Cellular and Molecular Bioengineering (© 2019)
https://doi.org/10.1007/s12195-019-00600-4

BMES BIOMEDICAL ENGINEERING SOCIETY

Methods Paper

Producing Collagen Micro-stripes with Aligned Fibers for Cell Migration Assays

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(*Received 20 February 2019; accepted 14 September 2019*)

Associate Editor Pinar Zorlutuna oversaw the review of this article.

Abstract
Introduction—The orientation of collagen fibers in native tissues plays an important role in cell signaling and mediates the progression of tumor cells in breast cancer by a contact guidance mechanism. Understanding how migration of epithelial cells is directed by the alignment of collagen fibers requires *in vitro* assays with standardized orientations of collagen fibers.
Methods—To address this issue, we produced micro-stripes with aligned collagen fibers using an easy-to-use and versatile approach based on the aspiration of a collagen solution within a microchannel. Glass coverslips were functionalized with a (3-aminopropyl)triethoxysilane/glutaraldehyde linkage to covalently anchor micro-stripes of aligned collagen fibers, whereas microchannels were functionalized with a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) nonionic triblock polymer to prevent adhesion of the collagen micro-stripes.
Results—Using this strategy, microchannels can be peeled off to expose micro-stripes of aligned collagen fibers without affecting their mechanical integrity. We used time-lapse confocal reflection microscopy to characterize the polymerization kinetics of collagen networks for different concentrations and the orientation of collagen fibers as a function of the microchannel width. Our results indicate a non-linear concentration dependence of the area of fluorescence, suggesting that the architecture of collagen networks is sensitive to small changes in concentration. We show the possibility to influence the collagen fibril coverage by adjusting the concentration of the collagen solution.
Conclusion—We applied this novel approach to study the migration of epithelial cells, demonstrating that collagen micro-stripes with aligned fibers represent a valuable *in-vitro* assay for studying cell contact guidance mechanisms.

Keywords—Collagen fibers, Alignment, Cell migration, Contact guidance.

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INTRODUCTION

Type I collagen is a predominant structural component of the extracellular matrix (ECM) and composed most of the connective tissues associated with bone, ligaments, tendon and skin.[24] As such, this ECM component dictates the biological microenvironment for normal cell functions and provides strength and mechanical integrity. The topographical features of type I collagen have been shown to drive cell migration through a contact guidance mechanism.[29] First evidence of this phenomenon was reported by Weiss in the sixties using grooves architectures to culture connective-tissue cells.[34] Among many other physicochemical cues,[3,16,26,31] the alignment of ECM fibers has emerged as a key aspect of the ECM structure in dictating cell behaviour.[7] Matrix orientation has also been observed to be implicated in many prominent diseases such as breast cancer.[25] Indeed, the specific arrangement of bundles of aligned collagen fibers perpendicular to the tumor boundary called "tumor-associated collagen signature" can be used as a prognostic marker independent of tumor grade and size.[8] To model and study how the alignment of collagen fiber direct cell contact migration, it is therefore necessary to develop

Published online: 25 September 2019

in-vitro assays with anisotropic type I collagen architectures.

To address this challenge, a multitude of methods have been designed to exert uniaxial stretching or deformation on collagen gels during their polymerization.[10,11,30] Other conventional approaches for the fabrication of anisotropic collagen include extrusion systems,[19] pre-stretched polydimethylsiloxane (PDMS) mold,[4] microtransfer molding[23] and microfluidic channels.[15] While some of these methods have started to give interesting results on the migration of metastatic cells,[5] most of them present difficulties for controlling the adhesion of collagen fibers to the substrate and the obligation to perform migration assays within narrow microchannels, imposing a spatial confinement to moving cells. In addition, current microfluidic methods require micropumps to control the flow conditions used to align collagen fibers.[13] Therefore, we believe developing a simple and low-cost strategy to functionalize flat culture substrate with micro-stripes of aligned collagen fibers may help biologists and biophysicists willing to investigate cell contact guidance mechanisms in standardized conditions.

Here we introduce an easy-to-use and versatile approach that uses microchannels to produce micro-stripes of aligned collagen fibers on flat culture substrates. We developed a robust linkage method based on a (3-aminopropyl)triethoxysilane (APTES)/glutaraldehyde linkage to anchor firmly aligned collagen fibers to PDMS-coated glass coverslips, whereas microchannels were coated with a nonionic triblock polymer to prevent adhesion of collagen. The contrast of adhesion between the surface and the microstamp allows to peel off microchannels after complete polymerization of the micro-stripes without affecting their mechanical integrity. This strategy ensures to expose micro-stripes with oriented collagen fibers to cell culture. This novel platform was validated by conducting migration assays on epithelial cells, demonstrating that micro-stripes with oriented collagen fibers represent a valuable assay for studying cell contact guidance mechanisms.

MATERIAL AND METHODS

Microchannel Fabrication

Microchannels were created using a silicon master fabricated by deep reactive-ion etching from a chromium photomask (Toppan Photomask, Corbeil Essonnes, France). The silicon surface was passivated with a fluorosilane (tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane, Gelest) in vapor phase for 30 min. During this step, a mix of 1:10 ratio of curing agent and PDMS monomers (Sylgard 184 Silicone Elastomer Kit; Dow Corning, Midland, MI) was prepared and degassed using an automatic conditioner mixer (Thinky ARE-250, Thinky Corp.). Then the silanized silicon master was molded with the degassed PDMS and cured for 4 h at 60 °C. The PDMS microchannels were gently peeled of the silicon wafer and a sharpened blunt needle was used to punch holes at the inlet and outlet.

Preparation of Polydimethylsiloxane Coated Coverslips

PDMS substrates of 3 MPa were prepared from the commercially available Sylgard 184 silicone elastomer kit (Dow Corning, Midland, MI) by mixing the base and the curing agent as 10:1 w/w ratio.[9,32] Pre-polymer solutions were mixed thoroughly for at least 5 min, degassed, and spin-coated at 5000 rpm on 25 mm glass coverslips or glass fluorodishes (World Precision Instruments Inc.). PDMS was then cured for 4 h at 60 °C. Samples were stored at room temperature in a vacuum desiccator.

Cell Culture

Keratocytes were harvested from scale of *Central American cichlid Hypsophrys Nicaraguensis*.[16] A Fish scale was deposited onto a glass coverslip of 25 mm previously washed in a 30% ethanol solution and dried with a nitrogen flow. The scale was sandwiched between two 25 mm diameter glass coverslips and cultured in Leibovitz's Media (Leibovitz L-15 medium, Thermofisher) supplemented with 10% Fetal Bovine Serum (FBS, Thermofisher), 1% antibiotic-antimycotic (100X, Thermofisher), 14.2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Gibco HEPES buffer 1 M, Thermofisher) and 30% deionized water at room temperature for 12 h. Individual keratocytes were then dissociated by incubating the tissue in a trypsin-EDTA solution (1 mL per glass slide, 0.25%, Thermofisher) for 5 min and resuspended in 4 mL of L-15 Leibovitz complete medium. Suspended cells were then deposited on microprinted coverslips to investigate their migration behaviour. Experiments were performed between 2 and 8 h after cell seeding. All experiments on fish epithelial keratocytes were performed in accordance with institutional guidelines and regulations approved by the ethical committee from the University of Mons.

Confocal Reflection Microscopy Imaging

Unstained collagen fibers were imaged with confocal reflection mode with a Nikon Eclipse Ti-E motorized inverted microscope (Nikon C1 scanhead; Nikon,

Japan) equipped with ×40 DIC, ×40 Plan Apo (NA 1.45, oil immersion), ×60 Plan Apo (NA 1.45, oil immersion) and ×100 Plan Apo (NA 1.45, oil immersion) objectives, two lasers (Ar-ion 488 nm; HeNe, 543 nm) and a modulable diode (408 nm).

Cell Tracking

Time-lapse microscopy experiments of cell migration were performed in differential interference contrast (DIC) mode with a Nikon Eclipse Ti-E motorized inverted microscope (Nikon, Japan) at ×40 magnification. Tracking of migrating cells were obtained with NIS Elements Advanced Research 4.0 software (Nikon, Japan) and analyzed with the Prism 7b software (GraphPad Software, Inc.).

Isotropic Collagen Network

A sterile neutralize type I collagen solution was prepared on ice-cold with 100 mM HEPES in 2x PBS at pH 7.4 by using a high concentrated rat tail collagen solution (10 mg/mL, Corning). The neutralized collagen solution was then diluted with cell culture media to the appropriate concentration (from 0.5 to 4 mg/mL). Isotropic collagen networks were prepared on a flat PDMS-coated glass coverslip by casting a drop (V = 30 μL) of a neutralized collagen solution on a glass fluorodish (World Precision Instruments Inc.) of 35 mm in diameter. The drop of collagen was incubated during 30 min at room temperature (~22°C) in the dark. We used five different concentrations of collagen: 0.5, 1, 1.5, 2 and 4 mg/mL to modulate the network density.

Anchoring Collagen Fibers

A thin layer of PDMS (~ 60 μm thick) was spin-coated on a glass coverslip. Then, the PDMS substrate was irradiated under a plasma treatment for 90 s (Harrick, Ithaca, NY) Then, we used (3-aminopropyl)triethoxysilane (APTES 99%, Sigma Aldrich) as a linker between PDMS and collagen. The PDMS surface was incubated with 500 μL of a freshly 8% APTES solution in distillated water for 1 h at room temperature and then washed three times with sterile PBS. The silanol groups at the PDMS surface reacted with APTES to create a monolayer with the reactive functional groups on the surface. The APTES coated surface was then functionalized with a 3% glutaraldehyde solution (Sigma Aldrich) during an hour at room temperature and washed with sterile PBS. Finally, the aldehyde groups reacted with the amino groups of the protein to anchor collagen fibers to the PDMS surface through the formation of covalent bonds. The second step consisted to passivate the microchannels to avoid adhesion of collagen fibers with the PDMS microchannels. This strategy provides only collagen fibers on the PDMS-coated glass coverslip. We deposited 350 μL of a poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock polymer solution (Pluronic F-127 from BASF) on the PDMS microchannels for 15 min. Microfluidic channels were gently dried with a nitrogen flow and firmly placed on the PDMS-coated coverslip that was previously functionalized with APTES/glutaraldehyde.

Alignment of Collagen Fibers

A fresh solution of type I collagen from rat tail tendon of 2 mg/mL was prepared by diluting a stock solution of 10 mg/mL (Sigma-Aldrich) in 0.1M CH_3COOH solution diluted in 2x PBS (C = 0.02 N, Thermofisher). The collagen solution was first neutralized on ice with a NaOH solution (C = 0.1 N, Thermofisher) diluted in PBS 2x with HEPES and then diluted with PBS 2x to reach the target concentration. A drop of neutralized collagen solution (V = 30 μL) was deposited at the inlet of the microchannels. Then a plastic culture pipette of 25 mL connected to an automatic pipette controller (Eppendorf, Easypet 3) was firmly placed on the outlet with a 90°. The collagen solution was aspirated inside each microchannel up to reach the outlet. We estimated that the micropipette controller connected to a 25 mL plastic pipette imposed an initial flow rate of 7.98 ± 0.03 mL/s, leading to an aspiration step that takes around ~ 1–2 s, depending on the channel size. Additional experiments indicated that any other brands of micropipette controller (e.g. Drummond, model Pipet-Aid) with a flow rate ranging from 6 to 10 mL/s can be used to orientate successfully collagen fibers. After 30 min at room temperature in the dark, the collagen polymerization was completed. The microchannels were then gently peeled off the PDMS-coated glass coverslip and the collagen fibers remained on the surface thanks to the APTES/glutaraldehyde functionalization. Finally, the substrate covered with collagen microstripes was incubated during 15 min with Pluronic F-127 to passivate uncoated zones. The surfactant was washed 2 times with PBS and replaced by a fresh culture medium.

Fiber Orientation Analysis

The orientation of collagen fibers in micro-stripes obtained from 1, 2 and 4 mg/mL solutions were characterized with OrientationJ plugin of Fiji.[28] The result is a colour-coded image of the different collagen networks for visual representation of the orientation.

The colour map represents the angle with the horizontal axis.

Statistical Analysis

Differences in means between groups were evaluated by two-tailed Student's t-tests performed in Prism 7b (GraphPad Software, Inc.). For multiple comparisons the differences were determined by using an analysis of variance (ANOVA) followed by Tukey *post-hoc* test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ and n.s. not significant. Unless otherwise stated, all data are presented as mean ± standard deviation (SD).

RESULTS

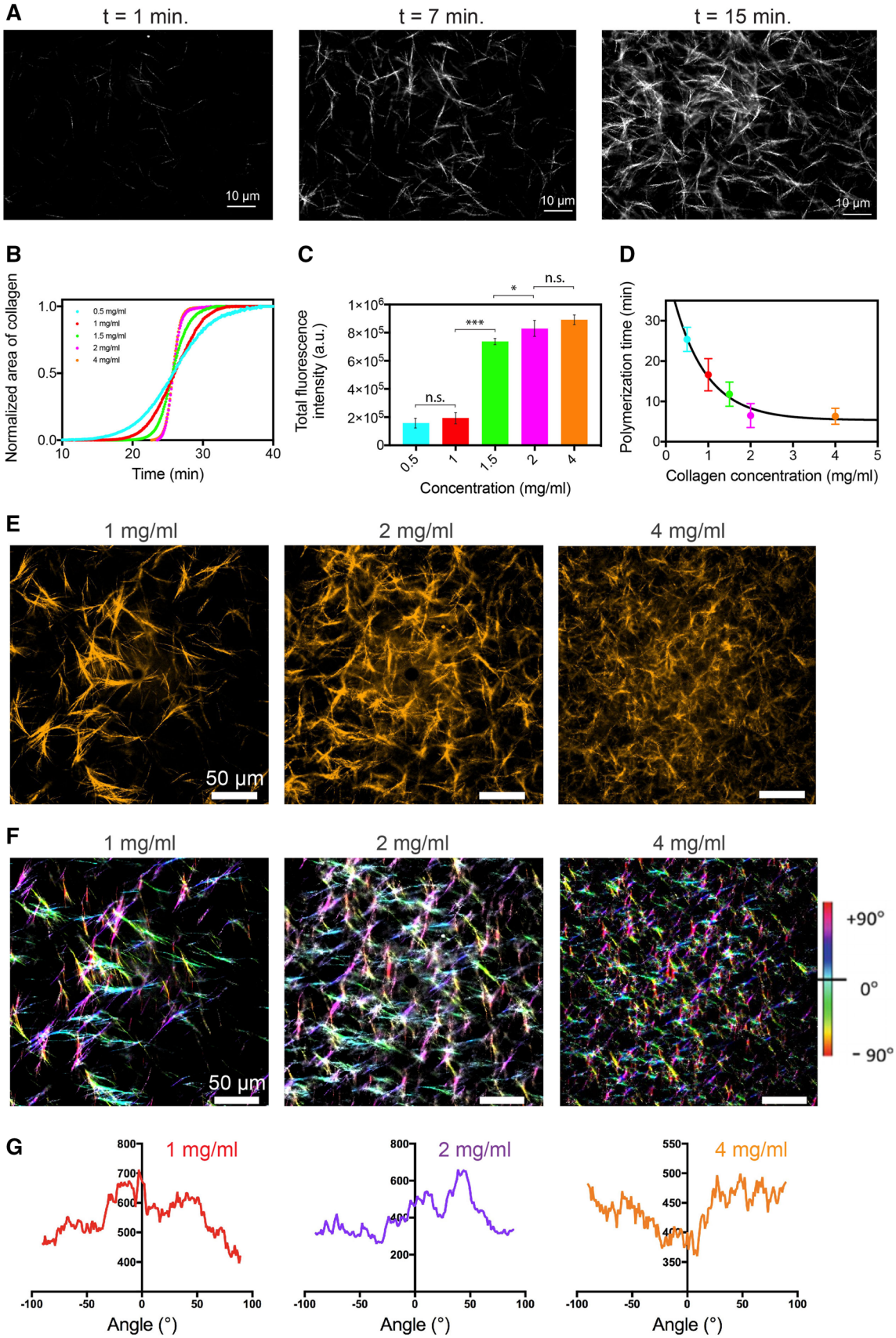
Isotropic Collagen Network

We first characterized the collagen network density as a function of the collagen concentration by using five different solutions: 0.5, 1, 1.5, 2 and 4 mg/mL. We deposited 30 μL of each collagen solution on a PDMS-coated fluorodish to the form isotropic collagen networks that were imaged with time-lapse confocal reflection microscopy during their polymerization (Fig. 1a and Supplementary Movie S1). The dynamics of polymerization was determined by quantifying the normalized fluorescence area vs. time for the whole range of concentrations (Fig. 1b, $n = 3$ for each). Our results showed a sigmoidal behaviour for each concentration, suggesting that the growth of collagen fibers is exponential until saturation occurs upon the complete polymerization. As shown in Fig. 1c, there was a significant densification of the collagen networks by increasing the concentration of the collagen solution. The total fluorescence intensity obtained for solutions of 2 and 4 mg/mL showed no statistical differences, suggesting that the maximal collagen network density was reached from 2 mg/mL. We evaluated the collagen polymerization time as a function of the solution concentration to estimate the time required for complete polymerization in microchannels. As expected, our results showed that the polymerization time decreased significantly with increasing collagen concentration (Fig. 1e). The fastest polymerization (~6 min) was achieved for collagen concentration of 2 and 4 mg/mL. Orientation of individual collagen fibers within the networks was then computed using the orientation plugin of Fiji software.[28] Figures 1f–g showed typical colour-coded images of three collagen networks (1, 2 and 4 mg/mL) for the visual representation of the orientation of the collagen fibers. The colour map represents the deviation angle of the collagen fibers with the horizontal axis. Qualitatively, all collagen networks obtained by casting a drop of collagen appeared isotropic regardless the concentration of the solution, as confirmed by the angular distribution (Fig. 1g). Taken together, our results demonstrate that a collagen concentration of 2 mg/mL allows to obtain the higher collagen fiber density with the lower polymerization time and that no preferential orientation of collagen fibers can be achieved by drop casting.

FIGURE 1. Isotropic polymerization of collagen networks showed higher density and faster polymerization dynamics with increasing concentrations. (a) Typical sequence of fluorescent images of collagen fibers during the polymerization reaction obtained using time-lapse microscopy in confocal reflection microscopy. The sequence was obtained for a solution of 2 mg/mL. The scale bar is 50 μm. (b) Normalized fluorescence area of collagen fibers as a function of the polymerization time for 0.5 mg/mL (in blue), 1 mg/mL (in red), 1.5 mg/mL (in green), 2 mg/mL (in purple) and 4 mg/mL (in orange). (c) Total fluorescence intensity of collagen fibers for 0.5 mg/mL (in blue), 1 mg/mL (in red), 1.5 mg/mL (in green), 2 mg/mL (in purple) and 4 mg/mL (in orange). Mean ± SD and $n = 8$ experiments for each condition. (d) Total polymerization time as a function of the collagen concentration (0.5, 1, 1.5, 2 and 4 mg/mL). Data are mean ± SD with $n = 8$ experiments for each concentration. (in purple) and 4 mg/mL (in orange). (e) Confocal reflection microscopy images of typical collagen networks polymerized from three different concentrations of collagen: 1, 2 and 4 mg/mL (from left to right). The scale bar is 50 μm. (f) Colour coded images of 1 mg/mL, 2 mg/mL and 4 mg/mL collagen networks for visual representation of the orientation of the collagen fibers. The colour map represents the deviation angle with the horizontal axis. (g) Mean distribution of angles for 1 mg/mL (in red, $n = 3$), 2 mg/mL (in purple, $n = 4$) and 4 mg/mL (in orange, $n = 3$) collagen networks. $*p < 0.05$, $***p < 0.001$ and n.s. is not significant.

Cell Migration on Isotropic Collagen Networks

To understand the role of the collagen network density on cell migration, we determined the migration speed of individual epithelial cells on isotropic collagen networks of 1, 2 and 4 mg/mL. We used fish epidermal keratocytes which represent an established model for cell migration because of their fast and persistent migration and simple and stable shape.[2,17] Using differential interference contrast (DIC) imaging, we recorded the displacement of individual keratocytes on each collagen network (1, 2 and 4 mg/mL) over 3500 s. For collagen solution of 1 mg/mL, the mean velocity was 0.097 ± 0.019 μm/s (Fig. 2a, $n = 6$), which is 3 to 5 times lower that the typical migration speed of isolated keratocytes.[20] We found a mean migrating velocity of 0.047±0.017 μm/s (Fig. 2b, $n = 14$) on 2 mg/mL collagen networks, which was statistically similar to that on 4 mg/mL collagen networks (0.049±0.020 μm/s, $n = 6$, Fig. 2c). Our findings demonstrated that increasing the collagen concentration from 1 to 2 mg/mL decreased two times the


A
t = 1 min.
t = 7 min.
t = 15 min.
10 μm
10 μm
10 μm
B
Normalized area of collagen
0.5 mg/ml
1 mg/ml
1.5 mg/ml
2 mg/ml
4 mg/ml
Time (min)
C
Total fluorescence intensity (a.u.)
n.s.
*
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n.s.
Concentration (mg/ml)
D
Polymerization time (min)
Collagen concentration (mg/ml)
E
1 mg/ml
2 mg/ml
4 mg/ml
50 μm
F
1 mg/ml
2 mg/ml
4 mg/ml
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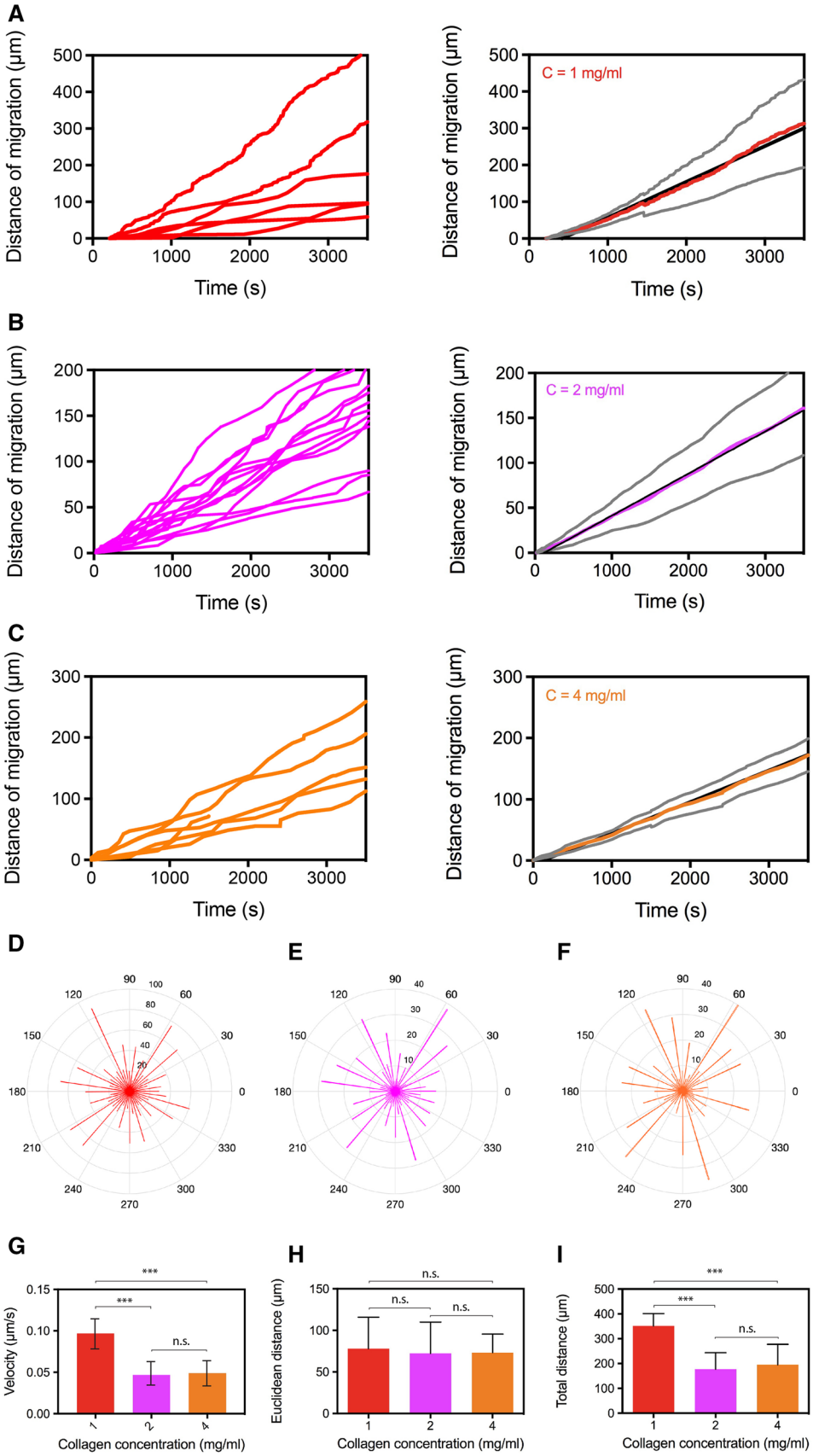
A
Distance of migration (μm)
Time (s)
C = 1 mg/ml
B
C = 2 mg/ml
C
C = 4 mg/ml
D
E
F
G
Velocity (μm/s)
Collagen concentration (mg/ml)
H
Euclidean distance (μm)
I
Total distance (μm)
n.s.
***

◀**FIGURE 2. The migration speed and the total migration distance of epithelial keratocytes decreased on dense isotropic collagen networks. Migration distance as a function of time on a collagen network obtained from a collagen solution of (a) 1 mg/mL, (b) 2 mg/mL and (c) 4 mg/mL. Superimposed raw trajectories from a collagen solution of (a) 1 mg/mL, (b) 2 mg/mL and (c) 4 mg/mL for $n = 6$, $n = 15$ and $n = 6$, respectively and mean distance ± SD. The black line corresponds to a linear fit. Angular distribution of the changes of direction during the migration of individual keraocytes on (d) 1 mg/mL, (e) 2 mg/mL and (f) 4 mg/mL collagen networks. (g) Migration velocity, (h) euclidean distance and (i) total distance of migration for individual epithelial keratocytes migrating on collagen networks of 1 mg/mL (in red), 2 mg/mL (in purple) and 4 mg/mL (in orange). ***$p < 0.001$ and n.s. is not significant.**

migration speed (Fig. 2d), whereas the migration speed on 4 mg/mL collagen networks remained to that observed on 2 mg/mL. Together, these results suggest that the collagen network density modulates the cell migration speed. Interestingly, we found that the Euclidean distance (i.e., the straight-line distance between two points) was statistically not different on the three collagen networks (Fig. 2e), whereas the total distance of migration was longer for the lower collagen concentration (Fig. 2f). These results demonstrated that the migration pathway was less persistent on 1 mg/mL collagen networks, suggesting that the collagen network density affect the migrating phenotype of epithelial keratocytes. Based on these observations, we selected an intermediate collagen concentration of 2 mg/mL to investigate the role of the collagen fiber orientation on epithelial cell migration.

Anchoring Collagen Fibers on Culture Substrates

A major drawback of other techniques to align collagen fibers is their low adhesion to the culture substrate, which constitutes a limiting factor for cell migration assays. Indeed, during migration cells generate traction forces by the actomyosin cytoskeleton that must be transmitted through focal adhesions to the ECM. To address this limitation, we developed a functionalization strategy to firmly anchor collagen fibers to biocompatible PDMS culture substrate. First of all, a thin layer of PDMS (~ 60 μm thick) was spin-coated on a glass fluorodish. This PDMS-coated glass fluorodish constituted the bottom surface of the microchannel into which collagen fibers will be aligned. The PDMS substrate was irradiated using an air/oxygen plasma treatment for 90 s to create silanol groups on the surface (Fig. 3a). Then, APTES was used as a linker between PDMS and collagen.[1,12] The silanol groups reacts with APTES to create a monolayer with the reactive functional groups on the surface. The PDMS devices were consistently incubated with freshly 8% APTES solution in distillated water for 1 h at room temperature and then washed three times with sterile PBS (Fig. 3b). The APTES coated surface was then functionalized with a 3% glutaraldehyde solution during an hour at room temperature and washed with sterile PBS (Figs. 3c and 4a, step 1). Finally, the aldehyde groups were reacted with the amino groups of the protein to anchor collagen fibers to the PDMS surface through the formation of covalent bonds (Fig. 3d). During the second step, the walls of the microchannels were passivated to avoid any adhesion between collagen fibers and the PDMS microchannel. This strategy ensured to peel off the microchannels after collagen polymerization to expose micro-stripes of oriented collagen fibers without affecting their mechanical integrity. To this aim, we incubated a 1% w/w solution of Pluronic F-127 (also known as Poloxamer 407, BASF) in sterile water on PDMS microchannels for 15 min (Fig. 4a, step 2). Then microchannels were gently dried with a nitrogen flow and firmly placed on the PDMS substrate previously functionalized with APTES/glutaraldehyde (Fig. 4a, steps 1 and 2).

Producing Micro-stripes of Oriented Collagen Fibers

To align collagen fibers, a fresh solution of type I collagen (rat tail tendon) of 2 mg/mL was prepared by diluting a stock solution of 10 mg/mL (Corning) in 0.1M CH_3COOH solution diluted in 2x PBS (C = 0.02 N). The collagen solution was first neutralized on ice with a NaOH solution (C = 0.1 N) diluted in PBS 2x with HEPES and then diluted with PBS 2x to reach the target final concentration. A neutralized collagen solution of 30 μL was deposited on the inlets of the microchannels (Fig. 4a, steps 3). Then a plastic pipette of 25 mL connected to an automatic pipette controller (Eppendorf, Easypet 3) was firmly placed on one of the outlets (Fig. 4a, step 3) by forming a 90° angle. The collagen solution was then aspirated inside each microchannel up to reach the outlet, leading to the prealignment of collagen fibrils with the flow imposed by the aspiration. We estimated that the micropipette controller imposed an initial flow rate of 7.98 ± 0.03 mL/s, leading to an aspiration step that took place in a couple of seconds. Collagen polymerization was completed at room temperature in the dark and then microchannels were gently peeled off (Fig. 4a, step 4). Thanks to the APTES/glutaraldehyde functionalization, collagen micro-stripes remained on the PDMS-coated glass fluorodish. Finally, the substrate covered with collagen fibers was incubated for 15 min with Pluronic F-127 to passivate uncoated areas. The surfactant was washed 2 times with PBS and replaced by a fresh culture medium.

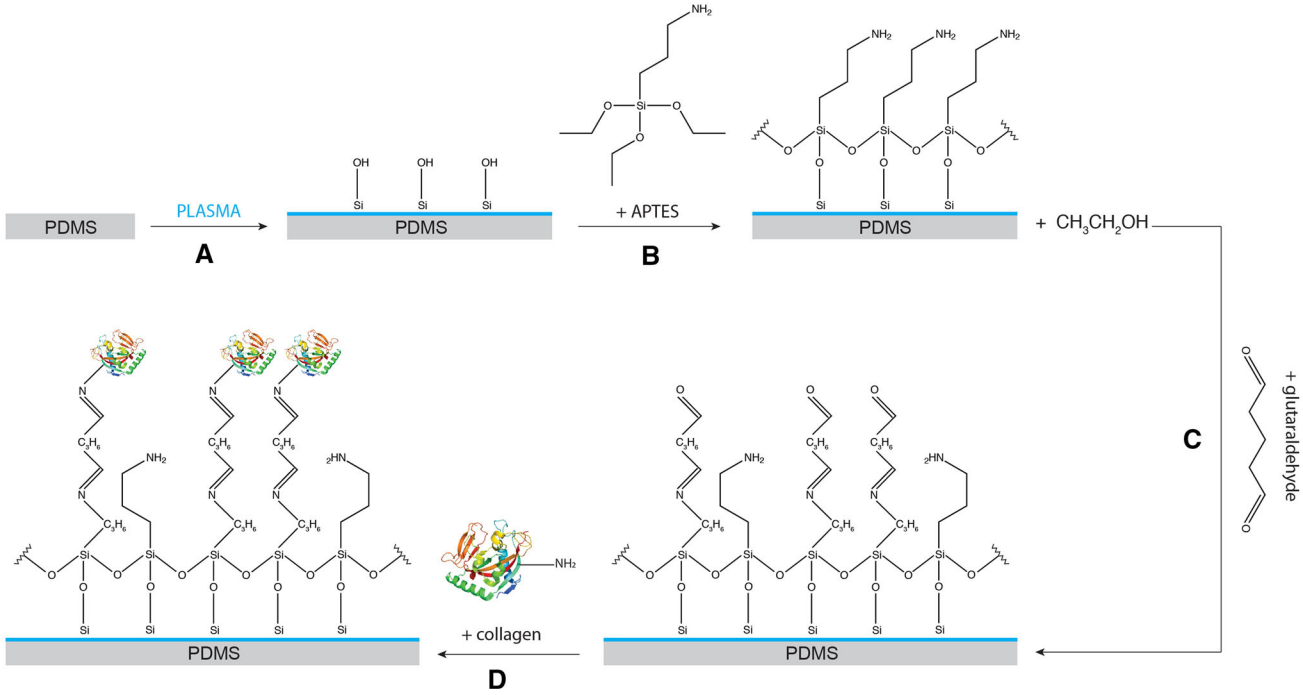

FIGURE 3. Anchoring collagen fibers to PDMS substrates. Representation of the four successive steps used to chemically functionalize PDMS: (step a) plasma irradiation to create silanol groups, (step b) functionalization with APTES as a linker between PDMS and collagen, (step c) functionalization with glutaraldehyde that anchors covalently 0 collagen fibers (step d) to PDMS-coated glass coverslips.

Characterization of the Collagen Fiber Alignment

A collagen solution of 2 mg/mL was aspirated in microchannels of three different widths (50, 100 and 200 μm) to form micro-stripes with oriented collagen fibers. As shown in Fig. 4c for an intermediate width of 100 μm, elongated collagen fibers grew up in the direction of the microchannel axis. We used the OrientationJ plugin of the Fiji image analysis software[28] to obtain the colour map (Fig. 4d) and the distribution of the angles formed by the collagen fibrils for each width of microchannels. As shown in Fig. 4e, the distribution of angles was found to be dependent on the microchannel width, with a mean angle of 0.8 ± 13.2° for the 50 μm wide microchannel. Our results indicated that the narrower the microchannel was, the more aligned the collagen fibers were.

Cell Migration on Collagen Micro-stripes with Aligned Fibers

We studied the migration of individual keratocytes on aligned collagen micro-stripes of 100 μm wide obtained from a collagen solution of 2 mg/mL. Keratocytes interacted with the collagen fibers by generating lamellipodial protrusions (filipodia-like). As shown in Fig 5a, time-lapse experiments indicated that cells polarized and migrated in the direction of the collagen fibers. Cells migrating on aligned collagen fibers did not maintain a constant shape such as the robust fan-shaped cells adopted by keratocytes migrating on homogeneously coated substrates, suggesting an active probing of the substrate. As shown in Fig. 5b, the cell area varied over time from 505 to 545 μm^2 during an experiment of 1000 s, corresponding to a mean cell area of 524 ± 13 μm^2, in agreement with previous results on homogeneously coated substrates. Using time-lapse microscopy, we determined that the distance of migration behaved linearly with time (Fig 5c) corresponding to a constant migration speed. We determined an average migrating velocity of 0.22 ± 0.03 μm/s (~ 13 μm/min), which is ~40% lower than commonly reported migrating velocities for isolated keratocytes but also 4 times higher than velocities found on our homogeneous collagen networks of 2 mg/mL. The instantaneous migration speed was constant during the whole experiment (Fig 5d).

DISCUSSION

Collagen fibrillogenesis is a complex process whose dynamics depends on the starting concentration. Our findings indicate that the polymerization time dropped when the concentration of collagen solution increased, suggesting that the polymerization of collagen net-

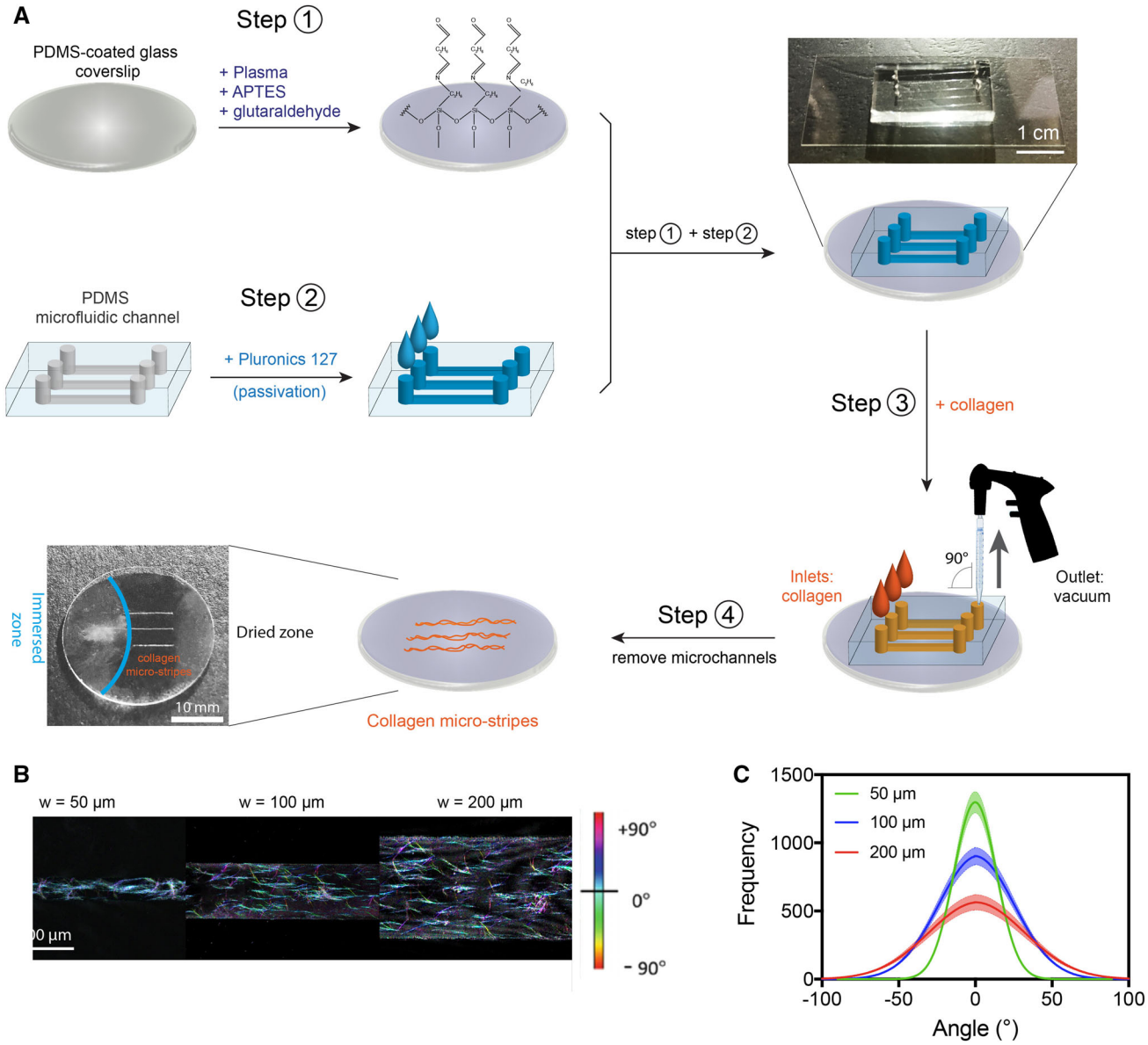

FIGURE 4. Aspiration of a collagen solution in microchannels form micro-stripes of well aligned collagen fibers. (a) Sketch of the successive steps required to form a microfluidic chamber. In step 1 a PDMS coated class coverslip is treated with Plasma, APTES and glutaraldehyde. In step 2 the PDMS microchannels are coated with Pluronics. Then the microfluidic chamber is assembled (step 1 + step 2). In step 3 collagen drops were deposited at the inlets and aspirated in the microchannels by placing an automatic pipette controller at the outlet. After polymerization, PDMS microchannels were removed in step 4 to expose collagen micro-stripes with aligned fibers. The micrograph in step 4 showed three parallel collagen micro-stripes of 100 μm wide obtained after the removal of the PDMS microchannels. (b) Confocal reflection image of oriented collagen fibers in 50, 100 and 200 μm wide micro-stripes. Images were colour-coded images to show a visual representation of the fiber orientation. The colour map represents the angle ranging from + 90°to − 90° with the horizontal axis. (c) Distribution of fibril angles in micro-stripes of 50 μm (in green), 100 μm (in blue) and 200 μm (in red) wide. Mean ± SD (n = 4 for each condition).

works is faster for higher concentrations. In addition, previous works have shown that higher concentrations of collagen are known to accelerate both the rate and number of collagen fibrils.[27] Interestingly, our results suggest the formation of two different architectures of collagen networks around a threshold at 1.5 mg/mL. These results are in agreement with recent reports that show a concentration dependence of the average connectivity in collagen networks.[14] The connectivity describes the number of fibers that meet at each network junction and is a key architectural parameter that governs the elastic response of collagen. Taken together our results indicate a non-linear concentration dependence of the area of fluorescence, suggesting that the architecture of collagen networks is sensitive to small changes in concentration.

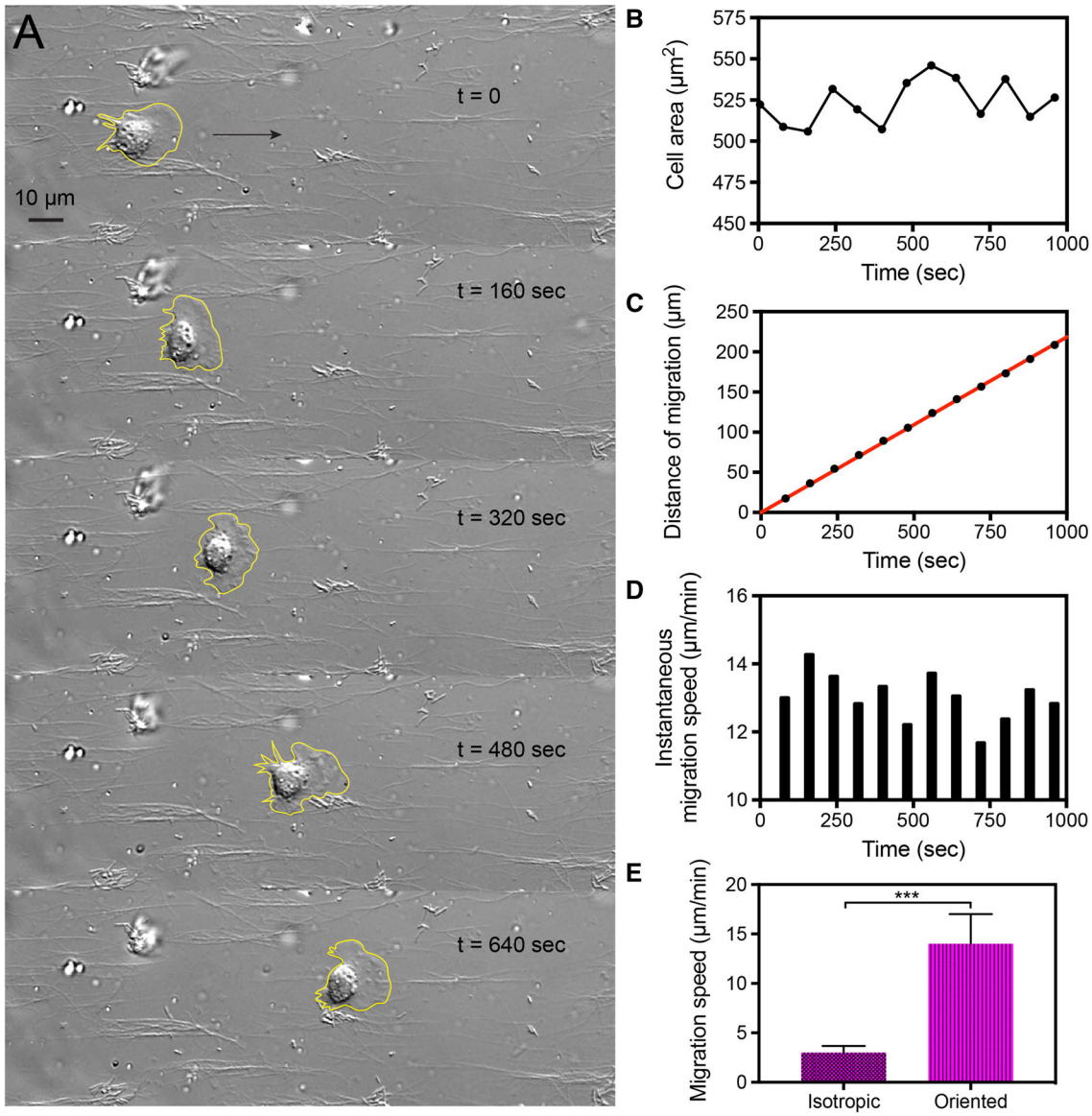

FIGURE 5. The migration speed of epithelial cells is enhanced on aligned collagen fibers. (a) Typical DIC image sequence of a single keratocyte migrating on a PDMS substrate covered with aligned collagen fibers. The cell contour is outlined in yellow. The scale bar is 10 μm and the total duration time is 640 s. Temporal evolution of (b) the cell area (n = 6), (c) the distance of migration (n = 6) and (d) the instantaneous speed (n = 6) of individual keratocytes migrating on aligned collagen fibers. (e) Mean cell migration speed for single keratocytes on isotropic (n = 11) and anisotropic (n = 13) collagen networks of 2 mg/mL. Mean ± SD and ***p < 0.05.

We found that the aspiration of a collagen solution with a pipette controller provides an appropriate the flow rate that enables the generation of highly aligned matrices of collagen. As observed in previous works,[18] the number of fibers near the bottom surface aligning in flow direction was reported to be higher for narrower microchannels. Interestingly, we show the possibility to influence the collagen fibril coverage by adjusting the concentration of the collagen solution.

Amino groups of the collagen fibrils interact with aldehyde groups present at the surface of the functionalized PDMS surface to form a covalent bonding. If one end of a collagen fibril attaches to the PDMS surface, the remaining part can be aligned by the streaming fluid in a position of low hydrodynamic resistance, i.e. parallel to the flow direction. Due to the covalent interactions of the collagen fibrils with the bottom surface, the PDMS microchannels can be removed without deteriorating the micro-stripes. Peeling off the PDMS microchannels allows to expose collagen micro-stripes with aligned fibers for cell culture assays. Collagen alignment was homogeneous for the whole length of the channel covering an area of several square millimeters.

Exposing long micro-stripes of aligned collagen fibers is a major advantage of this method for cell migration assays compared to collagen gels produced by other microfluidic alignment methods[6,15] that typically require to introduce living cells in 3D narrow microchannels.

By comparing isotropic collagen networks and collagen micro-stripes with aligned fibers, both formed from the same collagen solution, we showed that the orientation of collagen fibers significantly modulates the migration speed of epithelial cells. Indeed, we observed a four time increase of the migration velocity, suggesting that the orientation of collagen fibers is a key determinant of cell contact guidance. Experiments were performed on large collagen micro-stripes (w ~ 100 μm) to ensure that no lateral confinement was applied on migrating cells.[21,33]

Our findings on migrating epithelial cells demonstrate that collagen micro-stripes of oriented fibers can be considered as a valuable assay for studying cell contact guidance.[22] Using this platform, we envision that future experiments could be conducted *in vitro* to decipher the role of collagen fiber orientation in the cell contact guidance mechanism of breast tumor cells.

CONCLUSIONS

The versatility of this method enables easy-to-use and versatile preparation of collagen micro-stripes with aligned fibers. We envision that these micro-stripes can be useful for advanced cell biology and tissue engineering researches. In particular, collagen micro-stripes with aligned fibers can be considered as a valuable platform to understand the contact guidance mechanisms in cell migration. Ultimately, culture substrates with engineered collagen micro-stripes may provide a new opportunity to characterize *in vitro* the migration and persistence of breast cancer cells

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (https://doi.org/10.1007/s12195-019-00600-4) contains supplementary material, which is available to authorized users.

AUTHOR CONTRIBUTIONS

D.M., B.L.P and S.G. designed the study. D.M. and S.G. analyze data, wrote the main manuscript text and prepared figures. D.M., G.P., M.V., C.B., L.A., M.L. and E.V. contributed to the experiments and discussed the data. All authors improved the manuscript and the figure presentations.

FUNDING

This work was conducted with support from the Belgian National Fund for Scientific Research (F.R.S.-FNRS, Crédit de Recherches - J009916F). D.M, J.L., L.A., C.B., E.V. and M.L. are financially supported by FRIA (F.R.S.-FNRS). The Mechanobiology & Soft Matter group belongs to the French research consortium GDR 3070 CellTiss. The Interreg MAT(T)ISSE project is financially supported by Interreg France-Wallonie-Vlaanderen (Fonds Européen de Développement Régional, FEDER-ERDF).

ACKNOWLEDGMENTS

The authors thanks Thomas Boudou for insightful discussion, Robin Wilson and Daniel J. Cohen for their technical support with confocal microscopy.

CONFLICT OF INTEREST

Authors Danahe Mohammed, Gaspard Pardon, Marie Versaevel, Céline Bruyère, Laura Alaimo, Marine Luciano, Eléonore Vercruysse, Beth L. Pruitt and Sylvain Gabriele declare that they have no conflict of interest.

ETHICAL APPROVAL

All animal procedures on fish keratocytes obtained from the scales of *hypsophrys nicaraguensis* were carried out according to European Community Council guidelines for the care and use of animals in research, and with the agreement of the ethics committee of the University of Mons, Belgium. No human studies were carried out by the authors for this article.

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