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## Forces, growth and cell division

Ewa Zlotek-Zlotkiewicz

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PARIS DESCARTES UNIVERSITY

PHD THESIS

# Forces, growth and cell division

*Ewa Zlotek-Zlotkiewicz*

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# Forces, cell growth and cell division

Ewa Zlotek-Zlotkiewicz

June 27, 2016



PARIS DESCARTES UNIVERSITY

DOCTORAL THESIS

# Forces, growth and cell division

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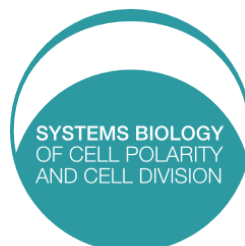
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# Contents

<b>Thesis summary</b>	<b>ix</b>
<b>1 Introduction</b>	<b>1</b>
1.1 Historical introduction into what is today's Mitotic Cell Rounding . . . . .	1
1.2 Importance of cell rounding in mitosis . . . . .	4
1.2.1 Mitotic Cell Rounding is universal . . . . .	4
1.2.2 Mitotic Cell Rounding has several functions in cell division . . . . .	6
1.3 Physical principles involved in Mitotic Cell Rounding . . . . .	12
1.3.1 Osmosis and osmotic pressure in cells . . . . .	12
1.3.2 Mechanical properties involved in cell shape acquisition . . . . .	13
1.4 Molecular mechanisms involved in Mitotic Cell Rounding . . . . .	15
1.4.1 Rounding up without confinement . . . . .	16
1.4.2 Rounding up under confinement . . . . .	20
1.5 Does cell volume change during mitosis? . . . . .	21
1.6 Hypothesis of volume increase as force generator in mitosis . . . . .	24
<b>2 Results</b>	<b>27</b>
2.1 Cells swell in mitosis (published) . . . . .	27
2.1.1 Failed calibration of FXm with silicon beads . . . . .	43
2.1.2 Cultured drosophila cells increase their area in mitosis under confinement . . . . .	44
2.1.3 Caveolin 1 has a role in volume regulation and mitotic cell rounding . . . . .	45
2.1.4 Anaphase and the volume undershoot . . . . .	47
2.2 Screen and isotropic confinement . . . . .	47
2.3 Nuclear envelope breakdown and volume increase . . . . .	49

<b>3 Discussion</b>	<b>53</b>
3.1 At the beginning, there was a hypothesis . . . . .	53
3.2 Cells swell in mitosis . . . . .	56
3.2.1 FXm method - the bad and the good . . . . .	58
3.3 Mechanisms behind the Mitotic Swelling . . . . .	59
3.4 Mitotic swelling and force generation . . . . .	61
3.5 Mitotic cell rounding in tissue homeostasis context . . . . .	64
3.6 Perspectives . . . . .	67
<b>4 Methods</b>	<b>69</b>
4.1 Cell confinement . . . . .	69
4.2 Volume measurement using FXm . . . . .	87
4.3 Volume measurements on patterns . . . . .	99
4.4 Volume measurements and media changes . . . . .	99
<b>A Review : Exploring the function of cell shape and size during mitosis. <i>Developmental Cell 2014</i></b>	<b>101</b>
<b>B Extra-lab PhD work</b>	<b>113</b>
B.1 Apprentis chercheurs - sharing science with teenagers . . . . .	113
B.2 Questions workshop during the Science Festival of Palestine . . . . .	116
B.3 Biology courses at the FdV Bachelor program and game designing with L'Arbre des Connaissances . . . . .	118
B.4 Intellectual self-defence in Bagnolet . . . . .	119
<b>Bibliography</b>	<b>123</b>
<b>List of Figures</b>	<b>141</b>

# Thesis summary

Most eukaryotic cells remodel their shape and round up at mitosis entry. Mitotic cell rounding (MCR) under confinement, as it happens in tissues, requires force generation. It is a way of generating sufficient space for the assembly and orientation of the mitotic spindle and is therefore of crucial importance for the outcome of the division. Cells in which this rounding is impeded present a higher number of errors in divisions. Such errors are deleterious for tissue homeostasis and in some cases might be a potential source of cancer transformation. Changes in cell volume at MCR have been a subject of scientific debate up to now, in part due to lack of adapted volume measurement methods.

The present PhD thesis report aims at summarizing the state of knowledge regarding the subject of MCR and describes the major outcomes of this PhD project. It describes also methods of studying cell rounding under confinement and live cell volume measurement (FXm for Fluorescence eXclusion method). At last the report contains a general public introduction and the description of the educational and scientific mediation activities that have been realized in parallel to the experimental work.

The principal goal of this PhD project was to study the mechanisms underlying MCR. In particular, the approach focused on the mechanism of force generation during rounding under confinement. In order to tackle the question, I co-developed and optimized novel techniques in cell biophysics, in particular controlled cell confinement and precise volume measurement of live cells. The development of professional skills in research, project development, teaching and scientific mediation constitute important side goals.

A major finding of the presented work is that cells swell during MCR. This work provides both, a versatile and simple method for live cell volume measurements and evidences that cells increase their volume of up to 30% during MCR. This volume increase is potentially a source of hydrostatic pressure that can generate forces reaching 100 kPa which is enough to deform almost any tissue in the human body (bone stiffness is of approx. 100 kPa). This volume change is driven by reversible water influx and is independent of the actomyosin cortex functionality. On the other hand, a functional and stiff cortex is necessary for reaching a round shape if cells are under soft confinement which is the second major outcome of this project.

This interdisciplinary work combines biological and physical approaches to study a fundamental process happening tens of billions of times per day in a human body. It unambiguously establishes that cells can increase their volume in mitosis and suggest that this might be a phenomenon underlying force generation in MCR. It also provides two ways of evaluating the robustness of cell rounding in mitosis; cell volume measurement by FXm and rounding under confinement. These two approaches can be further used to uncover molecular mechanisms underlying MCR. Once known, these in turn might become novel targets for cancer therapy, as most of tumor cells have to divide in stiffer environment and therefore provide more forces to round up and divide than normal cells.

**Eggs can swell...** As a proof of concept we realized, with Djineba and Antoine an experiment where a swelling egg generated pushing forces on a calibrated spring. Indeed it is possible to obtain chicken eggs, which are big, single cells, devoid of the egg shell. Such eggs are surrounded by a semi-permeable membrane and shrink in hyperosmotic media and swell put back to hypotonic solutions. When swelling, these eggs can generate forces...



Figure 1: Video of an egg swelling in a hypotonic medium (pure water).

# Chapter 1

## Introduction

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<b>1.1</b>	<b>Historical introduction into what is today's Mitotic Cell Rounding</b>	<b>1</b>
<b>1.2</b>	<b>Importance of cell rounding in mitosis</b>	<b>4</b>
1.2.1	Mitotic Cell Rounding is universal	4
1.2.2	Mitotic Cell Rounding has several functions in cell division	6
<b>1.3</b>	<b>Physical principles involved in Mitotic Cell Rounding</b>	<b>12</b>
1.3.1	Osmosis and osmotic pressure in cells	12
1.3.2	Mechanical properties involved in cell shape acquisition	13
<b>1.4</b>	<b>Molecular mechanisms involved in Mitotic Cell Rounding</b>	<b>15</b>
1.4.1	Rounding up without confinement	16
1.4.2	Rounding up under confinement	20
<b>1.5</b>	<b>Does cell volume change during mitosis?</b>	<b>21</b>
<b>1.6</b>	<b>Hypothesis of volume increase as force generator in mitosis</b>	<b>24</b>

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Cell shape is an intrinsic property of cells. Establishment of shape is an active, well controlled process and costs a lot of energy to the cell. In particular cells with different functions or from different tissues adopt different shapes. For example poorly adherent blood cells used to be transported in the blood-stream are roundish and cells that form epithelia are cuboid. Whatever their shape in interphase or origins, the vast majority of cells adopt the same shape at one particular moment of their life; they become all round as they divide. For the "Why?" question, we already have some cues that will be exposed in the introduction. As for the "How?" question, it is yet poorly studied. Known parts of the mechanism are described in the introduction and the results section of this PhD project contains novel information on how cells might round up during mitosis.

The following PhD project was developed with an interdisciplinary approach, studying cell division asking biology and physics questions and using novel technological approaches.

### 1.1 Historical introduction into what is today's Mitotic Cell Rounding

After a fascinating dive into history of science, I have to admit that it is rather difficult to find in the original literature who observed or who described the cellular shape throughout division for the first time. Clearly, new technical possibilities of the 19th century, as live cell observation using microscopy, raised a tremendous excitement in the scientific community. This resulted in a vast number of descriptions of



what we today know as cell division. Though cell division has been reported as soon as 1835 by Mohl in plants and 1841 by Remark in animals the notions of nascent cell biology were not helping to name things right. It was not clear what a cell was and how it was delimited from its environment. In fact in mid 1850s Brücke, Beale and Schultze agreed on the fact that cell membrane is unnecessary to the cell (Henneguy, 1896). Descriptions of indirect cell division at this time mostly and even exclusively concerned the nucleus. The global shape of the cell was mostly omitted. It is only in 1858 that Rudolf Virchow coined the " *omnis cellula e cellula* " pointing out the importance of mitosis, however not immediately accepted by the scientific community. But the interest in mitosis in science was growing and the first sequential description of stages of programmed cell division observed in fixed tissues came from Waclaw Mayzel (Mayzel, 1875) working in the reknown team of Prof. Hoyer in Warsaw.

At this time also started the interest in cell shape during division through the problem of predicting the plane of cell division. In plant cells, attempts to predict the division plane relative to cell shape (delimited by cell wall) were made by Sachs since 1878 and Errera since 1888 (Minc and Piel, 2012). Oskar Hertwig in 1884 predicted that cells divide in their center of mass and perpendicularly to their longest axis (Hertwig, 1884). This empirical rule named after him, is sometimes referred to as the "long-axis rule". It is still, after 132 years, an axiom in biology, even if it has been refined since then (Minc and Piel, 2012).

Scientists at the end of the 19th century could observe live cell divisions as in 1911 Burrows and Carrell published their results on how to extract and keep in culture cells from various organs and tumors (Carrel and Burrows, 1911). They even mention that: *The survival of cells outside of the body has been observed by many investigators ; especially by Ljunggren (in 1898), Jolly (in 1903), Carrel (in 1910), Volpino (1911), and others. These authors placed pieces of tissues in serum or other fluids and observed the survival of the cells and even some mitotic divisions.* The very first drawing of a cell undergoing mitosis and mitotic cell rounding, that I found was done by Lambert in 1913 (Lambert, 1913) and is representing a rat connective tissue cell in culture Figure 1.1.

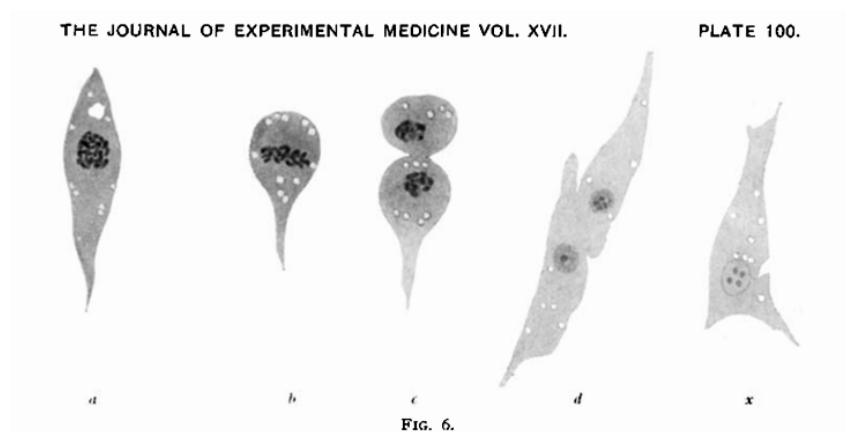


Figure 1.1: Reproduction of the drawing published in 1913 in an article by Robert A. Lambert (Lambert, 1913). This is the earliest drawing clearly showing Mitotic Cell Rounding that I found. It represents a rat connective tissue cell entering mitosis. (a) prophase, (b) metaphase, (c) telephase; (d) daughter cells, (x) interphase cell. The time-lapse is of 15 minutes.

In 1922, Strangeways (Strangeways, 1922) provided another, more detailed drawing of a mitotic cell in choroidal cells from chick embryos Figure 1.2.

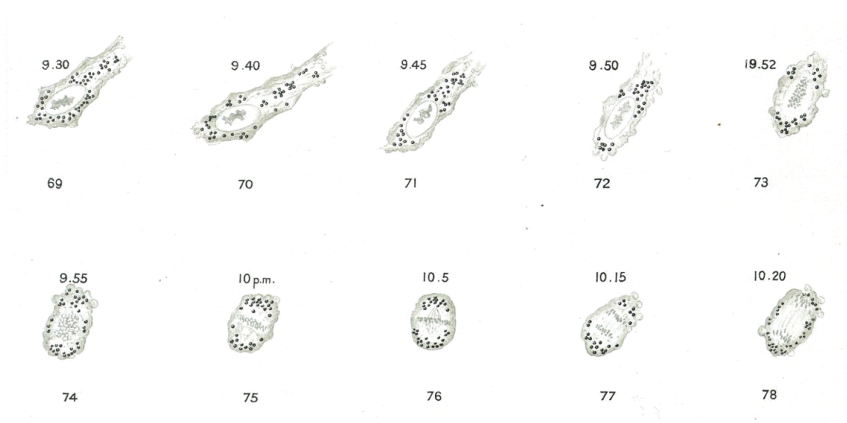


Figure 1.2: In his article on changes in cells during growth and cell division, Thomas S. P. Strangeways describes that: *The processes of the cytoplasm retract (fig. 72) and the cell assumes an oval or rounded shape (fig. 73)-the nucleoli become fainter (fig. 72) and then appear as hazy granules (fig. 73).* (Strangeways, 1922). Strangeways describes here choroidal embryonic cells. The numbering of figures seems incorrect.

The Parisian laboratory of Carrell published the first time-lapse stills illustrating the development of a *Sea Urchin egg* [Figure 1.3](#) where several cell divisions can be seen. This marked also a turning point in the methodology in cell biology. Dynamics of many processes can be from now-on visualised and analysed with more precision. This was the starting point of a new era in which cell imaging became a master approach in cell biology, as it is for this PhD project .

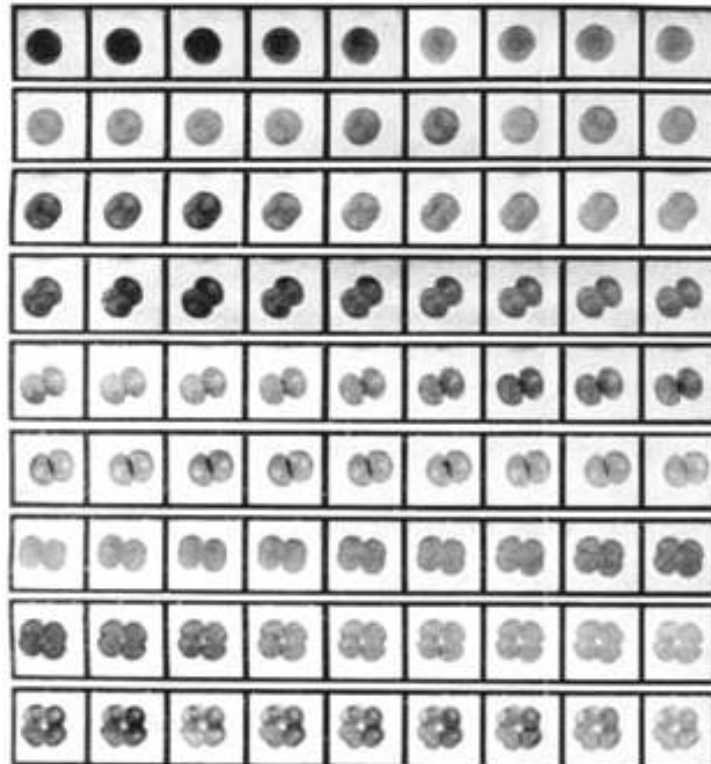


Figure 1.3: Stills by Julius Ries ([Ries, 1910](#)), realised in 1907, representing the fertilization and development of a Sea Urchin egg. It is one of the earliest microcinematographic time-lapse made, and therefore one of the first ones showing cell divisions.

Though mitotic rounding has been observed since more than 100 years, the mechanisms driving this process are still poorly understood. It is in fact only very recently that these mechanisms started to be

investigated.

Why do cells round up, and how they do it?

## 1.2 Importance of cell rounding in mitosis

The following part of introduction focuses on the question "why mitotic cell rounding is important?". First, it is universally observed in a variety of tissues and organisms. Second, it is important to allow sufficient space generation inside the dividing cell. This in turn is crucial for the outcome of mitosis, the orientation of the division plane relative to the tissue organisation and error-less chromosome segregation. The outcome of mitosis has strong consequences on tissue and organism homeostasis.

### 1.2.1 Mitotic Cell Rounding is universal

Cells round up for mitosis in different tissues, development stages, organisms across several phyla, *in* and *ex vivo*, in normal and cancer cells. The universality of this observation is the first argument pointing towards the importance of mitotic cell rounding.

The work of Lambert from 1913 mentioned above first reported the observation of mitotic rounding in rat cells coming for sarcoma (Lambert, 1913), Figure 1.1. Strangeways report again cell rounding in 1922 describing divisions of cells isolated from chick embryonic choroidal tissues Figure 1.2.

Modern biology provides a plethora of others examples of Mitotic Cell Rounding.

Observing it is pretty common in cell culture conditions. Canine epithelial cells (MDCK) form monolayers in culture dishes, where cells entering mitosis clearly round up (Wyatt et al., 2015). In the same culturing conditions human neuronal cells (hNPC) also round up (Keenan et al., 2010). In fact there are rare examples of cell lines that do not round up when seeded on tissue culture plates<sup>1</sup>. Mitotic MDCK cells also round up when cultured in 3D matrices, where they form cysts with internal lumen (Lzaro-Diguez et al., 2013) or when cultured as microspheres (Tuncay et al., 2015). Mice embryonic 3T3 fibroblasts also round up for mitosis when cultured in 3D matrices (Lesman et al., 2014). Cell rounding is therefore not a restrained to 2D environments and occurs under anisotropic 3D confinement.

More importantly, mitotic cell rounding has also be reported *in vivo* in mammals. In 1972 a particular case of mitotic cell rounding, the interkinetic nuclear migration (INM) Figure 1.4, has been described in early mouse thalamus development by (Smart, 1972). Interkinetic nuclear migration is a simultaneous movement and rounding of columnar cells in epithelial tissues during mitosis. In this process cells re-localize their center of mass towards the apical surface of the epithelial layer (Spear and Erickson, 2012). Furthermore *in vivo* works of Luxenburg et al. focus on mitotic cell rounding in the skin of mouse embryos (Luxenburg et al., 2015a) and (Luxenburg et al., 2011).

Mitotic cell rounding is not restricted to mammalian cells as it occurs in *Leishmania major* an intracellular protozoa infecting immune cells (Ambit et al., 2011). *Dictyostelium discoideum*, a soil-living amoeba also rounds up during mitosis (Plak et al., 2014), and so does cells of the developing starlet sea anemone, *Nematostella vectensis* (DuBuc et al., 2014).

Cells in widely studied model organisms in developmental biology such as *Drosophila melanogaster*, *Xenopus laevi* or *Danio reiro* provide examples of *in vivo* mitotic cell rounding. *Drosophila melanogaster* cells round up in the wing imaginal disc (Gibson et al., 2011) or in the notum of the pupae (Rosa et al.,

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<sup>1</sup>*Potorous tridactylis* epithelial kidney cells (Ptk2) cells are known for staying flattened till late metaphase, still they overgo an important change in shape. Normal rat kidney epithelial cells (NRK) also stay pretty flat during mitosis.

2015). In particular, the work of (Rosa et al., 2015) shows that MCR is less visible at the apical surface than in the middle plane of the epithelial layer of the notum. This could be a reason for underestimating the universality of the phenomenon *in vivo*. In *Xenopus laevis* a nice example of mitotic rounding is shown in unicellular and multicellular angiogenic sprouts by the work of (Aydogan et al., 2015). Mitotic cell rounding can also be observed *live* during the *Danio reio* neural tube formation (Žigman et al., 2014), (Geldmacher-Voss et al., 2003). The Figure 1.4 shows several of above described examples of rounded cells in mitosis.

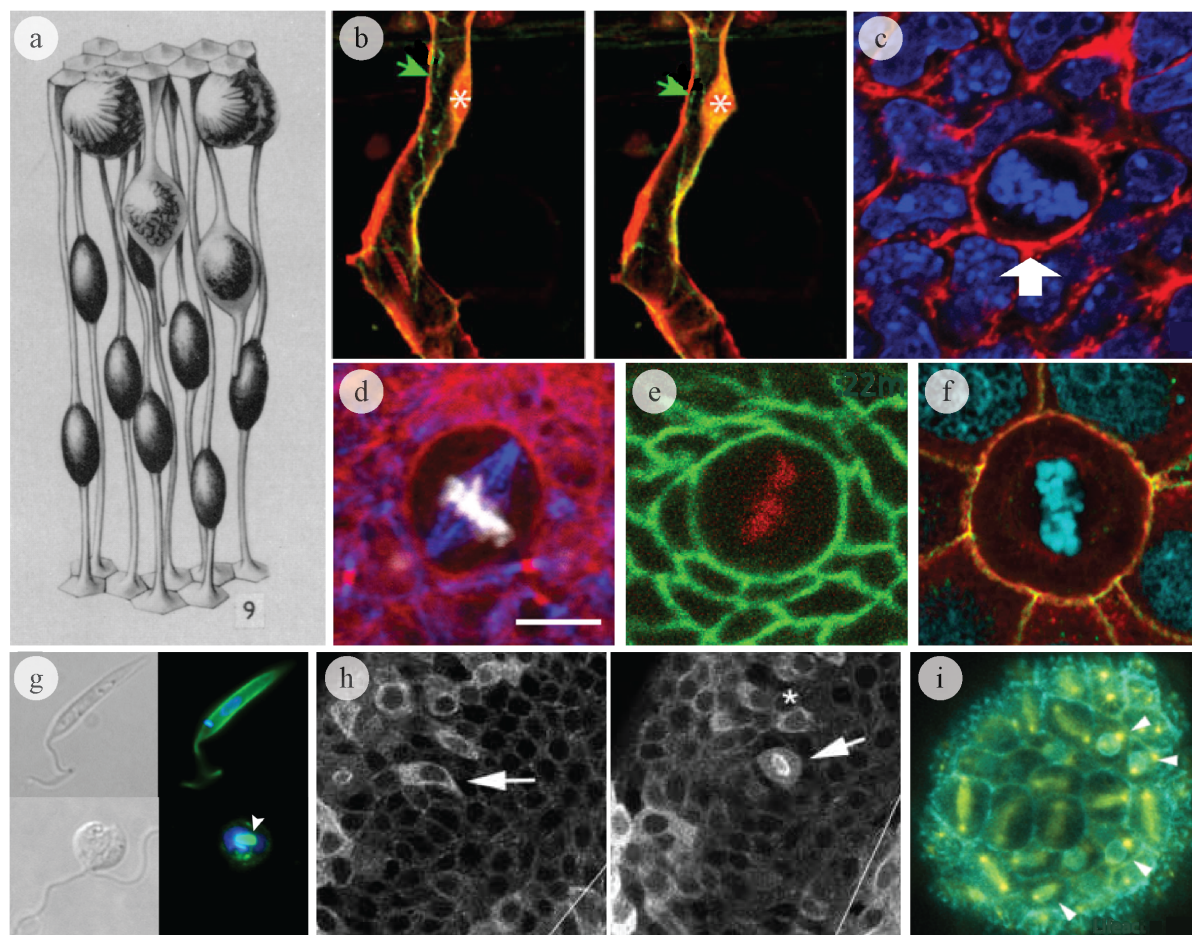


Figure 1.4: Images of various examples of mitotic cell rounding. **a)** Intekinetic nuclear migration in mouse brain, drawing in (Smart, 1972), **b)** An endothelial cell dividing in angiogenic sprouts in *Xenopus* (Aydogan et al., 2015), **c)** A metaphase cell in mouse skin (Luxenburg et al., 2011), **d)** A middle plane of the *Drosophila* notum showing a metaphase cell (Rosa et al., 2015), **e)** A mitotic cell in *Drosophila* wing imaginal disc (Gibson et al., 2011), **f)** Dividing cell in microspheres formed by MDCK cells (Tuncay et al., 2015), **g)** Cell division in *Leishmania*, the first panel shows the organism prior to mitosis (Ambit et al., 2011), **h)** A cell, prior and during mitosis in neural plate of a Zebrafish (Geldmacher-Voss et al., 2003), Several dividing cells during the development of *Nematostella* (DuBuc et al., 2014). **c-f and h)** show how surrounding neighbour cells seems to be deformed by the rounded cell in mitosis.

Mitotic rounding implies a profound and active remodelling of cell shape occurring in various organisms. The striking universality of this observation suggests a role of rounding up in mitosis. Moreover, the fact that neighbouring cells are frequently deformed around the dividing cell lets presume that it is an active process involving force generation. Indeed, counteracting this force enough to impede cell rounding by mechanical or biochemical cues threaten the outcome of cell division (Tse et al., 2012; Lancaster et al., 2013; Matthews et al., 2012; Kunda et al., 2008; Cadart et al., 2014), reinforcing arguments for the potential MCR importance.

### 1.2.2 Mitotic Cell Rounding has several functions in cell division

Segregation of the DNA into two distinct daughter cells is a tedious and risky task with crucial outcome. Normally, a mistake in DNA segregation that would pass through the scrutiny of the mitotic checkpoints, will lead to the death of the cell carrying the defect. If for any reason those control mechanisms fail in recognising the error or inducing cell death, such a cell might be the onset of a tumour. One lost or additional chromosome or even its part in the nascent cell can potentially be detrimental for the whole organism.

After the synthesis of the new copy of the genome in S phase and a non mandatory passage though the G2 phase the DNA is reorganised and condensed into chromosomes when entering into the M phase [Figure 1.5](#). Those will be shortly liberated into the cytoplasm by the Nuclear envelope breakdown (NEB). Mitotic cell rounding is starting roughly at that time (not yet precisely characterised). At this point, chromosomes are condensed but still bulky and are localized in the dense cytoplasm. The lack of protective nuclear envelope exposes the genome to a plethora of dangers and makes it vulnerable. Lengthening the time spent in mitosis may induce errors in chromosomes segregation and correlates with the frequency of mitotic mistakes and disorders in the next cell cycle ([Daum et al., 2011](#); [Uetake and Sluder, 2010](#)). It also has been reported that the duration of mitosis alters cell fate, changing the balance between neurons and progenitors production in the developing brain ([Pilaz et al., 2016](#)). In order to segregate chromosomes into daughter cells, microtubules assemble into the mitotic spindle, which binds and pulls apart the two sets of chromosomes. Catching chromosomes in the crowded environment of the cytoplasm is already challenging. But additionally the spindle, a massive and delicate structure, often needs to be repositioned in the cell. This has to be done cautiously and with precision to preserve its integrity. All this as fast as possible.

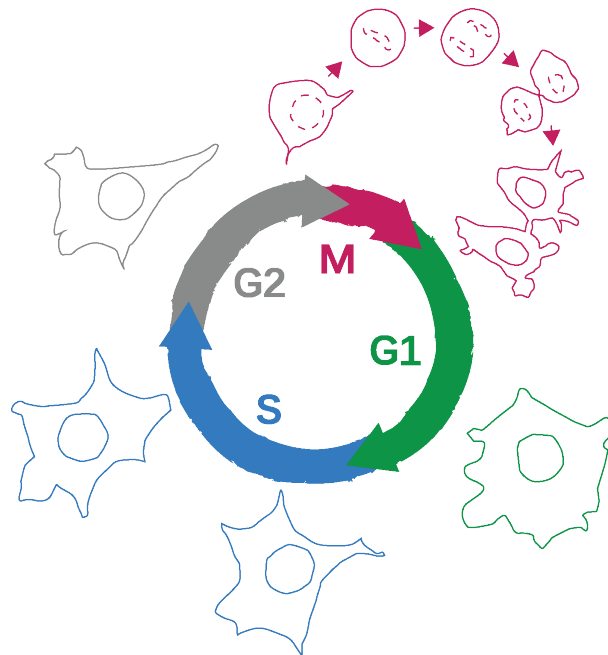


Figure 1.5: Cell cycle phases. After a division, the cell enters into the G1 phase, which is followed by the S phase where the DNA is duplicated. Before entering again into division most cells go through the G2 phase. During most of its cell cycle (about 24h), the adherent HeLa cell, whose contour is presented in this scheme, stays spread. Its shape over-goes dynamic changes, as protrusions are constantly formed and retracted, but the most prominent shape changes happen during division. Over several tens of minutes, the cell rounds up, elongates, splits into two new spheres that spread again.

One way of facilitating this complex task is to provide a optimal space, big enough to organise

chromosomes segregation by the spindle but small enough to keep everything in the reach of the spindle. This could be promoted by acquiring a spherical shape.

### Proper mitotic space generation

Cell rounding is particularly easy to observe in cultured adherent cells, as the shape change is drastic. Cell shapes go from flattened with irregular contours to smooth and spherical. This change, although less spectacular, can also be seen in cells growing in suspension that are rounded prior to mitosis.

Interphase adhering cells on rigid substrates have a flattened nucleus and cell body. Entry into mitosis implies profound changes in the cell architecture and behaviour. One of the major promoters of those changes is Cyclin Dependent Kinase 1 (CDK1) (Gavet and Pines, 2010b). It indirectly launches early in prophase centrosomes separation, the nuclear envelope breakdown, chromosomes condensation and cell rounding. Roughly at the same time, cell rounding starts, centrosomes are placed on the opposite sides of the nucleus and a radial gradient of ranGTP is created at the chromosomes. This gradient functions as a bait and promotes rapid and directional microtubules growth towards the chromosomes. This is thought to "splash" chromosomes around creating the characteristic rosette of chromosomes (Magidson et al., 2011; Chaly and Brown, 1988; Mosgler et al., 1991; Nagele et al., 1995). Chromosomes are placed such that their spatially imposing arms are excluded toward cell periphery while centromeres, that have to be bound by microtubules, are pointing toward the cell center and aligned on a circular plane. This results in a toroidal arrangement of chromosomes that is thought to facilitate search and capture of kinetochores by microtubules. Indeed, in order to segregate one and only one copy of each chromosomes to the daughter cells, each chromosome has to be caught by microtubules emanating from only one of the centrosomes. Such non-random spatial localization of chromosomes has been in fact predicted by the search and capture theory (Kirschner and Mitchison, 1986; Wollman et al., 2005; O'Connell et al., 2009; Paul et al., 2009; Kapoor et al., 2006), reviewed in (Heald and Khodjakov, 2015). It takes into account that in order to expose all kinetochores to one and only one pole of microtubules, kinetochores have to be placed avoiding occulting one another. Computer modeling has shown that catching all chromosomes if they were randomly scattered through the cell would necessitate much longer mitosis than observed.

Such a neat organisation is costly in space. A complex cell shape with potentially unreachable nooks that could hide chromosomes will hinder reaching kinetochore capture by the spindle.

Cells therefore adapt their shape in order to provide the space required for spindle assembly and chromosome capture. Both of these have specific biochemical and mechanical properties that set a range of dimensions in which their functions can be fulfilled. The spherical shape offers the best volume to surface ratio. This also provides a smooth geometry without nooks that might hide chromosomes or constrain the spindle.

Such theoretical assumptions are indeed supported by recent works where cell rounding is biochemically and mechanically impeded (Lancaster et al., 2013; Tse et al., 2012). Obtaining a spherical shape is not sufficient for a successful division as showed by a study in spheroids where cells still round up but show mitotic defects (Desmaison et al., 2013). In particular the work of (Lancaster et al., 2013) shows how cell shape influences mitosis outcome. If minimal space requirements of the spindle and the metaphase plate are not satisfied, cells fail to build a functional spindle and rapidly catch all chromosomes. This results in cell death or mitotic mistakes such as multipolar divisions.

Rounding might therefore be a way to meet the spatial needs for organising the assembly of the mitotic spindle and chromosome segregation.

### Spindle and metaphase plate protection

In this section I will describe the metaphase plate and the spindle particular biochemical and mechanical properties that influence their geometries. In certain cases the shape of these structures can be adapted, but only to a certain point, and as the DNA and the spindle are precious and delicate they have to be handled carefully.

Microtubules are hollow tubes of 25  $\mu\text{m}$  with a stiffness (flexural rigidity  $EI \sim 10^{-24} \text{Nm}^2$ ) comparable to carbon nano-tubes. This makes them the stiffest components of the cell cytoskeleton. They are also dynamic structures that grow and shrink and their stiffness can be modulated by intrinsic, length and composition, or extrinsic factors such as binding proteins or drugs (Hawkins et al., 2010). Mitotic spindles are composed of 3 main groups of microtubules with proper characteristics and functions, k-fibres that link centrosomes to chromatids, spindle microtubules that localise in the central part of the spindle and astral microtubules that link the centrosomes with cell periphery. K-fibres are bundles of 20-30 microtubules linking in a stable and rigid manner chromosomes to the centrosomes. They enable force transmission necessary for chromosome positioning and separation (Helmke et al., 2013; Mogilner and Craig, 2010). Spindle microtubules that connect both poles without binding directly to chromosomes determine spindle length and resistance to tension (Mogilner and Craig, 2010). They also facilitate chromosome congression (Magidson et al., 2011). Finally, astral microtubules, emanating from centrosomes maintain the link of the spindle with the cell periphery, keeping the spindle under tension necessary for its functions and promoting its positioning in the cell (Mogilner and Craig, 2010).

The assembled spindle can adapt to various geometrical constrains. Some of them are the dimensions and shape of the metaphase plate with which it has to interact. Indeed, the amount of DNA and the number of chromosomes, as well as the dimensions and geometry of the metaphase plate influence spindle morphology (Dinarina et al., 2009; Dumont and Mitchison, 2009). However, the spindle has its optimal range of functional dimensions. For example, in *X. laevis* egg extract, correct spindles formation is observed if chromatin is disposed into circles of 14 to 18  $\mu\text{m}$  diameter (Dinarina et al., 2009). Smaller discs disturb spindle formation, bigger discs induce spindle poles splitting (Dinarina et al., 2009). The spindle can also be constrained by the dimensions of the cell. In this case it can adapt its overall geometry, the distance between the two centrosomes (the pole to pole distance), its height and width and the angles of microtubules nucleation at the centrosomes. It has been shown that the volume of the cell can itself be a parameter influencing the spindle size (Good et al., 2013; Hazel et al., 2013), but the cell geometry can be as important. When cells get confined and mitotic rounding is impeded spindles adapt to the imposed shape change (Dumont and Mitchison, 2009; Lancaster et al., 2013). If, as in (Lancaster et al., 2013) the confinement strongly impedes cell rounding spindle space requirements are unsatisfied. In confined HeLa cells spindle pole to pole distance can increase from 9  $\mu\text{m}$  to 15  $\mu\text{m}$ . As cells get flattened, the distance between centrosomes and the cell cortex also increase, resulting in the lengthening of astral microtubules (from roughly 4  $\mu\text{m}$  to 7  $\mu\text{m}$ ). Furthermore, as chromosomes get scattered in the flat cells, the spindle adapts by widening in order to bind all chromosomes. But microtubules have a limit length of about 15  $\mu\text{m}$ . Capturing chromosomes that are beyond this limit implies extending the range of reach of microtubules. This is obtained by the creation of a new, ectopic pole of microtubules. These difficulties increase with the amount of DNA to segregate as diploid Rpe1 cells will start to have troubles with spindle formation at 5  $\mu\text{m}$  confinement whereas HeLa cells harbouring around 80 chromosomes fail to form proper spindles as soon as confinement reaches 8  $\mu\text{m}$  (Lancaster et al., 2013) and unpublished data.

The space taken by chromosomes depends on the amount of DNA that constitutes the genome. In animal cells it can vary from 20Mbp in the nematode *Pratylenchus coffeae*, 3.2Gb in human cells but much more in polyploid cancer cell lines for example, and reach up to 130Gb in the fish *Protopterus aethiopicus*. With the amount of DNA the size of the metaphase plate shape vary, meaning that the more DNA a cell has, the more space it needs to handle it. This space may be limited by geometrical constraints of the cell shape. However, as the spindle, the metaphase plate also can adapt, to a certain extent, to such

constraints. In the study of (Lancaster et al., 2013) cell height is limited by uni-axial confinement between two surfaces. As the height available for the cell decreases the metaphase plate adapts, decreasing its height and increasing its width from roughly 15  $\mu\text{m}$  to 21  $\mu\text{m}$  in HeLa cells confined to 5  $\mu\text{m}$  height. If further confined, the metaphase plate eventually loses its cohesion, breaks and chromosomes get scattered over the cell. Such disposition of chromosomes strongly interfere with chromosomes catching by microtubules, lengthens the timing of mitosis and result in an increased number of corrupt divisions (Lancaster et al., 2013).

During division chromosomes and the spindle associate so that both properties of the metaphase plate and the spindle are linked. The mitotic form of chromatin, highly condensed and wrapped up into chromosomes has a Young's modulus of 0.21 kPa (Poirier et al., 2002) and the spindle Young's modulus is estimated to be 5 kPa in *Xenopus* egg extract (Itabashi et al., 2009) which makes them pretty stiff in the context of the cell (Brckner and Janshoff, 2015). Microtubules bind to the metaphase plate and develop forces sufficient to stretch and elongate condensed chromosomes (Civelekoglu-Scholey and Scholey, 2010). The cohesion of this structure depends on the properties of both major components but can also be modified by the environment. For example in *Drosophila*, the spindle is surrounded by a meshwork of large proteins (Johansen and Johansen, 2009) and in *Xenopus* an isotropic matrix containing lamins (Schweizer et al., 2014). Any change in the rigidity or disintegration of the complex of chromosomes with the spindle increase the duration of metaphase and the risk of aneuploidy which may be deleterious for the tissue or even the whole organism (Ding et al., 2009; Schweizer et al., 2014). The spindle is a big, imposing and quite rigid but yet fragile object that needs a lot of space to form and function. Adapting the internal space of the cell, removing dangerous angles and widening narrow passages which happens during rounding facilitates the manoeuvres. In a cell that has an imposed geometry the spindle assembles taking the maximal space available until it reaches its optimal size. The interaction of astral microtubules with cell boundary will then influence the overall position of the spindle in the cell. For example in cells dividing in long microchannels, spindles will position diagonally, so to interact on both sides with the cell cortex (Clotilde Cadart personal communication). Furthermore, spindles position in a range of optimal angles in reference to the interaction surface (Lzaro-Diguez et al., 2015). The spindle and the metaphase plate optimise their position to have less constraints (Fernandez et al., 2011). But the position of the spindle at the end of mitosis is crucial and its position is far from being left to hazard. Its position is therefore tightly regulated and cell shape plays a key role in this process.

In conclusion, metaphase plate and the spindle have typical sizes and properties that are important for their proper functioning. Their dimensions can be adapted to a certain point but trespassing threshold limits have dramatic consequences for the cell and potentially the whole organism. Above a certain threshold they reorganise, the metaphase plate breaks and the spindle splits, creating new poles.

A round shape provides space that is important for the fast congression of chromosomes into a toroidal metaphase plate, the formation of the spindle and a timely capture of all chromosomes to ensure an accurate segregation of the genome into the daughter cells. Perturbation of cell rounding first leads to lengthening of the metaphase, but when it comes to alterations in the structures of the spindle, the division is blocked and aborted in the best case or in the worst scenario continues and ends up with wrongly segregated DNA. The latest might be a tumorigenesis onset.

### Spindle positioning

The position of the spindle and the metaphase plate determines the localization of the cleavage furrow and thus the plane of division. The cell can position its spindle in space, guided by a plethora of internal and external cues and for various reasons. This positioning has to be robust and stable to avoid spindle damaging. The importance of this process is translated into an abundant literature describing mechanisms involved in this phenomenon (for review see (Gillies and Cabernard, 2011; Morin and Bellaïche, 2011)).



First, a precise positioning, is crucial for obtaining a basic symmetric division. This is further important for size homeostasis within the cell lineage. However in some cases, asymmetric divisions are also necessary, and dependent on a robust positioning of the spindle. At the tissue scale the orientation of divisions plays an important role in the homeostasis of the tissue, its expansion and growth, its regeneration, cell specification, morphogenesis and shape maintenance.

In morphogenesis in particular the orientation of the spindle can determine cell fate. First early in development, tissue growth is obtained by an intense multiplication of cells. Epithelial tissues, at the very beginning formed by monolayers of cells, require divisions in the plane of the tissue in order to grow. Such divisions maintain both daughter cells in the same tissue layer. Later in development it might be important to switch to a perpendicular spindle positioning in order to evacuate one of the daughter cells from the current tissue and induce differentiation (Haydar et al., 2003; Asami et al., 2011; Lizarraga et al., 2010; Mora-Bermúdez et al., 2014) Figure 1.6.

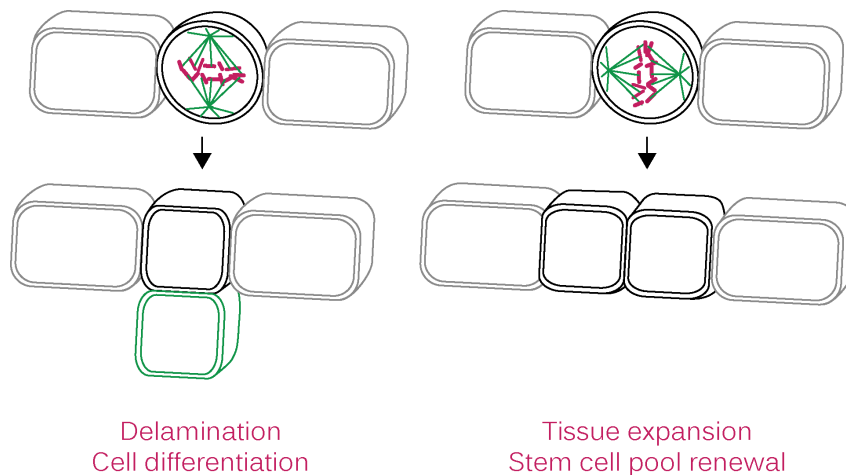


Figure 1.6: Sketch representing different outcomes of mitosis depending on the final positioning of the spindle and therefore the division plane. On the left the division plane is perpendicular to the tissue plane leading to the displacement of one of the daughter cells out of the tissue plane. On the right, after a division in the plane of the tissue, the two daughter cells stay in the same plane. The direction of spindle position can have important consequences for cell differentiation, survival and overall tissue structure.

The same principle stays important later, in developed tissues, to maintain the stem cells pool. In skin wound healing and repair the positioning of the spindle has been shown to be decisive in stem cell fate determination (Yang et al., 2015). If a division is symmetric or asymmetric depends on the division plane. The first leads to stem cell pool renewal as two new stem cells are generated, the second provide one stem and one differentiated cell (Yang et al., 2015). Along this line, the positioning of the spindle plays a pivotal role in the local distribution of biochemical cues, implicated in cell differentiation (Pearson and Bloom, 2004; Siller and Doe, 2009). And mutations in proteins playing a role in spindle positioning have been linked to disease, as it is the case for mutations in inversin that leads to renal cystic disease (Werner et al., 2013b).

Examples underlying the importance of spindle positioning are abundant and so it is for the mechanisms that have been implicated in this process. They will be shortly summarised here after, but with a particular importance given to those that depend on cell shape.

How the spindle is positioned is a balance of "passive" accommodation to available space and active sensing of mechanical and biochemical cues present in the environment and the cell itself.

The spindle is a massive object positioned in a non random manner in the crowded cell body, filled with the cytoplasm and macromolecular structures that have considerable visco-elastic properties (Schweizer

*et al.*, 2014). On the one hand it will tend to position following space availability in such a way that it will take the maximal space available both *in vitro* and *in vivo* (Fernandez *et al.*, 2011; Strauss *et al.*, 2006) taking into account its spindle geometry (Lzaro-Diguez *et al.*, 2015). On the other hand, it has to stay in contact with the cell periphery in order to maintain an internal tension and read polarity cues for an appropriate positioning. Even if cells round up and become spherical and thus symmetric during mitosis the positioning of the spindle is still tightly regulated. It can be determined in metaphase by intrinsic and extracellular cues and cues kept from before mitosis (Gibson *et al.*, 2011). The Hertwig's rule that predicts that the cell will divide perpendicularly to its longest axis is fulfilled in a lot of cases, but exceptions exist. For example sea urchin eggs in rectangular, elliptic or square wells divide along an axis dependent on the forces generated by astral microtubules interacting with cell cortex (Minc *et al.*, 2011). Spindle positioning is therefore taking into account both, polarity and geometrical cues. Intracellular conserved landmarks, such as polarity markers, influence the localization of molecular motors that pull and orient the spindle (Gillies and Cabernard, 2011). The localization of those internal cues is governed in *Drosophila* by a gradient of proteins forming the core planar cell polarity pathway (PCP) (Gillies and Cabernard, 2011). In mice their localization can depend on cell-cell interaction molecules (Lechler and Fuchs, 2005) linking internal cues with external requirements. Cells can also adapt to mechanical cues present during metaphase. An example are cultured cells that dynamically reorient their spindle according to forces transmitted by retraction fibres tethering the cell to the substrate (Fink *et al.*, 2011).

It is generally accepted that the principal way of moving around and positioning the spindle is pulling on them at the cortex (Kotak and Gönczy, 2013). Astral microtubules that are in proximity to the cortex can be bound by dynein, a molecular motor specialized in microtubule pulling. Dynein is distributed toward the cortex at mitosis and its precise localisation is refined by molecular cues such as Numa (Kotak and Gönczy, 2013). As each microtubule interacting with the cortex exert a pulling force on the whole spindle, the number and type of microtubules that interacts with the cortex will influence its positioning (Mora-Bermúdez *et al.*, 2014). Moreover it has been shown that the ERM proteins, specifically activated during cell rounding (Carreno *et al.*, 2008; Kunda *et al.*, 2008) are also important for spindle positioning (Machicoane *et al.*, 2014). The proximity of the cortex is therefore essential in establishing its interactions with microtubules. Furthermore the cortex has to be rigid enough to counteract and resist to pulling forces generated by the microtubules. There are evidences that both microtubules and the cortex regulate each others properties and cooperate to provide a mechanically stable entity (Théry *et al.*, 2005; Kaji *et al.*, 2008; Toyoshima *et al.*, 2007).

Not only the spindle with chromosomes is bulky and delicate, but it has to be moved quickly, precisely and in a compact environment. An optimised geometry, such as a sphere, provide at the same time space and keeps peripheral landmarks sufficiently close to guide the motion.

### **Tissue morphogenesis and homoeostasis**

In an organism cell division is a routine of an extraordinary importance. Cell division is as important for tissue morphogenesis as for maintaining its homoeostasis (Morin and Bellaïche, 2011; Gillies and Cabernard, 2011). As already mentioned in [section 1.2.2](#) cell rounding plays a role in the positioning of the spindle that in turn is important for various processes maintaining tissue homoeostasis. It is important for growth and morphogenesis in tissues during the development as well as regeneration and repair of already established tissues.

Cell division orientation can directly, dynamically and in a global way influence tissue morphogenesis. The active cell division axis positioning has been shown to be important as early as for gastrulation in embryos of shrimp (Hertzler and Clark, 1992), zebrafish (Concha and Adams, 1998; Gong *et al.*, 2004) or chicken (Wei and Mikawa, 2000). In early *Drosophila* embryos the rapid elongation of the germband is dependent on the alignment of cells divisions along the anteroposterior axis. Inhibition of cell divisions

at this time affects the elongation of the local tissue and the whole embryo (Silva and Vincent, 2007).

Later on in tissues, cell rounding due to its importance in chromosome segregation and spindle positioning has a key role in maintaining tissue homeostasis. How rounding may affect cell size distribution, tissue organisation and integrity is described in more details in (Cadart et al., 2014), annexed to the manuscript and in section 3.5.

## 1.3 Physical principles involved in Mitotic Cell Rounding

Cells have to deal with a large number of biochemical and mechanical stresses during their life time. Though there is growing number of known molecules involved in how mitotic cells might be able to round up, this process cannot be described without using physical notions. Indeed, cells changing their shape in tissues have to deal with mechanical constraints. In the following sections, elementary notions that can help describing mitotic cell rounding in tissues are briefly presented.

### 1.3.1 Osmosis and osmotic pressure in cells

Cells are made of molecules that interact, form polymers, gels and other structures in water. This implies using biochemistry, molecular biology, physics and maths to understand and describe it. Osmosis is the phenomenon of spontaneous movement of solvent molecules, from lower concentrations (hypotonic) to higher concentrations (hypertonic) of solute, through a semi-permeable membrane. As cells have a phospholipid membrane that is in some extent permeable to water, but poorly permeable to ions or other polar molecules, cells are subjected to osmosis. In order to maintain their functions, cells have to react to both, hyper- and hypoosmotic environments.

A plant cell, with its rigid cell wall, placed in a hypotonic solution, will not change shape much. It is the protoplast Figure 1.7 inside the wall that will increase its volume until it fills up all the space inside the cell wall. The volume increase is a result of water influx driven by the difference of concentrations between the internal and external media which in turn generate an osmotic pressure. As the protoplast fills up the space, the osmotic pressure is counteracted by the cell walls.

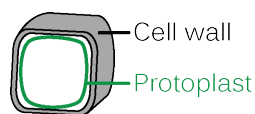


Figure 1.7: A cell plant where the protoplast (in green), the cytoplasm of the cell surrounded by a membrane, is enclosed by a rigid cell wall (in gray).

The osmotic pressure of a cell in a given medium is given by the Van't Hoff formula (1.1):

$$\Delta P = RT(C_b - C_a) \quad (1.1)$$

Where  $\Delta P$  stands for osmotic pressure difference,  $R$  is the gas constant,  $T$  the absolute temperature and  $C_a$  and  $C_b$  concentrations of osmolytes of the hypo- and hypertonic solutions. This formula is of course very general and has been refined to take into account the complexity of cells. It is nevertheless useful to relate the osmolytes concentration, this is the amount of internal osmolytes in the cell volume, to the osmotic pressure of the cell.

Because the cellular membrane is slightly permeable to some ions, even in an isotonic environment, cells have to actively pump out specific ions in order to maintain their internal number and avoid volume increase. Cells therefore constantly regulate and control, balancing passive and active mechanisms, the traffic of water and molecules through their plasma membrane.

In order to grow and modulate their shapes, plant cells (but also other organisms bearing a cell wall) have to modify the boundaries imposed by the wall. In order to achieve it, plant cells may increase their turgor pressure and locally modify the mechanical properties of their cell wall. The growth of a plant cell is therefore resulting from an interplay between the elasticity and extensibility of the wall (0.1-1  $\mu\text{m}$  thickness) and the internal osmotic pressure (Cosgrove, 2005). Plant cells can develop in this way impressive pressures in the range of 0.2 to 2.0 MPa (largely accepted values in the field). Tires in cars should be inflated to about 0.2 MPa.

Animal cells however are not surrounded by a wall as plant cells, and their membrane and cortex (properties detailed in subsection 1.3.2) cannot, by far, sustain forces as high as developed by plant cells. Nevertheless it is possible that animal cells use osmotic pressure to push against and deform their environment in order to maintain or change their shape (Charras et al., 2005; Moendarbary et al., 2013).

### 1.3.2 Mechanical properties involved in cell shape acquisition

There is a large number of physical models describing cell properties. These models vary a lot in the way they take into account the contribution of different cell structures. A cell can be described as a volume of liquid with particular visco-elastic properties containing a peripheral shell-like cortex tightly linked to a semipermeable membrane. Cytoplasm is described as visco-elastic, as its reaction will depend on the time-scale of the perturbation. Its reaction to slow deformation will be as of a viscous material, fast deformations of the cytoplasm result in a response of a material with elastic, solid-like properties. The cytoplasm is surrounded by a tensile actomyosin cortex, a mesh made of actin filaments and passive or actively contracting crosslinkers. This protein shell is tightly bound to the cell membrane via proteins interacting with both actin and the cell membrane. Due to the fact that it can be actively remodelled over short time-scales and contracted by a molecular motor, Myosin II, the cortex was seen as having a dominant role in cell shape acquisition. However a further refinement in cell description is to consider that the cytoplasm is a poroelastic material (Mitchison et al., 2008; Charras et al., 2009). This model consider that the cell can be described as a sponge, where the structure, made by the cytoskeleton and organelles is infiltrated by water and small solutes. Hydrodynamic forces can be therefore distributed in an inhomogeneous manner throughout the cell. This depends on the flow rate of intracellular water modulated by the local pore sizes of the structural mesh. This in turn may describe how cell shape changes arise, generated by local differences in pressure (Charras et al., 2005; Moendarbary et al., 2013).

It is now becoming evident that cell shape change is a result of an interplay between active cytoskeleton reorganisation and intracellular pressure regulation.

The cortex of cells in a steady state is under tension ( $\gamma$ ), which means that there is a certain amount of local stress that it is supporting. The measured cortical tensions in animal cells vary from 35  $\mu\text{N m}^{-1}$  in neutrophils (Evans and Yeung, 1989), around 400  $\mu\text{N m}^{-1}$  in mouse fibroblasts (Tinevez et al., 2009) to several  $\mu\text{N m}^{-1}$  in Dictyostelium (Schwarz et al., 2000)<sup>2</sup> (Clark and Paluch, 2011). This tension over the whole area of the cortex promotes a spherical cell shape to reduce the surface area. In a steady state the cortical tension is balanced by a hydrostatic pressure in the cytoplasm. The value of this hydrostatic pressure for a spherical cell is given by the Laplace law (1.2).

<sup>2</sup>It is several orders smaller than tension present in plant cell walls, as the lower range limit of pressures sustained by plant cell walls is 0.3 MPa, what in a round cell of 20  $\mu\text{m}$  radius would result in about 3  $\text{N m}^{-1}$

$$\Delta P = 2\gamma/R \quad (1.2)$$

If a spherical cell of a  $R = 10\mu\text{m}$  has a cortex under a  $500 \mu\text{N m}^{-1}$  tension, the hydrostatic pressure balanced by this tension is of  $100 \text{ Pa}$ <sup>3</sup>.

Cell boundary and therefore cell shape is defined by the localization of the cell membrane. The very compliant cell membrane (surface tension  $\sim 40 \text{ nN m}^{-1}$  in L929 cells (Tinevez et al., 2009)) is in turn bound to the actin cytoskeleton which determines cell shape. The cortex tension in L929 interphase cells is of  $\sim 450 \mu\text{N m}^{-1}$  (Tinevez et al., 2009).

The observed cell rounding at mitotic entry has been correlated with cell stiffening in sea urchin eggs (Mitchison and Swann, 1955; Yoneda and Dan, 1972) and *Xenopus laevis* eggs (Hara et al., 1980). In cultured cell lines, RhoA has been shown to induce an increase in the contractility and stiffness of the cortex (Matthews et al., 2012; Maddox and Burridge, 2003). Furthermore, Moesin promote cell stiffness increase by tethering the cortex to the membrane (Kunda et al., 2008). This cortical stiffening has also been shown in NRK cells, that round up very poorly in mitosis (Matzke et al., 2001). Recently works using Atomic Force Microscopy focused on measuring forces developed by cells during mitotic rounding and confirmed that the hydrostatic pressure and the cell cortex tension were increasing in mitosis (Stewart et al., 2011; Fischer-Friedrich et al., 2014; Ramanathan et al., 2015). In HeLa cells cortex tension increases sharply at mitotic cell rounding. In interphase it is  $\gamma \approx 200 \mu\text{N m}^{-1}$ , in early prophase  $\gamma \approx 400 \mu\text{N m}^{-1}$  and reaches its maximal tension at late metaphase (Ramanathan et al., 2015). If a mitotic HeLa cell is dynamically confined by a flat AFM cantilever, the measured transient tension is of  $\gamma \approx 2100 \mu\text{N m}^{-1}$  and it quickly stabilizes at  $\gamma \approx 1800 \mu\text{N m}^{-1}$  (Ramanathan et al., 2015). The tension in the cortex implies a tendency to minimize the surface area of the cell promoting a round shape if there are no mechanical impediments. This tension is increased by the contraction of the cortex mediated by molecular motors. This in turn is linked to an increase in the cellular pressure. The later has been measured to be  $P \approx 0 \text{ kPa}$  in interphase,  $P \approx 0.15 \text{ kPa}$  in early prometaphase and pic at  $P \approx 0.8 \text{ kPa}$  in late metaphase (Ramanathan et al., 2015). Increasing the cortical tension and internal pressure can result in an increase of the stress in the linkers tethering the membrane to the cortex. Above a certain threshold this stress leads to the detachment of the membrane (Alert et al., 2015) and a consequent drop in cell pressure (Tinevez et al., 2009). In interphase Dictyostelium, that has higher cortex tensions than human cells in culture, the critical pressure that induces membrane detachment is of  $500 \text{ kPa}$  (Merkel et al., 2000; Alert et al., 2015). In the absence of MyosinII, that critical pressure is of  $1500 \text{ kPa}$ . This is because this critical pressure (1.3) depends on two factors, the energy necessary to rupture all links ( $A$ ) and the cortex tension described by the Laplace law (1.2). After membrane detachment of the cortex cell shape is not guided by the cytoskeleton any more.

$$P^* = A - 2\gamma/R \quad (1.3)$$

Under confinement cell shape change implies a deformation of the environment. As shown in (Lancaster et al., 2013) cells under gels of  $15 \text{ kPa}$  rigidity are strongly flattened even when entering mitosis. In fact an increase of internal pressure of  $1 \text{ kPa}$  enables cells to deform the gel above of about  $1 \mu\text{m}$ . Under  $15 \text{ kPa}$  gels increasing cell height of  $1 \mu\text{m}$  is enough to provide the minimal space necessary for spindle assembly. However increasing gel over this threshold limit sharply increases the number of multipolar divisions due to insufficient cell height.

Additionally, in flat cells the curvature of the cortex is getting low, and as described by (1.3) this will lower the critical pressure needed to detach cell membrane from the cortex. In fact work of (Cattin et al., 2015) using Atomic Force Microscopy shows that mitotic cells start to bleb under a threshold cell height. Moreover both under gels and under the cantilever cells can expand in lateral directions, leading to an underestimation to the total pressure they can develop.

<sup>3</sup> $\Delta P = (2 * 500\mu\text{N m}^{-1})/10\mu\text{m} = 100\text{Pa}$

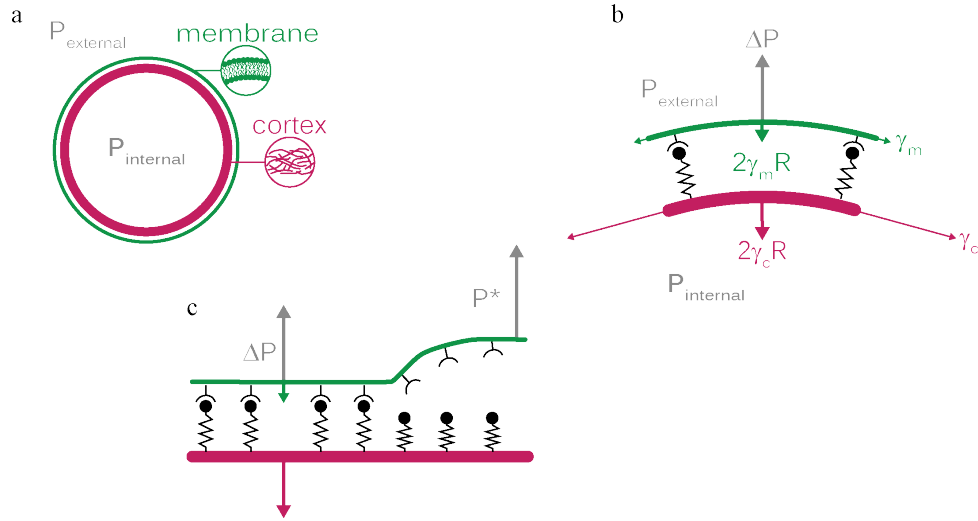


Figure 1.8: Sketch representing a simplified view of some of the forces present in cells that play a role in mitotic cell rounding. **a)** The plasma membrane is represented by a green line, the cortex by a pink one. **b)** Ligands between the membrane and the cortex are symbolized by springs. Forces in steady state are: internal and external pressures ( $P_{internal}$  and  $P_{external}$ ), membrane and cortical tensions ( $2\gamma_m R$  and  $2\gamma_c R$ ) that result in the inward directed forces ( $\gamma_m$  and  $\gamma_c$ ) that counteract the force generated by the difference of pressures ( $\Delta P$ ). **c)** If the force applied on the membrane is higher than the total amount of forces present in the ligands tethering the membrane to the cortex, the membrane detaches. Adapted from (Alert et al., 2015)

A stiff and homogeneous cortex is definitely important for the acquisition of a spherical shape under anisotropic confinement, as treating cells with drugs disturbing cortex organisation impedes gel deformation or cantilever counteraction. The mechanics behind cell rounding under confinement are however not yet fully understood. Preliminary data on molecular motors important in mitotic cell rounding are presented in the next section. Next, we ask if cells in tissues could potentially deform much stiffer environments using osmotic pressures.

## 1.4 Molecular mechanisms involved in Mitotic Cell Rounding

Even if the phenomenon of mitotic cell rounding has been observed a long time ago and in an era of general interdisciplinarity in science, it has been poorly studied. It appears today that understanding mitotic cell rounding *in vivo* needs combined biochemical and physical approaches and expertises. To do so it seems important to place cells in a context of confinement where forces developed by cells can be visualised and measured. Nevertheless it wasn't the predominant approach by now and acquired information on molecular mechanisms rarely take into account forces generation in this process. Thus several biochemical cues implicated in mitotic cell rounding are already known, they are rarely validated in a context mimicking mechanical constraints present in tissues.

Mitotic cell rounding happens in a complex environment and is critical for tissue homeostasis. It can be therefore predicted that mechanisms regulating it will be tightly controlled, redundant and will necessitate the coordination of all cell structures.

As cells round up at a particular time in the cell cycle, it seems logical that this will be regulated by Cyclins and Cyclin-Dependent Kinases (CDKs). In particular the main event promoting mitotic entry is the nuclear localisation of the complex of the active form of CDK1 with B-type Cyclins (Rhind and Russell, 2012). How the activity of this complex is regulated is a largely studied problem with no clear

answers yet. What is important for this introduction is that this complex regulates hundreds of proteins (Rhind and Russell, 2012) important for mitotic progression and among them those important for cell shape changes.

As it is often suggested cell cytoskeleton is seen as the backbone structure of the cell. In this context it seems logical to focus on its role during the MCR. The cell disposes of actin filaments, strong, flexible and dynamic structures that can sustain varying kinds of strain forces depending on the way they are assembled and organised. Microtubules are as dynamic as actin filaments but can organise into structures of higher rigidity but weaker pulling forces and high compression resistance. The third type of structures in the cell cytoskeleton are intermediate filaments, somehow in the shade of actin filaments and microtubules, which are starting only now to be studied. They are flexible and elastic, rather stable, tension bearing and help in maintaining the organelles and the nuclear lamina. At last, there are septins, claimed to be the fourth type of cell cytoskeleton, that could also take part in cell shape changing. A tightly orchestrated reorganisation of the cytoskeleton will be of primordial importance for any change of cell shape.

The external boundary of the cell, the membrane is strongly reorganised during mitosis. Not only there is a drastic diminution of surface happening at rounding of a spread cell (about 30%) but the membrane folding, composition and its biochemical and mechanical properties undergo a drastic change during mitosis.

As the membrane itself is not mechanically resistant enough to restrain the cell to a certain shape. Proteins from the Ezrin-Radixin-Moesin family are pinning cell membrane to the cytoskeleton and have fundamental importance for shape maintenance. These proteins can also regulate the organisation of the cytoskeleton.

The ion channels regulating cell volume are predicted, to have a potent role in any cell shape adaptation and as it will be discussed later, a very particular one in MCR. In this line it is possible that the biochemical properties of the nucleoplasm and the DNA could have a role to play as they become exposed to the cytoplasm after the NEB.

Such a rapid and extended change of shape and internal organisation cannot be done without a secure and robust supply of energy, which point out toward a potential role of mitochondria, themselves being strongly reorganised in mitosis.

And at last but not least, how the cell reacts, copes, interacts with its environment and the properties of the environment itself, how it is bound to other cells or tissue structures will impact cell rounding at mitosis onset.

Something would not be important for mitotic cell rounding?

In the following section the major molecular pathways known to be implicated in mitotic cell rounding will be presented in more details.

### 1.4.1 Rounding up without confinement

#### Actin needs to be remodelled

Actin is the most studied cell component in the context of mitotic cell rounding. The predominant interest in actin is not surprising, as it undergoes a drastic remodelling at mitotic entry. In adherent interphase cells, actin filaments can be associated into thick cables crossing the whole cell called stress fibres or shorter filaments creating meshes in the cell body or at the cell cortex. At mitotic entry in a very short lapse of time this heterogeneous network of filaments is reorganised into a homogeneous

and tight mesh right beneath the cell membrane (Ramanathan et al., 2015). As already mentioned in subsection 1.3.2 this reorganisation result in the increase in cortex tension in mitosis.

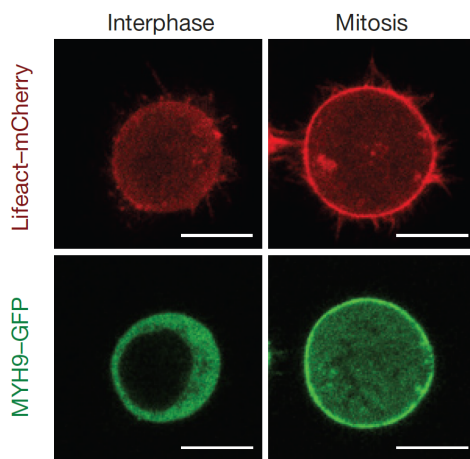


Figure 1.9: Trypsinized interphase cells (**left panels**) and mitotic cells (**right panels**) stained for actin (**upper panels**) and myosin (**bottom panels**) (Ramanathan et al., 2015). The organisation of actin and myosin is strikingly different in rounded interphase cells and cells in metaphase.

As the cell rounding starts, the existing actin organisation has to be completely remodelled and it implies an important de-polymerisation of existing structures. Consequently, cultured or *in vivo* cells present defects in MCR if actin severing is affected (Fujibuchi et al., 2005; Luxenburg et al., 2015b).

Adherent cells in tissue culture, if no mechanical constrains are applied, can round up provided they can detach from the substrate and reorganise actin filaments to retract their margin (Fujibuchi et al., 2005; Lancaster et al., 2013). Rap1 transient deactivation at mitotic entry leads to the release of focal adhesions, structures where actin interacts with integrins to anchor the cell to the substrate. Cells with constitutively active Rap1 do not detach from the substrate and do not round up at mitosis entry (Dao et al., 2009; Lancaster et al., 2013). A similar phenotype is observed in cells with knocked down Inversin as cells fail to disassemble their focal adhesion and consequently reorganize their actin cytoskeleton. This result in asymmetric and flattened metaphase cells (Werner et al., 2013b). In the same line, Trask, a substrate of src kinase relocates from membrane to the cytosol at mitosis promoting detachment. Its overexpression leads to cell rounding (Bhatt et al., 2005).

After this depolymerisation wave of actin filaments, a spherical cortex beneath the membrane has to be rebuilt, it is therefore necessary to bring back actin polymerisation. Hence, Eps8, an actin capping protein is locally degraded at the cell cortex and its persistence induces a delay in cell rounding (Werner et al., 2013a). The activity of cofilin, another actin severing protein, also has been reported to be shut down in mitosis (Kaji et al., 2008).

Following deadhesion, the rebuild cortex has also to be actively retracted, a process facilitated by RhoA activity as suggested by (O'Connell et al., 1999) and demonstrated in (Maddox and Burridge, 2003; Matthews et al., 2012). Mitotic cell rounding is perturbed in cells where RhoA activity is inhibited. Furthermore if RhoA activity is down-regulated those cells fail to assemble a stiff cortex and are softer than control cells. (Maddox and Burridge, 2003; Matthews et al., 2012)

Rho-kinase, one of downstream effectors of RhoA acts on actin cytoskeleton organisation through the activating phosphorylation of myosin light chain (MLC) (Kimura et al., 1996) promoting myosin driven contractility of the actin cytoskeleton. Rho-kinase also enhances interactions of the actomyosin cortex with cell membrane at cell-cell junctions (Kimura et al., 1998) and inhibits actin filaments severing (Maekawa et al., 1999).



The cytoplasmic fraction of actomyosin also may play a role in cell rounding. The bulk actin fraction of the *Xenopus* egg extract present an increase in gelation and contraction in mitosis (Field et al., 2011).

An intriguing example of affected MCR, are cells undergoing mitosis with unreplicated genomes (MUG). Those have a perturbed ranGTP gradient and do not round up at mitosis (O'Connell et al., 2009), but the mechanism behind it is unknown.

Further more the overall lengthening and bundling of actin filaments might facilitate cell rounding as actin filaments are bound to the membrane. This in turn could modulate their compliance and decrease the curvature of the membrane promoting cell rounding (Théry and Bornens, 2008). This points towards the importance of a tight link between cell acto-myosin cortex and cell membrane.

In some cases mitotic cells round up even if the acto-myosin cortex is highly disorganised. This is the case in cells where actin organisers Arp2/3 and mDia1 or the chaperone complex BAG3-HSPB8 activity are perturbed (Bovellan et al., 2014; Fuchs et al., 2015). Treating cells with actin polymerisation inhibitors such as Cytochalasin D or Latrunculin A does not prevent mitotic cells from rounding as long as they are not under confinement (Lancaster et al., 2013). It is therefore important to test the above mentioned molecular mechanisms in a context mimicking cell confinement in tissues.

### **A tight link between the acto-myosin cortex and cell membrane needs to be maintained**

Before even describing what is known about the importance of a tight link between acto-myosin cortex and the cell membrane, it has to be pointed out that the membrane itself undergoes profound changes at cell division.

The change in geometry happening in an adherent cell that round up implies membrane reorganisation as the surface to volume ratio decreases sharply. A rounded cell will have a much smaller surface area than a spread cell of the same volume. Indeed membrane characteristics are important and drastically altered during mitosis (Cauvin and Echard, 2015; Atilla-Gokcumen et al., 2014).

First, the basic composition in lipids that regulate signalling and mechanical properties of the membrane (van Meer et al., 2008) is altered in mitosis (Atilla-Gokcumen et al., 2014). This change in lipid composition influences mechanical properties of the cell membrane which becomes stiffer at cell division (Atilla-Gokcumen et al., 2014). Components of the membrane, in particular phosphoinositides play a role in signalling regulating mitotic entry and actin polymerisation (Campa et al., 2015; Atilla-Gokcumen et al., 2014; Echard, 2012).

Proteins known for having a role in membrane trafficking in interphase appear to have functions also in mitosis (Royle, 2011). Membrane trafficking, how cell membrane is internalized and restored during cellular processes, plays a role in membrane tension and shape regulation. It seems that endocytosis is not slowed down, but is less important due to reduced cell surface of the rounded mitotic cell (Tacheva-Grigorova et al., 2013), and cells where endocytosis is impeded fail to round up at mitotic entry (Boucrot and Kirchhausen, 2007). In contrary, exocytosis appears to be slowed down in mitosis and could facilitate cell surface reduction (Boucrot and Kirchhausen, 2007). On the other hand an induced volume increase up to 200% of cells in 50% hypotonic media is not affected by blocking exocytosis, meaning that cells have large and easily accessible membrane reservoirs (Groulx et al., 2007).

This points out that the spatial organisation of the membrane is also altered at MCR (Figard and Sokac, 2014). And in fact interphase cells have a smoother surface whereas round mitotic cells have a rough surface with a lot of villi, spikes, protrusions and wrinkles Figure 1.10 (Porter et al., 1973; Erickson and Trinkaus, 1976).

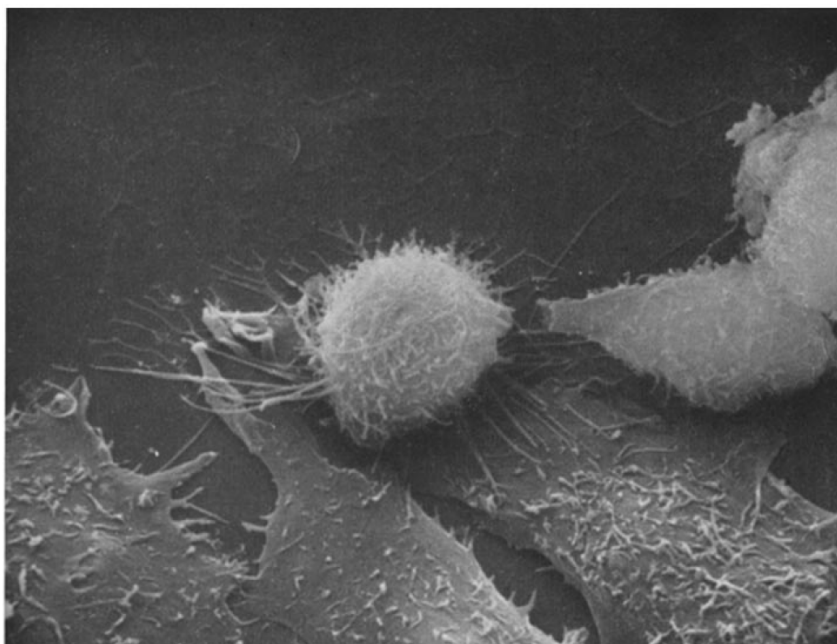


Figure 1.10: This image shows how different is the surface of spread, interphase cells and rounded mitotic cells. Interphase and mitotic Chinese Hamster cells imaged by scanning electron microscopy (Porter et al., 1973).

Even if the membrane thickness is only of 30 Å, the membrane bears a crucial role in most cellular processes. This was not always the point of view of scientists as in 1860's the cell membrane was seen as unnecessary by some of the greatest biologists of that times. Through the cell cycle and for any shape change, the interaction of the acto-myosin cortex with the membrane is crucial. In fact it is rather difficult to imagine how would look a spread cell with stress fibres and the cortex without the membrane pinned to it. It is thus not surprising that molecules involved in the linking of the membrane to the cortex play an important role in mitotic cell rounding.

One of the main players involved in this process is the highly conserved Ezrin-Radixin-Moesin (ERM) proteins family, that crosslinks actin filaments to the plasma membrane proteins (Bretscher et al., 2002). Interestingly ERMs are absent in plants and fungi that possess cell walls. In *Drosophila* this family is represented by one protein, Moesin. Its activation requires phosphoinositides (Cauvin and Echard, 2015), accumulated in mitosis (Atilla-Gokcumen et al., 2014), and phosphorylation by Slik (SLK in humans) (Carreno et al., 2008). Adherent *Drosophila* cells are less rigid and do not round up at mitotic entry when depleted of ERMs or SLK, moreover the artificial activation of Moesin leads to rounding of interphase cells (Carreno et al., 2008; Kunda et al., 2008). Activation of Moesin in cells lacking Myosin II activity led to cell rounding, providing an additional argument for the non-essential role of MyoII in MCR. Mitotic cells mutant for Moesin in (Kunda et al., 2008) are particularly flat and with a relaxed cortex, but these cells acquire even more far-fetched shapes later in metaphase. In both (Kunda et al., 2008) and (Carreno et al., 2008) the localisation of cell contractility actors as actin, myosin and RhoA is inhomogeneous. It is therefore pointing at the importance of ERM proteins for assembling a stiff and homogeneous cortex for shape acquisition and maintenance.

### Microtubules and intermediate filaments have their word to say

Tubulin is phosphorylated and microtubules are largely reorganised at mitotic onset (Piras and Piras, 1975). Microtubules might also be involved in cell rounding as cells depleted in PSKs, kinases regulating MTs dynamics that are activated in mitosis, round up less efficiently (Wojtala et al., 2011). PSKs can be

involved in the interaction between microtubules and actin and it is in line with the proposal of (Kiyomitsu and Cheeseman, 2012) that cortical dynein mediates MTs interactions with the cell membrane putting the spindle under tension. Microtubules also interact with Moesin that is implicated in cell rounding at mitosis onset (Solinet et al., 2013). Overall, the spindle rigidity due to its intrinsic properties (described in more details in section 1.2.2) and its interaction via astral microtubules with the cortex and the membrane can generate tension that could facilitate the generation and maintenance of a spherical shape.

The role of intermediate filaments in MCR is poorly studied by now, but it has been reported that vimentin stiffens the actin cortex (Guo et al., 2013) and that keratin filaments are disrupted during mitosis (Lane et al., 1982).

As septins gain in interest, it seems that they could also have a role in cell rounding. Septins among other functions can regulate the interplay between actin cytoskeleton and membrane in cell shape change. They are implicated in bleb retraction, cortical contraction and assemble on a damaged and unstable cortex (Tooley et al., 2009; Gilden et al., 2012). Moreover human lymphocytes T depleted of Septin7 seem less round and rigid in mitosis and are less efficient in their volume regulation upon a hypo-osmotic shock (Gilden et al., 2012).

### 1.4.2 Rounding up under confinement

The situation is indeed very different when cells are in a mechanically demanding environment such as tissues or tissue mimicking materials. One of the most obvious difference is that in order to maintain tissue integrity, mitotic cells cannot entirely detach from their substrates. Next, as cells in tissues are subjected to mechanic stresses due to the presence of other cells and tissues components, cell detachment would not be enough to acquire a round shape as in culture conditions.

#### Once remodelled, actin needs to provide a stiff cortex

As discussed in section 1.3, in conditions where cells have to generate forces in order to acquire a given shape, building a stiff cortex is crucial. Indeed, cells bearing an insufficient or weakened actin cortex at mitosis fail to round up under confinement (Luxenburg et al., 2011; Lancaster et al., 2013). In the article of (Lancaster et al., 2013) I showed as a part of preliminary results for this PhD project, that HeLa cells need a strong actin cortex and in particular actin polymerisation to round up, but only under confinement. Cells treated with Cytochalasin D or Latrunculin A, both perturbing actin polymerisation, round up and over-go mostly bipolar divisions in control conditions. Cells not treated with those drugs but under a soft gel (15 kPa) divided mostly into two cells. Successful bipolar spindle formation in these conditions is due to sufficient space generation through mitotic rounding. The particular stiffness of 15 kPa is a threshold for HeLa cells as cells confined with stiffer gels fail to round up. These cells do not generate enough space for spindle assembly which leads to multipolar divisions. In contrast, cells that have weakened actin cortex by these drugs and are under soft confinement undergo multipolar divisions or fail to divide. This shows that assembling a robust and stiff actin cortex is necessary for rounding up under confinement.

In this line, (Luxenburg et al., 2011) previously showed that depletion of Srf, a transcription factor involved in actin contractility regulation, leads to mitotic rounding defects in mouse skin (Figure 1.4). This work shows that the deleterious effects on skin morphogenesis of knocking down Srf were caused by weaker cell cortex at mitosis and consequent misalignments of the spindle in respect to the tissue geometry. Recently (Luxenburg et al., 2015a) showed that actin remodelling by Wdr1 and cofilin is essential for the assembly and maintenance of a rigid cortex.

Proofs of the importance of cell cortex rigidity for mitotic rounding *in vivo* were also obtained in the

*Drosophila notum*, a dorsal epithelial layer in the developing fly. Downstream effectors of Ect2, previously implicated in RhoA activity regulation (Matthews et al., 2012), have been shown to have a role in mitotic rounding in tissues (Rosa et al., 2015). In this work authors show that cells reorganise their acto-myosin into an isotropic and stiff cortex upon nuclear release of Ect2 into the cytoplasm at the nuclear envelope breakdown. In the *Drosophila notum*, cells expressing a dominant negative mutant of Rho do not achieve round shape in mitosis. This study further identify Ect2 as a key player of the acto-myosin cortex reorganisation, that activates Rho pathway to increase MyosinII contractility and the Cdc42/aPKC/Par6 complex regulating the localization of Dia, involved in actin bundling.

Recently (Ramanathan et al., 2015) confirmed the master role of CDK1 in regulating the assembly of a stiff acto-myosin cortex and maintaining cortex rigidity in mitosis. Authors use uni-axial confinement of mitotic cells in order to study the regulatory pathways and mechanical properties of the mitotic cortex. First they unambiguously show that actin and Myosin II are enriched in mitotic cells. Then the role of an unperturbed actin cortex for resisting deformation is confirmed as in (Lancaster et al., 2013). Authors also confirm the importance of RhoA regulation of the mitotic cell rigidity, as already shown in (Matthews et al., 2012; Maddox and Burridge, 2003). In line with (Rosa et al., 2015) they demonstrate that RhoA downstream effector DIAPH1 but not Arp2/3 is necessary for the assembly of a cortex resistant to deformation. The overall conclusion is that the backbone structure important in resisting to deformation is a bundled actin cortex and that MyosinII actin-crosslinking and contractility functions are important for long term shape maintenance.

Works of (Ramanathan et al., 2015) and (Stewart et al., 2011) bring insightful measurements of tension and pressure carried by the cell cortex. In these experiments however, cells are never isotropically confined. As mentioned in subsection 1.3.2 it is possible that forces measured in such conditions are underestimated. If cells are generating additional forces through increasing their osmotic pressure for example, unconfined cells would increase their volumes. It is however not trivial to measure volume changes in cells, and in particular when cells drastically change their shape.

## 1.5 Does cell volume change during mitosis?

Volume is an important characteristic for every cell and is therefore extremely well regulated. Mechanisms regulating cell volume are intensively studied and constitute an extremely large and dynamic field. Therefore a large quantity of information is available on how cells maintain, increase or decrease their volumes adapting their volume to changes happening in their environment. However little is known on if cells change their volume in mitosis. This is an intriguing question as any change in cell volume might point out to mechanisms regulating cell division. As already mentioned, mitotic cells need a certain amount of space in order to divide correctly. Increasing volume might provide additional space for division. However, cells are small and complex objects, refractive to light. They constantly move and change their shapes, are sensitive to chemicals, strong illumination and mechanical manipulation. This makes measuring live cell volumes during mitosis challenging.

**Measuring cell volume through mitosis is technically challenging.** Several approaches can be used to measure cell volume changes that cannot be used in cells quickly changing their shape. The first set of methods measures water fluxes that change the concentration of a given molecule in the cell. Cells are loaded with molecules that cannot cross the plasma membrane and the concentration of the molecule is measured by patch-clamp (Alvarez-Leefmans et al., 1992) or in fluorescence (Crowe et al., 1995). The first option is harsh for cells and the second necessitate important single cell calibration and can be biased by cell shape change. Scanning ion conductance microscopy (SICM) (Korchev et al., 2000) can be used to reconstruct cell shape and therefore calculate cell volume. The drawback here is that it is

restricted to adherent and still cells as the method is slow. Measurements of cell diameter using forward scatter (FSC) is a high through-output method that can be used to discriminate cells based on their size. But obtained measures depend on cell refractive index that may be different for different cell types and on the used apparatus and is limited to cells in suspension (Tzur et al., 2011). The method that is often taken as a gold standard is based on the Coulter counter. In this method the cell, suspended in a liquid, when passing through an aperture excludes a volume of this liquid equal to cell volume. The electrical conductivity of the aperture is monitored and enables calculating the volume of the excluded medium from the variation in the conductivity (Bryan et al., 2012). In this accurate method a single cell can be monitored over a long period of time. The method is cell shape independent, but as it is limited to cells growing in suspension, cells are mostly round.

Recently reconstructing cell shape using high speed and precision imaging of cell periphery (Boucrot and Kirchhausen, 2008) has also been used to reconstruct cell volume. However, staining the membrane implies the reconstruction of all dense, dynamic and highly curved membrane structures that can account for a considerable amount of volume. This can be tedious even for a fixed object but is highly hampered if the measured object moves or changes its shape. In particular, such reconstructions might highly overestimate the volume of very thin structures such as lamellipodia or thin protrusions well visible in fluorescence but bearing very small volumes. Additionally, any error in the z resolution may greatly influence measures of cells, particularly in the situation where the measured cell present an important change in height. Furthermore the illumination intensity that is improving image resolution is limited as illuminating cells implies photo-toxicity which in turn may perturb cell shape and volume.

Yet another method recently adapted to measure single cell volume over time is the dual suspended microchannel resonator (SMR). In this method the passing cell is changing the resonant frequency of the resonator in a density-dependent manner, which yields the buoyant mass of the cell. A second passage near the resonator of the same cell but suspended in a liquid of a different density gives the volume of the cell (Bryan et al., 2014). Volumes obtained with this method are very precise and shape independent but again are limited to cells in suspension.

**Measurements of volume changes during mitosis are sparse.** Those technical issues result in a pretty poor understanding and description of cell volume changes in particular over time and linked with shape changes. This is why measures of cell volume evolution through mitosis were sparse. The two earliest works that measure cell volume changes through mitosis are (Cameron and Prescott, 1961) and (Abrahamsohn and Sesso, 1983). The first one confines the protozoa *Tetrahymena pyriformis* between two glass coverslips and observes a volume increase of about 30%, based on the surface change of cells in division. The work of (Abrahamsohn and Sesso, 1983), is the only one that I know that measures mitotic cell volume in tissues. Authors use 0.5  $\mu\text{m}$  sections of rat pancreas to reconstruct volumes of acinar cells. Cell volume measurements are done in an indirect way by projecting the image of the section on a paper and cutting out cells based on their well defined contours, section by section. All pieces of paper representing one cell are then carefully manipulated and weighted, giving the volume of the cell in mg [Figure 1.11](#). Authors using this method are able to show that cells increase their volumes of about 30% from late interphase to late metaphase.

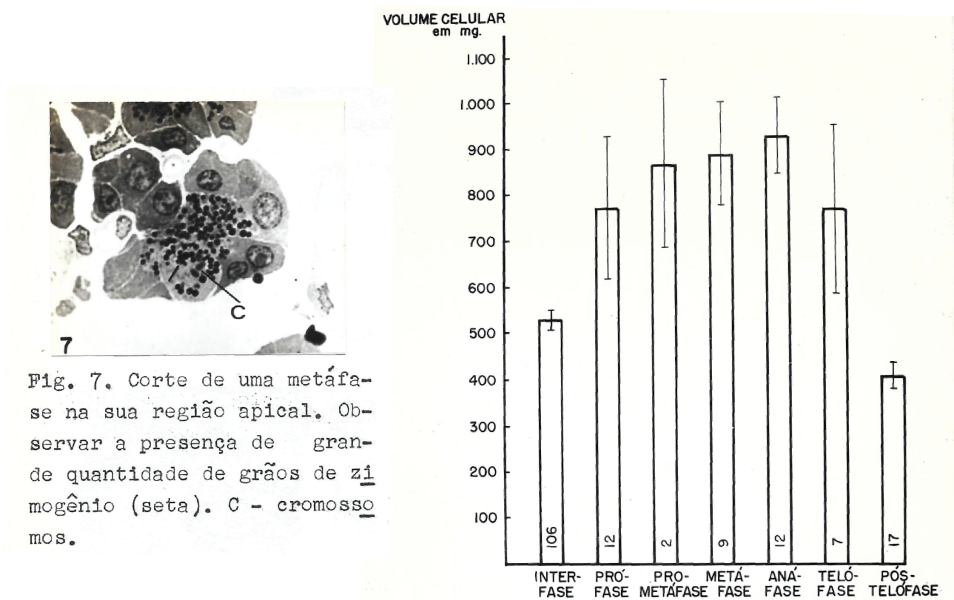


Fig. 7. Corte de uma metáfase na sua região apical. Observar a presença de grande quantidade de grãos de zimogênio (seta). C - cromossomos.

Figure 1.11: Figures from the doctoral thesis of Professor Paulo Abrahamsohn that served in the article (Abrahamsohn and Sesso, 1983). The **left panel** shows a photography of a section of a rat pancreas, where one of the acinar cells is in metaphase. Its contours are smooth and its shape is rounded and more regular than the shape of neighbouring, interphase cells. The **right panel** represent the volume of cells at various cell cycle phases and in mg. (Courtesy of P. A. Abrahamsohn)

In the works of (Boucrot and Kirchhausen, 2008; Habela and Sontheimer, 2007; Huang et al., 2012) an attempt to measure cell volume over mitotic cell rounding is done using confocal imaging and 3D reconstruction. Due to the complexity of the approach only few cells and few time points are taken. Authors observe a decrease of cell volume in metaphase of about 20-30 %. In these approaches the labelling of the membrane gives a very rough estimation of its localisation and therefore a large error in cell volume. For a spherical cell of a diameter of 18  $\mu\text{m}$ , an error of 1  $\mu\text{m}$  in the estimation of the diameter will result in an over estimation of cell volume of 18% or an under estimation of 16%. The error is even more important if the volume to surface ratio of the cell shape is smaller in particular at structures as lamellipodia that have a thickness of around 200 nm.

The only recent study that shows that cells might swell in mitosis is the work of (Fischer-Friedrich et al., 2014). In their approach cells are confined and cell volume is calculated from 3D reconstructions of confocal stacks of cells with fluorescent markers associated to the membrane. The graph representing volume of cells in mitosis shows a slight increase of cell volume of about 10% but it is at the edge of resolution of their measurement and authors interpret their result as not significant.

To date no methods enabled precise measurements of cell volume through mitosis. As for the question of force generation during mitotic cell rounding raised, the idea that cells could use osmosis to regulate their internal pressure raised the need of a shape independent, non invasive method that could assess cell volume changes over time. Two independent methods enabling this have recently been developed and published back to back. These two work show that several cell types can swell in mitosis (Son et al., 2015; Zlotek-Zlotkiewicz et al., 2015). The first one uses an optimised dual SMR already mentioned to track cell volume of two cell types growing in suspension over time (Son et al., 2015). The second we develop a method based on fluorescent dye exclusion (FXm) where volumes of suspended or adherent cells can be tracked over long periods of time and with high temporal resolution (Zlotek-Zlotkiewicz et al., 2015). We show that nine different cell lines transiently increase their volume at mitotic cell rounding, that this process is shape change independent, associated with mitotic entry and independent of the acto-myosin cortex. It is further detailed in the results part of this manuscript.

Both works show a transient volume increase of 10 to 30% at mitotic entry pointing toward the possibility that volume regulation during mitosis has an important role to play. This role could be to change internal concentrations of molecules, regulating cell signalling or could be a way of generating forces at mitotic cell rounding. In the next part I will focus the second possibility.

## 1.6 Hypothesis of volume increase as force generator in mitosis

When part of a tissue, a rounding cell has to change not only its own shape but also deform surrounding cells or tissue structures. As every tissue has its proper functions it also has adapted structural, biochemical and mechanical properties [Figure 1.12](#). It is important in biochemical and mechanical approaches to consider this environment in order to understand cellular processes.

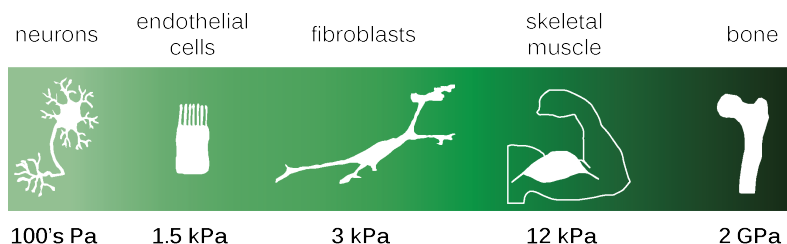


Figure 1.12: Scheme representing approximative rigidities of different cells and tissues in a human body. (Adapted from ([Butcher et al., 2009](#)))

The predominant regulator of cell shape is traditionally the actomyosin cytoskeleton. Cells deprived of MyoII activity or actin can still acquire a round shape if unconfined. In fact it is not that surprising, as trypsinized cells also round up. Rounding *per se* might therefore not be that important. Instead, how cells generate forces to deform their environment enough to round up is generally poorly studied and by now neither biochemical nor mechanical considerations can fully explain this phenomenon. As earlier described cytoskeleton can bear tensions that can counteract not more than 1 kPa ([section 1.3](#)). Forces generated by mitotic cells under uniaxial confinement are of about 70 to 90 nN ([Stewart, 2012](#); [Fischer-Friedrich et al., 2014](#)) and ([Ramanathan et al., 2015](#)). These forces are the result of an increase of internal cell pressure and cortex tension, respectively by 150 and 800 Pa and roughly  $1 \text{ mN m}^{-1}$ . These values can explain how cells confined under 15 kPa gels can deform the gel surface in order to increase their height of 1  $\mu\text{m}$ . But cells might need to deform surrounding tissues of more than that when they change their shapes embedded in tissue [Figure 1.4](#). Gels can also, as the atomic force microscopy set-up, be treated as an uniaxial confinement as they are not bound to the surface of the coverslips and cells can potentially expand on the side. In this case, the forces applied on the cantilever or the gel surface would not be the total forces generated by cells. This in turn would mean that measured in this way forces are underestimated and that cells could in principle develop higher forces if they were confined in all directions. An appealing hypothesis is that cells could regulate their osmolality in order to generate osmotic pressures that would be high enough to deform their cytoskeleton and their environment.

As already mentioned, cells tightly regulate their volume, which is necessary to maintain their homeostasis. Volume regulation is obtained by a complex interplay of the activity of a vast panel of ion channels at the cell surface and the regulation of internal osmolytes. Ion channels play roles in nearly any process happening in a cell and have been linked to cytoskeleton dynamics regulation, cell migration or cell cycle and proliferation regulation. The mis-regulation of ion channels activity or expression is related to a vast number of pathologies called channelopathies ([Kass, 2005](#); [Lieve and Wilde, 2015](#)), including cancer development ([Huang and Jan, 2014](#)). This points towards their importance in tissue homeostasis. Proteins forming ion channels are in general conserved and have redundant functions, which together with

their omni-presence renders studying and understanding them particularly challenging.

Though, their role in global or local cell volume regulation is widely appreciated, the literature regarding them as potential force generators is pretty marginal. How cells react to hyper- or hypotonic solutions is a dynamic field and the literature is particularly abundant (for a review on cell volume regulation of cell proliferation see (Lang et al., 2000)). The role of ion channels in cell rounding has already been observed, as (Wondergem et al., 2001) propose that cell rounding may be due to osmotic swelling. Authors of the study observe that mitotic, and not interphase cells, present a specific efflux of  $\text{Cl}^-$  which is known as a compensatory mechanism activated at cell swelling in hypotonic medium. Interfering with this compensatory mechanism, or placing cells in hypertonic media, inhibits cell proliferation. In fact, placing cells in 10 % hypertonic solution, doesn't affect the G2/M transition but arrests HeLa cells in metaphase and has been described as a synchronization method (Wheatley, 1974). The importance of volume regulation in animal mitotic cells is known since 1952, when (Hughes, 1952) observed that mitotic cells can deal with about 20 % changes in media osmolarity. Above these 20% cells and their spindle swell if placed in hypotonic media which result in scattering of chromosomes, and have disorganised spindles in hypotonic media (Hughes, 1952).

This need for compensatory mechanisms leading to water efflux of the cell could be activated by an increase of intracellular pressure. This increase of pressure might be created by the actomyosin cortex, but could also result from ion uptake or intracellular osmolytes regulation that would lead to water influx and therefore volume increase. Such a volume change would in turn result in cell density decrease, as dry mass production is much slower than volume regulation. Indeed it has been already observed that mitotic HeLa and Hep-2 cells can be isolated by density gradient centrifugation due to their smaller density (Wolff and Pertoft, 1972). Whereas cells in interphase had densities of about 1.055 g/ml, the fraction of cells of densities between 1.040 and 1.046 g/ml contained 71 % of mitotic cells.

An interesting preliminary result that we obtained is the fact that we can observe cell expansion at mitosis in cells confined with gels (Zlotek-Zlotkiewicz et al., 2015) Fig. 2H, Figure 2.4 and Figure 2.5. This is a first indication that cells may increase their volumes in such type of confinement. It also indicates that uni-axial confinement does underestimate forces that can be developed by cells in mitosis. The hypothesis we make is that, if cells use osmotic pressure to deform their environment, they should increase their internal number of osmolytes. In conditions where cells are not confined, and therefore do not have to counteract any mechanical forces, this increase of internal number of osmolytes should translate in a volume increase.

In order to measure volumes of live cells that are moving and changing shapes we used a volume measurement method based on fluorescent dye exclusion that will be described in more details in the Results section [chapter 2](#).





# Chapter 2

## Results

<b>2.1 Cells swell in mitosis (published)</b> . . . . .	<b>27</b>
2.1.1 Failed calibration of FXm with silicon beads . . . . .	43
2.1.2 Cultured drosophila cells increase their area in mitosis under confinement . . . . .	44
2.1.3 Caveolin 1 has a role in volume regulation and mitotic cell rounding . . . . .	45
2.1.4 Anaphase and the volume undershoot . . . . .	47
<b>2.2 Screen and isotropic confinement</b> . . . . .	<b>47</b>
<b>2.3 Nuclear envelope breakdown and volume increase</b> . . . . .	<b>49</b>

### 2.1 Cells swell in mitosis (published)

The following article summarizes the experiments we performed in order to measure cell volume through mitosis and establish that various cell types swell during mitosis.

The method used to obtain these results share the principle with the method presented in ([Bottier et al., 2011](#)). But has been independently developed in the lab by Maël Le Berre roughly at the same time. Maël Le Berre had first preliminary results showing volume evolution of live cells in November 2010, whereas the article of Bottier et al. was published in August 2011.

I obtained the first results, confirming the preliminary results of Maël Le Berre, and showing that HeLa cells increased their volume at the very beginning of my PhD. It took nearly two years to do all the necessary controls, perform the experiments in other cell types, improve the analytical approach and publish the paper. In fact the paper met with very critical reactions of the first referees, which made us rethink the way we presented our results and led to the bach-to-back publication with ([Son et al., 2015](#)). The obtained results are only the beginning of a quest for the molecular mechanism behind mitotic cell volume increase.

There are several results that did not find their place in the article. One of them is a failed trial to calibrate the method using silicon beads. I observed that a cell line with Caveolin 1 knocked down showed significantly smaller cell volumes than the control. Moreover these cells presented rounding defects, though they showed a relative volume increase similar to control cells. Cells with particularly long metaphases gradually loosed their volume. And possibly the most exciting one; some cells not only do overshoots from prophase to metaphase but also do fast undershoots at anaphase, loosing more volume then they gained. These results will be shortly discussed after the main article.

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# Optical volume and mass measurements show that mammalian cells swell during mitosis

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The extent, mechanism, and function of cell volume changes during specific cellular events, such as cell migration and cell division, have been poorly studied, mostly because of a lack of adequate techniques. Here we unambiguously report that a large range of mammalian cell types display a significant increase in volume during mitosis (up to 30%). We further show that this increase in volume is tightly linked to the mitotic state of the cell and not to its spread or rounded shape and is independent of the presence of an intact actomyosin cortex. Importantly, this volume increase is not accompanied by an increase in dry mass and thus corresponds to a decrease in cell density. This mitotic swelling might have important consequences for mitotic progression: it might contribute to produce strong pushing forces, allowing mitotic cells to round up; it might also, by lowering cytoplasmic density, contribute to the large change of physicochemical properties observed in mitotic cells.

## Introduction

As cells enter mitosis, they dramatically change their shape and round up (Lancaster and Baum, 2014). This change is driven by translocation of the cdk1 substrate Ect2 from the nucleus to the cytoplasm coupled to a loss of adhesion and to actomyosin cytoskeleton remodeling (Matthews et al., 2012). Mitotic rounding has recently been shown to be associated with transient pressure increase providing force to push on the cell surroundings (Stewart et al., 2011). Evidences demonstrate that rounding is crucial to achieve robust chromosomal segregation and so is vital for normal cell division (Lancaster et al., 2013; Cadart et al., 2014). This rapid change of shape might therefore be important not only for tissue morphogenesis (Kondo and Hayashi, 2013) and homeostasis (Nakajima et al., 2013) but also during cancer progression, because tumor growth generates high solid stress (Stylianopoulos et al., 2012), which can induce mitotic arrest (Desmaison et al., 2013). We and others have hypothesized (Cadart et al., 2014) that mitotic cell rounding might be driven, at least under confinement, by an osmotic swelling, a phenomenon that can produce very large forces and is crucial for the growth of walled cells such as plants and fungi but has not been evidenced yet in mammalian cells. If this hypothesis was true, mitotic cells should increase their volume during mitosis. This important point has been debated so far in the literature.

Accurate volume measurements during mitosis of adherent cells are particularly demanding, as cells undergo, both in culture and in tissues, important changes in shape. So far,

volume measurements have produced a limited number of results (Habela and Sontheimer, 2007; Boucrot and Kirchhausen, 2008; Huang et al., 2012; Fischer-Friedrich et al., 2014) and led to contradictory conclusions. Data from confocal reconstructions showed a volume decrease at mitotic entry for adherent cells (Habela and Sontheimer, 2007; Boucrot and Kirchhausen, 2008), whereas atomic force microscopy measurements of nonadherent cell height combined with confocal microscopy showed a volume increase (Fischer-Friedrich et al., 2014). For spread cells with complex shapes, volume calculation from 3D reconstruction of the cell boundary could lead to large errors. Even for spherical cells in suspension (Tzur et al., 2009), a very good estimate of cell diameter is needed, as volume depends on the cube of this measure; a 10% increase of volume of a sphere of 8- $\mu\text{m}$  radius results in an increase of radius of only 0.25  $\mu\text{m}$ . Lastly, impedance-based Coulter counter volume measurements are precise but limited to populations of cells in suspension and do not allow temporal tracking of individual cells (Gregg and Steidley, 1965; Tzur et al., 2009; Bryan et al., 2012). As a consequence, there is no consensus on cell volume changes during mitosis. Grover et al. (2011) introduced a new method to measure single-cell volume and density using the suspended microchannel resonator. In this issue, Son et al. advanced the method to enable dynamic measurements and showed that suspended lymphocytic leukemia and pro-B-cell lymphoid cells transiently increase their volume by more than 10% in mitosis.

To measure cell volume of single cells for several hours, we adapted the fluorescence exclusion method (FXm) first proposed

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Abbreviations used in this paper: FXm, fluorescence exclusion method; GUVs, giant unilamellar vesicles; PDMS, poly(dimethylsiloxane); RI, refractive index.

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Supplemental Material can be found at:  
<http://jcb.rupress.org/content/suppl/2015/11/19/jcb.201505056.DC1.html>

by Gray et al. (1983) and more recently used by Verkhovsky and co-workers for migrating cells (Bottier et al., 2011; Gabella et al., 2014). We recorded single-cell volume during mitosis for a broad range of cell lines (from adherent cells to cells in suspension). We unambiguously observed a transient and significant cell volume increase during cell division that could reach up to 30% for certain cells. Using quantitative phase microscopy, we demonstrate that the dry mass is constant during mitosis, which results in a cell density drop, and that an intact actomyosin cortex is not necessary to regulate this process. This suggests that mitotic volume increase relies more directly on a transient activation of some ion pumps, which remain to be precisely identified.

## Results and discussion

### FXm

Cells were grown in poly(dimethylsiloxane) (PDMS) chambers with a ceiling of constant and well-defined height (see Materials and methods). Fluorescent dextran, a hydrophilic polysaccharide that does not cross the cell membrane, was added to the cell culture medium. In such conditions, cells and other objects that exclude the dye appeared as areas of lower fluorescence intensity (Fig. 1 A and Fig. S1, A–C). The height of the chamber was adjusted according to the size of the cells to optimize the signal to noise ratio of the imaging ( $h \in [15:25] \mu\text{m}$  depending on the cell type). As the height of the chamber is constant, the drop in fluorescence at a given pixel and time point ( $\Delta I(x, y, t)$ ) is proportional to the height of the object ( $h$ ):  $\Delta I(x, y, t) = \alpha \times h(x, y, t)$  (see Materials and methods and Fig. S1, D and E). Calibration was performed for each field of view and every time point by extracting the coefficient  $\alpha$  using the value of fluorescence for the central region of the supporting pillars bound to the coverslip (see Materials and methods and Fig. S1, D and E). Cell volume was finally obtained by integrating the fluorescence intensity drop over all the pixels occupied by the cell (Fig. 1 A and Fig. S1 I; see Materials and methods). With this method, a single image with a low-magnification objective enabled us to extract the volume of a large number of individual cells.

We characterized the precision of our method using giant unilamellar vesicles (GUVs; see Materials and methods) of a perfect spherical shape. We measured the radius of GUVs made with rhodamin-conjugated lipids to calculate their volume and compare it to our FXm measurement (Fig. S1 F). The volumes calculated with FXm were in very good agreement with volumes derived from GUV diameter. Based on this comparison, the precision of the absolute volume assessment was high, exhibiting an error between 10% and 14% depending on the objective used (see Materials and methods and Fig. S1 H). The error for the absolute volume is, however, largely overestimated, as it includes the error for GUV diameter measurement. The relative precision is much lower (1%), as it only includes local variations (see Materials and methods). The method is thus well suited to measure temporal variations in volume. As FXm relies on integration of the fluorescent signal over the whole height of the chamber, it is preferable to use objectives with low numerical aperture to conduct measurements in chambers several micrometers deep. We calculated the volume of GUVs by FXm and by measuring their diameter using different objectives to ensure that our volume measurement remained accurate even for 20 $\times$  objectives with numerical apertures up to 0.75 (Fig. S1 G). Nev-

ertheless, for such objectives, the volume measured by FXm depended more on the accuracy of the focal plane choice. For low-aperture objectives, FXm measurements were completely insensitive to very large variations in focusing, bringing further robustness to the method for long-term imaging of live, moving, and changing-shape cells (Fig. S1 G).

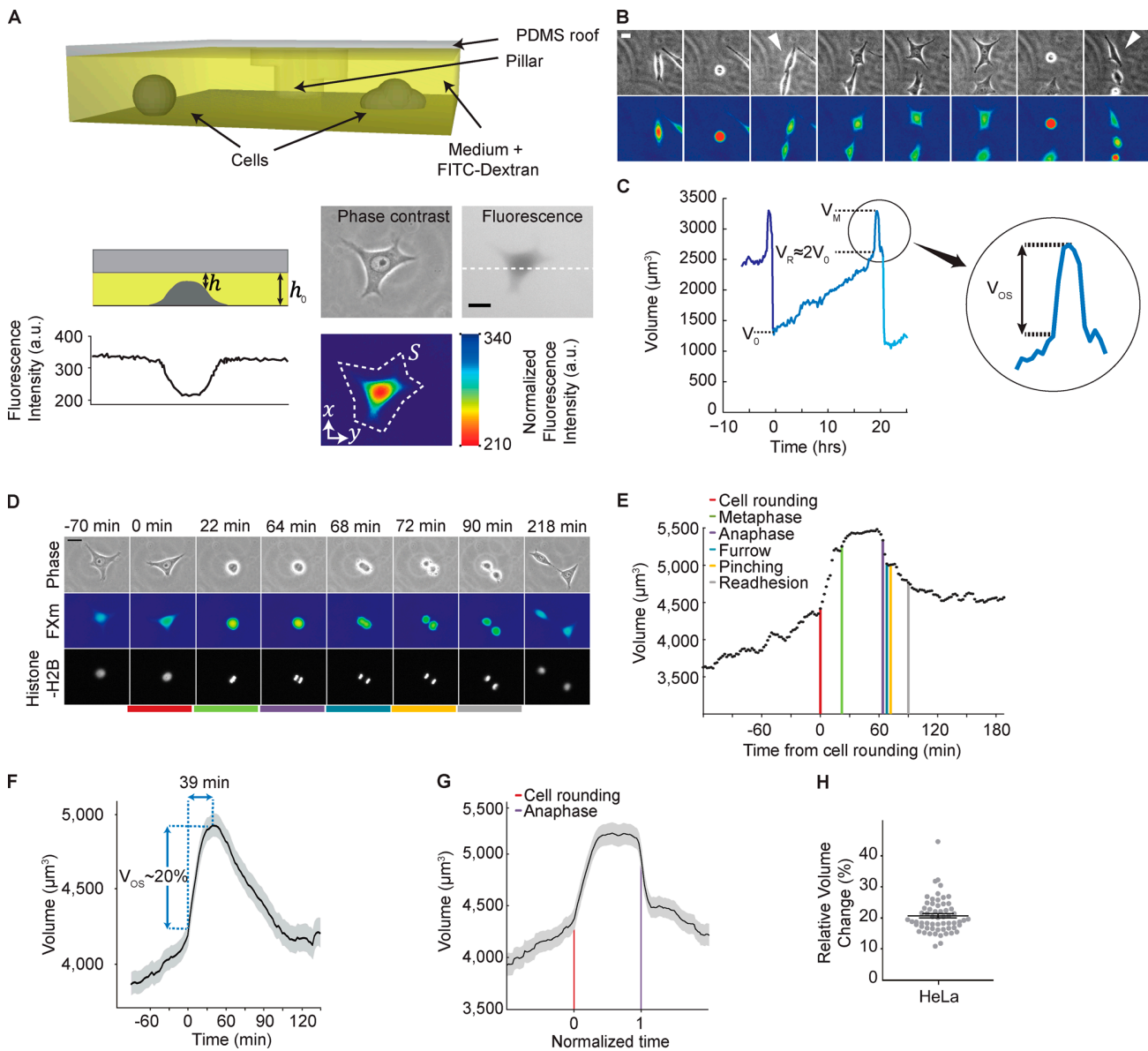
Chamber height was adapted so that during mitosis, at maximal cell height, cells would not touch the chamber ceiling, preventing any mechanical perturbation. To ensure that proper growth conditions were met, we checked that the duration of mitosis and of the entire cell division cycle was similar inside and outside of the volume measurement chambers for HeLa cells (Fig. S2, A and B). To further confirm that cell growth was not perturbed inside chambers, we measured the distributions of cell size at birth and before division for three different cell types and observed that cell size was doubling during one cell division cycle (Fig. S2 C).

### Cells increase their volume during mitosis

We recorded the volume of proliferating HeLa cells and observed that individual cell volume grew in a complex nonlinear manner to roughly double before the next mitosis. Instead of plateauing, cell volume displayed a sharp increase at mitotic onset ( $20.7 \pm 0.7\%$ ,  $n = 60$ ; Fig. 1, B and C). This was followed by a volume loss at mitotic exit, so that the summed volume of the two daughter cells at birth corresponded to the volume of their mother at mitotic entry (Fig. 1 C). To further study this transient mitotic volume increase, we monitored HeLa cells expressing histone H2B-mCherry (Fig. 1 D) and performed faster time-lapse recordings throughout mitosis. Cell volume increased concomitantly with cell rounding, reached a maximum during metaphase, and started to decrease shortly before anaphase (Fig. 1, D and E). This observation proved to be robust when averaged over many single cells (Fig. 1, F–H), showing that mitotic entry was accompanied by a sharp volume increase and mitotic exit by an equivalent loss of volume. We call this transient increase in volume “mitotic swelling.”

### Mitotic swelling is independent of cell adhesion or change in cell shape

Because mitotic entry and exit correspond, in HeLa cells, to a drastic change of cell shape, we asked whether the increase in volume was caused by the change in shape or to the mitotic state of the cell. First, HeLa cells expressing histone H2B-mCherry were placed in chambers coated with PLL-g-PEG to prevent cell adhesion. In these conditions, cells were already rounded before mitotic entry. We detected mitotic entry using the fluorescently labeled histones (Fig. 2 A). These prerounded cells showed the same transient mitotic volume increase ( $17.5\% \pm 0.8\%$ ,  $n = 14$ ) as flat and adherent HeLa cells ( $20.7 \pm 0.7\%$ ,  $n = 60$ ; Fig. 2, B and C). We then induced rounding of interphase HeLa cells using PBS-EDTA treatment. The volume of interphase cells remained constant before and after this induced rounding ( $1.2\% \pm 1.2\%$ ,  $n = 21$ ; Fig. 2, D–F). This shows that neither the change of shape itself nor the loss of adhesion alone affected cell volume or our measurements. Collectively, these results show that there is a fast and transient volume increase tightly linked to the mitotic state of the cell and independent of its shape. Using multiple drugs to perturb mitosis, Son et al. (2015) confirmed the strong link between volume change of the cell and its mitotic state.

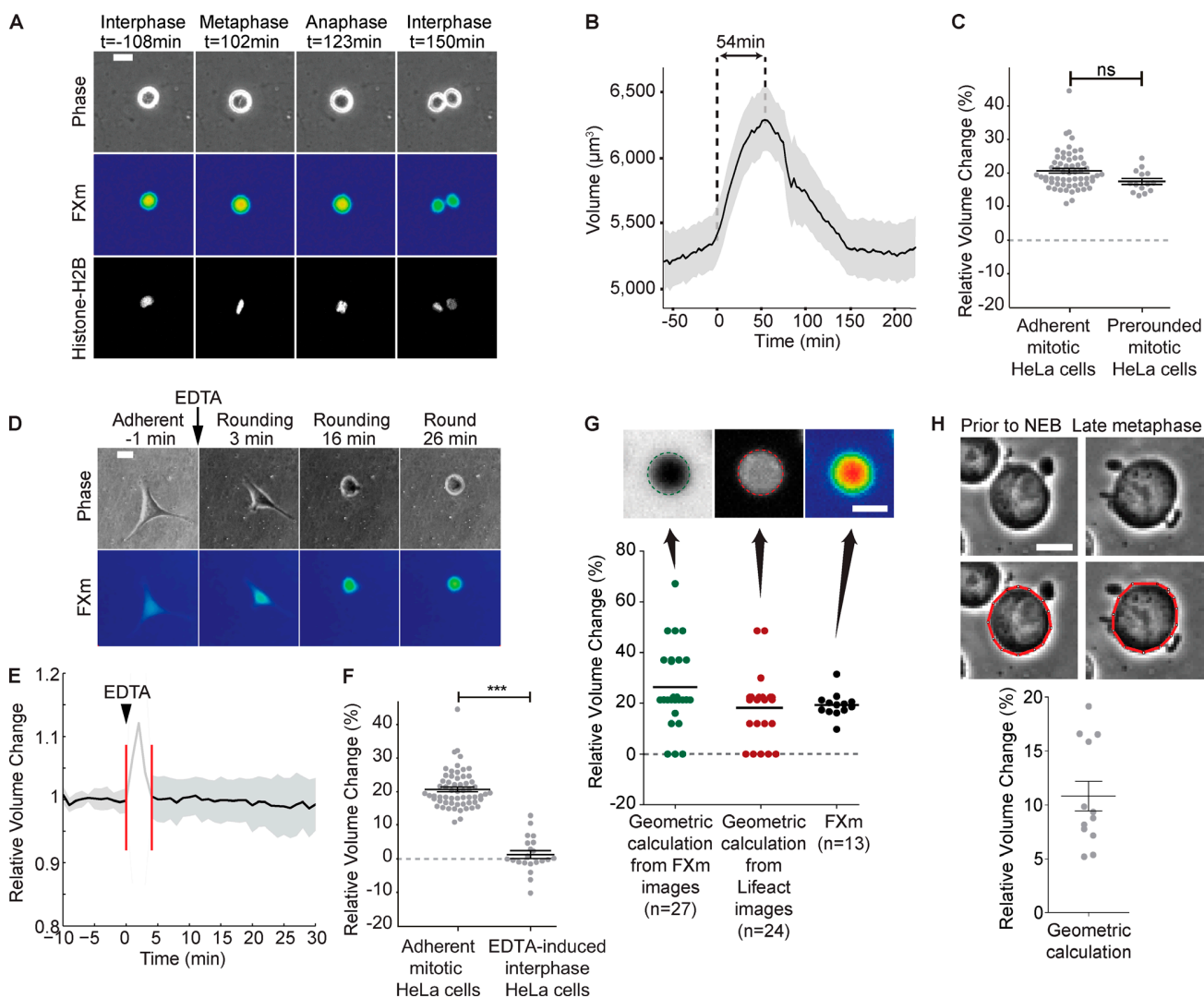


**Figure 1. Cell volume increases transiently during mitosis.** (A) Sketch of the thin PDMS chamber filled with fluorescent dextran for volume measurements, cross section of the chamber with the corresponding profile of fluorescence, and images of a cell in phase contrast and fluorescence in gray levels and false colors. Dashed lines on the grayscale image correspond to the sketch and profile on the left and on the color scale image show the integration area for calculation of cell volume. (B) Time-lapse images of HeLa cells in phase contrast and FXm. Arrows correspond to cells whose volume is shown in C. (C) Whole cell cycle volume trajectory, where arrows correspond to cells shown in B.  $V_0$ , cell volume at birth;  $V_R$ , volume at rounding; and  $V_M$ , maximum volume reached during mitotic swelling. Close-up shows the volume overshoot occurring during mitosis ( $V_{OS} = V_M - V_R$ ). (D) Time-lapse images of a HeLa cell stably expressing histone H2B-mCherry, in phase contrast, with FXm and in fluorescence for mCherry (Video 1). (E) Absolute volume as a function of time for the HeLa cell and the two daughters shown in D, with a time lapse every 2 min. Colored lines indicate successive mitotic events. (F) Mean volume overshoot for  $n = 25$  mitotic HeLa cells in one single experiment. Time registered at cell mitotic rounding (time = 0), and SEM is shown in gray. (G) Mean volume as in F, with time registered at cell mitotic rounding (time = 0) and normalized for mitosis length (anaphase at time = 1). Mean overshoot is 21.02%, and SEM is shown in gray. (H) Relative volume change in mitosis ( $V_{OS} = 20.72\%$ , SEM = 0.73,  $n = 60$ ,  $N = 2$ ). Bars, 20  $\mu\text{m}$ .

**Mitotic swelling can be detected based on measurement of the cell diameter**

To check if the mitotic volume overshoot could be seen by measuring cell diameter, we imaged rounded HeLa Life-Act-mCherry cells cultured in nonadhesive chambers (Fig. 2 G). Fitting the perimeter of cells to extract their radius and calculate their volume showed that this method was sufficient to measure a volume increase during mitosis and provided the same estimate for the mean volume increase, but with much more

variability in the measures compared with FXm (Fig. 2 G). We also reproduced the geometry studied in Fischer-Friedrich et al. (2014) by confining cells between two surfaces (Lancaster et al., 2013; Le Berre et al., 2014). In this geometry, cell height was constant and only the surface area changed with the volume. Similarly, we observed a significant increase of cell surface in mitosis (Fig. 2 H). Blebs were not taken into account in these measures, and this could explain the smaller volume increase obtained in this configuration.

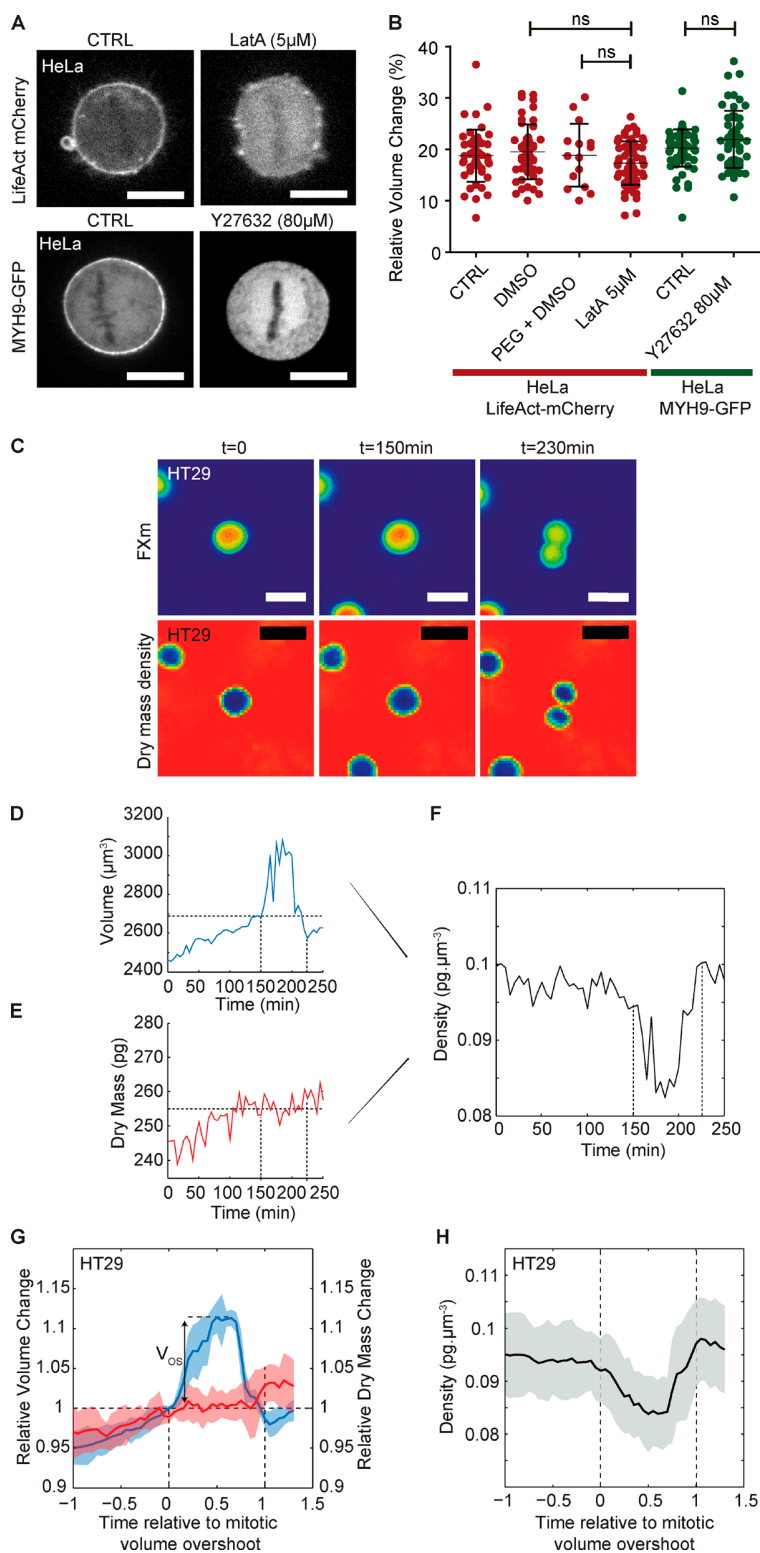


**Figure 2. Mitotic swelling is independent of cell shape, adhesion, or geometry.** (A) Time-lapse images of a prerounded cell before entering mitosis (–108 min) and at metaphase, anaphase, and after cell division (150 min; phase contrast, FXm, H2B-mCherry; Video 2). (B) Mean volume of H2B-mCherry expressing HeLa cells ongoing division in conditions to prevent cell adhesion ( $n = 14$ ,  $N = 3$ , overshoot = 17.49%, SEM is shown in gray). (C) Volume change for adherent cells entering mitosis (same as Fig. 1 H) and for prerounded cells entering mitosis ( $n = 14$ ,  $N = 3$ , overshoot = 17.49% [SEM = 0.88]). (D) Time-lapse images of an adherent cell just before adding EDTA, during early detachment, while rounding up, and completely rounded (phase contrast, FXm; Video 3). (E) Mean relative volume change of interphase HeLa cells during EDTA-induced detachment and rounding (volume before EDTA injection is normalized to 1; see Materials and methods and Video 3;  $n = 21$ ,  $N = 2$ ,  $\Delta V = 1.27\%$ , SEM is shown in gray). (F) Volume change for adherent HeLa cells entering mitosis (same as Fig. 1 H) and for detaching interphase cells after EDTA treatment ( $n = 21$ ,  $N = 2$ ,  $\Delta V = 1.27\%$  [SEM = 1.15]; \*\*\*,  $P < 0.0001$ ). (G) Mitotic volume overshoot for prerounded HeLa cells cultured in PLL-g-PEG-coated chambers. Volume calculations were done by fitting the maximal diameter with a circle from FXm and Lifeact images at mitotic onset and before anaphase and compared with FXm measurements. (H) Volume overshoot of HeLa cells confined between two plates. Volume is obtained by fitting the periphery of the cell before nuclear envelope breakdown and at late metaphase. Bars, 20  $\mu\text{m}$ .

### Swelling is independent of the actomyosin cytoskeleton

The actomyosin cortex plays an important role in mitotic cell shape and was previously proposed to counteract osmotic forces during mitotic rounding (Stewart et al., 2011). We thus tested if perturbing the cortex had an effect on volume increase during mitosis. We used drugs to destabilize filamentous actin (latrunculin A) or impede myosin II activity (Y-27632). We used HeLa cells expressing LifeAct-mCherry to monitor the effect of latrunculin A treatment and could completely remove the actin cortex of mitotic cells inside the chambers used for FXm during the whole duration of the experiment (Fig. 3 A, top; note that a high dose

of latrunculin A [5  $\mu\text{M}$ ] was required, probably because of long-term titration of this hydrophobic drug by the PDMS walls of the chamber). Similarly, HeLa cells expressing MYH9-GFP (myosin II A heavy chain) were used to check that treatment with Y27632 completely removed the cortical myosin II staining (Fig. 3 A, bottom). Neither complete removal of the actin cortex nor inhibition of myosin II activity significantly affected the mitotic swelling (Fig. 3 B; note that because treated cells tend to round up, we included a control with cells cultured on PLL-PEG substrate and thus having a round shape before mitosis). Together, these results show that, at least for HeLa cells, mitotic swelling does not depend upon and is not regulated by the actomyosin cortex.



**Figure 3. Mitotic swelling is independent of actin cortex integrity and contractility and is accompanied by a drop in cell density.** (A) Images of LifeAct-mCherry HeLa cells treated with 5  $\mu$ M latrunculin A and MYH9-GFP HeLa cells treated with 80  $\mu$ M ROCK inhibitor Y27632. Bars, 20  $\mu$ m. (B) Relative mitotic volume change for HeLa cells treated with latrunculin A (red) and Y27632 (green), with three types of controls: untreated cells (CTRL), addition of DMSO only, and cells in nonadherent chambers with DMSO. (C) Images of an HT29 cell undergoing mitosis with FXm and dry mass density imaging. Bars: (top) 20  $\mu$ m; (bottom) 24  $\mu$ m. (D and E) Volume and dry mass for the cell in C. (F) Density of the cell in C calculated from the volume and dry mass. (G) Mean volume and dry mass in mitosis for HT29 cells. Volume and mass are normalized to 1 and time is set to 0 at volume overshoot start, and time is set to 1 at the end of the overshoot (D–F, dashed line). Light blue and red areas show the SD for volume and mass, respectively ( $n = 8$  for G). (H) Mean cell density for HT29 cells, where time is set to 0 and 1 at volume overshoot start and end, respectively. The SD is shown as the gray area.

**Mitotic swelling is associated with a decrease in cell density**

To assess if mitotic swelling was caused by increasing cell mass, we simultaneously measured the volume and the dry mass of single HT29 cells using quantitative phase microscopy (Bon et al., 2009; Zangle and Teitell, 2014) combined with FXm (Fig. 3 C; see Materials and methods). The obtained density is

the ratio between cell dry mass and volume and is different from the density measured by Son et al. (2015) in this issue, as the latter is based on the total cell mass. Our measurements showed that although cell volume increased (Fig. 3 D), dry mass was constant during mitosis (Fig. 3 E), resulting in a drop of cell density (Fig. 3, F and H). This observation was confirmed using HeLa cells (Fig. S2, D–F). Therefore, osmolites that caused



mitotic swelling did not contribute to cell dry mass and were likely small osmolites such as ions. Son et al. (2015) similarly observed a drop in density and further showed that this resulted from water entry and not endocytosis.

### Mitotic swelling is observed in a large range of mammalian cells

As other studies have reported a volume decrease during or before mitosis (Habela and Sontheimer, 2007; Boucrot and Kirchhausen, 2008; Huang et al., 2012), we extended our observation to other cell lines. We studied both adherent and nonadherent cells, with different morphologies, derived from different organisms and tissues. All of the cell types we observed showed a significant increase in volume during mitosis, but with different amplitudes (from 10% to over 20%; Fig. 4, A–D). None of the cell lines we assayed (and none of the single cells we measured) showed a decrease in volume during mitosis. Adherent cells (HeLa,  $V_{OS} = 20.7 \pm 0.7\%$ ,  $n = 60$ ; S180,  $V_{OS} = 22.1 \pm 1.3\%$ ,  $n = 19$ ; L929,  $V_{OS} = 18 \pm 0.7\%$ ,  $n = 40$ ) showed a larger volume increase than poorly adhesive rounded (HT29,  $V_{OS} = 15.4 \pm 0.7\%$ ,  $n = 44$ ) or nonadherent cells (Raji,  $V_{OS} = 11.3 \pm 0.4\%$ ,  $n = 67$ ; Fig. 4, A–D). Note that Raji cells can be compared with L-1210 and FL5.12 cells used by Son et al. (2015) and that similar swelling rates were obtained using both methods (Raji 11%, L-1210 12%, and FL5.12 11%). Aside from the variability from one cell line to another, cell lines with larger cell size tend to display larger swelling ( $P = 0.01$ ; Fig. 4 F), resulting in similar relative volume increase ( $P = 0.49$ ; Fig. 4 E). Within a given cell line (for HeLa, HT29, and Raji cells), at the single cell level, we did not observe any correlation between volume increase and size of the cell at mitosis onset (Fig. S3, A–D) or the duration of mitosis (Fig. S3 E). This might be caused by smaller spread of sizes within one cell line and the existence of another source of variability masking the tendency observed between different cell lines. Altogether, our data suggest that mitotic swelling is a universal phenomenon for cultured mammalian cells.

In conclusion, most mammalian cells, both *in vivo* and *in vitro*, undergo a drastic change of shape at mitosis. This mitotic rounding has been proposed to play an important role in developmental processes and to be involved in morphogenetic events (Kondo and Hayashi, 2013). We have recently proposed that mitotic rounding is important to generate enough space for robust spindle assembly and chromosome segregation (Lancaster et al., 2013; Cadart et al., 2014). It is thus a crucial phenomenon for the fidelity of the mitotic process and thus for the survival of proliferating cells. Mitotic cell rounding is associated with cell cortex reorganization and stiffening (Matthews et al., 2012; Lancaster et al., 2013). It was recently shown that stiffening and increase in contractility associated to mitotic rounding could produce pushing forces (Stewart et al., 2011). In the context of a dense tissue, these pushing forces might be essential to achieve a spherical shape and secure enough space for robust spindle assembly and orientation. These pushing forces were attributed both to the increase in surface tension caused by increased myosin recruitment at the cell cortex (Fischer-Friedrich et al., 2014; Ramanathan et al., 2015) and an osmotic swelling (Stewart et al., 2011). This last aspect is crucial because osmotic swelling can produce very large forces, even with very small changes in osmolarity (10% increase in osmolarity corresponds to 80 kPa for a mammalian cell whose osmolarity is  $\sim 330$  mOsm; see Materials and methods). It would also imply that new players, such as ion pumps, are involved, as suggested by Son et

al. (2015) using ethylisopropylamiloride, which inhibits Na-H transporters. In the absence of resisting forces, an increase in osmolarity of the cell should very rapidly translate in a corresponding increase in volume.

Precise cell volume measurements are challenging to perform during dynamic changes in cell shape and over long periods of time and were so far performed either with techniques restricted to cells with a simple and constant shape (Tzur et al., 2009) or using 3D surface reconstruction. Here, using a precise and simple technique, we unambiguously demonstrate that all cultured mammalian cell lines that we assayed displayed a significant volume increase during mitosis. In this issue, Son et al. (2015), using a suspended microchannel resonator based on physical measurement of the cells' buoyant mass, observed a similar cell volume increase in mitosis for suspended cells. This volume increase, although hardly measurable by other means, is important enough to produce potentially very large pushing forces if cells were confined.

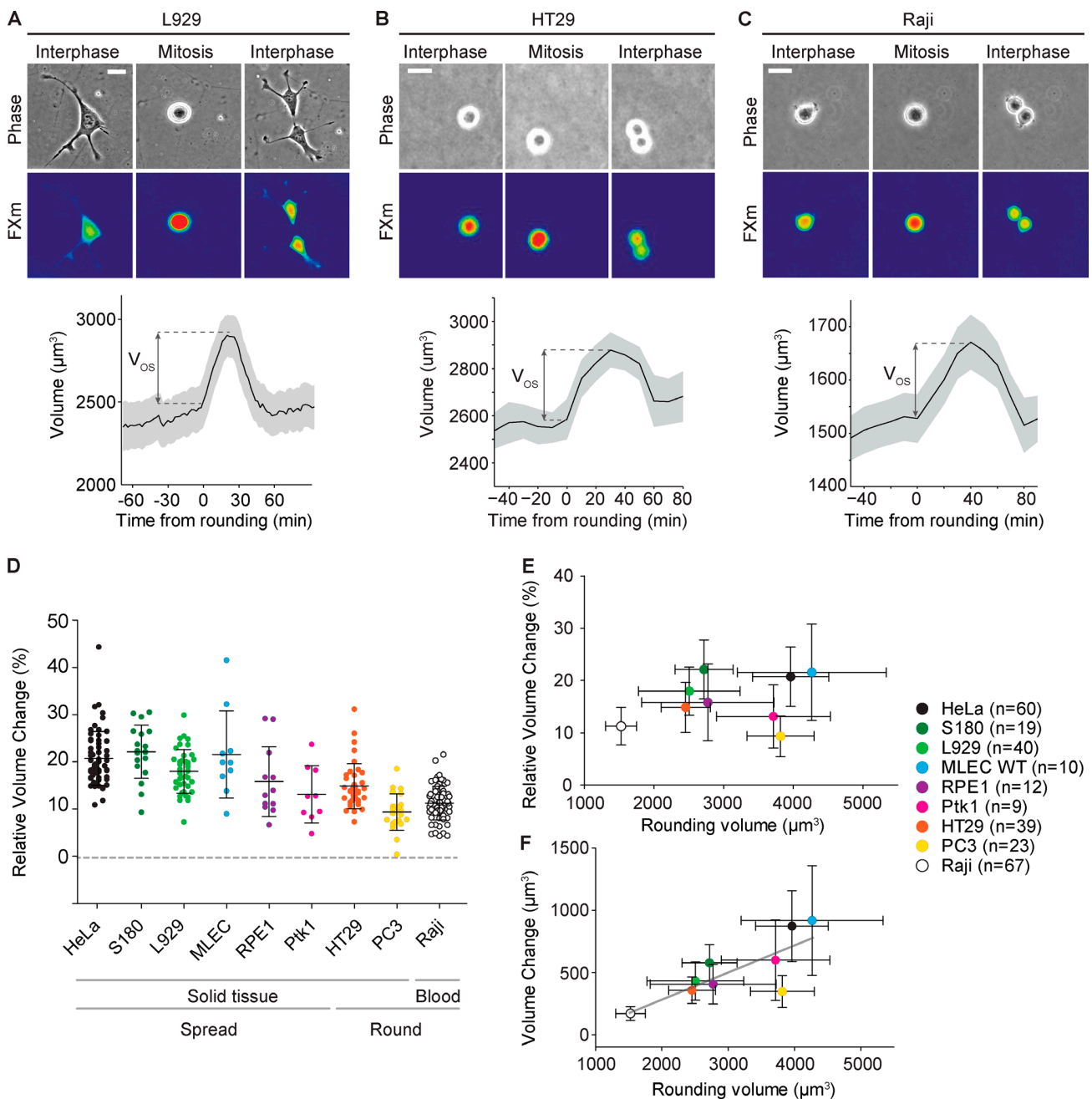
Our observation that the actin-myosin cortex is neither involved in mitotic swelling nor restrains it could seem surprising, as the cortex has been proposed to counteract internal osmotic pressure (Stewart et al., 2011). However, current measures available for cortical stiffness indicate that surface tension caused by the activity of the actomyosin cytoskeleton could account for changes in cell volume up to only 1% (Salbreux et al., 2012). As recently proposed through physical modeling, the actomyosin cortex could also have a regulatory role through mechanosensitive ion channels (Jiang and Sun, 2013). This does not seem to account for the mitotic swelling in the cell lines we assayed. Accordingly, the most recent measures showed that the actomyosin-dependent rounding pressure came purely from the surface tension (Fischer-Friedrich et al., 2014; Ramanathan et al., 2015). Indeed, in these atomic force microscopy-based experiments, cells were not constrained laterally, and thus any increase in osmolarity exceeding what the surface tension could bear translated in an increase in volume, which was observed (Fischer-Friedrich et al., 2014). On the contrary, in a context in which cells would be constrained in all directions (such as in a dense tissue), an increase in osmolarity could also generate strong pushing forces. According to the volume increase we measured ( $\sim 10\%$  or more for most cell lines), the corresponding osmotic pressure ( $\sim 100$  kPa) could potentially deform almost any tissue (cartilages reach stiffness of the order of 100 kPa; Swift et al., 2013).

Our study thus contributes to clarify a debated point and unambiguously shows that cells transiently increase their volume during mitosis. This increase results in a dilution of the cytoplasm, which might have a strong impact on out-of-equilibrium biochemical processes taking place during cell division. It also suggests that most cells are potentially able to exert strong pushing forces, most likely via activation of ion pumps. Determining which of these pumps could help design new drugs to specifically affect dividing cells embedded in stiff environments such as solid tumors.

## Materials and methods

### Cell culture

Cells were cultured in appropriate media adapted to each cell line. Media were supplemented with 10% FBS (Life Technologies) and 1% 100 $\times$  penicillin-streptomycin solution (Life Technologies).



**Figure 4. Mitotic swelling is observed for a large set of cell lines.** (A) Phase and FXm images of a L929 cell, and mean volume of fibroblastic L929 cells undergoing mitosis ( $n = 38$ ). (B) Phase and FXm images of an adherent HT29 cell, and mean volume of HT29 cells undergoing mitosis ( $n = 16$ ). (C) Phase and FXm images of a floating Raji cell, and mean volume of Raji cells undergoing mitosis ( $n = 67$ ). For A–C, time is set to 0 as cells start to round up, and the gray area displays the SEM. (D) Relative increase of volume in mitosis for different cell lines. For each cell, the volume increase is taken between mitotic entry and the maximal volume reached during mitosis. Cells were divided into three categories, from solid tissue with spread or rounded morphology and from blood with a rounded morphology. (E and F) Relative and absolute volume overshoot per cell line as a function of their rounding volume at mitotic entry. The gray line in F shows the linear regression ( $R^2 = 0.63$ ). Two-tailed  $t$  test:  $P = 0.49$  (E) and  $P = 0.01$  (F).

**Cell lines**

HeLa are cancerous epithelial cells derived from human cervix. S180 and L929 are mouse sarcoma fibroblast cell lines. MLEC are mouse endothelial cells derived from lung. RPE1 and Ptk1 are noncancerous epithelial cells derived from human retina pigment and potoroo kidney, respectively. HeLa, S180, L929, MLEC, RPE1, and Ptk1 are all adherent cells displaying spread morphologies.

HT29 and PC3 are cancerous human cells derived from colon and prostate, respectively. They grow in adherent culture but exhibit rounded shapes. HT29 cells (HTB-38; ATCC) were provided by D. Vignjevic, Institut Curie, Paris, France. PC3 cells were provided by L. Ghenim-Ziman from the CEA, Grenoble, France.

Raji are human B lymphocyte cells cultured in suspension and have spherical shapes (a gift from C. Hivroz, Institut Curie, Paris, France).

### Chip fabrication

Masters were fabricated using a conventional photolithography procedure or micromilling approach. The designs of the brass master molds were created using Catia software (Dassault Systemes). Brass masters were fabricated with a micromilling machine (MiniMill/3; Minitech) using a 100- $\mu\text{m}$ -diameter milling cutter (Minitech). Height profiles and surface roughness were measured with a vertical scanning interferometric profilometer (WYCO NT1100; Veeco). Surface roughness of the brass master and heights variation within one chamber did not exceed 0.2 and 0.3  $\mu\text{m}$ , respectively. The variability in chamber height, set by the height of the pillars, could generate errors on the absolute cell volume up to 2%, a contribution that is already included in the error on the absolute volume (see Volume measurement section).

A 10:1 mixture of PDMS Sylgard 184 or RTV614 silicone elastomer and curing agent was poured onto the brass master and cured at 65°C for 2 h. Inlet and outlet were punched with a 0.75-mm puncher before bonding. Glass-bottomed Petri dishes (homemade or Fluorodish) and PMDS chips were cleaned with isopropanol before a 30-s exposure to oxygen plasma for bonding. Finally, chambers were exposed for 10 min to UV light for sterilization.

Chambers were incubated with 50  $\mu\text{g}/\text{ml}$  fibronectin in PBS (Life Technologies) for 1 h for adherent cell types, washed with PBS, and then incubated overnight with medium at room temperature. Chambers were washed with medium before cell insertion. Cells were resuspended in medium supplemented with 1 mg/ml Alexa Fluor 488 Dextran (MW 10 kD; Life Technologies) to a concentration of  $\sim 500,000$  cells/ml (depending on the cell type) and then injected in the chamber. Finally, the chamber was immersed in medium to prevent evaporation.

### Drug and adhesion experiments

For EDTA-induced cell rounding experiments (Fig. 2, D–F), the medium was first replaced with PBS at least 20 min before adding 1 mM EDTA in PBS. PBS and PBS-EDTA have equal osmolarity. For experiments with prereduced HeLa cells (Fig. 2, A–C), cells were prereduced by placing them in chambers prepared as previously described but coated with PLL-g-PEG (1%) instead of fibronectin after plasma treatment.

Experiments with latrunculin A from Invitrogen and Y27632 from Calbiochem were performed in chambers preincubated with the final concentration of drug one night prior seeding the cells to prevent drug depletion caused by hydrophobicity or PDMS. Images of the cells were taken before launching acquisition to confirm the effect of the drug (Fig. 3 A).

### Live-cell imaging

Acquisitions were performed on a Ti inverted (Nikon) or an Axio Observer microscope (Carl Zeiss) at 37°C, with 5%  $\text{CO}_2$  atmosphere, using a 10 $\times$  dry objective (NA 0.30 phase), a 20 $\times$  dry objective (NA 0.5 phase), or a SFluor 20 $\times$  objective (NA 0.75 without phase ring) for the mass measurements. Images were acquired using MetaMorph (Molecular Devices) or Axio Vision (Carl Zeiss) software. The excitation source was either a mercury lamp (Intensilight; Nikon) or a LED illumination (SPECTRA X light; Lumencor or Zeiss Colibri). Images were acquired with a CoolSNAP HQ2 camera (Photometrics) for fluorescence or a PHASICS camera (PHASICS) for mass measurements.

### GUV measurements and FXm precision

GUVs were used rather than glass beads because the refractive index (RI) of such beads (RI > 1.47) was too different from that of water (RI = 1.33) and thus generated optical artifacts. On the other hand, the refractive index of GUVs was close to that of cells or external media (RI < 1.37; Curl et al., 2005). GUVs were prepared by regular electroformation (Carvalho et al., 2013) with EPC

(L- $\alpha$ -phosphatidylcholine) from Sigma-Aldrich, and rhodamin-PE. (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N) from Avanti polar lipids was used for membrane labeling. GUVs were placed in hypo-osmotic conditions to ensure their spherical shape, and osmolarity was controlled with a freezing point osmometer (Löser). To prevent vesicle adhesion and deformation, the surface of the chip was blocked with BSA or PLL-g-PEG. Volume measurements were performed at room temperature. Geometrical volume calculations were achieved by fitting the equatorial perimeter with a disk and extracting the radius from the surface of the disk from images of labeled GUVs obtained with a 20 $\times$  objective. This procedure was performed by the user and not by automatic means. Errors on the geometrical calculations were calculated from a fitting error of the size of one pixel. GUVs with volumes <300  $\mu\text{m}^3$  were excluded as they generated segmentation defects and would require lower chambers and higher magnification for proper volume calculation. Precision on FXm was obtained for each objectives by calculating the standard deviation for a normal distribution centered on  $V_{\text{geom}} = V_{\text{FXm}}$  (Fig. S1 H). We finally obtained for a 10 $\times$  with a 0.3 NA SD = 14%, for a 10 $\times$  NA 0.5 SD = 13%, for a 20  $\pm$  NA 0.5 SD = 9.5%, and finally for a 20 $\times$  objective with a 0.75 NA SD = 10%. These errors include the errors caused by the diameter estimation of the GUVs (likely accounting for most of the error here), the variability on the chamber height, the inhomogeneity of the light illumination, and the image analysis procedure. The relative accuracy (<1%) corresponds to the SD of cell volume over 10 min for a fixed region of interest for cells in PBS.

### Volume measurement

Images were analyzed with a custom-made MatLab program (Math-Works). Calibration was performed similarly to Bottier et al. (2011), using the sustaining pillars of multiple heights (Fig. S1, A–E), with the difference that calibration was performed on the same chip where the cells were cultured, and for every field of view and every time point. It gives a linear relationship between the fluorescence intensity and the space filled with the dye:  $I = \alpha \times h + I_0$ . To extract the coefficient  $\alpha$ , calibration using pillars was performed in initial experiments to check for the stability of the method, but was not generally required (Fig. S1, D and E). In general, pillars with a single height were used, and the coefficient was extracted using the region of the supporting pillars bound to the coverslip for  $I_0$ , and the intensity of the background in the absence of any object, which represents the fluorescence value for the maximal height of the chamber.

Even though a low-magnification objective and LED light source were used, the illumination was never perfectly homogeneous. To extract the background value, cells and pillars were first excluded using an automatic segmentation procedure based on thresholding; the background  $I_B(x,y)$  was then fitted with a quadratic polynomial function. Finally, the difference between the background and the image was integrated over the area manually defined by the user corresponding to the cell and divided by  $\alpha$  to obtain the cell volume:

$$V_{\text{cell}} = \iint_s \frac{I_B(x,y) - I(x,y)}{\alpha} dS.$$

Note that the initial segmentation procedure was used only to exclude cells and pillars to correct for illumination defects. The region used to integrate the cell volume was defined manually and kept constant through time (Fig. S1 I). Importantly, the position of the edges of this region did not matter as long as the region encompassed the whole cell, because the value of the background around the cell after subtraction was zero; the measure was thus completely insensitive to changes in cell shape.

### Coupled mass and volume measurements

Mass images were acquired on a PHASICS camera, whereas FXm images were acquired on a regular CoolSNAP HQ2 camera as described in the Live-cell imaging section. For each time point images were acquired with both cameras successively. Later generation of mass images requires acquisition of empty fields; this was performed after time-lapse acquisition by removing the cells with TryPLE. The refraction increment used to calculate dry mass from the optical thickness is  $\alpha = 0.180 \mu\text{m}^3 \cdot \text{pg}^{-1}$  as considered in Barer (1952) and suggested by the company. The dry mass of the cells was calculated as done for the volume by subtracting the background and integrating the mass on the same area used for volume calculation (see above).

### Calculation of osmotic force developed at mitosis

We assume that the fast volume change observed at cell rounding is exclusively caused by ion entry generated by active ion pumping, followed by water influx to equilibrate cytoplasm osmolarity with the surrounding medium. Physiological culture medium has typical osmolarity of 330 mOsm. A 10% volume change thus corresponds to the import of 33 mOsm of osmolytes. By using the Van't Hoff equation, we can calculate the pressure a cell would be able to generate in a confined environment that would not allow any volume increase:  $P = DC \cdot RT$ , where  $R$  and  $T$  are the gas constant and the temperature, respectively. This gives an osmotic pressure of  $\Pi \approx 80$  kPa for a 10% increase in cellular volume.

### Online supplemental material

Fig. S1 shows calibration of the fluorescence exclusion method (FXm). Fig. S2 shows that the cell division cycle and mitosis durations are not perturbed in the volume measurement chambers and cell density drops in mitosis for HeLa cells. Fig. S3 shows that mitotic swelling is constant for a given cell line. Video 1 shows the whole-cell division cycle for HeLa cells in the FXm chamber, corresponding to the time-lapse images shown in Fig. 1 B. Video 2 shows mitosis of a nonadherent HeLa cell, corresponding to Fig. 2 A. Video 3 shows cell rounding induced by PBS-EDTA, corresponding to Fig. 2 D. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.201505056/DC1>.

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The authors declare no competing financial interests.

Author contributions: M. Le Berre conceived the cell volume measurement method. S. Monnier, E. Zlotek-Zlotkiewicz, M. Le Berre, G. Cappello, and M. Piel conceived the experiments. S. Monnier and E. Zlotek-Zlotkiewicz performed the experiments and analysis. S. Mon-

nier and M. Le Berre wrote the analysis algorithms. G. Cappello, M. Le Berre, and M. Piel supervised the work. S. Monnier, E. Zlotek-Zlotkiewicz, and M. Piel wrote the paper.

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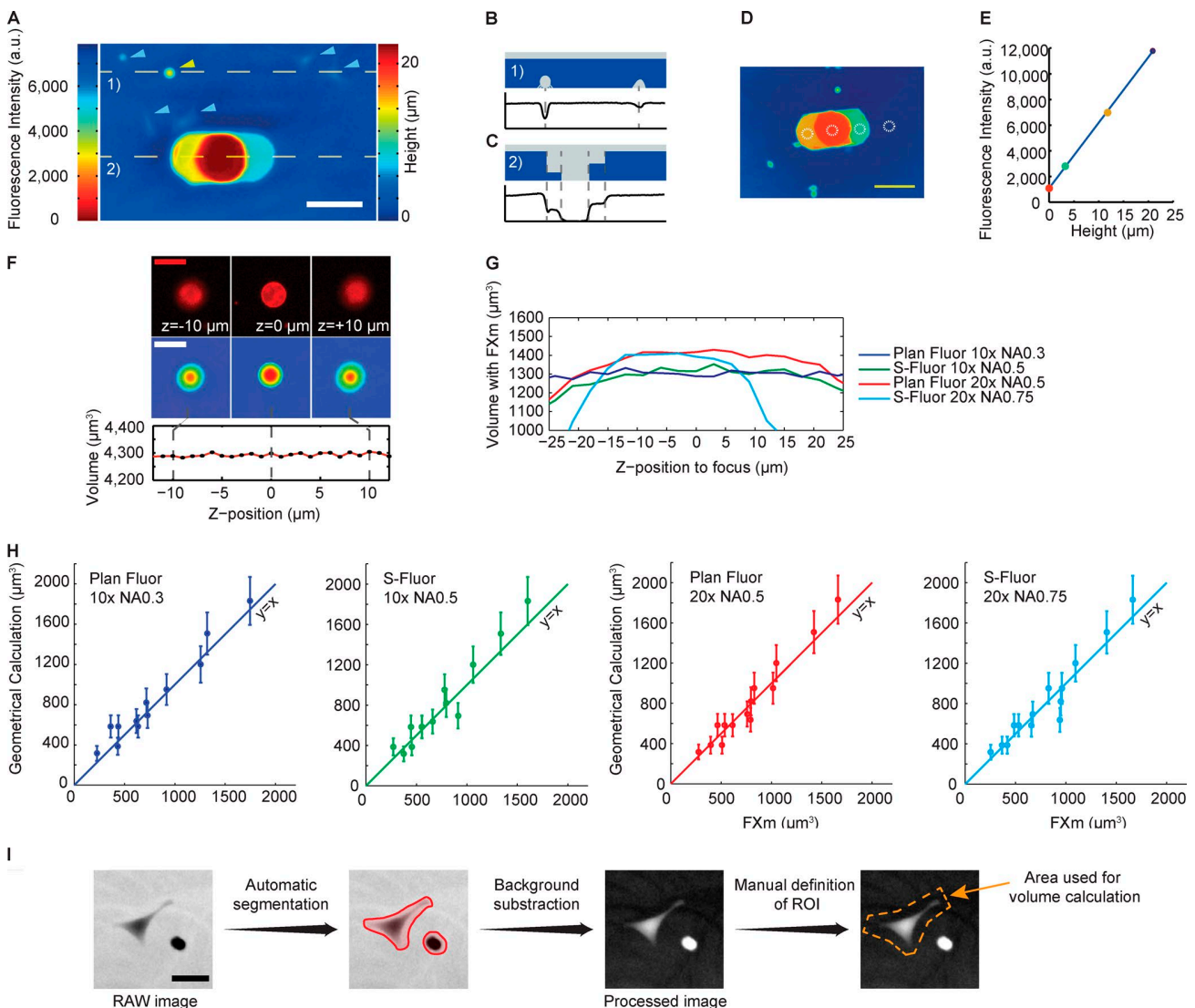
Zlotek-Zlotkiewicz et al., <http://www.jcb.org/cgi/content/full/jcb.201505056/DC1>

Figure S1. **Calibration of the fluorescence exclusion method (FXm).** (A) Fluorescence/height image of the chamber used for FXm. The field contains six HeLa cells (arrowheads; five interphase cells in light blue and one mitotic cell in yellow) and a large pillar (bottom). Bar, 100  $\mu\text{m}$ . (B) Sketch of the cross section (top) and intensity linescan (bottom) corresponding to the cells in mitosis and interphase (dashed line 1) in A. (C) Sketch (top) and linescan (bottom) of a pillar, including intermediate steps of 13 and 6  $\mu\text{m}$  (B, dashed line 2). (D) FXm image of a calibration pillar. Bar, 20  $\mu\text{m}$ . (E) Plot of the fluorescence intensity versus height for the pillar in D with linear fit  $I = \alpha \times h + I_0$ . (F) Fluorescent images of a rhodamin-labeled GUV (top) and the same GUV viewed in FXm (bottom) at three different Z-positions. (bottom graph) Volume measured with FXm as a function of the position in Z. Bar, 20  $\mu\text{m}$ . (G) Volume of the same GUV obtained with FXm as a function of Z-position for multiple objectives. Z = 0 is determined when the object is in focus. (H) Volumes obtained with FXm versus volumes calculated from the equatorial diameter of the same GUV for multiple objectives (see Materials and methods). (I) Image processing for volume calculation. Red line displays the result of automatic segmentation of objects to be excluded to further perform a quadratic fit of the background. After background fitting, the image was inverted, and the area integrated for volume measurement was manually defined and is displayed in yellow dashed line (see Materials and methods). Bar, 20  $\mu\text{m}$ .

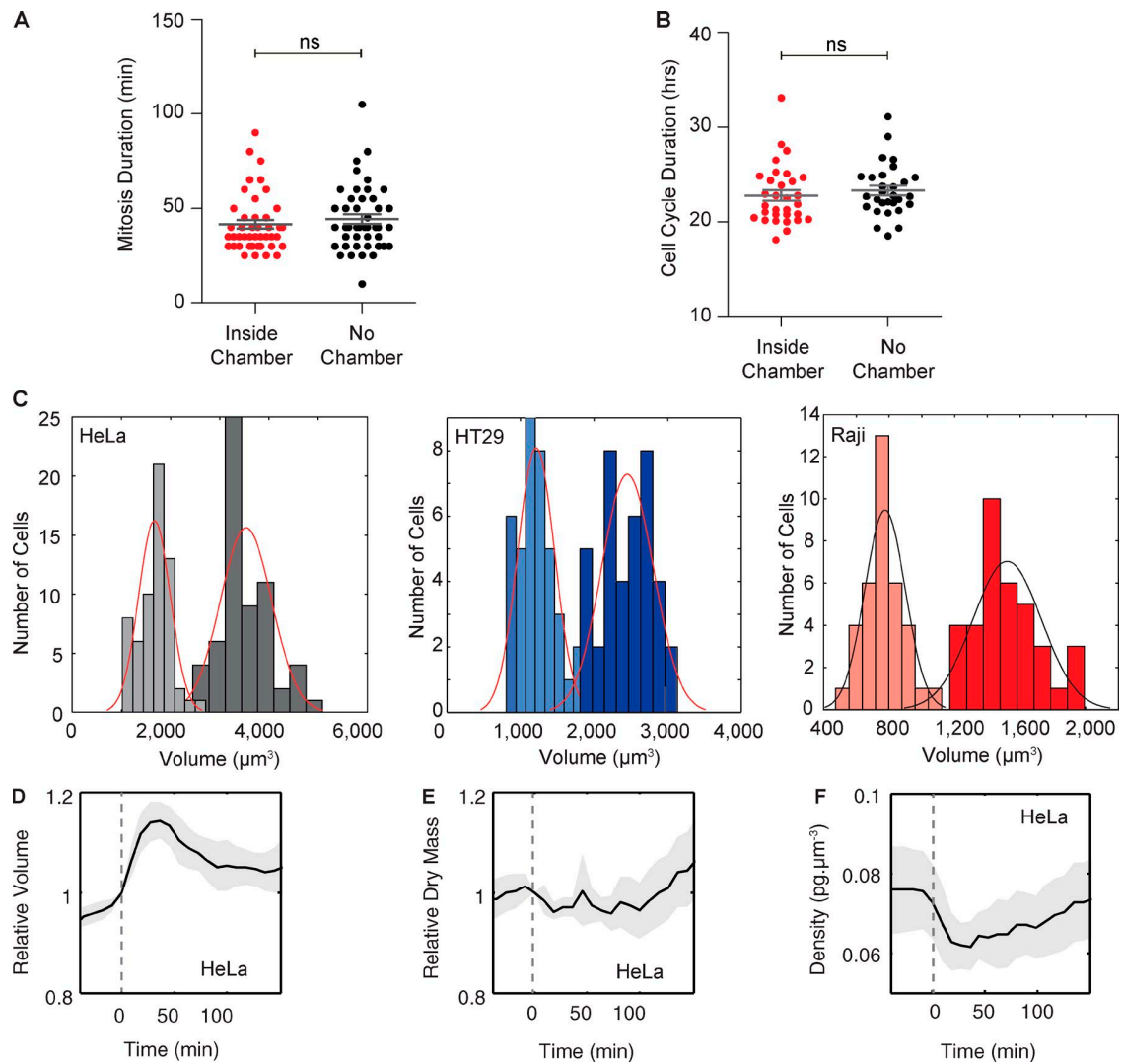


Figure S2. **Cell division cycle and mitosis durations are not perturbed in the volume measurement chambers, and cell density drops in mitosis for HeLa cells.** (A) Mitosis duration (from DNA condensation to anaphase) inside ( $n = 43$ ) and outside volume measurement chambers ( $n = 46$ ) for HeLa cells (two-tailed  $t$  test:  $P = 0.43$ ). (B) Cell division cycle duration inside ( $n = 31$ ) and outside chambers ( $n = 29$ ; two-tailed  $t$  test:  $P = 0.5$ ). (C) Distributions of volume for newborn cells (light colors) and cells at mitosis entry (dark colors) for HeLa cells (gray); distribution of premitotic cells is centered on  $V_M = 3530 \mu\text{m}^3$  (FWHM = 17.7%), newborn size at  $V_n = 1,670 \mu\text{m}^3$  (FWHM = 22.8%), human colon carcinoma cells (HT29, blue) at  $V_M = 2,458 \mu\text{m}^3$  (FWHM = 18.4%) and  $V_n = 1,210 \mu\text{m}^3$  (FWHM = 24.4%), and human B lymphocytes (Raji, red) at  $V_M = 1,520 \mu\text{m}^3$  (FWHM = 16.5%) and  $V_n = 780 \mu\text{m}^3$  (FWHM = 19.2%). Data were fitted with a normal density function. (D) Mean relative volume through mitosis. (E) Mean cell relative dry mass through mitosis. Volume and mass are normalized to 1 at volume overshoot start. (F) Mean cell density obtained from volume and dry mass measurements. For D–F, time is set to 0 at volume overshoot start and light gray area shows the SD ( $n = 5$  and  $N = 1$ ).

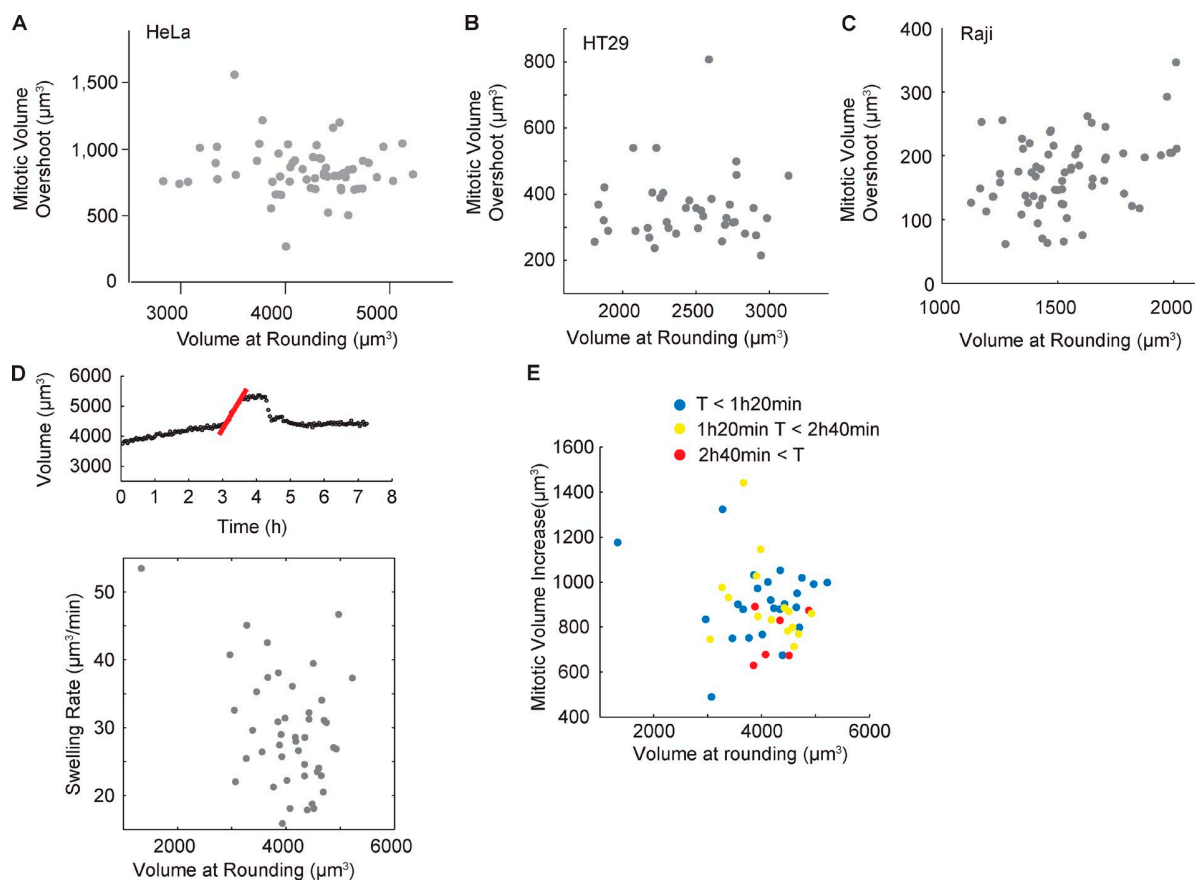
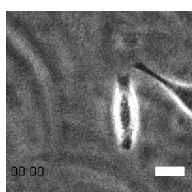


Figure S3. **Mitotic swelling is constant for a given cell line.** (A–C) Absolute mitotic volume overshoot as a function of the volume at rounding onset for HeLa ( $n = 60$ ), HT29 ( $n = 39$ ), and Raji cells ( $n = 67$ ), respectively. (D) Top panel shows a typical volume overshoot in mitosis and the fit of the initial increase performed to extract the swelling rate. (bottom) Swelling rate as a function of the volume at mitotic onset for HeLa cells ( $n = 45$  and  $N = 2$ ). (E) Mitotic volume swelling as a function of volume at mitotic onset, where the color code displays total overshoot duration (blue  $< 1$  h 20 min, yellow between 1 h 20 min and 2 h 40 min, and red  $> 2$  h 40 min;  $n = 45$  and  $N = 2$ ).

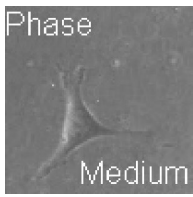


Video 1. **One cell division cycle for HeLa cells in the FXm chamber, corresponding to the time-lapse images shown in Fig. 1 B.** Top field displays phase-contrast images, and bottom field displays the FXm images after background removal. Time lapse is 10 min, and the total length of the movie is 31 h 10 min. Bar, 20  $\mu\text{m}$ .



Video 2. **Mitosis of a nonadherent HeLa cell, corresponding to Fig. 2 A.** Top left field shows phase images, top right images are H2B-mCherry signal, bottom left displays raw images obtained with FXm, and bottom right images are FXm after background removal. Frame rate is 3 min, and the total length of the movie is 5 h 36 min. Bar, 20  $\mu\text{m}$ .





Video 3. **Cell rounding induced by PBS-EDTA, corresponding to Fig. 2 D.** Left field shows phase images, in the middle are raw images obtained with FXm, and right are FXm images after background removal. Media is replaced by PBS at  $t = 33$  min, and PBS-EDTA is injected at  $t = 84$  min. Frame rate is 1 min, and the total length of the movie is 1 h 43 min. Bar, 20  $\mu\text{m}$ .

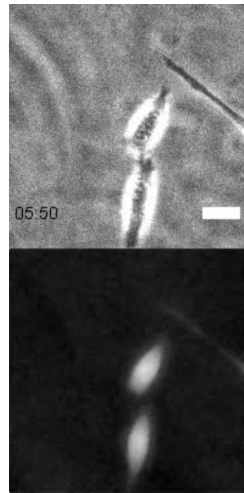


Figure 2.1: Video of an entire HeLa cell cycle corresponding to Figure 1 B in the JCB article

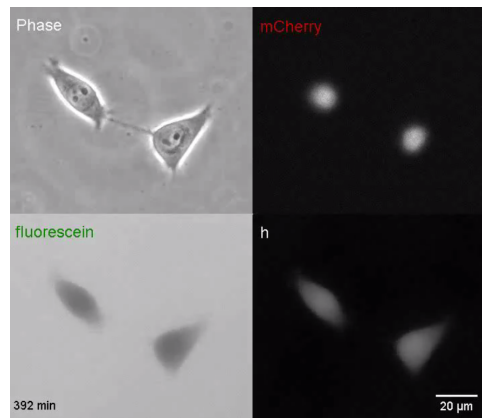


Figure 2.2: Video of a HeLa cell undergoing mitosis corresponding to Figure 1 D

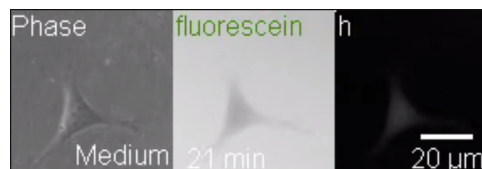


Figure 2.3: Video of a cell treated with EDTA to induce rounding, corresponding to Fig.2 D

### 2.1.1 Failed calibration of FXm with silicon beads

One of the approach to calibrate our method was to measure the volume of polystyrene beads of 20  $\mu\text{m}$  (74491 Sigma-Aldrich) using both confocal microscopy and FXm. The approach failed because of important differences in refractive properties of the beads ( $RI$  1.6), the PDMS ( $RI$  1.4) and the medium ( $RI$  1.3). Even if beads were placed in 75% glucose dilution ( $RI$  1.5) measured volumes were erroneous, probably because the refractive index of the PDMS was lower then the sugar solution, inducing artefacts in fluorescence. As the interpretation of obtained results was complicated, we switched to tensed, spherical GUVs that have a refractive index similar to cells. This was satisfying and therefore used in the published article as a reference for FXm.

### 2.1.2 Cultured drosophila cells increase their area in mitosis under confinement

As confined HeLa cells (Zlotek-Zlotkiewicz et al., 2015), drosophila cells increase their area when entering mitosis Figure 2.4 and Figure 2.5. The drastic difference in the cell periphery organisation can be easily observed. Unfortunately I was not able to perform 3D shape reconstructions to see if cells in such conditions were deforming the gel.

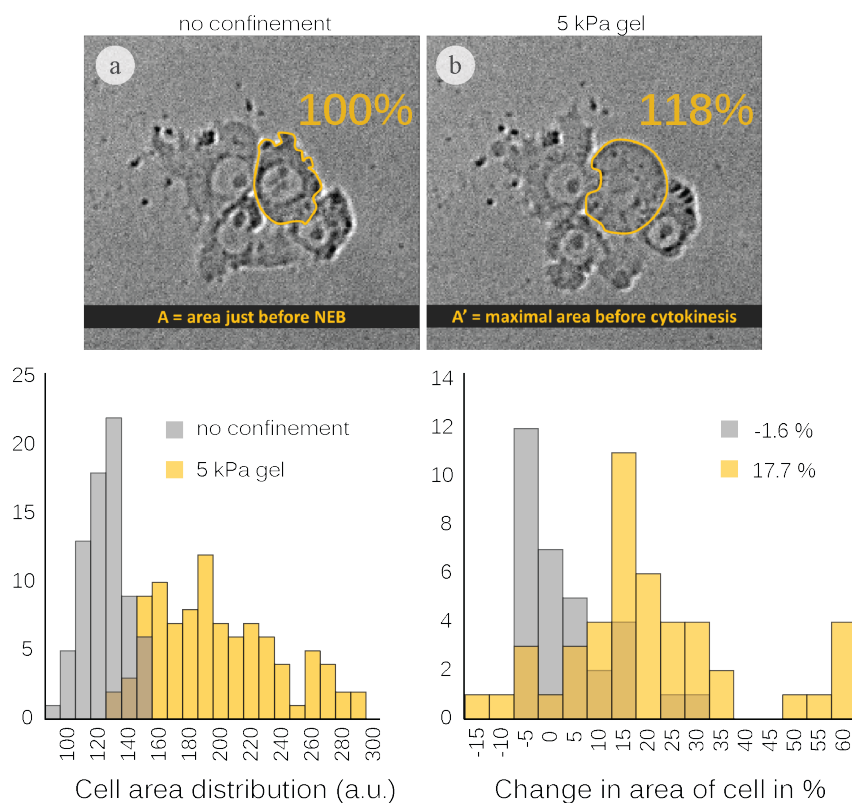


Figure 2.4: **a)** Shows a drosophila S2 cell just before mitosis and the outline of its contour defining cell area  $A$ . **b)** The same cell in late metaphase, just before anaphase onset, with again its contour showed in yellow. The contour is smoother and indicates an increase in cell area of 18%. Below are graphs representing the distribution of cell areas (left) and changes in cell area during mitosis (right) in unconfined cells and cells under 5 kPa gels

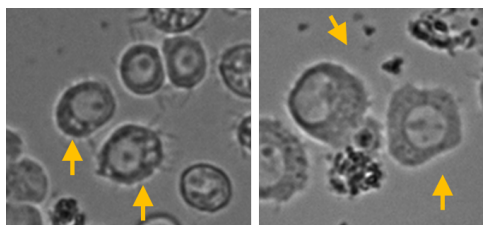


Figure 2.5: S2 cells dividing under a gel of 5 kPa.

### 2.1.3 Caveolin 1 has a role in volume regulation and mitotic cell rounding

MLEC stands for Mouse Endothelial Lung Cells and have been obtained from the lab of Christophe Lamaze and through Cédric Blouin. I measured the volumes of live cells both from WT mice and mice with a knocked down gene encoding for Caveolin 1. Caveolin 1 is important for the biogenesis of small invaginations of the plasma membrane, called caveolae. Their role in plasma trafficking and signalling is well known, but they also have a role in cell response to mechanical stresses ([Lamaze and Torrino, 2015](#)). Furthermore they are involved in a complex manner in tumorigenesis and tumour progression ([Lamaze and Torrino, 2015](#)). In response to a stretch (osmotically induced or mechanic), caveolae disappear from cell surface, potentially disrupting Caveolin 1 functions.

Interestingly, on a population level MLEC cells deprived of Caveolin 1 are much smaller than control cells [Figure 2.6](#). This does not change much the relative volume overshoot that these cells undergo [Figure 2.6](#). Control MLEC cells totally round up for mitosis, Caveolin 1 KO cells however often remain flattened during cell division [Figure 2.7](#). These preliminary data point out toward a role of Caveolin 1 in cell volume regulation and mitotic cell rounding. How the lack of Caveolin 1 is interfering with cell rounding is intriguing. Is the biogenesis of cell membrane affected, would an insufficient membrane area limit cell volume? What is happening with caveolae during mitotic rounding up, do they participate in the cell surface reduction? Are the mechanisms behind mitotic membrane reorganisation directly involved in mitotic shape change?

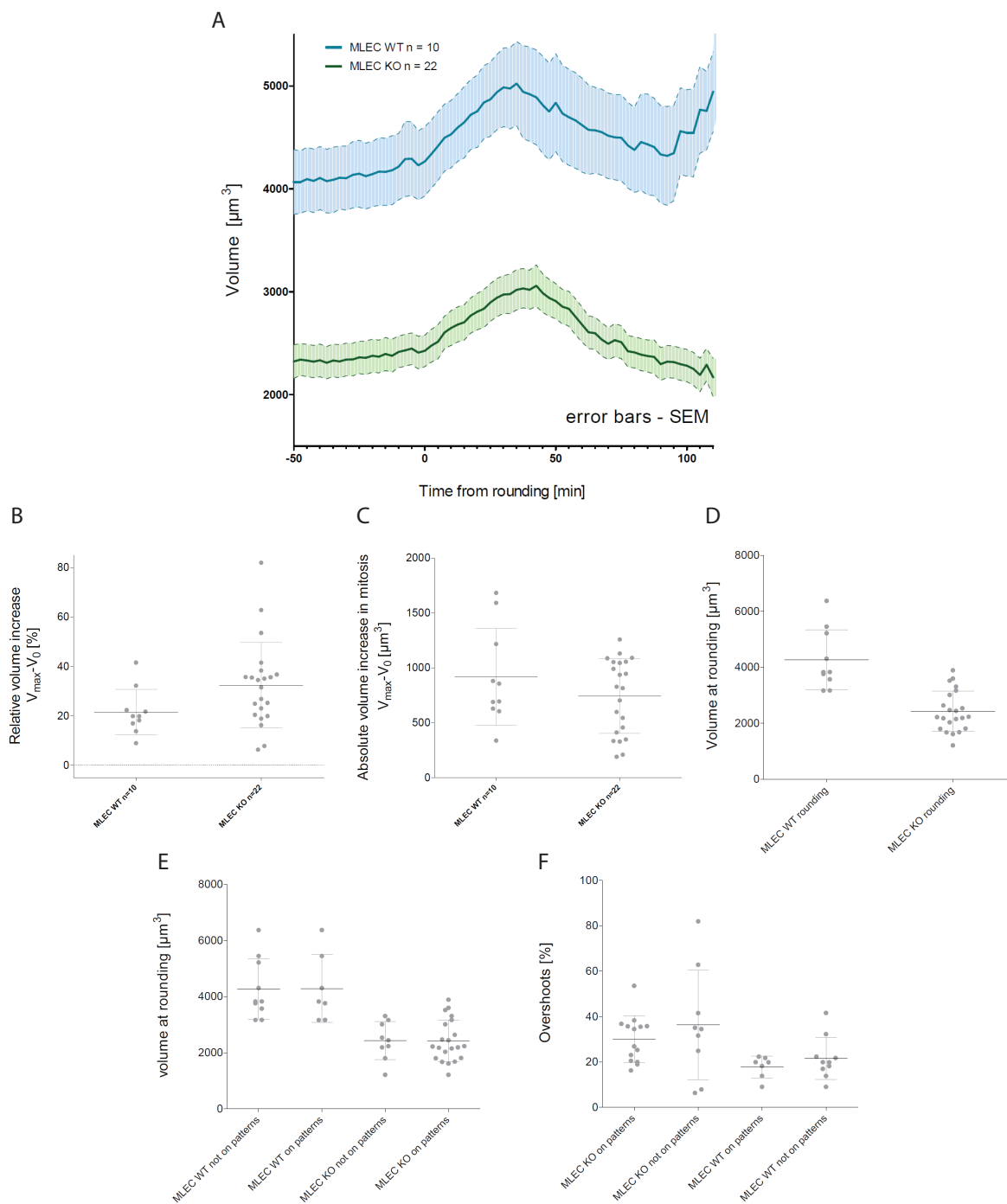


Figure 2.6: **a)** Curves of volume of MLEC cells (wt and Cav1 KO) during division. Cells lacking Caveolin 1 are significantly smaller, but increase their volume of the same amount as control cells. **b and c)** Mitotic volume increase of MLEC cells, **d)** Volumes of MLEC cells at the rounding time point, **e and f)** Mitotic volumes overshoots of MLEC cells measured on micropatterns or standard surface.

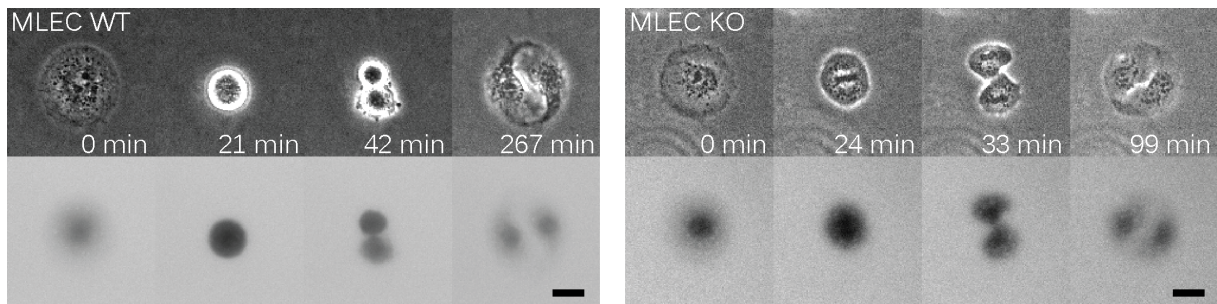


Figure 2.7: Wild type and Caveolin 1 deprived MLEC cells overgoing mitosis on disc micro-patterns. Whereas the WT cells round up, the KO cells remain flattened during the division. In KO cells the metaphase plate stays in the focal plane and is easy to observe.

### 2.1.4 Anaphase and the volume undershoot

As showed in (Zlotek-Zlotkiewicz et al., 2015), cells increase their volume at high rates when entering mitosis. This volume is then lost before cytokinesis. The volume decrease starts at anaphase, just before chromosome separation. Interestingly, cells often decrease their volume more than they increased it in metaphase creating a undershoot in volume. This undershoot is then quickly re-equilibrated.

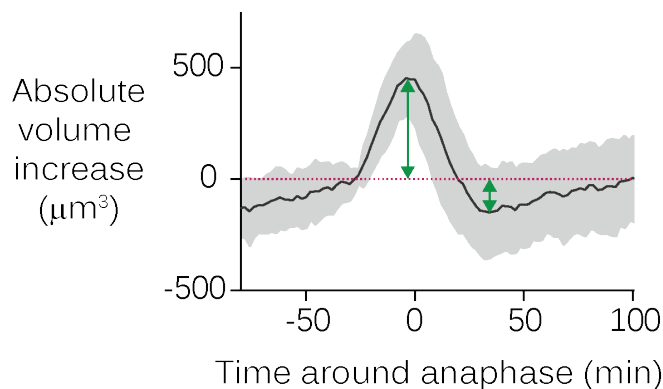


Figure 2.8: The volume of L929 cells ( $n=38$ ) overgoing mitosis. The 0 on the y axis has been aligned with the rounding up of cells, the 0 on the x axis is aligned with anaphase onset. The first green arrow shows the overshoot of volume, the second, the undershoot.

Why there is an undershoot and is it playing an active or passive role in cell division? It is tempting to speculate that this volume decrease is important for cytokinesis. It might be for example more important at the furrow. This could help the actomyosin ring contraction. It might also be a result of the overall contractility increase of the cell. What is the mechanism behind it? Is it controlled by ion channels (the VRAC) or could it be a modification of the aquaporins that play a role? What would be the result of inhibiting it, would a cell be able to process through the cytokinesis?

## 2.2 Screen and isotropic confinement

Cell volume increase in mitosis could be governed by ion channels regulation. We had in the lab two approaches, squeezing and volume measurement to asses mitotic swelling. As drugs against ion channels have often side effects that could blurry the outcome of experiments we thought about performing siRNA screens. Human cells have far to redundant and to many genes encoding ion channels so I ought to use

drosophila cells that have 85 genes for ion channels <sup>1</sup>. Another important property of these cells is that performing siRNA is easy and can be done directly in the plate with cells. This would be an important amount of work with the actual set-ups but additionally we wanted to test by RNA interference other candidates involved in cytoskeletal organisation. I had already scaled-up both methods from 6 to 24 conditions per experiment but it was not enough. The first thing to do was therefore to increase the through output of our set-ups to 96 conditions per experiment and imagine how obtained data could be analysed automatically [Figure 2.9](#). Indeed, the analysis in both set-ups is time consuming and necessitate a time lapse with an important resolution in time. This would generate data quantities difficult to manipulate and impossible to analyse by a human. Probably the best option would be to use cells stained for DNA and membrane with the same fluorophore. This would permit automatic detection of metaphase plates and therefore the localization of cells in metaphase. A segmenting step could then detect the area and the shape of these cells. Under gels of 5 kPa, control S2 cells increase their area of 18 % [Figure 2.4](#). It seems therefore possible to measure variations in conditions where the volume increase is affected by a siRNA. Any significant change in the mean metaphase volume should also be measurable.

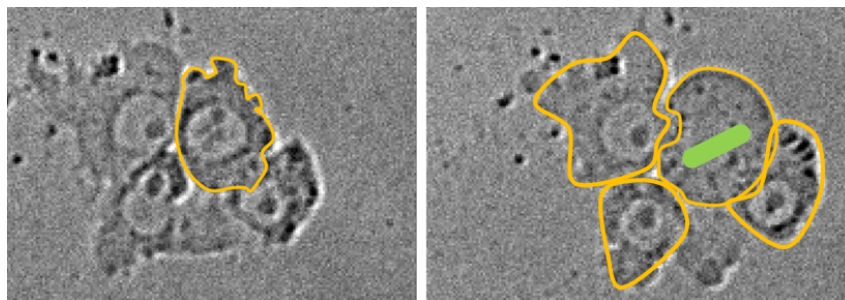


Figure 2.9: Sketch representing how could be automatized the analysis of cell shape and cell cycle in a screen. The detection of metaphase plates would indicate cells in metaphase, the detection of cell membrane would provide the shape of cells and their area.

Optimising the set-up turned out to be much more technical and complex then expected and could hardly be developed as a side project of a PhD in biology. I will however briefly present the two most promising methods that I was working on. For the confinement set-up, I would take advantage of the fact that drosophila plated on surfaces covered by siRNA uptake them spontaneously. SiRNAs could therefore be "printed" on a the bottom of a flat plate that would then be confined with one piece of gel [Figure 2.10](#). For the volume measurement, a new 96 well plate could be designed and fabricated. The plate would be covered with an adapted lead that would carry pillars with micro-spacers at their bottom. Once the plate closed these pillars would create one volume measurement chamber per well [Figure 2.10](#).

<sup>1</sup><http://flybase.org>

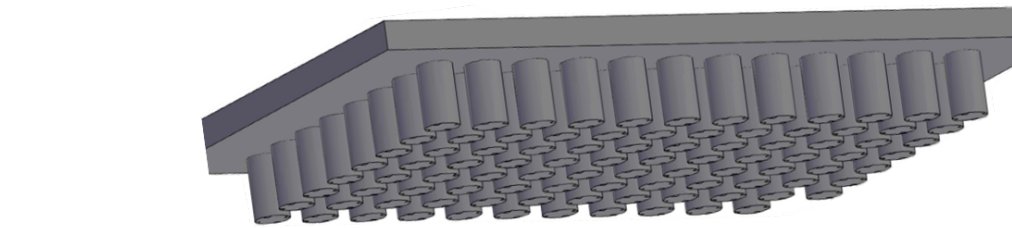
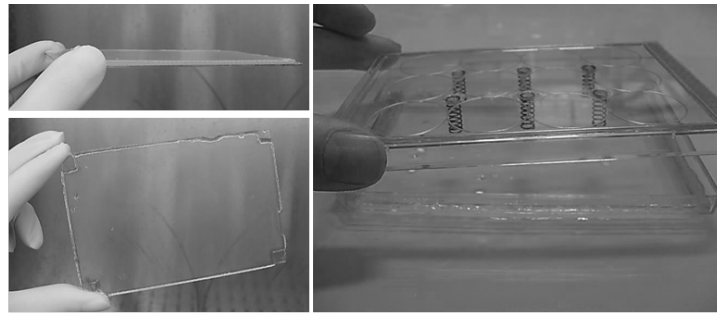


Figure 2.10: The **upper** picture shows a polyacrylamide gel polymerised on a glass surface that was used to confine cells plated on the bottom of a rectangular dish (image on the right). Soft springs were used to press down the gel layer. The **bottom** image represents a sketch of a cover that could be used to create individual volume measurements chambers in a 96 well plate. 4 spacers are visible on each of the pillars.

In parallel to the development of the techniques enabling the screen I started a preliminary screen with only few chosen candidates. Candidate genes were chosen from 3 categories, those implicated in cytoskeleton organisation, ion channels and cell cycle controllers.

The first experiments however showed that S2 cells after the siRNA protocol were not supporting confinement as well as in control conditions. This added another level of difficulty to the experiments. At this point I concentrated my efforts on other parts of the project.

## 2.3 Nuclear envelope breakdown and volume increase

As suggested by Buzz Baum, mitotic cell swelling could be driven by the release of nuclear content during the Nuclear Envelope Breakdown. In order to have a preliminary idea, I compared the timings of the NEB and cell volume increase [Figure 2.11](#). As swelling can begin before the NEB it doesn't seem that it is a resultant of the release of the nuclear content into the cytoplasm. Observing the NEB in phase is however imprecise. The NEB is a very sharp transition that is readily seen in phase contrast by the disappearance of a dark halo around the nucleus and the change in the aspect of the DNA. Nevertheless it is possible that some leakage could be present in cells where the NEB seemed to occur after the beginning of volume increase. Therefore experiments with more precise determination of the NEB could be of interest.



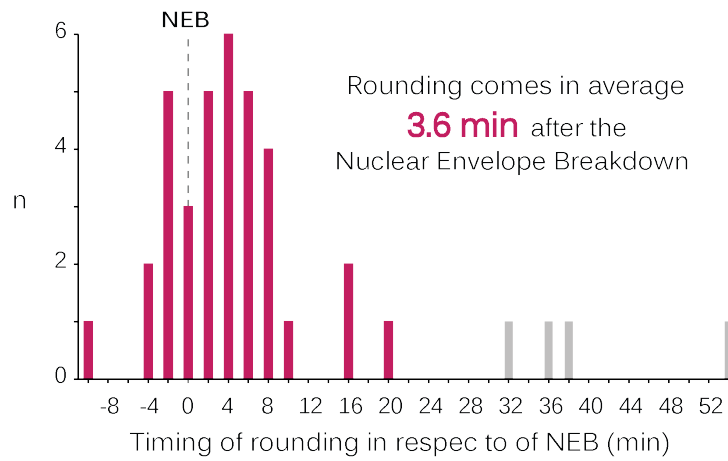


Figure 2.11: Preliminary results quantifying the timing of mitotic cell rounding in reference to the nuclear envelope breakdown. The mean time at which cells start rounding up is 3.6 min after the Nuclear envelope breakdown, over 39 cells, 8 start to round up before the Nuclear Envelope Breakdown.

The preliminary results of a mathematical model built by Pierre Recho also suggested that the amount of volume increase could be linked to the amount of Lamin A and C around the nucleus. Cells with nuclei containing a concentration in osmolytes higher than in the cytoplasm would be under osmotic pressure. In order to counteract this pressure, nuclei would build a stronger lamin cortex. One of the assumptions was therefore that cells producing higher volume overshoots in mitosis would have more Lamin A/C. As cell types we used for volume measurements might have different amounts of Lamin A/C, I first tried to quantify it. I realised a western blot using antibodies against Lamins A/C and actin or Myosin II as loading controls [Figure 2.12](#). As used cell lines came from different organisms and have different functions in different tissues, they have varying relative amounts of Lamin A/C, actin and Myosin II. It resulted impossible to conclude anything on the quantity of Lamin A/C in these cell lines.

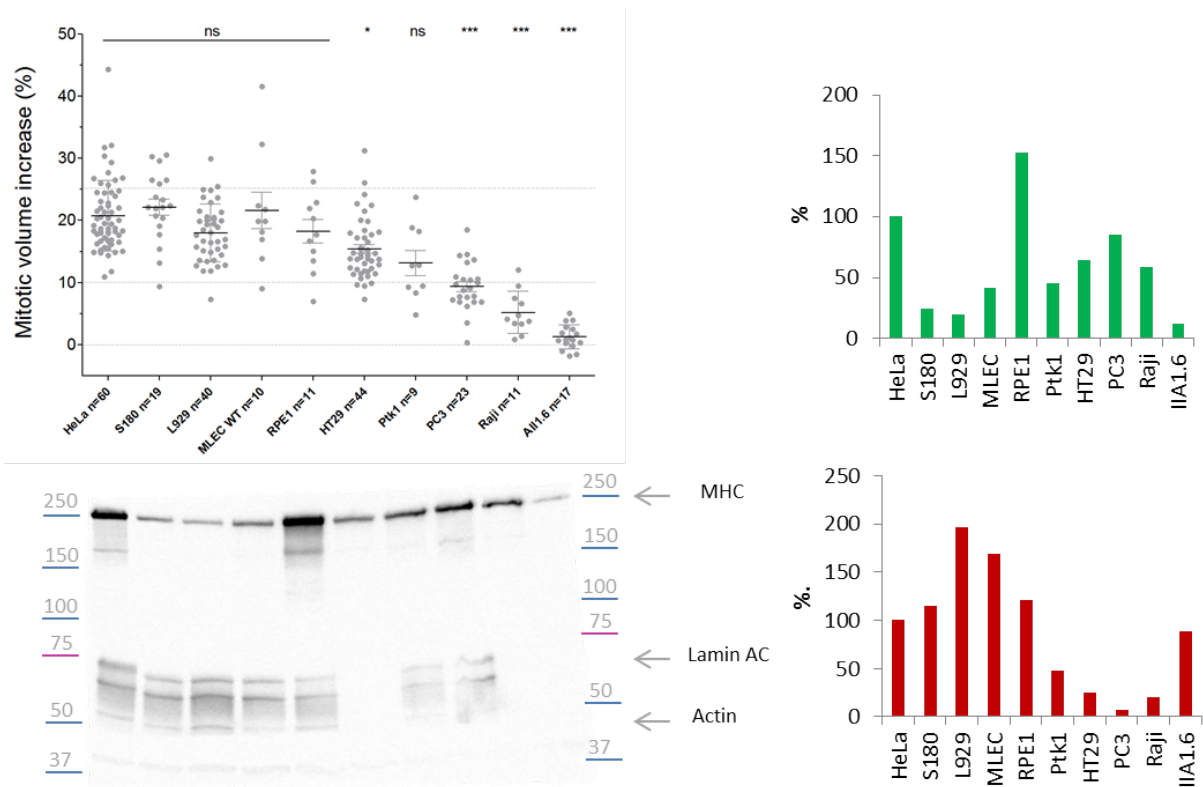


Figure 2.12: Preliminary results that aim to compare the amount of Lamin AC in cell lines with known mitotic volumes overshoots. Upper left panel represents a version of the figure from (Zlotek-Zlotkiewicz et al., 2015), where the mean volume increase is compared to HeLa cells. Compared to the figure in the article, there is one additional cell line, IIA1.6, but these data, due to their poor quality were not included in the paper. The bottom left panel shows the Western Blot realized on whole cell extracts of cells, loaded in the order corresponding to the upper left panel and at the same concentration of proteins, with two loading controls, MHC and Actin. The two right graphs represent the quantification of Lamin AC, based on densitometry, compared to HeLa cells and taking as loading control MHC (in green) or Actin (in red).

Overcoming this difficulty might come from using siRNAs against Lamin A/C in one cell line and comparing mitotic swelling. Unfortunately I didn't finish these experiments. SiRNA of Lamin A/C of L929 cells worked, but only in 40 % of cells. Cells would have to be first stained for Lamin A/C and sorted in order to increase the chances of seeing any difference in swelling dynamics. The volume measurement was postponed and never realised, but would be of interest.



# Chapter 3

## Discussion

<a href="#">3.1 At the beginning, there was a hypothesis</a>	53
<a href="#">3.2 Cells swell in mitosis</a>	56
<a href="#">3.2.1 FXm method - the bad and the good</a>	58
<a href="#">3.3 Mechanisms behind the Mitotic Swelling</a>	59
<a href="#">3.4 Mitotic swelling and force generation</a>	61
<a href="#">3.5 Mitotic cell rounding in tissue homeostasis context</a>	64
<a href="#">3.6 Perspectives</a>	67

### 3.1 At the beginning, there was a hypothesis

The starting point for this project was the observation that cells round up for mitosis even in mechanically demanding environments such as tissues or synthetic gels mimicking tissues.

The fact that cells round up at mitotic entry is known since first live observations of dividing cells, several decades ago. Since then it has been observed not only in tissue culture conditions but also *in vivo* in a large variety of organisms and tissues. Several facts point toward the supposition that MCR is important for cell division. First, the drastic shape change itself, then, the fact that the spherical shape provides the biggest possible volume to surface ratio. Third, the fact that, when rounding up in tissues cells deform their environment, indicates that this is an active process that requires force generation. This prediction is verified by a growing body of evidences.

The elegant search and capture theory proposed by Kirschner and Mitchison in 1986 ([Kirschner and Mitchison, 1986](#)) and its refinements [section 1.2.2](#) suggest that space is a crucial parameter for a timely and accurate cell division. We ([Lancaster et al., 2013](#)) and ([Tse et al., 2012](#)) further showed that cells indeed need space in order to correctly assemble the mitotic spindle and segregate chromosomes into daughter cells. Furthermore the role of Mitotic Cell Rounding in spindle positioning, is crucial for tissue homeostasis in a variety of ways ([subsection 1.2.2](#)). Despite its relevance and ubiquity, mitotic cell rounding is not yet understood. Cell deadhesion and margin retraction, cortex reorganisation, its stiffness and contractility have been shown to be important for this process in tissue culture conditions. There are evidences that the cytoskeleton, its linking with the plasma membrane, ion channels, septins, microtubules or membrane trafficking mediate it ([section 1.4](#)). But results do not converge into a clear mechanism. The fact that a round shape is the default shape of cells as and that trypsinising (detaching) adherent cells is enough to induce rounding might be an indication that the approach used to study

MCR has to be reconsidered. Maybe in order to study mitotic cell rounding we have to place cells under specific conditions, where the importance of space generation is primordial. Such conditions might be in my opinion controlled confinement. What if the main goal of cell rounding was to push on the environment in order to secure sufficient space inside the cell? Then studying cells in standard tissue culture conditions where there is nothing that limit the available space, nothing to push against, is probably not optimal. It would be as studying a weight lifter, without the weight. We could probably rip him, or her, of several muscles and bones, without perturbing to much his or her ability to stand up (as it is a purely imaginary person, he or she would feel no pain and would recover just fine from our experiment). It is only observing how he or she lifts the weight, that it would be possible to deduct which are the most important muscles or bones for this exercise.

As studying cells directly in tissues is difficult, we thought about mimicking tissue cell confinement using gels. We performed experiments where cells growing on culture dishes were placed under soft polyacrylamide gels. In our set up HeLa cells were dividing mostly into two cells in gels of 15 kPa or softer. Under stiffer gels, the occurrence of multipolar divisions and mitosis-linked death increased [Figure 3.1](#).

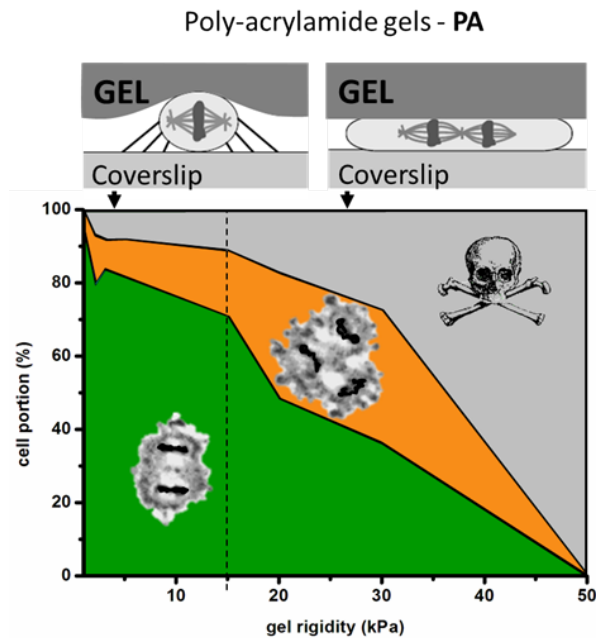


Figure 3.1: Graph representing outcomes of divisions of HeLa cells under gels of different rigidities. Cells under gels of less than 15 kPa undergo mostly bipolar divisions (green), cells under gels of more than 15 kPa present more mitotic mistakes with a growing number of multipolar divisions (orange) and cell death (gray). Graph adapted from unpublished results obtained by Daria Bonazzi.

In our understanding, bipolar divisions were possible as far as cells could deform the gel enough to provide sufficient space for the spindle formation. If the gel was too rigid, or the cell not strong enough, the mitotic cell was staying flat which hindered spindle assembly. We observed that 15 kPa was a threshold stiffness for HeLa cells above which cells had much more difficulties to undergo bipolar divisions and used it in further experiments. In order to affect actin cortex organisation we used Cytochalasin D and Latrunculin B, both inhibiting actin polymerisation. Whereas controls and unconfined cells treated with drugs mostly divided into two, cells that were treated and confined under the gel presented a drastic increase in the occurrence of multipolar divisions and mitotic cell death.

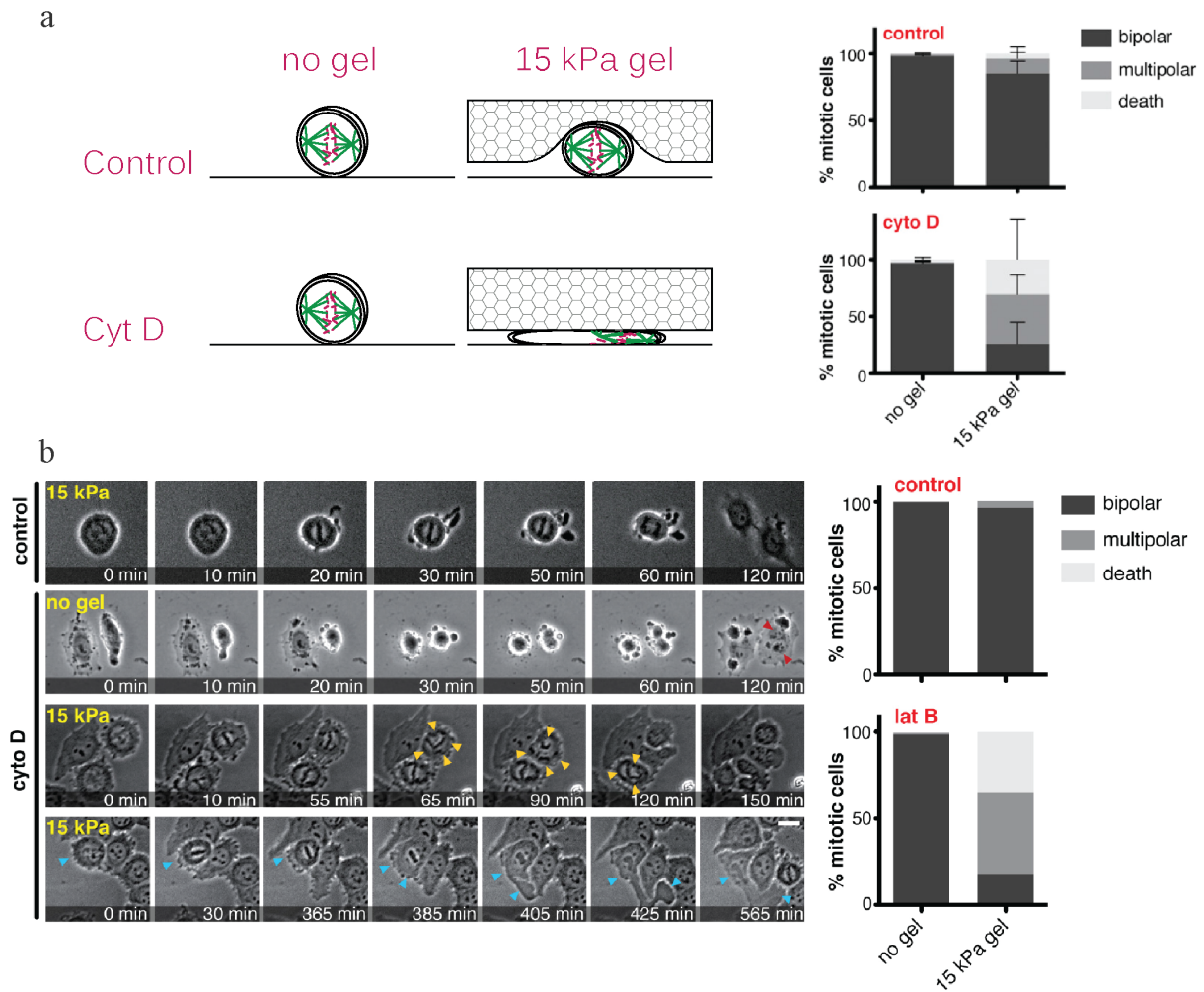


Figure 3.2: **a**) sketch representing the setup used in (Lancaster et al., 2013), where cells were treated with Cytochalasin D under confinement of 15 kPa. **b**) shows phase images of cells in the experiment. Panels on the right show the quantification of mitotic outcomes in the experiments with Cytochalasin D and Latrunculin B. Adapted from (Lancaster et al., 2013).

We observed that perturbing cell cortex organisation had dramatic consequences only when cells were placed under confinement. As treated but unconfined cells could round up, we proposed that this experiment is a proof of concept; important cell rounding mechanisms can be invisible if cells are not placed under confinement.

But this observation also pointed toward the fact that cells develop important forces in order to deform their environment and generate space for the division. Indeed cells could deform gels of 15 kPa. We cannot adjudge what is the pressure developed by cells in our experiments. First, the properties of the gel at the surface in contact with cells could not be measured. Second, this kind of confinement is not isotropic and doesn't reflect the total forces developed by cells as cells can expand under the gel. In these experiments cells deform gels of about 1  $\mu\text{m}$  which could be explained by the measurements of force generation done by (Stewart et al., 2011) and (Ramanathan et al., 2015). However cells in tissues might have to deform surrounding tissues much more. In order to do that it seems that the cortex cannot be the sole motor of MCR and another force generating mechanism has to be involved.

Although osmotic pressure is a well studied force generator in plants and that it inspires bioengineering (Bruhn et al., 2014), it is rarely seen as such in animal cells. Works measuring force generation by osmotic pressure in animal cells are sparse. In 1987 (Oster and Perelson, 1987) proposed that cell migration and in particular cell protrusion extension could be driven by osmotic pressure generated by actin network

swelling. Recently (Stroka et al., 2014) proposed that ion channels could act as an "Osmotic engine" in cells migrating under confinement. In this model water is actively pumped into the cell at cell front and out of the cell at the rear, generating forces driving cell movement. But compared to plants, osmotic pressure in animal cells do not appear as an attractive force generation mechanism.

We hypothesised that though the cortex is important for cell shape control, in the case of cell rounding it is not the sole force generator, and that rounding force could be provided by the regulation of cellular osmolality. If cells could actively increase the number of internal osmolytes, this would result in an increase of osmotic pressure. This increase of osmotic pressure would drive water influx to balance cell osmolality important for cell functioning and cells are perfect osmometers, at least in a certain range. However any external mechanical impediments the cell would encounter in this process, would counteract the osmotic pressure and water influx. A incompressible environment would not allow any water influx leaving the cell with an increased osmotic pressure. If a softer environment the water influx would be possible as long as the osmotic pressure in the cell would be higher than the pressure applied by the environment. In the case of an environment without any mechanical constraints the increase of the osmotic pressure would not be counteracted leading to water influx and cell volume increase.

Therefore, if cells used osmotic pressures to deform their environment, we should observe in basic tissue culture conditions an increase of volume at mitotic cell rounding.

If so, placing cells, that would increase their volume of 10 % in cell culture conditions, under isotropic confinement where this volume increase is not possible, would lead to osmotic pressure generation. From the Van't Hoff Equation 1.1 we obtain that this pressure might be of about 80 kPa<sup>1</sup> pressures in tissue confinement conditions where cells could not increase their volume.

As discussed in section 1.5 no adequate method was available that would enable volume measurements of adherent cells drastically changing their shapes in mitosis. Developing an adapted volume measurement method was therefore necessary for verifying our hypothesis. The method we used to obtain our results was developed by Maël Le Berre and we further developed it with Sylvain Monnier and Clotilde Cadart.

## 3.2 Cells swell in mitosis

As presented in the section 2.1 section, we could follow individual cell volume evolution through several cell cycles. We observe that after birth, cells roughly double their volume before the next division. When entering mitosis however, cells further increase their volume. This change in volume is much faster than the cell cycle-long volume increase and is reversed at anaphase.

The amount of volume increase we observed ranged between 11% in the lymphoblast-like, round Raji cells to 20% in epithelial-like, adherent HeLa cells. Whereas bigger cell lines tend to increase their volume by more  $\mu\text{m}^3$ , no such trend can be observed in the relative change of volume. The work of (Son et al., 2015), published back to back with our results, also show that two cell lines increase their volume entering mitosis. Son et al. use resonant microchannels to measure single cell buoyant mass with high precision. Measuring the same cell twice, in two solutions of different densities enables cell volume calculation with a precision of 1%. The two cell lines used by Son et al. (L1210 and FL5.12) are comparable to the Raji cell line we use in our work and, in accordance with our results, increase their volume of 12 and 11 %. Both papers, (Son et al., 2015) and (Zlotek-Zlotkiewicz et al., 2015) show therefore, on 11 different cell lines and using two unrelated methods, that cells can swell in mitosis. In addition, we never observed a cell that would decrease its volume in mitosis. These results are contradictory to the works of (Boucrot and Kirchhausen, 2008; Habela and Sontheimer, 2007; Huang et al., 2012) showing volume decrease in

<sup>1</sup> $\pi = RT(\Delta C)$ , where  $\Delta C$  is 33 mOsm/L (10 % of media osmolality being 330 mOsm/L),  $\pi = 8.3L.kPa/mol.K * 298.15K * 33mOsm/L = 81.6kPa$

metaphase in 3 cell lines (BSC1, D54MG and Vandy-MB-11). We argue that methods used in these work, that are based on 3D reconstruction of cell shape, or signal intensity measurements of an intracellular probe, are not adequate for volume measurements of living cells (discussed in [section 1.5](#)).

As the predominant view in the field is that it is the actomyosin cortex that is the principal motor of cell shape change, we decided to affect it using drugs. Latrunculin A is an inhibitor of actin polymerisation and Y-27632 is an inhibitor of the kinase ROCK that inhibits actin depolymerisation and induces MyosinII driven cell contractility. The effects of these drugs was monitored by confocal imaging. LatA treated cells had no actin based cortex and in Y-27632 treated cells MyosinII was missing from cell cortex. In both cases mitotic volume overshoots were similar to controls, clearly indicating that actin or MyosinII neither regulate nor are necessary for mitotic cell swelling in unconfined conditions. The situation is very different under confinement, and particularly if the mechanical constraints are not isotropic, where a stiff cortex seems crucial for deforming the environment [subsection 1.3.2](#).

The fast rate of this volume increase and its decrease pointed toward a fast and reversible mechanism. The volume increase we observed was concomitant with a decrease of cell density meaning that it is not due to mass production but rather water influx. ([Son et al., 2015](#)) also observed that during mitosis the buoyant mass of the cell was constant in normal medium, resulting in a decrease of cell density. The density drop measured by ([Son et al., 2015](#)) in L1210 cells is of 0.993% (from  $\sim 1.067$  to  $\sim 1.060$  g ml<sup>-1</sup>) is in agreement with the measures of ([Wolff and Pertoft, 1972](#)) on HeLa cells, where it is 0.991 % (from  $\sim 1.055$  to  $\sim 1.046$  g ml<sup>-1</sup>, whereas our measures of HT29 cells the density drop from  $\sim 1.067$  to  $\sim 1.060$  g ml<sup>-1</sup> that corresponds to 0.894 %. This points toward an error of 10 % in the volume and/or dry mass measurement. As the 10 % error of volume measurement by FXm is strongly overestimated, I suppose that this error comes mostly from the dry mass measurements. The calculation of the dry mass was done taking 0.180 pg/ $\mu\text{m}^3$  as the optical thickness, which is suggested by the company and ([Barer, 1952](#)). However the density measures were done in volume measurements chambers made of PDMS which could create a bias in the absolute dry mass measure.

Mitotic Volume Overshoot is specific to mitotic entry and independent of cell shape change. Indeed, changing the shape of adherent, interphase HeLa cells by detachment did not change cell volume. Furthermore mitotic swelling is similar in pre-rounded HeLa cells to the swelling of adherent cells and happens at mitotic entry. As we could often see a diameter increase in rounded cells when they entered mitosis we thought to quantify it. Mitotic volume increase in big HeLa cells can be easily quantified, although with high errors, measuring the diameter of round cells at mitotic entry. This was even more striking when cells were confined between two surfaces as in ([Lancaster et al., 2013](#)). Flattened cells under a stiff roof cannot round up but still increase their volume what is observed as an increase in the cell area. As the volume of blebs present in such conditions was uncertain, their volume was not taken into account, which might explain why the obtained mean relative change in volume is lower than in unconfined conditions.

The timing of swelling has not been quantitatively correlated in our experiments with any particular cell process. An attempt was made to estimate the timing of rounding in reference to the NEB observed in phase [Figure 2.11](#). Volume increase starts in average 3 minutes 36 seconds after the NEB but can as well start before the NEB indicating that these two processes are not tightly coupled. The only visible correlation of the volume overshoot timing was the one with the beginning of cell rounding. Cells start to swell exactly when they commit to round up. Even if cell surface is very dynamic, as cells constantly protrude and change their shape, it is possible to point out a moment when they start to retract their protrusions in order to round up. The cell surface is smoothing out and cells become more homogeneous and refringent in phase. Whereas mitotic cell rounding is obvious in adherent cell types, cells growing in suspension also change their morphology and get smoother, more homogeneous and rounder at mitosis. Pointing out the moment of rounding up is very qualitative, my attempts in finding a quantitative way of determining it failed because of the important noise in measures such as the cell boundary shape or



the length of the longest axis.

Interestingly, we observed that strongly adherent cell lines generate bigger Volume Overshoots than poorly adherent and round cell lines, growing in suspension. It is tempting to speculate that such differences in mitotic swelling might be linked to cell origin. Cells dividing in stiffer tissues might need to generate more forces to round up than cells from softer tissues. This might explain why epithelial cells as HeLa cells swell more than blood cells as Raji, that are not surrounded by cells or an extracellular matrix.

In the following sections I will first discuss the characteristics of our volume measurement method. Second, I will focus on the mechanism that can underlie it and finally will discuss how this volume increase might generate forces for mitotic cell rounding.

### 3.2.1 FXm method - the bad and the good

The FXm method has several pitfalls. First, it has several sources of errors. It is relatively complex to estimate the absolute error of obtained values as they cannot (as it is working now) be compared with more precise volume measurements methods as the one presented in (Son et al., 2015). One such source of error is the estimation of the chamber height which is defined by measures done on the mould used for fabrication. A constant monitoring of the chamber height throughout the experiment would increase the accuracy of the method. In order to do that, Maël Le Berre set up a method based on light reflection and it shall be implemented. Another source of error is the inhomogeneity of the illumination, as it can impact the volume calculation. It is not simple to obtain a homogeneous illumination, and more irregular it is, more difficult it is to fit a background for the calculation of the volume. Second, due to the properties of the PDMS used to fabricate measurements chambers the use of some drugs or compounds can be compromised, as they will be absorbed by the PDMS. This also results in an uncertainty concerning the actual drug concentration in the chamber, rigorous controls are necessary and not that easy. As FXm implies the integration of the fluorescence of the whole chamber height it works the best with objectives of low apertures, therefore mostly 10x and 20x magnifications. This is not compatible with simultaneous observations needing high resolutions. It can be done changing objectives and acquiring images of different magnifications, however this considerably increases the complexity of the method. The technique is also being adapted in order to follow cell volumes over several cell cycles, on adhesive micropatterns or after siRNA treatment. Maël Le Berre and Sylvain Monnier wrote the script that we further refined and optimised but it is not yet a straightforward tool and it is being finalised by a programmer external to the lab.

On the other hand FXm has a lot of remarkable features. The first is that no other method exist that could be used to measure the volume of adherent cells over time and with precision. Furthermore, it is a low cost, fast and rather simple technique that provides a good absolute volume measurement and an excellent measure of relative changes in volume. It enables following individual, adherent or suspended cells over long periods of time even if cells move or change their shape. As it is to be used with low magnitude objectives, one image provides information about a considerable amount of cells. The volume is calculated from only one image and does not need high illuminations. This is a benefit for cell conditions and do not generate small amount of data. It is also highly adaptive and could be used in a plethora of other experiments as it provides volume measurements based on cell height profiles. Though we used it for tracking individual cells, it can be used to measure the volume of groups of cells or even monolayers of cells. Adapting the height of the chamber can be useful to follow the volume of bigger or smaller objects, as tissues in development, blood platelets, bacteria and others. At last, as it is low cost and easy, it can be implemented in education as part of a practical course.

### 3.3 Mechanisms behind the Mitotic Swelling

The principal result of this project is that unconfined cells swell when entering mitosis. This, in our opinion, is an indication that cells could use the mechanism behind this volume increase to generate pressures in order to deform their environment and provide space they need for division. To test that hypothesis it will be necessary to know the molecular mechanism of mitotic cell swelling.

The mechanism underlying mitotic swelling is unknown. From our results it seem independent of the actomyosin cortex. As for the regulation and timing, it seems plausible that the induction of swelling is a matter of CDK1 signalling. As already mentioned CDK1 is the master regulator of mitosis onset in mammalian cells. In particular, the timing of events regulated by CDK1 is very robust. It would be therefore of high interest to first compare the timing of CDK1 activation, by following its nuclear relocalization or directly its activity (Gavet and Pines, 2010a), and swelling onset. If the swelling always happens after CDK1 activation, inhibition of CDK1's effectors for the swelling might point toward the mechanism activating the mitotic volume increase. As CDK1 is necessary for mitotic entry, its inhibition blocks the G2/M transition. Using low doses of CDK1 inhibitor however, extend the timing of mitosis and (Son et al., 2015) showed that the mitotic volume increases at slower rates, but of the same amount that in control cells. This potentially links the regulation of swelling with CDK1, but does not indicate how this swelling is obtained.

Cell cycle progression and in particular the G2/M transition governed by CDK1 is regulated by changes in ion concentrations and membrane polarisation (Hoffmann et al., 2009; Cone and Cone, 1976; Lang et al., 2006; Hu et al., 2012). Maintaining the membrane polarisation by active ion pumping might take up as much as 40% of the total cell energy (Djamgoz et al., 2014). Due to their manifold functions, in particular in cell proliferation and cell death regulation, ion channels are implicated in a vast group of channelopathies and in many cancers (Pedersen et al., 2013). As already mentioned section 1.6 ion channels are also main players of the constant volume adaptation that takes place in the cell. They might therefore be responsible for increasing the volume of cells entering mitosis. Moreover affecting cell volume acts on cell proliferation. The later is often being promoted in swollen and inhibited in shrunken cells (Hoffmann et al., 2009). Indeed ion channels are extensively implicated in the regulation of cell proliferation and cell death (Lang et al., 2007). There is actually 1995 proteins, across all families, recognised as ion channels in humans<sup>2</sup>. As the equilibrium between internal and external concentrations of ions and osmolytes is particularly complex I will not provide an extensive review of ion channels functions in cell proliferation here (for a review of extraordinary quality please see (Hoffmann et al., 2009)). The very general picture would be that for activating cell proliferation, the equilibrium of  $Cl^-$  and  $K^+$  ions has to be changed. This in turn induces  $Ca^{2+}$  oscillations that are a prerequisite for cell proliferation, they induce actin fibres depolymerisation and activate  $Na^+/H^+$  and subsequent  $Na^+/K^+/H^+$  exchangers. But how ion balance is being modified in mitosis is not known.

$Na^+$  exchangers are known for participating in regulatory cell volume increase and are therefore candidates for driving mitotic volume increase (Hoffmann et al., 2009). Indeed in (Son et al., 2015) the treatment with EIPA, a weak inhibitor of the main  $Na^+/H^+$  exchangers (Masereel et al., 2003) reduces swelling in mitotic cells (from 12% to 2.5%). Furthermore in (Stewart et al., 2011) treatment of cells with high concentrations of EIPA decreased the measured force at mitotic cell rounding of 60%. These experiments could be further refined using drugs that induce a stronger decrease of internal  $Na^+$  concentrations (ten Hove et al., 2000) than EIPA. In normal conditions,  $Na^+$  levels are highly regulated in cells (Murphy and Eisner, 2009) it might therefore be surprising that  $Na^+$  levels would not be immediately reduced by  $Na^+/K^+$  ATPases (Murphy and Eisner, 2009; Coppini et al., 2013) the predominant mechanism mediating  $Na^+$  efflux. Extremely high  $Na^+$  levels are however observed in diseases as ischemia or heart failure (Murphy and Eisner, 2009) where the  $Na^+$  concentration can increase

<sup>2</sup><http://www.guidetopharmacology.org/GRAC/IonChannelListForward?class=LGIC>

from 8 mM to 40 mM. The regulation of  $Na^+$  might therefore increase internal osmolality and lead to water influx and cell volume increase in mitosis. Additionally, NHE1 interacts directly with proteins of the Ezrin-Radixin-Moesin family (Hoffmann et al., 2009) involved in cell rounding and phosphatidylinositols (PtdIns(4,5)P2) (Hoffmann et al., 2009) that are altered in mitosis (Atilla-Gokcumen et al., 2014). Another  $Na^+$  exchanger, the NHE4 involved in regulatory volume increase, has been found to be activated in an actin dependent manner (Hoffmann et al., 2009). A large number of ion channels, regulative proteins and small molecules are involved in the regulation of internal ion concentrations. Both, internal and external cues are important. As the regulation mechanisms are often entangled dissecting molecular mechanism leading to mitotic cell volume increase shall be complex and fascinating.

Ion channels and their regulation could therefore be the principal actors of mitotic cell swelling. Elucidating their regulation may however be difficult, especially in mammalian models where channel functions are very redundant. As such, studies in systems as the *Drosophila*, where there is 85 channels<sup>3</sup> or *Caenorhabditis* with 39 channels<sup>4</sup> might be helpful.

Several approaches could be taken to gain further informations about the ions implicated in mitotic swelling. Among them siRNA approaches in *Drosophila* for example or using drugs to inhibit different ion channels at several times in mitosis. It might also be of interest to strongly confine cells and use fluorescent probes of particular ion concentrations. Such probes exist for a vast number of ions (Yin et al., 2015). Approaches based of ratiometry can be difficult to calibrate, but if HeLa cells, that increase their volume up to 30%, are flattened to 3.5  $\mu\text{m}$  changes in ions concentrations might become visible. It should be enough, as changes of 1% in  $Na^+$  concentrations can be detected (Knpfel et al., 2000) and for  $Ca^{2+}$  changes as low as 0.1% are visible (Knpfel et al., 2006). If such an approach would work it would provide direct informations on the fluxes of principal ions during cell swelling.

The recently identified LRRC8 (Syeda et al., 2016) is responsible for the the already mentioned  $CL^-$  efflux important for cell proliferation and particularly present during mitosis (Wondergem et al., 2001). As LRR8 mediate ion efflux it is potentially counteracting mitotic cell volume increase. It would be of interest to test the effect of the inhibitor (DCPIB) on volume increase. If cell swell even more after DCPIB treatment, it could be potentially used to study molecular mechanisms driving mitotic volume increase.

Preliminary results therefore point toward a decisive role of ion channels in the mitotic cell swelling. As inhibiting  $Na^+$  fluxes reduces swelling by 80% (Son et al., 2015), other ion channels or other mechanisms of volume increase may be implicated. Among them is the Nuclear Envelope Breakdown that releases the negatively charged DNA and nuclear proteins in the cytoplasm. Preliminary results of a mathematical model developed by Pierre Recho indicate that the NEB could result in volume increase, but it is contradictory to the results obtained by (Son et al., 2015). Authors of this study use a CDK1 inhibitor, in low doses to slow down mitosis. As the NEB is a discrete process, they expect that if the swelling is due to the release of nuclear content, the rate of volume increase should not change. They observe that the total time of metaphase is longer, and so it is for the the phase of volume increase. It is however difficult to completely rule out the participation of the NEB in these experiments. The onset of swelling is much more diffused in treated cells and it cannot be excluded that the dynamics of the NEB are not affected. Together with my observations that volume increase may start before the NEB is visible in phase contrast, it doesn't seem that the NEB has a decisive role in mitotic swelling. Because (Boucrot and Kirchhausen, 2007) showed that endosomal recycling is slowed down in mitosis, (Son et al., 2015) also take it into account. They follow cell divisions in constant, denser medium and see that the buoyant mass of the cell is changing during mitosis. If cells were increasing their volume by accumulation of endosomes containing the surrounding medium, the buoyant mass of the cell would not change.

<sup>3</sup><http://flybase.org>

<sup>4</sup><http://www.wormbase.org>

### 3.4 Mitotic swelling and force generation

Cell shape in round, suspended cells is therefore a resultant of 3 main parameters; cortical tension, the strength of the link between the cortex and the membrane and the internal pressure. Pressure and cortical tension can drive cell rounding until the threshold pressure where the membrane detaches from the cortex. The later is a limiting factor for shape generations for cells in conditions with no mechanical confinement.

In (Zlotek-Zlotkiewicz et al., 2015) we propose that the volume increase in mitosis, observed in conditions with no confinement, might reflect a pressure increase in cells dividing in tissues. As mitotic cells show a decrease in density we and (Son et al., 2015) assume that the volume increase is mostly due to water influx. This water influx might be triggered by an increase of cell osmolality. If a cell with a starting internal concentration of osmolytes ( $C_i$ ), equal to the concentration of the external medium ( $C_i = C_e = 330\text{mosm/L}$ ) increases its osmolytes number of 10%, a difference in concentrations will be created ( $\Delta C = 33\text{mosm/L}$ ). Normally, this difference is balanced by water influx, but if the cell is confined and cannot increase its volume, this increase in internal number of osmolytes generates an osmotic pressure. From the Vant Hoff's law (3.1), where  $R = 8.3 \frac{\text{L}\cdot\text{kPa}}{\text{osmol}\cdot\text{K}}$ ,  $T = 298.15\text{K}$  and  $\frac{\Delta C=0.033\text{osmol}}{\text{L}}$  we obtain that such pressure would be of  $\sim 80\text{ kPa}$  (3.1).

$$\Delta P = RT\Delta C \quad (3.1)$$

This pressure, under confinement where it can be counteracted, might deform tissues surrounding the dividing cell. It is however not clear how cells can exert forces on their environment and how this environment reacts to such constraints. Indeed cells are interacting with each others and with the extracellular matrix. For instance, forces created during both, cell rounding (Kondo and Hayashi, 2013) and apoptosis (Monier et al., 2015) have important consequences on the tissue during morphogenesis. Furthermore cells in tissues are tightly linked and share mechanical stresses present in the tissue. This interaction comports both biochemical and mechanical complex and dynamic cues. Recently, important measurements of mitotic cell mechanics have been made that improve the description of mechanics of mitotic cell rounding. These measurements however are limited to uni-axial confinement. Therefore further reasoning on cells confined in 3 dimensions stays purely speculative until it will be tested and measured *in vitro* and *in vivo*.

In the presence of an external confinement that could counteract intracellular pressure, the cortex and its tethering to the membrane would be less important and shape would be adapted to this external confinement. In the work of (Kunda et al., 2008) cells depleted in Moesin do not round up in normal conditions, but acquire a round shape if treated with Concavaline A. Concavaline A is a lectin that interacts with a large number of molecules at the cell surface resulting in their cross-linking and stiffening of the external cell surface. Cell rounding may therefore be acquired without a functional cortex-membrane complex.

We hypothesise, that an already round cell entering in mitosis surrounded by a mechanically isotropic matrix, could deform its environment by increasing its internal pressure. This could be independent of the actomyosin cortex as the scaffold would be provided by the external environment. Such increase in pressure could result in a small change of cell diameter. A 10% volume increase of a cell of  $20\text{ }\mu\text{m}$  diameter (corresponding to a rather big HeLa cell) would result in an increase of the diameter of  $0.65\text{ }\mu\text{m}$ . This seems insignificant compared to cell the size of the spindle  $\sim 14\text{ }\mu\text{m}$  (Cai et al., 2009).

Rounding up for mitosis becomes crucial for a cell with a complex shape and embedded in a mechanically anisotropic environment Figure 3.3. Recent measurements indicate that the anisotropic stress in aggregates of mammary cells is of  $3.4\text{ kPa}$  (Campàs et al., 2014). Autors also measure stresses in

mesenchymal tooth cells both, in aggregates and ex vivo in a mandible of a E711 embryo, obtaining values of 1.5 and 1.6 kPa respectively. The measures are done based on the deformation of incompressible droplets with an interfacial tension of  $26 \text{ mN m}^{-1}$ , mitotic cell maximal cortex tension was measured to be  $\sim 1.8 \text{ mN m}^{-1}$  (Ramanathan et al., 2015). Droplets embedded in the tissue represent only the anisotropic forces applied on the surface of the droplets. There is however in the tissue an additional, global isotropic pressure that cells have to bear.

In the case of a mitotic cell rounding up in a tissue, increasing only the cortex tension will not be sufficient, it has also to increase its internal pressure. This in turn facilitate the acquisition of a spherical shape and the deformation of the surrounding tissue [Figure 3.3](#).

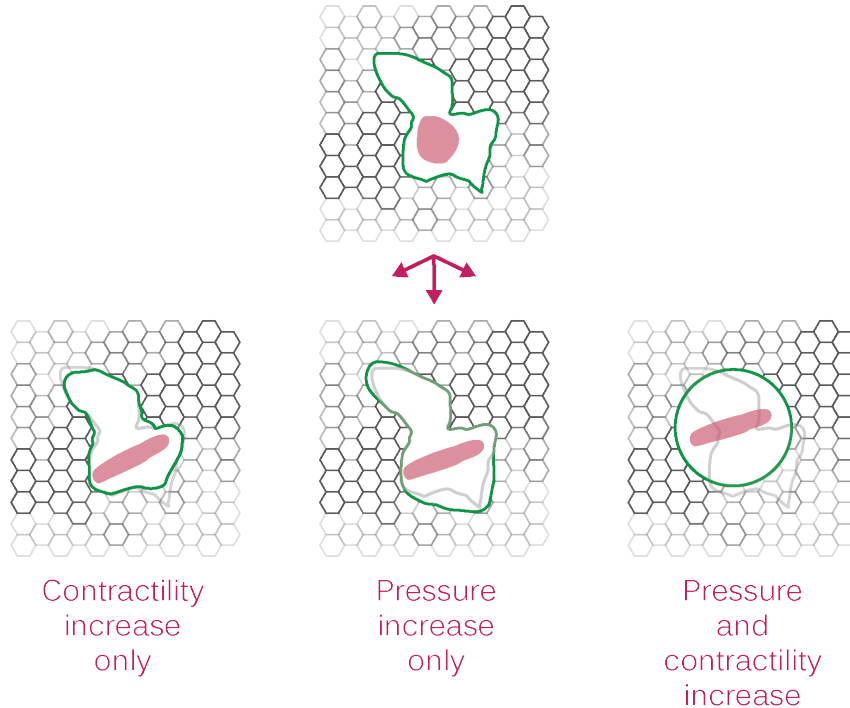


Figure 3.3: Sketch summarising the idea that, in order to round up under anisotropic confinement present in a tissue, cells might have to both, increase their internal pressure and cortex rigidity. The anisotropic rigidity of the tissue is symbolized by a mesh with different color intensity. Cells increasing only their contractility or would deform only slightly their surroundings without rounding up. Cells that would not have a stiff cortex would not be able to achieve a round shape under anisotropic confinement, even if they would increase their volume by increasing the osmotic pressure. Cells might need strong pressures and a stiff, shield-like cortex to round up even under anisotropic mechanical constrains.

The view of the cell as a sac of proteins surrounded by a semi-permeable membrane is dominant, however it may not be the only possible way to explain cell rounding. Little is known about the role in shape acquisition of the bulk actin present in the cell. It is probable that the water influx in mitosis might change not only the osmotic pressure but the global mechanical proprieties of the cell (Moeendarbary et al., 2013; Sachs and Sivaselvan, 2015). Ion fluxes also can be involved in cell mechanics regulation by acting on the biochemical properties of proteins. If actin filaments through the whole cell and not only on its periphery are important for maintaining cell shape it might be that osmotic changes involve much more complex processes than water influx.

In conclusion, in culture conditions, cell shape is a resultant of a balance between cell pressure, cortex tension and the tethering of the membrane to the cortex and the external hydrostatic pressure. In tissues this external pressure can be much higher and anisotropic. In order to understand how mitotic cells round up in tissues it is therefore important to take into account the mechanical properties of their environment

Figure 3.4.

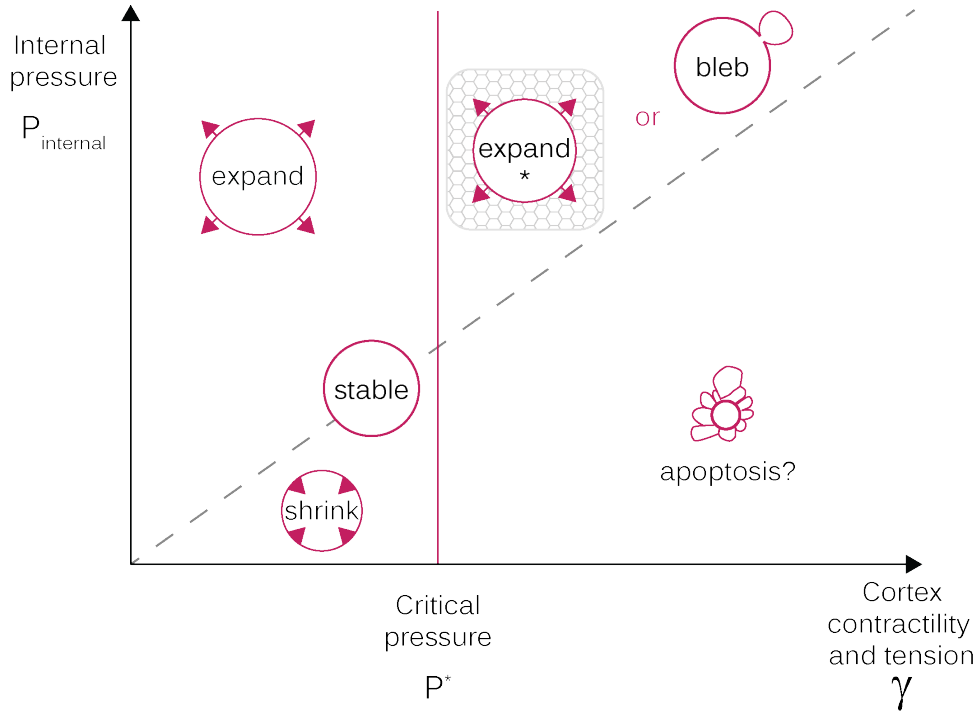


Figure 3.4: Graph summarising the potential interplay of cortical and membrane tension with internal pressure. As the tensions supported by the membrane are much lower than those supported by the cortex, for simplicity, the graph mentions only the cortical tension. At a steady state, if the internal pressure ( $P_{internal}$ ) is counteracted with the cortical tension ( $\gamma$ ) a cell has a stable shape (gray dotted line). If the pressure increases, the cell should expand until the critical pressure ( $P^*$ ) that can be sustained by the links tethering the cortex to the membrane is achieved. Above this pressure, if a cell is not surrounded by any matrix, the membrane detaches from the cortex and blebs are observed. If the cell is surrounded by a deformable matrix, the cell might expand. If the internal pressure decreases, it might be dominated by the cortex tension, which would result in shrinking. As actin contractility regulation has been involved in apoptosis (Coleman et al., 2001), it might be that the decrease of internal pressure with the increase of cortex contractility result in the detachment of the cell membrane and the strong blebbing observed in apoptosis.

$$\begin{aligned}
 P = T \leq P^* &\implies \text{stable cell shape} \\
 P > T \leq P^* &\implies \text{cell expansion} \\
 P > T > P^* &\implies \text{blebbing if not confined or expansion if external constraints} \\
 P < T \leq P^* &\implies \text{cell shrinking}
 \end{aligned}$$

Measuring hydrostatic pressure alone in cells embedded in tissues is problematic. A recently developed fluorescent protein can be used to detect high hydrostatic and osmotic pressures changes (50 MPa) (Watanabe et al., 2013), this is however out of the range important for mitotic rounding in animal cells. Until a further refinement of such probes or other techniques is not available, dissecting the role of the cortex tension and internal pressure for shape change will not be straightforward.

It is possible however that refinements of already used methods could be done. For instance, measuring cells in PDMS, stiff wells as showed in Figure 3.5 could give a better approximation of total forces that a cell is developing when swelling. An alternative method would be to embed cells in tissue mimicking matrices of different and controlled mechanical properties. Tools enabling measurement of forces applied

by the cell on its environment are available. For collagen matrices, the displacement of collagen fibres giving the force needed to such deformation may be followed in bright field or fluorescence (Kopanska et al., 2015). Using small fluorescent beads in hydrogels as done in (Legant et al., 2010) is also a potentially interesting approach. With this techniques, displacements in the  $\mu\text{m}$  range and forces of several kPa can be measured in 3D and in environments of finely tuned mechanical properties. In fact some preliminary data were obtain by embedding cells in collagen matrices by (He et al., 2015), showing that collagen fibres get reorganised as cells round up in mitosis, but no precise force measurement has been done.

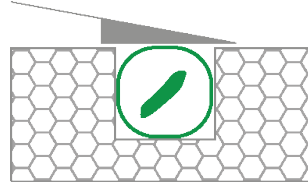


Figure 3.5: A scheme where a well in PDMS is of the volume close to the cell is G2. When entering mitosis and rounding up, the cell would generate forces that could be measured by AFM.

If measuring forces developed by mitotic cells could be done in such set-ups, they could provide important data on cell mechanics and our hypothesis. But they could also be used to test potential candidates important in mitotic rounding regulation. We observed different amplitudes of mitotic volume increase in cells from tissues of different rigidities. If these differences in volume overshoot correlate with the amount of forces that a cell could develop, it would indicate that there are stronger and weaker cells. It might be that blood cells do not have to develop forces as high as epithelial cells because they do not have to withstand the same loads. This would point out toward a fascinating possibility that mitotic cell strength can be regulated on a genetic level. This in turn would be important for the description and the understanding of the importance of mitotic cell rounding in tissue organisation.

### 3.5 Mitotic cell rounding in tissue homoeostasis context

Demonstrating that mitotic cells may increase their volume sets a ground for the hypothesis that cells might use osmotic pressure to generate forces when confined. This in turn could explain how mitotic cells can deform their surrounding in order to obtain the space and geometry that is necessary for an optimal cell division. We suppose that a cell with a soft cortex, if it is surrounded by regions of different rigidities, will adapt to its environment instead of imposing a spherical shape. In contrary, if a cell has a very contractile cortex, but no internal pressure can counteract it, the cell should collapse. We propose that in order to impose a spherical shape to its environment, a cell has to assemble a homogeneous and resistant cortex and generate outwardly directed pressure [Figure 3.3](#).

As mentioned in [subsection 1.2.2](#) mitotic cell rounding is important in tissue morphogenesis. Coordinated in space divisions may lead to tissue growth, shaping, invagination of monolayers and cell differentiation. I believe that understanding how cells can generate forces that deform their environment will be of interest to better understand processes enrolled in development. But on the single cell level, the role of rounding can be crucial for proper chromosomes segregation, spindle positioning, cell differentiation, tissue homoeostasis or morphogenesis (described in more details in ([Cadart et al., 2014](#))). Defects in rounding may engender mitotic delay and errors in chromosome segregations ([Lancaster et al., 2013](#)). These in most cases result in cell death. But exceptions happen, leading to aneuploid (with irregularities in the inherited DNA) cells ([Rieder and Maiato, 2004](#)). Escaping cell death after an erroneous division is one hallmark of cancer onset, if the new DNA set result in an up-regulation of cell proliferation, it is another hallmark of tumorigenesis ([Hanahan and Weinberg, 2011](#)) [Figure 3.6](#). Understanding mechanisms underlying mitotic cell rounding might therefore be important in the light of cancer onset.

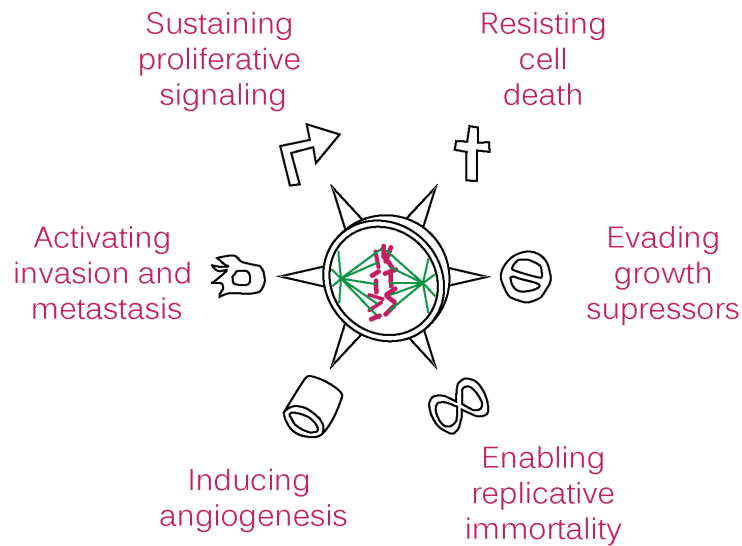


Figure 3.6: The six hallmarks of cancer, adapted from (Hanahan and Weinberg, 2011). Mitotic Cell Rounding potentially plays a role in at least three of these hallmarks. If cancer cells can round up and divide in conditions that would normally induce a cell cycle arrest in normal cells, they sustain proliferative signalling. If they divide with errors due to defects in rounding and survive, they resist cell death that would be imposed to a normal cell. If cells enter in mitosis ignoring tissue stiffness they might need to evade growth suppressors. Furthermore, it is probable that the same mechanism of force generation used in cell rounding might be used in migration and promote invasion and metastasis.

Furthermore it is becoming clear that cells can sense the stiffness of their environment and adapt not only their migration (Pelham and Wang, 1997) or differentiation (Engler et al., 2004) but also their proliferation rate (Aragona et al., 2013; Halder et al., 2012). What if cells could predict if they will be able to round up in a given environment in order to decide to divide or not. In fact, we observed that highly transformed HeLa cells enter mitosis even under extreme confinement of 3.5  $\mu\text{m}$ . In contrast, Rpe1 cells that are not transformed do not enter mitosis under such confinement (personal communication of Maël Le Berre). Could it mean that cancer cells might overcome a mechanical checkpoint that would normally prohibit mitotic entry in an unfavourable environment?

Once a tumour is developing in a tissue, its uncontrolled growth and resultant cell multiplication convey it new mechanical properties (Stylianopoulos et al., 2012; Plodinec et al., 2013). Tumours are in general stiffer than the surrounding healthy tissue (Nagelkerke et al., 2015). It might therefore be even more important for a tumour cells than a normal cell to be able to round up in a stiff environment. We imagine that understanding which molecular mechanisms are important for rounding up under confinement will first help understanding how cancer cells might deal with such constraints and second, might point toward a novel therapeutic targets. If cell rounding up for division is more difficult in a tumour than in the healthy tissue, affecting it even lightly, could be detrimental for cancer cells.

First, mutations or deregulations of proteins implicated in actin cortex strengthening in mitosis are linked to cancer progression. It is the case of Eps8, that has to be degraded in early M phase in order to facilitate cell rounding and mitotic progression (Werner et al., 2013a). As proposed by (Werner et al., 2013a) Eps8 could be eliminated from the actomyosin cortex in order to promote Moesin and Ect2 dependent reorganization of actin (Kunda et al., 2008; Matthews et al., 2012) (subsection 1.3.2).

As mutations in ion channels can be involved in all the hallmarks of cancers (Prevarskaya et al., 2010) it is tempting to speculate that their role in mitotic cell rounding might be important. For example an increased activity of channels involved in mitotic rounding under confinement might confer to tumour cells the ability to divide even in very stiff environment. As the contribution of ion channels to tumour



development is now established, important efforts are made to discover drugs that could target cancer cells by affecting ion channels functions (Leanza et al., 2015) Figure 3.7. It is however important to keep in mind that it is not possible to study changes in ion regulation without taking into account cytoskeleton, and particularly actin organisation as they are tightly linked (Hoffmann et al., 2009).

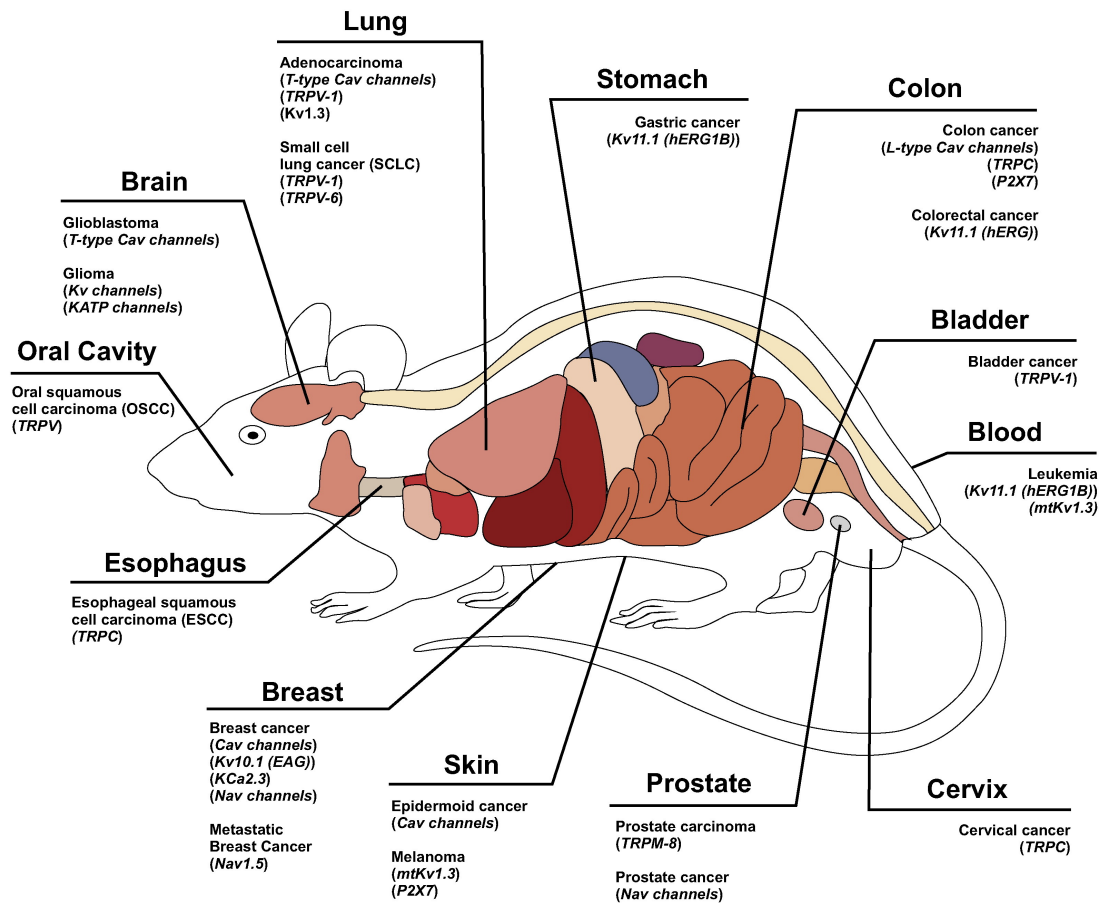


Figure 3.7: This is a figure from (Leanza et al., 2015) summarizing ion channels with altered expression in cancers arising in different tissues. Listed channels pharmacological targeting provide significant results in the diminution of tumor sizes or metastasis.

*In vitro* models such as cell spheroids can be used to mimic and study tumour cell behaviour. In (Delarue et al., 2014) authors demonstrate that cancer cells growing into spheroids can react and adapt their growth to mechanical changes in their environment. In time-scales of hours after a mechanical stress, cells alter gene expression leading to a decreased cell proliferation. Cells in spheroids however adapt to the mechanical stress and recover their proliferation rates over few days. Once potential candidates important for the mitotic volume increase will be identified, an approach based of spheroids could bring insightful informations. For example by mixing two population of cells, where one would be deficient in swelling. This might show if molecular mechanisms important for volume increase in non-confined mitotic cells are providing cell proliferation advantages in confined environments mimicking tissues. Spheroids might also be used to test effects of drugs affecting volume increase. Furthermore, the resistance to mechanical stress of the whole spheroid could be altered if mitotic volume increase is perturbed. This could be measured as in (Monnier et al., 2016).

Furthermore, recent work of (Staunton et al., 2016) showed that cells stiffen in a Rho dependent manner during the invasion of collagen gels. It is possible that cells might need to regulate the interplay between cortex stiffness and their internal pressure in others, cancer-related processes.

In conclusion, uncovering molecular mechanisms enabling cells to round up under confinement is expected

to impact our understanding of processes as tissue morphogenesis and diseases as cancers.

### 3.6 Perspectives

In this work we provide a novel technique that could be applied not only to studying mitosis in mammalian cells. It is currently applied in the team to study cell growth regulation and dynamics. This is a fascinating field that missed tools that would enable live following of individual cell volumes through several cell cycles. FXm can be adapted to follow changes in volume regulatory mechanisms activated upon different kinds of stimuli as osmotic variations in the external media, infection or drugs. It can be used to track height changes in migrating cells or excited neurons. Moreover it can be adapted to study organisms of different sizes as bacteria or developing embryos. These bring new possibilities and therefore might potentially bring answers to an important number of questions:

Are cells using osmotic pressures to create forces? How cells decide when to divide? How cells sense their size? How cells adapt their volume? What is the interplay of cytoskeleton and osmotic pressure in cell shape regulation? Does size matter?

Results of this PhD project bring also novel data about the mechanism of cell rounding. Cells transiently increase their volume when entering mitosis in un-confined environments. This has never been shown prior to our and (Son et al., 2015) results. Furthermore our results indicate that this volume increase is mediated by water influx. This in turn leads to the speculation that it could be driven by a change in cell osmolarity. It is possible that cells use this as a tool in tissue conditions. Cells could swell to deform their environment and achieve a optimal shape for mitosis. We show that volume increase is independent of actomyosin cortex functionality. But under anisotropic confinement such a cortex is crucial for acquiring a spherical shape. Cells could therefore combine two mechanisms to generate forces enabling them to round up under confinement. First the actomyosin cortex stiffening that increases surface tension and second osmotic pressure increase to push the cortex. That could be compared to a stiff shield to counteract external confinement.

Turgor pressure importance in plants physiology is widely studied and appreciated. The importance of differential hydrostatic pressures over the tissue for plant morphogenesis has been proposed based on first experimental data (Beauzamy et al., 2014) but techniques to study internal hydrostatic pressures in cells are only being developed. Wall-less plant cells have similar mechanical properties than animal cells (Durand-Smet et al., 2014). However plants can develop much higher internal pressures to drive tissue morphogenesis thanks to resistant to tension external layers (Beauzamy et al., 2015). Our results point toward the fact that animal cells, as plant cells, could use osmotic pressures to generate forces if surrounded by resistant environment. As the ability of such a force generation is important for diverse processes, uncovering the molecules governing it is of great interest. As our results do not provide the molecular mechanisms underlying it, they are an open question in cellular biophysics.



# Chapter 4

## Methods

<a href="#">4.1 Cell confinement</a>	69
<a href="#">4.2 Volume measurement using FXm</a>	87
<a href="#">4.3 Volume measurements on patterns</a>	99
<a href="#">4.4 Volume measurements and media changes</a>	99

The following sections describe principal methods developed or optimised during this PhD; cell confinement and volume measurement by FXm. As the general protocols of the two methods are published or being prepared for publication I include these below. Some modifications can be applied to the two methods and will be briefly described.

### 4.1 Cell confinement

The methods of cell confinement have been published in *Methods in Cell Biology* in 2014 ([Le Berre et al., 2014](#)).

# Methods for Two-Dimensional Cell Confinement

# 14

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## CHAPTER OUTLINE

<b>Introduction</b> .....	<b>214</b>
<b>14.1 Slide Design for Geometrical Confinement</b> .....	<b>216</b>
<b>14.2 Geometrical Confinement Slide Fabrication</b> .....	<b>217</b>
14.2.1 Materials .....	217
14.2.2 Equipment .....	218
14.2.3 Fabrication .....	218
14.2.3.1 Slide Treatment .....	219
<b>14.3 Soft Confinement Slide Fabrication</b> .....	<b>220</b>
14.3.1 Materials .....	220
14.3.2 Equipment .....	220
14.3.3 Method .....	221
<b>14.4 Dynamic Cell Confiner</b> .....	<b>222</b>
14.4.1 Materials .....	223
14.4.2 Design and Fabrication .....	225
14.4.3 Handling .....	225
<b>14.5 Multiwell Confiner</b> .....	<b>226</b>
14.5.1 Materials .....	226
14.5.2 Fabrication .....	227
14.5.3 Handling .....	228
<b>14.6 Discussion</b> .....	<b>228</b>
<b>General Conclusion</b> .....	<b>229</b>
<b>References</b> .....	<b>229</b>

## Abstract

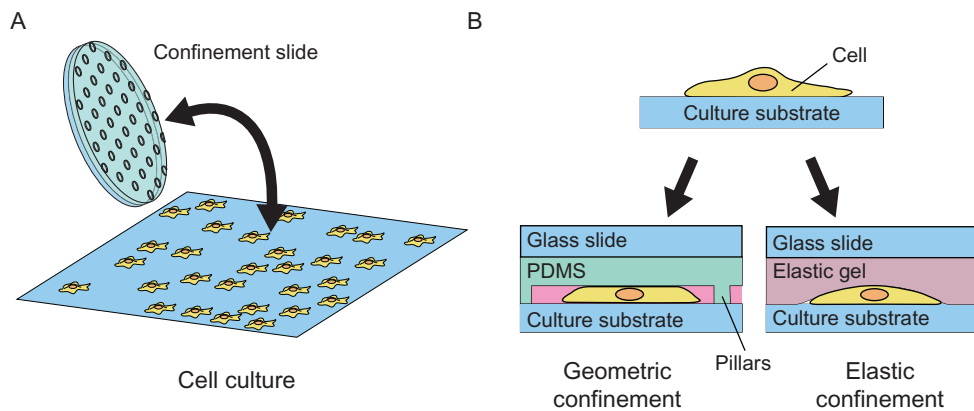
Protocols described in this chapter relate to a method to dynamically confine cells in two dimensions with various microenvironments. It can be used to impose on cells a

given height, with an accuracy of less than 100 nm on large surfaces ( $\text{cm}^2$ ). The method is based on the gentle application of a modified glass coverslip onto a standard cell culture. Depending on the preparation, this confinement slide can impose on the cells a given geometry but also an environment of controlled stiffness, controlled adhesion, or a more complex environment. An advantage is that the method is compatible with most optical microscopy technologies and molecular biology protocols allowing advanced analysis of confined cells. In this chapter, we first explain the principle and issues of using these slides to confine cells in a controlled geometry and describe their fabrication. Finally, we discuss how the nature of the confinement slide can vary and provide an alternative method to confine cells with gels of controlled rigidity.

## INTRODUCTION

Numerous methods have been used to study cells in a confined environment. The most common methods have certainly been three-dimensional (3D) cell cultures, which involve embedding cells in a gel or another 3D material (see [Justice, Badr, & Felder, 2009](#)). Although these methods offer environmental conditions for cells that are close to those of intermediate tissues *in vivo*, the various physical parameters of the cell environment (geometry adhesion, environment elasticity, etc.) are not homogeneous and well defined, making it difficult to decipher their respective roles. Moreover, observation is challenging since cells can migrate in the three dimensions, limiting the possibilities of high-resolution microscopy. To circumvent these limitations, other methods have been developed. Among them, many have been driven to a large extent by technological developments that allow controlling forces or geometries at the micrometer scale, for example, apparatuses such as atomic force microscopes, microplates, or micromanipulators that have been designed by physicists to manipulate single cells and constrain them in various manners (see [Mitrossilis et al., 2010](#)). The main advantage of these approaches is the possibility to dynamically measure and control forces on a single cell. On the other hand, statistical and molecular analysis of a single cell is challenging. Microfluidic channels offer a different strategy to answer specific questions using simple ways to confine cells in a given geometry (see [Velve-Casquillas et al., 2010](#)). These channels allow the observation of many cells at the same time and offer tools to control specifically the microenvironment of the cell (chemical gradients, temperature, electric fields, etc.). Even though these microchannels are suitable to obtain statistics and build complex environments, the dynamic change of cell confinement and molecular analyses remain challenging in these conditions.

Here we describe an intermediary method that allows confining a population of adherent cells in a culture without the need to detach them from their original culture substrate. Because the confinement is applied on a population of cells, statistical data are easy to obtain, and because confinement can be released, the cell material is usable for further study (immunofluorescence, Western blot, qPCR [quantitative polymerase chain reaction], or other molecular analysis). The method is based on the gentle application of a confining slide on the cultured cell ([Fig. 14.1A](#)) using a

**FIGURE 14.1**

Principle of dynamic confinement with confinement slides. (A) Confinement slides are glass coverslips modified to provide a homogeneous confinement. They can be applied reversibly on the cell culture. (B) Slides can be modified either with a microstructured layer or PDMS, providing a geometrical confinement, or with a soft gel, providing an elastic confinement.

suction cup device or a modified multiwell plate. The confinement slides are made of a glass coverslip, which is covered by a layer of material ensuring confinement. We will show how to use the microstructured layer of polydimethylsiloxane (PDMS), which is a relatively stiff biocompatible silicone rubber, to confine cells between two parallel planes (Fig. 14.1B). In this case, cells can move in two dimensions and the height of the cells is precisely imposed (geometric confinement). Alternatively, we will also show how to use a layer of polyacrylamide (PAM) gel to impose on the cell an environment of controlled stiffness (elastic confinement). In this second case, cells are covered by a gel of controlled rigidity and have to push on the gel to define their own space. In both cases, the rigid glass slide provides homogeneous confinement with no long-range deformation and the soft material (PDMS rubber or gel) ensures the robustness of the confinement by absorbing the local substrate inhomogeneities due to the possible presence of dust or particles. Except microfabricated molds, which need photolithography equipment, the fabrication of the confinement slides and handling devices is simple enough to be done in a cell biology lab with a small investment. Thus, this confinement method is well adapted to study of the roles of geometry and cell environment in cell processes such as migration (see [Le Berre et al., 2013](#)), division (see [Lancaster et al., 2013](#)), or adaptation (see [Le Berre, Aubertin, & Piel, 2012](#)).

The first section describes how to design microfabricated molds to produce confinement slides for geometric confinement. The following two sections specify the fabrication of the slides (for geometric confinement or gel confinement, respectively). Finally, two sections explain methods to handle these slides (either dynamically with a suction cup device or in multiwell plates to assay multiple conditions at the same time) followed by a discussion of how these slides can provide more complex configurations of confinement.

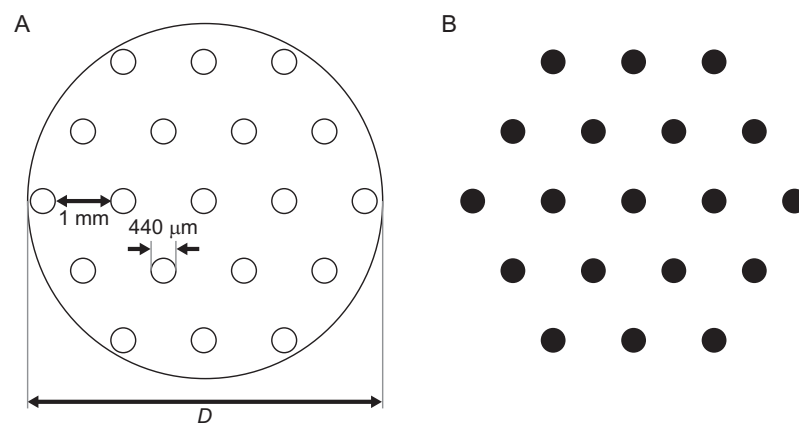
### 14.1 SLIDE DESIGN FOR GEOMETRICAL CONFINEMENT

For geometrical confinement, microspacers have to be molded under the confinement slides in a PDMS layer in order to control the distance between the slide and the culture substrate.

To get a robust confinement, the geometry of these spacers needs to be controlled. If the pillars are too close, many cells will be squashed by the pillars, but if they are too sparse, the glass slide sags in between the pillars, and the thickness of the cells is weakly controlled. In addition, if the surface of the pillars is large, a large proportion of the cells will be squashed, but, if the pillars are too small, they deform under pressure, giving an uncontrolled confinement height.

An optimal geometry of these spacers has been determined by *in silico* mechanical simulations to get pillars as far as possible from each other while making negligible long-range deformation of the confinement geometry ( $<100$  nm when a 10-kPa pressure is applied on the slide) and a small amount of squashed cells (6%) (see [Le Berre et al., 2012](#)). Typically, spacers of  $440\ \mu\text{m}$  in diameter spaced by 1 mm in a hexagonal network provide meet these specifications (see [Fig. 14.2A](#)). Note that this geometry is adapted to confine cells to thicknesses above  $3\ \mu\text{m}$ . Below this height, sagging is more pronounced, and a capillary effect often sticks the PDMS layer on the culture substrate, squashing the cells on the whole surface.

Based on this geometry, we need a mold to imprint the structures on the confinement slides. This mold can be produced by photolithography of a layer of photoresist deposited on a silicon wafer by using standard microfabrication protocols. These protocols require a photomask that is used to shade a layer of photoresist from



**FIGURE 14.2**

Optimal geometry of the pillars for geometrical confinement. (A) Dimensions of the pillars in a round slide of diameter  $D$ . Typically,  $D=10$  mm for the suction cup device, and  $D=18$  mm for a 6-well plate, and  $D=12$  mm for a 12-well plate. The number of pillars has to be adapted depending on  $D$ . (B) Photomask to produce a negative photoresist layer (such as SU8) corresponding to the design in (A).



the applied UV light and define the shape of the spacer after development of the photoresist. With this fabrication mode, the thickness of the photoresist layer corresponds to the height of the pillars and, therefore, the thickness of the cells after confinement. If manipulated carefully, a mold can be used many times. Thus, it is not necessary to have access to a microfabrication facility, and the mold, which is technically easy to fabricate, can be created by a specialized collaborator or a microfluidic foundry company. The mold provider usually needs a drawing of the photomask in DXF (Drawing eXchange Format) or GDSII (Graphic Database System II) format, which can be done easily with CAD software like AutoCAD (Autodesk) or L-Edit (Tanner). But due to the poor resolution required to produce the mold, the mask can also be printed on a plastic sheet with a high-resolution printer by using a simple drawing software like Adobe Illustrator. This way, many academic microfabrication facilities can make such a mold for less than \$200. (For more details on mold fabrication, see, for example, [Velve-Casquillas et al., 2010.](#))

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## 14.2 GEOMETRICAL CONFINEMENT SLIDE FABRICATION

This section describes the fabrication of stiff confinement slides for geometrical confinement.

### 14.2.1 Materials

- A mold to imprint slides (see previous section 14.1).
- 10-mm-diameter standard glass coverslips.
- PDMS (RTV615, General Electrics or Sylgard 184).
- Wash bottle of isopropanol.
- Scalpel blade.

For slide treatment (optional):

- Parafilm.
- Wash bottle of pure water.

Adhesive:

- Fibronectin solution 50  $\mu\text{g}/\text{ml}$  in phosphate buffered saline (PBS) (30  $\mu\text{l}$  per slide) (Fibronectin from Sigma).

Nonadhesive:

- P11-g-PEG solution 500  $\mu\text{g}/\text{ml}$  in HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer 10 mM, pH 8.6 (30  $\mu\text{l}$  per slide) (pLL-g-PEG (20)-[3.5]-(2) from SuSoS).

For antiadhesive coating of the mold (optional):

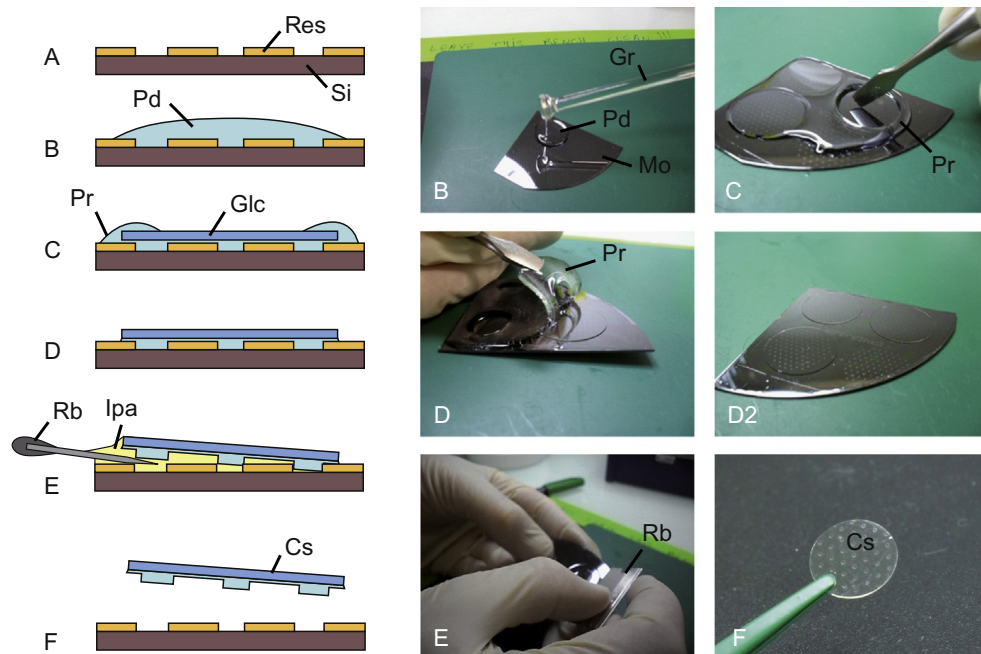
- Trimethylchlorosilane (TMCS) (Sigma).
- Fume hood.

### 14.2.2 Equipment

- Plasma cleaner (for example, PDC-32G, Harrick).
- Pressurized, filtered, and oil-less air stream.
- Hot plate.

### 14.2.3 Fabrication (Fig. 14.3A–F)

1. Prepare 10 g of PDMS mix with a ratio of PDMS/cross-linker (A/B) of 8/1 (w/w), avoiding introducing too many bubbles during mixing. PDMS can be mixed either manually with a simple swizzle stick or with an automatic planetary mixer (e.g., ARE-250, Thinky). If the PDMS is mixed manually, bubbles can be removed by centrifugation (2 min at 1000 rpm). Be careful in



**FIGURE 14.3**

Confinement slide fabrication. The schemes and corresponding photos summarize the fabrication process: (A) Start with a microfabricated mold (Mo) made of a silicon wafer (Si) covered by a patterned layer of photoresist (Res). (B) A drop of PDMS (Pd) is placed on the mold, for instance, with the help of a glass rod (Gr). (C) A plasma-activated glass coverslip (Glc) is pressed onto the drop of PDMS with the help of tweezers in order to keep only a residual layer of PDMS under the coverslip. The excess PDMS should make rims around the coverslips (Pr). (D) After baking, the PDMS rims (Pr) are removed. (D2) No residual PDMS should be visible on the mold at this stage. (E) To unmold the confinement slide (Cs), the slide is wet in isopropanol (Ipa) before a razor blade (Rb) is introduced between the mold and the slide. (F) After unmolding, the confinement slide (Cs) is rinsed with isopropanol and air dried.

this case that PDMS is well mixed because unreticulated PDMS is very difficult to remove and can make your mold unusable.

2. Place the 10-mm glass coverslips on a big glass slide and treat it in the plasma cleaner for 2 min at maximum power in order to activate one side of the slide (when the plasma is on, the plasma may produce a dense pink glow). This step allows the glass slide to stick onto the PDMS layer in the next step.
3. With a glass rod or equivalent, distribute drops of PDMS on the mold in the places you want to put the plasma-treated coverslips (Fig. 14.3B).
4. Place the coverslips on top of the drop and push gently with tweezers everywhere on top of the coverslips to keep only a very thin layer of PDMS under the coverslip. If you push too hard on the coverslip, it will break, and, if you push too gently, the residual layer of PDMS will be too thick, and the pillars will collapse. To gauge the right pressure, notice that it corresponds to the minimum pressure where you see a small white star appearing under the tweezers. Do not use sharp tweezers: You will break your coverslips! The ejected PDMS should form a rim partially covering the slide (Fig. 14.3C).
5. Bake the mold with the coverslips on the hot plate at 95 °C for 15 min. At this step, if the PDMS is baked too long or not long enough, it will stick too strongly to the mold, and it will be very difficult to unstick confining slides without breaking them.
6. Remove the PDMS rim with tweezers (Fig. 14.3D).
7. Place a droplet of isopropanol on the mold; it will help to unmold the slide.
8. Use a razor blade to gently unstick the coverslip from the mold. To avoid scratching the mold (molds made of photoresist are very easy to scratch) or breaking the coverslip, use the following method: At the beginning, place the razor blade on the edge between the mold and the coverslip at an angle of 20°, and then carefully slide the blade under the confining coverslip parallel to the mold surface (Fig. 14.3E).
9. Rinse the coverslip with isopropanol.
10. Dry the confining coverslip with an air gun (otherwise it will leave stains and inhomogeneities on the confining slide surface) (Fig. 14.3 F).
11. Gently clean the mold with a clean wipe soaked with isopropanol.
12. *Optional:* If the mold becomes too sticky and coverslips are difficult to detach, use an antiadhesive agent. For this step, work under a fume hood: TMCS is very toxic. Carefully clean the mold with isopropanol and a soft cleaning paper wipe (e.g., Kimtech, Kimberly-Clark), and put it in a petri dish. Seed two or three drops of TMCS around the mold, and close the petri dish. The drops will evaporate and react with the surface. After 5 min, open the petri dish, and vent the remaining TMCS vapors for 5 min, then bake the mold for 10 min at 70 °C to stabilize the surface.

### 14.2.3.1 Slide treatment

The coverslip can be used either without any treatment (it is not toxic to the cells) or with a chemical treatment to promote or avoid cell adhesion. However, we advise treating the confining slide systematically in order to avoid trapping air bubbles

between the slide and the substrate, which can kill the cells (PDMS is very hydrophobic and often retains air bubbles on its surface).

1. For nonadhesive treatment only: Place the confinement slide on a big glass slide with the PDMS side facing upward, and treat it in the plasma cleaner for 30 s maximum at maximum power in order to activate the PDMS surface.
2. On a piece of parafilm, drop 30  $\mu$ l of the fibronectin solution (for adhesive treatment) or of the pLL-g-PEG solution (for nonadhesive treatment).
3. Place the confinement slide on the drops, the structured slide in contact with the solution, and wait for 30 min.
4. Rinse extensively with pure water.
5. Dry the slide with an air jet.

---

### 14.3 SOFT CONFINEMENT SLIDE FABRICATION

This section describes the fabrication of soft confinement slides for elastic confinement.

#### 14.3.1 Materials

- a. Coverslips (any glass coverslip size can be used).
- b. Ethanol 70%.
- c. Deionized water.
- d. Acrylamide 40% (Biorad).
- e. Bis-acrylamide 2% (Biorad).
- f. 10 mM HEPES buffer pH 7.6.
- g. Irgacure 0.5% in 10 mM HEPES pH 7.6 (Irgacure 2959, CIBA). The Irgacure stock solution has to be protected from light.
- h. Aminopropyltrimethoxysilane (APTMS) (Sigma) 4% solution in acetone.
- i. Clean and smooth PMMA (polymethyl methacrylate) plate (Good Fellow) (Also available under the commercial name of “plexiglas”).
- j. PDMS (RTV615, GE).
- k. Wash bottle of isopropanol.

#### 14.3.2 Equipment

- l. Ultrasonic bath.
- m. Plasma cleaner (e.g., PDC-32G, Harrick).
- n. UV lamp (e.g., Delolux 03 S).
- o. Fume hood.
- p. Vacuum bell cloche.

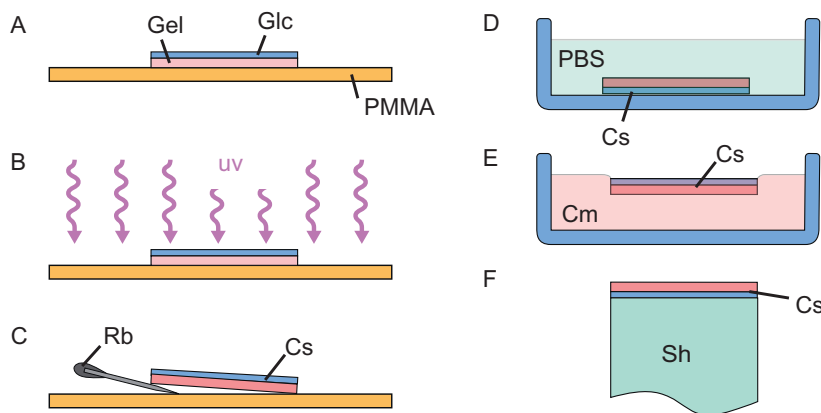
### 14.3.3 Method

#### 1. Coverslip surface preparation

- Wash the glass coverslip with ethanol 70% and dry it.
- Expose the dry coverslips to air plasma for 30 s, maximum power.
- Incubate clean slides in a solution of APTMS (4% in acetone) for 30 min under sonication.
- Wash with distilled water, and air dry (slides can be stored for several weeks at room temperature).
- Place the slide on a drop of glutaraldehyde solution (0.5% in PBS) for 30 min. It is convenient to do it on a laboratory parafilm. Drops of 100  $\mu$ l are sufficient for coverslips of 18 mm. Keep track of the side of the coverslip that was treated. Coverslips should be used right after this step. (Glutaraldehyde is toxic and an irritant, and it has to be manipulated under a fume hood.)
- Wash coverslips with distilled water, and air dry.

#### 2. Gel polymerization (Fig. 14.4A–E)

- Prepare the PAM mix in a stock solution of Irgacure (0.5% in 10 mM HEPES pH 7.6). The concentration of acrylamide and bis-acrylamide depends on the gel rigidity you want to obtain (see Table 14.1 and Boudou, Ohayon, Picart, & Tracqui, 2006).



**FIGURE 14.4**

Fabrication of confinement slides with gels of controlled rigidities. (A) First a droplet of the gel precursor mix (Gel) is deposited on a PMMA plate and covered by a pretreated glass coverslip (Glc). (B) The gel is polymerized under UV. (C) After polymerization, the confinement slide (Cs) can be detached from the PMMA plate with a razor blade (Rb). (D) The gel is incubated in PBS to remove any remaining diffusible molecules. (E) Before the experiment, the gel is incubated in the appropriate culture medium (Cm) upside down on the surface of the medium to avoid getting the back side wet in the medium. (F) When positioned on the confinement apparatus, the dry side of the slide sticks naturally to the PDMS surface of the slide holder (Sh).

**Table 14.1** Quantities of acrylamide, bis-acrylamide, and irgacure solution used to get 1 ml of gel depending on the required rigidity

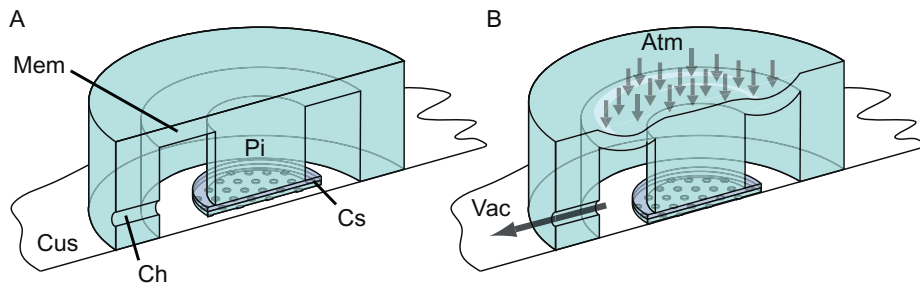
Young's modulus (kPa)	Irgacure 0.5% ( $\mu$ l)	Acrylamide 40% ( $\mu$ l)	Bis-acrylamide 2% ( $\mu$ l)
2	840.0	125.0 (5%)	35.0 (0.07%)
5	784.5	185.5 (7.5%)	30.0 (0.06%)
10	767.0	185.5 (7.5%)	47.5 (0.95%)
15	749.5	185.5 (7.5%)	65.0 (0.13%)
20	714.5	185.5 (7.5%)	100.0 (0.2%)
30	650.0	250.0 (10%)	100.0 (0.2%)

- b. Put drops of the PAM solution on the PMMA plate, and gently place the coverslips on them with the treated surface toward the PAM solution (the coverslip should float on the drop). The volume of the drops can be adapted according to the thickness of the gel you want to obtain (200  $\mu$ l for an 18-mm coverslip will result in gels of  $\sim$ 0.8 mm during the fabrication, but note that the gel will swell to  $\sim$ 2 mm after incubation) (Fig. 14.4A).
- c. Place the setup under a UV lamp for 10 min (Fig. 14.4B).
- d. Carefully detach the coverslips with gel from the plastic using a razor blade or a scalpel (Fig. 14.4C), and quickly place it in sterile PBS for incubation overnight (Fig. 14.4D).
- e. Before experiments with cells, gels have to be incubated at least 2 h in the cell culture medium that will be used during the experiment. If drugs will be used in the experiment, the gel has to be incubated in the medium containing the appropriate concentration of the given drug. To avoid wetting the back side of the confinement slide, it can be deposited upside down on the medium surface. This way, the slide will be incubated floating on the surface (Fig. 14.4E).

## 14.4 DYNAMIC CELL CONFINER

The application of the confinement slide on the cultured cells can be done in several ways. In all cases, a slight pressure ( $\sim$ 10 kPa) has to be applied on the slide to get a robust and uniform contact with the substrate of the cultured cells, and tangential movement must be eliminated to avoid shearing the cells. A practical way to proceed is to use the so-called cell confiner that is described in this section.

The cell confiner is a device that acts as a suction cup, as shown in Figure 14.5. It is composed of a soft empty part that forms a sealed cavity when it is placed on a surface. When a vacuum is created in the sealed cavity, the device is stuck on the substrate and cannot move. In the center of the empty part, a piston holds the confinement slide, which can be displaced vertically by changing the strength of the vacuum in the device. After the threshold strength of the vacuum is applied, the confinement slide comes into contact with the cultured cells.

**FIGURE 14.5**

Principle of a dynamic cell confiner. (A) The cell confiner acts as a suction cup, which can be stuck onto a cell culture substrate (Cus). The pressure in the device is controlled through a connection hole (Ch). When a vacuum is applied in the device (Vac), the atmospheric pressure (Atm) maintains the device on the culture substrate. A piston (Pi) in the central part of the confiner holds the confinement slide (Cs) face to the culture substrate. The position of this piston relative to the substrate can be precisely tuned by controlling the strength of the vacuum in the device, thanks to the deformation of the membrane (Mem) holding the piston. (B) The confinement slide can be pressed gently onto the cell population by aspirating air through the connection hole.

By using this device, confinement slides can be applied onto any substrate provided that it is flat. The culture substrate just has to be large enough to accept the device (the schematic shown in Fig. 14.6A is adapted to 35-mm-diameter petri dishes).

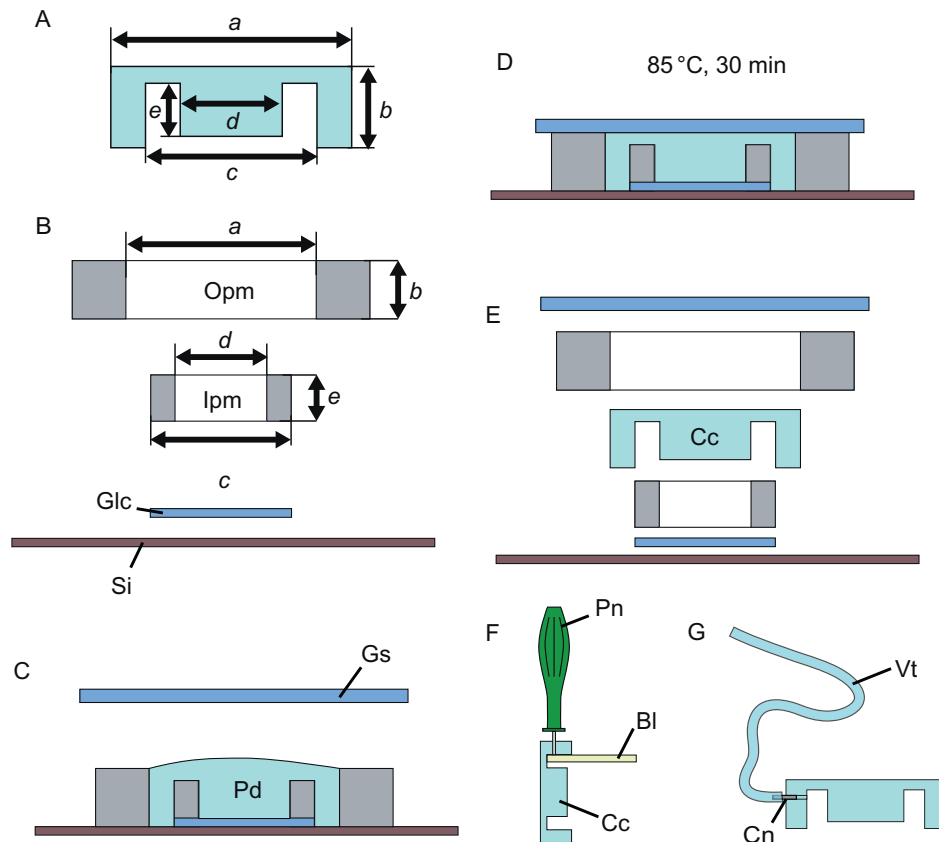
### 14.4.1 Materials

#### *For fabrication:*

- Machined confiner mold: This mold, intended to mold the PDMS device, can be fabricated in stainless steel in a standard workshop. In the simplest version, the mold is composed of two stainless rings, a silicon wafer (which can be replaced by a glass slide), and a 0.5-mm-thick, round glass coverslip of 16 mm diameter (which can be created by gluing several thinner glass coverslips together), according to the layout shown in Figure 14.6B.
- PDMS (RTV615, GE).
- A standard glass slide (typically 1 × 3 in., 1 mm thick).
- A hot plate.
- A 0.75-mm puncher to make the vacuum inlet hole in the device (Elveflow).

#### *For handling:*

- The cell confiner.
- A confinement slide.
- 70% alcohol.
- Absorbing paper.



**FIGURE 14.6**

Cell confiner fabrication. (A) Dimensions of the cell confiner. Typically, for a cell confiner adapted to 35-mm dishes:  $a=22$  mm;  $b=7$  mm;  $c=16$  mm;  $d=8$  mm;  $e=5$  mm. (A) A mold can be easily fabricated by assembling on a silicon wafer (Si) a 0.5-mm glass coverslip (Glc) and two rings forming the outer part of the mold (Opm) and the inner part of the mold (lpm). (B) After the mold has been filled with PDMS (Pd), a glass slide (Gs) is pressed on the top of the structure to flatten the device surface and produce its final shape. (C) The device is baked for 30 min at  $85^{\circ}$ . (D) After cooling down, the structure can be disassembled to retrieve the molded cell confiner (Cc). (F) A 0.75-mm hole is punched into the wall of the device with a puncher (Pn) by inserting a blade (Bl) below the drilling zone for protection. (G) This hole is used to insert a fluid connector (Cn) plugged into the vacuum tube (Vt).

- Removable adhesive tape (e.g., Scotch Magic Tape, 3 M).
- A precision vacuum generator or controller (e.g., Elveflow VG1006). Check before the experiment that you have all the connections plugged into the device. Sometimes, the culture medium is aspirated into the controller by the vacuum, which can cause severe damage to the controller. To avoid this, we strongly recommend placing a security reservoir between the device and the vacuum controller.



- Adapted tubing to plug into the vacuum generator, including a 0.51-mm ID tygon tube (Saint-Gobain) and Stainless Steel Tubing 0.025" OD (see, e.g., microfluidic kits, Elveflow) as a connector to plug the tube into the device (see Fig. 14.6G). It is best to bend the tube to avoid it taking up too much space in the petri dish.
- A sharp pair of tweezers.

### 14.4.2 Design and Fabrication

The design shown in Figure 14.6A is for a 35-mm petri dish. This particular geometry has been optimized to get a working pressure of  $-10$  kPa ( $-100$  mbar), which is sufficient to handle the device easily, but does not notably change the pressure around the cells. For other formats of cell cultures, the device can be scaled up or down: The working pressure of the device is independent of the device size if the proportions are respected.

The suction cup device can be molded with PDMS in a simple mold provided the top and bottom surfaces of the mold are flat enough to get a good optical transparency. Here, we describe a simple method to make a mold based on the simplest pieces, which can be done in any workshop.

1. Prepare a mix of PDMS/cross-linker (10/1 w/w) with no bubbles. (If you do not have a planetary mixer, you can remove bubbles either with a vacuum bell cloche for 2 h or by centrifuging PDMS for 2 min at 1000 rpm.)
2. Stack the different parts of the mold directly on a (cold) hot plate (Fig. 14.6B).
3. Pour PDMS very slowly into the mold, avoiding bubble formation (Fig. 14.6C).
4. Cover the filled mold with a glass slide, and press on it until there remains only a residual layer of PDMS between the mold and the slide (Fig. 14.6D).
5. Heat up the hot plate to  $85$  °C for 30 min (Fig. 14.6D).
6. Cool down the molded part before unmolding. To make unmolding easier, use a razor blade and isopropanol to reduce adhesion of the PDMS on the mold.
7. Punch the inlet hole with the 0.75-mm puncher as shown in Figure 14.6 F.

### 14.4.3 Handling

1. At least 1 h before the experiment, incubate the confinement slide in the cell culture medium that will be used for the experiment to equilibrate the PDMS with the medium. The structured side (PDMS side) of the slide should face upward. Be careful not to lose the orientation of the slide during manipulation; it can be difficult to recover. Note that PDMS absorbs small hydrophobic molecules from the medium. So, if drugs are used in the experiment, these drugs have to be present during this incubation step.
2. Before the experiment, clean the cell confiner with 70% alcohol, and dry it carefully with absorbing paper. Be careful not to leave the device in contact with the alcohol for a long time, or it will absorb the alcohol and become toxic to cells.
3. Clean the bottom face of the device with removable adhesive tape to remove dust particles.

4. Plug the device into the controllable vacuum source, and tune the controller to  $-3$  kPa ( $-30$  mbar). At this pressure, and with a good seal, the device sticks to the substrate, but the confinement slide does not yet touch the cells.
5. Prepare an absorbing paper to dry the back side of the confinement slide.
6. Pick up the confinement slide with tweezers, and dry its back side on the absorbing paper by placing it onto the paper. (If the back side is not totally dry, it will not stick onto the device and can be lost during handling.)
7. Place the confinement slide onto the piston of the cell confiner, structured side up. The slide should stick naturally onto the piston.
8. Put the device in contact with the cell culture substrate. Take care not to deform the device to achieve a good seal of the device cavity. Also, be careful not to push on the center of the device, or cells could be crushed by the device. After the device is well sealed, the membrane sustaining the piston should be slightly deformed. The step of placing the device must be carried out quickly, otherwise the medium will fill the device and enter the vacuum outlet. If the medium enters the vacuum tube, the device may not work properly due to the capillary pressure of the meniscus in the tube. If the medium has entered the tube by accident, the device and the tube must be cleaned and dried to avoid the formation of a meniscus in the tube.
9. At this point, cells can be observed before confinement. The petri dish will not move during the confinement operation, and several cells can be observed simultaneously.
10. To confine cells, slowly decrease the pressure down to  $-10$  kPa ( $-100$  mbar). By observing the cells during this time, one should see the confinement slide touching cells at approximately  $-5$  kPa.
11. Confinement can be released if the pressure is increased back to  $-3$  kPa.

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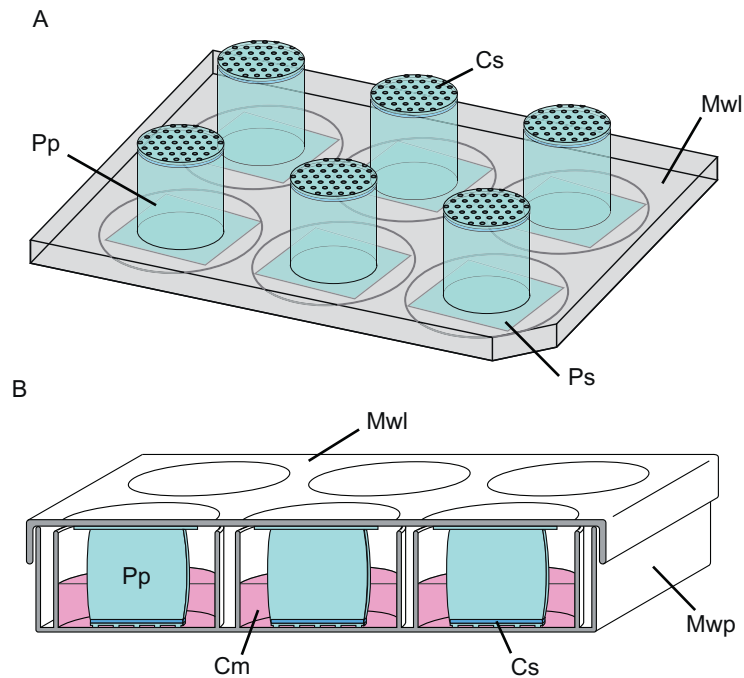
## 14.5 MULTIWELL CONFINER

If a large amount of confined cells or multiple conditions are required, a modified multiwell plate can be used to apply confinement slides on cells (Fig. 14.7). In this case, it is not the force induced by a vacuum that holds the slide on the cells, but the pressure induced by the deformation of a soft pillar. In this case, big PDMS pillars are stuck on the lid of the multiwell plate and hold the confinement slides. When the lid is closed, the pillars push the confinement slides onto the culture substrate and confine the cells. Many wells can be processed simultaneously, and confinement slides of larger surfaces can be used; however, the speed of confinement is not controlled, and it is not convenient to observe what happens during the confinement application. For this reason, this method is not applicable to the study of short-time events. In contrast, it is well adapted to long-term experiments and molecular analysis (e.g., overnight time-lapse imaging, immunofluorescence).

### 14.5.1 Materials

*For fabrication:*

- A 12 (or 6) well multiwell plate (TPP).
- A 48 (or 12) well multiwell plate.

**FIGURE 14.7**

Multiwell plate confiner. (A) A lid of a multiwell plate (Mwl) is modified by introducing large PDMS pillars (Pp) to hold the confinement slides (Cs). These pillars typically have the height of the plate, which can be slightly increased with a PDMS sheet (Ps). (B) When the multiwell plate (Mwp) containing cells and culture medium (Cm) is closed with the modified lid, the confinement slides are applied on the cells adhering to the bottom of the wells. Pillars that are slightly higher than the well depth deform and apply pressure on the confinement slides.

- PDMS (RTV 615, GE).
- Wash bottle of isopropanol.
- Removable adhesive tape (Scotch Magic Tape, 3 M).
- One sheet of gel-pack film (DGL Film X8 17 mils, Gel-Pack).

*For handling:*

- Adhesive tape.

### 14.5.2 Fabrication

1. Prepare the PDMS mix with a ratio of 35:1 w:w PDMS:cross-linker. Due to the small concentration of cross-linker, the PDMS has to be mixed extensively to get a good reticulation.
2. Pour the PDMS mix into the mold, which can be either a smaller multiwell plate or a custom-made mold. For example, for pillars to be used in 12-well plates, use

a 24-well plate as mold and fill them with PDMS. Pour the PDMS in excess, leave it in the vacuum bell cloche to get rid of bubbles, and carefully close with the lid of the plate avoiding bubble creation. For confinement slides including PAM gels, which have a higher thickness, spacers of appropriate thickness can be stuck on the plate lid to reduce the thickness of the final pillars.

3. A small amount of isopropanol will help get the pillars out of the mold. After unmolding and cleaning, the pillars should be soft and sticky.
4. Clean the pillars carefully with isopropanol.
5. On the multiwell plate lid, place a gel-pack sheet on each well position in order to raise the pillars slightly.
6. Place the pillars on the gel-pack sheet at the center of each well position. Clean pillars will stick naturally. You can clean the PDMS and gel-pack sheet before use with the adhesive tape to remove dust.
7. Now the lid is ready to be used.

### 14.5.3 Handling

1. Place one confinement slide on each pillar of the modified lid.
2. Optional: sterilize the lid under UV (365 nm).
3. Optional: incubate the pillars in culture medium to equilibrate the PDMS.
4. Gently close the multiwell plate with the modified lid.
5. While pushing on the lid, fix the lid to the multiwell plate with adhesive tape. During this step, avoid moving the lid laterally: This will shear cells and kill them. Pull on the adhesive tape while applying it to the closed plate to put the plate under tension.
6. Verify under a microscope that the cells are well confined.

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## 14.6 DISCUSSION

In this chapter, we have discussed how to confine cells in between two parallel surfaces separated by a defined gap in a reversible manner. Alternatively, we explained an adaptation of the method to confine cells below a soft PAM gel of controlled stiffness. However, since the two surfaces can be treated independently, and because the treatment of the confinement slides can be very versatile, this method is not limited to these two configurations in its principles; an infinity of confinement configuration can be imagined. The method can be easily combined with others to build more complex environments for specific studies. For the geometric confinement, for example, the flat roof can be replaced by more complex geometric features by using advanced photolithography methods (see [del Campo & Greiner, 2007](#)). Thus, it might be possible to introduce various textures or restrictions into the geometric landscape of the cell environment. Another parameter that can be varied is the level of adhesion. This can be done by mixing in a radiometric manner pLL-g-PEG with pLL-g-PEG-RGD, which is a modified version of pLL-g-PEG modified with the RGD motif and is responsible for integrin-mediated adhesion (see [Barnhart, Lee, Keren, Mogilner, & Theriot, 2011](#)). In this case, the

adhesion level of cells can be modulated independently on both sides of the cell. Confinement can also be combined with other methods. For example, it is possible to print adhesive micropatterns on the bottoms of the wells to restrict geometric cell adhesion. This allows a more reproducible organization of the cell under confinement (see Azioune, Carpi, Tseng, Théry, & Piel, 2010).

## GENERAL CONCLUSION

Confinement parameters are taking on increasing importance in cell biology studies, and tools that allow the control of the different confinement parameters independently while remaining simple enough to be used in a biological routine are crucial. We described in this chapter a versatile way to confine a population of cells in a controlled environment (geometry, stiffness, adhesion, etc.), which is easily combinable with specific observation techniques and molecular biology protocols. We hope that these protocols will help the community answer certain questions and will lead to new and innovative ideas about how to study cells in complex environments.

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## 4.2 Volume measurement using FXm

# Measuring live cell volume changes using Fluorescence eXclusion method

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Cells are small refractive objects constantly adapting their shapes. This renders the measurement of their volume challenging. Due to the importance of volume regulation in cells, several methods have been already developed to follow volume changes in live cells. However these methods all bear important drawbacks. They are often too slow to measure dynamic changes of volumes on short time scales, as scanning ion conductance microscopy (SICM). Other methods, as the Coulter counter, necessitate cells that are detached or growing in suspension. Cell shape reconstruction from z-stacks of confocal images is possible in principle but loaded with important errors. Here we provide a description of a method enabling cell volume measurements in live cells. The method is based on the principle of fluorescent dye exclusion. It can be used for cells of different sizes, adherent or growing in suspension, over several cell cycles and is independent of cell shape changes.

## Contents

1. Introduction: .....	1
2. Method .....	4
2.1. Protocol.....	4
2.1.1. Material.....	4
2.1.2. General comments.....	4
2.1.3. PDMS chamber preparation (Day -1).....	4
2.1.4. Cell injection (Day 0) .....	5
2.2. Imaging.....	7
2.3. Data Analysis.....	7
3. Discussion.....	9
Bibliography .....	10

### 1. Introduction:

Volume is a basic physical feature of all objects, however it has been poorly investigated in cell biology so far, mostly due to the hardness to precisely measure it. Major cellular processes have been shown to be linked volume regulation. In confined environment, tumor cell migration depends on osmotic control and volume regulation through ion pumps activity and localization (Stroka et al., 2014). During division, mammalian cells tightly regulate their volume and exhibit a transient swelling, again through osmotic processes controlled by ion pumps (Son et al., 2015; Zlotek-Zlotkiewicz et al., 2015). These volume modifications whether they are global or local can induce forces and osmotic processes could be an engine to actuate cells. On longer time scales, cellular volume seems to follow cell growth and could be a decisive parameter to answer a fundamental question in cell biology: how are cell growth and cell cycle regulation coupled (Bryan et al., 2012; Gregg and Steidley, 1965).

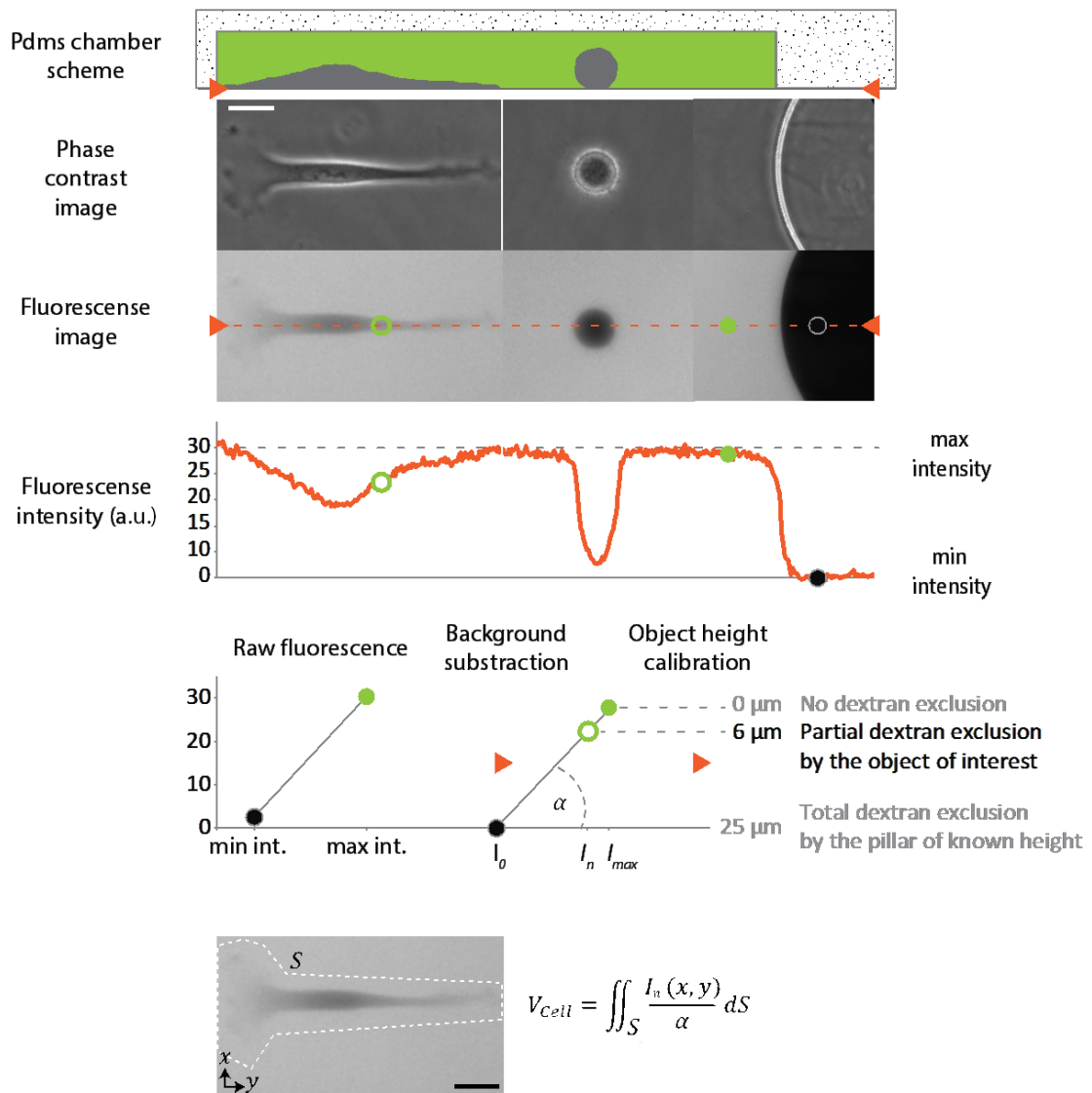
Accurate measurements of cellular volume were lacking mostly because of the technical hurdle to assess such a parameter. Indeed, cells constantly modify their shape to accomplish multiple functions, which makes cell volume very challenging to extract. The Coulter counter is a high throughput technic based on impedance, it gives cell volume independently of the cell shape, but it is limited to populations of cells in suspension (Bryan et al., 2012; Gregg and Steidley, 1965). 3D reconstruction of cell shape from confocal stacks of labeled membrane implies repeated exposures to excitation light and is not suitable for experiments over long time periods. Moreover, accuracy is limited by labelling artifacts and the volume reconstruction precision (Boucrot and Kirchhausen, 2008; Habela and Sontheimer, 2007). Finally suspended microchannel resonator allows very high precision of cell mass, density and volume measurements, but is limited to cells in suspension and requires very specific instrumentation (Bryan et al., 2014; Son et al., 2015).



Animal cells are small objects with sizes of several tens of micrometers. Cell shape is delimited by a semi-permeable cellular membrane. The plasma membrane is further pinned to cell cytoskeleton that is responsible for the over-all architecture of the cell. Cell shape is an important characteristic of cells as it is implicated in cell functions. Indeed cells from different tissues, performing different functions, acquire and maintain specific shapes. Cells therefore actively regulate their shapes, constantly adapting it to their environment. In culture conditions, adherent cells strongly bind to the substrate, which impose a flattened shape, cells growing in suspension stay roundish. In both cases the cell surface is covered by membrane structures that can be very thin and have complex shapes. Moreover cells constantly probe their environment and extend protrusions that are thin structures of varying sizes. As small as these structures can be, their volume can be important due to their big number. Observing live cell volume changes over short periods of time requires a fast method that would not interfere with cellular functions and that would be independent of cellular shape. In order to study cell growth, such a method must tackle several additional challenges: (i) the size measurement method chosen must allow accurate and repetitive measurements without perturbing cell's physiology; (ii) the experimental set-up must be stable over several days because the typical cell cycle length of an animal cell is ranging from 15 to 20 hours; (iii) the low frequency at which complete cell cycles occur in these cells makes it more difficult to obtain a satisfying number of observations and a method enabling measurement on several single cells in parallel might be preferable; (iv) a steady state of growth all throughout the experiment (>30h) must be guaranteed, which requires for example that sufficient amounts of nutrients are provided or that cells in excess are removed to prevent density-related effects on cell growth and proliferation.

In addition to these requirements, compatibility of the method with traditional cell biology tools to assess biochemical processes within the cell such as fluorescent microscopy or transient drug treatments would very much improve the output of the experimental set-up.

Here we describe a method to measure the volume of tens of single cells using simple microfabricated culture chambers. This technique is based on the principle of fluorescence exclusion or displacement (Bottier et al., 2011; Zlotek-Zlotkiewicz et al., 2015) and allow to extract volume of suspended or adherent cells with sizes ranging from few microns to 25 $\mu$ m. Cells are cultured in a poly(dimethylsiloxan) (PDMS) chamber of controlled height with regular culture media supplemented with fluorescent dextran (See figure 1). Cell membrane is impermeable to dextran which prevents its entry into cells. This results in the exclusion of fluorescence and appears dark on a classic fluorescent image. The fluorescence displaced by a cell is linearly proportional to its cellular volume (see figure 1 and (Zlotek-Zlotkiewicz et al., 2015), this principle can be seen as an optical analogue of Archimedes' principle. It is important to note that cell with high endocytic activity are not suitable for this method as dextran would be taken up inside the cell and would not exclude the fluorescence.



**Figure 1** Method principle. Cells are placed in PDMS chambers of calibrated height. Cell volume is calculated based on the fluorescent dye exclusion. The bigger and higher the cell is, the more dye is excluded and the lower the fluorescence signal is. Cell volume is obtained after background subtraction. The calculation is made integrating the total fluorescence intensity of the cell area.

## 2. Method

### 2.1. Protocol

#### 2.1.1. Material

Puncher (diameter: 0.75 or 1mm)

Bottom glass petri dishes (35mm, from WPI or Ibidi)

PDMS and curing agent (Sylgard 184 or RTV-615)

PBS (1X)

Cell culture medium

Dextran conjugate with fluorescent dye in PBS (FITC, Alexa488, Alexa-647... from LifeTechnologies or sigma Aldrich)

Plasma Cleaner

Oven @ between 65 and 80°C

Scalpel or razor blade

#### 2.1.2. General comments

This protocol describes the preparations steps for one chamber to be bound to a bottom-glass petri dish. The dish can be homemade or commercial; it can also be a 6-well plate with glass bottom. The height of the chamber should be adapted accordingly to the size of measured cells. Indeed to increase the contrast of the excluded fluorescence the ceiling should be as low as possible, but it should also be larger than the maximum cell diameter to prevent cells from confinement.

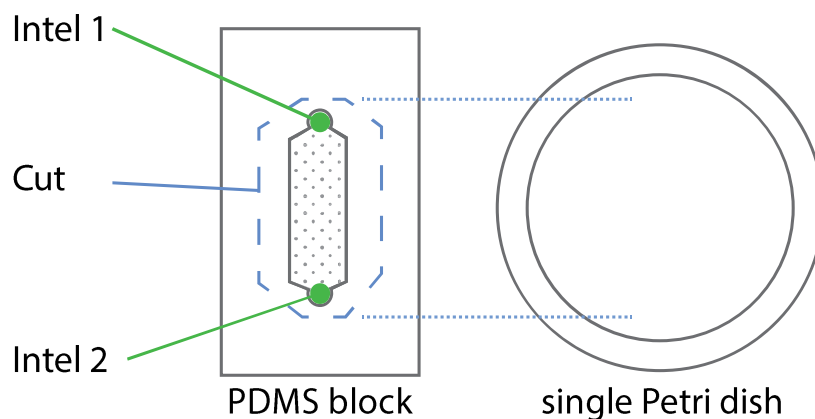
In order to fabricate your custom chamber you will need a mold. Molds micromilled in metal or done by photolithography can be used. Pillars should be placed such that there is one pillar in each field of view. The distribution of the pillars has to be calibrated in order to avoid chamber collapse. The general rule for chambers of rigidities that will be used in this protocol is that the distance between two pillars should not be bigger than 10 times the height of the chamber.

#### 2.1.3. PDMS chamber preparation (Day -1)

- Punch 0.75 or 1 mm diameter inlets and outlets in the PDMS chip (1:10) and cut the PDMS chip to make it fit in 35 mm diameter bottom glass petri dishes (Fig. 2).
- Clean the PDMS chip ethanol and air gun (optional) and with scotch tape to remove dusts.
- Activate PDMS chip and the glass surface for 30 sec in the plasma cleaner. This step is done in air plasma cleaner in our laboratory, but might need optimization if you use another plasma treatment.
- Put the PDMS chip on the glass surface immediately after plasma treatment and check carefully that the chip is well stuck: the change in reflection of the light under the PDMS pillars indicates that they are well stuck. If needed, use a soft tool (for example a plastic pipette tip or tweezers) to gently press on the chamber to force the contact between the glass and the pillars.
- Incubation in the oven for additional 15 min, tend to reinforce the binding (optional).
- For best coating, inject fibronectin (50 µg/mL in PBS) within less than 15 minutes, and under the hood. When this step is done right after plasma treatment it is sufficient to put a drop of 20-50 µl over one of the inlets, the solution is easily aspirated by the chamber. It is also possible to store the chips after plasma bonding, but then before injecting fibronectin, make the PDMS hydrophilic by

plasma activation (vacuum for 1min and then plasma-clean for 30 sec). *This step enables: (1) treating surfaces for efficient coating (surfaces become hydrophilic which helps fluid spreading throughout the chip and avoiding air bubbles); (2) sterilizing the dishes.*

- Incubate for 30 min to 1 h in wet chamber to avoid fibronectin to dry out.
- Rinse with culture medium under the hood, in order to avoid unsticking of pillars, it is preferred to rinse chambers using the procedure for placing cells in the chamber (Fig 3.). Cover the chip with medium and incubate overnight. *If a few bubbles were made during the process, they should disappear during the incubation overnight. If the experiment requires the use of hydrophobic drugs, the medium should already contain those drugs to load the PDMS with the drugs and avoid drug depletion later during the experiment.*



**Figure 2** How to punch out the inlets and cut out the chamber. Our chambers have mostly two inlets, here in green, that have to be punched out before the binding of the chamber to the Petri dish. The total size of the chamber should easily fit inside the Petri dish. In this way the total surface of the chamber can be in contact with the glass surface.

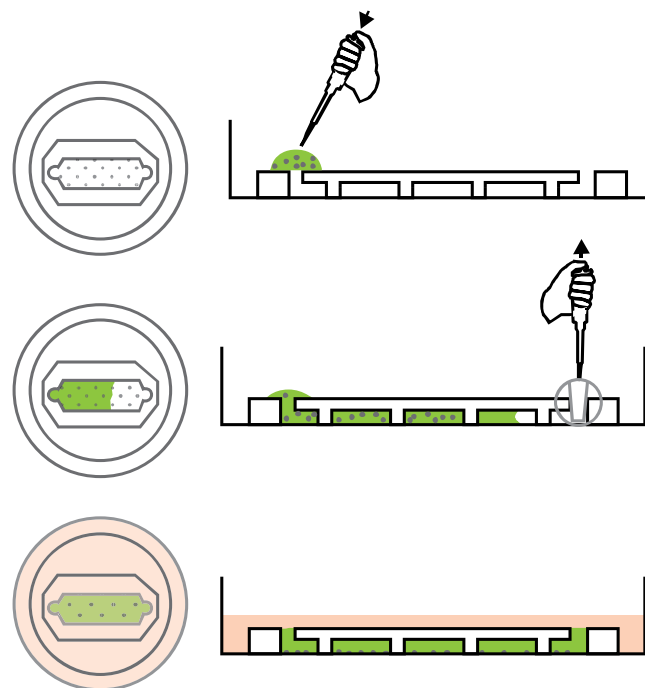
#### 2.1.4. Cell injection (Day 0)

**Figure 3** Injecting cells into chambers shows the main steps of cell injection.

- Pre-warm medium and Trypsin/EDTA.
- Prepare 50  $\mu\text{L}$ /chamber of FITC-Dextran supplemented medium (1 mg/mL), reserve in the incubator.
- Remove all medium around the chambers in the dish and dry the surface of the chamber.
- In order to wash the chamber. Place a drop of medium above the inlet and make it flow into the chamber by drug out the medium in the other outlet with a 10  $\mu\text{L}$  cone. *You should see the drop decreasing its size. Avoid sucking it completely to prevent air flow in the chamber, this creates air bubbles that are very difficult to get rid of.*
- Detach cells in culture with TrypLE, Trypsin or EDTA depending on the cell type and the time required for adhesion. *Using EDTA results in shorter adhesions times for most cells.*
- Re-suspend cells with warm media, calculate the volume needed to be centrifuged and re-suspended in the 50  $\mu\text{L}$  of Dextran supplemented medium to obtain a final concentration ranging from approx.  $0.5$  to  $2 \cdot 10^6$  cells/mL. *Cell concentration has to be adapted to final density required for*

the experiment. Obtaining single cell volumes using this method can be done only on cells that are clearly separated. If two cells are too close one to the other calculating their volumes might be impossible. For short time scale experiments as those following volumes over cell divisions, you can admit that if you have 100 cells that are isolated from others, during a movie of 10h you will be able to observe about 20 divisions. The probability that a cell will enter mitosis during your movie depends on the cell cycle length. If you have a fast migrating cell line, then some cells will move out of the field before over going mitosis. Finally, other cells can migrate and come into contact with your cell. For long term experiments, the final density of cells should also be taken into account. It is possible to have an idea of the concentration to use by placing a drop of your cell solution in a cell counting chamber. Cell density there will be comparable to the one in the volume measurement chamber.

- Centrifuge the volume of cells needed, re-suspend within the 50  $\mu$ L of pre-warmed dextran supplemented medium. Re-suspend well in order to avoid groups of cells binding to each other.
- Deposit a 5 $\mu$ L drop of cells above the inlet and drug out the medium from the other outlet (**Figure 3**)
- Check cell density under a microscope.
- Once the cell density is correct cover completely the chamber with medium to stop the flow (**Figure 3**).
- Aspirate all medium in the petri dish and replace by new one completely covering the chamber. *This step is important first to wash dextran remaining outside of the chamber and avoid parasite fluorescence signals coming from outside the chamber, second to completely cover the chip with medium to prevent evaporation during the image acquisition under the microscope since PDMS is porous to gaz.*



**Figure 3** Injecting cells into chambers. First a drop containing any solution or the medium with suspended cells is placed over one of the inlets. Second, the droplet is drug into the chamber by applying suction on the other inlet. The entry of the medium into the chamber should be easily seen. Third, the whole chamber is entirely covered by medium to avoid evaporation trough the experiment.

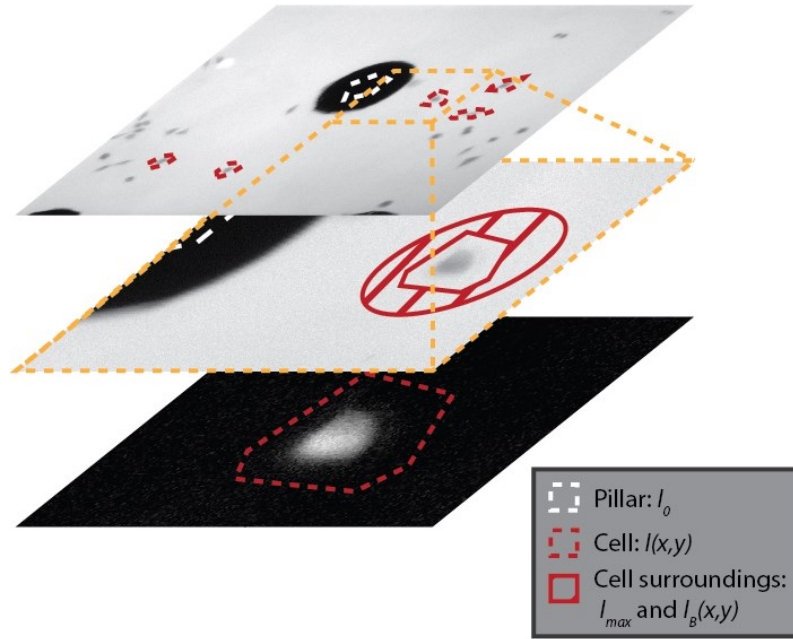
## **2.2. Imaging**

Ready chambers should be placed under a microscope with homogeneous fluorescence illumination. The homogeneity of the illumination can be roughly estimated by placing on a glass slide a drop of diluted fluorescent dextran and placing a coverslip above it. Illumination intensity should be optimized, the lowest possible for preserving the cells, but high enough to have a correct signal to noise ratio. Cells can be imaged in phase or in other wave lengths at the same time. The time lapse is depending on the time resolution you need. Cell division lasts for about an hour in HeLa cells, imaging them every 5 min gives a correct resolution in time and data quantity. Imaging cells over longer periods of time might be every 10 min.

## **2.3.Data Analysis**

Image analysis is performed with MatLab supplemented with the Image Analysis Toolbox. According to the kind of analysis to be performed, the procedure needs to be adapted, but the main steps are roughly the same.

First an area corresponding to the center of the pillar or to the edge of the chamber is selected by the user; the mean value of fluorescence  $I_0$  in this area is stored for each timepoint and will be used for the calibration of the fluorescence as it corresponds to a height of  $0\mu\text{m}$  (See figure 4). This highlights the significance to check whether pillars are correctly bound to glass, to insure that the height of the chamber is correctly set and the value of fluorescence really corresponds to a zero height.



**Figure 4** Cell volume calibration steps. First an area corresponding to the center of the pillar is chosen. Then the area around the cell to be analyzed is determined. Third, a large area around the cell is defined in order to take it as background.

Second, an area around the cell  $S$  to be analyzed is defined (See figure 4); this area could be modified for each time points, or kept the same if the cell stays at the same place. This area can be larger than the cell since the value of height outside the cell should be null, however a larger area will induce a larger noise though. The mean fluorescence around this area  $I_{max}$  (See figure 4) and the fluorescence of the pillar  $I_0$  will be used to calibrate the relationship between fluorescence and height by extracting the slope  $\alpha$  since we have shown it to be linear (Zlotek-Zlotkiewicz et al., 2015).

$$I_{max} - I_0 = \alpha \cdot h_{chamber}$$

$I_{max}$  is integrated locally around each cell because the illumination is usually not homogeneous throughout an entire field of view. The slope  $\alpha$  is then calculated for every timepoint and each cell. To further correct for illumination defect, the background around each cell is fitted with a quadratic polynomial equation  $I_B(x, y)$  the difference between the background and the image gives then positive values for the cell is then positive and null around the cell (See figure 4). In order to fit the background, the image is previously segmented to exclude all objects (pillar, border, cells, or dusts) in the field and is performed using dilation and erosion procedures.

Finally, to obtain the volume of a cell, the signal is integrated over the area defined around the cell:

$$V_{cell} = \iint_S \frac{I_B(x, y) - I(x, y)}{\alpha}$$

### 3. Discussion

The study of cell growth regulation in animal cells has recently been renewed by sophisticated techniques aiming at dynamically measuring a size parameter on single cells over the entire cell cycle (Mir et al., 2014; Park et al., 2008; Son et al., 2015). To date, three main techniques enable mass measurement on single cells over long periods of time: SLIM (spatial light interference microscopy) (Mir et al., 2014), SMR (Suspended microchannel resonator) (Son et al., 2015) and Pedestral Resonant Sensor (Park et al., 2008). These methods are reviewed and compared in (Popescu et al., 2014). Although these methods were successful in characterizing the exponential properties of single animal cells growth, many studies still lack rigorous control of growth parameters to ensure that experiments are made at steady state.

The versatility of this method allows studying phenomena ranging from seconds (drug treatments, osmotic shocks), minutes or hours (mitosis) up to several days (cell cycles). Indeed, it enables repetitive volume measurement on single cells over long periods of time (up to 50hrs) while maintaining good growth conditions through time. It is moreover fully compatible with classical cell biology methods such as fluorescence imaging or transient drug treatment and thus can be applied to a wide range of biological questions

It brings interesting possibilities compared to other available methods. The main drawbacks are the fact that cells have to be isolated in order to obtain correct results.

**Table 1** Comparison of different available methods for live cell volume imaging

	<b>FXm</b>	<b>3D reconstruction</b>	<b>SRM</b>	<b>Coulter counter</b>
<b>Single cell</b>	oo	ooo	ooo	x
<b>Suspended Cells</b>	oo	oo	ooo	ooo
<b>Adherent Cells</b>	oo	ooo	x	o
<b>Accuracy</b>	oo	oo	ooo	ooo
<b>Throughput</b>	oo	o	o	ooo
<b>Easy-to-use</b>	oo	oo	o	ooo
<b>Instrumentation</b>	oo	oo	o	ooo
<b>long term culture</b>	oo	x	ooo	ooo



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### 4.3 Volume measurements on patterns

Micropatterning is a technique widely used in cell biology and biophysics to deposit molecules in a certain pattern on a surface. It can be used to create adhesive patches of defined shape and dimensions on an otherwise non-adherent surface (Azioune et al., 2010). Cells plated on such a surface will adhere only on micropatterns. Obtaining micropatterns in our volume measurement chambers is however not possible. The treatment of the coverslip renders it globally non adhesive and prevents volume measurement chamber adhesion. After several trials, the easiest way I found to combine patterns and volume measurements was to use cell confinement (section 4.1). In such a set-up the bottom coverslip carries adhesive patterns and the top one micropillars of 15  $\mu\text{m}$ . The obtained effect is very satisfying and could be used in experiments with MLEC and Rpe1 cells. Using micropatterns in volume measurements has diverse advantages. First cells do not move, this simplify the analysis and also increase the number of cells that can be measured in one field. Randomly plated cells often interact with each-other and if they are too close, it is impossible to discern their volumes. Micropatterning will also greatly facilitate the automatic detection of cells for volume analysis and may reduce measurements errors.

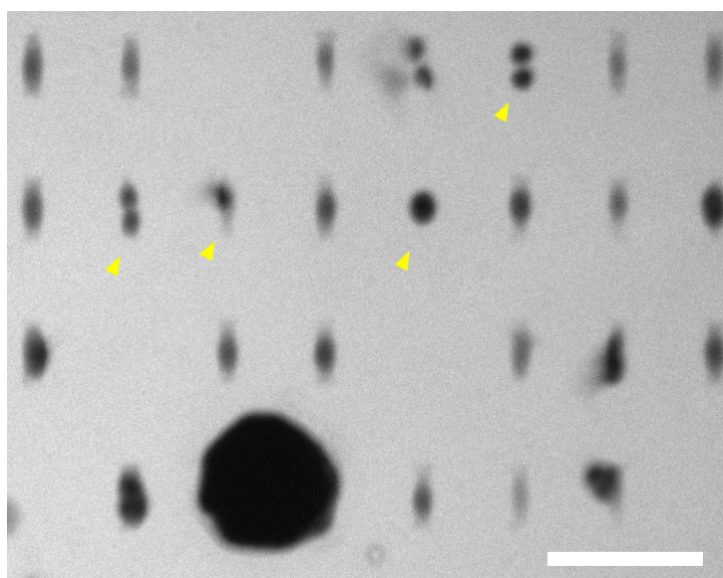


Figure 4.1: RPE1 cells plated on rectangular micropatterns and confined under a coverslip with spacer micropillars of 15  $\mu\text{m}$  height. Arrowheads point toward cells that divide during the video [Figure 4.2](#). Scale bar 100  $\mu\text{m}$

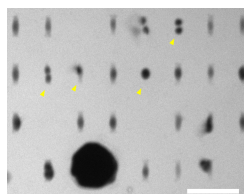


Figure 4.2: Video corresponding to [Figure 4.1](#) [Play the video](#)

### 4.4 Volume measurements and media changes

The media can be successfully changed in volume measurement chambers. It has been used in (Zlotek-Zlotkiewicz et al., 2015) in the experiments with EDTA, where cells were first in normal media, then were placed in PBS for equilibration and finally in PBS with EDTA to induce cell rounding by

detachment. The chambers need to be modified in two ways [Figure 4.3](#), first the inlet is made large (3-5 mm), then a second piece of PDMS is added in order to rise the level of the inlet of the chamber above the surrounding media. The medium is changed by aspiration of the liquid present in the two inlets and replacing it with the new medium in only one inlet. This creates a pressure difference between the two inlets and causes a flow. The flow intensity depends on the geometry of the chamber, the diameter of the inlet in the chamber and the difference in the height of the two columns of liquid. Although it can be modulated in a certain range it is a very rough approach and it will be important to optimise volume measurements chambers in order to induce fluxes of different solutions at a controlled rate and pressure and over short and long periods of time.

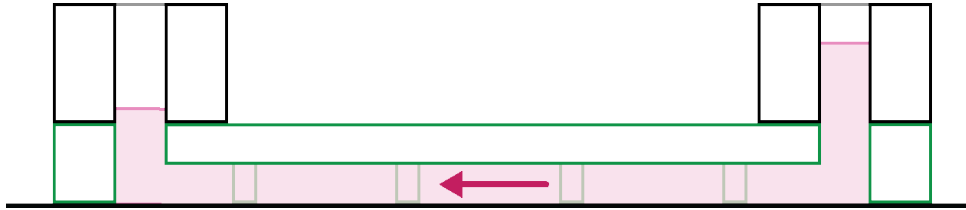


Figure 4.3: Scheme of the modification of the measurement chamber in order to easily change the medium. The inlets are punched bigger, and additional pieces of PDMS with large holes are placed above both inlets. The created sinks enable changing media with a pipette without moving the chamber and losing positions.

## Appendix A

**Review : Exploring the function of  
cell shape and size during mitosis.**

*Developmental Cell 2014*

# Exploring the Function of Cell Shape and Size during Mitosis

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Dividing cells almost always adopt a spherical shape. This is true of most eukaryotic cells lacking a rigid cell wall and is observed in tissue culture and single-celled organisms, as well as in cells dividing inside tissues. While the mechanisms underlying this shape change are now well described, the functional importance of the spherical mitotic cell for the success of cell division has been thus far scarcely addressed. Here we discuss how mitotic rounding contributes to spindle assembly and positioning, as well as the potential consequences of abnormal mitotic cell shape and size on chromosome segregation, tissue growth, and cancer.

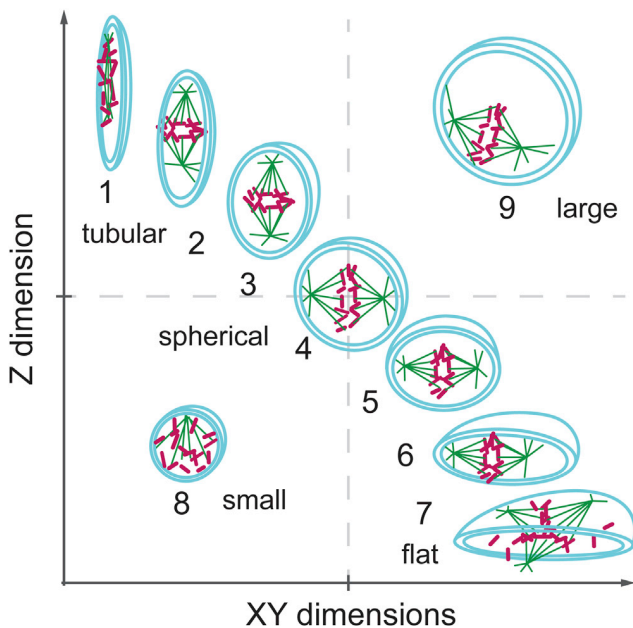
## Introduction

Since Walther Flemming first described the segregation of chromosomes in 1882, mitosis has become one of the best-studied processes in cell biology. How the mitotic spindle forms, captures chromosomes, and segregates them equally into two daughter cells is now understood with minute molecular detail. However, it has only relatively recently become clear that mitosis involves not only spindle formation but also a complete reorganization of cell architecture that affects almost all cellular processes. One of the most striking examples of this is the dramatic change to cell geometry that occurs when cells enter mitosis. Researchers working with cell lines in tissue culture have long observed that cells abandon their flattened, spread morphology at mitosis and round up to become spherical (Aubin et al., 1979; Cramer and Mitchison, 1997; Harris, 1973). This phenomenon, known as “mitotic cell rounding,” appears to be nearly universal in metazoan cells and other eukaryotes lacking a rigid cell wall. It has been observed in organisms as diverse as *Dictyostelium*, hydra, flies, and mice (Kunda et al., 2008; Luxenburg et al., 2011; McConnell, 1930; Zang et al., 1997). The fact that this process is so well conserved through evolution suggests that it plays an important role in cell division.

However, it is only in recent years that mitotic rounding has become the focus of detailed study, with many works attempting to unravel the molecular mechanisms that underlie it. It is now becoming clear that mitotic rounding is a complex process, requiring the precise coordination of multiple signaling pathways. To become spherical, cells must first decrease adhesion to their substrate by disassembling focal adhesion complexes (Dao et al., 2009). In parallel, changes to intracellular osmotic pressure cause water to enter the cell, allowing it to swell up like an inflated balloon (Stewart et al., 2011). Finally, mitotic rounding also requires the complete reorganization of the actin cytoskeleton. Interphase actin structures, such as stress fibers and lamellae, are disassembled upon entry to mitosis and actin filaments are recruited to the cortex, directly underlying the plasma membrane (Kunda and Baum, 2009; Kunda et al., 2008; Maddox and Burridge, 2003). The mitotic

cortex appears to differ from the interphase cortex in that it is thinner (Clark et al., 2013) but also more rigid, thereby imparting an increased stiffness to mitotic cells (Kunda et al., 2008; Maddox and Burridge, 2003; Matthews et al., 2012). Actin is required for both the changes to cell geometry and the increase in stiffness that occur at mitotic entry, but the precise interplay between shape and mechanics during this process is not yet fully understood. Recent studies have revealed several players that control actin remodeling at mitosis. These include the small Rho GTPase RhoA and its activator, Ect2, as well as members of the ezrin/radixin/moesin (ERM) family of actin/membrane crosslinkers (Carreno et al., 2008; Kunda et al., 2008, 2012; Maddox and Burridge, 2003; Matthews et al., 2012). However, the regulation of actin during mitosis has been widely reviewed elsewhere (Kunda and Baum, 2009; Lancaster and Baum, 2014) and so will not be discussed further here.

In this Perspective, we turn our attention to another fundamental question in the field: why do cells change shape to become round at mitosis? Given that mitotic spindles are able to form and segregate chromosomes in cell-free systems such as *Xenopus* egg extract (Hannak and Heald, 2006; Heald et al., 1997), it has been easy in the past to dismiss mitotic rounding as unnecessary for chromosome segregation. However, recent work has demonstrated that this is not the case and that in fact, mitotic rounding is essential for faithful chromosome segregation during cell division (Lancaster et al., 2013). This is because mitotic spindle formation requires a defined geometric space and becoming spherical at mitosis is the key mechanism that allows cells to generate this space. Here, we review the recent evidence that rounding is required for many aspects of mitosis including chromosome capture, spindle formation, and stability. In addition, we consider how cell geometry affects the positioning of the spindle and division plane within the cell and how this may lead to the symmetric or asymmetric segregation of cytoplasmic content between two daughter cells. Finally, we explore the wider significance of mitotic rounding and speculate on the role it may play in vivo on cell fate determination, tissue growth, morphogenesis, and cancer.



**Figure 1. Impact of Cell Geometry on Mitotic Spindle Assembly and Chromosome Capture**

Diagram representing altered mitotic cell geometry. (1) Below a threshold dimensions in [XY] chromosome capture and spindle assembly are altered, even if the [Z] dimension is large. (2) For [XY] dimensions permissible for chromosome capture and spindle assembly, a large [Z] dimension can induce spindle centering defects. (3 and 5) A slight shift from a spherical shape, in either [XY] (5) or [Z] (3), can strongly affect the cell division plane (Hertwig rule; see Minc et al., 2011; Minc and Piel, 2012). (4) Optimal cell geometry and size for spindle assembly and positioning. (6) Flattening cells increases their size in [XY] dimensions, hampering the contact of the astral microtubules with the cell cortex, which can cause spindle mispositioning defects, and (7) induce chromosome capture defects and spindle poles splitting below a threshold that depends on the cell type (see Lancaster et al., 2013). (8) Small cell, which prevents spindle assembly; (9) large cell, which causes spindle positioning defects.

### Mitotic Rounding Contributes to the Fidelity of Chromosome Segregation

The essential goal of mitosis is the accurate and symmetrical segregation of genetic information. To address the question of whether mitotic rounding plays any role in this process, a recent study by Lancaster et al. (2013) forced HeLa cells to enter mitosis with an artificial flattened shape induced either by overexpression of adhesion regulator Rap1 (Dao et al., 2009) or by mechanical confinement in chambers of limited height (Lancaster et al., 2013; Le Berre et al., 2012; Tse et al., 2012). Both treatments significantly alter cell geometry at mitosis, preventing cells from rounding up and forcing them to adopt a flat shape with an increased spread area but severely decreased height (Figure 1). Altering the dimensions of mitotic space in this way had a profound effect on the efficiency of spindle formation and led to frequent chromosome segregation errors and aneuploidy (Lancaster et al., 2013). Thus cell rounding was revealed to be one of the many mechanisms that facilitate efficient and faithful segregation of genetic material at mitosis. Specifically, this study identified two main aspects of spindle formation that were perturbed when mitotic rounding was inhibited: chromosome capture and bipolar spindle stability.

### Chromosome Capture: Finding Chromosomes Is Easier in a Limited Geometric Space

To achieve the symmetrical segregation of the genetic material at mitosis, all chromosomes must be captured by microtubule binding to kinetochores and arrayed to form a bipolar spindle. Biorientated attachment of every chromosome is ensured by the spindle assembly checkpoint (SAC), a signaling cascade that senses unattached kinetochores and acts together with a force-sensing correction mechanism to delay chromosome segregation and anaphase until all chromosomes are correctly attached (Foley and Kapoor, 2013). The reliability of this process is essential for tissue homeostasis as SAC slippage or prolonged activation results in chromosome missegregation errors leading to chromosome instability (Bakhom et al., 2009; Ganem et al., 2009; Ganem and Pellman, 2012; Silkworth et al., 2009), aneuploidy (Daum et al., 2011), and cell-cycle arrest (Uetake and Sluder, 2010). Mitotic rounding is required for successful and timely satisfaction of the SAC, as artificially flattened cells show a significant delay in anaphase onset compared to wild-type, spherical cells (Lancaster et al., 2013). This is due to severe defects in chromosome capture by microtubules: while control HeLa cells were able to capture all chromosomes within 10 min, flat cells still showed unattached kinetochores at the same time point (Lancaster et al., 2013 and Figure 1). A simple explanation for this is the limited range of microtubules emanating from centrosomes, which have a maximum reach of about 15  $\mu\text{m}$  in somatic cells (Collins et al., 2012; Lénárt et al., 2005; Picone et al., 2010; Piehl and Cassimeris, 2003; Varga et al., 2009; Wühr et al., 2008). In flat cells, chromosomes are scattered over a larger area than in control cells; therefore, some of the chromosomes can be reached only by microtubules emanating from one pole or are out of reach of microtubules entirely (Lancaster et al., 2013). The chromosome capture defects observed in flattened cells can be rescued by altering microtubule dynamics to elongate microtubules, demonstrating that microtubule reach is of critical importance for chromosome capture (Lancaster et al., 2013). Thus, the round shape of mitotic cells enables efficient chromosome capture by limiting the space dimensions in which chromosomes can be found by microtubules. Indeed in very large cells, such as the *Xenopus* oocyte, additional mechanisms are required to ensure that chromosomes are gathered in a limited space (Field and Lénárt, 2011). The spindle is thus of optimal size to catch all chromosomes in the constrained volume of a rounded somatic cell (Collins et al., 2012; Domnitz et al., 2012; Lancaster et al., 2013).

Changing geometry to become spherical at mitosis ensures that all chromosomes are captured quickly at mitosis by bringing them together with spindle microtubules in a small enough space. On the other hand, it is likely that there is also a minimal space requirement for chromosome capture. In 1986, Kirschner and Mitchison proposed the “Search & Capture” theory, whereby microtubules randomly emanate from centrosomes and become stabilized following kinetochore attachment (Kirschner and Mitchison, 1986). Though microtubules are indeed stabilized at kinetochores, recent mathematical models predict that random search and capture is not fast enough to achieve bioriented attachments to all chromosomes in realistic time scales (Paul et al., 2009) and might require additional biochemical or geometrical biases (Kapoor et al., 2006; Lénárt

et al., 2005; Magidson et al., 2011; O'Connell et al., 2009; Wollman et al., 2005). Several mechanisms have been proposed to explain the high efficiency of chromosome capture including a microtubule-stabilizing gradient of RanGTP that promotes microtubule polymerization toward the center of the cell (Wollman et al., 2005) and the positioning of chromosomes at the cell periphery (Magidson et al., 2011). A specific spatial configuration of chromosomes at prometaphase is instrumental for fast and robust capture, with condensed chromosomes being moved from their central position at nuclear envelope breakdown toward the cell equator (Magidson et al., 2011). This ring-like arrangement of chromosomes facilitates correct attachment but is space consuming. Rounding up at mitosis is likely to allow cells to create a perfectly sized space for chromosome capture, which is both small enough to prevent chromosomes becoming "lost" outside the reach of microtubules but also large enough to allow the specific chromosomal formations that facilitate efficient capture.

### **Bipolar Spindle Stability: Maintenance of a Bipolar Spindle Requires Space**

Forcing cells to enter mitosis with a flattened interphase-like shape imposes two major changes to cell dimensions without necessarily altering total cell volume. First, it greatly increases the length and width of the cell, resulting in defects in chromosome capture as described above. Second, it decreases cell height. This height restriction severely affects the structure of the mitotic spindle. In HeLa cells flattened to a height of 7  $\mu\text{m}$  or less, spindle poles often split, creating additional, ectopic poles lacking centrioles and leading to multipolar divisions and cell death or aneuploidy (Figure 1; Lancaster et al., 2013). This demonstrates that spindles have minimal space requirements, under which they cannot maintain bipolarity. This is probably due to length restrictions of kinetochore microtubules and to mechanical constraints on spindle pole positioning. Mitotic rounding functions to provide the optimal geometry to establish and maintain a bipolar spindle and, therefore, allow for correct chromosome segregation (Lancaster et al., 2013).

### **The Importance of Cell Shape on Spindle and Division Plane Positioning**

As well as being essential for chromosome capture and spindle stability, rounding also plays a third crucial role during mitosis. It allows the spindle to accurately find the center of the cell. While spindle positioning is not important for successful chromosome segregation per se, the location of the spindle determines the position of the cleavage furrow at cytokinesis and hence the division of cytoplasmic contents between the two daughter cells (Green et al., 2012). In artificially flattened cells, defects in spindle positioning are frequently observed with many cells showing unstable, "wandering" spindles that move about within the cell (O.M. Lancaster, M.L.B., B. Baum, and M.P., unpublished data). In a rounded metaphase cell, the spindle is positioned in the cell center by a balance of cortical pulling forces exerted on astral microtubules emanating from the spindle poles (Collins et al., 2012). This requires the microtubule motor dynein/dynactin, which is recruited at the poles through its interaction with the G $\alpha$ i/LGN/NuMa cortical complex (Kotak et al., 2012; Woodard et al., 2010). Two mechanisms regulate dynein localization at the cortex to center the spindle (Kiyomitsu and Cheeseman,

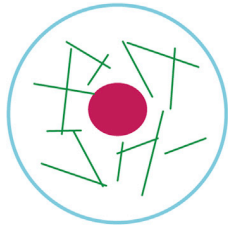
2012). First, polo-like kinase at the spindle pole inhibits the interaction of dynein/dynactin with LGN/NuMa, leading to negative regulation of force-generators as the pole comes closer to the cortex. Second, a Ran-GTP chromosome-derived signal stabilizes G $\alpha$ i/LGN/NuMa at the poles via inhibition of NuMa on the lateral cortex (Kiyomitsu and Cheeseman 2012). In addition, spindle mispositioning correction mechanisms also operate at anaphase to increase the robustness of division symmetry (Kiyomitsu and Cheeseman, 2013). These spindle-centering mechanisms rely on a balance of forces achieved by the symmetrical interaction of astral microtubules from both poles with the cell cortex. Rounding is likely to facilitate these spindle-cortical interactions, by ensuring that the cortex remains in close proximity to the spindle in all directions. In flattened cells, with an elongated axis, astral microtubules are unable to reach the cortex in every direction, resulting in an unstable mispositioned spindle (O.M. Lancaster, M.L.B., B. Baum, and M.P., unpublished data).

Mitotic rounding may also help cells sense their external environment to position the spindle. Indeed, the earliest identified factor that positions the division plane was cell shape, leading to the famous Hertwig rule: cells divide perpendicularly to their longest axis. This rule has since been verified in a very large number of cases, and some cells have been shown to be extremely sensitive even to small deformations in shape, due to microtubule-length-dependent pulling forces on spindle poles (Minc et al., 2011). Tissue-culture cells are able to align their spindle according to their interphase long axis, through a mechanism dependent on mitotic rounding. Rounding enables cells to retain a "memory" of their interphase shape through force-sensing retraction fibers that maintain connections with the substrate (Fink et al., 2011). In slightly confined cells, which have no spindle assembly defects, but cannot perfectly round up at mitosis, this force-sensing mechanism is disrupted and cells are no longer able to sense and align their spindle with their interphase long axis (O.M. Lancaster, M.L.B., B. Baum, and M.P., unpublished data). Thus, spindle orientation seems even more sensitive to mitotic cell shape than chromosome capture or spindle bipolarity (Figure 1). In tissues, the spindle orientation process is more complex, with spindles aligning relative to internal and external polarity cues. In addition, the orientation process can exploit the Hertwig rule by imposing an anisotropic shape to the dividing cell (Mao et al., 2011, 2013) but, alternatively, external constraints randomly imposing an elongated shape to dividing cells might be detrimental to sensing of these cues (Luxenburg et al., 2011). How various chemical and mechanical cues contribute to orient the division plane and might compete with each other was recently discussed (Minc and Piel, 2012) and will not be further discussed here. However, it is clear that mitotic rounding also has an important role in setting the division axis in this context. Depending on the tissue, it could either shelter the dividing cell from external constraints to allow other cues to dominate determination of the division plane or it could allow the dividing cell to align with force fields to reduce tension in a growing tissue (Caminho et al., 2013; Fink et al., 2011; Ranft et al., 2010).

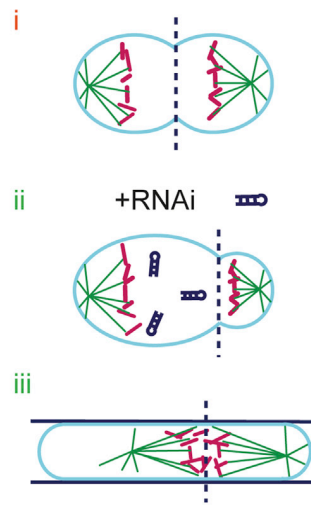
### **Cell Shape and Spindle Positioning in Symmetrical and Asymmetrical Cell Division**

The role of cell shape in positioning the mitotic spindle means that any defects in cell shape control are likely to result in gross asymmetries in daughter cell size following cytokinesis. Indeed,

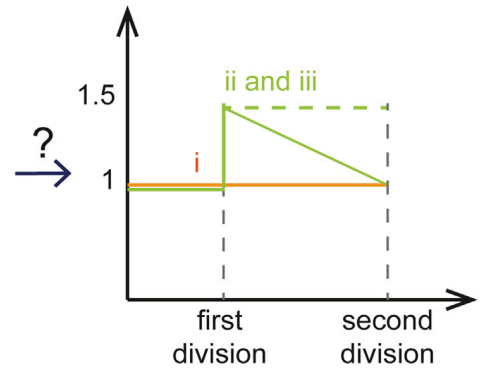
**A** Symmetrically dividing cells



— microtubules  
— chromosomes



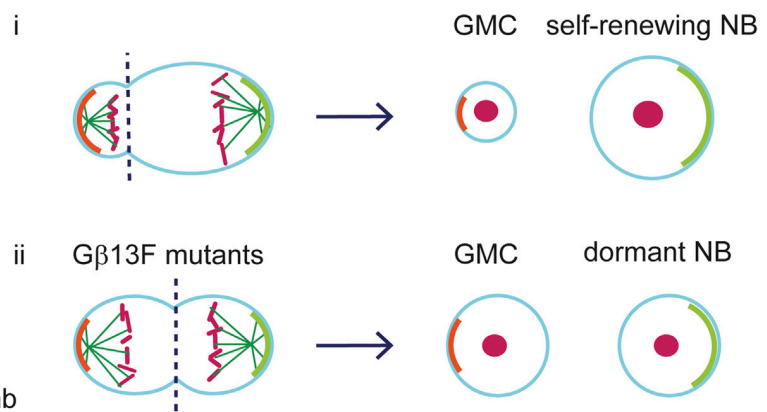
size asymmetry in pairs of daughter cells



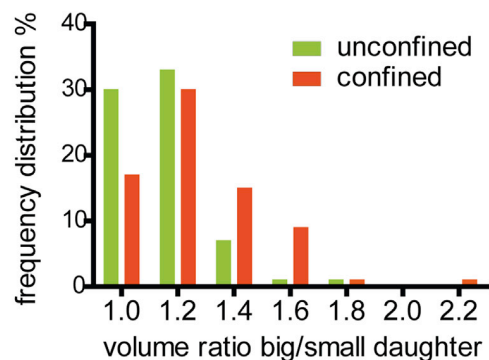
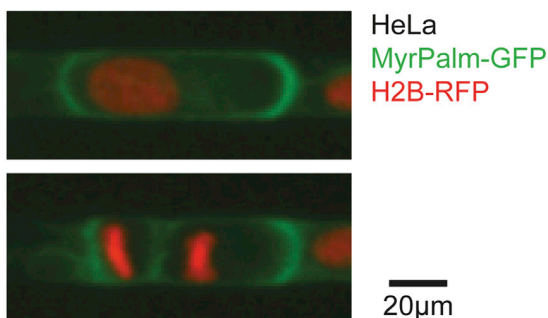
**B** Asymmetrically dividing cells



drosophila neuroblast (NB):  
— fate determinants: Brat-Pros-Numb  
— Gαi/Pins/Mud



**C** Geometrically induced asymmetrical division in confining micro-channels



**Figure 2. The Symmetry of Division**

(A) In symmetrically dividing cells (A<sub>i</sub>), the precision of the symmetry of division must be accurate to ensure size homeostasis in the population (graph, orange line). Asymmetric divisions can be artificially induced chemically by RNAi (A<sub>ii</sub>) or mechanically (A<sub>iii</sub>). Such artificially induced asymmetrical divisions would enable studying the consequences of asymmetrical division of cytoplasmic content and testing whether cells can correct for abnormal sizes at birth (graph, green filled line, volume ratio big daughter/small daughter comes down to 1) or not (green dashed line).

(B) Asymmetrically dividing cells are observed in developmental or stem cell divisions where the newborn daughter cells have asymmetrical fates. A common model is *Drosophila* neuroblasts (NB), which divide asymmetrically both in size and segregation of fate determinants. Among other cortical polarity markers, the *Gαi/Pins/Mud* complex is crucial for asymmetrical positioning of the spindle. In normal conditions, the division leads to (B<sub>i</sub>) a small daughter that inherits the fate

(legend continued on next page)



in artificially flattened cells where the spindle is unable to center, cell division often results in one daughter of much larger size than the other (O.M. Lancaster and M.L.B., unpublished data; Tse et al., 2012, see also Figure 2C). The long-term consequences of these size asymmetries are unknown but may be severe given the precision with which division symmetry is normally regulated. Recent studies have quantified a difference of 7% mean volume (Tzur et al., 2009) and  $7.5\% \pm 4.9\%$  in dry mass (Sung et al., 2013) in pairs of newborn sister cells. Mechanisms ensuring the symmetry of division must therefore be tightly regulated and robust to guarantee such precision in volume distribution.

Although most cell divisions require the production of two equal-sized daughters, there are also many examples of asymmetric division throughout development and stem cell biology. These divisions are usually termed asymmetric because they give rise to two daughters of different fates (Horvitz and Herskowitz, 1992). However, they are frequently also accompanied by dramatic asymmetries in the size of the resulting daughter cells. For example, oocytes divide asymmetrically at meiosis to give rise to a large gamete and a very small polar body, ensuring that maximum cytoplasmic content and resources are inherited by the future zygote (Fabritius et al., 2011; Li and Albertini, 2013). The *C. elegans* zygote undergoes an asymmetrical division to produce a larger anterior somatic daughter cell and a small germline-precursor posterior cell (Gönczy, 2008; Siller and Doe, 2009). Similarly, during *Drosophila* development, neuroblasts divide into one large apical daughter that self-renews into a neuroblast and a smaller basal daughter, which inherits fate-determining proteins that induce differentiation into a ganglion mother cell before dividing one last time to form two neurons (Figure 2B).

As in symmetrical division, positioning the mitotic spindle through microtubule-cortex interactions is key to determining daughter cell size during asymmetric division. In mammalian polar body extrusion, the mitotic spindle is translocated from the cell center to the cortex through an actin-dependent mechanism (Fabritius et al., 2011; Li and Albertini, 2013). Asymmetrical division in the *C. elegans* zygote and *Drosophila* neuroblast require the homologous form of the G $\alpha$ /LGN/NUMA complex—G $\alpha$ /GPR/LIN-5 and G $\alpha$ /Pins/Mud, respectively—for spindle positioning. In both models, the key player is a heterotrimeric G protein, operating through a receptor-independent pathway involving GoLoco domain-containing proteins such as Pins and GPR (Fuse et al., 2003; Gotta and Ahringer, 2001; Izumi et al., 2004; Miller and Rand, 2000; Srinivasan et al., 2003; Yu, 2004; Yu et al., 2005). However, the mechanisms leading to the spindle off-centering after its alignment along the polarity axis are different. In the *C. elegans* zygote, G $\alpha$ /GPR/LIN-5 promotes the localization of dynein heavy chain (Couwenbergs et al., 2007; Kozlowski et al., 2007; Nguyen-Ngoc et al., 2007) to pro-

duce a net pulling force toward the posterior pole by a mechanism that is not fully understood (Galli and van den Heuvel, 2008; Grill et al., 2001). In the *Drosophila* neuroblast, the mechanism, also not yet fully understood, is more likely to rely on asymmetrical regulation of microtubule nucleation resulting in astral microtubules extending more at the apical pole than at the basal pole (Fuse et al., 2003; Kaltschmidt et al., 2000). All of the mechanisms described above rely on a balance of cortical-pulling forces to position the spindle. Therefore, perturbations to cell dimensions and shape are likely to affect these distance-dependent signals and the efficiency of microtubule-based forces. The role that cell geometry may play in defining daughter cell size during both symmetrical and asymmetrical division is therefore an interesting question for future research.

### Impact of Cell Geometry on Cell Fate, Tissue Morphogenesis, and Disease

Most of our knowledge of the function of cell shape in mitosis comes from studies carried out in mammalian tissue-culture cell lines. However, as interest in this field grows, we anticipate that future work will seek to uncover the wider role of mitotic shape in vivo. Multiple examples of mitotic shape changes in adult and developing tissues have been observed (Kondo and Hayashi, 2013; Kunda et al., 2012; Luxenburg et al., 2011; Meyer et al., 2011; Nakajima et al., 2013), and it seems likely that a process that has such an impact on spindle assembly and positioning in tissue culture is also likely to be crucial for cell division in tissue homeostasis, development, and disease. Here we speculate on the role that mitotic cell geometry may play in three different in vivo processes: cell fate determination, tissue homeostasis, and cancer.

### Volume Asymmetry and Cell Fate

It is clear that cell shape at mitosis plays a role in spindle and division plane positioning and hence, in the distribution of cytoplasmic volume between the two daughter cells. One interesting question arising from this is what happens when defects in cell shape induce failure to correctly segregate cytoplasmic contents? How would a normally symmetrically dividing cell resolve an asymmetrical division and, conversely, what would be the consequences of forcing an asymmetrical division to become symmetric in terms of volume distribution? Although the asymmetrical segregation of cell fate determinants during asymmetrical division has been well studied, the function of cytoplasmic volume differences is not clear. One interesting idea arising from this is that either symmetrical or asymmetrical distribution of volume and cell components (i.e., organelles) could play a role in determining daughter cell fates (Figure 2B).

When addressing the question of the symmetry of volume division, it is worth noting that cytoplasm distribution does not necessarily reflect the distribution of subcellular components.

determinants Brat, Pros, and Numb and gives a ganglion mother cell (GMC) and a large daughter self-renews into a new neuroblast. Conversely, G $\beta$ 13F mutants (B<sub>i</sub>) asymmetrically segregate fate determinants but show defects in asymmetrical spindle positioning and thus divide symmetrically in size. At the embryonic stage, neuroblasts don't regrow after each division, and these abnormal symmetrical divisions fasten size reduction of neuroblasts, which leads to premature stopping of proliferation and entering into a dormant state.

(C) Asymmetrical divisions can be artificially induced by confinement in microchannels. Overlay of fluorescence images of a HeLa cell expressing MyrPalm-GFP (plasma membrane) and H2B-mCherry (showing chromosomes), undergoing mitosis under confinement in a microchannel (images on the left): at mitotic entry, the cell takes a cylindrical shape (top image), the elongated shape prevents correct spindle positioning (metaphase plate is shifted to the left), and cytokinesis give rise to two asymmetrically sized daughter cells (bottom). Right: histogram of volume ratios in pairs of daughter cells (big/small daughter cell) just after cytokinesis showing an increase of asymmetrical divisions when confined in microchannels of 102  $\mu\text{m}^2$  cross-sectional area.

In asymmetrically dividing cells, various mechanisms exist to specifically localize one fate determinant in one of the daughter cells. For example, in *Drosophila* neuroblasts, differential localization of fate determinants depends on cortical polarity markers previously asymmetrically recruited along an axis of polarity (reviewed in Gönczy, 2008; Neumüller and Knoblich, 2009). Similarly, in the *C. elegans* embryo, cytoplasmic P granules are inherited only by the posterior daughter cell, dependent on an anterior-posterior gradient of polarity factors and a position-dependent phase-transition phenomenon (Brangwynne et al., 2009; Griffin et al., 2011). Alternatively, some components are equally distributed between the two daughters, regardless of volume distribution, resulting in symmetrical amounts but asymmetrical concentrations if volume was asymmetrically distributed. This is the case for all spindle-associated structures, with the most evident example being chromosomes. However, other organelles are also able to exploit this mechanism, such as the SARA endosomes in *Drosophila* wing epithelial cells, which associate with the spindle machinery at mitosis, ensuring that both daughter cells inherit the same signal levels (Bökel et al., 2006). Finally, many cytoplasmic components, if freely diffusing at mitosis, will be distributed proportionally to volume. This is true for two important organelles, Golgi and mitochondria. Both organelles, which are linked to the microtubule cytoskeleton in interphase, detach from microtubules at mitosis and become fragmented throughout the cytoplasm, allowing uniform partitioning during cell division (Kashatus et al., 2011; Yadav and Linstedt, 2011; Yadav et al., 2012; Yamano and Youle, 2011). The way in which the endoplasmic reticulum is reorganized at mitosis is still debated (Lu et al., 2009; McCullough and Lucocq, 2005; Puhka et al., 2012; Puhka et al., 2007), and it will be interesting to know whether this crucial organelle for cell growth is distributed proportionally to daughter cell size or not.

This last category is intriguing because it suggests an important role for size at birth in both symmetrically and asymmetrically dividing cells: the distribution of volume mirrors that of cell components involved in key cellular functions including energy production, protein synthesis, nutrient uptake (membrane), cell-cycle timing (e.g., cyclins located in the cytoplasm), and aging (damaged proteins). Therefore, it is likely that any asymmetries in volume division would have consequences on the daughter cells' ability to grow and proliferate. The best approach to address this hypothesis would be to induce asymmetry of volume distribution in normally symmetrical cell division (Figures 2A and 2B). This could be achieved either by genetic manipulations that perturb spindle positioning (Figure 2A; see also Kiyomitsu and Cheeseman, 2012) or by altering cell geometry (Figure 2C; see also Tse et al., 2012). Alternatively, organelle distribution could be modified directly—for example, by using RALA knockdown, which disrupts mitochondrial segregation (Kashatus et al., 2011). In these knockdown cells, a decrease in the number of metabolically active cells in the population was measured. Among other plausible explanations for this effect is the repeated unequal distribution of mitochondria at mitosis (Kashatus et al., 2011). These approaches would allow us to determine the long-term implications of division asymmetries for cell growth.

Another interesting question is whether a cell population would eventually be able to recover size homeostasis following

induced division asymmetries. This is a long-standing problem in the field of cell growth and proliferation. If cell growth occurs exponentially, then any minor error in the symmetry of division will be rapidly magnified and would be corrected only if a size-homeostasis maintenance mechanism exists (Conlon and Raff, 2003; Lloyd, 2013; Mitchison, 2003). The measurement of a small error in the symmetry of division (Sung et al., 2013; Tzur et al., 2009) recently led the authors to hypothesize that there is a need for a mechanism controlling cell size (Sung et al., 2013), at least for cell types that were shown to grow exponentially (Godin et al., 2010; Mir et al., 2011; Park et al., 2010; Sung et al., 2013; Tzur et al., 2009). It has been suggested that this mechanism relies on a differential reduction in growth rate after the G1/S transition with a more pronounced reduction for big, fast-growing cells (Kafri et al., 2013; Son et al., 2012). However, the nature of any size-adjusting mechanism that would drive this size-asymmetry detection and correction mechanism remains elusive. It is not known whether, for example, it would relate to cell volume (Tzur et al., 2009), mass (Park et al., 2010; Sung et al., 2013), or density (Godin et al., 2010; Grover et al., 2011; Son et al., 2012).

In asymmetrically dividing cells, few studies have focused on the consequences of unequal daughter cell size. Computational modeling studies predict that active asymmetrical segregation of aged or damaged proteins in daughter cells results in increased robustness in a cell population (Erjavec et al., 2008). The idea that asymmetrical distribution of damaged protein occurs in stem cell division to preserve one of the two daughter cells has been verified in intestinal stem cells (Rujano et al., 2006), reproducing previous findings in budding yeast cell division (Aguilaniu et al., 2003; Shcheprova et al., 2008). In this study, asymmetrical distribution of the damaged content relied on an active mechanism involving centrosome-associated structures (Rujano et al., 2006) but it would be interesting to discover whether asymmetry in size could also participate in preventing aging, allowing one daughter cell to maintain its undamaged, self-renewing properties. Alternatively, the asymmetrical inheritance of organelles could promote growth advantages in one daughter cell. Neumüller and Knoblich hypothesized that asymmetric ribosome biogenesis could function as a mechanism to regulate growth and self-renewal (Neumüller and Knoblich, 2009). Several studies in *Drosophila* germline stem cells support this idea. For example, the U3 snoRNP component, required for preribosomal maturation, is inherited by the self-renewing daughter cell (Fichelson et al., 2009). Conversely, the microRNA-inhibiting pathway Mei-26 protein is inherited by the differentiating daughter cell where it restricts growth (Neumüller et al., 2008). To further explore the possibility that asymmetrical size contributes to specifying asymmetrical fate during stem cell divisions, techniques that allow us to artificially modify the symmetry of volume at division are required. In the *C. elegans* neuroblast lineage, perturbation of the division plane can be used to increase the size of the anterior daughter cell, which induced differentiation into a neuroblast, rather than following an apoptotic program (Ou et al., 2010). In *Drosophila* neuroblasts, cell size is thought to impact cell proliferation rather than differentiation. Wild-type embryonic neuroblasts do not regrow after each division and thus gradually become smaller through consecutive divisions until they reach a dormant state. Disruption of Gβ13F and Gγ1,

components of the heterotrimeric G protein complex recruiting polarity proteins at the cell cortex, perturb only the symmetry of spindle positioning and not the segregation of fate determinants (Fuse et al., 2003; Izumi et al., 2004; Kitajima et al., 2010), hence providing tools to help understand the role of the asymmetry of volume. Interestingly, in G $\beta$ 13F mutants, the size of neuroblasts decreases faster as volume is equally distributed between the neuroblast and the GMC daughters and they become dormant faster than in the wild-type condition, thus leading to neural development defects (Fuse et al., 2003; Kitajima et al., 2010). This led the authors to hypothesize that unequal-size division serves to maintain stem cell properties by minimizing volume reduction in the neuroblast lineage (Fuse et al., 2003). Further studies are required to reveal the precise interplay between mitotic cell shape and volume segregation in these systems and to determine any eventual consequences on cell fate.

#### **Mitotic Rounding in Tissues: Homeostasis and Morphogenesis**

Mitosis in a tissue presents extra challenges compared to mitosis in a tissue culture dish. Cells must accomplish successful chromosome segregation in a crowded environment while maintaining tight connections with neighboring cells. To help achieve this, mitotic rounding is therefore likely to be of even greater importance. Round swollen mitotic cells have been frequently observed in tissues, where dividing cells often appear to actively deform their interphase neighbors (Kunda et al., 2012; Luxenburg et al., 2011; Meyer et al., 2011; Nakajima et al., 2013). Furthermore, this cell shape change at mitosis has been shown to play a role in several processes key to maintaining tissue integrity and homeostasis. For example, in mouse epithelia, failure of cells to form a mitotic actin cortex and round up leads to defects in spindle orientation, resulting in differentiation defects in a system where cell commitment relies on polarized cell division (Luxenburg et al., 2011). Likewise, in the pseudostratified epithelium of the fly imaginal disc, mitotic actin is required to position the spindle parallel to cell junctions and thus maintain epithelial integrity (Morin et al., 2007; Nakajima et al., 2013). Not only is mitotic rounding important for processes occurring inside the dividing cell, but the drastic change of shape also affects neighboring cells and produces forces that can have implications for overall tissue morphogenesis. For example, mitotic rounding has been recently shown to contribute to morphogenesis in the fly tracheal placode, where it is sufficient to initiate tissue invagination (Kondo and Hayashi, 2013). However, there have been no studies analyzing whether mitotic rounding is required for faithful spindle assembly, chromosome segregation, and division in tissues. We would argue that mitotic rounding is likely to be critical in this context, not only to generate the necessary space for spindle formation, but also to withstand the high forces exerted by neighboring cells in a tissue. Measurements using both atomic force microscopy and optical stretching have demonstrated that rounding is also accompanied by cell stiffening, with cells in mitosis being significantly stiffer than their interphase counterparts (Kunda et al., 2008; Matthews et al., 2012; Stewart et al., 2011). Like mitotic shape changes, this stiffening is dependent on cortical actin (Matthews et al., 2012). Actin is essential to generate force during mitotic rounding, as HeLa cells constrained under a polyacrylamide gel are unable to round

up and deform the gel when treated with actin depolymerizing drugs, resulting in mitotic catastrophe (Lancaster et al., 2013). In addition, forces generated by increasing internal osmotic pressure (Stewart et al., 2011) are also likely to be used by cells in tissue to acquire a round shape independently of external mechanical cues. This hypothesis remains to be tested and leaves us with many questions waiting to be tackled. For example, how stiff would a cell have to become in order to be independent of external mechanical cues in tissues? How does rounding change in different tissues given the inherent variability in tissue stiffness? Can cells adapt to grow in tissues of a different stiffness? What are the consequences of failing to round up during a division in a tissue, considering that multipolar divisions either are deleterious to cell survival or can participate to cancer onset/development? Importantly, mitotic rounding mechanisms required for cells to divide in tissues are likely, due to differences in mechanical environment, to differ from the mechanisms identified in tissue culture cells.

#### **Mitotic Rounding in Cancer**

If cell division in a mechanically stiff tissue is a challenge, then division in an environment of overgrowth, such as in a tumor, must present further difficulties. Tumors exhibit distinct nano-mechanical stiffness profiles, with regions that are both stiffer and softer than healthy tissue (Plodinec et al., 2012) and mitosis in such an environment may prove particularly challenging. We predict that cancer cells in these tumors would have to be more efficient in space generation at mitotic rounding. Mitosis is frequently observed in tumor samples, where it can be quantified as an indicator of prognosis and mitotic cells frequently appear round and swollen compared to their neighbors (Elston and Ellis, 1991; Roux et al., 2013). We hypothesize that mutations that promote mitotic swelling and stiffening would be advantageous in such a tumor environment by enabling cancer cells to continue to divide under extreme physical pressure. In addition, mitotic rounding may also assist cancer cell division at sites of metastasis, where cells may have to complete division in an environment both mechanically and biochemically very different from that of the primary tumor (reviewed in Matthews and Baum, 2012). Creating a protective space in which to build a mitotic spindle, independent of the external mechanical environment, would provide an advantage at all stages of cancer progression.

In addition to dealing with the problems of completing mitosis in different environments, building a spindle in a cancer cell is likely to be intrinsically more difficult than in a noncancer cell due to chromosomal and centrosomal aberrations. Chromosome capture and spindle assembly may require greater physical space when chromosomes are duplicated or damaged. An increased number of chromosomes generates a larger metaphase plate and bipolarity can be lost above a certain plate length (Dinarina et al., 2009). DNA content appears to correlate with how well cells are able to cope with mitosis under mechanical confinement (O.M. Lancaster, M.L.B., B. Baum, and M.P., unpublished data). HeLa cells suffer multiple mitotic defects when constrained in a PDMS chamber of 5  $\mu$ m high, which prevents rounding, while nontransformed RPE1 cells are able to divide successfully under a 5  $\mu$ m constraint (O.M. Lancaster, M.L.B., B. Baum, and M.P., unpublished data). RPE1 and HeLa cells differ significantly in chromosome number, since

RPE1 cells are diploid while HeLa cells contain 82 chromosomes with large amounts of foreign DNA from the human papilloma virus integrated into their genome (Adey et al., 2013). In addition, binucleated HeLa cells face a greater struggle to divide under 5  $\mu\text{m}$  confinement, with almost no cells able to assemble a bipolar spindle in these conditions (Lancaster et al., 2013). These data suggest that the space required to build a fully functioning mitotic spindle scales with the number of chromosomes to be segregated and that in aneuploid cancer cells, such as HeLa, generating this space through mitotic rounding is likely to be of greater importance.

Another common characteristic of cancer cells is alteration to the number of centrosomes (reviewed in Godinho et al., 2009). This is somewhat paradoxical because, while increased centrosome number has been shown to initiate tumorigenesis (Basto et al., 2008) and correlate with tumor aggressiveness (D'Assoro et al., 2002; Giehl et al., 2005; Levine et al., 1991; Lingle et al., 1998; Pihan et al., 2003), it also results in the recurrent presence of multipolar spindles in tumor samples (Quintyne et al., 2005). If these multipolar spindles are maintained until anaphase, they are likely to be highly deleterious for cancer cells, as multipolar division often results in death (Ganem et al., 2009). Therefore, cancer cells have developed a number of mechanisms to form a bipolar spindle in the presence of extra centrosomes, including inactivation of some centrosomes, asymmetrical distribution of centrosomes at anaphase, and clustering of several centrosomes to form one pole (Ganem et al., 2009; Logarinho et al., 2012; Quintyne et al., 2005). Spindles with extra centrosomes are likely to be less stable and be less able to resist external constraints (Kwon et al., 2008). We observed that inducing overduplication of centrosomes by overexpression of Plk4 (Basto et al., 2008) in RPE1 cells dividing under confinement induced formation of multipolar spindles while this was not the case if cells were not confined or in confined cells with a normal number of centrosomes (O.M. Lancaster, M.L.B., B. Baum, and M.P., unpublished data). Thus, it seems that mitotic cell rounding can facilitate bipolar spindle assembly and successful division in cells with extra centrosomes. This may be because a larger metaphase plate imposes stronger forces on the poles and antagonizes clustering forces. Thus, it seems likely that a robust mitotic rounding provides an advantage for cancer cells with additional centrosomes and chromosomes.

The challenges faced by cancer cells during cell division, including segregating aneuploid chromosomes, resolving multipolar spindles, and dividing in environments of high mechanical stress, suggest that any adaptations that enhance mitotic rounding in these conditions would be highly favorable. Cancer cells frequently have altered mechanical properties compared to nontransformed cells (Guck et al., 2005; Suresh, 2007), but how this may be specifically regulated during mitosis has not been studied. Likewise, oncogenic transformation is often associated with changes to the actin cytoskeleton (reviewed in Nürnberg et al., 2011). Many of the proteins that regulate actin at mitosis, including Ect2, RhoA, and the ERM family, are also known as proto-oncogenes and are frequently overexpressed in a variety of cancers (Bruce et al., 2007; Fields and Justilien, 2010; Heiska et al., 2011; Vega and Ridley, 2008). Many of the changes to actin regulation in cancer cells have been associated with cancer invasion and motility (Olson and Sahai, 2009; Yama-

guchi and Condeelis, 2007) and are often viewed solely in this context. However, it is possible that oncogenic mutations to ERM or actin regulatory proteins serve the additional purpose of promoting mitotic rounding and thus facilitating cancer cell division in the crowded tumor environment. Our preliminary observations suggest that there may be fundamental differences in mitotic rounding between different cell types and between cancer and noncancer cells (O.M. Lancaster, M.L.B., B. Baum, and M.P., unpublished data). This hypothesis demands further testing. Indeed, identifying mechanisms specific to force production during mitotic rounding under the confining conditions of tissues might offer new opportunities to prevent cancer cell proliferation.

### Conclusion

Cells have long been known to assume a spherical shape at mitosis, but the function of this mitotic change in geometry has, until recently, remained a mystery. New evidence suggests that becoming round at mitosis is essential for faithful segregation of genetic content, by allowing cells to efficiently capture chromosomes and construct a bipolar spindle. In addition, a uniform mitotic shape allows cells to position the spindle in the center of the cell, resulting in symmetrical division into two equal-sized daughter cells. Further studies are required to address the long-term consequences of the symmetrical or asymmetrical division of cell volume, and to expand our knowledge of the role of shape and size in mitosis to the in vivo environment, in both normal developing tissue and in disease contexts such as cancer.

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# Appendix B

## Extra-lab PhD work

<b>B.1</b>	<b>Apprentis chercheurs - sharing science with teenagers</b>	<b>113</b>
<b>B.2</b>	<b>Questions workshop during the Science Festival of Palestine</b>	<b>116</b>
<b>B.3</b>	<b>Biology courses at the FdV Bachelor program and game designing with L'Arbre des Connaissances</b>	<b>118</b>
<b>B.4</b>	<b>Intellectual self-defence in Bagnolet</b>	<b>119</b>

Placing this chapter in a PhD manuscript can be controversial. I want to argue that the work of scientists should not be restrained to the experiments they were able to publish in high impact factor journals. I see education through science and sharing my expertise as fundamental parts of science. I greatly benefited from all below presented actions, both on a professional and a personal level, and I sincerely think that more PhD candidates and scientists could benefit from such outreach projects if some of the pressure from our environment was released.

Presenting these extra-lab activities is therefore a kind of manifesto promoting that the PhD is a professional training that does not have to be restrained to the bench and that it can and should be successfully adapted to the motivations and needs of every PhD candidate. The following experiences bring additional important and concrete professional skills as conception and animation of diverse events with a wide range of public, scientific mediation in various contexts, innovative teaching or game designing. These can be added to my CV, strongly influence my career plans and are an important source of motivation and satisfaction.

### B.1 Apprentis chercheurs - sharing science with teenagers

*Les Apprentis Chercheurs* is a program created by *L'Arbre des Connaissances*, an organisation created ten years ago by scientists to promote the dialogue between science makers and society. The aim of this particular program is to bring closer pupils of 13-15 years to science in a hands on approach. From March to June, on Wednesdays, participating laboratories host two apprentices. In Curie there is an important particularity, the high-school participant is from Bondy in the suburb and the college participant is from the 5th district of Paris, which creates a rich mix.

I had the joy of organising the participation of our team in this program for two years. Both years I had three main goals; show to the apprentices that scientists are people like others, and that they could and should ask and find answers on themselves for any question. We therefore spent quite some time at the beginning training to ask questions and interviewing my colleagues. I also avoided giving them ready



answers or solutions, what at the end resulted in the totally merited feeling of accomplishment that this was their project. The first year, I took the subject that was already used previously in the lab: the hydrophilic and hydrophobic notions. The whole team participated in the project bringing the different expertises and approaches. The second year I decided to make them work on my project. The team was still participating, but this time the two young scientists were at the heart of the project. The outcome was a proof of concept of my PhD project.

The project started by finding a model that could bring us informations on the behaviour of cells but in which measuring volume changes or forces could be done without the microscope. Chicken eggs were just what was needed, single cells, big, readily available and inexpensive. After some bibliography on eggs, the Archimedes law was used to measure egg volumes. This implicated tackling general notions such as the need of theoretical preparation before any experiment and measurement errors. But also notions of biology and physics as what are eggs, density or fluid displacement. Eggs have a membrane beneath the eggshell, the next step was therefore to get rid of the eggshell in order to obtain something that could be comparable to cells. As the eggshell is made mostly of calcium carbonate it is easy to dissolve it in acetic acid. The concentration of acetic acid had to be optimised, as placing eggs in a solution too weak took too long to dissolve the eggshell and placing them in a solution that is too strong led to denaturing proteins in the egg. At this point additional notions of chemistry and physics were acquired as there was a chemical reaction happening on the egg surface that resulted in the generation of a gas. It took about 30 hours to dissolve eggshells in a 3% solution of acetic acid. Eggs without their eggshell were slightly transparent and bouncy.

As in my project we hypothesise that cells change their volume using osmolarity, the next step was to see how eggs react to osmotic stresses. Eggs were therefore weighted and placed in hypo- or hypertonic solutions. Eggs in hypertonic solutions shrunk and lost weight and eggs in hypotonic solutions appeared swollen and gained weight. The additional notion of osmolarity was acquired. Furthermore, in my project, the swelling of cells could be a way of force generation. Here the apprentices faced a technical problem of how to measure forces generated by an egg that is swelling in a hypotonic solution. Several ideas came up and even if after some test it appeared more complex than expected, a set-up that could be used to maintain an egg in place was constructed. A measurement instrument, with a calibrated spring, was also built step by step. Finally a previously shrunken egg was placed in the set-up filled with pure water and forces generated during its swelling were measured. The set-up built allowed to obtain quantitative data, that showed that swelling eggs exert forces on their environment and can deform a spring when swelling.

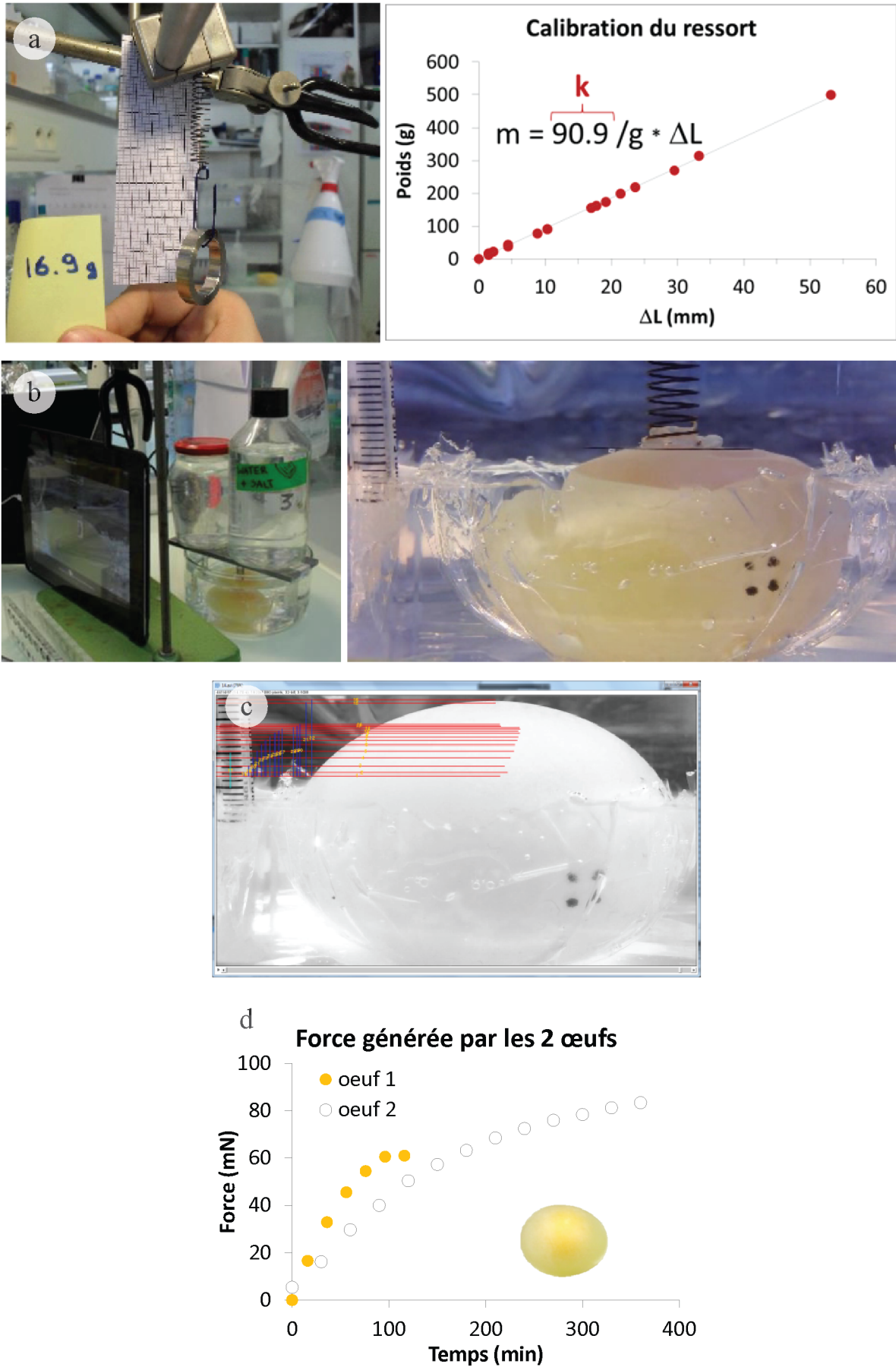


Figure B.1: a) Calibration of the spring with objects of various weight and calculus of the spring constant, b) the set-up c) image analysis, measuring the height of the egg, the syringe is used as scale, d) converting the deformation of the spring to generated forces.



Figure B.2: Video of an egg swelling in a hypotonic medium (pure water).

This project was a big source of satisfaction for the participants, the Curie Institute and organisers but also for me. I had a great time adapting my PhD project to this program and was very proud that it resulted in the obvious empowerment of my two apprentices and a proof of concept that could be integrated into my PhD. I reinforced my scientific mediation skills and also get experience in working with a new public.

## B.2 Questions workshop during the Science Festival of Palestine

With *Atelier des Jours a Venir* I participated in the conception and the animation of a workshop on question asking for primary schools that was part of the program of the annual Science Festival of Palestine. We discussed about scientists and asking questions in several Palestinian schools and animated debates with adults in the evenings. Our note published in the Science magazine shortly describes our standing point.

Participating in these workshops was a great experience. First of all it opened a particularly reach way of getting in contact with another culture and broadening my mind. We met with a lot of enthusiasm and had fascinating discussions with the kids at school, their teachers and their parents at evenings. I felt empowered as a scientist as I realised how much the scientific approach to solving problems is easy and important to share. On a personal level, being in a territory with such complex geopolitics was an important experience for me. Altogether this experience strongly inspired my professional, pots-Phd plans.

to envisage a future where both the climate change problem is resolved and today's fossil fuel industry persists.

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## Sharing ideas too soon

THE IN DEPTH ARTICLE "A radical change in peer review" (J. Mervis, 18 July, p. 248) suggests asking grant applicants to review their competitors. This initiative from the National Science Foundation (NSF) may save time and money, and the NSF's "novel scoring system aimed at dissuading a reviewer from downgrading a competitor's proposal in order to boost their own" may represent an advance in the peer-review procedures. However, this "radical alternative" to the traditional peer review system raises questions about intellectual property.

In the new peer-review system, grant applicants will share their novel ideas, which have not yet been scientifically tested, with their competitors for the grant. Although the "reviewers of grant applications are expected to maintain confidentiality and avoid using ideas in those applications without permission in their own research and for their own gain" (1), stealing ideas occurs in the research field (2). This unethical behavior might increase with the proposed system. The peer-review process certainly needs to evolve, but changes should be accompanied by mechanisms that minimize the risk of unethical behavior.

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Workshop in Nablus (An-Najah National University, West Bank).

#### OUTSIDE THE TOWER

## Kids' questions transcend conflict

“Are nails growing at the same speed on each finger?”  
“Is there a volcano in our land?”  
“Why does the moon walk with us at night, while stars stay still?”

These questions were asked by children last November, during the Science Days of Palestine, an event we initiated in 2010. This time, in several Palestinian cities in the West Bank and Gaza, we introduced 300 kids aged 8 to 12 to the questioning process of scientific research.

First, to their surprise, instead of the gray-haired men they expected, the children met young scientists, both men and women. We explained the role of questions in science. Then, through games and observation of their environment, pupils raised a hundred questions, genuine, profound, poetic, and meaningful to their lives (1). Their surprise grew when we did not provide answers. Instead, we invited them to ask peers, teachers, and parents to search books and Web sites, or to set up simple observations and experiments. Kids were thus left positively frustrated, keen to learn and explore further.

*Outside the Tower is an occasional feature highlighting science advocacy projects led by scientists and citizen scientists. How do you advocate for science? Tell us at submit2science.org.*

We held similar activities in Israel, Egypt, former Yugoslavia, and impoverished suburbs of Western cities (2–4). These costless activities (a pen and a paper suffice) can substantially change the attitude of students, and indirectly, teachers. They are encouraged to collectively explore the unknown, in close connection with their daily experience, thereby allowing them to find unexpected solutions. We hope more scientists will join us in sharing with children the very first step of their research practice, which is to ask open questions. In particular, in difficult situations where conflict lies too close, curiosity may spark enthusiasm, divert from violence, and lead toward innovative and pragmatic answers, which may eventually contribute to peace.

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## B.3 Biology courses at the FdV Bachelor program and game designing with L'Arbre des Connaissances

I took the opportunity of making a *monitorat* during the three years of my PhD. The *monitorat* is a governmental measure to help PhD students broadening their professional experiences during their doctorate. It consists on spending one sixth of the year time on an extra-laboratory work in science communication, cooperation with the industry or education and it brings an additional pay.

For the first two years of my PhD I assisted Antoine Taly in conceiving and animating biology courses and workshops for the interdisciplinary bachelor program *Frontieres du Vivant* at the *Centre de Recherches Interdisciplinaires*. The approach of this program is based on the fact that sciences are interconnected and that education goals and therefore methods have to be adapted to the present, 2.0 and the future 3.0 world. The courses of course had to teach students basics of biology, but it was not the principal objective. Indeed, notions of biology were learned by students through problem solving, project building and pair to pair learning, as one of the CRIs motto is *None of us is as smart as all of us together*. Every class was based on a different scenario and none of it turned out to have a predictable outcome. Students learned biology notions through learning to cooperate and use all available sources with criticism.

This experience strongly motivated me to pursue innovative teaching through my career and is a source of important professional experience that I will use through my career.

During the third year of my PhD I decided to work with *L'Arbre des connaissances*, organising the already mentioned program *Les Apprentis Chercheurs*. As there was no *monitorat* created to work in this organisation I had to build a proposal that was accepted and financed by the University.

As I already knew part of the program *Les Apprentis Chercheurs* I worked on another action developed by *L'Arbre des connaissances*, the development of debate games for high schools. The first game was already established when I joined the team, but a guide for animators was missing. The idea being to develop games that could be easily implemented in classrooms, it was important to provide a simple user guide that would help high school teachers to organise the game in their class. My first mission was therefore to conceive such guide [Figure B.3](#). I also actualized the bibliography concerning the subject of the game, enhancing humans. My second mission was to bring my expertise and help in the coordination of the conception and development of the second game, placed in the domain of synthetic biology.

This experience let me know more about how organisations of this type work, and how a game is designed.



Figure B.3: The first page of the guidebook developed with *L'Arbre des connaissances*, the totality is available on the <http://www.jeudebat.com/nos-actions/humain-augmente/>.

## B.4 Intellectual self-defence in Bagnolet

I was invited by Anne Lise Le Brun, to help her conceive and animate a workshop on Intellectual self-defence. The workshop was designed to be part of a special week in one of the college in Bagnolet, Paris suburbs. The article by Elise Graton describes very well the spirit of the workshop and the project.

For me it was yet another experience in innovative teaching. It was also very inspiring to meet the teachers of the school that were full of enthusiasm and very creative.



## Des vampires dans la salle des profs

**LABORATOIRE D'APPRENTISSAGE** Avant Noël, le collège Travail Langevin de Bagnolet dans la banlieue parisienne propose à ses élèves des ateliers à la place des cours. Les mesures de sécurité renforcées en raison des récents attentats ont entravé certains projets, l'enthousiasme des élèves, des enseignants et des parents n'en a pas pour autant souffert

Anne-Lise Le Brun grimpe quatre à quatre les marches du collège de son fils de quatorze ans. Son atelier d'autodéfense intellectuelle commence dans dix minutes et elle doit encore faire quelques photocopies. Elle passe en trombe à côté d'un groupe d'élèves assemblés devant un grand tableau dans le hall d'entrée. Y est affichée la liste des 27 ateliers proposés à tous les 385 élèves des classes de sixième à la quatrième durant la semaine précédant les vacances de Noël. C'est déjà la troisième fois qu'a lieu la dite « semaine décloisonnée » au collège Travail Langevin dans la petite ville de Bagnolet à l'est de Paris.

« Salle 6 ! », lance Anne-Lise. À toute vitesse, elle pousse les tables contre les murs de la salle, puis dispose quelques chaises en cercle au milieu. « Pardon, mais c'est quoi l'autodéfense intellectuelle », demande Thiziri, 12 ans, à peine entrée dans la salle. « À votre avis, c'est quoi ? » demande Anne-Lise à la ronde. « C'est de la bagarre avec la tête », propose Issa. « Ah d'accord, donc au lieu de taper l'autre, tu le fixes droit dans les yeux », blague Hajar, tout en plissant des yeux.

Les douze élèves inscrits à l'atelier ne sont pas encore tous présents. En attendant, Anne-Lise, traductrice et parent engagé, propose un jeu pour faire connaissance : à deux, il s'agit de collecter le plus d'informations sur son partenaire sans parler, seulement par le mime. Les enfants affichent un air sceptique, mais acceptent sans discuter de tenter l'expérience.

Au moyen de questionnaires, ils méditent ensuite en groupe sur leur relation aux médias numériques. Tous les élèves possèdent au moins un téléphone portable, un ordinateur, parfois aussi une tablette. La discussion débouche sur le thème du harcèlement moral via Facebook, ce qui offre une transition parfaite vers le sujet suivant : les théories de la conspiration. Grâce à son fils, Anne-Lise sait à quel point les adolescents peuvent être sensibles à toutes sortes de complots. Très en vogue sont actuellement les Reptiliens. Après l'attentat contre Charlie Hebdo, la rumeur courut aussi que celui-ci avait été fomenté pour remonter la popularité de François Hollande.

Anne-Lise souhaite aborder la problématique avec humour et présente un épisode de la séquence télévisée parodique « Le complot » intitulé « Et si le rap français était un complot des homosexuels ? » On y affirme entre autres que les rappers évoquent si souvent le nombre 93 pour la simple raison qu'en 1993, l'homosexualité a été décriminalisée dans de nombreux pays.

« C'est vrai ou c'est faux ? » demande Rawan, visiblement perplexe, à peine l'épisode fini. « N'importe quoi ! », réagit Issa. « Le 93, c'est nous ! » En effet, Bagnolet se trouve dans le

département 93 de Seine Saint-Denis. De nombreux rappers proviennent de ce secteur socialement défavorisé – et revendiquent leurs origines banlieusardes. Anne-Lise convie alors les élèves à créer leur propre complot farfelu – sur le thème : nos profs sont des vampires !

De nouveau, les élèves lui jettent un regard incrédule, puis Thiziri lance sur un ton conspiratif : « C'est quand même bizarre ... qu'on n'ait pas le droit d'entrer dans la salle des profs ? » « Génial ! », jubile Anne-Lise. Inspiré, Foune embraye : « S'ils sont capables de boire des trucs dégoûtants comme du café froid, pourquoi pas du sang ? » Mohamed suit : « Le fait qu'ils corrigent nos devoirs en rouge n'est certainement pas lié au hasard. » La première journée touche déjà à sa fin. « Si jamais j'arrive à persuader mes parents de notre thèse », annonce Thiziri avec un sourire malicieux, « pas sûr que je sois là demain ».

Le personnel enseignant et surveillant, les travailleurs sociaux des maisons de quartiers environnantes et les quelques voisins et parents participant à la semaine décloisonnée se retrouvent à la cantine autour d'un repas de Noël. Dans la queue du buffet, le graffeur Gilbert informe de l'avancée des travaux de décoration des toilettes chroniquement dégradées. François, architecte, est un peu déçu : son groupe était censé examiner à la loupe la rue menant au collège, pour ensuite en faire le dessin idéal. « À la place, nous nous consacrons au bâtiment du collège ». Le plan Vigipirate est à son niveau d'alerte maximum et toutes les excursions en dehors du collège annulées.

Isabelle Voydyllo, qui enseigne le latin et l'ancien grec, a par contre le droit de convoquer son groupe à 6 heures du matin au collège : « L'astronomie est ma passion ». Elle met son télescope personnel à la disposition des élèves qui, entre deux bâillements, auront l'opportunité d'explorer Jupiter. Le principal-adjoint Laurent Kaufmann, censé faire une apparition des plus décontractées dans la parodie de pub du collège que le groupe vidéo va tourner, a été prié de ne pas oublier ses lunettes de soleil.

« Lorsque nous avons démarré ce projet 'Made in Bagnolet' il y a trois ans, nous ne savions pas où nous allions », raconte Kaufmann. En répartissant les élèves en petits ateliers, classes d'âges mélangées, il s'agissait de créer une situation d'enseignement différente. Le concept fait ses preuves. Certains enseignants s'en inspirent le reste de l'année. Et la relation souvent tendue entre profs et élèves profite aussi.

Sur le chemin du retour, Anne-Lise ne tarit pas d'éloges sur l'engagement du collège. Cela fait quatre ans qu'elle et sa famille ont quitté Paris et ses loyers immodérés pour Bagnolet. La gentrification est bien sûr ici aussi une réalité. « Les nouveaux venus plus aisés mettent malheureusement tout en œuvre pour continuer d'envoyer leurs enfants dans des écoles parisiennes », regrette Anne-Lise. Son propre fils aimerait d'ailleurs lui aussi retourner à Paris l'année prochaine – rejoindre le lycée interacadémique Louis-le-Grand ou l'école d'art Boule. Le bon élève s'ennuie-t-il ou bien s'agit-il simplement de contredire sa mère ? Il laisse volontiers planer le doute. Anne-Lise n'a en tout cas pas l'intention d'entraver ses projets – « même si l'apprentissage dans une école où 43

nationalités sont représentées n'en est pas moins riche ! »

Le lendemain matin, Ewa Zlotek-Zlotkiewicz, biologiste cellulaire invitée par Anne-Lise pour l'épauler, assume l'animation de l'atelier avec la « parabole des polygones » – un jeu développé par l'économiste américain Thomas Schelling mêlant mathématiques et sociologie sur le thème de la mixité sociale. « Les attentats ont relancé la discussion à ce sujet », estime Ewa. « Et il ne suffit pas de brandir le drapeau français pour régler le problème ».

Le jeu commence : des triangles jaunes et des carrés bleus en sont les protagonistes. La vie est belle tant qu'ils sont entourés d'au moins un tiers de leurs semblables. Concentrés, les élèves déplacent de-ci de-là les formes géométriques en papier disposées dans tous les sens sur une grille, jusqu'à ce qu'apparaisse un tableau plutôt monotone. Ce faisant, les élèves parlent de leur environnement. « Les gens sont très gentils ici », trouve Thiziri. Il n'y a que la cité des Capsuleries où personne n'aime trop s'attarder : « trop de dealers ».

Le soir-même a lieu la réunion des parents des élèves de troisième. « Pourvu que Lucian n'oublie pas sa carte d'identité », espère Anne-Lise. Dar Al-Islam, le magazine francophone de propagande djihadiste, a récemment appelé à tuer des enseignants. L'établissement se voit en conséquence obligé de contrôler à l'entrée si les visiteurs appartiennent bien au collège. La principale, Madame Petit, a d'ailleurs dû demander une autorisation spéciale auprès de la préfecture pour que la réunion ne soit pas simplement annulée.

Margaux de l'association SynLab, un laboratoire voué entre autres à la recherche de formats éducatifs innovants, salue Anne-Lise en partant. Son atelier s'est employé à la transformation du foyer du collège. De bonnes idées ont surgi : un cercle de discussion réservé aux élèves, la création d'une station de radio, faire venir des personnalités. « Les élèves commentaient leurs propres propositions d'un 'enfin c'est n'importe quoi' », se souvient Margaux. « Et nous : mais si ! Exprimez-vous, travaillez avec les adultes, utilisez vos moyens ! » Anne-Lise comme toujours est ravie. Mais ne court-on pas le risque de trop promettre aux élèves ? « Nous leur proposons des outils pratiques qui puissent leur permettre de transformer les contraintes – qu'ils se posent souvent eux-mêmes – en opportunités », répond Margaux. « Et durant cette première phase de travail, on doit pouvoir **s'autoriser** à rêver ».





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# List of Figures

- 1 Video of an egg swelling in a hypotonic medium (pure water). . . . . x
  
- 1.1 Reproduction of the drawing published in 1913 in an article by Robert A. Lambert (Lambert, 1913). This is the earliest drawing clearly showing Mitotic Cell Rounding that I found. It represents a rat connective tissue cell entering mitosis. (a) prophase, (b) metaphase, (c) telephase; (d) daughter cells, (x) interphase cell. The time-lapse is of 15 minutes. . . . . 2
  
- 1.2 In his article on changes in cells during growth and cell division, Thomas S. P. Strangeways describes that: *The processes of the cytoplasm retract (fig. 72) and the cell assumes an oval or rounded shape (fig. 73)-the nucleoli become fainter (fig. 72) and then appear as hazy granules (fig. 73).* (Strangeways, 1922). Strangeways describes here choroidal embryonic cells. The numbering of figures seems incorrect. . . . . 3
  
- 1.3 Stills by Julius Ries (Ries, 1910), realised in 1907, representing the fertilization and development of a Sea Urchin egg. It is one of the earliest microcinematographic time-lapse made, and therefore one of the first ones showing cell divisions. . . . . 3
  
- 1.4 Images of various examples of mitotic cell rounding. **a)** Intekinetic nuclear migration in mouse brain, drawing in (Smart, 1972), **b)** An endothelial cell dividing in angiogenic sprouts in *Xenopus* (Aydogan et al., 2015), **c)** A metaphase cell in mouse skin (Luxenburg et al., 2011), **d)** A middle plane of the *Drosophila notum* showing a metaphase cell (Rosa et al., 2015), **e)** A mitotic cell in *Drosophila* wing imaginal disc (Gibson et al., 2011), **f)** Dividing cell in microspheres formed by MDCK cells (Tuncay et al., 2015), **g)** Cell division in *Leishmania*, the first panel shows the organism prior to mitosis (Ambit et al., 2011), **h)** A cell, prior and during mitosis in neural plate of a Zebrafish (Geldmacher-Voss et al., 2003), Several dividing cells during the development of *Nematostella* (DuBuc et al., 2014). **c-f and h)** show how surrounding neighbour cells seems to be deformed by the rounded cell in mitosis. . . . . 5
  
- 1.5 Cell cycle phases. After a division, the cell enters into the G1 phase, which is followed by the S phase where the DNA is duplicated. Before entering again into division most cells go through the G2 phase. During most of its cell cycle (about 24h), the adherent HeLa cell, whose contour is presented in this scheme, stays spread. Its shape over-goes dynamic changes, as protrusions are constantly formed and retracted, but the most prominent shape changes happen during division. Over several tens of minutes, the cell rounds up, elongates, splits into two new spheres that spread again. . . . . 6

1.6	Sketch representing different outcomes of mitosis depending on the final positioning of the spindle and therefore the division plane. On the left the division plane is perpendicular to the tissue plane leading to the displacement of one of the daughter cells out of the tissue plane. On the right, after a division in the plane of the tissue, the two daughter cells stay in the same plane. The direction of spindle position can have important consequences for cell differentiation, survival and overall tissue structure. . . . .	10
1.7	A cell plant where the protoplast (in green), the cytoplasm of the cell surrounded by a membrane, is enclosed by a rigid cell wall (in gray). . . . .	12
1.8	Sketch representing a simplified view of some of the forces present in cells that play a role in mitotic cell rounding. <b>a)</b> The plasma membrane is represented by a green line, the cortex by a pink one. <b>b)</b> Ligands between the membrane and the cortex are symbolized by springs. Forces in steady state are: internal and external pressures ( $P_{internal}$ and $P_{external}$ ), membrane and cortical tensions ( $2\gamma_m R$ and $2\gamma_c R$ ) that result in the inward directed forces ( $\gamma_m$ and $\gamma_c$ ) that counteract the force generated by the difference of pressures ( $\Delta P$ ). <b>c)</b> If the force applied on the membrane is higher than the total amount of forces present in the ligands tethering the membrane to the cortex, the membrane detaches. Adapted from (Alert et al., 2015) . . . . .	15
1.9	Trypsinized interphase cells ( <b>left panels</b> ) and mitotic cells ( <b>right panels</b> ) stained for actin ( <b>upper panels</b> ) and myosin ( <b>bottom panels</b> ) (Ramanathan et al., 2015). The organisation of actin and myosin is strikingly different in rounded interphase cells and cells in metaphase. . . . .	17
1.10	This image shows how different is the surface of spread, interphase cells and rounded mitotic cells. Interphase and mitotic Chinese Hamster cells imaged by scanning electron microscopy (Porter et al., 1973). . . . .	19
1.11	Figures from the doctoral thesis of Professor Paulo Abrahamsohn that served in the article (Abrahamsohn and Sesso, 1983). The <b>left panel</b> shows a photography of a section of a rat pancreas, where one of the acinar cells is in metaphase. Its contours are smooth and its shape is rounded and more regular than the shape of neighbouring, interphase cells. The <b>right panel</b> represent the volume of cells at various cell cycle phases and in mg. ( <i>Courtesy of P. A. Abrahamsohn</i> ) . . . . .	23
1.12	Scheme representing approximative rigidities of different cells and tissues in a human body. (Adapted from (Butcher et al., 2009)) . . . . .	24
2.1	Video of an entire HeLa cell cycle corresponding to Figure 1 B in the JCB article . . . . .	43
2.2	Video of a HeLa cell undergoing mitosis corresponding to Figure 1 D . . . . .	43
2.3	Video of a cell treated with EDTA to induce rounding, corresponding to Fig.2 D . . . . .	43
2.4	<b>a)</b> Shows a drosophila S2 cell just before mitosis and the outline of its contour defining cell area A. <b>b)</b> The same cell in late metaphase, just before anaphase onset, with again its contour showed in yellow. The contour is smoother and indicates an increase in cell area of 18%. Below are graphs representing the distribution of cell areas (left) and changes in cell area during mitosis (right) in unconfined cells and cells under 5 kPa gels . . . . .	44
2.5	S2 cells dividing under a gel of 5 kPa. . . . .	44

2.6	<b>a)</b> Curves of volume of MLEC cells (wt and Cav1 KO) during division. Cells lacking Caveolin 1 are significantly smaller, but increase their volume of the same amount as control cells. <b>b and c)</b> Mitotic volume increase of MLEC cells, <b>d)</b> Volumes of MLEC cells at the rounding time point, <b>e and f)</b> Mitotic volumes overshoots of MLEC cells measured on micropatterns or standard surface. . . . .	46
2.7	Wild type and Caveolin 1 deprived MLEC cells overgoing mitosis on disc micro-patterns. Whereas the WT cells round up, the KO cells remain flattened during the division. In KO cells the metaphase plate stays in the focal plane and is easy to observe. . . . .	47
2.8	The volume of L929 cells (n=38) overgoing mitosis. The 0 on the y axis has been aligned with the rounding up of cells, the 0 on the x axis is aligned with anaphase onset. The first green arrow shows the overshoot of volume, the second, the undershoot. . . . .	47
2.9	Sketch representing how could be automatized the analysis of cell shape and cell cycle in a screen. The detection of metaphase plates would indicate cells in metaphase, the detection of cell membrane would provide the shape of cells and their area. . . . .	48
2.10	The <b>upper</b> picture shows a polyacrylamide gel polymerised on a glass surface that was used to confine cells plated on the bottom of a rectangular dish (image on the right). Soft springs were used to press down the gel layer. The <b>bottom</b> image represents a sketch of a cover that could be used to create individual volume measurements chambers in a 96 well plate. 4 spacers are visible on each of the pillars. . . . .	49
2.11	Preliminary results quantifying the timing of mitotic cell rounding in reference to the nuclear envelope breakdown. The mean time at which cells start rounding up is 3.6 min after the Nuclear envelope breakdown, over 39 cells, 8 start to round up before the Nuclear Envelope Breakdown. . . . .	50
2.12	Preliminary results that aim to compare the amount of Lamin AC in cell lines with known mitotic volumes overshoots. Upper left panel represents a version of the figure from (Zlotek-Zlotkiewicz et al., 2015), where the mean volume increase is compared to HeLa cells. Compared to the figure in the article, there is one additional cell line, IIA1.6, but these data, due to their poor quality were not included in the paper. The bottom left panel shows the Western Blot realized on whole cell extracts of cells, loaded in the order corresponding to the upper left panel and at the same concentration of proteins, with two loading controls, MHC and Actin. The two right graphs represent the quantification of Lamin AC, based on densitometry, compared to HeLa cells and taking as loading control MHC (in green) or Actin (in red). . . . .	51
3.1	Graph representing outcomes of divisions of HeLa cells under gels of different rigidities. Cells under gels of less than 15 kPa undergo mostly bipolar divisions (green), cells under gels of more than 15 kPa present more mitotic mistakes with a growing number of multipolar divisions (orange) and cell death (gray). Graph adapted from unpublished results obtained by Daria Bonazzi. . . . .	54
3.2	<b>a)</b> sketch representing the setup used in (Lancaster et al., 2013), where cells were treated with Cytochalasin D under confinement of 15 kPa. <b>b)</b> shows phase images of cells in the experiment. Panels on the right show the quantification of mitotic outcomes in the experiments with Cytochalasin D and Latrunculin B. Adapted from (Lancaster et al., 2013). . . . .	55

3.3	Sketch summarising the idea that, in order to round up under anisotropic confinement present in a tissue, cells might have to both, increase their internal pressure and cortex rigidity. The anisotropic rigidity of the tissue is symbolized by a mesh with different color intensity. Cells increasing only their contractility or would deform only slightly their surroundings without rounding up. Cells that would not have a stiff cortex would not be able to achieve a round shape under anisotropic confinement, even if they would increase their volume by increasing the osmotic pressure. Cells might need strong pressures and a stiff, shield-like cortex to round up even under anisotropic mechanical constrains. . . . .	62
3.4	Graph summarising the potential interplay of cortical and membrane tension with internal pressure. As the tensions supported by the membrane are much lower than those supported by the cortex, for simplicity, the graph mentions only the cortical tension. At a steady state, if the internal pressure ( $P_{internal}$ ) is counteracted with the cortical tension ( $\gamma$ ) a cell has a stable shape (gray dotted line). If the pressure increases, the cell should expand until the critical pressure ( $P^*$ ) that can be sustained by the links tethering the cortex to the membrane is achieved. Above this pressure, if a cell is not surrounded by any matrix, the membrane detaches from the cortex and blebs are observed. If the cell is surrounded by a deformable matrix, the cell might expand. If the internal pressure decreases, it might be dominated by the cortex tension, which would result in shrinking. As actin contractility regulation has been involved in apoptosis (Coleman et al., 2001), it might be that the decrease of internal pressure with the increase of cortex contractility result in the detachment of the cell membrane and the strong blebbing observed in apoptosis. . . . .	63
3.5	A scheme where a well in PDMS is of the volume close to the cell is G2. When entering mitosis and rounding up, the cell would generate forces that could be measured by AFM.	64
3.6	The six hallmarks of cancer, adapted from (Hanahan and Weinberg, 2011). Mitotic Cell Rounding potentially plays a role in at least three of these hallmarks. If cancer cells can round up and divide in conditions that would normally induce a cell cycle arrest in normal cells, they sustain proliferative signalling. If they divide with errors due to defects in rounding and survive, they resist cell death that would be imposed to a normal cell. If cells enter in mitosis ignoring tissue stiffens they might need to evade growth suppressors. Furthermore, it is probable that the same mechanism of force generation used in cell rounding might be used in migration and promote invasion and metastasis. . . . .	65
3.7	This is a figure from (Leanza et al., 2015)@summarizing ion channels with altered expression in cancers arising in different tissues. Listed channels pharmacological targeting provide significant results in the diminution of tumor sizes or metastasis. . . . .	66
4.1	RPE1 cells plated on rectangular micropatterns and confined under a coverslip with spacer micropillars of 15 $\mu\text{m}$ height. Arrowheads point toward cells that divide during the video Figure 4.2. Scale bar 100 $\mu\text{m}$ . . . . .	99
4.2	Video corresponding to Figure 4.1 <a href="#">Play the video</a> . . . . .	99
4.3	Scheme of the modification of the measurement chamber in order to easily change the medium. The inlets are punched bigger, and additional pieces of PDMS with large holes are placed above both inlets. The created sinks enable changing media with a pipette without moving the chamber and losing positions. . . . .	100

B.1 a) Calibration of the spring with objects of various weight and calculus of the spring constant, b) the set-up c) image analysis, measuring the height of the egg, the syringe is used as scale, d) converting the deformation of the spring to generated forces. . . . . 115

B.2 Video of an egg swelling in a hypotonic medium (pure water). . . . . 116

B.3 The fist page of the guidebook developed with *L'Arbre des connaissances*, the totality is available on the <http://www.jeudebat.com/nos-actions/humain-augmente/> . . . . . 119