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3D Analysis of Multi-cellular Responses to Chemoattractant Gradients

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Dr. Ronald Myers

Editorial Office

JoVE

Dear Dr. Myers,

Following your invitation, we are pleased to submit the attached the manuscript "Convenient method to build a 3D culture device for studying cellular sensing to chemoattractant gradient" for your consideration. This manuscript describes in detail the method to build an in-vitro platform for incorporating cells into a controlled 3D environment permitting introduction of spatially graded and dynamically controlled gradients of chemoattractants and growth factors. This method will be helpful to anyone interested in studying cellular behavior in 3D, with a great potential to be used for various investigations of single cells and small tissue-like samples in complex biomimetic environments. My co-authors and I look forward to assisting you during the review process, and to sharing this protocol with the readers of the JoVE. Should you have any questions, please let me know. I look forward to hearing from you.

Thank you very much for considering our paper.

Yours (on behalf of all authors),

Andre Levchenko

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TITLE:

3D Analysis of Multi-cellular Responses to Chemoattractant Gradients

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KEYWORDS:

gradient sensing, organs-on-a-chip, chemotaxis, stereolithography, extracellular matrix, development, organoids

SUMMARY:

We describe a method to construct devices for 3D culture and experimentation with cells and multicellular organoids. This device allows analysis of cellular responses to soluble signals in 3D microenvironments with defined chemoattractant gradients. Organoids are better than single cells at detection of weak noisy inputs.

ABSTRACT:

Various limitations of 2D cell culture systems have sparked interest in 3D cell culture and analysis platforms, which would better mimic the spatial and chemical complexity of living tissues and mimic in vivo tissue functions. Recent advances in microfabrication technologies have facilitated the development of 3D in vitro environments in which cells can be integrated into a well-defined extracellular matrix (ECM) and a defined set of soluble or matrix associated biomolecules. However, technological barriers have limited their widespread use in research laboratories. Here, we describe a method to construct simple devices for 3D culture and experimentation with cells and multicellular organoids in 3D microenvironments with a defined chemoattractant gradient. We illustrate the use of this platform for analysis of the response of epithelial cells and organoids to gradients of growth factors, such as epidermal growth factor (EGF). EGF gradients were stable in the devices for several days leading to directed branch formation in breast organoids. This analysis allowed us to conclude that collective gradient sensing by groups of cells is more

sensitive vs. single cells. We also describe the fabrication method, which does not require photolithography facilities nor advanced soft lithography techniques. This method will be helpful to study 3D cellular behaviors in the context of the analysis of development and pathological states, including cancer.

INTRODUCTION:

In physiological environment, cells are embedded in an extracellular matrix (ECM) and exposed to a plethora of biomolecules. Interactions between cells and the surrounding microenvironment regulate intracellular processes controlling diverse phenotypes, including migration, growth, differentiation and survival[1,2]. Much has been learned about cellular behaviors in a conventional 2D cell culture. However, with the advent of intravital imaging and experimentation with cells embedded in 3D hydrogels, important differences in cell behaviors have been recognized in the simplified 2D in vitro cultures vs. 3D tissue-like environments. While cells interact with ECM fibers and sense their mechanical properties within the 3D matrix, the material stiffness of the gel is not a fully independent variable in a 2D in vitro system. The dimensionality alters focal adhesion formation, resulting in different cell morphology and behavior. Furthermore, cells on a 2D surface are exposed to fewer signaling cues than cells open to all directions in 3D.

These limitations have increased the interests for 3D systems that represent the spatial and chemical complexity of living tissues and better predict in vivo tissue functions. They have been developed in many forms from organoids as self-assembling cellular microstructures to cells randomly interspersed in ECM[3,4]. Recent advances in microfabrication technologies have facilitated the advent of various types of 3D culture systems[5-9] for studying phenotypic changes and cellular responses to soluble signals; however, technological barriers limit the widespread use in research laboratories. In many cases, the fabrication processes require photolithography techniques and background knowledges for soft lithography. Moreover, various factors must be controlled to successfully build a device and to achieve an optimal function of the device over a long period of time.

Our method describes how to construct a 3D PDMS device for incorporating cells and multicellular organoids into a 3D microenvironment with defined chemoattractant gradients and then analyze epithelial responses to EGF[10]. Our data reveal that the capacity of organoids to respond to shallow EGF gradients arises from intercellular chemical coupling through gap junctions. It suggests the potential of organoids for more precise detection of weak and noisy spatially graded inputs. The fabrication process does not require a cleanroom facility nor photolithography techniques. However, the 3D PDMS device includes necessary factors of 3D physiological environment. This method will be helpful to study 3D cellular behaviors and it has great research potential with different cell types, chemoattractants, and ECM combinations.

PROTOCOL:

All animal work was conducted in accordance with protocols reviewed and approved by the Institutional Animal Care and Use Committee, Johns Hopkins University, School of Medicine.

1. Fabrication of the mesofluidic device

1.1. Design the mask of the mold for PDMS device using a 3D CAD software.

1.2. Print the mold using stereolithography equipment with a thermal resistant resin.

NOTE: The procedures described here were carried out by a commercial 3D printing service.

1.3. Mix thoroughly a PDMS monomer solution with the curing agent in a 10:1 ratio. 3 mL of PDMS mixture was required for fabrication of the device. The total volume depends on the number of the molds to be fabricated.

1.4. Degas the mixture by applying a vacuum with the use of an in-house laboratory vacuum or vacuum pump in a vacuum desiccator for 1 hour.

1.5. Dab the surface of the mold with an adhesive tape to remove dust, and then pour the PDMS mixture to the mold. If bubbles are introduced in the process, repeat degassing in the vacuum desiccator. Bubbles trapped between pillars of the mold can be removed by poking them with a sharp needle.

NOTE: The degassing process at room temperature should be done within 1 hour or less**.**

1.6. Cure the PDMS by heating at 80 °C for 2 hours. Allow the mold to cool down at room temperature.

1.7. Cut the boundary between the mold and PDMS with a blade and then peel off PDMS from the mold carefully.

1.8. Trim the PDMS part to fit the 22 mm x 22 mm coverslip and punch a hole at the inlet.

NOTE: The protocol can be paused here.

1.9. Clean the PDMS part and 22 mm x 22 mm coverslip by wiping the surfaces with low-lint tissues and 70% ethanol. Then remove large dust particles by dabbing with an adhesive tape.

1.10. Sterilize the PDMS part and coverslip by either autoclaving (121 °C for 8 minutes wet, 15 minutes dry) or exposure to UV light for 1 hour.

1.11. Treat the bottom surface of the PDMS part and cover slip with corona discharge gun for 5 min in the tissue culture hood and then bond them together.

CAUTION: Lay any non-conducting material such as polystyrene foam on the bottom and remove all conducting materials during this process. Inject collagen into the device immediately, within 5 minutes, after the treatment to increase the bonding between collagen gel and glass coverslip.

2. Cell preparation: primary mammary organoid isolation

NOTE: The details of mammary organoid isolation can be found in a previous work[11]. Any kind of single cell or organoid can be prepared according to their own isolation/detachment protocols.

2.1. Mince the mammary gland tissue of the mice with a scalpel until the tissue relaxes and shake it for 30 min at 37 °C in 50 mL of collagenase/trypsin solution (10 mL per mouse) in DEME/F12 supplemented with 0.1 g of trypsin, 0.1 g of collagenase, 5 mL of FBS, 250 µL of 1 µg/mL insulin, and 50 µL of 50 µg/mL gentamicin.

2.2. Centrifuge the collagenase/trypsin solution at 1,250 x *g* for 10 min. Remove the supernatant by aspiration. Disperse cells in 10 mL of DMEM/F12, and centrifuge at 1,250 x *g* for 10 min. After removing DMEM/F12 by aspiration, re-suspend cells in 4 mL of DMEM/F12 supplemented with 40 µL of DNase (2 units/µL).

2.3. Shake the DNase solution by hand for 2-5 min and then centrifuge at 1,250 x *g* for 10 min.

2.4. Separate organoids from single cells through four differential centrifugations (pulse to 1,250 x *g* and stop the centrifuge 3-4 s after it reaches the intended speed). Between each pulse, aspirate the supernatant and resuspend the pellet in 10 mL of DMEM/F12.

2.5. Re-suspend the final pellet in the desired amount of growth medium of DMEM/F12 with 1% penicillin/streptomycin and 1% insulin- transferrin-selenium-X for collagen preparation.

3. Collagen preparation and injection

NOTE: Other types of ECM gels can be prepared according to their own gelation protocols.

3.1. Prepare collagen type I solution (3.78 mg/mL), 10x DMEM, 1 N NaOH solution, normal growth medium on ice.

NOTE: Keep on ice until the procedure is completed.

3.2. Add 6 µL of 1 N NaOH solution, 200 µL of growth medium and 50 µL of 10x DMEM into a pre-chilled microcentrifuge tube and mix the solution thoroughly.

3.3. Add 425 µL of collagen into the pre-mixed solution and mix thoroughly by pipetting.

3.4. Centrifuge the collagen mixture briefly (less than 5 seconds) to separate and remove bubbles from the mixture.

3.5. Keep the neutralized collagen solution on ice for 1 hour to induce fiber formation.

3.6. Add 50 µL of cell suspension into the collagen mixture and mix thoroughly.

NOTE: The final collagen concentration is 2 mg/mL.

3.7. Inject the solution using 200 µL pipet through the inlet of the PDMS device until the whole chamber is filled in. If the collagen mixture overflows through the gaps of pillars, cut out the excess collagen gel with sharp surgical blade after gelation.

3.8. Keep the device in the incubator at 37 °C with 5% CO_2 for 1 hour to induce gelation.

3.9. Fill the reservoirs on both sides with growth medium and keep the device in the incubator at 37 °C with 5% CO2 until confocal imaging is performed.

4. 3D imaging and quantification

4.1. Install a live-cell imaging culture chamber to a confocal microscope and pre-set the temperature and CO_2 to 37 °C and 5%, respectively. To get humidity up, add water in the reservoir of the chamber and place wet wipes in the chamber if necessary.

4.2. Place the PDMS device in the chamber and add EGF to one of the reservoir as a 'source' of the EGF in the gradient formation. The other reservoir will serve a 'sink' needed for development of the spatially graded EGF distribution. 2.5 nM of EGF was added in the sink for making the gradient of 0.5 nM/mm in this study.

4.3. Set the range of Z-stack covering the volume of organoids of interest and start the imaging.

CAUTION: To avoid phototoxicity, try a lower laser intensity in confocal imaging or use multi-photon imaging techniques.

4.4. Reconstruct a 3D image from 2D image stacks using either commercial software or a custom-made program. Measure the length and angle of branches extending from the organoids or migration of individual cells. Here, perform quantification by drawing a freehand outline around the organoid body using ImageJ.

REPRESENTATIVE RESULTS:

EGF is an essential regulator of branching morphogenesis in mammary glands and a critical chemoattractant guiding the migration of breast epithelial cells in invasive cancer growth. We used the mesoscopic fluidic devices described above to study the response of cells to defined EGF gradients (**Figure 1A,B**)[10]. The device yields a culture area 5 mm wide, 10 mm long, and 1 mm tall. The sides of the culture area are separated from open wells by hexagonal pillars, which are used to trap the liquid ECM and cell mixture within the cell culture area through surface tension. We note that, given the size of this 3D culture, it would be very difficult, expensive, and time consuming to construct an appropriately sized mold by photolithography[10]. By using a novel application of a standard technique, stereolithography, we quickly and inexpensively produced

the mold. Moreover, by modifying the design, an exponential gradient (**Figure 1C**, i) or multiple linear gradients in a chip (**Figure 1C**, ii) can be induced as well as a stable gradient with continuous flow (**Figure 1C**, iii).

Both in silico (**Figure 2A,B,C**) and in vitro (**Figure 2D,E,F**) tests demonstrated the formation of a stable linear EGF gradient across the cell culture area which lasted for approximately two days without replenishing the 'source' and 'sink' reservoirs. The time-dependent diffusion of the proteins was simulated using a commercialized multiphysics software. The 3D geometry of the chamber configuration was recreated on the software and the average concentration within the chamber volume was determined and plotted. These results suggested that EGF, a 6.4 kDa protein, can form a stable diffusion-based gradient within the collagen gel prepared as described above over a relatively short period of time. We also determined that similar profiles of proteins of 1, 10, and 150 kDa could be formed using this method.

Mammary organoids formed multiple branches in the presence of spatially uniform 2.5 nM EGF and the branch formation displayed no directional bias over 3 days (**Figure 3A,D**). However, if EGF was added in the form of a linear gradient of 0.5 nM/mm, branch formation displayed a significant directional bias (**Figure 3B,E**). However, we detected no such bias for single cells in the same gradient. In order to determine if multicellular communication was necessary for the gradient response, we explored if blocking intercellular gap junctions with various inhibitors would prevent the biased branch formation. The gap junction inhibitors indeed suppressed the ability of the organoids to respond to the gradient (**Figure 3C,F**). As explored more extensively in the original study describing this method[10], this result supports the hypothesis that the communication through gap junctions is necessary for the EGF gradient response in mammary organoids[10].

FIGURE & TABLE LEGENDS:

Figure 1. Mesofluidic device with defined EGF gradient. (A) 3D view of PDMS chip: (i) top view and (ii) front view of a mold (unit: mm) (iii) an array of molds (red) filled with PDMS (transparent) (iv) PDMS chambers pilled off from the mold (v) a schematic diagram of an assembled device (vi) a real picture of the device filled with collagen gel and culture medium. **(B)** A schematic diagram of the mesofluidic chamber with organoids embedded in a collagen gel and reservoirs of high (red) and low (red) EGF concentration resulting in EGF gradients (This figure has been modified from[10]). **(C)** Potential designs for inducing an exponential gradient (i), four different linear gradients in a chip (ii), and a stable gradient with continuous flow (iii). Red regions indicate sources or sinks filled with culture medium and grey regions indicate chambers filled with gel/cells mixture.

Figure 2. Evaluation of ligand gradients across the chip. (A) Simulation of ligand concentration across the chip showing gradient profiles. Simulated gradient profiles of **(B)** 1 kDa protein and **(C)** 70 kDa protein. **(D)** Image of the device with a linear gradient of 10 kDa Dextran Blue used to visualize the diffusion of EGF. Hexagonal dotted lines indicate pillars at the side of gel area.

Experimental gradient profiles of 1, 10 and 150 kDa dextran across the chip (This figure has been modified from Ellison et al. 2016[10]) **(E)** after 8 hours and **(F)** after 48 hours.

Figure 3. Cellular response to EGF gradient. (A) Non-directional branching of organoids in collagen with a uniform 2.5 nM EGF. **(B)** Directional branching of organoids in collagen with a linear gradient of 0.5 nM/mm of EGF. **(C)** Non-directional branching of organoids in a linear gradient of 0.5 nM/mm of EGF with a gap junction blocker. **(D-F)** Angular histograms of organoid branching directions corresponding to (A)-(C), respectively.

DISCUSSION:

The fabrication of PDMS molds was performed using a commercial 3D printing service, but can also be accomplished by a high end 3D printer in-house. Among various 3D fabrication methods, stereolithography is recommended for high resolution mold generation. Because PDMS curing occurs at a high temperature (80 °C), the materials should be sufficiently thermally resistant, which should be explicitly specified, if printing is outsourced. A thermal post-cure can be discussed with the printing service company to increase the thermal resistance of the part. The details of the printing service and materials are specified in the **Table of Materials**.

The mechanical property of cured PDMS depends on the curing temperature. If the PDMS mixture is kept at room temperature for a long time before curing in an oven, it becomes too elastic even after the complete curing. Thus, the degassing process at room temperature should be done within 1 hour or less. Degassing refers to a cyclic application of vacuum that can help remove any micro-bubbles within PDMS.

The pH of the collagen solution is critical not only for gelation but also for cell viability. If collagen is not neutralized before mixing with cells, the cell viability will be low. The amount of 1 N NaOH solution for neutralizing the collagen mixture depends on the pH of the original collagen solution and it can be calculated theoretically. However, it is recommended to add the NaOH solution to the collagen solution gradually, checking the pH readings of the medium using phenol red indicator or using pH testing tapes.

Degassing the gel is important throughout the entire process. If bubbles form during gelation after the collagen mixture is injected into the device, they can be also removed by placing the device on ice for 10 min or more. By dropping the temperature, the size of bubbles reduces and the voids are eventually occupied by the gel mixture. The same method can be used when bubbles are trapped between pillars after adding culture medium to the reservoirs.

We used this technology to create mesofluidic devices that permit embedding of large multi-cellular constructs into a physiologically relevant extracellular matrix environment and integrating it with defined gradients of growth factors and other soluble bioactive molecules. High resolution stereolithography allowed us to produce high-quality, low-cost molds. The fabrication process does not require cleanroom facilities nor photolithography techniques, and thus allows a simple but effective generation of a 3D environment for cell culture and experimentation, which has multiple advantages vs. the traditional 2D experimentation. The

device illustrated here was designed to have a culture area of 5 mm wide, 10 mm long, and 1 mm tall. The relatively large culture area with thicker height permits growth of mesoscopically large organoids or spheroids, ranging in size from tens to hundreds of micrometers. It also permits the analysis of branched epithelial morphogenesis, and analysis of collective and single cell migration within the 3D environment. The sides of the device are open wells that allow the use of standard pipettes to change media or to add drugs without the need for complicated interfacing with the liquid control devices, usually used for mainstream microfluidic devices. Moreover, the simple configuration of a PDMS chamber and a cover glass at the bottom is universally compatible with diverse microscopic systems. The use of the devices showed the biased branching direction of morphogenesis in normal breast tissue, in response to the imposed EGF gradient as a representative example for the usage of the device. However, this usage can also be extended to different tissues, cell sources, chemoattractants, and drugs for the analysis of cell/tissue behavior in 3D. The use also can be extended to accommodate more realistic tissue modeling, which would accommodate vascular network formation from individual cells within the gel along with incorporating of other cell types, including mesenchymal stromal and immune components and diverse epithelial cell types.

Although stereolithography offers the finest resolution among 3D printing techniques, the minimum feature size is still limited to several tens of micrometers. Therefore, this protocol is not sufficient to replace photolithography in fabricating molds at a scale of several micrometers. Another limitation of this protocol is the relatively low depth of high quality imaging in the z direction compared to the x-y direction, which is an inherent issue of imaging tissue-scale constructs, using conventional microscopy. However, even with these limitations, the described method can provide a powerful and flexible analysis platform that is easy to fabricate and use.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure1

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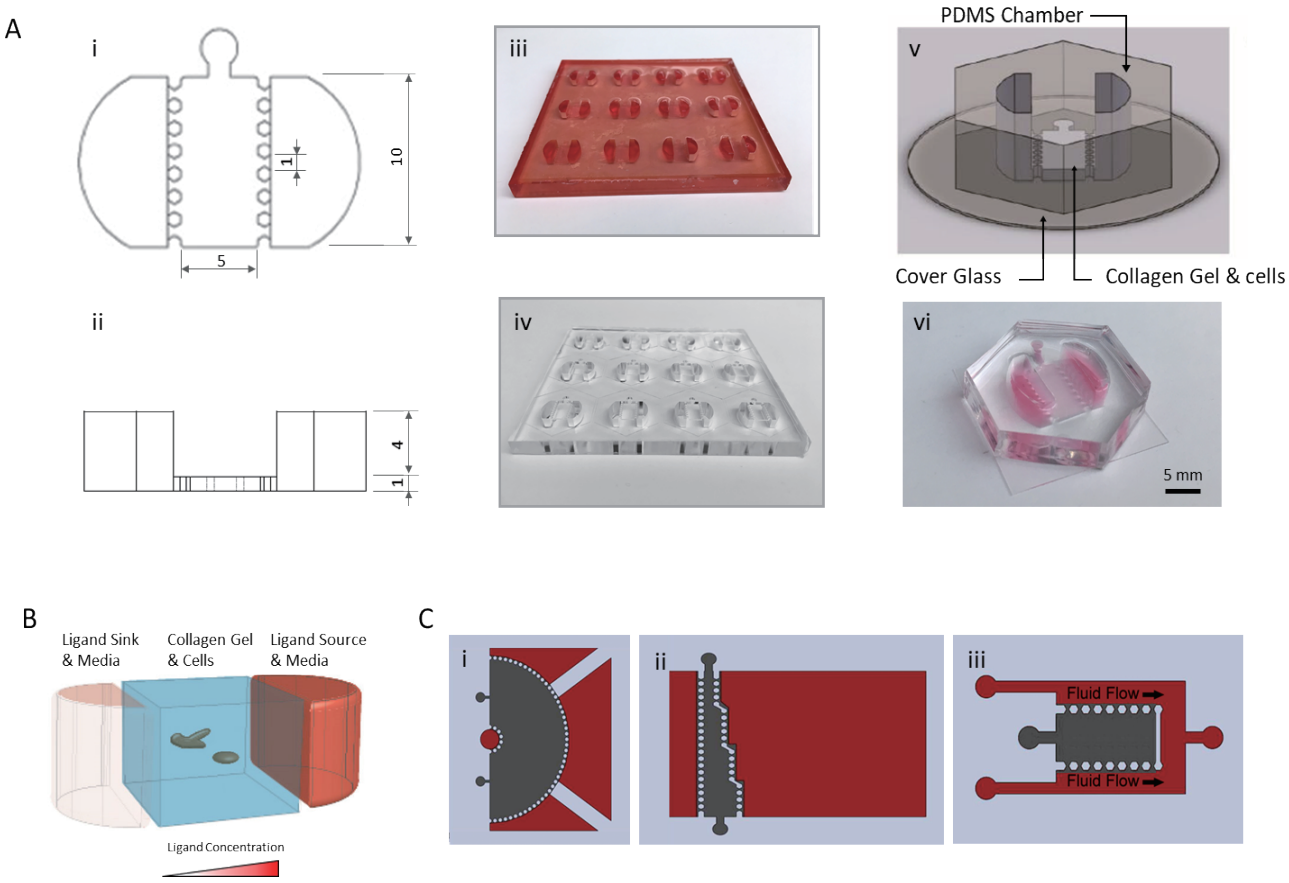

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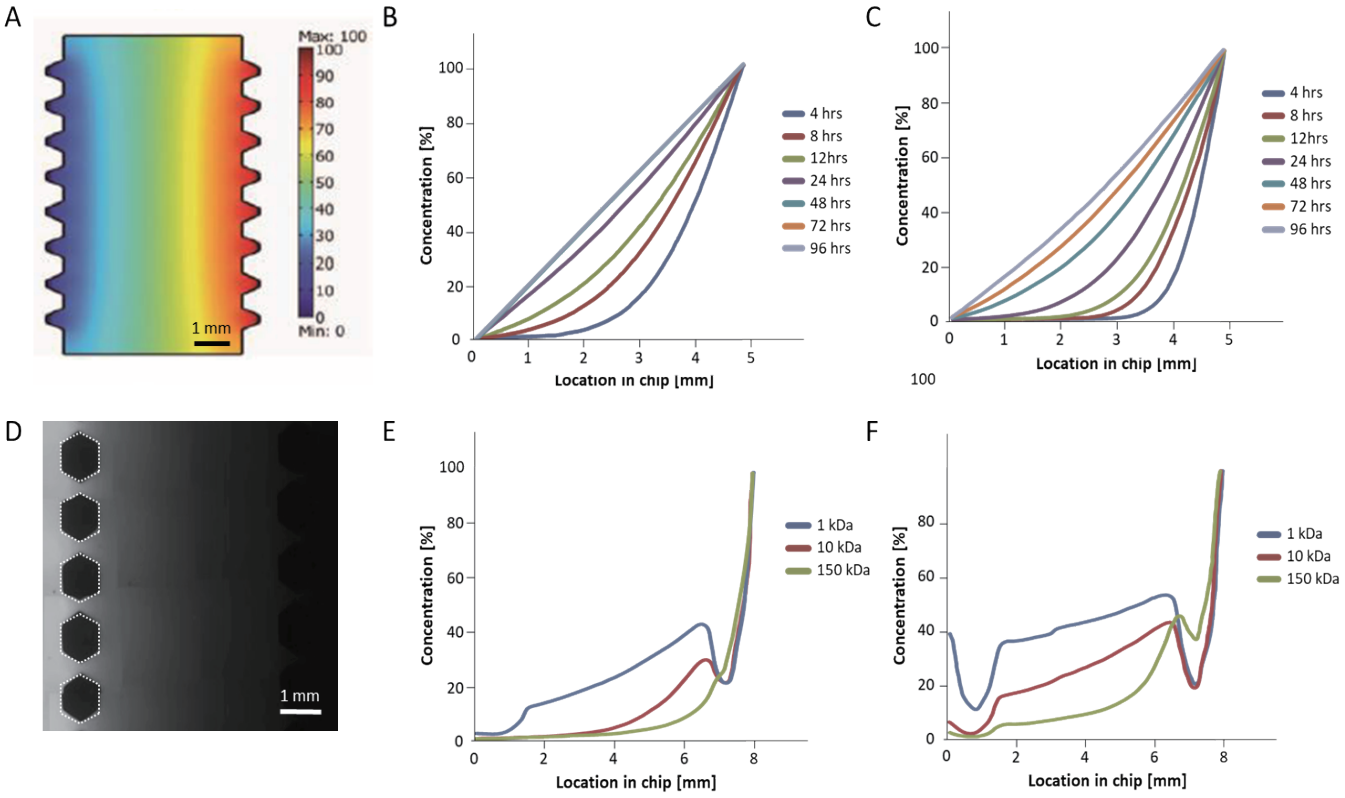

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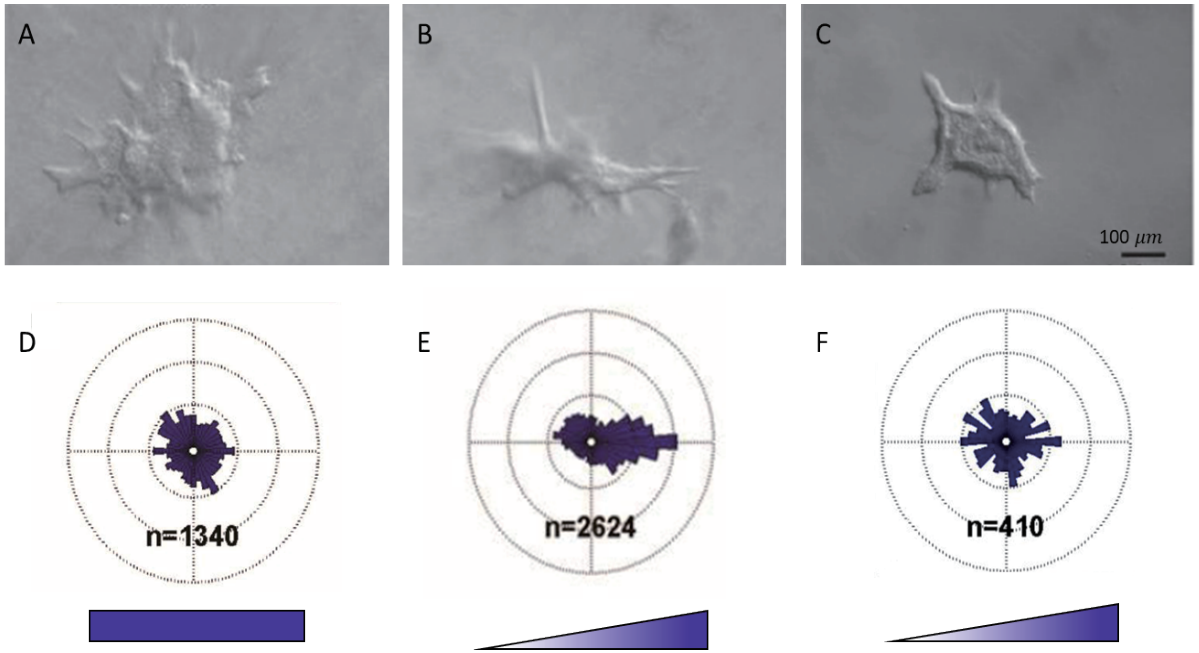

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
22mm x 22mm coverslip	Fisher Scientific	12-542-B	
Collagen I, Rat	Fisher Scientific	CB-40236	
Collagenease	Sigma-Aldrich	C5138	
COMSOL Multiphysics 4.2	COMSOL Inc		Used for simulating diffusion dynamics
10x DMEM	Sigma-Aldrich	D2429	
DEME/F12	Thermo Fisher	11330032	
DNase	Sigma-Aldrich	D4623	
EGF Recombinant Mouse Protein	Thermo Fisher	PMG8041	
Fetal Bovine Serum (FBS)	Life technologies	16140-071	
Fiji-ImageJ			Used for measuring branching length and angles
Gentamicin	GIBCO	5750-060	
IMARIS	Bitplane		
Insulin	Sigma-Aldrich	19278	
Insulin-Transferrin-Selenium-X	GIBCO	51500	
Low-lint tissue	Kimberly-Clark Professional	Kimtech wipe	
Mold Material	Proto labs	Accura SL5530	
Mold printing equipment	Proto labs	Stereolithogrphy	Maximum dimension: 127mm x 127mm x 63.5mm, Layer thnickness: 0.0254mm
Mold printing Service	Proto labs	Custom	https://www.protolabs.com/
NaOH	Sigma-Aldrich	S2770	
Penicillin/Streptomycin	VWR	16777-164P	
Spinning-disk confocal microscope	Solamere Technology Group		
Sylgard 184	Electron Microscopy Sciences	184 SIL ELAST KI	PDMS kit
Trypsin	Sigma-Aldrich	T9935	

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Author(s): Tae-Yun Kang, David Ellison, Sung Hoon Lee, Andrew J. Ewald, Andre Levchenko

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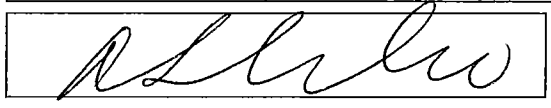
Article Title: Convenient method to build a 3D culture device for studying cellular sensing of chemoattractant gradients

Signature:  Date: 10/10/2018

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Responses to the Editorial Comments

Article ID:	JoVE59226
Title:	An easy-to-implement method for 3D analysis of multi-cellular response to chemoattractant gradients
Author:	Tae-Yun Kang, David Ellison, Sung Hoon Lee, Andrew J. Ewald, Andre Levchenko

We appreciate the constructive comments of the reviewer. The manuscript has been revised based on these comments. Please find our specific responses to the referee's comments below.

Summary of response:

We have responded to the comments and revised the manuscript accordingly.

Editorial Comment 1:

1. Note that all text written in Equation Editor will be formatted differently from the rest of the text; it is therefore advised that all inline text is formatted as 12 pt Calibri.

Answer: All inline text has been formatted as 12 pt Calibri.

Editorial Comment 2:

2.1: How is the mammary gland tissue obtained, the same way as in reference 11?

Answer: The same method was used in the reference 11. We have added the note mentioning that the details can be found in the reference.

[Section related to the response]: Protocol

2. Cell preparation-Primary mammary organoid Isolation[11] (**Note 1**: The details of mammary organoid isolation can be found in the reference[11], **Note 2:** Any kind of single cells or organoids can be prepared according to their isolation/detachment protocols)

Editorial Comment 3:

4.4: Which software are you using here? It's not apparent if it's in the Table of Materials. Also, how are lengths and angles measured?

Answer: Fiji-ImageJ was used for the quantification. We have added the explanation in

the protocol and specified the software name in the table of materials.

[Sentence related to the response]: Protocol

4.4. Reconstruct a 3D image from 2D image stacks using either commercial software or custom made program. Measure the length and angle of branches extending from the organoids or migration of individual cells. Here, the quantification was performed by drawing a freehand outline around the organoid body using an open source image processing program..

Editorial Comment 4:

Results/Figure 2A-C: How was the simulation here done? This portion of the Figure doesn't appear to be from reference 10. Please include at least a brief explanation.

Answer: We have added a brief explanation of the simulation in the Result section and specified the software name in the Table of Materials.

[Section related to the response]: Representative results

Both in silico (Fig. 2A-C) and in vitro (Fig. 2D-F) tests demonstrated the formation of a stable linear EGF gradient across the cell culture area which lasted for approximately two days without replenishing the 'source' and 'sink' reservoirs. The time-dependent diffusion of the proteins was simulated using a commercialized multiphysics software. The 3D geometry of the chamber configuration was recreated on the software and the average concentration within the chamber volume was determined and plotted.

Dear Editorial Board,

We modified some figures from our previous paper published in PNAS and they were cited in the figure legend. PNAS allows the use of original figures for noncommercial and educational purpose without requesting permission. We have added the link to the editorial policy that allows re-prints as below.

https://www.pnas.org/page/about/rights-permissions

Thank you very much for considering our paper.

Yours (on behalf of all authors),

Andre Levchenko