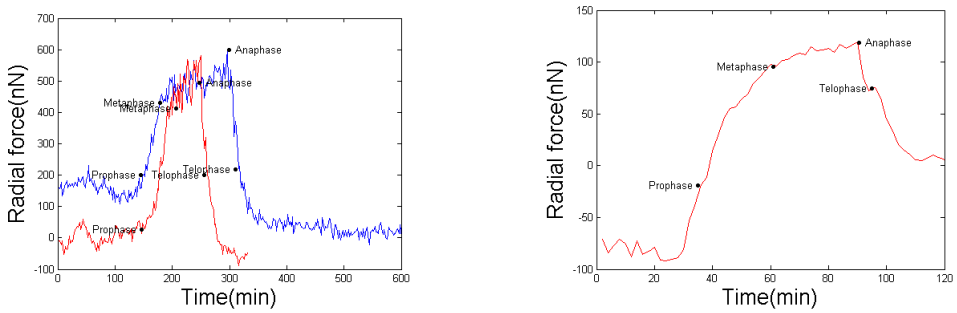
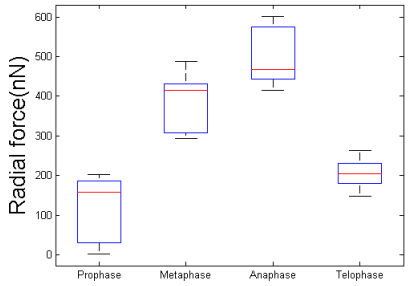
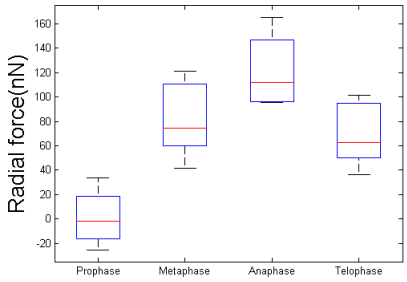


Figure 13: *Left: Two plots of the total radial directed force over time for two cells on high stiffness pillars. The beginning of the different phases of the cell cycle are annotated. When the cell has not entered the prophase, the forces are randomly oriented. Some of them are therefor facing outwards. When the chromosomes start condensing the forces point radially outward from the nucleus. As the chromosomes condense further, this force increases. It reaches its maximum at the moment when the two daughter nuclei are separated at the beginning of the anaphase. After the nucleus has divided the forces decrease again, until eventually they are randomly oriented and low in absolute value. Right: Plot of the total radial outward directed force over time for a cell on low stiffness pillars. The force before and at the beginning of the prophase is negative because the cell pulls strongly on its environment. At the end of the M-phase the force has not dropped to its initial level because the cell has not started spreading yet.*



(a) High stiffness, E = 2023 kPa



(b) Low stiffness, E = 2314 kPa

Figure 14: *Boxplots of the total radial force in the beginning of the four different phases on two different pillar stiffnesses. The amount of cells imaged for high stiffness pillars in prophase, metaphase, anaphase and telophase are respectively 6, 6, 5 and 5. The amount of cells imaged for low stiffness pillars in prophase, metaphase, anaphase and telophase are respectively 6, 7, 6 and 5.*

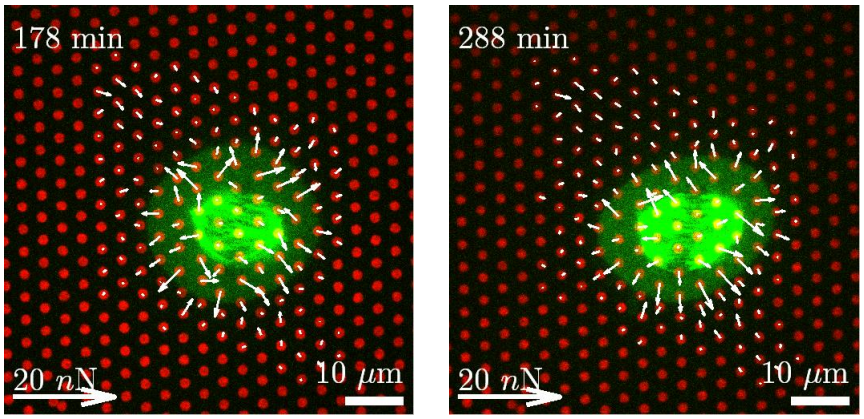
The duration of M-phase differs for each observed cell and ranges from one hour to three hours. Also the duration of the phases within the M-phase is not constant. For some cells the metaphase takes more than an hour while for others this takes only 15 minutes. It is possible that one cell has a long prophase but short metaphase, while another cell has a short prophase and a long metaphase. No dependence of the cell cycle duration on pillar stiffness has been found.

4 Discussion and conclusion

Just before the cell enters prophase it polarizes and pulls at the perimeter. The axis along which these forces are exerted shows the orientation of the spindle and to which directions the two daughter cells will move. The direction in which the two daughter cells will move is determined by the centrosomes, because from here the spindle forms. Previous experiments have found that actin filaments are used to generate pulling forces to position the nucleus.

When the cell starts exerting the outward directed force in the metaphase, the nucleus takes up most of the cells volume. The pillars at the edge of the nucleus and thus at the edge of the cell can not be deflected outward by pulling forces since the cell does not extend that far. The deflections need to be explained by pushing forces. Of the filaments that are present in the cell the microtubules are the most likely candidates because of their high stifness and persistence length of 5200 μm.

Microtubules are present near the nucleus, because the mitotic spindle consists of microtubules. Experiments with cells expressing tubulin-GFP show that the microtubules are not only located near the nucleus, but are spread throughout the whole cell when the cell exerts pushing forces. This shows that it is possible that microtubules are used to generate these pushing forces.



(a) Metaphase (b) Anaphase

Figure 15: *Images of a dividing cell expressing tubulin-GFP at the beginning of the metaphase and anaphase. Most of the microtubules are located near the nucleus because the spindle consists of microtubules. Microtubules can also be found throughout the rest of the cell, reaching the cell cortex.*

We propose a model (figure 16) in which microtubules emanate from the two centrosomes in all directions to the cell cortex to produce the pushing forces. Polymerization at the plus end of the microtubules creates this force. The pushing forces on the centrosomes are neccesary for the movement of the chromosomes during the different phases of mitosis and are balanced by forces on the cortex. During the prophase the condensing chromosomes need to be pushed toward the center. Microtubules are used to push to every direction, including towards the center. This force towards the centers is used to align the chromosomes. On the outside of the cell this is visible as outward pushing forces on the pillars. During anaphase microtubules are not only used to pull on the chromosomes, but also for pushing on the two centrosomes to aid in this movement. The structure of microtubules emanating in all directions is still present. In addition to the pushing forces this structure creates, there is now also a force that pulls the chromosomes towards the spindle poles. In the telophase a new nuclear envelope forms around both nuclei. At this point the division of the nucleus is done succesfully and there are no more pushing forces on the centrosomes neccesary. According to this model, the pushing forces will decrease as the chromosomes segregrate and will dissappear when the two new nuclei have formed. The data is in agreement with this model. The total radial force depends on the stiffness of the pillars. The model presented here will need to be worked out further to explain this dependence.

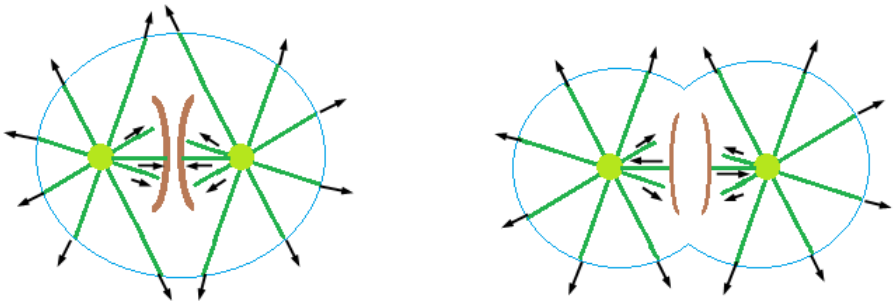


Figure 16: *Schematic of the centrosomes during M-phase. Microtubules extend from the centrosomes and push on the cell cortex by polymerization on the plus end. In the left image the chromosomes are condensed and microtubules push them towards the center. After chromosome segragation the microtubules still push against the cell cortex, but the force is now directed from two centers.*

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5 Appendix

```
function [prophase, metaphase, anaphase] = CellPhaseM(file,pos)

% ------------------------------------------------------------------
% find the frames at which the pro, meta and anaphases begin
%
% INPUT
%
% file:   name of the file
% pos:    position of the cell in file
% ------------------------------------------------------------------

% read in the file and find parameters
[data,par]=iqreadMartin(file,1);
n_c=par(1,par(2,:)==3);if isempty(n_c);n_c=1;end
n_z=par(1,par(2,:)==4);if isempty(n_z);n_z=1;end
n_t=par(1,par(2,:)==5);if isempty(n_t);n_t=1;end
n_p=par(1,par(2,:)==6);if isempty(n_p);n_p=1;end

prophase=[];
metaphase=[];
anaphase=[];

h   = waitbar(1,'...');
for j=1:300
    % make black and white image of the cell
    [d,p] = getsub(0,0,j,pos,data,par);
    dproj = projz(d,p,'max',0);
    Cell = squeeze(dproj(:,:,2));
    Pil = squeeze(dproj(:,:,1));
    BW=CellbwMartin(Cell);


    % calculate properties of the shape of the cell
    props = [regionprops(BW,'Eccentricity','Area')];
    plength(j)=length(props);
    Area=max([props.Area]);
    ind=find([props.Area]==Area);
    Eccentricity(j,:)=props(ind).Eccentricity;

    waitbar(j/n_t,h,sprintf('... %d%%',round(100*j/n_t)));

end
delete(h);

% check in which phase the cell division is
% prophase if number of objects>1, condensing creates holes in BW
% image of cell
```

```
% metaphase chromosomes form bar shape, eccentricity>0.9
% anaphase if number of objects>1 after metaphase
for j=1:n_t
    if isempty(anaphase) & ~isempty(metaphase) & ~isempty(prophase)
        if plength(j)>1 & plength(j-1)>1 & plength(j-2)>1 & plength(j-3)>1
            anaphase=j-2;
        end
    end

    if isempty(metaphase) & ~isempty(prophase)
        if Eccentricity(j)>0.9 & Eccentricity(j-1)>0.9 & Eccentricity(j-2)>0.9
            metaphase=j-2;
        end
    end

    if isempty(prophase)
        if plength(j)>1 & plength(j-1)>1 & plength(j-2)>1
            prophase=j-3;
        end
    end
end
end
```

```
function ForceDirection(file,defl,pos,frame,Telophase,c1,c2,c3)

% ------------------------------------------------------------------
% calculates the direction of forces with respect to the cellcenter
%
% INPUT
%
% file:         name of the file
% defl:         structure with pillar deflections
% pos:          position of the cell in file
% frame:        frame for which c1 is calculated
% Telophase:    frame at which the telophase start
% c1:  xy coordinates of the cellcenter before telophase
% c2:  xy coordinates of the first cellcenter after telophase
% c3:  xy coordinates of the second cellcenter after telophase

% ------------------------------------------------------------------

% read in the file and find parameters
[data,par]=iqreadMartin(file,1);
n_x=par(1,par(2,:)==1);
n_y=par(1,par(2,:)==2);
n_c=par(1,par(2,:)==3);if isempty(n_c);n_c=1;end
n_z=par(1,par(2,:)==4);if isempty(n_z);n_z=1;end
n_t=par(1,par(2,:)==5);if isempty(n_t);n_t=1;end
n_p=par(1,par(2,:)==6);if isempty(n_p);n_p=1;end

set(0,'defaulttextinterpreter','tex');
h   = waitbar(1,'Time...');

for j=1:n_t
    % use new cellcenter after telophase
    if j==Telophase
        c1=c3;
    end

    % determine direction of forces
    if ~isempty(defl(j).xydefl)

        % calculate drift by comparing positions of 1 pillar that is
        % present in all images for 2 different frames
        xref1=defl(frame).xyref(252,1);
        xref2=defl(j).xyref(252,1);
        xdif=xref2-xref1;

        yref1=defl(frame).xyref(252,2);
        yref2=defl(j).xyref(252,2);
        ydif=yref2-yref1;

```

```
50          % correct cellcenter for drift after telophase
51          if j==Telophase
52              c1(1)=c1(1)-xdif;
53              c1(2)=c1(2)-ydif;
54
55              c2(1)=c2(1)-xdif;
56              c2(2)=c2(2)-ydif;
57
58          end
59
60          for k=1:length(defl(j).xydefl(:,1))
61              if isnan(defl(j).absdefl(k))
62                  angle(j,k)=NaN;
63              else
64                  % determine angle of force
65                  xdefl=defl(j).xydefl(k,1);
66                  ydefl=defl(j).xydefl(k,2);
67                  x=defl(j).xyref(k,1)-xdif;
68                  y=defl(j).xyref(k,2)-ydif;
69
70                  d=abs(ydefl/xdefl);
71                  theta(j,k)=180*atan(d)/pi;
72
73                  dx(1)=x-c1(1);
74                  dy(1)=y-c1(2);
75                  dr(1)=abs(dy(1)/dx(1));
76
77                  % shift theta(j,k) so it starts pointing to the right
78                  if xdefl>0
79                      if ydefl>0
80                          theta(j,k)=theta(j,k);
81                      elseif ydefl<0
82                          theta(j,k)=360-theta(j,k);
83                      end
84                  elseif xdefl<0
85                      if ydefl>0
86                          theta(j,k)=180-theta(j,k);
87                      elseif ydefl<0
88                          theta(j,k)=180+theta(j,k);
89                      end
90                  end
91
92                  % after telophase the angle is
93                  % calculated for 2 nuclei
94
95                  if j>Telophase
96
97
98                      dx(2)=x-c2(1);
```

```
99                      dy(2)=y-c2(2);
100                     dr(2)=abs(dy(2)/dx(2));
101
102                     dx(1)=x-c1(1);
103                     dy(1)=y-c1(2);
104                     dr(1)=abs(dy(1)/dx(1));
105                     theta2(j,k)=180*atan(dr(2))/pi;
106
107                     % shift theta2(j,k) so it starts pointing to the right
108                     if dx(2)>0
109                         if dy(2)>0
110                             theta2(j,k)=theta2(j,k);
111                         elseif dy<0
112                             theta2(j,k)=360-theta2(j,k);
113                         end
114                     elseif dx(2)<0
115                         if dy(2)>0
116                             theta2(j,k)=180-theta2(j,k);
117                         elseif dy(2)<0
118                             theta2(j,k)=180+theta2(j,k);
119                         end
120                     end
121                     angle2(j,k)=theta(j,k)-theta2(j,k);
122                 else
123                     angle2(j,k)=0;
124                 end
125
126                 % determine angle from centre
127                 theta1(j,k)=180*atan(dr(1))/pi;
128
129                 % shift theta1(j,k) so it starts pointing to the right
130                 if dx(1)>0
131                     if dy(1)>0
132                         theta1(j,k)=theta1(j,k);
133                     elseif dy(1)<0
134                         theta1(j,k)=360-theta1(j,k);
135                     end
136                 elseif dx(1)<0
137                     if dy(1)>0
138                         theta1(j,k)=180-theta1(j,k);
139                     elseif dy(1)<0
140                         theta1(j,k)=180+theta1(j,k);
141                     end
142                 end
143                 angle1(j,k)=theta(j,k)-theta1(j,k);
144
145                 % Determine to which nucleus the pillar is closest
146                 if j>Telophase
147
```

```
148                         if angle2(j,k)~=0 & dr(2)<dr(1)
149                             angle(j,k)=angle2(j,k);
150                         else
151                             angle(j,k)=angle1(j,k);
152                         end
153                     else
154                         angle(j,k)=angle1(j,k);
155                     end
156                 end
157             end
158         end
159
160         waitbar(j/n_t,h,sprintf('Time... %d%%',round(100*j/n_t)));
161     end
162     delete(h);
163
164     % save in mat-file
165     save(['Angles',file(1:end-4),'_pos',num2str(pos),'.mat']);
166     end
```

```
function GetPillarIndex(file,defl,frame,pos,x,y)

% ------------------------------------------------------------------
% finds the indices of pillars that the cell exerts forces on
%
% INPUT
%
% file:   name of the file
% defl:   structure with pillar deflections
% frame:  frame for which the mask is made
% pos:    position of the cell in file
% x,y:    x,y coordinates of manually selected pillars
% ------------------------------------------------------------------

% read in the file and find parameters
[data,par]=iqreadMartin(file,1);
n_x=par(1,par(2,:)==1);
n_y=par(1,par(2,:)==2);
n_c=par(1,par(2,:)==3);if isempty(n_c);n_c=1;end
n_z=par(1,par(2,:)==4);if isempty(n_z);n_z=1;end
n_t=par(1,par(2,:)==5);if isempty(n_t);n_t=1;end
n_p=par(1,par(2,:)==6);if isempty(n_p);n_p=1;end

x=round(x);
y=round(y);
j=frame;

% make a black and white image of the cell
[d,p]=getsub(0,0,j,pos,data,par);
[dproj,pproj] = projz(d,p,'max',0);
Cell=squeeze(dproj(:,:,2));
BW=CellbwMartin(Cell);
% make a mask around the cell to select pillars
props = regionprops(BW,'Area','PixelIdxList','Centroid');
[~,cellindex]=max([props(:).Area]);
I=false(size(Cell));
I(props(cellindex).PixelIdxList)=1;
pixels= props(cellindex).PixelIdxList;

xy=defl(j).xy;
[ymask,xmask]  = ind2sub(size(I),pixels);
% now take out only the pillars within the mask + some pixels
for i=1:length(xy)

    if min((xy(i,1)-xmask).^2+(xy(i,2)-ymask).^2)<5000
        ind(i)=i;
    else
        ind(i)=NaN;
    end
```

```
50      if min((xy(i,1)-x).^2+(xy(i,2)-y).^2)<500
51          ind1(i)=i;
52      else
53          ind1(i)=NaN;
54      end
55  end
56  % remove nans
57  index=cat(1,ind,ind1);
58  index=index(~isnan(index));
59
60  % save in mat-file
61  save(['Angles',file(1:end-4),'_pos',num2str(pos),'.mat'],'index','-append');
62
63  end
```

```
function [absforce,absdefl] = DeflPlot(file,pos,defl,ind,angle,p1,p2,p3,p4)

% ------------------------------------------------------------------
% converts pillar deflection into forces and plots force vs time
%
% INPUT
%
% file:   name of the file
% pos:    position of the cell in file
% defl:   structure with pillar deflections
% ind:    indices of selected pillars
% angle:  array with angles of pillars w.r.t. cellcenter
% p1:     frame where prophase starts
% p2:     frame where metaphase starts
% p3:     frame where anaphase starts
% p4:     frame where telophase starts
% ------------------------------------------------------------------

% read in the file and find parameters
[data,par]=iqreadMartin(file,1);
n_x=par(1,par(2,:)==1);
n_y=par(1,par(2,:)==2);
n_c=par(1,par(2,:)==3);if isempty(n_c);n_c=1;end
n_z=par(1,par(2,:)==4);if isempty(n_z);n_z=1;end
n_t=par(1,par(2,:)==5);if isempty(n_t);n_t=1;end
n_p=par(1,par(2,:)==6);if isempty(n_p);n_p=1;end

norm=2*(p1-1);

for j=1:n_t
    % calculate the force exerted on the pillars
    deflabs=[];
    for i=1:length(ind)
        deflabs1(i)=defl(j).absdefl(ind(i)).*0.138*65.9.*(1+0.04.*(defl(j).abs
        deflabs(i)=(deflabs1(i)*cos(angle(j,ind(i))*pi/180));
    end

    for i=1:length(deflabs)
        if ~isnan(deflabs(i))
            adefl(i)=deflabs(i);
        end
    end

    % add up all forces per frame
    absdefl(j,:)=adefl(:);
    absforce(j)=sum(adefl);

    index(j)=(j)*2;
    index(j)=index(j)-norm;
```

```
50 end
51
52 % plot force vs time and annotate the four phases
53 plot([index],absforce);
54 set(gca,'XTick',-norm:2*n_t/6:2*n_t-norm);
55 xlabel('Time(min)','FontSize', 20);
56 ylabel('Force(nN)','FontSize', 20);
57 title('Plot of Force vs Time (cellphase)','FontSize', 20);
58 text(p1*2-norm,absforce(p1),'Prophase \bullet', 'HorizontalAlignment','right')
59 text(p2*2-norm,absforce(p2),'Metaphase \bullet', 'HorizontalAlignment','right'
60 text(p3*2-norm,absforce(p3),'\bullet Anaphase', 'HorizontalAlignment','left');
61 text(p4*2-norm,absforce(p4),'Telophase \bullet', 'HorizontalAlignment','right'
62
63 end
```