Temporal Characterization of Neuronal Migration Behavior on Chemically Patterned Neuronal Circuits in a Defined *in Vitro* Environment

Anupama Natarajan,[†,‡,⊥] Alec S. T. Smith,[†,⊥] Bonnie Berry,[†,‡] Stephen Lambert,[§] Peter Molnar,[§,‖] and James J. Hickman*,[†]

[†]NanoScience Technology Center, University of Central Florida, 12424 Research Parkway, Suite 400, Orlando, Florida 32826, United States

[‡]Burnett School of Biomedical Sciences, University of Central Florida, 6900 Lake Nona Boulevard, Orlando, Florida 32827, United States

[§]College of Medicine, University of Central Florida, 6900 Lake Nona Boulevard, Suite 101, Orlando, Florida 32827, United States

[‖]Department of Zoology, Institute of Biology, Savaria Campus, University of West Hungary, H-9700 Szombathely, Hungary

Ⓢ *Supporting Information*

ABSTRACT: Directed control of neuronal migration, facilitating the correct spatial positioning of neurons, is crucial to the development of a functional nervous system. An understanding of neuronal migration and positioning on patterned surfaces *in vitro* would also be beneficial for investigators seeking to design culture platforms capable of mimicking the complex functional architectures of neuronal tissues for drug development as well as basic biomedical research applications. This study used coplanar self-assembled monolayer patterns of cytophilic, *N*-1[3-(trimethoxysilyl)propyl] diethylenetriamine (DETA) and cytophobic, tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (13F) to assess the migratory behavior and physiological characteristics of cultured neurons. Analysis of time-lapse microscopy data revealed a dynamic procedure underlying the controlled migration of neurons, in response to extrinsic geometric and chemical cues, to promote the formation of distinct two-neuron circuits. Immunocytochemical characterization of the neurons highlights the organization of actin filaments (phalloidin) and microtubules (*β*-tubulin) at each migration stage. These data have applications in the development of precise artificial neuronal networks and provide a platform for investigating neuronal migration as well as neurite identification in differentiating cultured neurons. Importantly, the cytoskeletal arrangement of these cells identifies a specific mode of neuronal migration on these *in vitro* surfaces characterized by a single process determining the direction of cell migration and mimicking somal translocation behavior *in vivo*. Such information provides valuable additional insight into the mechanisms controlling neuronal development and maturation *in vitro* and validates the biochemical mechanisms underlying this behavior as representative of neuronal positioning phenomena *in vivo*.

KEYWORDS: neurons, chemical patterns, in vitro, migration
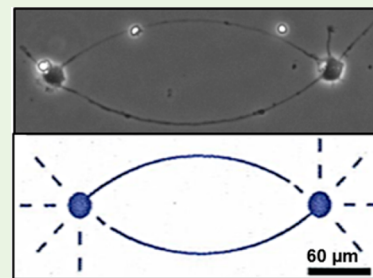
■ INTRODUCTION

The central nervous system is a highly organized network of interconnected neurons with polarized neuritic distribution, which form the basis for unidirectional signal transmission. Controlled neurite outgrowth and neuronal migration therefore play important roles in the developing brain as well as in the repair and remodeling of adult neuronal tissues.[1] The *in vivo* mammalian cortex is a complex network of neurons and supporting cell types composed of six layers. Correct development of this laminated structure is reliant on the directed migration of postmitotic cortical neurons in waves from the margin of the embryonic cerebral ventricles toward the developing neocortex.[2,3] Migrating neurons move to the cortical plate through migration pathways which are either radial or tangential to the ventricle.[3,4] In rats, controlled neuronal migration begins around E12 and peaks at E18 in the CA1 hippocampal region and neocortex.[5] Failure of neurons to migrate to their correct locations in tissues such as the hippocampus and cortex can lead to severe brain defects[6] and may interfere with essential physiological events such as long-term potentiation and memory formation.[7]

Radial migration is the dominant neuronal migration pathway *in vivo*,[4,8] and is facilitated by two complementary motility mechanisms: glial-guided migration and somal translocation. Glial-guided migration requires the close association of a migrating neuron with one or more supporting radial glia via the extension of pseudopodia-like structures from the neuronal membrane.[9,10] Somal translocation is characterized

by a leading process which extends from the cell's soma and attaches to a target site (typically the pial surface *in vivo*).[11,12] Interaction of microtubules extending from the microtubule organizing center (MTOC) with the motor protein dynein then facilitates the transport of the nucleus toward the target site independent of radial glia presence.[13]

Directed neuronal migration and positioning is regulated by temporally and spatially modified extracellular cues[14] such as changes to the extracellular matrix composition,[15−18] local cell adhesion molecules and related cell surface proteins,[19−21] soluble and membrane-bound factors and their receptors,[22,23] and neurotransmitters and ion channels.[24,25] A complex interplay of these intra and extracellular cues regulate neuronal migration mechanisms by activating cytoskeletal reorganization in order to modulate cell motility and neuritic development.[26−28] Since current *in vitro* systems lack the complexity of living tissues, many of the physical and chemical cues used to control and direct neuronal migration *in vivo* are either missing or significantly altered in culture. It therefore remains unclear which extrinsic cues are necessary and sufficient to control the migration of isolated neurons *in vitro*.

In order to effectively model complex cortical tissues *in vitro*, it is therefore necessary to develop culture platforms capable of controlling the spatial arrangement of neurons, the direction of neuritic outgrowth and the synaptic connections formed between different cell populations. Application of self-assembled monolayers (SAMs) with differing cell adhesion properties provides a simple, repeatable, and low cost method of regulating these variables on standard glass coverslips as well as within more complex body-on-a-chip platforms.[29,30]

Chemically patterned substrates have long been used to assess the ability for geometric cues to influence neuronal outgrowth[31,32] and network formation[33,34] in culture. Pioneering early work on the development of neuronal polarity *in vitro* demonstrated that neuritic identity is not predetermined, and the first neurite to develop axonal characteristics typically becomes the axon, while the others form dendrites.[35,36] Application of patterned chemical surfaces can therefore be used to induce the differentiation of neurites into axons and dendritic trees, by controlling the formation of axonal characteristics in a single neurite, and therefore confer a controlled polarized neuronal morphology on to cultured cells similar to that observed *in vivo*.[37,38] However, analysis of temporal changes in neuronal morphology during *in vitro* neuronal migration is less well studied. This phenomenon represents a key stage in the formation of high-resolution artificial neuronal networks. Determination as to whether this process is intrinsically or extrinsically controlled as well as the physiological changes underlying its occurrence *in vitro* is therefore of considerable interest to investigators seeking to emulate complex neuronal tissues in controlled laboratory environments.

This study focuses on the migratory abilities of individual primary rat hippocampal neurons cultured on chemically patterned substrates. We chose to study neural migration on a two-cell circuit pattern made using a combination of DETA, an analogue of spermine[39−41] shown to promote the survival and maturation of primary neurons in long-term culture,[42−47] as a cytophilic adhesion surface and 13F, a cytophobic surface shown to prevent the adhesion of cells in culture,[48] with which to make two-cell circuit patterns. The patterned combination of these two chemicals creates cell-adhesive and cell-repellant regions for the directed migration of cells. Cultured neurons were found to migrate toward designated adhesion sites via a somal translocation-like mechanism, mimicking the radial migration phenomenon observed *in vivo*.[11] This is the first report of a somal translocation mechanism *in vitro*. Migration of the neurons was found to proceed through stages characterized by distinct cytoskeletal morphology and dynamic behavior. Immunocytochemical characterization of actin filaments and microtubules at each migration stage revealed defined intracellular cytoskeletal organization during *in vitro* somal translocation. It was also observed that cultured hippocampal neurons were restricted in the distance they could effectively migrate across chemically patterned hydrophobic surfaces, which could have important implications in cultured neuronal network applications. The acquired data provide an in depth understanding of the physiological development and migration limitations of neurons on defined patterned chemical substrates. This information will aid in the development of more biologically relevant and functionally competent artificial neuronal networks for disease modeling and drug development applications as well as increase our understanding of fundamental processes during neuronal migration.

■ MATERIALS AND METHODS

Surface Cleaning. Glass coverslips were cleaned using an acid wash protocol described previously.[48] Briefly, ceramic racks containing glass coverslips were soaked in a solution of 50/50 methanol (HPLC grade)/hydrochloric acid (reagent grade) for 30 min. They were then rinsed with deionized ultrafiltered (DIUF) water three times and placed in a beaker containing concentrated reagent grade sulfuric acid (Fisher Scientific, Pittsburgh, PA) overnight. The racks containing the coverslips were rinsed thoroughly and boiled in DIUF water for a further 30 min. The racks and coverslips were then rinsed in two solutions of acetone and oven-dried at 110 °C for 10−15 min or until dry. The contact angle of each surface was confirmed to be less than 5° before undergoing surface modification.

Surface Modification and Photolithographic Patterning of Acid Washed Coverslips. The method used to chemically modify culture surfaces was based on previously published protocols.[49,50] Briefly, ceramic racks containing the acid washed glass coverslips were immersed in 0.1% (v/v) DETA (*N*-1[3-(trimethoxysilyl)propyl] diethylenetriamine) in HPLC-graded toluene solvent and heated to just below the boiling temperature for 30 min before being allowed to cool to room temperature. HPLC-graded toluene was then used to rinse the coverslips and they were then reheated as before for 30 min. Following DETA treatment, the coverslips were oven-dried overnight.

After DETA treatment, surfaces were ablated by exposure to a 193 nm Ar/F excimer LPX2001 laser (Lambda Physik, Ft. Lauderdale, FL) combined with a beam homogenizer (Microlas, Ft. Lauderdale, FL) for 30 s. Patterns were made on the DETA surface photolithographically by exposing the DETA monolayer to Ar/F laser irradiation through a quartz photomask possessing the desired pattern. The ablated region was then backfilled by exposing the surface to 0.1% (v/v) tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (13F) in a chloroform solution. Racks containing ablated coverslips were immersed in the 13F solution for 5 min, rinsed in chloroform, and oven-dried for 2 h.

Metallization and XPS Analysis. Characterization of prepared surfaces was achieved using electroless copper metallization.[51] The patterned and backfilled glass coverslips were immersed in a $PdCl_4^{-2}$ solution for 10 min. The coverslips were rinsed with DIUF water and immersed in a solution of dimethylamine borane (DMAB) for 10 min. The substrates were rinsed in DIUF water and immediately immersed in a copper solution containing formaldehyde for 3 min. These reactions allowed the copper to bind to the amine groups of the DETA, and the pattern could then be visualized under a Polyvar-MET metallurgical microscope (Reichert Jung, Buffalo, NY).
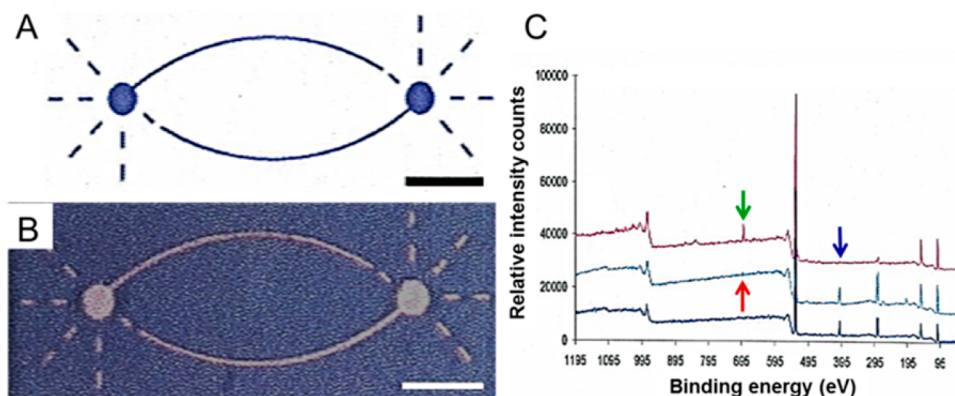
Figure 1. Neuronal circuit geometry and characterization of chemically patterned surfaces. (A) Schematic representation of the two-neuron circuit design utilized in this study. Each solid dot has a diameter of 20 μm and the distance between the two dots is 200 μm. Each dashed line contains two 10 μm bars separated by a 10 μm gap. The two continuous lines promoting axon development have a maximum separation distance of 75 μm, and each line is 2 μm in width. Scale bar = 50 μm. (B) Metallization of the DETA/13F pattern described in part A. Amine groups from the deposited DETA were labeled with a Pd−Cl catalyst and metalized with copper in order to visualize successful pattern formation. Scale bar = 50 μm. (C) Representative XPS survey traces for a DETA control surface (bottom), a 13F on DETA surface (middle) and a 13F on ablated DETA surface (top). The scan of the 13F on DETA surface indicates a negligible fluorine presence (red arrow), suggesting very little incorporation of 13F into the DETA monolayer. The scan of the 13F on ablated DETA surface indicates very little nitrogen presence (blue arrow), but a substantial fluorine presence (green arrow), indicating that the DETA monolayer has been successfully ablated and replaced with the backfilled 13F.

Surface analysis of chemically modified coverslips was completed using X-ray photoelectron spectroscopy (XPS) and contact angle measurements. Three spots on a coverslip were analyzed using XPS. The XPS scans were made using a Kratos AXIS165 system with an AlKα incident photon beam and were operated at a takeoff angle of 90°. For each sample, survey scans and high-resolution scans for the individual peaks of fluorine (1s), oxygen (1s), carbon (1s), silicon (2p), and nitrogen (1s) were performed. Using a standard curve fitting technique for the high-resolution peaks, the individual elemental compositions, the relative values of fluorine with respect to the rest of the elements, the ratio of nitrogen to silicon were calculated.

The surface energy of the different monolayers was assessed using contact angle measurements. Measurements were made using a CAM 200 digital goniometer (KSV Instruments, Ltd., Linthicum Heights, MD). A static drop (5 μL) of deionized water was applied to the sample surface at three different points and an average of the readings then calculated.

Optimized element ratios from XPS analysis and contact angle measurements for promoting maximal levels of cell adhesion and neuritic development are defined in the results section. Surfaces that produced results outside of these ranges were discarded and not used in culture.

Cell Culture. Primary hippocampal neurons were obtained from day 18 (E18) Sprague−Dawley rat fetuses and prepared as previously described.[49,52] Time-pregnant rats were euthanized by exposure to an excess of CO_2; this procedure complied with standards laid out by the Institutional Animal Care and Use Committee of The University of Central Florida and conformed to NIH guidelines. Fetuses were removed from the mother and transferred to an ice-cold dissection medium consisting of Hibernate E (Brain Bits LLC, Springfield, IL) supplemented with B27 (2% v/v; Invitrogen, Grand Island, NY), glutamax (1% v/v; Invitrogen), and antibiotic/antimycotic (1% v/v; Invitrogen). The hippocampi were dissected out and transferred to a digestive enzyme bath consisting of 2 mg/mL Papain (Worthington, Lakewood, NJ) dissolved in Hibernate E. The enzyme bath was incubated in a shaking water bath at 37 °C and 100 rpm for 12 min before being spun down and the tissue resuspended in fresh Hibernate E. The tissue was mechanically dissociated using a fire polished glass Pasteur pipet and the cell suspension again spun down and resuspended in a serum-free feeding medium consisting of neurobasal medium supplemented with B27 (2% v/v; Invitrogen), glutamax (1% v/v; Invitrogen), and antibiotic/antimycotic (1% v/v;

Invitrogen). The cells were then counted and plated onto sterilized surfaces at a density of 75 cells/mm^2. We have determined this plating density to be optimal in acquiring the highest number of 2-cell networks; fewer than 75 cells/mm^2 will result in too few cells migrating onto the patterns whereas more than 75 cells/mm^2 will result in the pattern being overwhelmed by too many cells or too much debris.

Time-Lapse Recordings. Time-lapse recordings were started immediately after the cells were plated. Cells were observed under a Zeiss-Axiovert 100 inverted microscope equipped with a Plan-Neofluar 10× objective lens (Zeiss, Oberkochen, Germany). A humidified incubation chamber was used throughout data recording in order to maintain a constant 37 °C and 5% CO_2 culture environment. Time-lapse images were captured with a Hamamatsu C8484-05G digital charge-coupled device camera (Hamamatsu Photonics, Shizuoka, Japan). Time-lapse experiments were performed under the control of Okolab software (OKO-lab, Ottaviano, Italy). Live cell image sequences were exported and processed using NIH ImageJ software to create videos.

Immunocytochemistry. Neuronal cultures to be assessed immunocytochemically were first washed with phosphate buffered saline (PBS) and the neurons then fixed and permeabolized by treatment with 0.25% gluteraldehyde in a 1×BRB80 buffered solution (80 mM PIPES buffer, pH 6.85, plus 2 mM $MgCl_2$ and 2 mM EGTA) for 30 s, followed by a second treatment with 0.25% (v/v) glutaraldehyde plus 0.1% (v/v) Triton X-100 in a 1×BRB80 buffer solution for 15 min. Aldehyde autofluorescence was reduced by addition of 0.2% (v/v) sodium borohydride in PBS for 30 min. The neurons were washed twice with PBS and blocked with a blocking solution containing 2% (v/v) bovine serum albumin plus 0.1% (v/v) Triton X-100 in PBS for 10 min.

Neurons were incubated for 30 min with a mouse monoclonal antibody against β-tubulin (T0198; Sigma-Aldrich, St. Louis, MO) diluted in blocking buffer for 30 min. Cells were washed 3 times with PBS and incubated for 20 min with a secondary antibody solution consisting of a chicken antimouse, affinity purified secondary antibody (A21201; Invitrogen) diluted in blocking buffer. Finally, cells were incubated in a PBS solution containing rhodamine conjugated Phalloidin (R415; Invitrogen) and Hoechst dye (H1339; Invitrogen), to label actin and nuclei respectively, before being mounted onto glass microscope slides for imaging. Immunolabeled cells were imaged using a Zeiss Axioscope confocal microscope coupled to UltraVIEW LCI software (PerkinElmer, Waltham, MA).

Statistical Analysis. Data from successful translocation events (n = 20) was collected from three independent cultures plated on different days using fetuses from different litters. Immunocytochemical investigation of migrating cells was performed in triplicate ($n = 3$) for each migration stage described. Significant difference in average neurite length between neurons plated on 13F incorporated onto ablated DETA and those plated on 13F deposited onto clean glass was calculated using a two-tailed Mann−Whitney test with a Gaussian approximation of the p-value conducted using GraphPad Prism software.

■ RESULTS

Neuronal Circuit Pattern Geometry Design. A two neuron circuit design (Figure 1A) was selected for use in this study as a means to reduce cell−cell interaction and thereby provide the best chance of studying individual cell behavior in response to geometric cues without confounding influences from surrounding neurons and glia. The two-neuron circuit design was optimized previously as a means to study synaptic connectivity in vitro[53] and was adapted from earlier work on neuronal patterning[54] and the use of geometric cues to establish neuronal polarity in vitro.[38] Dashed lines were used to induce dendritic tree development and comprised two 10 μm strips, each 2 μm wide, separated by a 10 μm gap.[38] The somal adhesion site constituted a solid dot with a 20 μm diameter. The distance between the two somal adhesion sites was 200 μm. The continuous lines connecting the 2 somal adhesion sites were designed to encourage the development of a single process from each neuron toward the soma of the other neuron in the circuit. These axon pathways were 2 μm wide and separated by a maximum distance of 75 μm. The lines connecting the 2 somal adhesion sites were designed to form an arc shape to eliminate any angular geometric influence on the development of the neuritic arbor from the cultured neurons.

Patterned Substrate Surface Chemistry and Analysis. Coplanar self-assembled monolayer (SAM) patterns were used to generate the described neuronal circuit design on glass coverslips. The aminosilane DETA (trimethoxysilylpropyldiethylenetriamine) was used as the foreground patterning molecule since it has previously been shown to promote neuronal adhesion and outgrowth.[49,50,55] A hydrophobic fluorinated silane, 13F (tridecafluoro-1,1,2,2-tetrahydroctyl-1-dimethylchlorsilane) was then used as the background repellent molecule to minimize neuronal adhesion outside the desired pattern.[46,48,54,56] DETA surfaces were patterned by ablating the monolayer using deep UV lithography through a quartz photo mask possessing the two-neuron circuit geometric features described above. The DETA-patterned glass was then backfilled with 13F to form DETA-13F coplanar monolayer patterns.

Correct deposition of chemical patterns on glass surfaces was verified using an electroless copper deposition method.[51] The amine groups of the DETA were labeled a with Pd−Cl catalyst and then metalized with copper. Labeled surfaces were visualized using a metallurgical microscope to see whether the copper had deposited correctly onto the DETA pattern and not the surrounding 13F surface (Figure 1B).

The fabricated surfaces were also characterized by X-ray photoelectron spectroscopy (XPS) and contact angle measurements to assess suitability for cell culture (Figure 1C). Since the surface area of the pattern was too small to effectively analyze by XPS, control coverslips were produced for each type of surface preparation present within the coplanar monolayer

pattern: 13F reacted with unpatterned DETA, 13F on ablated DETA, and unaltered DETA surfaces. Each control surface was assessed using XPS, and from the preliminary data (not shown) it was determined that optimal neuronal survival and maturation was achieved with a nitrogen to silicon element ratio between 1300 and 1700, and a contact angle value of 48° ± 3. For 13F on ablated DETA surfaces, a contact angle value of 75° ± 10 was desirable to prevent neuronal adhesion. As an additional control, 13F was also incorporated onto acid-washed glass to facilitate comparisons with pure 13F surfaces; such surfaces had contact angles of 95° ± 10. These XPS and contact angle ranges were used in the selection of surfaces for application in all subsequent experiments.

Neuronal Circuit Formation by Somal Translocation. Neuronal circuit formation on SAM patterns was visualized using time-lapse microscopy which was started immediately after the hippocampal neurons were plated. Initially, neurons were uniformly distributed across the entire surface, with the majority of cells located on the 13F background area at a range of distances from the DETA patterns. Subsequent videographic data analysis revealed how a typical two-neuron circuit was formed on the patterned surface by directed neuronal migration (Figure 2 and Supplementary Video 1). It was observed that the DETA dashed line geometric cues provided specific migration pathways which cultured neurons followed toward the patterned somal adhesion site. Within the first 15 to 18 h of culture, neurons began to extend exploratory neurites across the culture surface. On patterned surfaces, the dashed DETA dendrite line patterns were detected by random exploratory neurites extending from nearby cells. Once the guidance cue was detected, other neurites retracted, and the single remaining neurite became a leading process which followed the DETA line toward the somal adhesion site. A distance of less than 50 μm from the dashed DETA cue was calculated in 95% of all successful translocation events observed ($n = 20$, Figure 3A). This behavior in response to geometric surface signals indicated that leading process outgrowth could be determined and controlled by altering external physical cues.

Neuritic extension toward the patterned somal adhesion site varied in speed considerably; observed neurites grew at a rate of 0.62 μm/min ±0.45 ($n = 19$, Figure 3B). This variability in neuritic outgrowth speed may have been influenced by the cultured cell's differing ability to overcome the broken nature of the DETA guidance cues. The broken line design was developed to encourage the formation of a dendritic morphology in neurites extending from cells adhered to the somal adhesion site.[38] However, the broken line likely inhibited growth cone development toward the adhesion site in migrating neurons as they navigated the 10 μm gap between the 2 patterned DETA lines. The ability for broken line patterns to retard and influence neuritic development has been reported previously[38] and provides further evidence for the ability of chemical patterns to influence neuritic development.

Once the growth cone reached the somal adhesion site, a delay of 11.83 h ± 11.26 was observed (Figure 3C). In all successful translocation events, net movement of the soma was restricted to a period following this delay, indicating that successful and directed migration in vitro first required the firm adhesion of the growth cone to a cytophilic domain. The observed delay likely corresponded to a period of traction force generation, cytoskeletal rearrangement, and transmission of positioning information necessary to initiate the migration
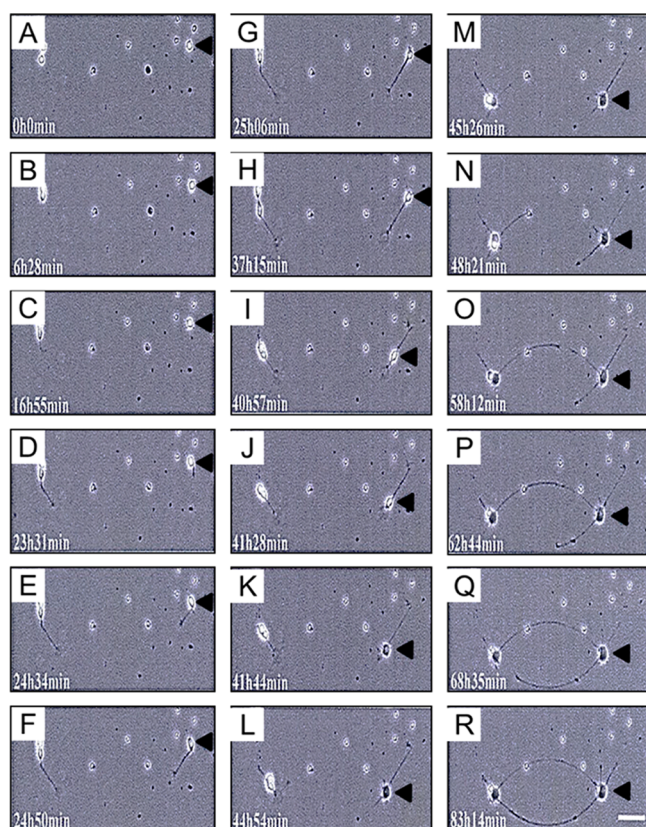
Figure 2. Representative images collected from a time-lapse sequence demonstrating two-neuron circuit formation by primary hippocampal neurons cultured on a chemically patterned substrate. (A–D) Stage 1: Within the first 24 h of culture, a neuron (black arrowhead) adheres to the 13F surface, close to the DETA dashed guidance line, and develops short multipolar processes. (E and F) Stage 2: One short process randomly detects the DETA guidance line and extends along it rapidly toward the somal adhesion site. (G and H) Stage 3: The neurite growth cone reaches the somal adhesion site, where it stops growing and adheres to form a circular attachment zone with a diameter close to that of the patterned adhesion site. (I and J) Stage 4: The cell soma then translocates along this leading process toward the somal adhesion site. (K and L) Stage 5: Once the cell reaches the patterned adhesion site, it develops a circular morphology with a diameter roughly equal to that of the somal adhesion site. (M–R) Once the migration event is complete, axonal outgrowth along the continuous DETA line toward the neuron on the other side of the pattern is initiated. Scale bar = 50 μm.

event. The substantial variance in the duration of this delay can likely be attributed to variation in migration distance, cell health, and surface hydrophobicity between the recorded data sets.

During the subsequent 24 h, translocation of the cell soma from its initial attachment point, along the leading process, to the patterned somal adhesion site was observed and occurred at an average speed of 0.5 μm/min $\pm$0.39 (n = 19, Figure 3D). Following somal adhesion to the patterned site, neurites extended along all radial DETA cues. Previous data[38] suggests that those neurites extending along the radial dashed lines formed dendritic processes, while those that grew along the continuous line toward the other cell in the network became axons.

The mode of cell migration observed on these *in vitro* patterned substrates and described above, was similar to the somal translocation mechanism which has been well

characterized *in vivo*.[11,12] For ease of assessment, this *in vitro* somal translocation process was subdivided into five distinct stages (Figure 2): Stage 1, multipolar short processes (Figure 2A–D); Stage 2, leading process extension (Figure 2E,F); Stage 3, growth cone adhesion (Figure 2G,H); Stage 4, somal translocation (Figure 2I,J); and Stage 5, somal adhesion (Figure 2K,L). The subsequent neuritic outgrowth along the patterned continuous lines facilitated a physical connection between the two adhered neurons, leading to the formation of a distinct two-neuron circuit (Figure 2M–R). Once the migrating neurons reached the somal adhesion site successfully, they were maintained in culture for a further 7 days and no detachment events were observed (n = 19).

In contrast, the migration behavior observed in neurons whose initial attachment site was close to the continuous axon lines differed substantially from those cells near the dashed dendrite lines. In both cases, cells began by extending a short path finding process toward the DETA lines. However, once neurites reached the continuous axon line, neuritic extension became bipolar and followed the path in both directions; during this event, the cell soma translocated to the line. The neuron continued to extend long processes bidirectionally along the continuous line and the cell body appeared to move back and forth with varied net displacement. Very few neurons which translocated to the continuous axon line were able to migrate to the somal adhesion site successfully, and in all such cases, the initial adhesion point was very close to the patterned adhesion site (data not shown). The majority of neurons which migrated to the continuous axon line developed a spindle-shaped morphology and were phase bright.

Neurons which initially adhered within the 13F region at a significant distance (>50 μM) from the DETA patterns, generally died within 3 days of plating. To further characterize the migration of neurons across cell repulsive, hydrophobic surfaces, neurons were plated on control surfaces composed of 13F incorporated on ablated DETA (contact angle = 75° $\pm$ 10) and 13F deposited onto acid washed clean glass surfaces (contact angle = 95° $\pm$ 10). A leading process length of 18.04 μm $\pm$ 10.44 (n = 19) was observed growing from neurons adhered to 13F on ablated DETA (Figure 4), as the cells tried to locate a cytophilic region to which they could migrate. After 2 to 3 days, unable to find a hospitable attachment site, this leading process retracted and the neuron subsequently remained stationary and eventually died. Leading process lengths measured from cells cultured on acid washed clean glass surfaces treated with 13F were shorter, compared with those on 13F incorporated on ablated DETA (Figure 4). On acid washed, 13F treated surfaces, 98.8% of all cells examined (n = 83) were found to have neuritic processes shorter than 60 μm. In contrast, 71.15% of cells cultured on 13F on ablated DETA surfaces had neurites of a similar length. The remaining 28.15% of cells examined (n = 104) had neurite lengths between 61 and 200 μm, indicating that the less hydrophobic nature of this culture surface permitted more extensive neuritic outgrowth.

Cytology and Migration Dynamics of Somal Translocation *in Vitro*. Morphological and immunocytochemical analysis of the hippocampal neurons was carried out to generate a more in depth understanding of their development during migration on the chemical patterns. During stage 1, rounded, phase-bright cells were temporarily positioned on the 13F surface and remained immobile; however, they were found to be rotating constantly, with multiple short processes
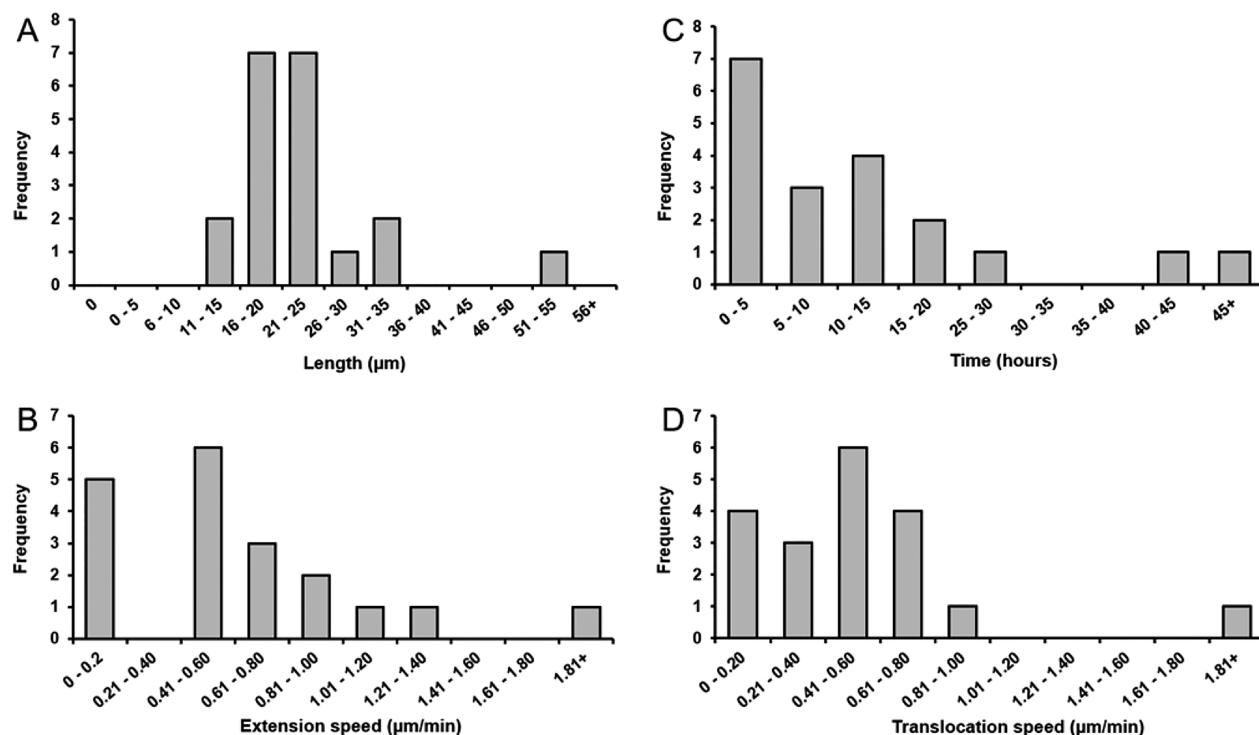
Figure 3. Overview of migration dynamics in a population of rat hippocampal neurons cultured on chemically patterned substrates. (A) Histogram illustrating the lengths of neuritic processes prior to cells locating DETA migration lines in cases of successful somal translocation ($n = 20$). (B) Histogram illustrating neuritic extension speed along DETA guidance cues in cases of successful somal translocation ($n = 19$). (C) Histogram illustrating the duration of growth cone adhesion to a somal adhesion site prior to initiation of a successful somal translocation event ($n = 19$). (D) Histogram illustrating speed of somal translocation during successful migration events ($n = 19$).
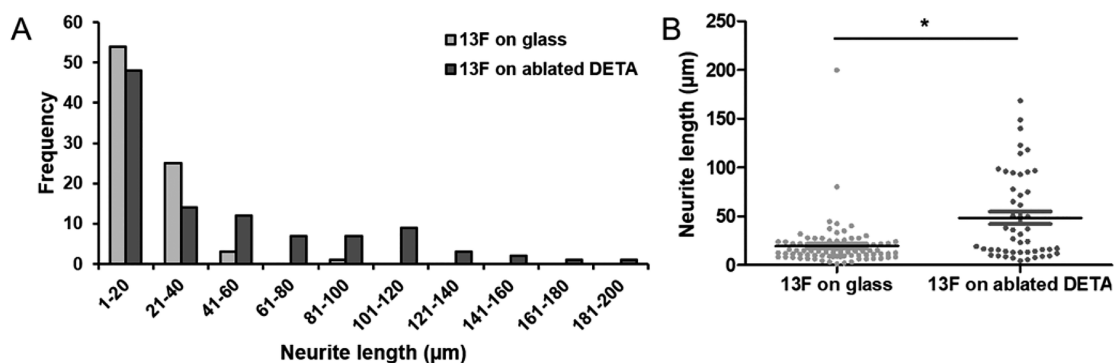

Figure 4. Analysis of neuritic outgrowth on hydrophobic culture surfaces. (A) Frequency distribution of neurite lengths on 13F incorporated onto ablated DETA surfaces (contact angle = $75° \pm 10$, $n = 104$) and 13F deposited onto clean glass surfaces (contact angle = $95° \pm 10$, $n = 83$). (B) Comparison of average neurite length in neurons plated on the two examined surfaces, *$p = 0.0001$.

dynamically extending and retracting from their somata in all directions, indicating the cell's inability to form successful and long-term attachments to such hydrophobic surfaces (Figure 5A). Immunostaining data from this time-point indicated that cultured cells had developed a dense cage of microtubules surrounding the nucleus (Figure 6A). Small actin positive protrusions were also observed, indicating that the extension of small exploratory neurites at this stage was actin driven.

Typically, within the first 24 h of culture, one of the short exploratory neuritic processes extending from the cell detected the nearby DETA dashed line cue (Figure 4B) and began to grow toward the somal adhesion site (stage 2). Within the extending leading process, a longitudinally oriented microtubule network was observed with actin filaments localized at

the tip of the growth cone and the rear of the soma, as has been documented previously for glial guided migration events[27,57] (Figure 6B). The microtubule cage around the nucleus persisted at this stage but appeared more organized and was oriented toward the developing growth cone. A dense cluster of microtubules, likely indicating the location of the MTOC, was positioned directly behind the nucleus at this stage.

During the rapid development of the leading process toward the somal adhesion site, the cell soma remained immobile but went from a rounded to a more spindle shaped morphology (Figure 5B). When the developing neurite reached the DETA somal adhesion site, the process extension stopped and the growth cone spread to form a circular structure conforming to
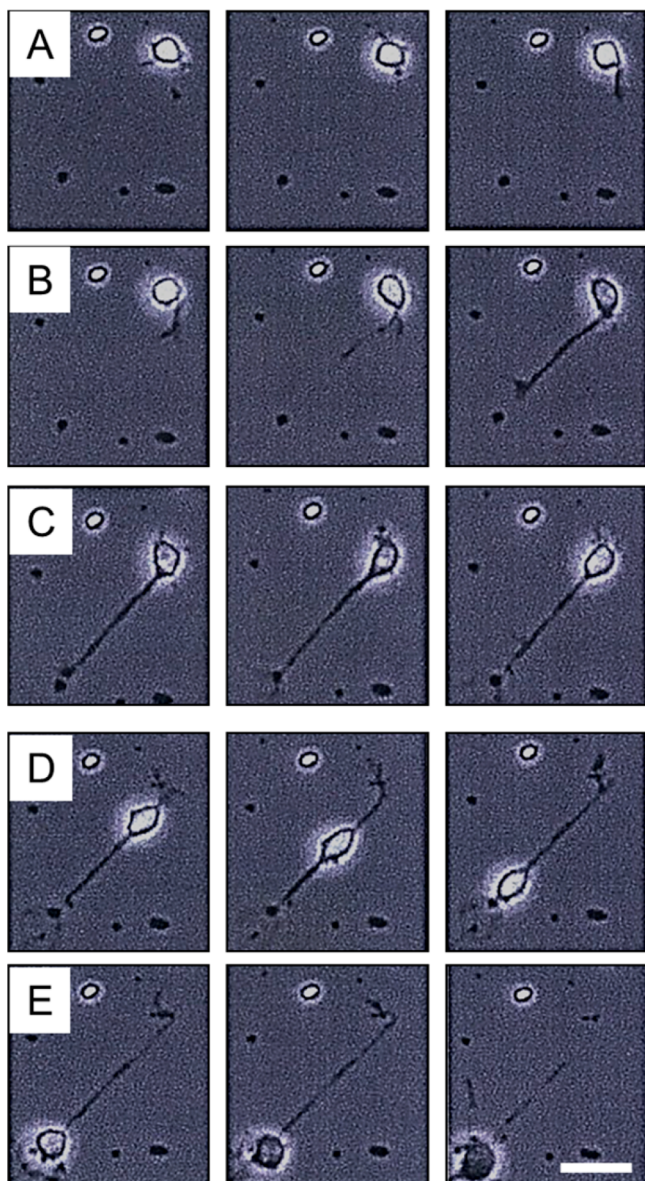
Figure 5. Representative cytology and migration dynamics of a single neuron cultured on a chemically patterned substrate. (A) Cellular morphology during the multipolar short process stage of *in vitro* migration (stage 1). (B) Cellular morphology during the leading process extension stage (stage 2). (C) Cellular morphology during growth cone formation (stage 3). (D) Cellular morphology during somal translocation (stage 4). (E) Cellular morphology during somal adhesion (stage 5). Scale bar = 25 μm.

Figure 6. Distribution of microtubules (light blue), actin filaments (red) and nucleus (dark blue) at each migration stage. (A) Stage 1: The nucleus is encircled by a tangled cage of microtubules. Short exploratory processes are formed by protruding actin filaments. (B) Stage 2: Nuclear cage transforms into a spindle shape oriented toward the developing growth cone. Actin filaments are localized to the tip and the external edges of the extending process. Microtubules are organized in longitudinally oriented filamentous arrays within the developing process. (C) Stage 3: The leading process forms a circular growth cone at the somal adhesion site composed of a dense actin network. Microtubules appear to have lost their filamentous structure at the central domain of the growth cone. (D) Stage 4: The cell's soma translocates along the leading process toward the somal adhesion site. During translocation, the nucleus remains within the caudal aspect of the spindle shaped migrating soma. A dense microtubule network is observed throughout the growth cone adhesion site while the tip of the trailing process is actin rich. (E) Stage 5: The cell soma reaches the patterned adhesion site which is now covered in an array of actin filaments and microtubules. Following adhesion, the cell's nucleus covers roughly half of the circular somal adhesion site. Scale bar = 20 μm.

the shape of the patterned adhesion area (stage 3, Figure 5C). The circular structure was formed almost exclusively from actin filaments while the microtubules maintained a loose, filamentous structure at the center of the growth cone (Figure 6C). At this stage in the migration event, the MTOC had migrated in front of the nucleus, close to the base of the leading process, assuming a position similar to that observed during neuronal migration *in vivo*.[13,27]

During somal translocation (stage 4), the soma migrated along the leading process toward the somal adhesion site (Figure 5D). The soma appeared phase bright with a symmetrical spindle morphology oriented toward the leading process. During the entire somal translocation event, a trailing process remained attached to the surface and its length
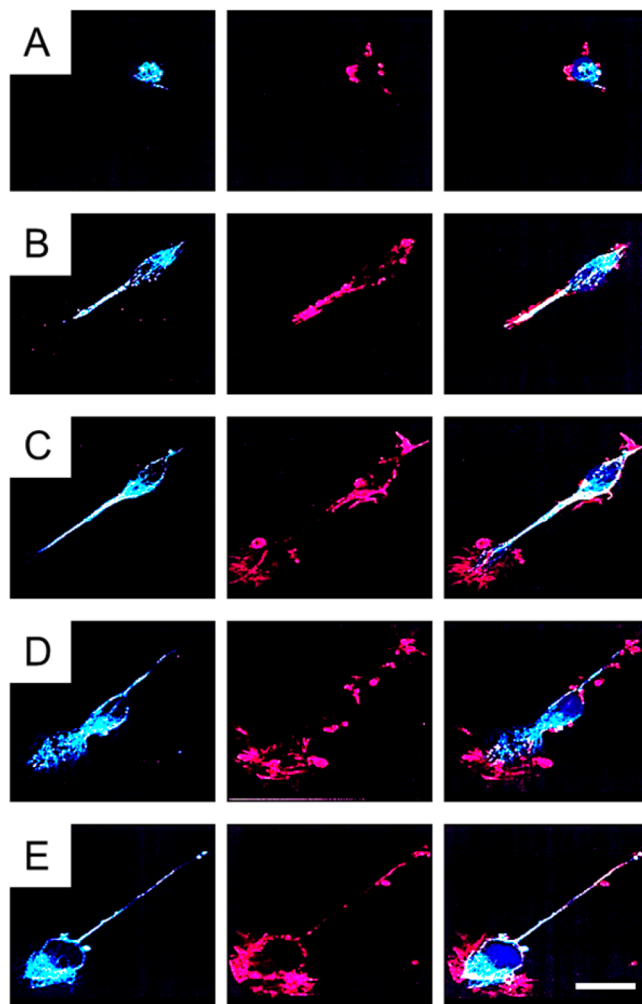
remained constant. As the soma reached the adhesion site, the MTOC (which remained at the base of the leading process throughout the migration event) and associated nuclear microtubule cage interspersed with the actin rich adhesion area to form a complex meshwork of cytoskeletal filaments (Figure 6D). When the cell soma reached the patterned adhesion site, it attached to the culture surface (stage 5, Figure 5E) and the actin and microtubule network surrounded the

arriving nucleus (Figure 6E). Similar results were achieved in all translocation events observed using time-lapse microscopy (n = 19) and in all migrating cells investigated immunocytochemically (n = 3 from each migration stage).

■ DISCUSSION

Targeted neuronal migration and controlled neuritic outgrowth are essential for the correct development of the mammalian nervous system. As such, the different forms of neuronal movement have been extensively studied and characterized *in vivo*, in order to better understand neural development in healthy and diseased states.[2−4,6,12] However, the mechanisms by which cultured neurons migrate across artificial culture surfaces have been less extensively studied. A more complete understanding of the physiological changes and limitations of neuronal cells during *in vitro* migration events is of considerable importance for the design and development of advanced functional neuronal tissue mimics for drug development and disease modeling applications.

Glial-guided migration has been shown to occur *in vitro*, and such studies have been used to better elucidate the morphological characteristics of migrating neurons *in vivo*. Somal translocation is employed by neurons at both early and late developmental stages and is believed to be important for correct neuronal positioning during the establishment of distinct cortical layers and the formation of specific neuronal circuits.[11,12]

This study demonstrates that primary neurons are capable of migrating from inhospitable culture surfaces to cytophilic domains with a high degree of accuracy. Moreover, such cells utilize somal translocation mode migration to respond to extrinsic geometric cues provided by chemical surface patterning. Previous work has demonstrated a neuritic predisposition to follow extrinsic cues.[31,32,38,53] Data collected in this study builds on this observation by demonstrating that such physical cues are sufficient to initiate neuronal migration and directed neurite outgrowth and that such behavior can occur successfully without additional biochemical cues and gradients or input from supporting cell types, known to influence neuronal migration *in vivo*.[28] These observations are supported by recently published findings that independently verify the capacity for neurons to exert a somal translocation mechanism when migrating on patterned culture surfaces.[58]

The neuronal circuit design employed in these experiments (Figure 1) was based on an earlier study[53] which sought to analyze synaptic connectivity between two hippocampal neurons. Embryonic hippocampal neurons were found to either adhere directly to the elliptical patterns or migrate to the pattern based upon the differential properties of the chemically treated culture surface. The pattern was characterized for optimum cell viability and neurite outgrowth with regard to line width and somal adhesion site diameter. The dashed lines near the solid dot were found to act as extrinsic cues initiating the migration of cultured cells toward the somal adhesion site (Supplementary Video 1), in addition to providing restricted pathways to encourage subsequent dendritic outgrowth in a second stage of development. Once the cell soma adhered to the somal adhesion site, the longer 2 μm line promoted axon development as has been demonstrated previously.[38]

Surface properties were found to play a significant role in cell migration behavior in this *in vitro* system. Two different types of self-assembled monolayers were utilized to control cell migration. The aminosilane DETA possesses hydrophilic

properties and has been shown previously to promote neuronal adhesion,[38,48,49,53] whereas the fluorinated silane 13F is hydrophobic and cell repellant.[38,53] It has been shown that although ECM proteins adsorb to the 13 F surface in amounts equivalent to DETA, they then tend to denature and this process inhibits a cells ability to attach and survive in the long term.[59] The surface wettability was found to play a substantial role in regulating neurite lengths in these cultures. Analysis of neurite growth on 13F control surfaces highlighted that neurites extended further before retracting on surfaces with lower contact angles (Figure 4). Shorter neurite lengths were also observed in cells cultured on patterned coverslips which is likely due to the high density of adhesion sites across the surface. In such cases, exploratory leading processes did not need to travel as far to reach a somal adhesion site as they did on control 13F surfaces, and so average neurite length was shorter.

On patterns, it was observed that cell somata never instigated translocation events until their developing growth cone had located and adhered to a cell permissive surface. This highlights the controlled nature of the migration events taking place in these cultures, and this two phase migration and development suggests the activity of a retrograde signaling mechanism from the growth cone to the cell soma necessary for instigating translocation *in vitro*. Furthermore, cells that were deposited further away from somal adhesion sites were found to move more slowly, taking longer to reach their final destination (data not shown). Given the apparent importance of retrograde signaling for initiating successful neuronal migration events, substantial increases in neurite length may well retard the speed at which cultured cells are able to respond to extrinsic cues.

Migrating neurons were also characterized by immunocytochemistry for microtubules and actin filament arrangement. The various morphological changes in migrating neurons *in vivo* are due to cytoskeleton reorganization, which is regulated by numerous molecular pathways.[28] As the main components of the cytoskeleton, actin filament reorganization is known to drive direct cell migration, while the microtubule network is necessary for the advancement of the cell body and subsequent retraction of the trailing process.[26] Immunostaining of cultures fixed at different stages during migration events in these patterned cultures demonstrated that similar cytoskeletal reorganization events occur *in vitro* and are necessary to facilitate targeted neuronal translocation in culture. Migrating neurons *in vitro* promoted microtubule development into a cage-like structure surrounding the nucleus, with the MTOC in a rostral position at the base of the leading process as well as a dense microtubule network at the tip of the developing growth cone, as occurs *in vivo*.[27,60] Actin filaments were concentrated in the lamellapodia of the leading process and spread across the surface of the somal adhesion site once it was reached by the developing growth cone. The extension of the actin network to cover the cytophilic DETA surface prior to translocation provides evidence for the cell probing and testing the adhesion site to ensure it could support the cell before initiating a migration event and further confirms the importance of retrograde signaling from the growth cone to the cell soma before such behavior is undertaken.

This study demonstrates that cells migrating on chemically engineered *in vitro* patterns do so using a somal translocation mechanism that mimics the well-established *in vivo* phenomenon. Furthermore, the data compliments previous observa-

tions demonstrating a similar ability for such neurons to undergo glial-guided migration;[9] together, these data provide evidence that neuronal cells are able to utilize multiple *in vivo* migration modes in defined *in vitro* environments depending on the specific parameters of the culture. Verification of hippocampal neurons' ability to respond to extrinsic geometric cues using a biologically relevant migration mechanism is important for the development of more organized and functionally relevant *in vitro* culture assays for drug development and disease modeling applications. Demonstration of such biologically accurate translocation behavior provides further validation for chemically patterned neuronal network technology,[49,53] as it shows that the self-assembly of such cell systems is achieved using appropriate signaling and motility mechanisms. Critically, the characterization of cell limitations with regards to survival on patterns, effective translocation distances, migration responses to bifurcating patterns, and neurite outgrowth on increasingly hydrophobic surfaces provides valuable data to investigators seeking to adapt current chemical pattern designs to improve *in vitro* neuronal network formation.

ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.8b00610.

> Chemically directed circuit formation of embryonic hippocampal neurons on DETA somal adhesion sites and neurite guides: within 15−18 h of plating, neurons deposited on cytophobic 13F surfaces translocated to cytophilic DETA adhesion sites; video shows dual neuritic extension, detection of somal adhesion sites, establishment of polarity and subsequent axonal extension over guides, and dentritic output over dashed DETA lines, and cells could actively search for, detect, and translocate to DETA surfaces within approximately 50 μm of initial deposition (AVI)

AUTHOR INFORMATION

Corresponding Author
*E-mail: jhickman@ucf.edu.

ORCID Ⓘ

James J. Hickman: 0000-0002-1621-3006

Author Contributions

[⊥]A.N. and A.S.T.S. contributed equally. All authors have seen and approved the submission of the manuscript.

Notes

The authors declare the following competing financial interest(s): J.J.H. has a potential competing financial interest, in that a company has been formed that potentially could market services for the type of device described herein in which he has a financial interest.

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