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Development of microfluidic devices with 3D collagen gels for Traction Force Microscopy

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RESUMEN

En las últimas décadas, se ha demostrado sobradamente que las células ejercen fuerzas de manera constante sobre las células y matriz que las rodea. Este comportamiento es fundamental en procesos fisiológicos y patológicos, como son la embriogénesis y la metástasis; por consiguiente, el estudio de cómo las células interactúan con su entorno es de vital importancia para entender estos procesos. Con el objetivo de contribuir en este campo de investigación, el presente proyecto de máster pretende adaptar un dispositivo de microfluidica dado a la técnica de *traction force microscopy*, que actualmente es el método más fiable para medir fuerzas celulares. Resulta de gran interés la cuantificación de las fuerzas ejercidas por las células sembradas dentro del dispositivo de microfluidica ya que este dispositivo en concreto permite el depósito de colágeno de manera tridimensional, lo cual se asemeja más al ambiente celular fisiológico que los cultivos celulares tradicionales en 2D.

La adaptación del dispositivo consistió en la introducción de microesferas fluorescentes en el colágeno, y la posterior toma de imágenes de las células mediante microscopía confocal. En ambas etapas fue necesaria la optimización de diversas características, como por ejemplo: concentración y tamaño de las esferas, viabilidad y marcaje celulares, complicaciones al realizar las tinciones fluorescentes, etc. Mediante varios experimentos y búsqueda de información, los puntos descritos fueron mejorando hasta que finalmente el dispositivo de microfluidica fue adaptado a *traction force microscopy* con éxito. Por tanto, los últimos ensayos generaron datos útiles para la realización del cálculo de las fuerzas celulares.

ABSTRACT

In the past few decades, it has been widely demonstrated that cells constantly exert traction forces on the cells and matrix surrounding them. This behavior is fundamental in physiological and pathological processes such as embryogenesis and metastasis, therefore studying how cells interact with their environment is vitally important to understand these processes. To contribute to this research field, the present master project aims to adapt a given microfluidic device to the traction force microscopy technique, which is currently the most reliable approach for measuring cell forces. It is of great interest to quantify the forces exerted by cells seeded inside this microfluidic device because this specific device allows the deposition of collagen in a 3D arrangement, which resembles better the cell's physiological environment than traditional 2D cell cultures.

Device adaptation consisted in introduction of fluorescent microbeads into the collagen matrix, and subsequent confocal microscopy imaging of the cultured cells. Both stages required optimization of diverse features, for example: bead size and concentration, cell viability and labeling, fluorescence staining complications, etc. After numerous experiments and information search, the aforementioned features were improved, and ultimately, the microfluidic device was successfully adapted to traction force microscopy. Thus, the final assays produced useful data for performing the cell force calculation.

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1. INTRODUCTION

Cells within a tissue interact with neighboring cells and with the surrounding extracellular matrix (ECM) through biochemical and mechanical signals. Cell-cell and cell-ECM interactions establish a 3D communication network that maintains the specificity and homeostasis of the tissue[1]. In this context, Mechanobiology studies how cells detect and respond to environmental mechanical stimuli[2]. Particularly, the cell cytoskeleton is responsible of these interactions. It generates intracellular mechanical forces that actively respond to the perturbations occurring in their underlying substrate[3]. In addition, intensity of such cell forces is adapted to and modulated by both environmental biochemical stimuli and physical properties[4]. For instance, even for cells with the same genetic material, they behave very differently depending on the attributes of their microenvironments[5].

Several studies suggest a ubiquitous role for cell forces in regulating cell signaling and function *in vivo* [6-8]. Therefore, a deeper knowledge of cell force generation and modulation is crucial to understand physiological and pathological events at tissue and organ levels[9]. Thus, obtaining new information on this concern appears as an important step in numerous fields of biology, including cancer research, regenerative medicine or tissue bioengineering[5]. Surprisingly little has been reported that sheds light on this issue, probably because there is limited access to approaches that precisely measure physiologic cellular forces in space and time[10].

In order to overcome this drawback, the present project develops a microfluidic device for cell culture under diverse controlled conditions and prepared for investigating the behavior of such cells. Furthermore, this platform is also adapted to fit in the Traction Force Microscopy technique (TFM), which is currently the most reliable method to measure the forces exerted by cells on their surrounding substrate[9]. In detail, the proposed platform consists in a microfluidic device containing cell-loaded 3D collagen hydrogel, which indeed assures a tissue-like surrounding with constant nutrient supply. The design of this approach is addressed toward reaching the most similar *in vitro* environment to the natural physiologic milieu, in order to obtain more realistic cell behavior data.

1. 1. Biological background

The minimal structural unit that defines living organisms is a single cell, for example fibroblasts and smooth muscle cells. By proliferating and interacting with each other, cells can build complex arrangements such as tissues that ultimately organize into even more complex multicellular living organisms[5]. The supporting substrate within tissues, termed extracellular matrix (ECM), materializes as a mesh of crosslinked proteins (collagen, proteoglycans, elastin, and other tissue-specific molecules) and carbohydrates surrounding the cells[3]. Indeed, it establishes their microenvironment, where cell-cell and cell-ECM interactions occur through biochemical and physical stimuli; for instance, diffusion of soluble molecules, electrical signal transmission and transduction of mechanical cues[11]. Moreover, key events in the life cycle of a cell, like proliferation, migration and apoptosis, are regulated by organizing principles that are determined by the cellular context[1].

As opposed to passive objects such as water droplets, living cells constantly probe, push and pull their environment (ECM and adjacent cells) by exerting forces on it[2] (figure 1).

Forces of this kind not only drive mechanical events like cell shape deformation but also are essential for mitosis and cell migration[12], and have a large influence on other cellular functions such as cell adhesion[13,14], gene expression[15] and differentiation[16]. Adhesion and migration then play a key joint role in embryogenesis[15], wound healing[17], inflammatory responses[18] and many other biological processes[5]. Moreover, a dysregulation of cell attachment has dramatic effects and can cause pathological states like developmental defects, cancer invasion and metastasis[19]. As an example, both fibroblasts' and myofibroblasts' mechanical activities have a critical purpose in wound healing by generating traction and contractile forces, respectively, to enhance wound contraction; but an excessive force usually results in tissue scarring[20].

In order to survive and grow, cells like fibroblasts and smooth muscle cells must attach to and spread on the surrounding substrate[9]. Once adhered to the ECM, these cells generate internal forces by their cytoskeleton, as roughly mentioned before, and these forces are then transmitted to the surrounding environment. The cytoskeleton is a highly dynamic cellular scaffolding structure fundamental to maintain and modify the cell's shape. Besides, it is composed of diverse polymers: filamentous actin (6 nm in diameter), intermediate filaments (10 nm), and microtubules (23 nm)[3]. These three cytoskeletal elements are not single proteins, but consist of many monomers able to span large distances within the cell. In particular, actin filaments together with myosin II proteins form the cytoskeletal contractile apparatus at non-muscle cells, which connects multiple parts of the cell membrane as well as the cell membrane to the nucleus[21]. This structure is responsible for generating the contractile forces at adherent cells so that the tension applied to the substrate is directed inward, towards the centroid of the cell[10,22-24]. Nevertheless there are instances in which this directionality is not true, such as when migrating cells extend protrusions at their leading edge[25]. In this case, traction forces are produced by actin polymerization alone.

All these in and outward traction forces are transmitted to the ECM through clusters of proteins located at the cell membrane, so-called focal adhesions (FA). These structures physically link the actin cytoskeleton with the ECM. Hence, FAs are sites of tight adhesion between the cell and the underlying ECM, where both forces and signals pass across[9]. Furthermore, once transmitted, forces manage many cellular functions involving ECM interaction, including cell migration, mechanical signal generation, and structural deformations and rearrangements of the ECM, as aforementioned. That is the reason why a detailed knowledge of cell traction forces is crucial to understand many fundamental biological processes[3].

1. 2. Cell force measurement approaches

The existence of cell traction forces was first demonstrated by the ability of adherent cells to wrinkle thin films of silicone rubber[26], as well as to drive matrix reorganization[27]. However, due to the inherent nonlinearity of wrinkling, there is currently no mathematical solution available for calculating cell forces determined through this approach[20]. Therefore, nowadays this technique remains as a qualitative tool and is not applicable to accurately quantify cell forces. Since then, various methods have been developed to quantitatively measure the direction and magnitude of traction forces exerted by cells on artificial substrates, with the intention of better understanding the cell-ECM interactions and their regulation. Albeit, such measurements remain a challenging problem owing to the small dimensions of the

adhesion sites (~μm^2) and the range of magnitudes of these traction forces (nano-Newton scale)[5]. Due to these concerns the precise characterization of physiological cell forces in space and time remains elusive nowadays[2].

Nevertheless, there are currently some established methods to perform traction force measurement on cell populations or on single cells. On the one hand, to measure forces of a cell population, many studies use the cell-populated collagen gel method, in which cells are embedded in a collagen gel and shrink the gel as a result of cellular traction forces; the forces are then indirectly estimated by changes in gel volume or area. However, cells are heterogeneous and the forces they generate are often widely variable, therefore cell population-based techniques only provide an estimation of the averaged forces of a group of cells[20].

On the other hand, traction forces of individual cells can be determined by a variety of approaches, including the aforementioned wrinkleable substrate assay, and also the micromachined cantilever beam array[28], the micropost force sensor array[23,29], and traction force microscopy[22] (figure 2). Cantilever beams fixed on micro-machined devices bend when an isolated cell exerts a traction force, and the bending degree is recorded and used to determine the force magnitude[28]. Such a technique can reliably determine the traction forces of individual cells, however it is limited in that it cannot resolve the traction force field within the whole cell area, but it can only obtain the forces in one direction. To overcome the former limitation, techniques based on micropost force sensor array (MFSA) have been developed to detect traction forces in all directions[23,29]. Each micropost or pillar placed in an MFSA functions as an individual force-sensing unit since it bends due to the locally applied cell's traction forces.

The development of traction force microscopy (TFM) by Dembo and Wang in 1999 has been a significant improvement to measure cellular forces[3], and at present, this technique provides the most reliable, comprehensive information on cell traction forces underlying an entire cell[9]. In this method, elastic hydrogels with flat surface, commonly polyacrylamide gel (PA), are used as a cell culture substrate (figure 3). Fluorescent microbeads are embedded into the gel in order to act as markers of its deformation, caused by movement of the also embedded cells in there[5,9,20]. The substrate's surface is further coated with ECM proteins to stimulate cell adhesion and spreading. Those cells deform the surrounding matrix, which is visualized by tracking the displacement of the beads situated in the vicinity of each cell. With that, a displacement field is determined around each cell. Finally, the displacement field values are used to calculate the cell traction forces by applying complex computational algorithms to solve the inverse problem.

In TFM, bead tracking is performed through fluorescent microscopy by imaging an isolated cell and its surrounding. A pair of images of the same cell, referred to as "force-loaded" (or deformed) and "null-force" (or non-stressed), are taken during TFM measurement[5,9,20]. The force-loaded image is taken while the adherent cell remains on the substrate, whereas the null-force image is taken after the cell has been removed by tripsinization or detergent treatment. The comparison of both images determines the displacement vector for every bead, which all together builds up the displacement field around a single cell; then, this data is employed in cell force calculation. There are different alternatives to this proceeding, such as taking images during cell movement instead of before and after exerting forces on the substrate[30], or like treating cells with a cytoskeleton inhibitor that will prevent them from force generation[31].

There are three strategies to execute TFM, each of which is unique in both how displacement field is extracted from images and how cell forces are subsequently estimated[9]:

Dembo and Wang 1999, Butler *et al.* 2002, Yang *et al.* 2006. Characteristically, the latter[33] introduces the finite element method in the TFM process to model the substrate as a 3D object, in order to improve the efficiency of computational calculation[20].

Almost all TFM studies have been applied to two-dimensional (2D) cell culture systems (figure 3). Regarding it has been shown that cellular traction forces in all three dimensions matter to fully understand cell–substrate interactions[34], recently some research groups are applying more natural 3D cell cultures to the TFM measurement technique[24,35]. The substrate of these cultures is made of PEG (polyethylene glycol) or collagen gel shaped into 500µm-tall disks, and cells are grown in its inside (figure 4). This new feature requires the use of confocal microscopy, so as to properly visualize the bead and cell localization in the axial plane (z axis). Confocal microscopy shows an individual section or slice of the z axis, with controllable thickness. This approach can only be performed by applying laser light to the sample at the excitation wavelength of the fluorochrome inserted in the sample, in this case, the fluorescent beads.

1. 3. *In vitro* cell cultures: 2D versus 3D environments

Although *in vivo* studies have been carried out to monitor cell-cell interactions and cell signaling within their native microenvironments, these studies are limited by expensive experimental manipulations (e.g., animal models), lack of control over local experimental conditions, and complex imaging setups[11]. Study of cell-cell interactions *in vitro* is advantageous due to more tightly controlled experimental conditions, higher experimental throughput, and lower costs. The traditional *in vitro* approach consists in culturing cells on flat plastin or glass plates, where only cell monolayers can be grown. In fact, tissue-specific architecture, mechanical and biochemical cues and physiological cell–cell communication are missed by planar, two-dimensional (2D) cell cultures, as a result of their simplified and highly biased conditions[1]. Indeed, this limits the culture's potential to predict the cellular responses of real organisms.

Cell culturing within 3D scaffolds mimics the specificity of real tissues better than conventional 2D cultures[1]. Cells cultured using traditional 2D tissue culture methods migrate different from cells in 3D environments, and moreover, they show different gene expression levels of a variety of proteins compared with their counterparts in native *in vivo* environments. In addition, natural cell-ECM adhesions differ in structure, localization, and function from classically described *in vitro* adhesions[36]. Hence, 3D cell cultures re-establish the physiological cell-cell and cell-ECM interactions, reducing the gap between cell cultures and physiological tissues[1].

The need for quantitative and physiologically relevant cellular systems has driven to develop several 3D assays, like suspending cells in a gel or plating cells on beads within a gel. However these fail in the ability to produce a well-controlled microenvironment with similar dimensions to tissue structures *in vivo* [37]. To overcome this obstacle, a hydrogel-incorporating microfluidic cell culture assay has recently been elaborated[11,37,38], which is a multipurpose platform (it has been used to study angiogenesis and tumour cell interactions, for example). Particularly, a microfluidic device or chip is a set of micro-channels molded into a material (glass, silicon or polymer), filled with a desired liquid, and connected together so as to achieve a desired function like mix, pump, redirect and/or allow chemical reactions[39]. This technology

has opened the door for creating more realistic *in vitro* cell culture methods that replicate many aspects of the true *in vivo* microenvironment, by enabling the containment of ECM-mimicking hydrogels inside the micro-channels and the introduction of biochemical gradients, shear stress or other factors, with precise spatial and temporal control. Besides, the microfluidic device can be custom-designed, i.e. its channel architecture is adaptable to the specific biological process in study and the applied methodology.

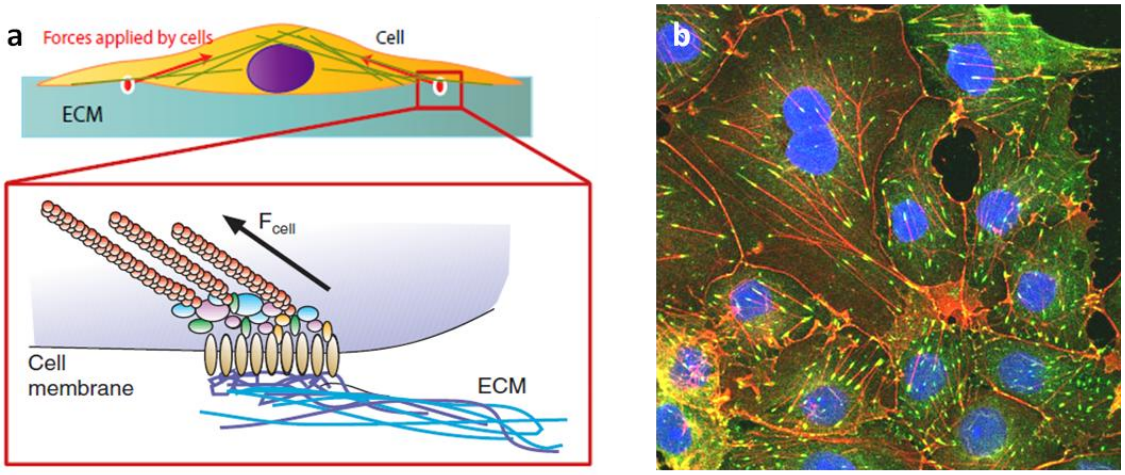

Figure 1. Cell adhered to the ECM. a. Cells' actin cytoskeleton (green) generates traction forces, which are transmitted to the ECM (blue) through focal adhesions (red spot and inset). b. Confocal fluorescence microscopy image of adherent cells. Cells' nuclei are dyed in blue, actin fibers in red, and focal adhesions in green. Figure adapted from refs. 10, 5.

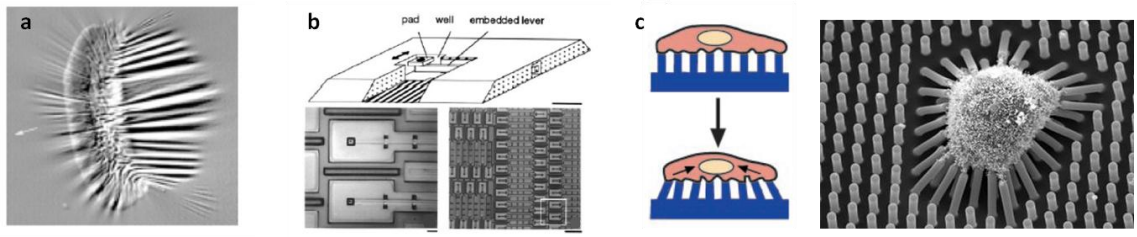

Figure 2. Techniques for measuring traction forces of single cells. a. A cell produces wrinkles on a silicone membrane. b. Different magnifications of a micromachined cantilever beam array. c. Scheme and phase contrast microscopy image of a micropost force sensor array. Figure adapted from refs. 23, 28, 40.

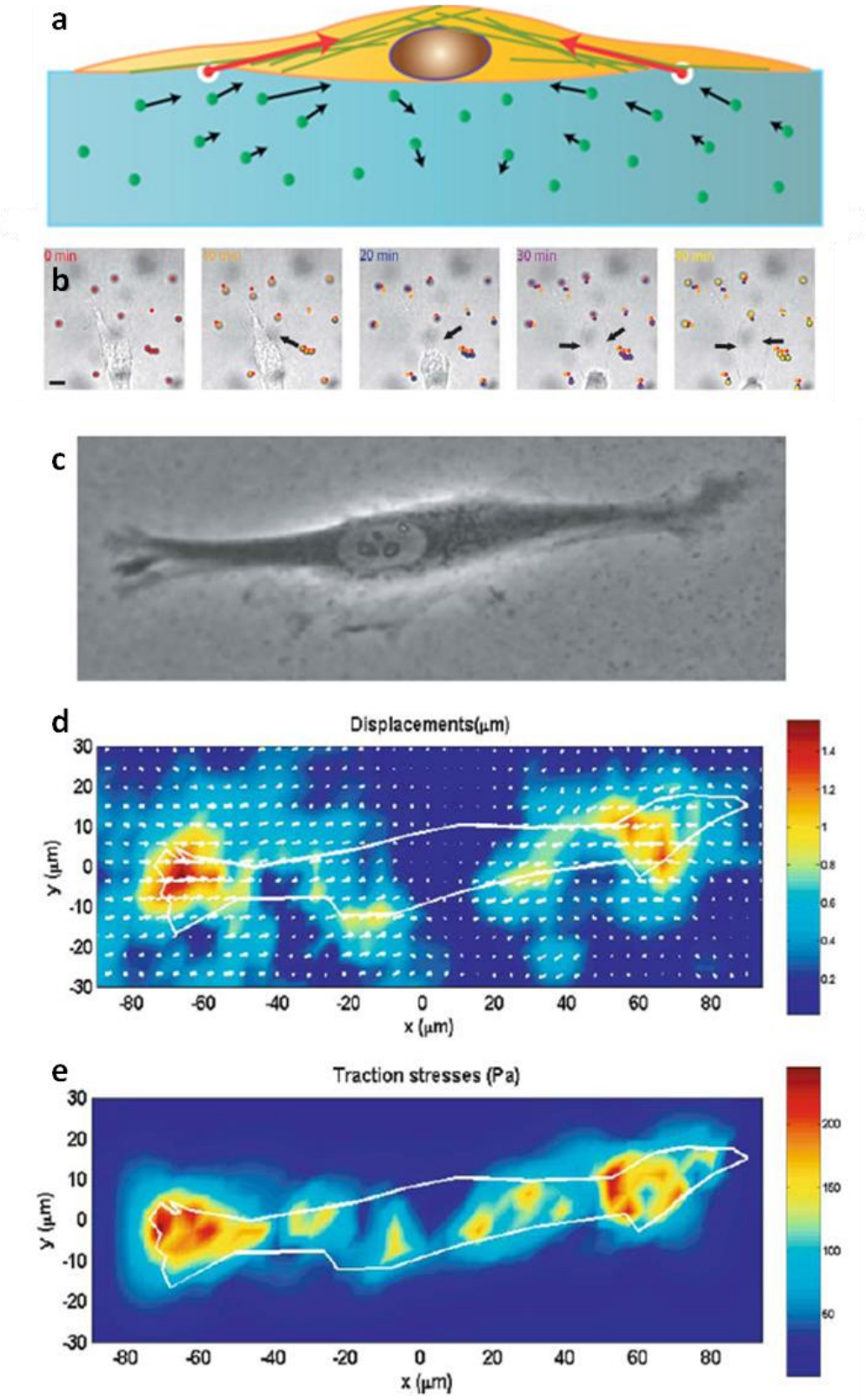

Figure 3. Traction force microscopy in two-dimensions. a. Schematic representation of the TFM: cell cultured on a gel loaded with fluorescent beads. b. Deformation of a collagen gel denoted by bead movement. Scale bar, 20μm. c. Human patellar tendon fibroblast cell on a polyacrylamide gel with embedded fluorescent beads. d. Substrate displacement field. e. Recovered cell traction force field. Figure adapted from refs. 5, 33, 41.

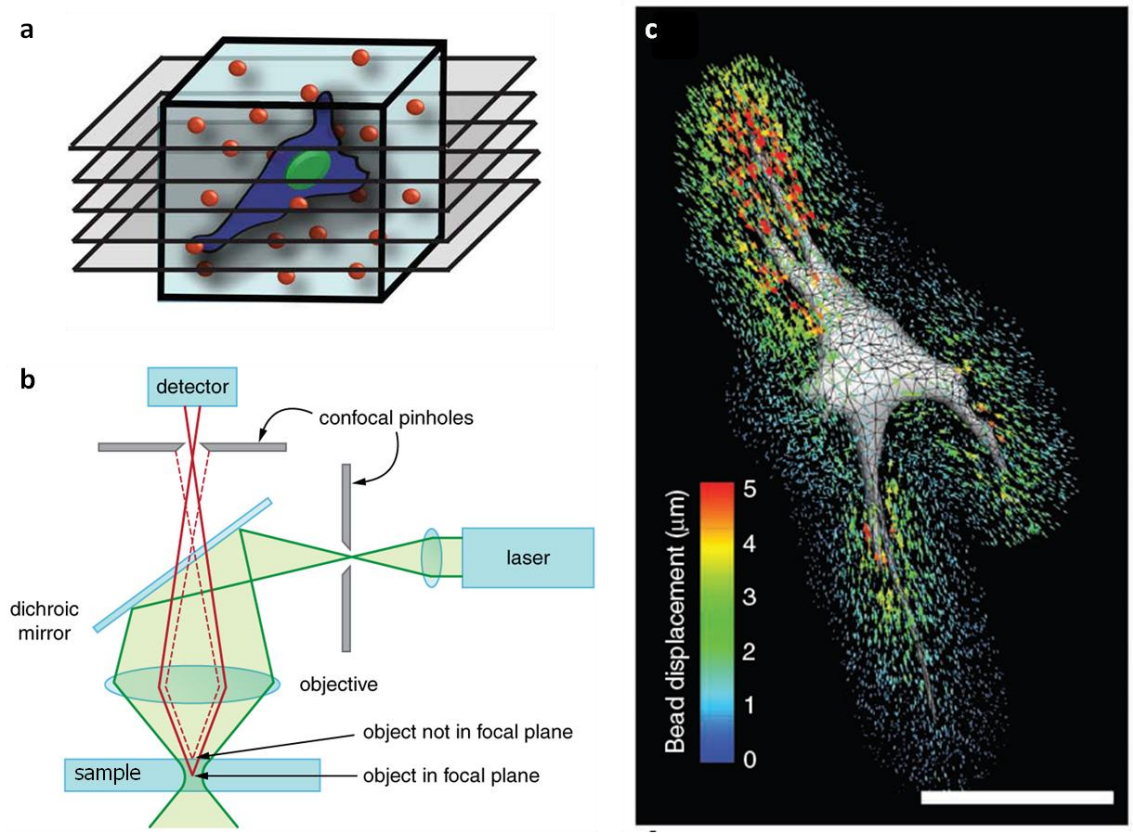

Figure 4. Traction force microscopy in three-dimensions. a. Schematic of the TFM: cell cultured within a bead-loaded gel; equidistant z-axis sections are also marked. b. Confocal microscope schematic: any confocal image corresponds to a specific focal plane or z-axis section of the sample. c. Example of bead displacement trajectories color coded by magnitude. Scale bar, 50μm. Figure adapted from refs. 24, 41.

2. PROJECT AIM

The aim of this project is to develop a microfluidic device containing a cell-loaded 3D collagen hydrogel, completely tailored for employing it in traction force microscopy. This platform is intended to recreate the cells' physiological environment by embedding them within a three-dimensional substrate made up of the most abundant protein in natural ECMs, collagen fibers; and also by guaranteeing a constant supply of nutrients owing to the microfluidic device. Therefore, theoretically the cell traction forces measured by the proposed strategy will be closer to the ones existing *in vivo* than the values obtained through previously published studies employing TFM.

This master project is part of the INSILICO-CELL project "Predictive modeling and simulation in mechano-chemo-biology: a computer multi-approach" (European Union Starting Grant), performed by the M2BE group, at I3A, Zaragoza. The main project seeks the answers to how cells interact with each other and with the environment to produce the large-scale organization typical of tissues, by combining *in silico* and *in vitro* models. This research is applied in three physiological processes, where the role of environment conditions is important and the main biological events are cell migration, cell-matrix and cell-cell interactions: bone regeneration, wound healing and angiogenesis. For this, the used *in vitro* model is the microfluidic device employed here, and traction force microscopy is the chosen technique to assess cell migration. Therefore, it is vital task in the INSILICO-CELL project to adjust the device to this technique in order to be able to perform cell migration measurements.

To achieve the master project's aim, the specific objectives are to:

I. Learn the microfluidic device fabrication process and cell culture inside it.

II. Optimize bead conditions within the collagen hydrogel.

III. Assess cultured cell conditions within the collagen hydrogel.

IV. Evaluate the traction forces exerted by cells cultured inside the microfluidic device by confocal microscopy.

3. DEVICE DESCRIPTION

The project's microfluidic device is fabricated in polydimethylsiloxane (PDMS) applying a proceeding adapted from a previous work[37]. The designed microfluidic channel architecture consists of two side channels and one central channel. As shown in fig. 5, all channels are connected in the middle region, termed culture chamber. At each end of the channels there is a drilling hole through the whole thickness of the device, which provides direct access from the external surface to the inside of the channels. Through both of its holes, the central channel is entirely loaded with collagen hydrogel, which arranges in a 3D manner in there. And the lateral channels are filled with cell culture medium in order to hydrate the polymerized collagen matrix and provide nutrients to the cells embedded in it, symmetrically from both sides. Ultimately, the whole device is fabricated on a compatible support for confocal microscopy.

Introducing collagen substrate into this microfluidic device is conceived to achieve a three dimensional matrix where fibroblast cells can attach like to their native environment, accomplishing a fusiform or elongated cell shape. Also, preparation of the collagen hydrogel solution is performed to specifically reach a pH 7.42, since that is the physiological pH. As well, hydrogel preparation conditions are adapted to obtain a small collagen pore size, significantly smaller than human dermal fibroblast cells.

Further, the collagen hydrogel injected into a microfluidic device must be laden with numerous fluorescent microbeads, in addition to cells. These beads are indispensable for the TFM technique since they act as markers of the deformation of the matrix (displacement field) produced by the forces exerted by cells. Therefore, the achievement of a homogeneous bead distribution all over the collagen matrix is crucial for acquiring meaningful data. The size of the beads is equally key as they should be bigger than the matrix pores so that each one remains immobile at one point of the hydrogel. Moreover, bead concentration, bead-cell interaction, thermal stability and other issues are also important concerns in this project.

These fluorescent markers are ultraclean polystyrene latex microspheres, inside loaded with fluorescent dye, supplied as aqueous suspension containing 2% solids. The manufacturer offers them in ten different colors, in a wide range of sizes and with several surface modifications; so accordingly there are multiple choices. Apart from dye color, the selection of the microsphere type is dictated by its behavior against the collagen solution. Hence, on one hand, carboxylate-modified beads (anionic) are chosen because their surface has a high density of carboxylic acids that firmly adhere to the amine groups vastly present on the collagen proteins of the substrate, whereas not bind to cell membranes. Thus, these beads act as faithful fiduciary markers of the local deformations of the collagen matrix induced by cell forces[41], therefore they are suitable for the project's TFM substrate.

On the other hand, the bead size selection was determined regarding the particle size used in previously published studies and human dermal fibroblast cell dimension. The most frequently used bead diameter size has been 0.2μm since TFM was conceived[22,24,42], however, these assays were performed employing elastic polymer substrates, commonly polyacrylamide gels. In contrast, few has been tested on collagen matrixes[35,41,43] and with very distinct TFM procedures. In these investigations, microsphere diameter varies between 1μm and 3.6μm. Knowing this data plus taking into account that all the mentioned bead sizes are truly smaller than fibroblasts (circa 150μm length x 25μm width, as averaged using own measured values), 1μm diameter beads was decided to be used in this project.

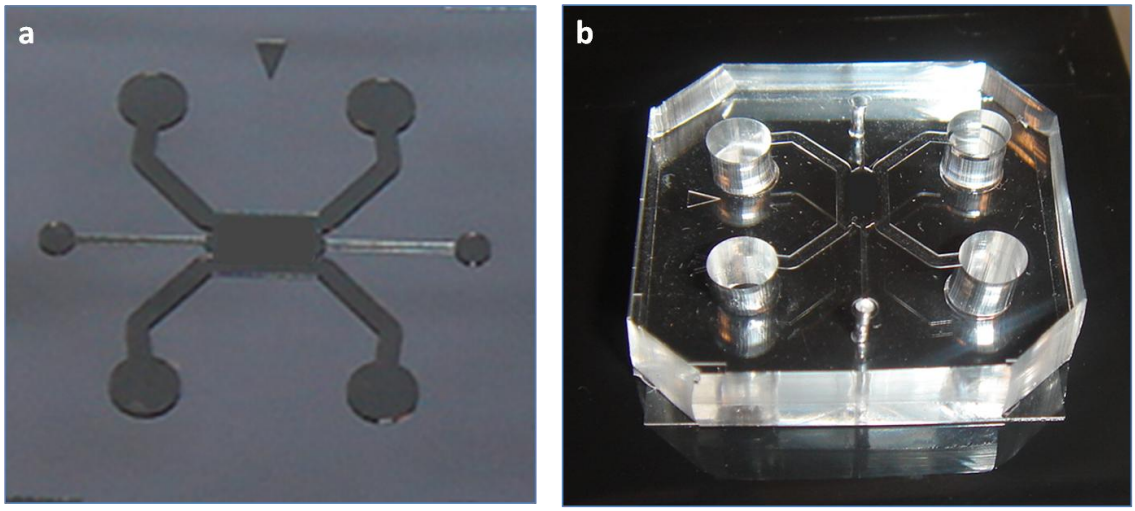

Figure 5. Employed microfluidic device. a. Channel architecture. b. Photograph.

4. MATERIALS AND METHODS

4. 1. Microfluidic device fabrication

To fabricate the PDMS core of the devices, Sylgard 184 silicone elastomer base and curing agent (Dow Corning) were mixed in a 10:1 weight ratio. The viscous solution was poured onto a silicon wafer mold, and cured overnight in an oven at 80°C. Cured PDMS was removed from the mold and shaped into individual round devices using a puncher. Channel-end holes were perforated through the full thickness of the device with size-specific biopsy punches. These were afterwards sterilized via wet autoclave and then dry autoclave. Finally, device assembly and coating of the microchannels were performed as previously described[37].

4. 2. Fibroblast cell culture

Human dermal fibroblasts (normal adult human dermal fibroblasts from Lonza) were maintained in cell culture medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% antibiotics penicillin, streptomycin and amphotericin (all from Lonza). Incubation was done by traditional two-dimensional culturing in plastic flasks and under standard growth conditions, i.e. 37ºC and 5% CO_2. Cell culture medium was changed every 2 days.

4. 3. Hydrogel solution preparation

First, polystyrene latex fluorescent beads (1µm diameter, carboxylate-modified, fluorescent red dyed FluoSpheres® from Molecular Probes) were briefly vortexed and mixed with culture medium supplemented with antibiotics penicillin and streptomycin (medium-PS, all purchased from Lonza) in a volume ratio of 1:1, all the time protecting them from light and maintaining them cooled at 4ºC. This solution was afterwards treated in a bath sonicator (Sonorex, from Bandelin electronic GmbH) for 2 minutes and then stored at 4ºC.

Second, a confluent fibroblast cell culture at passages 10-12 was washed twice with PBS (phosphate-buffered saline, Lonza) and treated with warm trypsin (Biochrom) to detach cells from the dish. Cells were centrifuged at 1500rpm during 5 minutes, and then the remaining pellet was resuspended in cell culture medium to a final density of 120,000cells/mL, as earlier calculated through cell counting at a Neubauer chamber (Incyto). Cells were resuspended at low density to seed them into the collagen matrix avoiding potential confounding effects of local matrix deformation by neighboring cells.

Third, on ice, a 200µL collagen solution at pH 7.4 and 2mg/mL was prepared as published[37]. Next, 20µL of the resuspended fibroblasts were added and well mixed, and 20µL were removed from the hydrogel mixture. Then, the beads-medium-PS tube was briefly vortexed again and 8µL of it were added to the previously prepared collagen solution, to achieve a 1:50 dilution by volume. From now on, the procedure was performed avoiding direct light in order to preserve the beads' fluorescence. With this, the solution was finally accomplished and 20µL of it were carefully injected into the central channel of a microfluidic

device. To conclude the proceeding, the collagen-loaded device was put in an incubator at 5% CO_2 and 37°C for 20 minutes and afterwards, its other two channels were filled with cell culture medium. The device was stored at the CO_2 incubator to allow collagen hydrogel polymerization and cell spreading within it. Cell culture medium was changed every day.

In order to assess cell viability within the collagen-beads hydrogel injected inside a microfluidic device, the Live/Dead Viability/Citotoxicity Kit (Invitrogen) was employed. Sample staining process was done after washing it three times with Dulbecco's PBS 1x (DPBS, from Lonza). Reaction solution was prepared just before treatment because calcein hydrolyzes rapidly if exposed to moisture. It consisted of vortexed 4µL of ethidium homodimer-1 (EthD-1) in 2mL of DPBS, plus 1µL of calcein. With this, dead cells were dyed in red by EthD-1 and live cells in green by calcein.

4. 4. Cell staining and microscopy imaging

To visualize cells by fluorescence microscopy, all channels of the microfluidic device were incubated with CellTracker Green (Invitrogen) at 1:200 dilution in medium-PS, for 30min, with previous and afterwards washes with NaCl solution. Channels were finally filled again with culture medium. Overall cell staining was performed just before microscopy imaging, since CellTracker fades out with time.

Another cell fluorescent staining was done using calcein AM, which is well retained inside live cells, producing an intense uniform green fluorescence. Samples were washed thrice with Tris Buffered Saline 1x (TBS, Sigma) and then treated with a solution consisting of 1mL TBS plus 0.5µL calcein AM. After 30min incubation time at room temperature and protected from light, samples were ready for microscopy imaging.

Phase images of cells and fluorescence images of cells and beads were recorded simultaneously with a 63x, NA 1.4 oil-immersion objective, on an inverted confocal microscope (TCS SP2, Leica). Three laser lines were used: an Argon 488nm laser to provoke collagen fibers' reflection, an Argon 496nm laser to excite CellTracker Green for cell cytoplasm labeling, and a Helium-Neon 543nm laser for the fluorescent red beads inside the collagen hydrogel.

To quantify the collagen hydrogel deformation, a 147 x 147 x 0.12 µm volume was imaged around each cell, in both horizontal planes and the axial plane, respectively. At least two image stacks were carried out: the deformed state of the hydrogel, and the non-stressed state, achieved by treating the cells with 25µM cytochalasin D (C2618 from Sigma) for at least 15min. Also, other samples were treated for 15 minutes with triton 1x (Sigma) diluted in PBS. Images were saved in 1024x1024 multipage TIFF format.

5. RESULTS

To begin with the project, progressive learning of the microfluidic device fabrication proceeding was carried out, and then collagen hydrogel injection required some training. Once the process was known (aprox. 74 hours), adaptation of the device to TFM was developed through numerous experiments.

First assays consisted in filling microfluidic devices with collagen hydrogels containing fluorescent beads and no cells, at different conditions. This was performed in order to find the optimal bead concentration, while comparing the behavior for different bead sizes. With this, it was shown that in fact 0.5µm-diameter beads were too tiny, therefore didn't get physically encapsulated in the hydrogel and they diffused within it in a Brownian motion (figure 6). As opposed to them, 1µm particles got well encapsulated in the collagen hydrogel and showed a uniform distribution along the three dimensions of the substrate. Bead concentration was notably differentiated at both tested sizes. And so, 1:100 and 1µm were determined as the optimal concentration and bead size conditions. The selected dilution factor is similar to the ones used in many other publications: 1:125[22,32], 9:100[30].

Before sampling, vortex mixing was applied to the bead solution during 10 seconds, as recommended by the manufacturer, in order to avoid particle agglomeration. However, observing fluorescent microscope images of 1µm particles at 1:100 dilution assays, single fluorescent dots seemed small accumulations of some beads instead of single beads; and this was not attributable to the collagen solution's pH, given that it was always mixed to reach a pH 7.42, while microspheres get neutralized and agglomerate at a pH inferior to 5.0[44]. To solve this problem, it was carried out a set of experiments to analyze the effect on bead aggregation of vigorous shaking via sonication, since this treatment generates millions of microscopic vacuum bubbles within the sample that collapse violently (cavitation) driving liquid into all openings and corners of the sample particles. This strategy was both also recommended by the manufacturer and applied in some previous studies[22,43]. Further search was performed in order to verify that temperature increase due to sonication (until 40°C, maximum) wouldn't affect bead properties. Polystyrene non-coated microspheres' glass transition temperature is 100-110°C and carboxylate-coated microspheres' is 120°C[45], beyond those values particles suffer deformation. So indeed, the carboxylic surface groups provide thermal stability to polystyrene beads, hence sonication temperature rising wasn't an obstacle for the project's experiments.

Here, 1µm beads were briefly sonicated after their suspension in culture medium supplemented with 1% penicillin and 1% streptomycin (medium-PS) in volume ratio 1:1, as described by Petroll *et al.*[43]. Two kinds of sonicator were compared, i.e. probe and bath sonicator, and the resulting data was that beads treated with a bath sonicator were the less aggregated, even though probe sonicator was applied at multiple times and intensities. This difference occurred owing principally to the distinct application system between the two instruments. The concluded optimal bath sonicator time was 120", since there was almost no bead accumulation.

Additional assays demonstrated that both bead sonication treatment and fluorescence were maintained after 12 days in medium-PS-bead samples stored at 4°C, protected from light. Those samples appropriately mixed with collagen solution were injected into microfluidic devices the sonication day, and also 5, 7 and 12 days later, with same results.

A further step in this project was to add human dermal fibroblast cells to the 1:100 bead-collagen solution (figure 7). The first approach consisted in checking the distribution and viability of the added cells at 120,000cells/mL density, and qualitatively validating the size dissimilarity between fibroblasts and microspheres. Indeed, cells were randomly distributed and in a extremely low density, just as needed in TFM, since each cell has to be isolated from others to measure its single mechanical influence on the surrounding substrate and avoid confounding effects exerted by neighboring cells. Moreover, size difference between fibroblast and beads was unconditionally confirmed; and there was evidence of good cell viability as plenty of cells adhered within the collagen matrix and spread acquiring an elongated shape, and after 4 days cells had proliferated.

To support this indirect viability evidence, a viability assay for mammalian cells (Live/Dead Viability/Cytotoxicity Kit from Invitrogen) was performed, which applies ethidium homodimer-1 (EthD-1) and calcein AM to the sample. Nonfluorescent cell-permeant calcein AM is converted to the intensely fluorescent calcein by intracellular enzymatic activity, which is only present in live cells. Further, EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells. Moreover, EthD-1 is excluded by the intact plasma membrane of live cells. Hence, this kit dyes dead cells in red by EthD-1 and live cells in green by calcein. As expected, this assay performed on sample devices revealed a 100% of viability, as all the cultured cells within a device were marked as living cells (figure 8).

Next step consisted in confocal microscopy visualization of the device's central channel, with a higher objective (63x instead of 40x), so as to closer observe cells. As aforementioned, confocal microscopes show only fluorescently marked structures. Therefore, cells were dyed in green in order to see them together with the red beads. Also, collagen fibers' reflection at 488nm was useful to visualize them as well.

To begin, some control samples were processed. With them, collagen fiber distribution was obtained: at an only collagen hydrogel sample device, fibers were randomly deposited all over the central channel; there was no difference between axial plane bottom and top fibers, nor between central and lateral situated fibers within the x axis (figure 9). The same distribution was achieved at the collagen-beads hydrogel sample device (figure 10). In addition, reflection spots and red bead spots colocalized, hence beads were wrapped by collagen fibers. However, beads didn't act like nucleation sites for collagen fibers, altering the collagen assembling network, as reported by Newman *et al.* [46].

Another control consisted in collagen-beads-cells hydrogel sample device, with cells not yet fluorescently marked (figure 11). This assay revealed a convergence of fibers towards the cells, which perhaps was evidence for local degradation-remodelation of the collagen matrix by the cell[31,41,43]. Furthermore, the number of beads surrounding each cell was observed insufficient along the axial plane. Therefore, another experiment was performed introducing cells into collagen solutions with distinct fluorescent particle concentrations, from volume ratio 1:50 to 1:1000. This assay revealed that at more concentrated solutions (1:50, 1:100 and 1:200), several beads gathered really close to the cells, particularly around the not yet spread cells, namely the round-shaped ones. Interestingly, this tendency diminished in accordance with bead concentration decrease, until the 1:500 dilution, in which beads no longer gathered (figure 12). The 1:50 dilution was selected as optimal regarding the number of beads surrounding each cell.

In order to better visualize the cell-bead interaction, cells were marked with CellTracker™ Green, which is a green fluorescent dye for the whole cell. With that, cells were stained, but surprisingly they had a rounded shape instead of elongated; and also beads looked thicker than in previous samples. The washing process of cell staining with CellTracker was modified by employing NaCl instead of PBS, so as to prevent cell shape alteration. PBS was also deleted from the protocol since it contains monobasic phosphates that occasionally attach to the collagen matrix, as a lab colleague reported, therefore these phosphates could be adhering to the beads and thickening them.

Confocal visualization improved a bit with the employment of NaCl, but cells were still round-shaped and, as well, fluorescence faded away rapidly with laser exposition. To overcome this obstacle, cells were dyed green with calcein, which specifically marks cell cytoplasm. This marker was the same as in the viability assay, thus only live cells would be stained. Indeed, all cells got stained, for a longer time, and were visually better defined, but even so they became round-shaped. This circumstance was not favorable to the project, because those rounded cells didn't change their shape due to treatment with a cytoskeleton inhibitor.

Despite this setback, some interesting confocal images were taken that revealed a colocalization of numerous beads and cell cytoplasm (figure 13). This demonstrates that the cultured fibroblasts were phagocyting the fluorescent beads[44,47], which were probably the beads previously localized at the collagen matrix remodeled by the cell. However, there were still diverse beads surrounding the cell, fact that is still useful for TFM.

Regarding that for an unknown reason cells lost their fusiform shape when stained with fluorescent dyes, transfection of the fibroblasts with GFP (green fluorescent protein) was decided to be done, in order to obtain GFP-expressing cells. This protein exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range, and it has been employed to label cells in diverse TFM publications[24,30,43]. However, this process has to be carried out by specialized scientists and requires a long time to get fully accomplished. Therefore, in order to continue with this project, subsequent experiments were performed without cell staining, so as to work on elongated cells.

Diverse assays involving collagen-beads-cells hydrogel sample device were prepared to take confocal images 5 minutes before and 15 minutes after treatment with triton X-100, which is a detergent that kills cells by disrupting their membranes (figure 14). In fact, triton influence was observed on the fibroblasts: their body lost elongation. Images stacks of this phenomena were taken every 30 seconds at the same z-axis section, situated near the centroid of a selected isolated cell. Bead movement could be observed, therefore, cells were changing their traction force exertion. However, collagen reflection images revealed that the detergent altered also the collagen matrix's reflection pattern.

Another experiment consisted in treating the sample with cytochalasin D (figure 15). This cell permeable micotoxin acts as an inhibitor of actin polymerization[48], therefore it halts cell movement and force generation. As expected, the effect on cell body was weaker than with triton, but collagen reflection remained unmodified. With the cytochalasin D and triton image stacks, bead spatial localizations and displacement vectors can be recovered through computational algorithms by other members of the research group; and then, this data will be used to calculate cell traction forces. Therefore traction force microscopy has been successfully applied to a microfluidic device.

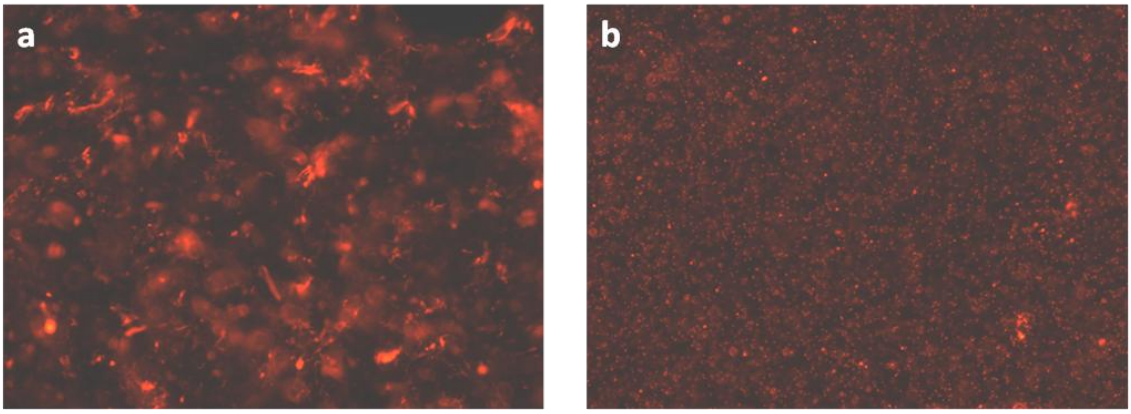

Figure 6. Bead distribution within the collagen hydrogel. a. Collagen hydrogel with 0.5µm-diameter beads. b. Collagen hydrogel with 1µm-diameter beads.

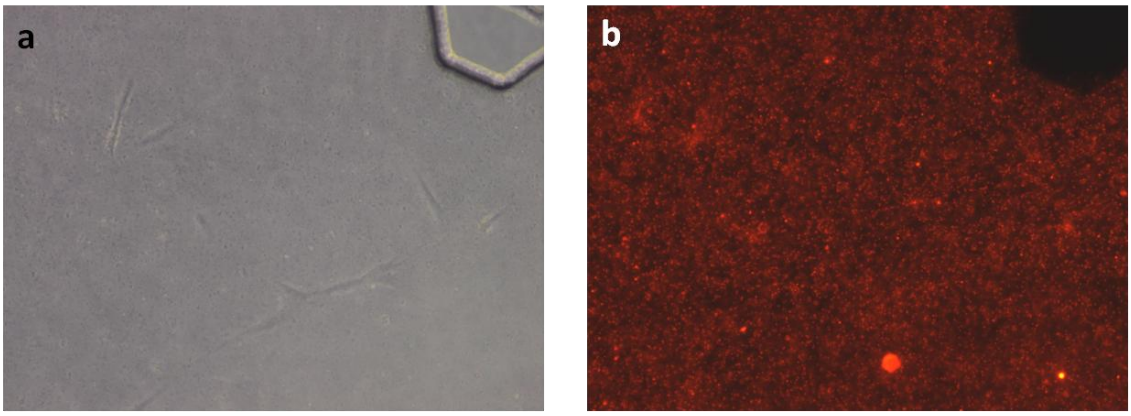

Figure 7. Cell distribution and shape within a collagen-beads hydrogel. a. Cells adopt an elongated shape. b. Bead distribution at 1:100 dilution.

Figure 8. Cell viability assay. a. Cells marked in green demonstrating they are alive. c. There is nothing labeled in red.

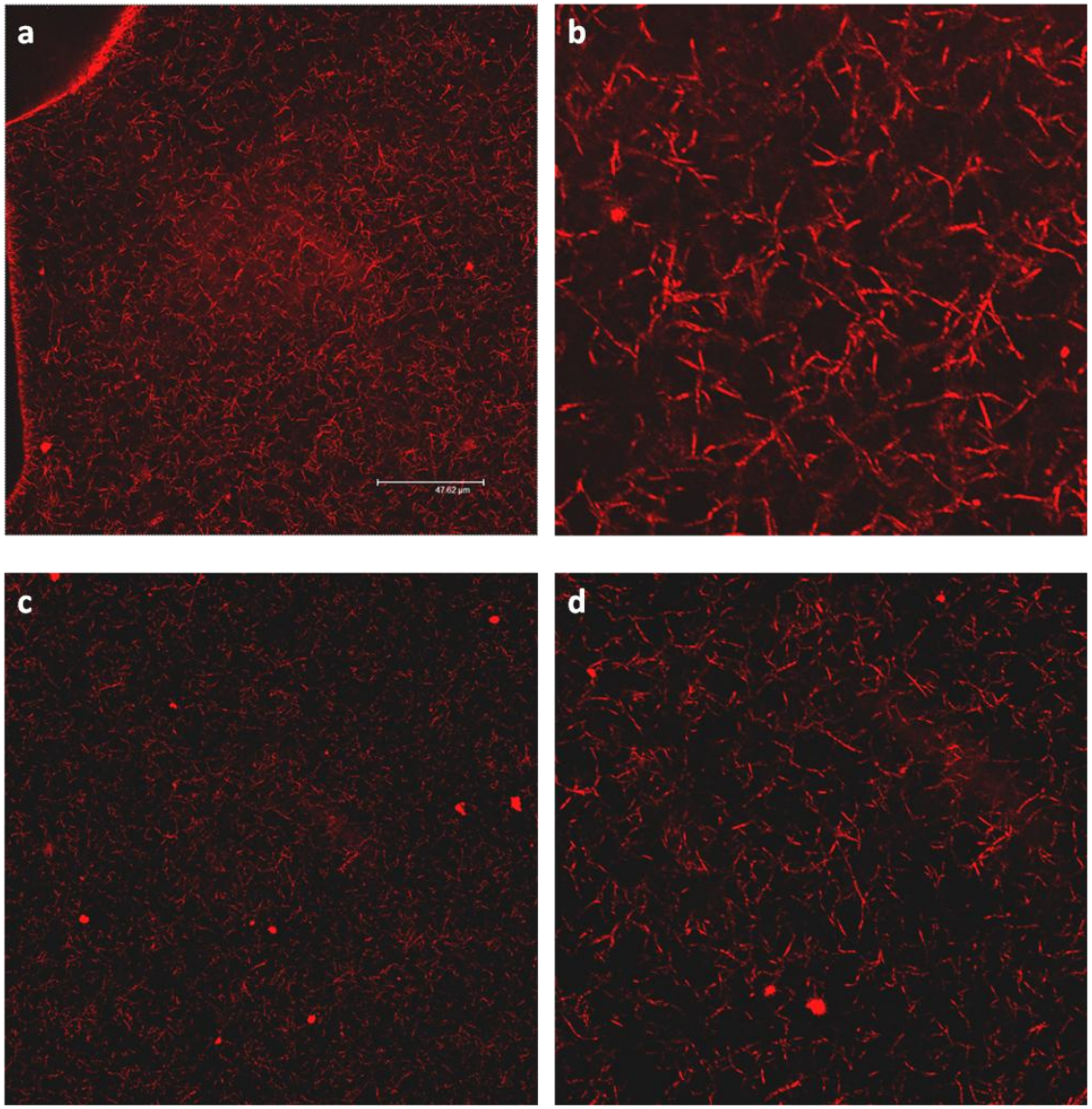

Figure 9. Collagen fibers distribution in a collagen hydrogel by confocal microscope with 63x objective. a. Fiber distribution near the central channel's posts. b. Zoom image of a. at 3.7x. c. Fiber distribution in the middle of the central channel. d. Zoom image of c. at 2.3x.

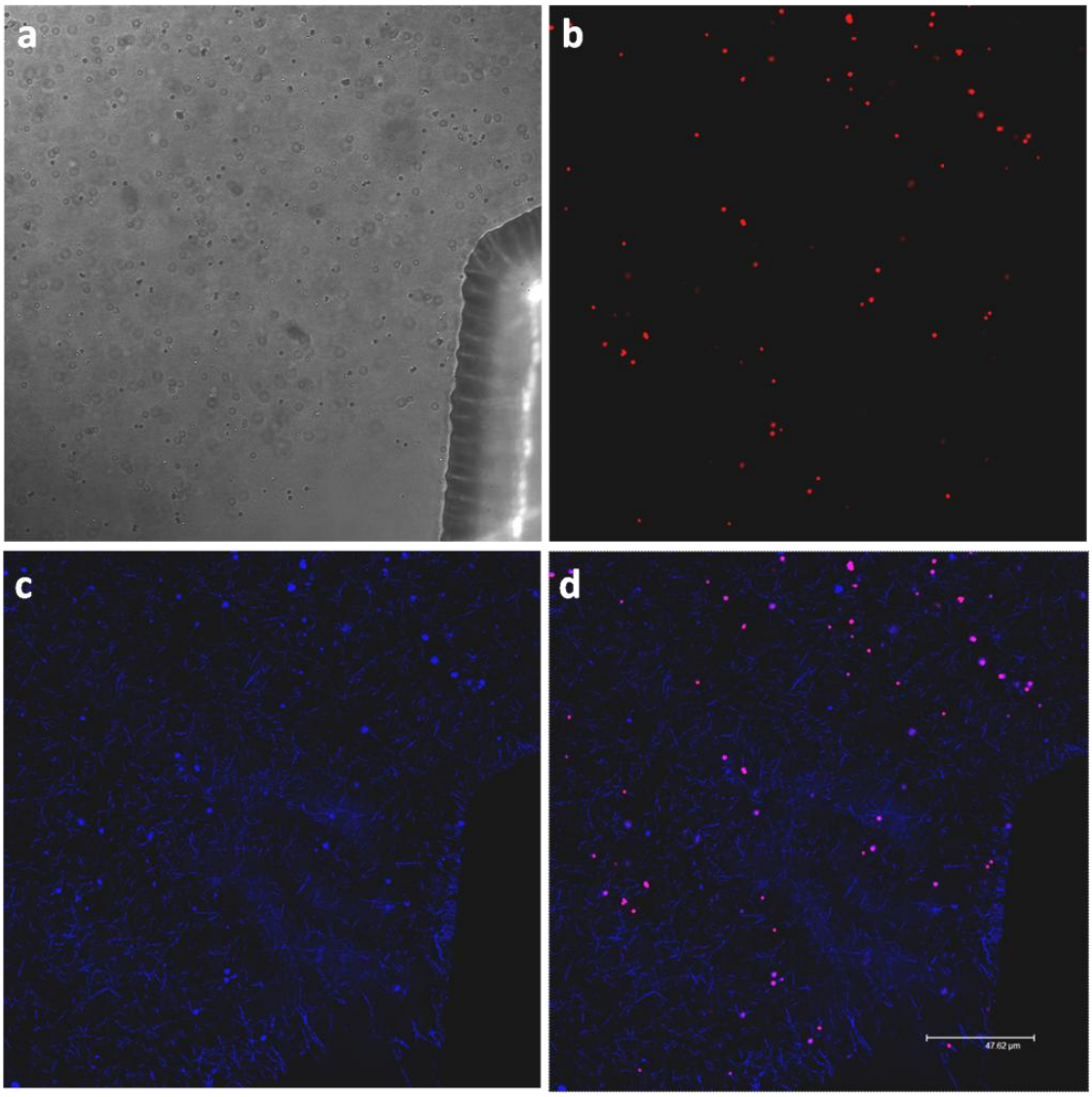

Figure 10. Confocal image of collagen-beads hydrogel. a. Phase contrast image (not confocal). b. Bead distribution. c. Collagen fiber distribution. d. Overlap of b. and c. to emphasize colocalization (colored in purple).

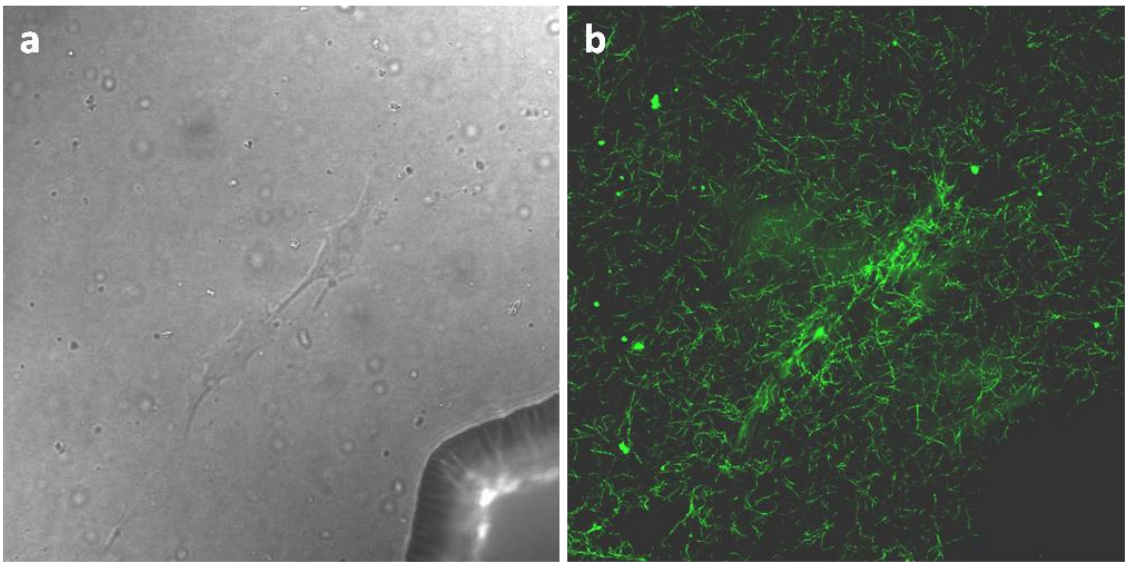

Figure 11. Collagen-cells hydrogel. a. Phase contrast image of two cells. b. Confocal image of collagen fiber distribution underneath the cells.

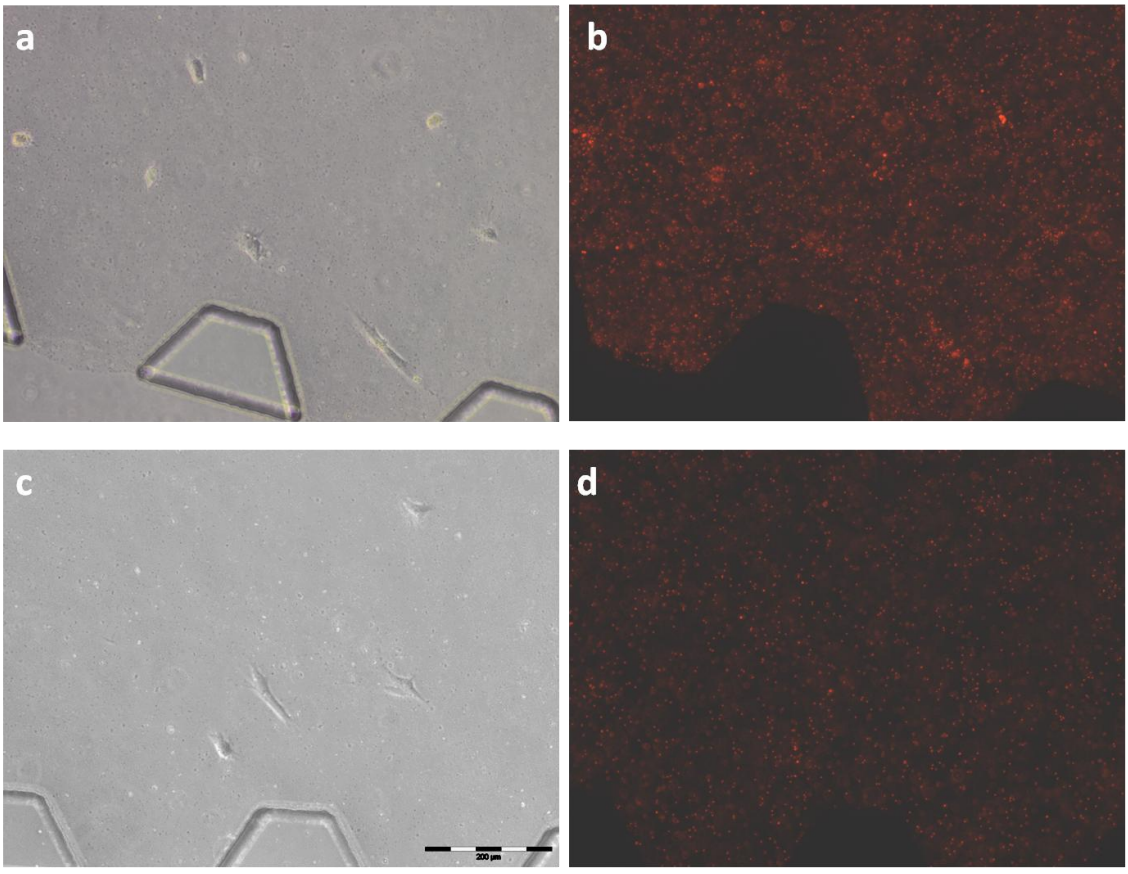

Figure 12. Gathering of beads near the cells. a. and b. Cells within a collagen hydrogel with beads at 1:200 dilution. c. and d. Cells within a collagen hydrogel with beads at 1:500 dilution. a. and c. Phase contrast images of cells. b. and d. Confocal images of beads.

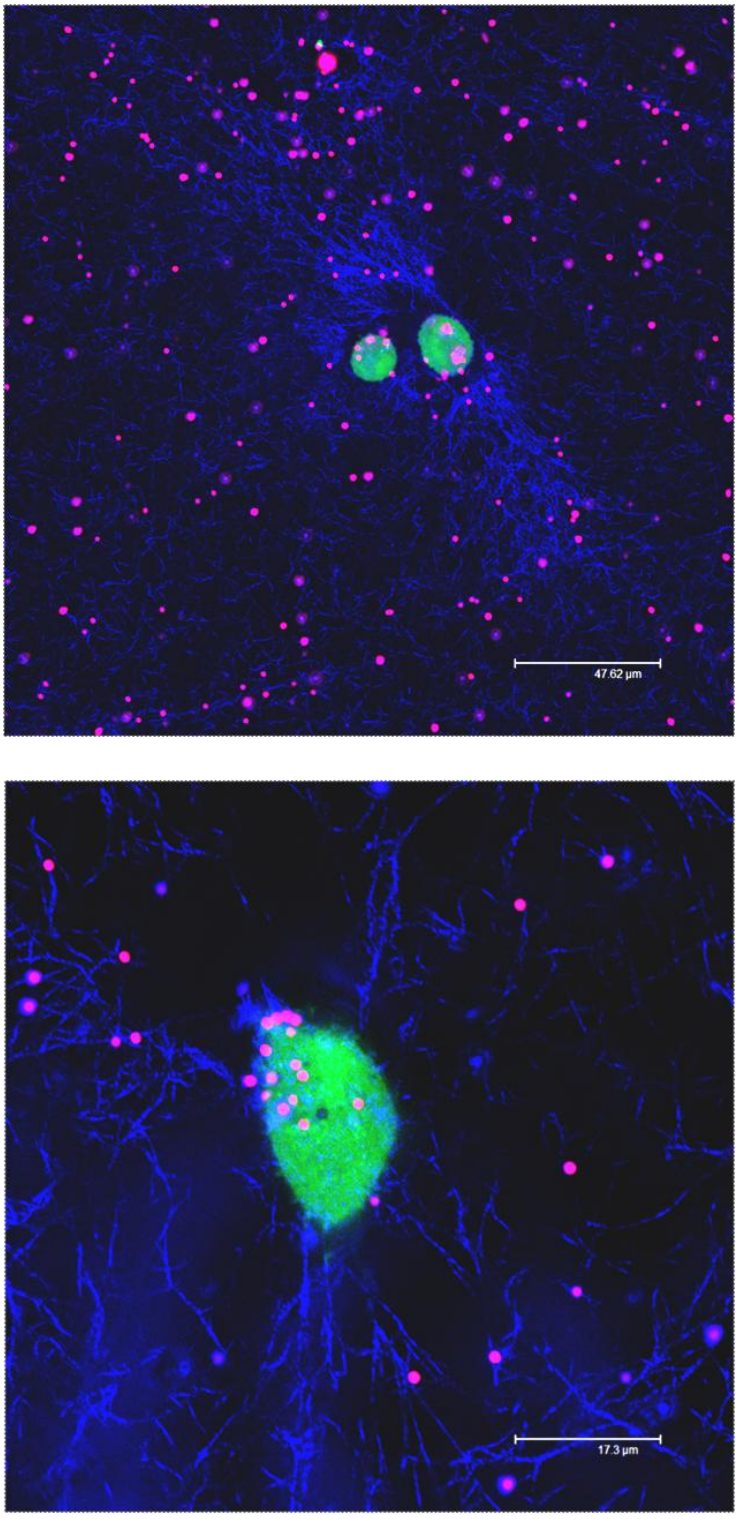

Figure 13. Calcein fluorescent staining of cells, in green. Two different samples.

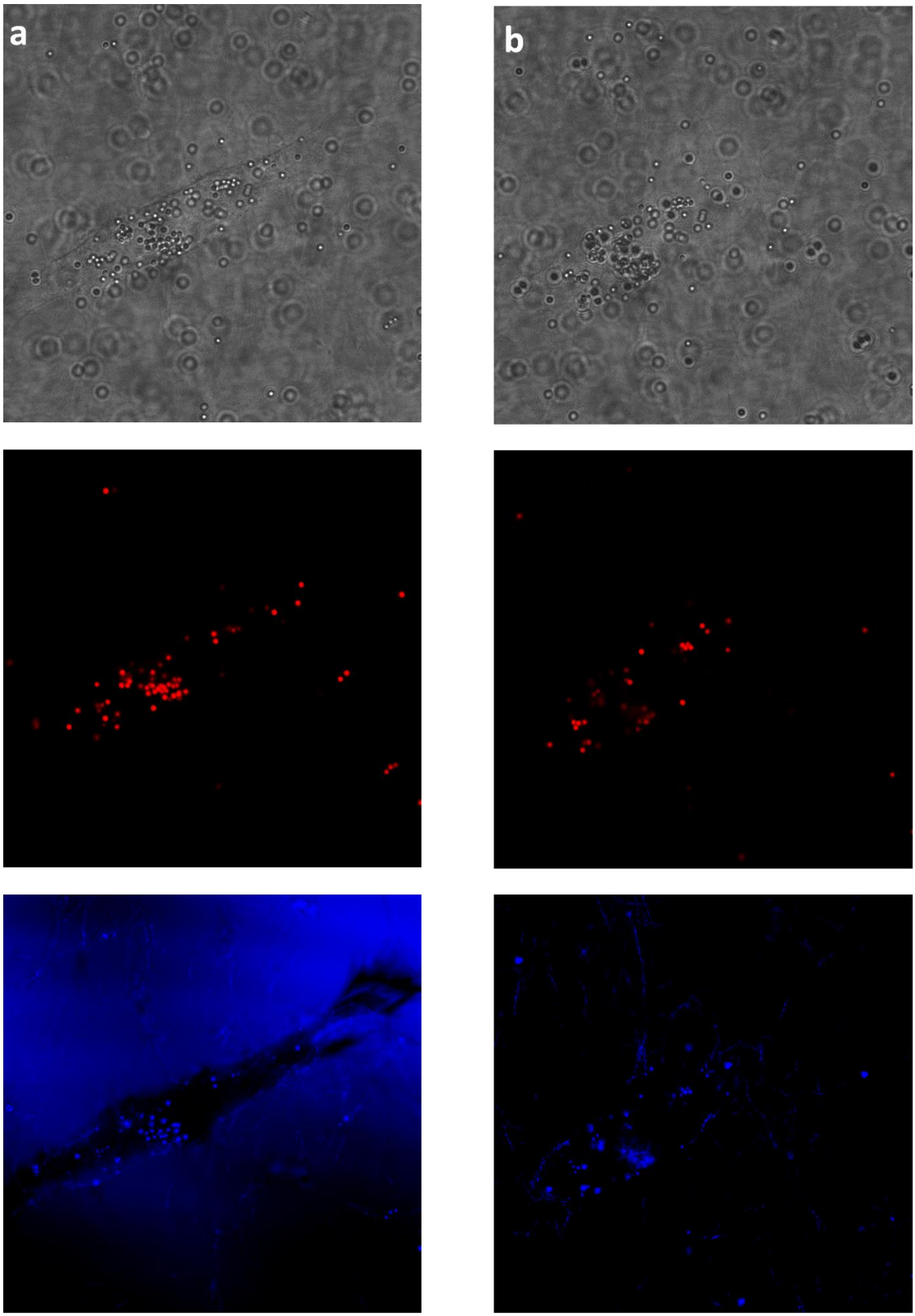

Figure 14. Triton detergent treatment. a. Image stack before. b. Image stack after 15 minutes administration of Triton 1x. a. and b. Upper images are phase contrast for cell visualization, middle images are confocal imaging of the embedded beads, and lower images are confocal imaging of collagen fibers' reflection.

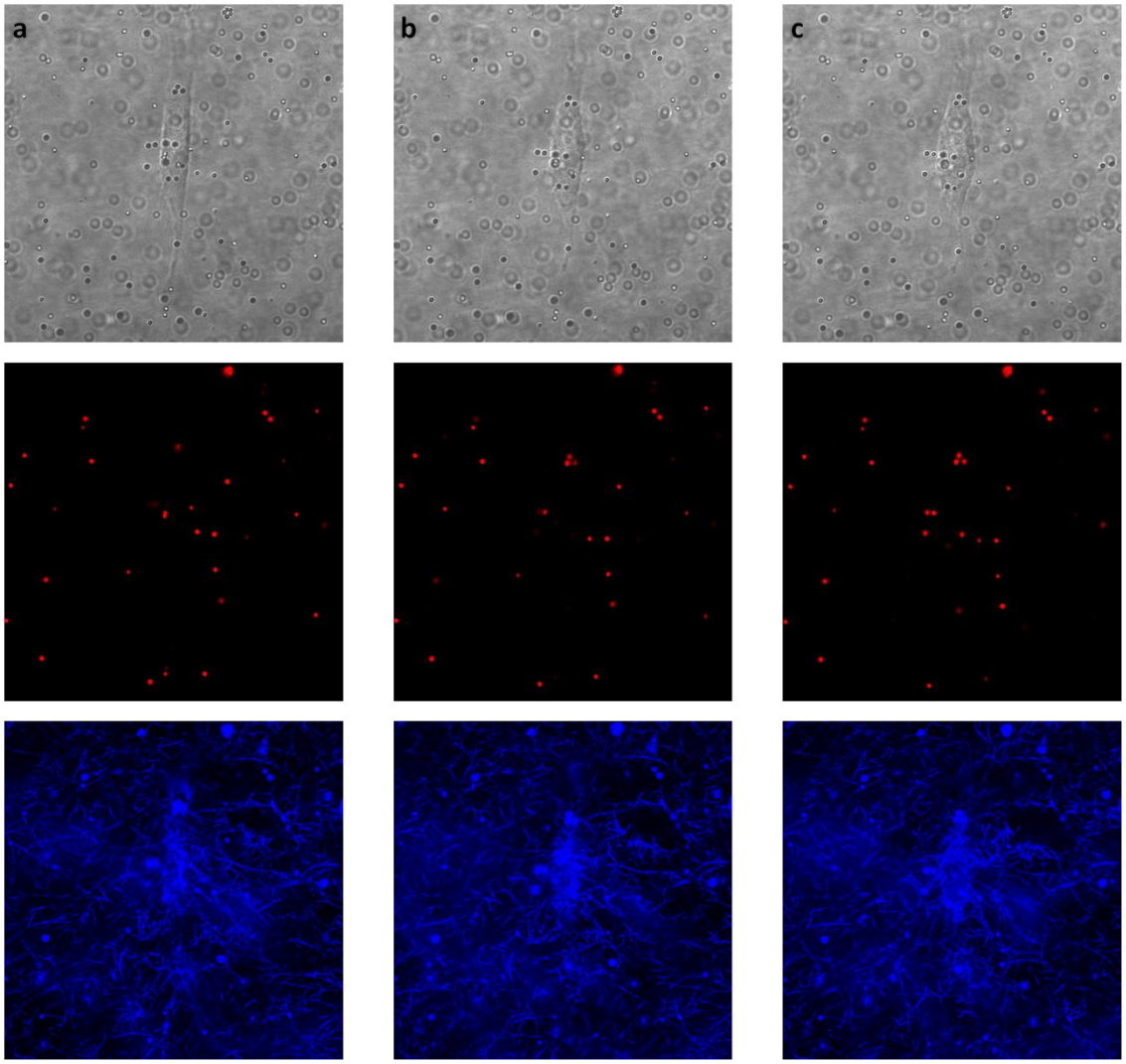

Figure 15. Cytochalasin D treatment. a. Image stack before treatment. b. Image stack after 15 minutes of cytochalasin D administration. c. Image stack after 10 minutes with medium-PS. a., b. and c. Upper images are phase contrast for cell visualization, middle images are confocal imaging of the embedded beads, and lower images are confocal imaging of collagen fibers' reflection.

6. CONCLUSIONS

The aim of this project has been almost achieved: the microfluidic device is now able to perform traction force microscopy. The overall proceeding has been optimized, except for the cell fluorescent labeling. To obtain GFP-expressing fibroblasts requires numerous attempts, thus needs weeks of work by specialized scientists. Therefore, in this project I performed traction force microscopy on microfluidic devices with unmarked cells, and obtained the necessary data for subsequent computational analysis.

All specific objectives were accomplished:

I. Microfluidic device fabrication and cell culture inside it proceedings were perfectly understood and learnt. Hence all microfluidic samples in this project were prepared by me.

II. Bead size, distribution, and concentration within the collagen hydrogel were optimized. So the best bead conditions are: 1µm diameter, 1:50 dilution and 120" at bath sonicator.

III. Cells embedded in collagen-beads hydrogel had 100% viability and spread adopting an elongated shape. In addition, some beads were phagocyted by cells. Nevertheless, TFM could be performed with cells in these conditions.

IV. Confocal microscopy imaging was carried out without cell labeling. Useful data was acquired from samples treated with actin-inhibitor cytochalasin D and triton detergent.

7. REFERENCES

1) Pampaloni F, Reynaud EG and Stelzer EHK. The third dimension bridges the gap between cell culture and live tissue. Nature Reviews. 2007; 8: 839-845.

2) Hersen P and Ladoux B. Push it, pull it. Nature. 2011; 470: 340-341.

3) Eyckmans J, Boudou T, Yu X and Chen CS. A hitchhiker's guide to Mechanobiology. Develop Cell. 2011; 21:35-47.

4) Discher DE, Janmey P, Wang Y. Tissue cells feel and respond to the stiffness of their substrate. Science. 2005; 310: 1139-1143.

5) Ladoux B and Nicolas A. Physically based principles of cell adhesion mechanosensitivity in tissues. Rep Prog Phys. 2012: 75.

6) Moore KA, Polte T, Huang S, Shi B, Alsberg E, Ingber DE. Control of basement membrane remodeling and epithelial branching morphogenesis in embryonic lung by Rho and cytoskeletal tension. Dev Dyn. 2005; 232: 268-281.

7) Keller R, Davidson LA, Shook DR. How we are shaped: The biomechanics of gastrulation. Differentiation. 2003; 71: 171-205.

8) Lee JY, Marston DJ, Walston T, Hardin J, Halberstadt A, Goldstein B. Wnt/Frizzled signaling controls *C. Elegans* gastrulation by activating actomyosin contractility. Curr Biol. 2006; 16: 1986-1997.

9) Wang JHC and Lin JS. Cell traction force and measurement forces. Biomechan Model Mechanobiol. 2007; 6: 361-371.

10) Chen CS. Mechanotransduction – a field pulling together? J Cell Sci. 2008; 121: 3285-3292.

11) Zervantonakis IK, Chandrasekhar RK, Chung S, Sudo R, Kamm RD. Microfluidic devices for studying heterotypic cell-cell interactions and tissue specimen cultures under controlled microenvironments. Biomicrofluidics. 2011; 5: 013406.

12) Lo CM, Wang HB, Dembo M and Wang YL. Cell movement is guided by the rigidity of the substrate. Biophys J. 2000; 79: 144–52.

13) Pelham RJ, Wang YL. Cell locomotion and focal adhesions are regulated by substrate flexibility PNAS. 1997; 94: 13661–5.

14) Giannone G, Dubin-Thaler BJ, Dobereiner HG, Kieffer N, Bresnick AR, Sheetz MP. Periodic lamellipodial contractions correlate with rearward actin waves. Cell. 2004; 116: 431–43.

15) Jacinto A, Woolner S, Martin P. Dynamic analysis of dorsal closure in Drosophila: from genetics to cell biology. Dev Cell. 2002; 3: 9–19.

16) Engler AJ, Sen S, Sweeney HL, Discher DE. 2006 Matrix elasticity directs stem cell lineage specification. Cell. 2006; 126: 677–89.

17) Martin P. Wound healing — aiming for perfect skin regeneration. Science. 1997; 276: 75–81.

18) Muller WA. Leukocyte – endothelial-cell interactions in leukocyte transmigration and the inflammatory response. Trends Immunol. 2003; 24: 327–34.

19) Thiery JP. Epithelial–mesenchymal transitions in tumour progression Nature Rev Cancer. 2002; 2: 442–54.

20) Li B, Wang JHC. Fibroblasts and myofibroblasts in wound healing: force generation and measurement. J Tissue Viability. 2011; 20: 108-120.

21) Sims JR, Karp S, Ingber DE. Altering the cellular mechanical force balance results in integrated changes in cell, cytoskeletal and nuclear shape. J. Cell Sci. 1992; 103: 1215–1222.

22) Dembo M, Wang YL. Stresses at the cell-to-substrate interface during locomotion of fibroblasts. Biophys. J. 1999; 76: 2307-2316.

23) Tan JL, Tien J, Pirone DM, Gray DS, Bhadriraju K, Chen CS. Cells lying on a bed of microneedles: an approach to isolate mechanical force. PNAS. 2003; 100: 1484-1489.

24) Legant WR, Miller JS, Blakely BL, Cohen DM, Genin GM, Chen CS. Measurement of mechanical tractions exerted by cells in threedimensional matrices. Nat Methods. 2010; 7: 969–971.

25) Prass M, Jacobson K, Mogilner A, Radmacher M. Direct measurement of the lamellipodial protrusive force in a migrating cell. J. Cell Biol. 2006; 174: 767-772.

26) Harris AK, Wild P, Stopak D. Silicone rubber substrata: a new wrinkle in the study of cell locomotion. Science. 1980 ; 208: 177-179.

27) Stopak D, Harris AK. Connective tissue morphogenesis by fibroblast traction. I. Tissue culture observations. Dev. Biol. 1982; 90: 383-398.

28) Galbraith CG, Sheetz MP. A micromachined device provides a new bend on fibroblast traction forces. PNAS. 1997; 94: 9114-9118.

29) du Roure O, Saez A, Buguin A, Austin RH, Chavrier P, Silberzan P, Ladoux B. Force mapping in epithelial cell migration. PNAS. 2005; 102: 2390–2395.

30) Franck C, Maskarinec SA, Tirrell DA, Ravichandran G. Three-dimensional traction force microscopy: a new tool for quantifying cell-matrix interactions. PlosOne. 2011; 6 (3).

31) Kraning-Rush CM, Carey SP, Califano JP, Smith BN, Reinhart-King CA. The role of the cytoskeleton in cellular force generation in 2D and 3D environments. Phys Biol. 2011; 8.

32) Butler JP, Tolic-Nørrelykke IM, Fabry B, Fredberg JJ. Traction fields, moments, and strain energy that cells exert on their surroundings. Am J Physiol Cell Physiol. 2002; 282: C595–C605.

33) Yang Z, Lin JS, Chen J, Wang JH. Determining substrate displacement and cell traction fields—a new approach. J Theor Biol. 2006; 242: 607–616.

34) Delanoë –Ayari H, Rieu JP, Sano M. 4D traction force microscopy reveals asymmetric cortical forces in migrating Dictyostelium cells. Phys Rev Lett. 2010; 105: 248103.

35) Koch TM, Münster S, Bonakdar N, Butler JP, Fabry B. 3D traction forces in cancer cell invasion. PlosOne. 2012; 7 (3).

36) Cukierman E, Pankov R, Stevens DR, Yamada KM. Taking cell-matrix adhesions to the third dimension. Science. 2001; 294: 1708-1712.

37) Shin Y, Kamm RD, Chung S et al. Microfluidic assay for simultaneous culture of multiple cell types on surfaces or within hydrogels. Nature Protocols. 2012; 7(7): 1247-1257.

38) Chung S, Sudo R, Vickerman V, Zervantonakis IK, Kamm RD. Microfluidic platforms for studies of angiogenesis, cell migration, and cell-cell interactions. Annals of Biomedical Engineering. 2010; 38(8): 1164-1177.

39) Velvé Casquillas G. Microfluidic reviews and tutorials. Elveflow company website: www.elveflow.com.

40) Beningo KA, Wang YL. Flxible substrata for the detection of cellular traction forces. Trends Cell Biol. 2002; 12(2): 79-84.

41) Bloom RJ, George JP, Celedon A, Sun SX, Wirtz D. Mapping local matrix remodeling induced by a migrating tumor cell using three-dimensional multiple-particle tracking. Biophys J. 2008; 95: 4077-4088.

42) Chang SS, Guo W, Kim Y, Wang Y. Guidance of cell migration by substrate dimension. Biophys J. 2013; 104: 313-321.

43) Petroll WM, Ma L, Jester JV. Direct correlation of collagen matrix deformation with focal adhesion dynamics in living corneal fibroblasts. J cell sci. 2003; 116: 1481-1491.

44) Molecular Probes. Product information: FluoSpheres Fluorescent Microspheres.

45) Herzog G, Kashem MMA, Benecke G et al. Influence of nanoparticle surface functionalization on the thermal stability of colloidal polystyrene films. Langmuir. 2012; 28: 8230-8237.

46) Newman SA, Forgacs G, Hinner B, Maier CW, Sackmann E. Phase transformations in a model mesenchymal tissue. Phys Bio. 2004; 1: 100-109.

47) Müller RH, Rühl D, Lück M, Paulke BR. Influence of fluorescent labelling of polystyrene particles on phagocytic uptake, surface hydrophobicity, and plasma protein adsorption. Pharma Res. 1997; 14 (1): 18-24.

48) Sigma. Product information: Cytochalasin D. Revision date 07-01-2013.