Published in final edited form as:

Integr Biol (Camb). 2010 November ; 2(0): 696–706. doi:10.1039/c0ib00044b.

A new chemotaxis device for cell migration studies†,‡

Waseem Khan Raja^{§,a}, Bojana Gligorijevic^{§,b,c}, Jeff Wyckoff^{b,c}, John S. Condeelis^{¶,b,c}, and James Castracane^{¶,a}

John S. Condeelis: john.condeelis@einstein.yu.edu; James Castracane: jcastracane@uamail.albany.edu

^aCollege of Nanoscale Science and Engineering, University at Albany, SUNY, Albany, NY

^bDepartment of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY 10461, USA

^cGruss-Lipper Biophotonic Center at Albert Einstein College of Medicine, Bronx, NY 10461, USA

Abstract

This study presents the design and optimization for *in vitro* use of a new versatile chemotaxis device called the NANIVID (NANo IntraVital Imaging Device), developed using advanced nano/micro fabrication techniques. The device is fabricated using microphotolithographic techniques and two substrates are bonded together using a thin polymer layer creating a sealed device with one outlet. The main structure of the device consists of two Pyrex substrates: an etched chemoattractant reservoir and a top cover, with a final size of $0.2 \times 2 \times 3$ mm. This reservoir contains a hydrogel blend with EGF which diffuses out through a small ($\sim 9 \cdot 10^3 \mu\text{m}^2$) outlet. This reservoir sustains a steady release of growth factor into the surrounding environment for several hours establishing a consistent concentration gradient from the device. The focus of this study was to design and optimize the new device for cell chemotaxis studies in breast cancer cells in cell culture. Our results show that we have created a flexible, cheap, miniature and autonomous chemotaxis device and demonstrate its usefulness in 2D and 3D cell culture. We also provide preliminary data for use of the device *in vivo*.

Introduction

Various types of cells and organisms respond to particular chemical gradients through a phenomenon known as chemotaxis. Chemotaxis-based cell motility plays a critical role in a variety of biological processes such as wound healing,¹ development,² inflammation^{3,4} and tumor metastasis.^{5,6} In the last few decades, extensive studies have been performed on cellular responses to a variety of chemical gradients using various types of *in vitro* assays.⁷ Various versions of Boyden's assays^{8,9} and Dunn's chamber^{10,11} were developed and used to study the chemotaxis of different cell types *in vitro*. These assays have limitations involving

†Published as part of a themed issue on Mechanisms of Directed Cell Migration: Guest Editors David Beebe and Anna Huttenlocher.

‡Electronic supplementary information (ESI) available: Additional data and supplementary movies 1 and 2. See DOI: 10.1039/c0ib00044b

This journal is © The Royal Society of Chemistry 2010

§These authors contributed equally to the manuscript.

¶Co-contributing senior authors, Tumor Microenvironment Network, NCI.

either endpoint analysis or cellular positioning for optimum responses and cannot be used directly for *in vivo* analysis. Soon,¹² in 2005 developed a chemotactic chamber based on a pipette assay to create a dam of growth factor below the cultured cells in 2D and demonstrated a more pronounced chemotaxis response of MTLn3 breast cancer cells in a nonlinear gradient of EGF. This finding using similar EGF concentrations was later confirmed in the MDA-MB-231 human breast cancer cell line.¹³ Pipette based assays have their own limitations and complexities.¹⁴ A variety of microfluidic based chemotaxis devices were also developed for studying chemotaxis in various cells types. Barkefors¹⁵ created a chemical gradient of VEGFA and FGF-2 in a microfluidic device, to study the migration of endothelial cells. Lin¹⁶ studied the chemotaxis of neutrophils in a microfluidic device in which the concentration gradient was generated from two inlet streams using a network of bifurcating and merging microfluidic channels. Saadi¹⁷ and Wang¹³ used the same dual inlet microfluidic devices to examine the migration of cancer cells in an EGF gradient under flow conditions. Walsh¹⁸ grew tumor cells inside PDMS and glass microfluidic devices to create a tumor microenvironment *in vitro* for targeted intratumoral therapeutics. In microfluidic devices, an external source is required that is connected through a tubing system. In most of these devices, cellular cultivation should be inside the device which makes them hard to implement and use directly for *in vivo* analysis.

Recently, cell migration was also investigated in 3D *in vitro* by creating a stable growth factor gradient in matrices of collagen, agarose gel and matrigel, or in living animals, using the *in vivo* invasion assay. In his studies of the interaction between tumor cells and macrophages, Goswami¹⁹ overlaid cultures with a 3D collagen matrix and measured their co-migration, discovering a paracrine loop signaling pattern between the two cell types. Mousseau²⁰ used a thin agarose membrane to separate wells which contain chemoattractant, cells and control solution and tracked cell migration in 3D over days. Abhyankar²¹ generated a biochemical gradient in a 2 cm-long covered channel filled with collagen gel and observed the migration of either neutrophils or MTLn3 cancer cells in linear and non-linear gradients. As of now, the only example of the chemotaxis assay in living animals is the '*in vivo* invasion assay',²² performed by placing a chemoattractant loaded needle within mammary tumors of rats, mice and humans. This assay was successfully used for collection and gene expression analysis of cancer cells.^{22–26}

Most available assays are created for use under constrained conditions (specialized dishes, either 2D or 3D migration, cultivation of cells inside microfluidic devices *etc.*) and cannot be implemented for *in vivo* use. They have limitations of being either labor-intensive or end-point measurements. The work presented here demonstrates the development of a new versatile chemotaxis device, in which PDMS, glass and a growth factor/hydrogel reservoir are combined in such a way that the device can be used directly in already established tissue cultures in 2D and 3D and can potentially be implemented for *in vivo* use. PDMS, glass and hydrogel are commonly used materials in the biomedical field. A variety of hydrogel systems are available for the release of targeted molecules in a controlled manner, such as, degradable polymer^{27,28} and stimuli responsive hydrogel.^{29–31} These hydrogel systems have associated limitations which make them technically hard to incorporate in our device, including undesirable byproduct release when polymer degrades, an extended curing time,

and organic solvents. Drug loading and fabrication techniques, slow response time in responsive polymer (in bulk) as well as the need for external stimuli agent make these systems less suitable for use in our device. To overcome these issues, an aqueous, solvent-based, UV-curable hydrogel system was employed in this study.^{32,33} The selected hydrogel system passively releases the targeted molecules due to the hydration resulting in desorption and diffusion of targeted molecules from the surface and pores of these hydrogels. This hydrogel system in the device makes it self sufficient in releasing the growth factor into the surrounding environment even in the absence of any external pumping source and stimulating factor. The device consists of two Pyrex (glass) substrates processed using photolithography and bonded together using a thin film of PDMS (Polydimethylsiloxane) with oxygen plasma treatment. Inside the device, the source chamber is loaded with an aqueous, solvent-based hydrogel blend (PEGDA/PEGMA) containing growth factors, which is UV crosslinked with a short curing time. The fabrication and drug loading technique for this hydrogel system can be easily incorporated into the device fabrication process flow. The hydrogel system acts as a growth factor reservoir for the device, which passively releases growth factor by hydration in the media solution. The released growth factor then diffuses out into the surrounding environment to establish a concentration gradient originating at the device outlet. These characteristics make it an autonomous tool for both *in vitro* and potentially *in vivo* characterization of cellular chemotaxis.

Results and discussion

The concept of the NANIVID was based on an idea to build an autonomous and self-sufficient device which is flexible and scalable in shape and size. The device should be microscopic, easy to handle and usable both in the *in vitro* assays, and *in vivo* experiments. Finally, the device should form an exponentially decaying (non-linear) gradient of growth factor from a small opening with the shape and range of the gradient controlled by changing the device design and chemoattractant concentration inside the device. The rate of chemoattractant release is controlled by the level of cross-linking inside the carrier polymer.

Critical issues with device fabrication for biological applications are compatibility with the biological system, integrity and bio-functionality. These issues were addressed and resolved by the selection of available biocompatible materials and an optimized process flow for NANIVID fabrication and integration. Some of the components were fabricated at the wafer level in order to reduce the cost and increase throughput of device fabrication shown in Fig. 1A. The same fabrication process flow was also used for the various designs and shapes of the device used in the *in vivo* experiments. The process flow for the device was designed such that a biological material (EGF-hydrogel) can be easily integrated inside the device at room temperature without denaturation of the proteins. The bonding step of the device shown in Fig. 1B was in two phases: the first was to create a glass top coated with PDMS and the second phase, which was less than 20 min (per 10 devices), includes loading and curing of the EGF-hydrogel system in the source chamber of the device and final bonding. The functionality of the device was verified by cellular response toward the device. The release of growth factor from the device was modulated and controlled by adjusting the hydrogel formula and device design.

Fig. 1C shows an exploded view of the NANIVID with its top cover opened and placed in front of the cancer cells. In the side view of the device, one can see the chemoattractant reservoir, source chamber and device outlet from which EGF diffuses. Iterations in the design of the etched chambers inside the device³⁴ led us to the current version of the NANIVID, presented in Fig. 1D. This version proved capable of releasing approximately 80% of the EGF loaded inside the hydrogel as a slow and steady gradient. Fig. 1D also shows the current *in vitro* version of the device, which consists of a source chamber in the back of the device where the growth factor/hydrogel system was loaded and cured. The middle region of the device is an open space for hydrogel swelling due to hydration as well as for EGF release inside the device shown in Fig. 1E. The front chamber of the device was for the collection of hydrogel desorbed EGF, from where the EGF would disseminate out through the device opening into the surrounding environment to create a concentration gradient shown in Fig. 2C. Hydrogel was in its liquid form during the device loading after which it was UV-cured in less than 3 min and turned solid. In its liquid state during the loading step, the hydrogel flows along the edges of the etched Pyrex substrate. The obstructing features in the source chamber and two anchoring regions shown in Fig. 1D will interrupt the flow of uncured hydrogel. The anchors will also stop the hydrogel from going into the front chamber and keep it in the source chamber even after hydration.

Optimization of EGF release from hydrogel

A hydrogel system made of PEGDA molecules only, when crosslinked in a tight mesh, results in most EGF molecules getting trapped inside.³⁵ In order to avoid this, we have produced a custom hydrogel blend by mixing 20% PEGDA (diacrylate) with varying concentrations of PEGMA (monoacrylate) which results in a more porous hydrogel network. By comparing both hydrogels under SEM, we observe that the blended hydrogel shows more porosity compared to PEGDA alone (Fig. S1A, ESI[†]). Hydrogel swelling assays were performed on both hydrogel systems with various concentrations. PEGDA alone showed slight changes in swelling from 10% up to 30% (Fig. S1B, ESI[†]). Conversely, the blended hydrogel showed significant changes in swelling compared with PEGDA alone. The hydrogel blend system consists of 20% PEGDA in varying concentrations of PEGMA. Results showed that as the amount of PEGMA in the blend increases, the hydrogel accommodates more solution as shown in Fig. S1B, ESI[†]. The addition of PEGMA in blended hydrogel systems significantly increased the amount of released EGF, reaching the plateau at 10% SI[†] relative to PEGDA alone (~3 nM EGF in the media, Fig. S1C, ESI[†]). After the initial fast release of the EGF arising from the surface desorption, a slow release phase starts from the pores of the hydrogel and slowly increases the EGF concentration in the media over the course of days. The bioactivity of EGF is critical to the device performance but can potentially be damaged by the UV-curing of the hydrogel or changes in temperature. This was tested by a simple 'up shift' assay where EGF in conditioned medium is added to cells to stimulate their protrusion response. Starved MTLn3 cells are known to protrude³⁶ in response to 1–10 nM EGF added globally to the media. We have loaded the hydrogel with various EGF concentrations and UV-cured it. Cured hydrogel was then placed

[†]Electronic supplementary information (ESI) available: Additional data and supplementary movies 1 and 2. See DOI: 10.1039/c0ib00044b

in media and incubated, after which conditioned media was used in the up shift assay (Fig. S1F, ESI[†]). The concentration of EGF in the conditioned media (calculated from the loading concentration and release data) agrees with the known protrusion cell response range measured using EGF directly.¹²

EGF release and gradient formation from the device

The source chamber of the etched substrate was loaded with hydrogel containing R-EGF and bonded to the glass substrate coated with PDMS film. Fig. 1E shows the device at different stages after hydration in the solution. It was found that the hydrogel reservoir after hydration released R-EGF inside the device, which then slowly diffused out through the device outlet as shown in Fig. 2C. To verify the NANIVID release EGF into the surroundings for longer periods of time, the R-EGF release experiment was performed for more than 60 h time. The capacity of the source chamber of the NANIVID is relatively low (~65 nl), so to enhance the concentration of R-EGF, 9 devices were placed per dish in the release experiments. Fig. S1G, ESI[†] shows the plot of R-EGF release over the time interval indicated, demonstrating that > 80% of the R-EGF has diffused out of the device by 60 h.

In the actual cell migration assays done in 2D, cells were starved and their movement was recorded. Therefore, all experiments were performed for the same duration and under the same conditions. R-EGF was used instead of EGF to visualize the concentration gradient from the NANIVID. Fig. 2B and C show the close up view of the NANIVID opening (top view) using a fluorescence microscope with the arrows defining the pointed opening of the device. The front of the device is coated with fluorescent beads as shown in Fig. 2B to help to find the device opening while Fig. 2C is the snapshot (2 h time point in the time lapse experiment) of the R-EGF diffusion from the device. The brightness intensity was measured along the line from the opening of the NANIVID and in the rectangular area ~ 100 μ m from the device opening as shown in Fig. 2C. The brightness was converted into EGF concentration using the standard curve as shown in the insert in Fig. 2D. The R-EGF gradient is plotted in Fig. 2D showing a nonlinear profile from the device opening. The R-EGF gradient profile was plotted for 0, 2, 3 and 4 h in the L15 + 0.8% BSA starvation media. The concentration gradient rose in the first two hours of the experiment which represents the initial fast release effect from the hydrogel and then, slows down because of the release from the pores of the hydrogel during the experiment. The final phase (6 h) shows that the EGF level eventually decreases due to the limited source. The amount of R-EGF was measured inside the rectangular area in front of the device opening and was plotted against time as shown in Fig. 2E.

Device-induced chemotaxis response in 2D and 3D

The final step in the device validation included measurements of 2D and 3D cell chemotactic migration towards the device. Fig. 3A shows the experimental setup for the 2D experiment done using mammary carcinoma MTLn3-Mena^{Inv} cells³⁷ grown on plastic. For clarity, cells and the device are not to scale (scale bars 25 and 400 μ m). White arrows represent the total displacement of the cell over the course of experiment (4 h, Movie S1, ESI[†]). On the right, a gradient map of the same area is shown to illustrate the EGF concentration at cellular level. In Fig. 3B, representative vector plots are shown for several

concentrations of EGF inside the device, where each cell represents one vector and each plot represents one experiment. Black arrowheads point to position of the device outlet. As the concentration of EGF inside the device increases, the directionality and chemotactic index (C.I.) of cells are increasing, reaching the maximum at the 3.5 μM internal reservoir concentration.

In order to quantify and confirm the chemotaxis of MTLn3-Mena^{Inv} cells, the directionality shown in eqn (1) and chemotaxis index (*i.e.* $\cos \theta$) were analyzed for all EGF loaded NANIVIDs.

$$D = \frac{\sqrt{[(x_2 - x_1)]^2 - (y_2 - y_1)^2}}{\text{Total path length}} \quad (1)$$

Directionality is a measure of the efficiency of directional migration, which is defined as the ratio between the displacement (direct distance from the starting position (x_1, y_1) to the final position (x_2, y_2) of the cell) and total path length covered by the cell³⁶ (see Fig. 3 in reference 36). This value will be 0 for random walk and 1 for the situation when the cell moves in a straight line. Chemotaxis index reflects if a cell migrates directly towards the chemotaxis source and varies between 0 for random walk and 1 for cells moving straight to the source.

Results (Fig. 3C) show that maximum response is reached at reservoir concentrations of 3–4 μM for directionality and 3–3.5 μM for chemotaxis index. At higher loadings, we see a biphasic effect with a statistically significant decrease in both measurements.

To eliminate the possibility that the chemotactic response to the device was due to its shape, coating or mechanical properties, a control experiment was performed using two devices within the same dish. Cells were prepared as before and two NANIVIDs loaded with 0 (left) and 4 μM EGF (right) were placed in the dish facing each other ($\sim 500 \mu\text{m}$ apart, Fig. S2 and Movie S2, ESI[†]). While the average chemotaxis index of the EGF-carrying device was 0.67, chemotaxis index of the control device -0.023 .

Experiments were repeated using 5 μM containing devices in human breast cancer cell line MDA-MB-231 (Fig. S3A, ESI[†]), demonstrating a similar chemotactic response (C.I. = 0.56) compared to MTLn3-Mena^{Inv}.

The device was next calibrated for use in 3D chemotactic migration assays. In this 3D assay, the device is placed $\sim 25 \mu\text{m}$ above the cells (Fig. 4A) where it acted as a source of EGF, forming a gradient in x , y and z . Due to the slower diffusion through collagen than in media, the gradient is shallower and the EGF concentration is lower in front of the device (Fig. 4B) compared to the measurements in L15 media. However, under these conditions EGF will accumulate and possibly bind to collagen over longer periods, possibly mimicking conditions in tissue. Cells migrate along the gradient both laterally and axially (Fig. 4C), orient and polarize towards the device (Fig. 4C, magnified cell inset). In Fig. 4D, we see that the total cell number in front of the device increases over 48 h when 5 μM or 6 μM EGF is loaded into the device. This suggests a directional migration in the xy plane towards the

device or alternatively, an increase in cell division due to the presence of the EGF. However, 2 nM EGF (maximal concentration in front of the device) used as a media supplement does not result in a significant increase in cell division (Fig. S4, ESI[†]). In addition, devices containing 10 μ M EGF show cell numbers similar to the control, which further demonstrates that the increase in cell number is not due to the cell division (Fig. 4D).

In the xz plane, cells invade through collagen closer to the device opening when 5 μ M or 6 μ M EGF is loaded in the device, an effect which disappears when we use 10 μ M devices as shown in Fig. 4E. This result agrees with previous studies¹³ showing a decrease in chemotactic response at high EGF concentrations.

Finally, we have used the device in several established *in vivo* assays to test if the components of the device are bio-compatible and if the device can be visualized and studied in *in vivo* conditions. Fig. 5A shows results of using the well established *in vivo* invasion assay,²² which is routinely used to collect invasive populations of tumor cells from the tumors using chemotaxis. In comparison to EGF in matrigel, hydrogel loaded with EGF performed equally well in terms of numbers of cell collected. Further, we have used Dendra2-labeled MTLn3 xenograft tumors and a mammary imaging window³⁸ to see if hydrogel loaded with EGF induces chemotactic responses in tumor cells in mammary tumors over longer times. Two areas were photoconverted in each of the animals tested: one <500 μ m from the injection site and the other one >1 mm away. Major blood vessels were absent in both of the chosen areas. 24 h later, photoconverted tumor cells (Dendra2-red) were visualized outside of the original photo-converted square, indicating the migration of dendra-red cells towards the hydrogel-EGF source (Fig. 5B,C). As a proof-of-principle towards future validation of the device *in vivo*, we have also modified the NANIVID to have a pointed, fluorescent tip for easier insertion into tissue (Fig. S5A, ESI[†]) and visualized it inside anesthetized transgenic mice with GFP-myeloid cells which are chemotactic to EGF. The device was traced to 50–150 μ m depth inside the mammary tumor (Fig. S5B, ESI[†]), and the tip of the device was associated with GFP positive cells through this volume suggesting migration toward the device.⁶

Experimental

Device fabrication

The device is fabricated using micro fabrication techniques.^{35,39} It consists of dual chambers designed to contain the chemoattractant source (EGF in hydrogel) and create a gradient at the outlet (Fig. 2C). Fig. 1A shows the process flow of fabrication of the dual chamber piece. A Pyrex glass substrate (No.0 thickness, ~100 μ m) was cleaned using Piranha solution (H₂SO₄/H₂O₂) and then, coated with a Cr/Au hard mask: a 5 nm layer of chromium and a 100 nm layer of gold on each side. Further, 2 μ m thick positive photoresist (*i.e.* 1813 photoresist, Shipley) was spin coated on both sides of the substrate. Photolithography was performed on the top of the substrate, with Au and Cr etchants used to open a window in the hard mask. Pyrex was then selectively etched in HF/HCl, 10: 1, to create features in the substrate. Photoresist and the hard mask were then stripped away and the etched substrates were diced.

The final device consists of the reservoir piece and top cover, bonded together using a thin film of PDMS (Dow Corning). Both glass pieces were cleaned for 10 min in acetone and 10 min in isopropyl alcohol followed by a thorough cleaning in ethanol and a drying step. Then, a thin layer of PDMS was produced by spinning PDMS solution at 2800 rpm on an acetate sheet and cured overnight at 65 °C. The PDMS film was transferred onto the top cover after an oxygen plasma treatment (Harrick Plasma) and was bonded together for two hours to maximize adhesion. The PDMS/glass top cover was then removed from the acetate sheet. Further, the reservoir piece and the flip side of PDMS were treated with oxygen plasma. Then, the mixture of EGF in hydrogel was UV-cured in the back of the reservoir and bonding was performed, as shown in Fig. 1B. In order to find the opening of the device by fluorescence, fluorescent polystyrene beads were coated on the front of the device shown in Fig. 2B. The bonded devices were kept in the freezer at -20 °C until used.

Optimization of EGF release from hydrogel

A hydrogel was made either from polyethylene (glycol) diacrylate (PEGDA Mw = 3.4 kDa, Glycosan BioSystems, Inc.) alone or PEGDA and (methoxy) polyethylene glycol monocrilate (PEGMA Mw~672 Da, Sartomer Company, Inc) blend. Dilutions were made in 1 × Phosphate Buffer Saline (PBS, Sigma-Aldrich) solution to the desired concentration. Various concentrations of PEGDA alone (*i.e.* 10% to 30%) and 20% PEGDA and varying concentrations of PEGMA (*i.e.* 0.5% to 15%), were tested to optimize EGF release (Fig. S1, ESI[†]). The polymer was cured under 365 nm UV light in the presence of 0.15% Irgacure-2959 (Ciba Corporation) as a curing agent.³⁵ EGF (Sigma-Aldrich) was mixed in the hydrogel solution at 104 μM in PEGDA alone and 10 μM in the blend solution along with the curing agent and cured under UV light on a glass cover slip pretreated with binding silane.³⁹ Measurements of released EGF were performed by placing hydrogel in L15 media containing 0.8% Bovine Serum Albumin (BSA, Sigma-Aldrich), used in later experiments as MTLn3 Mena^{inv} cell starvation media. Released EGF samples were collected at various time intervals and kept at -20 °C until the end of the experiment. Enzyme-Linked ImmunoSorbant Assay (ELISA, R & D Systems, Inc.) was used to measure the amount of the released EGF. ELISA was performed on collected samples placed in a 96-well plate along with controls and standard solutions according to company protocol and using a Tecan Infinite M200 microplate fluorometer (Durham, NC). The measured absorption spectra from the samples were back calculated using a standard curve (Fig. S1E, ESI[†]).

The hydrogel swelling ratio M_f/M_i , where M_i and M_f were the mass of dried and hydrated hydrogel respectively, was measured by a gravimetric method.

Cell lines and EGF biofunctionality test

MTLn3 Mena^{Inv} rat mammary carcinoma cells (ref. 38) were cultured in α-MEM (Invitrogen Corporation) supplemented with 5% fetal bovine serum (FBS, Gemini Bio-Products), 0.2% NaHCO₃ (Sigma-Aldrich), along with penicillin/ streptomycin (Gibco/Invitrogen Corporation). Cells were cultured in an incubator at 37 °C with 5% CO₂ at ~60% humidity.

To test the biofunctionality of the EGF released from UV-cured hydrogel, an EGF up shift assay³⁷ was done with previously starved MTLn3 Mena^{Inv} cells. The amount of cell spreading due to the EGF was measured by tracing cell areas before and up to 4 min after stimulation with the EGF released from the hydrogel. Cells were plated on a tissue culture dish and grown overnight. Cells were then starved in L15 + 0.8% BSA starvation media for 3 h before the EGF stimulation. In parallel, 10 μ L of hydrogel blend (20% PEGDA and 10% PEGMA) was mixed and cured with 1–6 μ M EGF. Cured hydrogel was placed in 1.3 ml of L15 + 0.8% BSA starvation media for 3 h, resulting in conditioned media containing released EGF. Starved cells were then placed under a microscope (Nikon Corporation) on a heated plate (Tokai Hit Co., Ltd) at 37 °C and bright field images were collected for 5 min, 4 images/min, with the addition of conditioned media at 1 min.

MDA-MB-231 human mammary carcinoma cells were cultured in DMEM (Invitrogen Corporation) supplemented with 10% FBS (Atlanta Biologicals, Inc.), along with penicillin/streptomycin. Cells were cultured in an incubator at 37 °C with 5% CO₂ at ~60% humidity.

EGF release and gradient formation from the device

To measure the release of EGF from bonded devices, fluorescent R-EGF (Mw = 6.8 kDa, Invitrogen Corporation) was used in place of EGF (Fig. S1F, ESI[†]). Similar diffusion rates from the hydrogel blends were expected due to small differences in the molecular weights of EGF and R-EGF. The solution was then cured in the reservoir of the device. The device was then bonded and hydrated by low vacuum treatment (for 60 to 120 s) in the presence of L15 + 0.8% BSA starvation media to remove any air bubbles from the reservoir. Absence of air bubbles was confirmed using an optical microscope with 10 $\times$ magnification. Samples were collected at various time intervals and kept at –20 °C until the end of the experiment. Collected samples were then transferred into 96-well plates and measured using a microplate fluorometer.

R-EGF loading was also used to measure the gradient formation from the device. The device was placed in a plastic dish, hydrated in L15 + 0.8% BSA starvation media and transferred to a MatTek dish. A thin layer of mineral oil (Sigma-Aldrich) was cast on the top to reduce evaporation and device was imaged using a wide field fluorescent microscope (DeltaVision Core, at 37 °C, 10 $\times$ objective, TRITC filter). Fluorescence intensity at increasing distance from the device was converted into EGF concentration using a standard curve obtained by serial dilutions of R-EGF under the same conditions. Similar experiments were performed with devices embedded in 3D collagen (see below).

Cell migration assays in 2D

Cancer cells (MTLn3 Mena^{Inv}; MDA-MB-231 cells) were cultured in standard culture media overnight in 35 mm tissue culture or matrigel-coated MatTek dishes (MDA-MB-231). Prior to device introduction, MTLn3 Mena^{Inv} cells were starved in L15 + 0.8% BSA for 2–3 h; MDA-MB-231 cells were starved in L15 + 0.8% BSA + 0.5% FBS for 24 h. Devices containing EGF at concentrations of 1–5.5 μ M were hydrated at low vacuum and positioned next to the cells. The device outlet and cells in front of it were then imaged for 3–4 h, 30

images/h, using a dry 20× objective at 37 °C. A thin layer of mineral oil was cast on top of the media to reduce evaporation.

Cell migration in 3D through collagen

Cell migration in 3D was measured using a collagen invasion assay similar to the one described in ref. 19 with some modifications. MDA-MB-231-GFP cells (150 k) were cultured on a 10 mm MatTek dish overnight in culturing media. Cells were then starved in L15 + 0.8% BSA + 0.5% FBS starvation media for 6–8 h before the collagen mixture was added. Collagen mixture was made by neutralizing collagen I (BD Biosciences) with 5% NaHCO₃ and buffering with 1M HEPES. A drop of collagen was cast over cells and incubated for 5 min to form a 25 µm thick layer (Fig. 4A). Hydrated devices were placed in the center of the dish and covered with more collagen. Starvation media (L15 + 0.8% BSA + 0.5% FBS) was added 1 h later and 3D image stacks were taken at 0 h, 18 h and 48 h in front and far from the device. Imaging was done using a Leica SP5 confocal microscope with the following channels: 488 nm laser line and 500–535 nm emission window for MDA-MB-231-GFP cells; 543/555–600 nm for the beads marking the device.

***In vivo* invasion assays**—*In vivo* invasion assays (using microneedles) were conducted as described previously²². In brief, micro-needles were filled with a mixture of matrigel, starvation media and either no EGF, EGF in solution or EGF inside cured hydrogel. The ratio between the volume of the hydrogel (5 µl) and space inside the micro-needle (40 µl) was equal to the ratio inside the device (8 nl hydrogel in 65 nl total volume). Needles were placed into the tumor cortex of an anesthetized animal for 4 h. After removal of the needles, cells were expelled out to microscope slides, labeled with DAPI and counted using a wide field microscope with 20× magnification.

Dendra2 photoconversion assays

Photoconversion assays were done as described in ref. 38. Briefly, Dendra2-MTLn3 cells were injected into SCID mice to form xenografts tumors. Mammary imaging windows were implanted at day 17–19 after injection. After 3 days of recovery, anesthetized animals were injected with a mixture of matrigel, hydrogel loaded with EGF and fluorescent polystyrene beads at the edge of the mammary imaging window. Animals were immediately imaged, delineating injection site. Photoconversion was done (0 h) in two avascular [36] areas in each of the animals, one positioned close (< 500 µm) and the other one far (> 1 mm) from the injection site. One day later (24 h), imaging was repeated in the same areas.

Intravital imaging of the NANIVID

In order to facilitate the insertion of the device into animals, we have slightly modified the device into a pointed tip. Transgenic mice (MMTV-PyMT-cfms-GFP) were anesthetized and prepared for imaging using skin-flap surgery.⁶ Using a 5× magnification lens with a lamp, the device was inserted into the tumor and animal placed on the microscope. The device was traced in red channel using fluorescent beads, and the presence of cells in green the channel inside the tumor tissue.

Data processing and statistical analysis

All images were processed using ImageJ v1.41 with a customwritten ROI Tracker plug-in for tracking cell perimeters and centroid in time (Movies S1, S2, ESI[†]). Data (x , y , z coordinates, segment path length, total path length, velocity, and cell area) were further analyzed and plotted using Microsoft Excel 2007. The cell migration data were analyzed by determining vector plot, directionality and chemotaxis index. Statistical analysis was done using Mathematica 7 (Wolfram). A one-sided mean difference test (t -test) with a null hypothesis was used to determine statistical significance. Results were considered to be significant at $p < 0.05$. In addition, analysis of variance (ANOVA) on the basis of 95% confidence interval was used for comparisons between groups.

Conclusions

PEG-based molecules are commonly used in tissue engineering and controlled release studies and were used in this study as the source reservoir for the chemoattractant. The main component of the hydrogel was PEGDA which was mixed with PEGMA to fabricate a loose network of hydrogel blend, resulting in a more nano porous network of hydrogel material. This customized hydrogel was able to accommodate more solution in the swelling assay and released more chemoattractant relative to PEGDA alone because of the weak crosslinking and the fact that more hydrogel surface area was exposed resulting in the diffusion of adsorbed growth factor from deep inside the gel. In addition to the improved EGF release from this customized hydrogel system, the EGF was found to be biologically active. The cellular response toward the released EGF was measured by an EGF stimulation assay and a change in cell morphology was observed in the dose response experiments. The increase in the cell area was directly related to EGF loading in the hydrogel system and the amount of released EGF was increased as the growth factor loading was increased in the hydrogel. In the experiments to characterize the growth factor release from the NANIVID and the cell migration assays, more than 80% of the devices were intact after soaking and hydrating in the L15 + 0.8% BSA starvation media. The EGF release rate from the NANIVID was lower than that of the bulk sample because the growth factor was trapped inside the reservoir and only able to diffuse through one opening, thus reducing the release rate into the surrounding environment. The optimized design of the NANIVID was shown to controllably release R-EGF over multiple days. In the growth factor gradient experiments, the bonded device was able to create a reproducible gradient profile of R-EGF and a steady increase in the profile was observed in the course of the experiment. Small fluctuations in the intensity profile were observed in the experiment, which was most likely a result of external factors such as vibration and temperature gradient changes. In order to further extend the duration of release for long lasting gradients, the device design can be changed by either increasing its length to enhance the capacity of source chamber or adding additional source chambers to accommodate more hydrogel.

In the 2D cell migration assays, the migration of MTLn3-Mena^{Inv} cells in devices loaded with $>1.5 \mu\text{M}$ is directional, compared to the random walk in devices with no EGF, as illustrated by vector plots. The chemotaxis index plot showed that all loading concentrations of EGF induced statistically significant degree of chemotaxis. However, NANIVIDs with

loading concentrations 3–3.5 μM (where maximal EGF concentrations reach 4–5 nM in front of the device) show maximal directionality and chemotaxis index, while other concentrations show significantly lower response. This biphasic effect was observed in both directionality and chemotaxis index at similar concentrations, suggesting that the NANIVID loaded with 3.5 μM EGF creates an optimal EGF gradient *in vitro* 2D culture for the MTLn3-Mena^{Inv} cell type.

A critical component of the NANIVID is the chemoattractant reservoir housed in the back of the device which is capable of keeping EGF in its native state. The hydrogelloaded reservoir is able to release the EGF in a controlled manner over multiple days if the volume is optimized. ELISA was performed to quantify the released EGF from the reservoir, while R-EGF diffusion assay was carried out using the NANIVID to both visualize and quantify the chemical gradient created by the release of EGF from the device. The biofunctionality of the released EGF was verified by an up shift assay using MTLn3-Mena^{Inv} cancer cells. NANIVIDs were loaded with hydrogel containing EGF and were then characterized using the MTLn3-Mena^{Inv} cell line. The same designs of the NANIVID were also tested in a 3D collagen-I matrix. Stimulated by the EGF gradient, cells migrated in 3D toward the device. Due to its transparency, the device is compatible with optical and fluorescent microscopy used in combination with intravital imaging techniques to document *in vivo* chemotactic behavior. Therefore, the NANIVID should be useful *in vivo* in probing the chemotactic potential of tumor cells and other migratory cell types in solid tissue *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Dr Yubing Xie at the College of Nanoscale Science and Engineering for valuable discussions and access to her lab facility. This project was funded by NIH Grant: U54-CA126511 (TMEN) to JC and JSC, U01-1105490 (MMHCC) to JSC, and DOD Grant BC075554 to BG.

References

1. Schultz G, Rotatori DS, Clark W. J Cell Biochem. 1991; 45:346–352. [PubMed: 2045428]
2. Locascio A, Nieto MA. Curr Opin Genet Dev. 2001; 11:464–469. [PubMed: 11448634]
3. Nick JA, Coldren CD, Geraci MW, Poch KR, Fouty BW, O'Brien J, Gruber M, Zarini S, Murphy RC, Kuhn K, Richter D, Kast KR, Abraham E. Blood. 2004; 104:3878–3885. [PubMed: 15339848]
4. Orr FW, Adamson IY, Young L. Am Rev Respir Dis. 1985; 131:607–611. [PubMed: 3994156]
5. Yamaguchi H, Wyckoff J, Condeelis J. Curr Opin Cell Biol. 2005; 17:559–564. [PubMed: 16098726]
6. Wyckoff J, Wang W, Lin EY, Wang Y, Pixley F, Stanley ER, Graf T, Pollard JW, Segall J, Condeelis J. Cancer Res. 2004; 64:7022–7029. [PubMed: 15466195]
7. Toetsch S, Olwell P, Prina-Mello A, Volkov Y. Integr Biol. 2009; 1:170–181.
8. Rupprecht TA, Plate A, Adam M, Wick M, Kastenbauer S, Schmidt C, Klein M, Pfister HW, Koedel U. J Neuroinflammation. 2009; 6:42. [PubMed: 20042073]
9. Alge-Priglinger CS, Andre S, Kreutzer TC, Deeg CA, Kampik A, Kernt M, Schoffl H, Priglinger SG, Gabius HJ. Mol Vis. 2009; 15:2162–2173. [PubMed: 19898636]
10. Wells CM, Ridley AJ. Methods Mol Biol. 2005; 294:31–41. [PubMed: 15576903]

11. Mahadevan VS, Campbell M, McKeown PP, Bayraktutan U. *Cardiovasc Res.* 2006; 72:60–68. [PubMed: 16860294]
12. Soon L, Mouneimne G, Segall J, Wyckoff J, Condeelis J. *Cell Motil Cytoskeleton.* 2005; 62:27–34. [PubMed: 16025469]
13. Wang SJ, Saadi W, Lin F, Minh-Canh Nguyen C, Li Jeon N. *Exp Cell Res.* 2004; 300:180–189. [PubMed: 15383325]
14. Ainla A, Jansson ET, Stepanyants N, Orwar O, Jesorka A. *Anal Chem.* 2010; 82:4529–4536. [PubMed: 20443547]
15. Barkefors I, Le Jan S, Jakobsson L, Hejll E, Carlson G, Johansson H, Jarvius J, Park JW, Li Jeon N, Kreuger J. *J Biol Chem.* 2008; 283:13905–13912. [PubMed: 18347025]
16. Lin F, Nguyen CM, Wang SJ, Saadi W, Gross SP, Jeon NL. *Biochem Biophys Res Commun.* 2004; 319:576–581. [PubMed: 15178445]
17. Saadi W, Wang SJ, Lin F, Jeon NL. *Biomed Micro-devices.* 2006; 8:109–118.
18. Walsh CL, Babin BM, Kasinskas RW, Foster JA, McGarry MJ, Forbes NS. *Lab Chip.* 2009; 9:545–554. [PubMed: 19190790]
19. Goswami S, Sahai E, Wyckoff JB, Cammer M, Cox D, Pixley FJ, Stanley ER, Segall JE, Condeelis JS. *Cancer Res.* 2005; 65:5278–5283. [PubMed: 15958574]
20. Mousseau Y, Leclers D, Faucher-Durand K, Cook-Moreau J, Lia-Baldini AS, Rigaud M, Sturtz FG. *BioTechniques.* 2007; 43:509–516. [PubMed: 18019343]
21. Abhyankar VV, Toepke MW, Cortesio CL, Lokuta MA, Huttenlocher A, Beebe DJ. *Lab Chip.* 2008; 8:1507–1515. [PubMed: 18818806]
22. Wyckoff JB, Segall JE, Condeelis JS. *Cancer Res.* 2000; 60:5401–5404. [PubMed: 11034079]
23. Wang W, Wyckoff JB, Wang Y, Bottinger EP, Segall JE, Condeelis JS. *BMC Biotechnol.* 2003; 3:13. [PubMed: 12914671]
24. Wang W, Goswami S, Lapidus K, Wells AL, Wyckoff JB, Sahai E, Singer RH, Segall JE, Condeelis JS. *Cancer Res.* 2004; 64:8585–8594. [PubMed: 15574765]
25. Wang W, Wyckoff JB, Goswami S, Wang Y, Sidani M, Segall JE, Condeelis JS. *Cancer Res.* 2007; 67:3505–3511. [PubMed: 17440055]
26. Patsialou A, Wyckoff J, Wang Y, Goswami S, Stanley ER, Condeelis JS. *Cancer Res.* 2009; 69:9498–9506. [PubMed: 19934330]
27. Barrett DG, Yousaf MN. *Molecules.* 2009; 14:4022–4050. [PubMed: 19924045]
28. Park CW, Rhee YS, Park SH, Danh SD, Ahn SH, Chi SC, Park ES. *Arch Pharmacol Res.* 2010; 33:427–432.
29. de Las Heras Alarcon C, Pennadam S, Alexander C. *Chem Soc Rev.* 2005; 34:276–285. [PubMed: 15726163]
30. L M, Wang Lu, Gao Chunmei, Ma Liwei, Cui Dapeng. *React Funct Polym.* 2010; 70:159–167.
31. Suedee R, Jantararat C, Lindner W, Viernstein H, Songkro S, Srichana T. *J Controlled Release.* 2010; 142:122–131.
32. Mellott MB, Searcy K, Pishko MV. *Biomaterials.* 2001; 22:929–941. [PubMed: 11311012]
33. Cuchiara MP, Allen AC, Chen TM, Miller JS, West JL. *Biomaterials.* 2010; 31:5491–5497. [PubMed: 20447685]
34. Raja WK, Gligorijevic B, Padgen MR, Eggers D, Condeelis J, Castracane J. *Microfluidics, BioMEMS, and Medical Microsystems VIII.* 2010
35. Raja WK, Gligorijevic B, Condeelis JS, Castracane J. *Microfluidics, BioMEMS, and Medical Microsystems VII.* 2009
36. Sidani M, Wessels D, Mouneimne G, Ghosh M, Goswami S, Sarmiento C, Wang W, Kuhl S, El-Sibai M, Backer JM, Eddy R, Soll D, Condeelis J. *J Cell Biol.* 2007; 179:777–791. [PubMed: 18025308]
37. Philippar U, Roussos ET, Oser M, Yamaguchi H, Kim HD, Giampieri S, Wang Y, Goswami S, Wyckoff JB, Lauffenburger DA, Sahai E, Condeelis JS, Gertler FB. *Dev Cell.* 2008; 15:813–828. [PubMed: 19081071]

38. Kedrin D, Gligorijevic B, Wyckoff J, Verkhusha VV, Condeelis J, Segall JE, van Rheenen J. Nat Methods. 2008; 5:1019–1021. [PubMed: 18997781]
39. Raja WK, Cady NC, Castracane J, Gligorijevic B, Rheenen Jv, Condeelis JS. Imaging, Manipulation, and Analysis of Biomolecules, Cells, and Tissues VI. 2008

Abbreviations

BSA	Bovine Serum Albumin
CSF-1	Colony Stimulator Factor 1
C.I.	Chemotactic Index
EGF	Epidermal growth factor
ELISA	Enzyme-Linked ImmunoSorbant Assay
FBS	Fetal Bovine Serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
NANIVID	NANo IntraVital Imaging Device
PBS	Phosphate Buffering Solution
PDMS	Polydimethylsiloxane
PEGDA	Polyethylene glycol diacrylate
PEGMA	Methoxypolyethylene glycol monoacrylate
R-EGF	Rhodamine labeled EGF
SEM	Scanning electron microscopy
UV	Ultra Violet light

Insight, innovation, integration

Current chemotaxis assays and microfluidic devices do not have the flexibility for use in 2D and 3D, and both *in vitro* and *in vivo*. We have combined microphotolithography, polymer chemistry and microfluidics to design a chemotaxis device that has the flexibility to be used beyond the capabilities of current chemotaxis devices. It is also autonomous and has the potential for use *in vivo*. New biological insights about the role of chemotaxis in embryonic development and disease progression will be possible with such autonomous devices.

Fig. 1.

Device design and fabrication: (A) microfabrication process flow of the device chamber fabrication in cross-section view. (B) Device bonding steps. Phase 1 includes cleaning of bottom and top and coating of top cover with PDMS. Phase 2 includes loading of the source chamber with hydrogel (green) and sealing with the top cover. (C) Schematic of device with top cover opened in front of cancer cells, showing the source chamber, chemoattractant reservoir and outlet from which EGF diffuses. (D) Top view of the device with the map of different chambers (source chamber, front chamber, obstructions, anchors and outlet). Insert shows magnified fluorescent image of the source chamber loaded with R-EGF hydrogel blend. (E) Top view of the device loaded with fluorescent R-EGF in hydrogel. Images present the device before (left) the hydration, 5 min after hydration (middle) and 100 min after hydration (right).

Fig. 2.

Visualization of the finished device and EGF release measurements with increasing distance and time: (A) Top view of the device in bright-field microscopy. Arrows point to the device outlet. White line marks the source chamber for hydrogel loading. (B) Fluorescent micrograph of the same device shows the front end of the device coated with polystyrene fluorescent beads. White arrows indicate the position of the device outlet. (C) Fluorescent micrograph of the device loaded with R-EGF which can be seen diffusing out. Image was taken at 2 h after the device was hydrated. White line indicates the length of the intensity profile in (D), dashed white box indicates area of measurement in (E). (D) Gradient profile of EGF at increasing distance from the device outlet. Measurements were done at 0 h, 2 h (dotted line), 3 h (full line) and 4 h (dashed line) after device hydration, along the white line from panel (C). Insert represents standard curve to convert fluorescence into R-EGF concentration. (E) Average concentration of R-EGF measured over 6 h after device hydration inside the rectangular area ($100 \times 200 \mu\text{m}$) shown in (C)

Fig. 3.

Device induces a chemotactic response in 2D cell culture conditions: (A) experimental setup and representative results. Device is positioned inside the dish at $t = 0$ and images were collected for 4 h. White arrows indicate total path lengths (for paths, see Movie S1, ESI[†]) and directions of cells in front of the device at the end of the experiment. For clarity, paths of cells which left the field of view, divided and detached from the surface were excluded and device is not shown to scale. On the right, gradient map is shown illustrating EGF concentration at the cellular level. (B) Representative vector plots of MTLn3 Mena^{Inv} cells exposed to increasing concentrations of EGF (0–5.5 μM in loaded in the device). (C) Directionality (left) and chemotaxis index (cos θ , right) of cell migration toward the devices. Bars represent the mean data ($\pm$ s.d.) from 3 experiments, 6–19 cells in each. Maximal effects are indicated by a star ($p < 0.05$).

Fig. 4.

Device induces cell migration along the EGF gradient in 3D collagen: (A) Experimental setup for measuring chemotactic effect in 3D. MDA-MB-231-GFP cells were cultured on a MatTek dish and overlaid with a thin layer of collagen (25 μm), device, and another layer of collagen (500 μm). Collagen was finally topped with media low in EGF (L15 + 0.8% BSA + 0.5% FBS). A stable EGF gradient forms over time in 3D as schematized by the curved lines. (B) R-EGF gradient with increasing distance from the outlet, measured in 3D collagen at 2 h after hydration. Insert illustrates gradient distribution from the outlet into 3D collagen using 16-color look-up-table (LUT, legend included). (C) Top image is a representative 3D stack (maximum projection, $z = 0\text{--}40\text{ }\mu\text{m}$) acquired at the end of the experiment (48 h) with a 5 μM loaded device. Cells (green) are polarized towards the device (red fluorescent beads point to device outlet). Bottom images represent xz views of the same dish at 0 h and 48 h, clearly showing cell migration through collagen above a yellow line. Above the bottom of the glass surface. (D) Increase in total number of cells over 18 h or 48 h within the measurement volume ($600 \times 600 \times 40\text{ }\mu\text{m}$) in front of the devices loaded with 0, 5, 6 or 10 μM EGF. Bars represent mean data $\pm$ s.e.m. from 3–10 separate experiments. Same images were used for (E). (E) Relative number of cells which have migrated inside the collagen above the 15 μm yellow line in C. Significant effects are indicated by one ($p < 0.05$) or two stars ($p < 0.01$).

Fig. 5. Device chemoattractant source induces cell migration along the EGF gradient *in vivo*: (A) Comparison of the number of cells collected into microneedles in the *in vivo* invasion assay with either EGF or hydrogel loaded with EGF as the chemoattractant. (B,C) Photoconversion and further tumor cell migration at different distances from the site of injection of hydrogel loaded with EGF; (B) Area was photoconverted >1 mm from the injection site. (C) Photoconversion area was <500 μ m from the injection site (yellow star). Scale 50 μ m.