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Magnetically attachable stencils and the non-destructive analysis of the contribution made by the underlying matrix to cell migration.

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Abstract

Cell migration is controlled by the integration of numerous distinct components. Consequently, the analysis of cell migration is advancing towards comprehensive, multifaceted *in vitro* models. To accurately evaluate the contribution of an underlying substrate to cell motility in complex cellular environments we developed a migration assay using magnetically attachable stencils (MATs). When attached to a culture surface, MATs create a defined void in the cell monolayer without disrupting the cells or damaging the underlying substrate. Quantitative analysis of migration into this void reveals the substrate's contribution to migration. The magnetically-guided placement of a microfabricated stencil allows for full experimental control of the substrate on which migration is analyzed. MATs enable the evaluation of intact, defined matrix, and make it possible to analyze migration on unique surfaces such as micropatterned proteins, nano-textured surfaces, and pliable hydrogels. These studies also revealed that mechanical disruption, including the damage that occurs during scratch assays, diminishes migration and confounds the analysis of individual cell behavior. Analysis of migration on increasingly complex biomaterials reveals that the contribution of the underlying matrix depends not only on its molecular composition but also its organization and the context in which it is presented.

1. INTRODUCTION

Cell migration is a multifactorial process controlled by many different molecular components that can be parsed into four distinct categories: 1) cell autonomous characteristics, including the genetic and epigenetic regulation of cell signaling molecules; 2) cell-matrix adhesion, including its regulation by the matrix composition and elasticity; 3) cell-to-cell interactions through the formation of cell-cell adhesions; and 4) soluble communication through factors such as small molecules, cellular metabolites, and secreted

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proteins, (Fig. 1a) [1,2]. The spatial and temporal integration of these fundamentally different elements determines the successful initiation, progression, and eventual completion of cell migration.

Because migration is the product of a multifactorial integration of many different mechanisms, the specific contribution of any single factor can only be studied within the context of the other participating components. The migratory response to a soluble cytokine, for example, can be augmented by the presence of mature cell-cell adhesions or the composition of the matrix to which the cells adhere. Although migration *in vitro* is frequently done with isolated cells, migration *in vivo* occurs within the context of a physical tissue architecture and densely organized cellular populations [3]. *In vivo* single cells rarely have the opportunity to migrate alone across a cell-free expanse of matrix because both the neighboring cells and the surrounding matrix inhibit motility. *In vitro* recreation of these *in vivo* conditions is challenging because existing assays do not enable investigators to control matrix conditions while analyzing the migration of dense cell populations [4].

Traditional scratch assays disrupt the matrix, and the accurate patterning of cells on fragile, bioactive substrates is technically challenging and does not accommodate many existing experimental strategies. Therefore, depending on the importance of cell-matrix interactions versus cell-cell contact, *in vitro* cell migration studies have generally been divided into two distinct approaches: 1) the analysis of single cells on intact matrix and 2) the analysis of densely organized cells engaged in cell-cell contact on a wounded or modified monolayer (Fig. 1f). A few exceptions to this general dichotomy are scatter assays [5], the dispersal of confluent populations from droplets [6,7], and the analysis of collective cell migration using electrically controllable substrates [8].

In order to overcome these limitations and study the permissive or non-permissive nature of the underlying substrate during migration, we created a non-destructive space-filling assay using magnetically attachable stencils (MATs). MATs are attached magnetically to a culture surface and kept in place during cell culture. The migration assay is initiated by removing the MAT to create a void into which the cells migrate (Fig. 1b-e & Video S1). The rate at which cells migrate into the void is the product of the composition of the substrate in the void, cell autonomous properties, soluble factors, and cell-cell interactions. MATs provide the ability to accurately and non-destructively pattern cells on a wide variety of substrates, including culture surfaces coated with matrix proteins, micropatterned and nanofabricated surfaces, pliable gels (Fig. 1f). In this way MATs integrate both cell-matrix and cell-cell interactions at high density and enable the investigator to determine the contribution of the underlying matrix to cell migration.

2. MATERIALS and METHODS

The following materials and methods were used unless stated otherwise in the text.

2.1 Materials

Rare-earth (NdFeB) magnets were obtained from K&J Magnetics. The 1/4"×1/8"×1/8" grade N42 magnets (B422) were used to attach MATs to t.c. plastic dishes. Polyacrylamide gels used identical magnets for stiff gels and 1/4"×1/8"×1/16" grade N42 magnets (B421) for soft gels. Standard MATs were cast in brass molds using polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning) mixed with magnetite (Pirox 200HP, Pittsburgh Iron Oxides LLC) at a 2:1 ratio by weight, homogenized in an AR-100 mixer (Thinky), and cured at 60°C in an oven. Glass and t.c. plastic substrates, specifically 6- and 12-well plates (Costar 3516 and 3513, Corning) were coated with collagen (rat tail type I, 354249, BD Biosciences), human plasma fibronectin, murine laminin (L2020, Sigma), poly-D-lysine

(P7280, Sigma), and FITC gelatin (G13187, Fisher). Collagen refers to collagen type I isolated from rat tails unless stated otherwise. Alexa fluor 555-conjugated bovine serum albumin (A555-BSA, A34786, Invitrogen) and fraction V BSA (A8412, Sigma) were used for blocking substrates. DME medium (11965, Gibco), Roswell Park Memorial Institute 1640 medium (RPMI, 21870, Gibco), Optimem (31985, Gibco) were used with cells. Polyacrylamide (PAA) substrates were made using acrylamide (40%, 161-0140, BioRad), bis-acrylamide (2%, 161-0142, BioRad), ammonium persulfate (248614, Sigma), and TEMED (161-0800, BioRad) on 22 mm diameter #1.5 glass coverslips (Electron Microscopy Services). Gelatin (G1890, Sigma) and collagen (rat tail type I, 354249, BD Biosciences) were conjugated to polyacrylamide surfaces by activating *N*-Sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH, 22589, Thermo Scientific) with black light blue UV bulbs (~340 nm) 3-4 inches from the PAA surface in a UV Stratalinker 2400 (Stratagene). Solidworks (Dassault Systems) and GibbsCAM (Gibbs and Associates) software was used in the design and fabrication of MAT molds for computer-numeric-control (CNC) milling. Images were calibrated to achieve actual distances using a micro-ruler (1 mm total length, 100 × 10 μm divisions, Graticules, Ltd., Tonbridge England). Nanofiber substrates came from Nanofiber Solutions LLC.

2.3 Cell culture

Cell cultures of human head and neck carcinoma HEP3, human lung carcinoma A549 cell lines, murine brain endothelial cells bEnd.3, and primary murine mammary carcinoma cells (MMC) were cultured in DME medium, RPMI, DME medium and according to the procedures of Ramirez *et al.* [9] DMEM and RPMI were supplemented with pen/strep, HEPES buffer, non-essential amino acids, and 10% fetal bovine serum. Cells were cultured at 37°C in a humidified 5% CO₂ incubator and passaged every 2-4 days.

2.4 Mold production

Brass molds were constructed in two layers in order to ensure a smooth contact surface free of tool marks that also transitions sharply without any curvature at the bottom edge. The bottom layer consists of a 3" × 3" × 0.25" piece of brass, with a 3 by 3 array of bolt holes surrounded by 2 reamed holes on each side that was later pressure-fitted with steel dowels serving as guide pins for the brass cubes. The top layer was made of an outer frame and a 3 by 3 array of 0.5" × 0.5" × 0.25" cubes of brass with rounded corners (3/32" diameter), a 5° taper all around, a central threaded hole, and two holes to each side for the guide pins. After grinding and polishing the base to a mirror finish, dowels were pressed into the base. Then the brass cubes were inserted over the dowels and individually fastened into place with screws (Fig. S1a). MATs were cast in the void between the cubes, resulting in a 2 by 2 array of connected MATs (Fig. S1b-d). Molds for larger arrays are under development and will utilize a replaceable mirror-finish plastic sheet sandwiched between the base and brass cubes.

2.5 Preparing and sterilizing MATs

MATs were fabricated according to the following steps. Polydimethylsiloxane prepolymer was made by mixing 10 parts base to 1 part curing agent by weight, as recommended by the manufacturer. Then magnetite was measured into the prepolymer to achieve a 33% magnetite mixture by weight. The magnetite was mixed briefly by hand and then homogenized in an AR-100 mixer. PDMS-magnetite mixture (PDMS-m) was poured into the brass molds and degassed by applying sufficient vacuum to cause large (2 mm) bubbles to rise, at which point the vacuum was released. This degassing procedure was repeated once. The PDMS-m was cured in the molds at 60°C for at least 4 hours, after which the connected 2 by 2 of MATs was removed from the molds and cut with a clean razor blade (Fig. S1, b-d). As expected, cured PDMS-m was noticeably stiffer than plain PDMS [10].

MATs were stored with the contact surface against the bottom of clean plastic petri dishes. To prepare MATs for use, the contact surface was sprayed vigorously with 70% ethanol to both remove dust and provide sterilization. Ethanol was immediately aspirated from the surfaces of the MATs in the cell culture hood. After this, MATs were placed upside down to dry. For greater sterilization MATs may be soaked in 70% ethanol for 10 minutes, following which they must be dried in an oven at 50–60°C for 2 hours in order to evaporate and remove ethanol from within the PDMS-m.

2.6 Magnetic attachment

MATs were attached to a culture surface by placing magnets underneath, in direct contact with the t.c. plastic. The magnetic force is determined by the strength of the magnet, amount of magnetite material in the stencil, and the distance between the magnet and stencil. For simplicity we chose to maintain 33% magnetite in the MATs, though this concentration could be decreased to achieve less force. Different magnetic arrangements were tested for their ability to facilitate alignment of the MATs. The arrangement of four small magnets rotated by 90° around a central point and pressed into plus-shaped grooves was chosen because it visually and magnetically facilitates alignment of Star MATs (Fig. S1, f and g). Magnet arrangements for 6- and 12-well plates were created this way. Magnet arrangements for 96-well plates were also created using a single 1/8" diameter 1/16" tall grade N52 magnets (D21B-N52) for each well. An appropriate level of force was determined empirically. The 1/4" × 1/8" × 1/8" grade N42 magnets (B422, K&J Magnetics, Inc.) were chosen based on their ability to prevent cell protrusions underneath MATs in 6-well plates by generating sufficient force to seal but not deform the MATs. The calculated magnetic field across the contact surface of the MATs using these magnets and 6- or 12-well plates was 0.17 ± 0.01 T. Stiff and soft PAA gels used fields of 0.14 ± 0.01 T and 0.10 ± 0.01 T by using arrangements of 1/4" × 1/8" × 1/8" and 1/4" × 1/8" × 1/16" grade N42 magnets (B422 and B421), respectively.

2.7 Protein patterning

Protein patterns were created around Star MATs (Fig. 3c-d) as follows. Tissue culture plastic was coated with 100 µg/ml FITC gelatin and rinsed three times with PBS. With PBS in the culture dish, the MATs were positioned near the culture surface and released, allowing the magnets to pull the MATs against the surface. A PBS solution containing 3 µg/ml human plasma fibronectin was added and allowed to incubate around the MATs for 2 hours at 37°C. After rinsing three times with PBS, the plate was removed from the magnets, the MATs were detached, and the culture surface was rinsed twice more with PBS. Then rabbit antibodies against human fibronectin were added at 2 µg/ml in PBS containing 0.5% BSA and incubated for 1 hour at 37°C. The surface was rinsed, and an anti-rabbit goat antibody labeled with Alexa-546 at 2 µg/ml in PBS containing 0.5% BSA was added for 1 hour at 37°C. After rinsing three times with PBS, the surface was imaged.

2.8 Micropatterned proteins

Microfluidic channels made of PDMS-m by casting the prepolymer over SU-8 patterns on silicon wafers provided by the Vanderbilt Institute for Integrative Biosystems Research and Education were sealed against 6-well plates with custom magnet arrangements. In Fig. 4, the microchannels created alternating lanes of collagen and A555-BSA. PBS containing 100 µg/ml collagen was pipetted into the microchannels and incubated for 2 hours at 37°C. The collagen solution was washed away by rinsing and aspirating fluid through the microchannels 3 times. After removal of the microfluidic channels, the substrate was rinsed twice with PBS. The entire surface was subsequently blocked with 3 µg/ml Alexa555-BSA in PBS for 1 hour at 37°C. Collagen bound to the surface in alternating lanes blocked most of the Alexa555-BSA binding and thus appears as dark red lanes in Fig. 4. The culture

surface was rinsed twice, filled with cell culture medium, and MATs were attached perpendicular to the direction of the lanes of collagen for cell patterning.

2.9 Nanofiber substrates

Parallel and randomly oriented poly(ϵ -caprolactone) nanofiber substrates (Fig. 5) in 24-well plates were graciously provided by Nanofiber Solutions (Columbus, OH). Wells were coated with 100 μ g/ml of collagen (rat tail type I, BD) in PBS for 2 hours at 37°C. The substrates were rinsed twice, fresh cell culture medium was added, and MATs were magnetically attached. The assembly of MATs, 24-well plate, and magnets was incubated at 37°C and 5% CO₂ for approximately 15 minutes while HEP3 GFP cells were trypsinized and counted. Cells were plated at a quantity of 250,000 cells per well (131,000 cells per cm²) and incubated for 8 hours prior to MAT removal. Images were taken immediately after removal. Plates were then incubated for 8 hours, after which the final images were taken.

2.10 Polyacrylamide substrates of varying elasticity

Polyacrylamide substrates of soft or stiff rigidity (Fig. 6) were made following the protocol of Pelham and Wang [11]. Briefly, 8% acrylamide and 0.05% bis-acrylamide (soft PAA) or 0.35% bis-acrylamide (stiff PAA) were cross-linked using 0.05% ammonium persulfate and 0.05% TEMED solution on 22 mm diameter glass coverslips. Spacers were used to create 170 μ m thick polyacrylamide gels. The polyacrylamide surface was coated with 1% gelatin for 3 minutes, then the gelatin was aspirated and dried in the vertical position for 45 minutes. After this the gelatin on the polyacrylamide was cross-linked with 0.5% glutaraldehyde in PBS for 45 minutes and washed with repeated changes of PBS over a 15-minute period. Prior to plating cells, the prepared soft and stiff substrates were incubated for 1 hour with cell culture medium. The elastic modulus of soft polyacrylamide as made above has been reported as 1 kPa and the hard polyacrylamide has been reported as 10 kPa [12] which is similar to the elastic modulus of muscle tissue [13]. In order to avoid distorting the soft substrate by compressing it significantly more than the stiff substrates, magnetic arrangements for soft substrates were assembled using 1/4" $\times$ 1/8" $\times$ 1/16" (length, width, height). The standard 1/4" $\times$ 1/8" $\times$ 1/8" magnets were used on stiff PAA substrates.

2.11 Matrix protein interfaces

Using large (8 mm $\times$ 4 mm $\times$ 5 mm) Block MATs, interfaces between matrix proteins were created (Fig. 9 a and b). A Block MAT was attached to each well of 6-well plates using the standard magnet arrangement, fiducial marks were made with the tip of stainless steel tweezers, and the wells were coated with the proteins onto which cells later migrated, specifically collagen 100 μ g/ml, poly-D-lysine 0.05 mg/ml, fibronectin 10 μ g/ml, and laminin 10 μ g/ml in PBS for 1 hour at 37°C. Wells were rinsed thrice with PBS, blocked with 0.5% BSA for 2 hours at 37°C, and rinsed twice with PBS. In each well a second Block MAT were placed adjacent to the first block MAT and the first Block MAT was carefully removed, exposing an uncoated area of t.c. plastic. Collagen (100 μ g/ml), poly-D-lysine (0.05 mg/ml), fibronectin (10 μ g/ml), or laminin (10 μ g/ml) were added to the wells thereby creating the matrix onto which the cells adhered. HEP3 cells were plated at 800,000 cells per well overnight. The medium was changed to Optimem containing 0.5% BSA 1 hour prior to removing the MATs. Cell migration was documented at hour 0, 8, and 16 through a 10x 0.25NA objective on an Axiovert 135 (Zeiss) microscope. To analyze migration across these matrix interfaces, fiducial marks were created on each end of the migration zone, individual 10x images were stitched together using the ImageJ plugin "Grid/Collection Stitching" [15], registered manually or registered automatically with the "Descriptor-based series registration" plugin [16], then cropped and quantified.

2.12 MAt migration assay

Extracellular matrix proteins (collagen 100 $\mu\text{g/ml}$ in PBS and/or fibronectin 10 $\mu\text{g/ml}$ in PBS) were coated onto glass or plastic culture surfaces for 1 hour at 37°C and 5% CO₂. After being coated, the culture dish was rinsed 3 times with PBS and securely affixed above an array of magnets (Fig. S1). Medium was added to the wells (1-2 ml for a 6-well plate), MATs were lowered into position slightly above the culture surface and released, allowing the magnetic force to pull MATs against the wet protein-coated surface and thus avoiding scraping proteins off the surface. Cells were added (800,000 - 100,000 cells per cm² depending on cell type) and allowed to adhere. After incubating for 16 hours or more, the culture dish was released from the array of magnets and MATs were subsequently removed to reveal the underlying void. Fresh cell culture medium containing 10% FBS was added. Images were taken immediately after removal of the MATs and at indicated times (generally 8, 12, 16, and/or 24 hours). For a direct comparison between MATs and scratches, the scratch and MAt assays were performed in the same well, thereby ensuring that identical culture conditions were applied to each. For figure 8 MATs were attached to half the wells of 12-well plates. Collagen was added to all wells for 1 hour at 37°C. After rinsing thrice, the wells were blocked with 0.5% BSA and for figure 8b the wells were additionally coated with human plasma fibronectin 10 $\mu\text{g/ml}$ in PBS for 1 hour at 37°C. Cells were serum-starved overnight and migration was analyzed in medium containing 10% serum.

2.13 Scratch migration assay

The culture surfaces were coated with matrix proteins as described for the MAt assay. Cells were added (800,000-100,000 cells per cm² depending on cell type). After achieving a confluent cell population, a void or “scratch” was created by dragging a plastic 200 μl pipette tip through dense cell population, according to the protocol of Liang *et al.* [14]. The culture was rinsed with PBS or medium to remove debris. Fresh cell culture medium containing 10% FBS was added. Images were taken immediately after void formation and at indicated times (generally 8, 12, 16, and/or 24 hours).

2.14 Image acquisition

The microscope systems used to capture images are as follows unless specified otherwise in the text:

The upright microscope is a BX61 (Olympus). Images were taken using a 10× 0.4NA objective via DIC contrast or fluorescence using an ORCA-ER camera (Hamamatsu) and Volocity (PerkinElmer).

The shared spinning disk confocal consists of a CSU-10 confocal unit (Yokogawa) on an Axiovert 200 (Zeiss). Images were recorded through the 63× 1.4NA oil-immersion objective with an ORCA-ER camera (Hamamatsu) using Metamorph (Molecular Devices).

The cell culture microscope is a TMS-F (Nikon). Images were taken with a 10× 0.25NA objective and recorded using a D90 SLR camera (Nikon) attached to the trinocular.

The shared inverted microscope is an Axiovert 135 (Zeiss). Images were taken via phase contrast or fluorescence with a 10× 0.25NA objective and recorded with a Retiga 200R camera (QImaging) using QCapture (QImaging)

The stereomicroscope is a Lumar V12 (Zeiss). Images were recorded through a NeoLumar 1.5× objective at 80× magnification using a Retiga Exi camera (QImaging) and Volocity (PerkinElmer) unless specified otherwise

2.15 Migration quantified by area using TScratch

For both MAt and scratch assays, we acquired data microscopically as digital images using phase contrast, differential interference contrast (DIC), and/or fluorescence. Generally, images captured the entire void and cells along both sides of the void. The void between cell monolayers was quantified as a percentage of the total image area using TScratch [17]. The average width of the void was calculated by multiplying the percent area from TScratch by the width (in μm) of the image. This is only possible when the height of the migration area is the same as the height of the image so that

$$\%area = \frac{width_{wound} * height_{wound}}{width_{image} * height_{image}} * 100$$

can be reduced and rearranged to

$$width_{wound} = \frac{\%area}{100} * width_{image}$$

By orienting the edge of the population of migrating cells vertically in the field of view, the height of the image and wound were always equal. The migration rate was subsequently calculated by dividing the difference of the wound widths in microns by the time (in hours) and as needed dividing this result by 2 to account for the migration of two cell boundaries towards each other.

2.16 Migration quantified by area using ImageJ

For time-lapse data, ImageJ macros were written to automatically prepare images and determine the open area. Briefly, the contrast was adjusted, edges were detected, and the images were thresholded to create a binary image. These binary images were dilated in order to connect any disconnected edges before running ImageJ's Analyze Particle command. Analyze Particle parameters were selected to identify the large cell population and ignore individual cells. The open areas were converted to average widths as described, negative values resulting from improper area selection were omitted, and the results are shown in Fig. 7d. **Cell boundaries** (Fig. S6b) were created using the results of this macro and then measured in ImageJ to get the width. The average **area of breakaway cells** per image (Fig. S6f) was determined by modifying the Analyze Particle's parameters to exclude the large cell population and identify individual cells. **Individual cell tracking** was done manually (Fig. 7e) using ImageJ's Manual Tracking plugin.

2.17 Statistics and graphs

Umbrella plots in Fig. 7b and GFP intensity over distance in Fig. 4b and d were created in Microsoft Excel. All other plots and statistical analyses were produced using the free, open-source statistics language R (r-project.org)[18]. Box and whisker plots were made using R's boxplot command (boxplot {graphics}). The box shows the 25th, 50th (median), and 75th percentiles, and the whiskers extend to the most extreme data point, which is no more than 1.5 times the interquartile distance. Individual data points were overlaid on the boxplots using the stripchart command (stripchart {graphics}) to visually show the number of replicates for each experiment and any outliers. P-values were determined using all replicates in an experiment with the R implementation of the Welch Two Sample t-test (t.test {stats}). Asterisks were used to demonstrate significance: * for p-values < 0.05, ** for p-values < 0.01, and *** for p-values < 0.001. Datasets comparing voids created with scratches and MAts were screened for trends caused by user error. For the data on intra-

experiment reproducibility with A549 cells (Fig. 6), the direction of scratching was found to influence the initial width of the void. This source of user-error was eliminated by restricting the dataset to scratches made by pulling toward the user rather than pushing the pipette tip away from the user. Interaction plots in Fig. 8 display means $\pm$ standard deviation.

2.18 Online supplemental material

Fig. S1 shows dimensions of Star, Microwell, and Dot MATs. Fig. S2 shows current fabrication and usage of MATs in 6-well plate format. Fig. S3 shows how submicron toolmarks on the MAT contact surface enable cell protrusions into the void. Fig. S4 shows the maintenance of FITC gelatin substrates using MAT and scratch assays with A549 cells. Fig. S5 demonstrates that MATs do not damage the matrix adhered to the surface of pliable hydrogels. Fig. S6 shows how a permissive matrix can facilitate the rapid dissemination of individual cells and a non-permissive or inappropriately organized matrix results in collective migration. Video 1 animates the steps of the standard MAT assay. Video 2 shows void-filling migration of HEP3 cells patterned with MATs on collagen-coated glass. Video 3 shows wound healing migration of HEP3 cells on collagen-coated glass that was scratched. Video 4 shows void-filling migration of HEP3 cells when MATs are attached before coating with collagen.

3. RESULTS

3.1 MAT production

Mat development was driven by the need for a migration assay in which the contribution of the underlying matrix can be defined by the investigator. The basic requirements of such an assay include 1) the deposition of a defined matrix, 2) plating cells adjacent to the defined substrate, 3) creating reproducible cell-free voids for accurate quantitation of cell migration, 4) avoiding damage to the substrate during the creation of the void, 5) adaptability to a variety of cell culture surfaces, and 6) ease of use. MAT assays are the first method to meet each of these requirements. MATs were designed to reproducibly create a void width similar to the typical void width of an *in vitro* traditional scratch assay [14]. Four different designs were created: 1) a general purpose, star-shaped MAT with four individual arms 650 μm wide, where each arm represents an individual void, 2) a microwell MAT composed of 4 small chambers separated by thin sidewalls designed to create voids of 200, 400, and 800 μm , 3) a dot MAT with an 800 μm radius created for use in high-throughput, 96-well format, and 4) a 2 mm wide block MAT that enables parallel coating of different matrixes (Fig. S1). Because of our prevalent use of the star-shaped pattern, hereafter “MAT” refers to that design unless otherwise specified.

MATs are molded in a one-step process from a mixture of magnetite and PDMS pre-polymer. A twotiered mold was needed to eliminate tool marks and achieve a crisp transition from contact surface to sidewalls (Fig. S2 and Methods). The bottom tier consists of a flat brass base polished to a flat, mirror finish. The top tier consists of an outer frame and inner cubes with tapered sidewalls positioned so that the void or empty space between cubes creates the desired star design (Fig. S2).

3.2 Patterning cells with microscale precision

Because MATs have a mirror finish on their contact surface and square edges at the transition from contact surface to sidewall, they can pattern cells with micron precision. After coating matrix protein onto a culture surface (Fig. 1b), we place the dish directly on top of an array of magnets and gently lower the MATs onto the culture surface (Fig. 1c). The magnetite-containing MATs are held to the culture surface during cell plating (Fig. 1d) and adhesion by means of the magnetic force generated by the underlying magnets. The field of the magnets

is constant, but the force they apply on the MAt is readily adjusted by adjusting the distance of the magnets from the surface, such that the force between the MAt and protein-coated surface excludes cells during adhesion and subsequent culture. Removal of the MAt creates a void in the monolayer which is imaged at the start, during, and/or after completion of the assay in order to quantify migration (Fig. 1e). Star MAts (Fig. 2a) create cell-free voids even with aggressive cancer cells, such as the human epidermoid carcinoma cell line HEp3 (Fig. 2b,c). To confirm the ability of a MAt to exclude submicron cell protrusions, confocal imaging was used to visualize the contact area between the MAt, the culture surface, and the adjacent cells. Three-dimensional reconstruction of this interface revealed complete exclusion of the GFP-expressing cells (green, Fig. 2c). In contrast, MAts generated with an unfinished surface containing submicron tool marks are unable to exclude invasive cells protrusions (Fig. S3b-d).

3.3 Protection of the underlying substrate

The ability of MAts to prevent damage to the underlying substrate and disruption of the adjacent cells was evaluated by comparing the integrity of the void in a MAt assay with the integrity of the void in a standard scratch assay [14]. For both assays the A549 cells (Fig. 3a-b and Fig. S4) or fibronectin (Fig. 3c-d) were allowed to bind to a collagen or FITC gelatin-coated surface. In the scratch assay, mechanical surface abrasion removed the cells and most, but not all, of the collagen substrate underlying the cells. This partial removal of the matrix left an irregular void (Fig. 3a, inset a') where cellular debris (arrowhead) and disrupted matrix (arrow) were clearly visible. The matrix disruption was very evident when scratching surfaces coated with both fluorescently labeled collagen and fibronectin (Fig. 3c). The disruption of the matrix was irregular across the "wound" with streaks of matrix remaining perpendicular to the void. Upon close examination the non-uniform disruption is particularly apparent in places where the fibronectin, but not the underlying collagen, were removed (insets c' and c''). In contrast, the voids created by MAts are uniform, without evidence of a disrupted matrix or dislodged cells (Fig. 3b,d). The uniformity of fibronectin patterned on the collagen surface is particularly striking (Fig. 3d and insets d', d''). Similar observations were made with collagen-coated surfaces covered with A549 cells (Fig. S4). These results demonstrate the unique ability of MAts to provide a defined void with intact matrix for migration of densely organized cells.

3.4 Intra- and inter-assay reproducibility in MAt and scratch migration assays

The intra-experimental reproducibility of MAts was determined by evaluating the average void width in 92 images from two independent experiments (Fig. 3e, f). The median initial width for repeated MAt experiments with A549 cells (Fig. 3e) was highly reproducible (MAAt experiment A: $665.5 \pm 4.7 \mu\text{m}$ and B: $668.1 \pm 12.3 \mu\text{m}$; values are given as mean $\pm$ SEM), while manual scratching was highly variable (scratch experiment A: $617.6 \pm 17.8 \mu\text{m}$ and B: $570.5 \pm 12.8 \mu\text{m}$) despite active precaution against user-induced error (see Methods).

The inter-experimental reproducibility of MAts was determined by evaluating initial void widths (Fig. 3f) in parallel experiments of A549 and murine mammary carcinoma cells (MMC). Both cell types display epithelial, pre-EMT (epithelial-to-mesenchymal transition) characteristics [19], but MMC cells exhibit greater cell-cell adhesion. This cell-cell adhesion can disrupt cells adjacent to the wound during void formation. Indeed, in scratch assays the initial void width for MMC cells was $300 \mu\text{m}$ greater than the void for A549 cells, compared to a mere $50 \mu\text{m}$ difference when using MAts (Fig. 3f). Considering the $600 \mu\text{m}$ width of the pipette tip, the action of scraping MMCs removed an additional $150 \mu\text{m}$ swath on each side of the scratched surface. This was also observed in Fig. S4b, where the area devoid of cells due to scratching was much wider than the area of disrupted matrix. In contrast, MAts created a consistent $650 \mu\text{m}$ void width with minimal ($< 25 \mu\text{m}$) removal of

cells adjacent to the stencil. Thus MATs significantly improve intra- and inter-experimental reproducibility.

3.5 Migration on micropatterned and microfabricated substrates

The structural complexity experienced by cells migrating in an *in vivo* microenvironment has been recreated *in vitro* using complex protein patterns [20-24] and complex physical topographies [25,26]. These features approximate the complexity of an *in vivo* environment, but the analysis of migration on patterned and textured substrates has been restricted to single cell migration because these delicate substrates are disrupted in scratch assays. MATs make it possible to analyze migration on patterned proteins and topographies because they are positioned with a gentle, controlled magnetic force and because the elastomeric stencil can conform to the textured surface.

3.5.1 Analysis of haptotaxis on micropatterned proteins (Fig. 4)—Parallel, 100 μm wide lanes of collagen and bovine serum albumin were printed microfluidically [27] onto a plastic culture surface using magnetically adhered channels. HEp3 cells expressing green fluorescent protein (GFP) were patterned in serum-free medium with MATs placed perpendicular to the lanes of protein (Fig. 4a). Cell culture medium was changed one hour prior to removing MATs in order to prevent HEp3 cells from detaching from the substrate. After removal of the MATs, we monitored migration into the void in the absence of serum. From the confluent populations, HEp3 cells migrated readily onto collagen-coated lanes but not onto BSA-coated lanes (Fig. 4c). Migration was quantified by tracking GFP-expressing cells in collagen vs. albumin-coated lanes. BSA-coated surfaces, that allowed for cell adhesion adjacent to the Mats, supported only limited migration when cells were given collagen as an alternate choice (Fig. 4b, d).

3.5.2 Analysis of topography orientation sensing (Fig. 5)—The response to matrix topography and its orientation was evaluated on aligned and randomly oriented poly(ϵ -caprolactone) nanofibers, a structural model that mimics the organization of white matter of the brain [26]. Using MATs, HEp3 cells were patterned on planar sheets of collagen-coated nanofibers. In order to reduce protrusions and premature migration into the void on nanofiber substrates, MATs were removed after incubating 8 hours rather than the normal 16 hours. The voids created by MATs were organized into three distinct configurations (Fig. 5a): 1) aligned: nanofibers = oriented perpendicular to the void and thus aligned with the direction of cell migration into the void, 2) counter-aligned: nanofibers oriented parallel to the void and thus orthogonal to the direction of cell migration, and 3) random: nanofibers oriented randomly. Migration was the greatest when the direction of migration was aligned with the nanofibers (61 $\mu\text{m/hr}$). This rate of migration was comparable to migration on a continuous, flat surface (not shown). Migration on random fibers was intermediate in speed (29 $\mu\text{m/hr}$) but significantly faster than in the counter-aligned configuration (15 $\mu\text{m/hr}$, Fig. 5b). These observations suggest that a permissive matrix (collagen type I) can be made non-permissive simply by organizing fibers perpendicular to the direction of migration.

3.5.3 Analysis of durotaxis (Fig. 6)—Traditional *in vitro* cell culture surfaces such as glass are extremely rigid (elastic modulus = 1-100 GPa) compared to *in vivo* tissues (0.1-10 kPa, elastic modulus) [12,13,28]. Since the migratory behavior of adherent cells is strongly influenced by matrix rigidity [29-32] pliable hydrogels are increasingly replacing rigid surfaces for the analysis of cell migration. However, the difficulty of patterning cell populations without damaging the pliable substrates limits their use in cell migration to the analysis of single cells. MATs overcome this limitation and enable evaluation of densely organized cell migration on pliable surfaces (Fig. S5). MATs were placed directly onto soft (1 kPa) vs. hard (10 kPa) polyacrylamide (PAA) gels [33]. To compensate for the different

elastic moduli and to prevent excessive compression of soft PAA, proportionally weaker magnets were placed underneath soft PAA gels (see Methods), enabling patterning of A549 cells on hydrogels of any stiffness (Fig. 6a). Interestingly, tumor cells displayed a more spindle-like morphology on pliable substrates than on rigid t.c. plastic (Fig. 6a-b). This spindle-like morphology is similar to the morphology of invasive cancer cells *in vivo* [9,20,34]. Quantitative analysis revealed that A549 cells on soft PAA exhibited significantly slower migration rates (9 $\mu\text{m/hr}$) than stiff PAA (11 $\mu\text{m/hr}$).

3.6 Rapid single cell dissemination on an intact, permissive substrate

Mechanical removal of cells during a scratch assay not only disrupts the monolayer and damages the underlying matrix but also orients the matrix orthogonal to the path of migration (Fig. 3). Orientation of the substrate can strongly impact cell migration. Consequently, we asked what specific impact matrix disruption had on cell migration. To answer this question, we used time-lapse microscopy of HEp3 tumor cells migrating in MAt and scratch assays to evaluate the impact of matrix disruption (Fig. 7). The undamaged matrix left in a void by the MAt allowed unrestricted migration on collagen-coated glass (Fig. 7a, left column and Video S2 vs. S4). Single cell tracking (colored lines) revealed individual cells migrating rapidly into the void. In contrast, migration across a scratch void was greatly diminished and occurred in two distinct phases (Fig. 7a, right column and Video S3). In the first 7.5 hours the cells migrated collectively into the void. In the 8th hour the cells began migrating parallel to the edge of the monolayer without moving further into the void (Fig. 7b). Repeating the experiment with a FITC gelatin substrate verified that the second phase was due to disruption of matrix proteins (Fig. 7c). Although void closure was not significantly different between the MAt assay and the scratch assay (Fig. 7d), single-cell tracking illustrated that the productive motility of individual cells in a MAt assay was at least twice the productive motility of individual cells in a scratch assay (Fig. 7e). This implies that although a similar number of cells can migrate into the void for both assays, damage to both cells and substrate in the scratch assay prevents effective migration of individual cells away from the leading edge. Indeed, a detailed analysis of individual “break away” cells shows that MATs enable a far greater number of cells to escape the leading edge and migrate into the void (Fig. S6f). These observations demonstrate that even in a densely organized cell population, individual cell migration is heavily dependent upon the local matrix. Moreover, the interpretation of a migration assay can change dramatically when considering this contribution of the matrix to cellular behavior.

3.7 Cell-autonomous properties and the underlying matrix

During the progression of cancer, tumor cells frequently gain phenotypic plasticity, such as through an epithelial-to-mesenchymal transition (EMT). This transition dramatically changes the cell's adhesive properties and autonomous migration behavior. HEp3 and A549 are both epithelial cells, but HEp3 cells exhibit mesenchymal behavior while A549 cells exhibit epithelial behavior. Although HEp3 cells would be expected to migrate faster than A549 cells, the divergence between the two cells lines is very matrix-dependent. The cell lines exhibit opposing behaviors in response to the availability of intact collagen matrix, and a 7-fold difference in their migration on collagen is reduced to less than 2-fold on plastic (Fig. 8a).

Similar to global phenotypic changes, the loss of a singular molecular component (specifically the collagen receptor $\alpha 2\beta 1$) can also have strong impacts on migration. MMCs that lack the $\alpha 2$ integrin subunit (MMC $\alpha 2^{-/-}$) exhibited diminished adhesion to collagen but continued to adhere and migrate normally on fibronectin-containing matrixes [9]. Interestingly, cells lacking integrin $\alpha 2$ migrated faster on t.c. plastic than on the fibronectin and collagen matrix. Conversely, wildtype cells expressing endogenous $\alpha 2$ migrated slower

on t.c. plastic than on the fibronectin and collagen matrix. While a migration deficiency on collagen-containing matrixes is not unexpected, the inverse disparity between wildtype and $\alpha 2$ -null cells (*i.e.*, $\alpha 2$ -null cells migrated faster on t.c. plastic than wildtype cells) suggests that $\alpha 2$ can contribute to migration in a cell-autonomous, ligand-independent manner. This is consistent with our previous *in vivo* studies demonstrating that $\alpha 2$ is a metastasis suppressor [9]. These results demonstrate that the availability of a specific substrate can, in fact, strongly impact the interpretation of cell-autonomous migratory behavior.

3.8 Matrix switching

Matrix switching during migration affects motility disproportionately. When a cell migrates away from its point of origin, it enters a new microenvironment and is exposed to new extracellular adhesive options. The nature of this new matrix can determine the rate of migration and the type of motility (*i.e.*, collective vs. single cell motility, Fig. 7, 8, and S6). However, independent of the permissive/non-permissive nature of each individual matrix, the act of switching from one matrix to another may affect migration. To address this possibility we created two adjacent areas coated with different matrix proteins (collagen, fibronectin, or laminin) or poly-D-lysine (PDL) using block MATs and subsequently analyzed the migration of HEp3 cells from one area to the other in a 16 hour period (Fig. 9a-c). The consequences of matrix switching were revealed by comparing it with migration on uniform collagen, PDL, and laminin (Fig. 9a).

As might be anticipated, when two adjacent areas were coated with the same matrix proteins (laminin, fibronectin, or collagen) there was no reduction in migration when compared to migration across a uniform coating of matrix protein. However, this was not true when two dissimilar coatings were coated adjacent one another.

When evaluating cells migrating to or from a PDL-coated area, we found that the cells moved at a rate similar to that seen on uniform PDL (approximately $6\mu\text{m/hr}$, Fig. 9b) which is much less than the rate of migration on collagen ($26\mu\text{m/hr}$). It appears that the limited HEp3 cell spreading on PDL (Fig. 9c, second column) and low motility on PDL limits the ability of these cells not only when they move onto the PDL surface but also when they must leave the PDL to enter the collagen-coated surface..

In contrast to PDL, fibronectin and laminin supported rapid migration onto adjacent collagen coated areas. However, when migrating cells switched from collagen to fibronectin or laminin there was an unexpected reduction in the rate of migration. The reduced rate of migration was surprising because it was even less than the rate of migration supported by a uniform matrix of fibronectin ($23.0\mu\text{m/hr}$) or laminin ($13.4\mu\text{m/hr}$, Fig. 9b) which was already significantly less than the migration of collagen ($26\mu\text{m/hr}$). The reduced migration indicates that switching from collagen to fibronectin or laminin adversely affected cell migration. Conversely the same is not true for cells switching from fibronectin or laminin to collagen.

4. DISCUSSION

4.1 MATs and related migration assays

The creation and design of MATs was driven by the need to define the contribution of the underlying substrate to cell migration. The mechanical damage created by a traditional *in vitro* wound healing assays prevents this model from evaluating substrate contributions. More recent assays which exclude cells by various means such as stencils [35-38], stoppers [39,40], barricade gels [41], microfluidic barriers [42,43], and ligand switchable substrates [8] can interrogate the contribution of a substrata but have significant limitations. Stencils and stoppers that auto-adhere to the plastic or glass culture surface cannot be applied to wet

or protein-coated surfaces. Barriers that are manually forced onto the culture surface are difficult to apply without damaging an underlying matrix while barrier gels place significant restrictions on the substrate and culture conditions used. Advanced methods utilizing microfluidic barriers, and ligand switchable substrates are promising but technically challenging and are difficult to integrate into a broader empirical investigation.

By using fixed magnets to orient and attach MATs to a culture surface, an exclusion zone (void) is created in a cellular monolayer. Similar to a scratch assay, documenting the movement of cells into this void provides a means to quantify the rate of migration. However, MATs are unique because they protect the underlying surface in the void and thereby allow full control over the composition of the matrix onto which the cells migrate. This application is not limited to classic analysis of protein-coated plastic and glass surfaces but is readily extended to protein micropatterns, nanotextured surfaces, and pliable substrata such as hydrogels. In the past, migration on such substrates was limited to single cells or non-patterned colonies and droplets of cells [6,7]. Consequently, MAT assays are not merely an improvement over scratch assays, they provide a unique, versatile method to enable investigations into the contribution of the substrata to cell migration.

4.2 Effects of matrix context

In several instances, providing a new substrate resulted in unexpected changes in migration. Switching from one matrix to another during migration (Fig. 9) caused a disproportionate reduction in motility. Such matrix-dependent phenotypes have been reported for primary cells during long-term culture [44] but have not been observed in established cell lines across such small temporal and spatial dimensions. Our observations suggest that at microscopic dimensions a change in the available matrix can influence migration in a dynamic manner that requires accounting for more than just the molecular identity and the supportive nature of the matrix. We expect further analysis of the effect of matrix switching on migration to reveal an unappreciated form of biological hysteresis [45-48].

Implementation of MAT assays using collagen absorbed to a variety of substrate demonstrated that a single matrix protein (collagen) can mediate very different rates of migration depending on the context of its presentation to the cell. When provided as a choice adjacent to a non-permissive matrix such as BSA (Fig. 4), collagen and promote rapid cell migration ($50 \mu\text{m/hr}$). Similar high rates of migration were achieved by physically aligning nanofibers (coated with collagen) with the direction of migration into the void ("Aligned" Fig. 5). In contrast, orienting the nanofiber surface, and thereby the matrix, parallel to the void causes cells to migrate along, rather than into the void ("Counter-aligned" Fig. 5). These experiments show that the rate of productive motility depends upon the organization of the extracellular matrix as well as its molecular composition.

4.3 MATs and migratory companion cells

Migration on a non-permissive matrix or adversely organized substrate can be facilitated by companion cells. For this reason (and others) the role of tumor-associated cells is under intense investigation. While it is generally accepted that fibroblast and immune cells can modify the tumor-associated matrix, MATs enables the analysis of this modification and its contribution to migration *in vitro*. As proof of this concept, HEP3 cells were sparsely interspersed within a monolayer of A549 cells and allowed to migrate collectively with the A549 cells into a void containing a BSA-coated surface ("Non-permissive" Fig. S6) or a collagen-coated surface ("Permissive"). Although BSA generally limits the migration of HEP3 cells these cells migrated across the BSA-coated surface by moving collectively with the A549 cells. Conversely, when presented with a collagen-coated surface, numerous HEP3 cells disseminated into the void as isolated individual cells free of adjacent A549 cells.

Thus, while A549 cells migrate much slower than the aggressive HEP3 cells, they can enable HEP3 cells to enter otherwise non-permissive locations. These observations demonstrate that it is possible to investigate complex interactions within cell populations and its contribution to the cell migration. Similar future studies can illuminate the role of such cell-cell interactions in development, normal physiology, and disease.

5. CONCLUSION

MATs provide experimental accuracy and versatility. The use of magnetic force to attach a stencil onto a substrate represents a new strategy in the arena of space-filling assays. This strategy works well on wet and dry surfaces, on simple and complex protein coatings, on rigid and pliable substrates, and on microfabricated textures. The strategy is applicable to standard culture wares, high-throughput plates, and microculture models by simply using different shapes of MATs (Fig. S2). Such versatility is crucial in the early stages of discovery when experimental conditions and parameters are routinely refined and must be controlled fully by the investigator.

MATs open up exciting new approaches to analyzing the migration of densely organized cells. These studies can range from the analysis of migration on defined, intact substrates to the evaluation on complex engineered substrata such as protein-coated hydrogels. Using MATs we identified specific migratory responses contributed by matrix modifications including inadvertent damage and specific matrix orientation, and by matrix switching. MATs can be customized to meet a wide variety of applications (Fig. S1). MATs readily incorporate microfluidic designs and microfabrication techniques, creating opportunities for protein patterns and interfaces that enable innovative investigations of cellular behavior. Unique problems that can be addressed with MATs include: a) determining the contribution of complex protein patterns and protein complexes to migration, b) evaluating the migration of densely organized cells on pliable substrata with defined matrix adhesions, c) determining the contribution of tumor-associated cells to migration onto substrate not permissive to tumor cells, d) deciphering how cells choose among the adhesive substrata available during migration, and e) evaluating the cryptic epitopes in the extracellular matrix. Most important, MATs allow the experimenter to define the composition of the substrate onto which the cells migrate. This control makes it possible to quantitatively determine the contribution that a defined matrix makes to cell migration in the context of complex microenvironment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

A555	Alexa fluor 555
BSA	bovine serum albumin
CN	collagen

CNC	computer numeric control
DIC	differential interference contrast
EGF	epidermal growth factor
EMT	epithelial-mesenchymal transition
FN	fibronectin
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
HNSCC	head and neck squamous cell carcinoma
LN	laminin
MATs	magnetically attachable stencils
MMC	murine mammary carcinoma
Pa	Pascals
PAA	polyacrylamide
PDL	poly-D-lysine
PDMS	polydimethylsiloxane
T	Tesla
t.c.	tissue culture
TEMED	N,N'-methylene bisacrylamide N,N,N,N-Tetramethylene-diamine

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Figure 1. Role of matrix in cell migration and its protection using MATs
(a) A schematic depiction of the elements that contribute to cell migration: Cell migration in tissues is determined by the integration of cell autonomous ability, soluble communication, cell-cell adhesion and matrix interaction. The presentation of a new or modified substrate and the acquisition of new matrix adhesive properties (lower right) have a powerful influence on cell migration. (b-e) Schematic depiction of the MAT assay: (b) The culture surface, such as tissue culture plastic or glass, is coated with relevant protein. (c) A magnet is placed underneath the culture surface and a MAT is subsequently attached. The magnet not only secures the MAT to the surface, it also provides sufficient force to seal the stencil on wet, protein-coated surfaces without disrupting the underlying substrate. (d) Cells are plated around the MAT. (e) The MAT is removed, thereby creating a void adjacent to the cells, and cell migration into the void is analyzed by comparing pictures between initial and final time-points. (f) Comparison of the MAT assay with scratch and single cell migration assays in their ability to address the four elements that contribute to migration.

Figure 2. MATs prevent cell protrusions into the void

(a) A Star MAT, inverted to show the surface that is pressed against the substrate, and (b) corresponding void created in a monolayer of GFP-expressing cancer cells (HEp3) on t.c. plastic. Scale bars = 1 mm. Image acquired on a Lumar stereomicroscope using the 0.8x objective at 12.5x total magnification. (c) GFP-expressing HEp3 cells (green) were plated on collagen-coated glass coverslips adjacent to a MAT labeled with Alexa-549. Confocal imaging was used to visualize the interaction between the MAT, the culture surface, and the adjacent cell monolayer. With the MAT in place, a z-stack of images was captured with the shared spinning disk confocal (see Methods) and reconstructed in 3D. Scale bar, 50 μ m.

Figure 3. MATs prevent damaging the matrix during void formation and improve its reproducibility

A scratch was used to mechanically create a void in a monolayer of A549 cells (a) or a matrix composed of fibronectin to collagen adsorbed to a glass surface (c + insets). Similarly a MAT was used to create a void in a monolayer of A549 cells (b) or a fibronectin matrix (red) bound to a layer collagen (green) after placement of the MAT (d + insets). Magnified inset a' reveals the cell debris (arrowhead), disrupted matrix (arrow), and irregularities along the cell front (asterisk) that are created by the mechanical removal of the cells in the scratch assay but which are absent in a void created by the MAT (b'). The ability of a MAT to protect the underlying matrix while providing a high-precision seal is shown through the detection of a fibronectin-free void when fibronectin (red) is adhered to the collagen after placing the MAT (d). (e) The intra-experimental variability of void formation for MAT and scratch assays was quantified for independent repeat experiments (A549 A vs. A549 B) with 12 void measurements each. (f) The inter-experimental variability of void formation for MAT and scratch assays was quantified using a parallel analysis of A549 and mouse mammary epithelial cells (MMC) with 45 void measurements each. For boxplot details and microscopy, see Methods. Scale bars, 50 μm .

Figure 4. Micropatterned substrates reveal preferential cell migration

MATs were adhered perpendicular to 100 μm -wide alternating lanes of BSA (bright red) and collagen (weakly red) micropatterned onto the t.c. surface using a magnetically adhered microfluidic channel network. HEp3 cells (green) were cultured adjacent to the MATs and their migration across the lanes was monitored at 0 and 8 hours after removal of the MATs (a and b, respectively). Motility was documented as the average cell intensity plotted over distance for both collagen-coated lanes (solid line) and BSA-coated lanes (dashed line) with the crisp cell boundary created by the MATs indicated with a blue line (c and d). Despite the cells' ability to adhere to BSA-coated t.c. plastic, no cells were observed in these lanes, showing the strong preference of HEp3 cells to migrate on collagen-coated t.c. plastic. Scale bars, 50 μm .

Figure 5. Oriented nanofibers increase or decrease migration

(a) HEp3 GFP cells (green) were patterned onto collagen-coated nanofibers using MATs. Nanofibers were either oriented perpendicular to the void and with the direction of migration (Aligned), oriented parallel to the void (Counter-aligned), or randomly distributed (Random). Migration into the void was imaged at 0 and 8 hours. (b) Migration rates were determined from the rate of void closure. For boxplot details, see Methods. Scale bars, 50 μm .

Figure 6. Substrate elasticity alters morphology and migration rate

Using MATs, the migration of A549 cells on FITC gelatin bound to polyacrylamide gels of low and high elastic modulus (a, 1kPa and 10kPa) was compared with migration on t.c. plastic (b, 2-4 GPa). (c) Migration rates were determined based on void closure (n = 7). Images for (a) were acquired via differential interference contrast on the BX61 microscope and via phase contrast for (b) on the cell culture microscope. For boxplot and microscope details, see Methods. Scale bars, 50 μm .

Figure 7. Consequences of matrix disruption for cell migration

(a) HEp3 cells were plated on collagen-coated glass coverslips, serum starved overnight, and imaged in serum free medium every 5 minutes for 16 hours as they migrated into voids created with a MAT or a scratch. (b) Umbrella plots show the trajectories of individually tracked HEp3 cells. (c) Substrate damage responsible for the inhibition of productive cell migration (upper panel) was visualized using FITC gelatin coated glass (lower panel) after 8 hours of migration. To improve contrast 150 $\mu\text{g/ml}$ of FITC-gelatin was used, and cells were imaged in serum-free medium. (d) Average migration rates for HEp3 cells patterned with MATs or scratches on collagen-coated glass were equal using standard area-based analysis. (e) Migration rates as determined by individual cell tracking. Images were acquired via

differential interference contrast and fluorescence microscopy using the upright microscope (see Methods). Frames were recorded every 5 minutes for 19 hours. For boxplot details, see Methods. Scale bars, 50 μm .

Figure 8. The contribution of cell-autonomous mechanisms to migration is strongly influenced by the underlying matrix

A549 and HEP3 cells display pre- and post- EMT migratory behavior, respectively. Using MATs, the migration of these cells on t.c. plastic was contrasted with their migration on collagen-coated plastic (n = 18). (b) The migration of murine mammary carcinoma cells from wildtype (MMC wt) and integrin $\alpha 2$ knockout mice (MMC $\alpha 2^{-/-}$) was similarly evaluated on a matrix of collagen + fibronectin vs. t.c. plastic (n = 10). Values of n are individual data points. Lines connect mean values. Error bars = SD.

Figure 9. Matrix switching during migration affects motility disproportionately to the nature of the matrix

(a) Using Star MATs HEP3 cell migration was analyzed on uniform substrates of collagen (CN), fibronectin (FN), and laminin (LN). The migration rates are displayed in (b) as from CN onto CN, from FN onto FN, and so on. (c) Using Block MATs, two adjacent matrixes were created as described in Methods. Cells were seeded onto one matrix, cultured to confluence, and subsequently allowed to migrate onto the adjacent matrix. Images taken at 0, 8, and 16 hours document migration and the morphological changes visible when HEP3 cells switch from CN onto adjacent poly-D-lysine (PDL), FN, and LN, or vice versa. (b) The quantitation of migration rates across these interfaces revealed significant matrix-specific effects and unexpected migration deficiencies that could only be contributed to matrix switching and not the matrix itself (CN vs. FN). Images were taken on the Axiovert 135 microscope. Microscope and boxplot details are described in Methods. Scale bars, 50 μm .