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Porous Substrates Promote Endothelial Migration at the Expense of Fibronectin Fibrillogenesis

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Abstract

Porous substrates have gained increased usage in cell studies and tissue mimetic applications because they can partition distinct cell types while still allowing important biochemical crosstalk. In the presented work, we investigated how porous substrates with micron and submicron features influence early cell migration and the associated ECM establishment, which can critically affect the rate of cell coverage on the substrate and the ensuing tissue organization. We showed through time-lapse microscopy that cell speed and migratory distance on membranes with 0.5 μm pores were nearly two-fold of those observed on nonporous membranes, while values on membranes with 3.0 μm pores fell in between. Although the cell directionality ratio and the persistence time was unaffected by the presence of pores, the cells did exhibit directionality preferences based on the hexagonal pore patterning. Fibronectin fibrillogenesis exhibited a distinct inverse relationship to cell speed, as the fibrils formed on the nonporous control were significantly longer than those on both types of porous substrates. We further confirmed on a per cell basis that there is a negative correlation between fibronectin fibril length and cell speed. The observed trade-off between early cell coverage and ECM establishment thus warrants consideration in the selection or the engineering of the ideal porous substrate for tissue mimetic applications and may help guide future cell studies.

Graphical Abstract

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The following files are available free of charge.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): TRG is a co-founder of SiMPore, a start-up company that is commercializing ultrathin silicon-based membrane technologies.

Supporting Information: Experimental details on the determination of sample size for the PRW model, time progression of cell motility, directionality ratio, and the angle autocorrelation, the comparison of mean differences in fibril length on the different substrate types, and the correlation of fibronectin fibril length and residence time (PDF)

Keywords

membrane; substrate; migration; motility; directionality; focal adhesion; fibronectin

INTRODUCTION

Many tissues consist of the basic structural unit where two compartments are separated by a permeable membrane. Often, one or both compartments are populated with distinct cell types that carry out specialized physiological tasks. Some classic examples include the blood vessel, blood brain barrier, intestine, mammary gland, alveolus in the lung, lobule in the liver, and nephron in the kidney.^{1–9} Porous substrates are thus indispensable parts of many organ-on-a-chip and tissue mimetic platforms.^{10–17} One example is the use of a track-etched membrane in the Transwell® cell culture insert, which is popularly used in chemotaxis studies and *in vitro* tissue barrier models^{18–20}.

While the microarchitecture of the porous substrate is known to influence cell migration and extracellular matrix (ECM) deposition, a deep understanding of the cell-substrate interplay that underlies these processes remains limited. This shortcoming stems not only from the vast complexity of cells but also due in part to the heterogeneity of ECM components and structures. One of the better-understood cell-substrate interactions is the anchoring of cells *via* focal adhesions (FAs), which enables the cells to sense their microenvironment during migration and tissue barrier formation and remodeling.^{21,22} FA formation initiates as the integrins on the cell surface bind to extracellular matrix (ECM) ligands.²³ As integrins cluster at the cell-ECM contact, the closely spaced cytoplasmic portions of the integrins serve as a recruiting platform to host the docking and interaction of proteins that either provide linkage to the actin cytoskeleton or signal the cells to proliferate, survive, or migrate.^{24–36} Since FAs enable the force coupling between the cell and the ECM, the coordinated assembly and disassembly of FAs can sometimes influence cell motility and directionality.³⁷

Like FAs, the formation of a FN fibril is initiated by FN-integrin binding.^{38–41} FN fibrils elongate as the tension exerted through the cells induces a conformational change (or a binding site exposure) of the bound FN to favor subsequent FN-FN assembly.^{38–41} Therefore, it is not unexpected that FA formation and FN fibrillogenesis co-localize through their associations with integrins and traction forces. Studies using fibronectin-null fibroblasts have demonstrated that cell migration and growth is severely hindered in the absence or the

functional inhibition of FN.^{42–45} We have previously shown that substrate disruptions, such as those presented on a porous membrane, negatively affected both FA formation and FN fibrillogenesis.⁴⁶ Since cell migration is often tied to FA turnover and ECM generation, we hypothesized that the substrate disruption presented by the porous membranes can significantly alter the migratory behaviors of cells.

Since changes in cell speed and migratory direction can critically influence the rate of cell coverage on the substrate and the ensuing tissue alignment, we believe this cell migration study will help guide the selection or the engineering of the ideal porous substrate for tissue mimetic applications. In the presented work, we explored how the two different regimes of substrate disruption (micron versus submicron) influence early cell migration and the associated ECM establishment. We patterned 3.0 μm and 0.5 μm pores on a 300 nm film of glass (SiO_2) in a hexagonal packing arrangement, with the center-to-center distance set at two pore diameters apart. We chose thin glass as the base material because it enables the direct observation of cell migration and the high-resolution imaging of FN fibrils, while the regular placement of pores may help reduce variable cell response that arises due to substrate structural heterogeneity.

MATERIALS AND METHODS

Fabrication of Ultrathin SiO_2 Membrane

SiO_2 membranes were fabricated using conventional microfabrication techniques, as detailed in our previous work.^{16,47} Briefly described, plasma enhanced chemical vapor deposition (PECVD) was used to deposit a 300 nm film of SiO_2 on a double-side polished silicon wafer (150 mm diameter). The wafer was then backside patterned with a mask that resulted in 5.4 x 5.4 mm square dies with 2 x 2 mm windows after the backside-etch (Figure 1A). The oxide membrane was front-side patterned with an ASML PAS 5500/200 i-line stepper to create 3.0 μm and 0.5 μm pores in a hexagonal packing arrangement, with the center-to-center distance set at two pore diameters apart (Figure 1C). There were no pores patterned within a 100 micron frame along the edge of the suspended membrane (Figure 1B). The pores were reactive ion etched into the SiO_2 film with a Drytek 482 Quad Etcher. To reduce wrinkling and to further increase the mechanical strength of the membrane, the membrane was pulled taut in a slightly tensile state through a 600 °C anneal in nitrogen.⁴⁷ The wafer was through-etched from the backside using ethylenediamine pyrocatechol (EDP) in a custom fabricated one-sided heated etch cell to reveal free-standing membranes.⁴⁸ A nonporous suspended SiO_2 membrane was also fabricated as the control substrate using the same fabrication procedures.

Migration Chamber Assembly

Restricted grade silicone sheets of 300 μm or 600 μm thickness (Silicone Specialty Fabricators, Paso Robles, CA) were custom-patterned using the Silhouette Cameo digital craft cutter (Silhouette America, Oren, UT) to form the hosting space for cells and media (Figure 1A and 1B).⁴⁹ The patterned silicone sheets were then bonded to the top and the bottom of each membrane chip using a handheld corona surface treatment wand (Nbond, Littleton, CO), as described previously.⁵⁰

Cell Culture

The cells and all cell culture reagents were purchased from Thermo Fisher (Carlsbad, CA). Pooled human umbilical vein endothelial cells (HUVECs) were cultured in M200 with 2% (v/v) GIBCO Large Vessel Endothelial Supplement (LVES) and 1% penicillin and streptomycin. The LVES contains fetal bovine serum (FBS), hydrocortisone, human epidermal growth factor, basic fibroblast growth factor, heparin, ascorbic acid and other proprietary constituents not specified by the manufacturer. Since the LVES contains FBS, there will be soluble FN present in the media. Cells were detached and sub-cultured using TrypLE as per manufacturer's instructions. The culture media was exchanged every 2–3 days and the cells were passaged at 80% confluence. HUVECs were used between passages 3–5.

Migration Assay

All substrates were coated *via* incubation with 1:100 dilution of Geltrex® (0.15 mg/mL concentration) for 30 minutes, then pipetted off and let dry for another 30 minutes at 37 °C. The Geltrex provides a good mimic of a basement membrane, and contain laminin, collagen IV, entactin, and heparin sulfate proteoglycan, but no fibronectin. The 1:100 dilution is recommended by the manufacturer to prevent the formation of a thick gel on the substrate. We have shown through scanning electron microscopy (SEM) and fluid permeability study that the Geltrex coating performed this way did not occlude the pores. Media was introduced to the bottom gasket, followed by the seeding of ~160 cells onto the 2 mm x 2 mm substrate area to minimize the frequency of cell collision, which may complicates the migration analysis. Cells were allowed to adhere to the coated substrates for three hours prior to imaging. 24-hour live imaging (at four frames per hour) was performed on the Tokai Hit WSKM stage top incubator (Tokai Hit CO.,Ltd, Shizuoka, Japan).

Migration Analysis

Cells were tracked manually using the ImageJ Manual Tracking plug-in (developed by Fabrice Cordelieres, Institut Curie, Orsay, France). The trajectories obtained were then analyzed using custom-written routines in MATLAB (MathWorks, Inc., Natick, MA), which is shared over GitHub (<https://github.com/gaborskilab/Cell-migration-analysis>). The migration metrics calculated from the trajectories were: pathlength (total distance traveled), average speed (pathlength/total duration of travel), displacement (start-to-end distance of migration), and the directionality ratio (displacement/pathlength). The directionality ratio describes the tendency of a cell to maintain the same direction of travel and takes on a value of one if the cell migrates in a straight line. The instantaneous speed and the persistence time are also determined by taking the regression of a cell's mean squared displacement (MSD) as a function of time in accordance to the persistent random walk (PRW) model, which states that:

$$MSD(t) = 2s^2P \left[t - P \left(1 - e^{-\frac{t}{P}} \right) \right] \quad (1)$$

where t is time, s is the instantaneous speed, and P is the persistence time (which represents the average time that a cell commits to the same direction of travel).^{51,52} The PRW model assumes the hypothetical scenario that a cell obeys a fixed probability of turning as it steps throughout migration. For example, if this probability is 1/8, then on average a cell will make a turn after taking eight steps, and the persistence time would be eight times the duration between steps. For each cell, only the first third of the MSDs were used for the regression (the first eight hours; equivalent to 32 data points since we image four frames every hour), and only cells with a coefficient of determination (r^2) > 0.99 were included in the analysis (~20% of HUVECs analyzed). Ideally, the higher the r^2 threshold for cell selection, the better the cell conforms to the PRW model. We included enough cells ($n \sim 30$) in the analysis until the median value of instantaneous speed fell within 5% of the average speed of the total cell population (see Supporting Information; Figures S1 and S2). Since the PRW model assumed cell migration to be a biased random walk, the persistence time obtained inherently did not follow the normal distribution, and the mean instantaneous speed measured often overestimated the true cell speed. As such, we report the population estimates of instantaneous speed and persistence time in terms of median rather than the mean. To assess the directionality of cell movement, we defined each step that a cell made based on the center of the cell from two successive time points. The direction of each step takes on a value between 0° and 360° . The step directions of all cells were pooled together and assessed *via* radial histogram.

Immunofluorescence

Immediately after the 24-hour migration, the cells were permeabilized with 0.1% Triton X-100 for 3 seconds, fixed with 3.7% formaldehyde for 15 minutes, blocked with 20 mg/mL BSA for 15 minutes, then stained with 1:100 dilution (to 10 $\mu\text{g/mL}$) of AlexaFluor488 conjugated anti-fibronectin, Clone FN-3 (Affymetrix eBioscience, San Diego, CA), either alone or together with 1:100 dilution (to 10 $\mu\text{g/mL}$) of eFluor570 conjugated anti-vinculin, Clone 7F9 (Affymetrix eBioscience, San Diego, CA). Triple wash with PBS was performed before and after each aforementioned step. The brief permeabilization before fixation was performed intentionally to wash out the unbound vinculin inside the cells, which tend to blur the visualization of distinct focal adhesions.

Fibronectin Fibril Length Analysis

For each 40X image of the fibronectin tracks, the fibril lengths were measured using custom-written MATLAB routines. A disk filter with a 10-pixel radius was used to blur the image to obtain a background for subtraction. The edges of the fibronectin fibrils in the background corrected image were then sought using the Laplacian of Gaussian method.⁵³ Briefly described, the second spatial derivative of image intensity was calculated and the edges were defined at wherever there were zero-crossings. The half perimeters of the edges were used as the approximation of fiber lengths. Since we are most interested in understanding how cell-substrate interactions affect the maturation of FN fibrils, we report the mean values in terms of fibrils whose lengths are greater than ($>$) a specific cutoff value in our analysis.

Correlation of Fibronectin Fibril Length versus Cell Residence Time

The outlines of a cell throughout all time points of its migration were traced manually *via* a custom-written MATLAB routine, and the corresponding cell footprints were summed together to produce a map of cell residence times $\propto$ the higher the overlapping of the footprints, the higher the residence times. The map of residence time was then overlaid onto the labeled fibronectin tracks for the assessment of spatial correlation.

RESULTS AND DISCUSSION

Cell Motility and Directionality

In order to study how substrate disruption affects cell migration, we compared HUVEC migration on porous SiO₂ membranes with that on a nonporous SiO₂ control. We examined motility parameters such as speed, pathlength, and displacement traveled, as well as directionality parameters such as the directionality ratio, persistence time, and the distribution of the directions of cell movement. In our previous work, we observed the fewest FAs on the 0.5 μ m pore membranes.⁴⁶ Since the 0.5 μ m and the 3.0 μ m pore membranes both have the same available area for contact (77% substrate area, 23% porosity), and cells on all substrates exhibited similar spread area, the lack of mature FAs is likely attributed to the more frequent substrate disruption on the 0.5 μ m pore membranes and not just due to the reduction in the available area for contact. We hypothesized that the difference in FA formation (in terms of number and mean size) would lead to notable variations in migratory patterns.

Indeed, our current study showed that cell speed and migratory distances (both pathlength and displacement) were significantly higher on the 0.5 μ m pore membranes, followed by those seen on the 3.0 μ m pore membranes and then the nonporous control (Figure 2A–C). The faster cell speed may be attributed to the cells having better grip along the numerous pore edges on the 0.5 μ m pore membranes. Alternatively, the higher cell motility observed may be due to a reduced FA formation. A prior study by Kim and Wirtz on fibroblast migration revealed a tight, biphasic Gaussian relationship between cell speed and the size of FA $\propto$ cell speed increases with the size of FA until a speed maxima is reached, and decreases with larger FA thereafter.³⁷ On the other hand, in our previous study we found that FN fibrillogenesis was lowest on 0.5 μ m pore membranes and highest on non-porous,⁴⁶ the opposite of cell motility reported here. This suggests yet another possible explanation is that fibrillogenesis slows cells down and will be explored more in the next sections. This inverse relationship could be another In the case of HUVEC migration on the 0.5 μ m pore membranes, the lack of mature FAs likely facilitated quicker substrate detachment to allow higher cell motility. The nearly two-fold increase in cell speed on the 0.5 μ m pore membranes corresponded to a four-fold increase in the area coverage by the cells (see Supporting Information; Figure S3), which suggests favorable use in applications where faster cell coverage is desired, such as the early endothelialization of a vascular graft or *in vitro* barrier model. The presence of pores, however, did not affect the HUVECs' ability to maintain (or change) their course of migration. Statistical analysis of the directionality ratio and persistence time, both metrics that describe the tendency of cells to maintain course, did not indicate any significant difference amongst the different substrate types (Figure 2D–F).

It is likely that there is still sufficient substrate adhesion for the cells to maintain course (or to anchor to turn).

Since FA formation is initiated through the anchoring of cell surface integrins with their respective ligands on the substrate, the geometry of substrate continuity likely dictates the size and orientation of FAs. The hexagonal packing arrangement of pores potentially gives rise to two favored geometries for FA growth $\frac{3}{4}$ the growth patch type A with limited dimensions but higher circularity and the growth patch type B that has a smaller width but infinite length (Figure 3A). In our previous study, we observed a preference of cell alignment on the 0.5 μm porous SiO_2 in the directions defined by the placement of the type A growth patches.⁴⁶ We speculated that the same directional preference could be seen in migration.

We defined each step of a cell's migratory trajectory based on the center of the same cell from two successive time points. Examination of the step directions revealed that HUVECs indeed preferentially migrate in the directions defined by the placement of the type A growth patches (Figure 3B). It is likely that the smaller width of the type B growth patch limited the growth of FAs, as there are no integrin engagements over the pores. The larger FAs on the type A growth patch may thus dominate the couplings of traction throughout the cell, and directionality emerged as the cell pulled itself forward through these points of traction.^{41,54,55} It is not unexpected from this perspective that the bias in step directions on the 0.5 μm porous SiO_2 is stronger than the bias on the 3.0 μm porous SiO_2 (Figure 3B). The smaller pore-to-pore spacing of the 0.5 μm porous SiO_2 likely promoted a more precise alignment of traction along the placement of the type A growth patches, whereas there are more potential sites for FA formation between pores on the 3.0 μm porous SiO_2 .

In contrast to our previous study where we did not see a preference of cell alignment on the 3.0 μm porous SiO_2 , we saw a preference in the directions of cell movement on both the 0.5 μm the 3.0 μm porous SiO_2 membranes in our current study. We believe this discrepancy arises due in part to the weaker directional bias on the 3.0 μm porous SiO_2 and mainly from the large difference in sample size $\frac{3}{4}$ whereas the cell alignment data is obtained from a single snapshot in time ($n = 30$ cells), the cell movement data is gathered from 96 steps from each cell over the 24-hour imaging, resulting in more than 3000 data points per condition.

Fibronectin Fibrillogenesis

We next investigated the relationship between HUVEC migration and FN fibrillogenesis. Immediately after the 24-hour cell migration, the substrates were fixed and labeled for FN. Since the Geltrex coating that facilitated the initial cell adhesion does not contain FN, any FN fibrils observed were initiated and elongated by the cells using soluble FN that is already present in the media and FN secreted by the HUVECs themselves. Consistent with our previous study, the FN fibrils were qualitatively the longest on the nonporous control and the shortest on the membranes with 0.5 μm pores (Figure 4A). To enable quantitative comparison, we automated the survey of fibril length distribution on the different substrate types with a custom image processing algorithm (Figure 4B). It is not uncommon to find fibrils longer than 10 μm in length, especially on the nonporous SiO_2 (Figure 4B and S5). However, due to the large number of short fibrils, the median fibril lengths tend to be small

(Figure 4C). Not unexpected, the lengths of FN fibrils increased over time on all substrate types, and statistical differences emerged amongst the different substrate types at the 24 h time point (Figure 4C).

This trend of fibril length differences is still retained even at day 17 (two weeks after the HUVECs have attained confluency) (Figure 5). While all fibrils at day 17 were generally longer and brighter compared to those during the 24-hour migration on the same substrate types, the fibrils formed on the 0.5 μm pore membranes were still shorter than those on the 3.0 μm pore membranes, and the nonporous control still produced the longest fibrils. Interestingly, very few fibrils bridged over the 3.0 μm pores during the 24-hour cell migration and even at day 17 (Figure 5B and 5E). These observations thus suggest that the presence of substrate is conducive to nascent fibril assembly. As described by Singh et al., "...cell-substrate attachment can promote separation of adhesion sites from sites of FN assembly. This provides a mechanism for extending compact FN dimers by applying tension through pulling against substrate-attached protein and may explain some of the observed effects of substrate on FN assembly."⁵⁶ In the context of the porous substrates, the cell-substrate contacts between pores define the sites of FN-substrate tether, and FN fibrils form preferentially along the tension between FN-substrate tether and the FN-bound integrins on the cell surface. The absence of ECM ligands (e.g. collagen or even FN itself) at the pores likely prevented the close placement of FN-substrate tether and the FN-bound integrins on the cell surface, thereby hindering fibril elongation. Therefore it is not unexpected from this perspective that FN fibrils formed on porous substrates tend to be shorter, and that the FN fibrils tend to form in the space between the pores.

Not unexpected, our immunofluorescence studies revealed a co-localization of FAs with the FN fibrils (Figure 6), indicating that the sites of FN-substrate tether are also likely the sites of FA formation. This result further reaffirmed our prior observations that FAs do not form over pores at 24 h.⁴⁶ Due to the limitations of standard fluorescence microscopy, we were not able to definitively evaluate whether the fibrils formed over or bordered the 0.5 μm pores.

Inverse Relationship between Cell Migratory Speed and Fibronectin Fibrillogenesis

Examination of the mean cell speed and the median fibril length revealed negative correlation, in which fibril length decreases as cell migration speed increases, or said differently, migration speed decreases as fibril length increases (Figure 7A). We speculated that the elongation of FN fibrils is disrupted by the frequent presentation of pores, and that the lack of FN-substrate tether or strong cell-substrate contact in this case promoted higher cell motility. However, it is also possible that the promotion of fibrillogenesis on the continuous and non-porous substrates slowed cell migration. We further confirmed the negative correlation between cell speed and fibril length on a per cell basis. We tracked the footprints of cells through the time-lapse imaging to produce a "heat map" of residence time, effectively the reciprocal of speed. Overlays of the FN label and the residence time map revealed that the longer fibrils do indeed co-localize with the higher residence times (Figure 7B). Detailed time-lapse microscopy of 3T3 fibroblasts by Ohashi et al. revealed that the end of the fibrils at the cell edge appeared immobile, likely attached to the substrate, whereas the

end near the cell center moved along with cell motion.⁵⁷ Similar observations were made in studies that relate fibroblast traction force to FN fibrillogenesis.⁵⁸ Consistent with these observations in fibroblasts, the majority of FN fibrils formed by the HUVECs tend to align along the directions of cell movement, and there were more fibrils at the cell edge (corresponding to the periphery of the residence time map) presumably because that is the preferential site of fibril initiation.^{57,58} Interestingly, prior work by Grigoriou et al. have shown an opposite relationship in which fibroblast migration is slower on substrates coated with short, globular FN, and faster on substrates coated with lengthier, fibrillar fibronectin.⁵⁹ We believe this contrast to our finding is due to differences in the initial presentation of FN on the substrates. In the work of Grigoriou et al., the FN fibrils were already patterned on the substrate, whereas in our work there was no FN present on the substrates initially. The FN fibrils that we observe and measure were assembled by the migrating cells.

CONCLUSIONS

In this study, we investigated how the micron and submicron features of the porous substrates influence cell migration and the associated ECM establishment. We showed that frequent substrate disruption, such as those presented on the 0.5 μm and the 3.0 μm porous SiO_2 membranes, promoted higher cell motility. The nearly four-fold increase in the area coverage by the cells on the 0.5 μm porous SiO_2 membrane thus suggests favorable use in applications where faster cell coverage is desired, such as the early endothelialization of a vascular graft or *in vitro* barrier model. The higher cell motility, however, comes at the expense of diminished FN fibrillogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Abbott NJ, Rönnbäck L, Hansson E. Astrocyte–endothelial Interactions at the Blood–brain Barrier. *Nat Rev Neurosci.* 2006; 7(1):41–53. DOI: 10.1038/nrn1824 [PubMed: 16371949]
2. Rivera MN, Haber DA. Wilms' Tumour: Connecting Tumorigenesis and Organ Development in the Kidney. *Nat Rev Cancer.* 2005; 5(9):699–712. DOI: 10.1038/nrc1696 [PubMed: 16110318]
3. Krenkel O, Tacke F. Liver Macrophages in Tissue Homeostasis and Disease. *Nat Rev Immunol.* 2017; 17(5):306–321. DOI: 10.1038/nri.2017.11 [PubMed: 28317925]
4. Banks WA. From Blood-Brain Barrier to Blood-Brain Interface: New Opportunities for CNS Drug Delivery. *Nat Rev Drug Discov.* 2016; doi: 10.1038/nrd.2015.21
5. Sun S, Schiller JH, Gazdar AF. Lung Cancer in Never Smokers — a Different Disease. *Nat Rev Cancer.* 2007; 7(10):778–790. DOI: 10.1038/nrc2190 [PubMed: 17882278]
6. Gjorevski N, Nelson CM. Integrated Morphodynamic Signalling of the Mammary Gland. *Nat Rev Mol Cell Biol.* 2011; 12(9):581–593. DOI: 10.1038/nrm3168 [PubMed: 21829222]

7. Hussell T, Bell TJ. Alveolar Macrophages: Plasticity in a Tissue-Specific Context. *Nat Rev Immunol.* 2014; 14(2):81–93. DOI: 10.1038/nri3600 [PubMed: 24445666]
8. Lopes Pinheiro MA, Kooij G, Mizze MR, Kamermans A, Enzmann G, Lyck R, Schwaninger M, Engelhardt B, de Vries HE. Immune Cell Trafficking across the Barriers of the Central Nervous System in Multiple Sclerosis and Stroke. *Biochim Biophys Acta - Mol Basis Dis.* 2016; 1862(3): 461–471. DOI: <https://doi.org/10.1016/j.bbadis.2015.10.018>.
9. Cerf-Bensussan N, Gaboriau-Routhiau V. The Immune System and the Gut Microbiota: Friends or Foes? *Nat Rev Immunol.* 2010; 10(10):735–744. DOI: 10.1038/nri2850 [PubMed: 20865020]
10. Hubatsch I, Ragnarsson EGE, Artursson P. Determination of Drug Permeability and Prediction of Drug Absorption in Caco-2 Monolayers. *Nat Protoc.* 2007; 2(9):2111–2119. DOI: 10.1038/nprot.2007.303 [PubMed: 17853866]
11. Huh D, Matthews BD, Mammoto A, Montoya-Zavala M, Hsin HY, Ingber DE. Reconstituting Organ-Level Lung Functions on a Chip. *Science (Washington, DC, U S).* 2010; 328(5986):1662–1668. DOI: 10.1126/science.1188302
12. Lee JS, Romero R, Han YM, Kim HC, Kim CJ, Hong JS, Huh D. Placenta-on-a-Chip: A Novel Platform to Study the Biology of the Human Placenta. *J Matern Neonatal Med.* 2016; 29(7):1046–1054.
13. Naik P, Cucullo L. In Vitro Blood–brain Barrier Models: Current and Perspective Technologies. *J Pharm Sci.* 2012; 101(4):1337–1354. DOI: 10.1002/jps.23022 [PubMed: 22213383]
14. Ma SH, Lepak LA, Hussain RJ, Shain W, Shuler ML. An Endothelial and Astrocyte Co-Culture Model of the Blood–brain Barrier Utilizing an Ultra-Thin, Nanofabricated Silicon Nitride Membrane. *Lab Chip.* 2005; 5(1):74–85. DOI: 10.1039/B405713A [PubMed: 15616743]
15. Chung HH, Chan CK, Khire TS, Marsh GA, Clark A, Waugh RE, McGrath JL. Highly Permeable Silicon Membranes for Shear Free Chemotaxis and Rapid Cell Labeling. *Lab Chip.* 2014; 14(14): 2456–2468. DOI: 10.1039/C4LC00326H [PubMed: 24850320]
16. Mazzocchi AR, Man AJ, DesOrmeaux JPS, Gaborski TR. Porous Membranes Promote Endothelial Differentiation of Adipose-Derived Stem Cells and Perivascular Interactions. *Cell Mol Bioeng.* 2014; 7(3):369–378. DOI: 10.1007/s12195-014-0354-7
17. Boyden S. The Chemotactic Effect of Mixtures of Antibody and Antigen on Polymorphonuclear Leucocytes. *J Exp Med.* 1962; 115(3):453–466. DOI: 10.1084/jem.115.3.453 [PubMed: 13872176]
18. Toetsch S, Olwell P, Prina-Mello A, Volkov Y. The Evolution of Chemotaxis Assays from Static Models to Physiologically Relevant Platforms. *Integr Biol.* 2009; 1(2):170–181. DOI: 10.1039/B814567A
19. Keenan TM, Folch A. Biomolecular Gradients in Cell Culture Systems. *Lab Chip.* 2008; 8(1):34–57. DOI: 10.1039/B711887B [PubMed: 18094760]
20. Srinivasan B, Kolli AR, Esch MB, Abaci HE, Shuler ML, Hickman JJ. TEER Measurement Techniques for in Vitro Barrier Model Systems. *J Lab Autom.* 2015; 20(2):107–126. DOI: 10.1177/2211068214561025 [PubMed: 25586998]
21. Geiger B, Spatz JP, Bershadsky AD. Environmental Sensing through Focal Adhesions. *Nat Rev Mol cell Biol.* 2009; 10(1):21–33. DOI: 10.1038/nrm2593 [PubMed: 19197329]
22. Romer LH, Birukov KG, Garcia JGN. Focal Adhesions: Paradigm for a Signaling Nexus. *Circ Res.* 2006; 98(5):606–616. DOI: 10.1161/01.RES.0000207408.31270.db [PubMed: 16543511]
23. Nagano M, Hoshino D, Koshikawa N, Akizawa T, Seiki M. Turnover of Focal Adhesions and Cancer Cell Migration. *Int J Cell Biol.* 2012; 2012doi: 10.1155/2012/310616
24. Stoker AW. Protein Tyrosine Phosphatases and Signalling. *J Endocrinol.* 2005; 185(1):19–33. DOI: 10.1677/joe.1.06069 [PubMed: 15817824]
25. McDonald PC, Fielding AB, Dedhar S. Integrin-Linked Kinase—essential Roles in Physiology and Cancer Biology. *J Cell Sci.* 2008; 121(19):3121–3132. [PubMed: 18799788]
26. Ishibe S, Joly D, Zhu X, Cantley LG. Phosphorylation-Dependent Paxillin-ERK Association Mediates Hepatocyte Growth Factor-Stimulated Epithelial Morphogenesis. *Mol Cell.* 2003; 12(5): 1275–1285. DOI: 10.1016/S1097-2765(03)00406-4 [PubMed: 14636584]
27. Mitra SK, Hanson DA, Schlaepfer DD. Focal Adhesion Kinase: In Command and Control of Cell Motility. *Nat Rev Mol cell Biol.* 2005; 6(1):56–68. DOI: 10.1038/nrm1549 [PubMed: 15688067]

28. Parsons JT, Parsons SJ. Src Family Protein Tyrosine Kinases: Cooperating with Growth Factor and Adhesion Signaling Pathways. *Curr Opin Cell Biol.* 1997; 9(2):187–192. DOI: 10.1016/S0955-0674(97)80062-2 [PubMed: 9069259]
29. Burridge K, Turner CE, Romer LH. Tyrosine Phosphorylation of Paxillin and pp125 FAK Accompanies Cell Adhesion to Extracellular Matrix: A Role in Cytoskeletal Assembly. *J Cell Biol.* 1992; 119:893.doi: 10.1083/jcb.119.4.893 [PubMed: 1385444]
30. Geiger B, Bershadsky A, Pankov R, Yamada KM. Transmembrane Crosstalk between the Extracellular Matrix and the Cytoskeleton. *Nat Rev Mol cell Biol.* 2001; 2(11):793–805. DOI: 10.1038/35099066 [PubMed: 11715046]
31. Gluck U, Ben-Ze'ev A. Modulation of Alpha-Actinin Levels Affects Cell Motility and Confers Tumorigenicity on 3T3 Cells. *J Cell Sci.* 1994; 107(7):1773–1782. [PubMed: 7983147]
32. Horwitz A, Duggan K, Buck C, Beckerle MC, Burridge K. Interaction of Plasma Membrane Fibronectin Receptor with Talin—a Transmembrane Linkage. 1986; doi: 10.1038/320531a0
33. Humphries JD, Wang P, Streuli C, Geiger B, Humphries MJ, Ballestrem C. Vinculin Controls Focal Adhesion Formation by Direct Interactions with Talin and Actin. *J Cell Biol.* 2007; 179(5): 1043–1057. DOI: 10.1083/jcb.200703036 [PubMed: 18056416]
34. Petit V, Thiery J. Focal Adhesions: Structure and Dynamics. *Biol Cell.* 2000; 92(7):477–494. DOI: 10.1016/S0248-4900(00)01101-1 [PubMed: 11229600]
35. Liu S, Calderwood DA, Ginsberg MH. Integrin Cytoplasmic Domain-Binding Proteins. *J Cell Sci.* 2000; 113(20):3563–3571. [PubMed: 11017872]
36. Galbraith CG, Sheetz MP. Forces on Adhesive Contacts Affect Cell Function. *Curr Opin Cell Biol.* 1998; 10(5):566–571. DOI: 10.1016/S0955-0674(98)80030-6 [PubMed: 9818165]
37. Kim DH, Wirtz D. Focal Adhesion Size Uniquely Predicts Cell Migration. *FASEB J.* 2013; 27(4): 1351–1361. DOI: 10.1096/fj.12-220160 [PubMed: 23254340]
38. Schwarzbauer JE, Sechler JL. Fibronectin Fibrillogenesis: A Paradigm for Extracellular Matrix Assembly. *Curr Opin Cell Biol.* 1999; 11(5):622–627. DOI: 10.1016/S0955-0674(99)00017-4 [PubMed: 10508649]
39. Mao Y, Schwarzbauer JE. Fibronectin Fibrillogenesis, a Cell-Mediated Matrix Assembly Process. *Matrix Biol.* 2005; 24(6):389–399. DOI: 10.1016/j.matbio.2005.06.008 [PubMed: 16061370]
40. Wierzbicka-Patynowski I, Schwarzbauer JE. The Ins and Outs of Fibronectin Matrix Assembly. *J Cell Sci.* 2003; 116(16):3269–3276. DOI: 10.1242/jcs.00670 [PubMed: 12857786]
41. Burridge K, Guilluy C. Focal Adhesions, Stress Fibers and Mechanical Tension. *Exp Cell Res.* 2016; 343(1):14–20. DOI: 10.1016/j.yexcr.2015.10.029 [PubMed: 26519907]
42. Sottile J, Hocking DC, Swiatek PJ. Fibronectin Matrix Assembly Enhances Adhesion-Dependent Cell Growth. *J Cell Sci.* 1998; 111(19):2933–2943. [PubMed: 9730985]
43. Sottile J, Hocking DC, Langenbach KJ. Fibronectin Polymerization Stimulates Cell Growth by RGD-Dependent and-Independent Mechanisms. *J Cell Sci.* 2000; 113(23):4287–4299. [PubMed: 11069773]
44. Hocking DC, Chang CH. Fibronectin Matrix Polymerization Regulates Small Airway Epithelial Cell Migration. *Am J Physiol Cell Mol Physiol.* 2003; 285(1):L169–L179. DOI: 10.1152/ajplung.00371.2002
45. Sottile J, Shi F, Rublyevska I, Chiang HY, Lust J, Chandler J. Fibronectin-Dependent Collagen I Deposition Modulates the Cell Response to Fibronectin. *Am J Physiol Physiol.* 2007; 293(6):C1934–C1946. DOI: 10.1152/ajpcell.00130.2007
46. Casillo SM, Peredo AP, Perry SJ, Chung HH, Gaborski TR. Membrane Pore Spacing Can Modulate Endothelial Cell–Substrate and Cell–Cell Interactions. *ACS Biomater Sci Eng.* 2017; 3(3):243–248. DOI: 10.1021/acsbomaterials.7b00055 [PubMed: 28993815]
47. Carter RN, Casillo SM, Mazzocchi AR, DesOrmeaux JPS, Roussie JA, Gaborski TR. Ultrathin Transparent Membranes for Cellular Barrier and Co-Culture Models. *Biofabrication.* 2017; 9(1): 15019.doi: 10.1088/1758-5090/aa5ba7
48. Striemer CC, Gaborski TR, McGrath JL, Fauchet PM. Charge-and Size-Based Separation of Macromolecules Using Ultrathin Silicon Membranes. *Nature.* 2007; 445(7129):749–753. DOI: 10.1038/nature05532 [PubMed: 17301789]

49. Yuen PK, Goral VN. Low-Cost Rapid Prototyping of Flexible Microfluidic Devices Using a Desktop Digital Craft Cutter. *Lab Chip*. 2010; 10(3):384–387. DOI: 10.1039/B918089C [PubMed: 20091012]
50. Yang, C., Wang, W., Li, Z. Optimization of Corona-Triggered PDMS-PDMS Bonding Method. *Nano/Micro Engineered and Molecular Systems*, 2009. NEMS 2009. 4th IEEE International Conference on; IEEE; 2009. p. 319-322.
51. Othmer HG, Dunbar SR, Alt W. Models of Dispersal in Biological Systems. *J Math Biol*. 1988; 26(3):263–298. DOI: 10.1007/bf00277392 [PubMed: 3411255]
52. Dunn GA. Characterising a Kinesis Response: Time Averaged Measures of Cell Speed and Directional Persistence. *Agents Actions Suppl*. 1983; 12:14.doi: 10.1007/978-3-0348-9352-7_1 [PubMed: 6573115]
53. Canny J. A Computational Approach to Edge Detection. *IEEE Trans Pattern Anal Mach Intell*. 1986; (6):679–698. DOI: 10.1109/TPAMI.1986.4767851 [PubMed: 21869365]
54. Ananthakrishnan R, Ehrlicher A. The Forces behind Cell Movement. *Int J Biol Sci*. 2007; 3(5): 303–317. DOI: 10.7150/ijbs.3.303 [PubMed: 17589565]
55. Berrier AL, Yamada KM. Cell–matrix Adhesion. *J Cell Physiol*. 2007; 213(3):565–573. DOI: 10.1002/jcp.21237 [PubMed: 17680633]
56. Singh P, Carraher C, Schwarzbauer JE. Assembly of Fibronectin Extracellular Matrix. *Annu Rev Cell Dev Biol*. 2010; 26:397–419. DOI: 10.1146/annurev-cellbio-100109-104020 [PubMed: 20690820]
57. Ohashi T, Kiehart DP, Erickson HP. Dual Labeling of the Fibronectin Matrix and Actin Cytoskeleton with Green Fluorescent Protein Variants. *J Cell Sci*. 2002; 115(6):1221–1229. [PubMed: 11884521]
58. Lemmon CA, Chen CS, Romer LH. Cell Traction Forces Direct Fibronectin Matrix Assembly. *Biophys J*. 2009; 96(2):729–738. DOI: 10.1016/j.bpj.2008.10.009 [PubMed: 19167317]
59. Grigoriou E, Cantini M, Dalby MJ, Petersen A. *Biomaterials Science*. 2017; :1326–1333. DOI: 10.1039/c7bm00333a [PubMed: 28612879]

Figure 1. Setup of the migration chamber. (A) The SiO₂ membrane-based migration chamber in a 24-well plate format. (B) A cross sectional view of the chamber. (C) SEM close-ups of the SiO₂ membranes. Left: 3.0 μm pore version; Right: 0.5 μm pore version.

Figure 2. Metrics of cell migration over 24 h on the porous substrates

Statistical differences are seen in the (A) speed, (B) pathlength, and (C) displacement (the start-to-end distance) of cell migration on nonporous, 0.5 μm pores, and 3.0 μm pores SiO_2 . (D) Detailed illustration of the cell migration metrics, including (E) the directionality and the (F) persistence time. Directionality ratio is defined as the ratio of displacement to pathlength and describes the efficiency of a cell in maintaining the same direction of migration. Since the persistence time does not assume normal distribution, the data is presented in terms of a box-and-whisker plot. The neck of each box denotes the median, the notch denotes the 95% confidence interval, the ends of each box denote the 25th and 75th percentile, and the whiskers correspond to the 1.0 multiple of the interquartile range. No statistical difference is seen in both the directionality ratio and the persistence time. Kruskal-Wallis one-way analysis of variance was used for the statistical analysis of persistence time ($\alpha = 0.05$). One-way ANOVA with a Turkey-Kramer *post hoc* procedure was used for the statistical analysis of all other migration metrics ($\alpha = 0.05$). All error bars represent the standard errors of the means. All data were obtained from cells ($n > 150$) pooled from 3–4 independent migrations on each substrate type.

Figure 3. Radial histogram of the step directions

(A) Schematic of the pore spacing and the geometry of potential FA growth. At a center-to-center distance of two pore diameters, the membrane has a porosity of 23% and 77% contact area. Growth patch type A and type B denote the two favored geometries of FA growth. Growth patch type A' and type B' are the symmetrical identity of growth patch type A and type B, respectively. The center of the same cell from two successive time points defined each step of its migration. (B) The distribution of the step directions for cell migration on membranes with 3.0 μm and 0.5 μm pores (n = 35 cells; 3000 steps), presented in the form of radial histogram.

Figure 4. Fibronectin fibril lengths on the different substrates

(A) Representative images of FN fibrils after 24 h on nonporous, 3.0 μm pore, and 0.5 μm pore membrane. (B) Representative histogram of the fibril length distribution seen on a nonporous control. The inset shows a cropped region from which the histogram is produced. The “skeleton”, branching points, and end points of the each identified FN fibril are marked in blue, red, and green, respectively. (C) The mean fibril lengths measured at the 6 h, 12 h, and 24 h time points. The data was obtained from 15–26 images on each substrate type ($n = 97$ –253 cells when pooled together). The error bars represent the standard error of the means. One-way ANOVA with a Turkey-Kramer *post hoc* procedure was used for the statistical analysis of all other migration metrics ($\alpha = 0.05$).

Figure 5. Formation of fibronectin fibrils on the different substrates over time
Fibronectin fibrils left behind by the cells during their 24-hour migration (A–C) and established two weeks after the HUVECs attained confluency (D–F). The white circles in (B) and (E) denote the 3.0 μm pores.

Figure 6. Co-localization of focal adhesions and fibronectin fibrils on the different substrates
 Representative cell at the border (gray) between the nonporous region and the region with 0.5 μm pores (**A**), on the 3.0 μm pores (**B**), and on the 0.5 μm pores (**C**). Note that the focal adhesions can only be found on the 0.5 μm porous SiO_2 region of the membrane in (**A**) because the cell moved from the nonporous region at the left to the 0.5 μm porous SiO_2 region at the right. Automated identification of fibronectin fibrils (marked by the white outlines) and focal adhesions (marked in red) were also given on the panel to the right (**D–F**) to help guide visual assessment.

Figure 7. Fibronectin fibril lengths versus cell speed on the different substrates

(A) Length of fibronectin fibrils versus cell speed over 24-hour migrations on nonporous, 3.0 μm porous, and 0.5 μm porous SiO₂. The data was obtained from 3–4 independent migrations on each substrate type ($n > 150$ cells when pooled together; 3–4 images of FN label per migration). The error bars represent the standard error of the means. (B) Representative “heat map” of cell residence times, obtained by summing the footprints of a migrating cell over the all time points of its migration on the membrane with 0.5 μm pores. The blue and red color correspond to a cell residence time of 0 h and 24 h, respectively. The outlines of fibronectin fibrils were delineated in white. Displayed at the center are the positions of cell center at different times, with the start and the end point marked by the green and red circles, respectively.