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Article

Study of cell migration under mechanical confinement in microfluidic lab-on-a-chip

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Abstract: Understanding cell migration is a key step to unravel many physiological phenomena and predict several pathologies, like cancer metastasis. In particular, mechanical confinement has been proved to be a key factor in the cellular migration strategy choice. As our insight in the field improves, new tools are needed in order to empower biologists' analysis capabilities. In this framework, microfluidic devices have been used to engineer the mechanical stimuli and to investigate cellular migration response in a more controlled way. In this work, we will review the existing technologies employed in the realization of microfluidic cellular migration assays, namely soft lithography of PDMS and hydrogels and femtosecond laser micromachining. We will give an overview of the state of the art of these devices, focusing on the different geometrical configurations that have been exploited to study specific aspects of cellular migration. Our scope is to highlight the advantages and possibilities given by each approach and to envisage the future developments in in-vitro migration studies under mechanical confinement in microfluidic devices.

Keywords: lab-on-a-chip, cell migration, microfluidics, PDMS, hydrogels, femtosecond laser microfabrication, two-photon polymerization

1. Introduction

Cell migration is a complex phenomenon that plays a key role in a variety of biological processes in multicellular organisms, including embryological development, wound healing and immune response. Pathological processes, such as tumour invasion and metastatic spread, also rely on cell migration [1]. For instance, cells that have evolved into a malignant phenotype can exploit the epithelial-to-mesenchymal transition (EMT) to initiate motion, invade the surrounding tissue and reach distant organs in the body [2]. In vivo, tissues are generally characterized by a dense three-dimensional scaffolding, the extra-cellular matrix (ECM), consisting of proteoglycans, occupying the extra-cellular space in form of a hydrated gel, and a variety of fibrous proteins, such as collagens, fibronectins, elastins and laminins [3,4]. Given such complex extra-cellular microenvironment and the high cellular density characterizing biological tissues, in vivo migration and spreading occur under varying degrees of confinement, leading motile cells to constantly adapt their shape and migration strategy.

Confinement is an important mechanical cue for motile cells, as it can trigger a variety of cellular responses [5]. For instance, fibroblasts and mesenchymal tumour cells respond to confinement by switching in-between different migration mechanisms in order to successfully navigate through their surrounding ECM. Particularly in the context of tumour metastasis, matrix of high stiffness and rigidity has been shown to promote tumour cell proliferation [6] and EMT [7], cause multinucleated cell division [8,9] and influence cell graft into secondary tissues and organs during dissemination [10]. Similarly, interstitial pressure has been shown to drive malignant cell outgrow and dissemination throughout the ECM [11].

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Dissemination can occur through single cell invasion or collective invasion, depending on whether the cells have transitioned to a fully mesenchymal phenotype, or whether they still retain epithelial characteristics [12,13]. In 3D environments, mesenchymal cells can migrate through the available gaps by using protrusions of different morphology and size driven by actin polymerization such as lamellipodia (thin, fan-like in shape) and filopodia (thin and slender extensions used by the cells to probe the environment) [14,15]. When the cell's focal adhesions are intact and binding to the surrounding ECM, cells can apply traction forces on the matrix and migrate through it using arm-like projections known as pseudopodia [16]. When the activity of cell adhesions is inhibited or suppressed, cancer cells can switch their migration strategy to an amoeboid based motility mode, thus acquiring a rounded morphology, diffuse distribution of adhesion proteins and spherical, actin-free protrusions (membrane blebs) to drive cell locomotion [17].

When escaping the primary tumour and moving through the ECM, tumour cells squeeze through $1-30\mu m$ wide pores [18,19], and $1-2\mu m$ wide openings between endothelial cells to access the bloodstream. Once they reach the bloodstream, cells may experience confinement as they navigate through vessels of $3-4\mu m$ diameter [12,13]. Such confined migration induces significant structural changes in the cell's cytoskeleton [20,21] and focal adhesions, while also affecting nuclear shape and the distribution of Lamin A/C (a protein present in the nuclear lamina whose role is to protect the nuclear envelope from external stress and potential rupture) [22–27] .

Because of its clinical relevance particularly in the context of cancer research, understanding the effects that confinement, and related mechanical cues such as stiffness and shear stress, can have on cellular behaviours may prove to be a valuable contribution to the development of strategies and therapies tackling metastatic spread at its early stages. Given its importance, many in vitro systems have been developed in order to study and characterize cellular motility and migration patterns, as well as to replicate the physiology of human tissues, and in particular ECM mechanical, structural and chemical characteristics.

The four main standard techniques used to study cell migration, being it in 2D or in 3D environments, are the scratch assay, the cell exclusion zone assay, the Boyden chamber and patterned lines [28]. In a scratch assay, initially used to study the wound healing for epithelial and mesenchymal cells [29], the target cells are seeded on a Petri dish or in a well plate. Once they attach to the surface and form a uniform monolayer, cells are removed from a certain discrete area (scratching) with the help of a pin tool or a needle. By observing the subsequent migration of the cells and acquiring several images of the edges of the "artificial wound", it is possible to infer the characteristics of their motility behavior. For instance, multiple assays can be parallelized in a multi-well plate, adding different molecules on the top of the scratch to study possible inhibition or promotion effects on the cell migration. Moreover, the assay plate can be covered with an ECM, in order to characterize its interaction with the migrating cells. This test is simple and straightforward and the time-lapse images acquired during the experiment can give important information on cell morphology during migration. However, it lacks reproducibility and standardization, as it is hard to guarantee that the monolayer condition and the scratching procedure are exactly the same within different experiments. Furthermore, this mechanical action can damage the deposited ECM, falsifying the results. During the years, several options have been presented and commercialized in order to create a reproducible scratching process, such as laser ablation or electrical wound, but these dedicated systems increase the complexity of the assay.

A similar assay, that aims to avoid the variability in the scratch process, is the cell exclusion zone assay [30]. In this test a mechanical barrier is placed in contact with the cell culture plate before the seeding. The target cells are then cultured around the barrier as in the scratch assay until they reach the uniform monolayer structure, and only then the barrier is removed. The collective migration of the cells towards the newly exposed surface is then studied like in the previous case. This second assay has the advantage of

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avoiding the scratching procedure, preventing possible damages to the ECM. Furthermore, the wounds created in this way were reproducible in dimension.

The Boyden chamber [31] is a system for transmembrane migration assay. It consists of two chambers separated by a porous membrane. The target cell population is seeded in the first chamber, while the solution to be tested is placed in the second one, generating in this way a chemical gradient and inducing a chemical-signal driven cell migration (chemotaxis). After a given time, the membrane is fixed and stained and the cells on the second chamber side of the membrane are counted using microscope observation. This system has been widely used and commercialized also in combination with multi-well plates in order to increase the parallelization level and the throughput of the analysis. The main advantage of this system is the possibility to test both adherent and non-adherent cells. Furthermore, the membrane can be coated with ECM proteins to mimic different migration conditions. "Moreover, choosing membranes with different average porous sizes makes it possible to investigate the effect of cell mechanical confinement on their three-dimensional migration properties, in contrast with previously described assays, that only allow the investigation of 2D unconstrained motion. However, by using this system it is not possible to observe the cells during their migration and, in case of too few cells counted out of the membrane, statistical analysis might not be applicable.

The last technique standardly used to study cell migration is the so-called "patterned lines". In this case a planar substrate is patterned with proteins that can drive cell adhesion, so that the seeded cells naturally stretches along the defined geometry (for instance lines). This long lines allows the study of cell migration imposing a lateral constrain induced by the limited contact area. This technique allows the engineering of the mechanical stimuli, by changing the shape of the patterned lines, but does not give hints of the behaviors of cells under mechanical confinement, as in the case of migration through pores.

In the last decades, microfluidics proved to be a new enabling technology for biological assays and in particular in the case of cell migration. One first big advantage of microfluidics is the precise and reproducible control on the fluidic environment, i.e. the possibility to induce precise flow rate, control the temperature and the composition of the buffer and even the ability to induce chemical gradients with different shapes thanks to engineered micromixer geometries [32]. Secondly, the system can be optionally embedded with sensors in order to automatically count the migrating cells or to measure their characteristics. Third, advanced fluidic geometries can be used to manipulate the cells in a gentle way [33], or to provide external stimuli on demand. Lastly, thanks to the modern microfabrication techniques, it is possible to shape with micrometric precision level the migration area, either in 2D or in 3D. This latter possibility is particularly interesting when studying cell migration under physical confinement, as it allows the engineering of the mechanical stimuli of the ECM, changing the environment geometry [34]. The main focus in this field of research is, nowadays, to realize customized systems, tailored on the specific need of the biologists, in order to empower their analysis capability, standardize the experimental procedure and investigate characteristics of the cells that could not be distinguished with standard techniques.

Microfluidic cell migration assays (MCMAs) can be realized with many different techniques and depending on the fabrication process the minimum feature size and the three-dimensionality level changes. Because of this, in the last years many different designs have been proposed, in order to study cell migration behaviors. Despite their designs or dimensions, or the biological problem they aim to study, it is possible to make a general classification depending on the material and/or the fabrication technique. In this framework we identify three main categories for the existing MCMAs platforms, namely those made in polydimethylsiloxane (PDMS), those made in hydrogels, and those made in glass and/or photopolymers, manifactured by ultrafast laser writing. We will discuss the different possible designs for the study of cell migration under mechanical confinement and highlight the possibilities offered by the different fabrication techniques in terms of design complexity and geometrical limits.

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2. PDMS-based microfluidic devices

PDMS is a widely used material in microfluidics [35]. It is an inexpensive, transparent and flexible silicon-based polymer and it is in general inert, non-toxic and non-flammable. It is widely used in replica-molding and soft lithography, processes that can be carried out by non-specialized personnel. The standard fabrication protocol for the realization of PDMS microfluidic devices is divided in three steps, schematically reported in Figure 1: the realization of the mold, the replica-molding in PDMS and the sealing of the patterned PDMS. In the first step, the microfluidic network is designed and realized with standard microfabrication techniques, as a negative copy. Depending on the employed technique, different minimum feature sizes can be achieved, down to few tens of microns. After that, the liquid PDMS is poured on the mold and is cured at a temperature of $60 - 80°C$. Afterwards, the PDMS membrane is peeled off from the mold and thus providing open channels on the surface. The microchannels are then sealed by sticking the PDMS membrane on the top of a flat surface. Following this type of procedure, the microchannels are arranged in an intrinsically planar configuration, but 3D fluidic networks can be obtained using a multilayer configuration or sacrificial layer approach [36,37]. It is important to notice that after the sealing on transparent substrate it is possible to optically access the migration area with a standard objective, allowing live imaging of the cells during their migration.

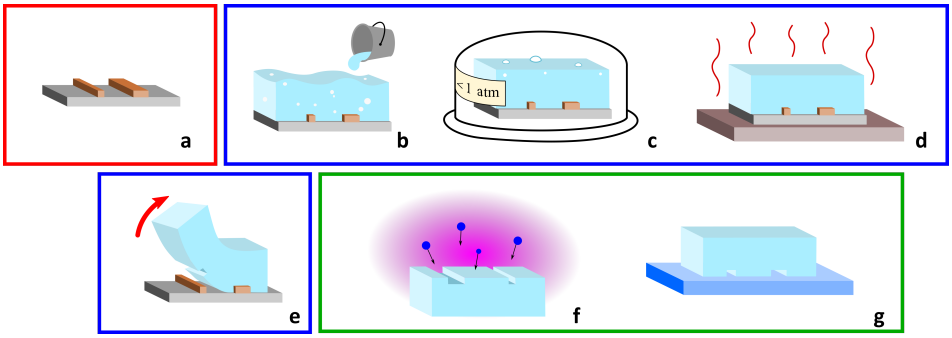

Figure 1. Procedure for PDMS microfluidic device realization: first the negative mold of the desired fluidic network is realized using, for instance, lithographic techniques (**a**); as a second step, the network is replicated on the PDMS: the liquid elastomer is poured on the mold (**b**), then it is degassed under low vacuum chamber (**c**), it is cured on an hotplate and finally it is peeled off the mold; last the PDMS is bonded on a sealing substrate, for instance glass: the surface is activated using a plasma treatment (**f**) and then the whole devices is permanently assembled

In order to study confined cell migration, it is possible to exploit different geometries, with increasing complexity. In the following, we will present the main strategies in order to give an overview of the implementation of microfluidic channels for migration assays, with a focus on the possibility to tailor the geometries addressing specific biological aspects.

2.1. Arrays of straight channel as cell migration assays

The most simple geometry of PDMS biochip used to study cell migration and chemotaxis consists in an array of straight channels connecting two reservoirs. As in Boyden chambers, the first reservoir is used for cell seeding, while the second contains the solution that is used to create a chemical gradient along the microchannels. Differently from Boyden chambers, using these MCMAs, it is easy to observe the cells migrating in the channel and study their motion, by putting the PDMS biochip on a standard microscope. A simple configuration of this type is presented by Rolli *et al.* [38], where the two reservoirs were connected by multiple parallel channels, in order to increase the throughput of the assay. They studied the migration velocity of human pancreatic epithelial cancer cells (Panc-1) both inside the microchannels and using a patterned fibronectin lines on a culture dish, comparing the migration strategies with and without mechanical confinement. Their results show how the motion of the cells inside the microchannels is faster and more similar to a constant sliding, compared to a push-and-pull motion observed for unconstrained cell migration. If

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the dimension of the channel is comparable to or smaller than the average cell diameter, the device can be used to simulate mechanical confinement. Using this type of configuration, it is possible to study which are the minimum dimensions of channel cross section that can be engaged by the cells during the migration and whether the increased contact surface, with respect to the 2D planar motion case, can influence the cell migration strategy. An example of this type of experiment is reported in Figure 2.a . Using this geometry, Fu *et al.* [39] compared the migration speed of two different human breast cancer cell lines (MDA-MB-231, highly metastatic, and MCF7, poorly metastatic), depending on the different cross section of the microchannels (fixed height of 5 μm and variable width from 4 to 12 μm). Taking advantage of the optical accessibility of the microfluidic platform, they were able to observe the deformation of the stained nuclei of the cells during the migration. As a second step of the experiment, they added a protein (59-Deoxy-59-methylthioadenosine) to inhibit chromatin condensation and reduce nuclear deformability and they measured a reduction in cell invasiveness, depending on the protein concentration. On the other hand, Tong *et al.* [40] designed a microfluidic device where the chemical gradient in the second reservoir is controlled and kept constant thanks to a hydrofluidic focusing approach, resulting in a stable concentration profile used to study multiple cell lines, including human osteosarcoma cells (HOS), human breast adenocarcinoma cells (MCF-7, MDA-MB-231) and non-tumorigenic mammary epithelial cells (MCF-10A). In order to study in detail the effect of the chemical gradient combined with mechanical confinement, Irimia *et al.* [41] realized a MCMA with a more complex geometry, reported in Figure 2.c. In their device the two reservoirs are connected by a wide channel, far from the migration area, and shaped so that a linear chemical gradient is established across the capillary channels, despite the input flow at the inlet of the two reservoirs, obtaining a robust and reliable device. They also included some microvalves in order to guarantee the proper cell upload in the system and in this way they were able to deliver chemical reagents to the front or to the rear of the migrating cell and dynamically study their response. With this device, they studied the migration of leukocytes in a highly confining ECM and their response to different drugs, both attractant or inhibitors.

Another important feature that can be added to PDMS-based MCMAs is the possibility to coat the capillaries surfaces in order to study whether specific proteins can promote or inhibit their migration properties. The same geometry presented above, i.e. a series of straight capillaries, has been used to perform comparative assays with or without coating, using for instance fibronectin [42] or collagene [43].

The shape of these straight PDMS channels can be engineered in order to induce controlled mechanical stimuli on the cells during their migration and study their response. Mak *et al.* [44] designed a microfluidic system with a series of capillaries that tapers from a width of 15 μm to 4 μm with 7 different possible angles, in order to investigate the cell invasiveness once facing different spatial constriction gradients. A brightfield microscope image of these channels is reported in Figure 2.b. They studied the behavior of bovine aortic endothelial cells (BAECs) as baseline and human breast adenocarcinoma cells (MCF-7, MDA-MB-231), verifying that depending on the tapering angle, these cell lines might invert their migration direction with an higher or lower probability. In a following article [45] the same team investigated the effect of a series of tapered constriction along the migration channel, as shown in Figure 2.d. This PDMS microfluidic device was used to identify different phases of cell migration (MDA-MB-231) once they met a constriction with a dimension smaller than its nucleus size. Furthermore they verified that the periodic modulation of the channel dimension, with a series of constrictions and widenings, can influence the morphology of the cell, leading to an increased spreading of their membrane.

2.2. Advanced geometries of fluidic networks

Up to now we presented some simple geometries consisting in a series of straight channels with a bi-dimensional geometry and we introduced possible variations on this scheme. In this section we will present advanced geometries that exploit the possibility to

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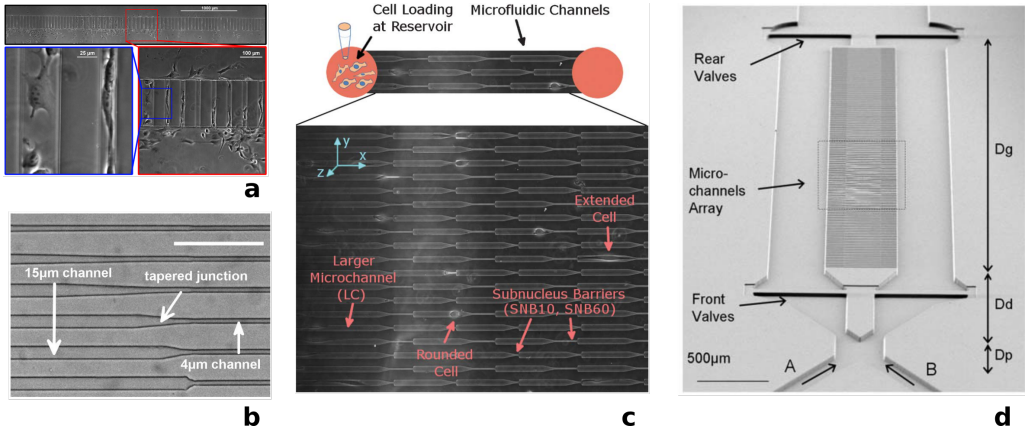

Figure 2. Examples of cellular migration assay realized with arrays of straight microchannels in PDMS: (**a**) array of microchannel connecting culture and chemoactractant chambers (reprinted from [40]; (**b**) advanced geometry integrating channel array, connection channels and valves (reprinted from [41]); (**c**) variable cross section channels with different tapering angles (reprinted from [44]; (**d**) array of microchannels with periodically modulated width (reprinted from [45])

explore three-dimensional complexity or to integrate several microfluidic components in the same device. Some of these examples are reported in Figure 3.

The first example is a 3D version of the periodic modulation of the straight channel cross section introduced above. Ma *et al.* [49] presented an array of 36 straight channels with a periodically modulated height, from 15 to 10 μm. They tested their device with adherent MHCC-97L liver cancer cells and suspended OCI-AML leukaemia cells, finding results analogous and complementary to those presented in [45]: upon periodic mechanical modulation the cancer cells showed an increased migration velocity, as well as an increased plasticity and a cytoskeleton structure alteration.

Raman *et al.* [46] fabricated a multilayer device with straight channels with a series of bendable PDMS micro-pillars as floor. In this way, they forced the cells to migrate anchoring on the pillars and studied the direction and the intensity of the traction force depending on the pillar deformation. In addition, they included, in the same MCMA, channels with different width in order to study the difference in migration behavior between 2D planar case and constricted case.

Boneschansker *et al.* [50] proposed an advanced geometry to study at the same time the migration of leukocytes towards and away from the chemoattractant, both with or without mechanical constriction. In this device the chemical gradient is established between two external channels connected by an array of capillaries with small (6-10 μm) and large (50 μm, i.e. not constricting) dimensions. A third channel is located in-between the two external channels, splitting the capillaries in a left-handed and a right-handed halves. This channel is used for the cell upload and it is equipped with a series of cell traps, in order to stop the cells in between of the chemical gradient, allowing in this way a migration in both directions. This device was used to study different types of cell lines, such as different neutrophil and leukocytes, and it highlights different migration patterns depending on the combinations of cell type and chemical agent tested.

An additional strategy is to use micropillars to mimick the ECM. In this case, a 2D array of pillar structures with different dimensions and spacing is used to study the migration of cells either atop of it or, in case of migration under constriction, within the array. Doolin and Stroka [51] presented a study on the migration of mesenchymal stem cells in different devices, characterized by different pillar spacing, showing different behavior and, for instance, different turning angles during the motion. Furthermore, in the same article an extended list of works using micropillars-based migration assays is presented. On the other hand, Davidson *et al.* [47] used pillars with variable diameter and arranged them in a channel-like fashion . In this way they obtained a device varying between a pillar array and

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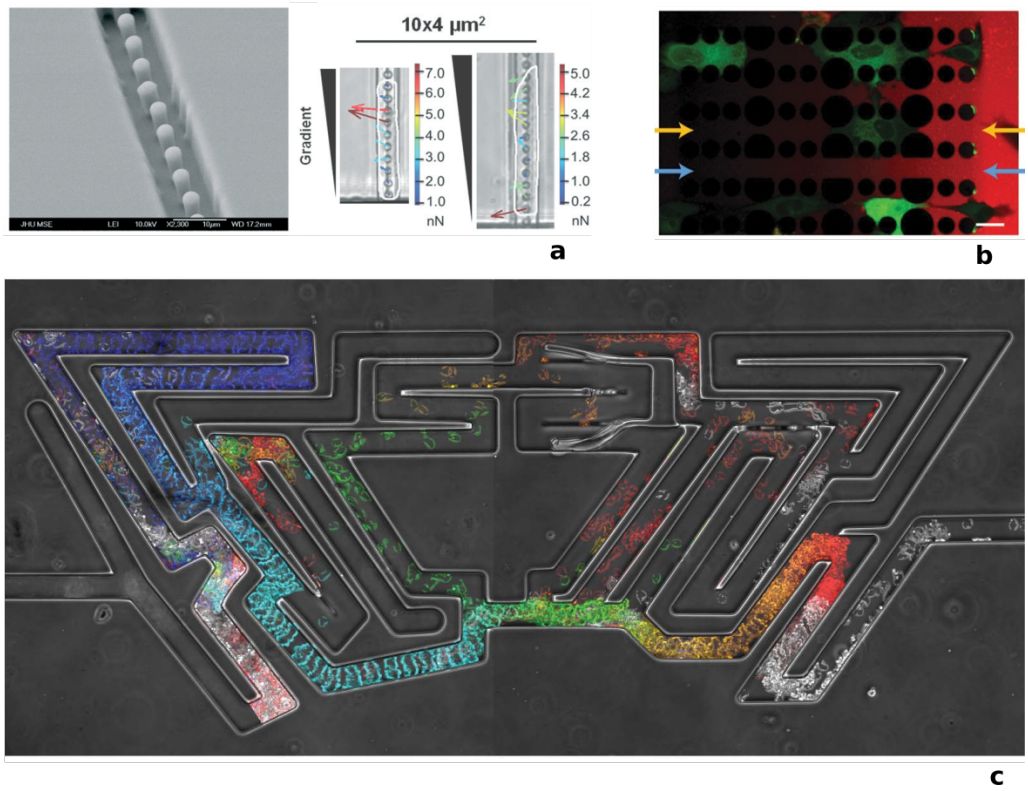

Figure 3. Advanced configurations of PDMS microfluidic assays: (**a**) PDMS pillar structures combined with constricting channels, used to study cell adhesion forces (reprinted from [46]); (**b**) Combination of microchannel and pillar structures, used to study nuclear rupture during cell migration through high confining constriction. The cells are reported in green, while the chemoactractant is reported in red. The arrows highlight the constricting channel and the connection channel used for chemoactractant diffusion, in orange and blue respectively (reprinted from [47]); (**c**) Fluidic maze used to investigate cellular migration decisions. The different time-steps are represented in different fake colors (reprinted from [48])

a straight channel array, in order to simulate the non-continuous spatial constraints found in the natural microenvironment formed by ECM fibers. In particular, this pillar-made channels present constriction points with a minimum dimension of 5 x 5 μm. This porous size is small enough to cause nuclear rupture and strong deformation during the migration and this feature has been used to study nuclear envelope damage and repair during cancer cell migration [24]. A particular technical aspect of this device was the inclusion of a parallel by-pass channel in between of the two chambers (seeding and collection) in order to balance the reservoir medium level and guarantee experimental robustness against operator buffer uploading errors.

One last example of high complexity MCMA are the so called “fluidic mazes”. A series of microfluidic channels are realized in between of the seeding and the collection chamber, including bifurcation, parallel channels with different fluidic resistance and dead-end channels. These complex network are used to study the “decisions” taken by the cells during the motion and to investigate the mechanism underneath these choices. For instance, it is possible to study the response of the cells to different degrees of fluidic resistance, to different mechanical stimuli [52] or the feedback mechanism based on self-induced chemical gradient, used to navigate the cells away from dead-end structures [48]. These last studies are extremely interesting and can help scientists to investigate the behavior of cell population migration, moving the focus of the assay from the single-cell motion mechanism to collective strategies.

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3. Hydrogels-based microfluidic devices

Hydrogels are a class of crosslinked polymeric materials, not soluble in water, that have been explored for microfluidic applications. They can have natural origin, like collagen, fibrin, gelatin or agarose, or they can be synthetic, like polyacrylic acid and polyethylene oxide. Hydrogels present a series of properties that make them interesting for the fabrication of microfluidic devices mimicking in-vivo environment [53], such as: (i) mostly inexpensive and easy to be used as fabrication material; (ii) optically clear over visible spectral range; (iii) permeable for small molecules, being in this way ideal as selective diffusion barrier; (iv) they guarantee good cell adhesion and proliferation on their surface; (v) their mechanical properties (such as their Young modulus) is comparable to the one of biological tissues and can be tuned depending on the fabrication procedure. From the fabrication point of view, they can be shaped exactly like PDMS using soft lithography procedures. Indeed almost all hydrogels are liquid above certain temperature, so that they can be poured on a mold and then peeled off once solidified. The obtained open-channels can be either closed in between two rigid slabs and sealed by pressure, or they can be attached to another hydrogel lid and fused together by partial melting of the contact interface. Alternatively, they can be injected in existing microchannels (for instance PDMS ones) and then solidified, acting as an embedded element in the fluidic network.

An example of the use of hydrogel (agarose) based MCMA for the study of chemotaxis, even if without mechanical confinement, is presented by Cheng *et al.* [54]. The device presents the typical scheme as a chemotaxis microfluidic device: it consists in a first channel for the chemical agent injection, a buffer channel to act as second edge for the chemical gradient and an intermediate channel for the cell migration analysis. The peculiarity of this device is that the three channels are not connected and the chemical gradient is established through the hydrogel matrix, leading to a linear chemical diffusion without liquid media exchange. Choi *et al.* [55] exploited the tunable mechanical properties of agarose to fabricate a gel confiner device with different stiffness, as shown in Figure 4.b . This device consists in a hydrogel lid that is placed on the top of some 2D seeded cells in order to apply on them a known pressure and study their migration once confined. In particular, the agarose concentration was calibrated in order to mimic the mechanical stiffness corresponding to brain, lung, skin or spleen. Wang *et al.* [56] instead realized some straight channel array using collagen-alginate hydrogel using replica molding approach. By changing the Ca^{2+} concentration in the solution, they were able to tune the stiffness of the structures, in a range of 0.3-20 kPa, comparable to most biological tissues up to tumoral ones. They investigated the motion strategies of MDA-MB-231 breast cancer cells depending on the combination of channel width and stiffness and observed how this last parameter influences the transition from mesenchymal to ameboid migration mode.

A different approach is to embed the cells directly in the hydrogel, exactly as they would be in a biological tissue in a real case scenario, like in the devices reported in Figure 4.b-d . Huang *et al.* [57] embedded the cells (MDA-MB-231) in a collagen matrix, pumped inside a microfluidic device, and they used the system to simulate interstitial flow inside a biological-like ECM. In this case the confinement on the cells was operated by the hydrogel matrix itself, that acts as ECM, and the microfluidic system was used in order to accurately control the experimental condition, in a lab-on-a-chip fashion, simulating tissue perfusion. In a similar way, Anguiano *et al.* [58] embedded in a collagen-Matrigel matrix some lung cancer cells using two parallel lateral channels to establish a chemical gradient. Before injecting the hydrogel, they marked with a fluorescent dye the collagen structures. In this way they were able to characterize the cell motility in this biological-like environment and identify the adhesion points between cells and ECM, thanks to specific fluorescent labelling. Furthermore, they studied the interaction between the cells and the ECM, the collagen fiber displacement and characterized the force exerted by the cells on the surrounding structure. Ayuso *et al.* [59] used a collagen-based hydrogel ECM to simulate the migration of C-6 glicoblastoma cells towards or away from a capillary clogged

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by a thrombosis. In this configuration, the intermediate hydrogel mimics the biological tissue, while the two parallel external microchannel act as parallel blood vessels, delivering nutrients to the cells for the whole duration of the experiment. By stopping the flow of one of the two channels, they simulated the thrombosis event, characterized the migration of the cells towards the working microchannel and studied the change of the morphology of the population along the migration direction.

In general, hydrogels are an interesting platform for the realization of microfluidic devices, thanks to their specific properties. The use of hydrogel matrices to simulate biological ECM in migration assays is a promising application, as they can faithfully replicate interesting characteristics such as stiffness or perfusion of the tissues. At the same time, being transparent at visible wavelengths and with a low light scattering, they allow for direct optical inspection and cell morphology study. On the other hand, as the internal structure of these hydrogel matrix is random, it is not possible to directly engineer the mechanical stimulus applied to the migrating cell. This aspect limits the analysis to a statistical observation of population characteristics, rather than probing the specific response of the cell to a given stimulus, like it was done instead in the case of hydrogel-based microchannels.

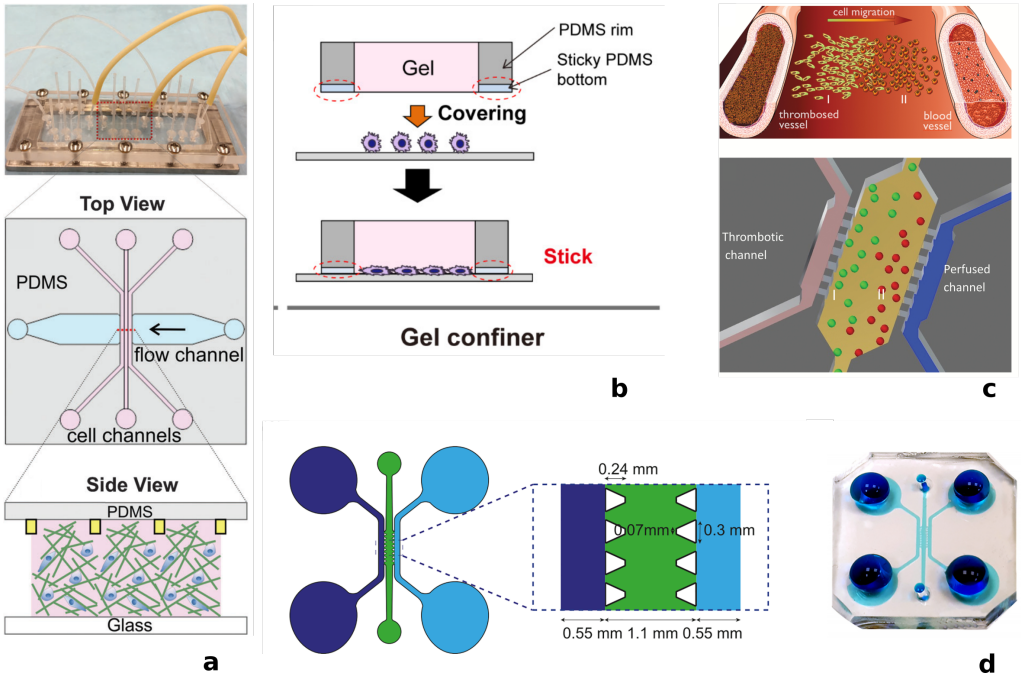

Figure 4. Examples of microfluidic migration assays realized with hydrogels: (**a**) Collagen matrix used to mimic biological ECM (reprinted from [57]); (**b**) Hydrogel lid used to apply controlled mechanical load on migrating cells (reprint from [55]); (**c-d**) Hydrogel-based matrix used to study chemotaxis (reprint from [59] and [58], respectively)

4. Microfluidic devices for migration assays realized by femtosecond laser micromachining

Femtosecond laser micromachining (FLM) of microfluidic devices for cell assays allows to fabricate chips with complex 3D geometries in several materials, as glass or polymer, with micrometric (and even nanometric) accuracy, and with functionalities that are impossible with the previous strategies. FLM is implemented by focusing a femtosecond laser beam inside a material, transparent to the laser wavelength. Due to the high intensities reached at the focal spot non-linear absorption phenomena takes place leading to a permanent modification of the material properties.

In the case of glasses, under specific irradiation conditions, it will lead to a local increase in

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the etching rate that will allow to obtain embedded microfluidic channels after a chemical etching process. This regime is exploited to fabricate 3D microfluidic devices, inside glasses like fused silica or Foturan [60,61] with no need of a clean room facility or a post gluing/bonding process to close the device. The technique is known wit the name of Femtosecond Laser Irradiation followed by Chemical Etching (FLICE)
In the case of photopolymers it will induce a local photo-crosslinking of the material and consequent polymerization of sub-micrometer features [62], thus allowing the fabrication of 3D structures with arbitrary geometries that could even be fabricated inside complicated microfluidic devices.

There are a few examples in the literature that exploit FLM for the development of microfluidic devices to study cell motility under a constrained environment. The first applications were dedicated to the fabrication of free-standing scaffold-like structures fabricated by two photon polymerization (2PP) in order to study human fibrosarcoma cell (line HT1080) migration [63]. The research team manufactured scaffolds with pore sizes ranging from single cell size to ten times larger (12 m to 110 m). This 3D structure allowed them to demonstrate that the migration speed through these structures is higher than in the 2D migration experiments, thus paving the way to more systematic studies of cell migration in 3D environments.
In order to have a more controllable chemical environment for the cell growth, Olsen *et al.* [64] fabricated similar structures (woodpiles-like) inside a commercial plastic microfluidic chip, designed for chemotaxis experiments, as shown in Figure 5.a. They applied this device to study dendritic cells migration under CCL21 chemoattractant gradient. To provide a better biological environment, the structure was filled with collagen in a concentration that still permitted to estabilish a chemoattractant gradient. They observed that dendritic cells were able to migrate through 8x8 μm^2 pores, while migration in woodpile structures with lower porosity was rarely seen. A more complicated configuration was presented some years after by the same group [65]. They fabricated linear channel constructs with profiles ranging from 10x10 m to 20x20m, which allowed a more precise control of the CCL21 gradient. They demonstrated that under these constrain conditions the chemoattractant gradient steepness governs the directed migration speed rather than the channel size.

A possibility is to take advantage of FLM versatility to fabricate both the microfluidic chip and the migrating assay constrains. Indeed, Sima and co-workers developed a fully FLM-fabricated device to study the migration potential in sub-micrometric constrictions of PC3 cancer cells under a chemical gradient [66]. The microfluidic device was fabricated in Foturan glass by the FLICE approach, with a subsequent thermal treatment to restore the glass optical transparency, to guarantee clear imaging of cell migration. Afterwards, 2PP was used to include a series of small sub-channels inside the microfluidic chip with a rectangular profile presenting lateral open widths ranging from 2 to 0.7 μ m (see Fig. 5.b). These interesting sub-micrometer features were possible thanks to the unique capabilities of 2PP structuring. A different approach was presented later on, by fabricating the whole device in glass using the FLICE technique [67]. They created long pillars inside the microchannel that, after the thermal treatment, deformed into nanochannels narrower than 1 μm with a height of 6.75 μm and a length of over 50 μm.

Additional exploitation of the FLM 3D capabilities was demonstrated by Ficorella and co-workers [20,68], in the fabrication of a lab-on-a-chip to study cell migration under micrometer size confinement, in channel constructs with different geometries as the ones reported in Fig. 5.d-f. The devices were fabricated with a hybrid approach: FLICE was first used for the fabrication of cell growth chambers and afterwards 2PP was employed for the realization of the channel-like constrictions and for the sealing of the microfluidic device. Beside the micrometric size of the constraints and the tailoring of their geometry, this approach allows for a high-resolution imaging of the migrating cells thanks to the thin and transparent device floor. This micro-constriction configuration has been used for more systematic biological studies like the adaptability of different cancer cell lines (MCF-10,

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MDA-MB-231) to micro-constrained environments and to study different migration behaviours depending on cancer cell invasiveness [20,21].

Although FLM fabrication is more expensive and time consuming than PDMS and hydrogels soft-lithography, the robustness of the devices fabricated by the former technique gives the possibility to clean and reuse them multiple times, thus making FLM an attractive alternative. Moreover, the possibility of this technique to micromachine several different materials with sub-micrometer accuracy and its inherent 3D capabilities, opens the possibility of including new functionalities that could improve in-vitro cell-migration assays. We envisage that in the future it will be a main player in this kind of biological experiments even moving to clinical diagnosis.

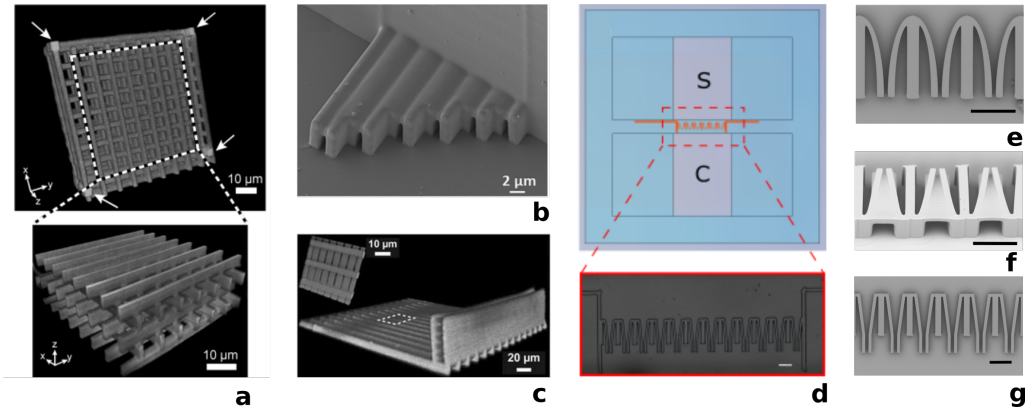

Figure 5. Examples of polymeric structures for cell migration assays realized with 2PP: (**a**) Confocal image of woodpile structure, integrated inside a microfluidic channel (reprint from [64]); (**b**-**c**) Arrays of straight microchannels realized inside a glass and plastic microchannels (reprint from [66] and [65], respectively); (**d**) example of glass-polymer microfluidic cell migration assay, completely realized with femtosecond laser micromachining (scalebar 100 m,); SEM images of different possible microconstriction geometries, realized with 2PP, with (**e**) elliptical profile, (**f**) three-dimensional profile and (**g**) combination of funnel profile and straight channels (scalebar 100 m, reprint from [68])

5. Conclusions

In this work, we reported some examples of the use of microfluidics devices to study the cellular motility, with a focus on migration under mechanical confinement. The realization of these devices requires specific competencies and a careful design. Their use can be less intuitive and straightforward than the standard methods used for cell motility analysis presented in the first Section, such as Boyden chambers or scratch assays. On the other hand, microfluidic devices offer several advantages, as a precise control of the biological and chemical environment, an easy interface, and the possibility of being tailored on the requirements of the specific biological experiment. Indeed, these MCMA devices constitute a powerful tool to perform more focused experiments and improve the quantity and the quality of information that can be extracted from biological studies.

From the point of view of the possible analysis, the great control on the geometry and on the characteristics of the MCMA allows the design of different kinds of experiments, choosing the type of ECM, of mechanical stimulus and of environmental conditions. Microfluidics enables the realization of specific assays, opening the possibility to inquire aspects of cell mechanics that would not be detectable with standard techniques. Furthermore, we envisage that in the next future the possibility to integrate them with embedded sensors, or new microscopy techniques, will improve the interpretation of cell behaviour.

The choice of the fabrication technology employed in the MCMA realization has some consequences on the possible characteristics of the final device. PDMS and hydrogels are inexpensive materials that can be used by unspecialized personnel to realize simple

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prototypes. The process of soft-lithography allows the realization of minimum feature sizes comparable to the one of the single cells (few microns), thus they can be used for the realization of MCMA to study mechanical confinement. At the same time, complex geometries can be realized to investigate specific aspects, such as the memory effect of a series of constrictions or the traction force that the cells exert on bendable pillars.

Still, this type of technology remains intrinsically planar and the shape of the single channel or constriction is dependant on the characteristics of the mold, thus it can't be easily changed. Additionally, it is hard to push the geometrical limit towards or below the micrometer level. Furthermore, these materials tends to adsorb proteins with time, and this strongly limits their use in the field of pharmacology and drug-related studies [69].

FLICE and other direct laser writing techniques, like 2PP, offer the possibility to realize custom made geometries with micrometric or sub-micron precision and the capacity of shaping the microchannels in a three-dimensional way. This allows the realization of structures for the analysis of cell migration under mechanical confinement with features hardly reproducible with other techniques. Moreover, the class of materials that can be microstructured (glass and polymers) proved to be biocompatible and mostly inert, thus allowing chemical and biochemical analysis.

As a main drawback, the realization of these devices requires dedicated equipment, thus the fabrication of MCMA with this approach is limited to specialized laboratories. Nevertheless, the devices fabricated by this technique offer the advantage of being reusable several times. This technique expresses its advantages in the prototyping and in the realization of customized devices, with strict requirements from the point of view of the design complexity. Furthermore, it allows the integration of other possible technological elements, such as micromechanical or optical components, in the same platform and fabricated with the same setup, pushing the devices from simple, single-task systems towards multi-analysis lab-on-a-chip platforms [60].

6. Patents

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Abbreviations

The following abbreviations are used in this manuscript:

EMT	epithelial-to-mesenchymal transition
ECM	Extra-cellular Matrix
MCMA	Microfluidic cell migration assay
FLM	Femtosecond Laser Micromachining
2PP	Two Photon Polymerization
FLICE	Femtosecond Laser Irradiation followed by Chemical Etching
PDMS	Polydimethylsiloxane

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