Patterning ECM microstructure to investigate 3D cellular dynamics under multiplexed mechanochemical guidance
[version 1; peer review: 1 approved, 2 approved with reservations]

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Abstract

**Background:** Biochemical and biophysical factors jointly regulate the cellular dynamics in many physiological processes. It is therefore imperative to include multiplexed microenvironment cues when employing in vitro cell-based assays to model physiological processes.

**Methods:** To meet this need, we have developed a modular platform of 3D cell culture, Modular Control of Microenvironment for Cell Migration and Culture Assay (MC3A), that incorporates directed chemical and mechanical cues in the forms of chemotaxis and contact guidance, respectively. Taking advantage of the functionalities of MC3A, we study the migration and morphology of breast cancer cells in 3D engineered extracellular matrix (ECM) following a serum gradient for chemotaxis.

**Results:** We show that when chemotaxis is facilitated by contact guidance in the same direction as the serum gradient, cells demonstrate dimensional-reduction in their motility and highly elongated ellipsoidal shape. When the direction of ECM alignment diverges from the direction of serum gradient, chemotactic motion is significantly suppressed, and cells are generally more protrusive and rounded in their morphology.

**Conclusions:** These examples demonstrate MC3A as a powerful tool for engineering complex microenvironments of cells that will advance the state-of-the-art of cell-based analysis in drug development, regenerative medicine, and other research areas in bioengineering.

**Keywords**
extracellular matrix, tissue engineering, contact guidance, chemotaxis, 3D culture
Introduction

Many physiological processes involve directed mechanochemical cues that regulate the motility, polarization, and morphogenesis of cells such as during wound healing, immune response, and cancer metastasis. It is, therefore, crucial to incorporate these extracellular signals when employing in vitro cell-based assays in applications such as drug screening and tissue regeneration. Here we present a modular platform to study cellular dynamics in a 3D extracellular matrix (ECM) where directed chemical and mechanical cues, in the forms of chemotaxis and contact guidance, are fully controlled in the microenvironment of cells.

As one of the most common types of directed chemical cues, chemotaxis is the process where a cell follows the gradient of chemoattractants to coordinate with other cells in the functions of multicellular organisms. Contact guidance, on the other hand, utilizes the substrate or tissue topography to direct the cells through mechanosensing pathways. Contact guidance strongly modulates the morphology and motility of cells, as have been observed in many cell types.

Although the effects of chemotaxis and contact guidance have been well studied separately, accurate representation of physiological conditions requires simultaneous presence and control of mechanochemical cues. One salient example is cancer metastasis, where the gradient of various growth factors drives chemotaxis, which facilitates the cancer cell dissemination. Concurrently, cells move through vast tissue space filled by fibrous ECM and the alignment of ECM fibers generates contact guidance. As a result ECM microstructure significantly correlates with tumor prognosis.

While it is desirable to program complex mechanochemical cues in cell-based assays, a reliable and user-friendly method has not been available to the broader community. To incorporate both biochemical and biophysical factors in the microenvironment of the cells, we have developed techniques to pattern the microstructure of 3D extracellular matrices. We have packaged these techniques into a portable modular platform: Modular Control of Microenvironment for Cell Migration and Culture Assay (MC³A). MC³A simultaneously controls mechanochemical factors for 3D cultured cells, with a form factor that is compatible with standard microscopy for live or fixed cell imaging.

To demonstrate the functions of MC³A, we study the migration and morphology of breast cancer cells in 3D ECM. We engineer the extracellular microenvironment to simultaneously establish a chemotactic serum gradient, and contact guidance that is either in converging or diverging directions of chemotaxis. These examples will show MC³A as a valuable tool to study cellular dynamics and functions in realistic tissue environments.

Methods

We used OEM, and 3D printed parts to build the prototype of MC³A and followed the manufacturer’s instructions to prepare the 3D ECM and cell culture. We analyzed the images using ImageJ (RRID:SCR_003070) plugins and homemade scripts compiled with MathWorks MATLAB (RRID:SCR_001622). As an open source alternative to MATLAB, GNU Octave (RRID:SCR_014398) can be employed to render the same results. Microscopy images were directly analyzed for ECM geometry and cell segmentation without any modifications. Microscopy images shown in Figure 3 and Figure 5 are contrast enhanced to facilitate visual inspection.

Cell culture

We culture RFP-labeled MDA-MB-231 human breast cancer cell line (GenTarget, San Diego, CA), following the standard protocol, in a high glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS; Avantor Seradigm, Radnor, PA) and 0.1% Penicillin-Streptomycin (Gibco Thermofisher, Waltham, MA), and maintained it at 37°C and 5% CO₂ incubator.

Prior to conducting experiments, for precisely 12 hours, we culture the cells in a serum-free medium containing Dulbecco’s modified Eagle’s medium supplemented with 0.1% Penicillin-Streptomycin Gibco Thermofisher, Waltham, MA, and maintained at 37°C and 5% CO₂ incubator.

To embed the cells in 3D collagen matrices, we suspend the cells at low density in neutralized collagen solutions. Highly concentrated rat-tail FITC-labeled collagen is diluted with 10× L15 medium (Dulbecco), DDI water, NaHCO₃ 7.5%, and neutralized with sodium hydroxide (NaOH, 1M) to a final concentration of 1.5 mg/mL with a pH of 7.4.

Sample preparation on the culture inserts

The culture assays are first corona treated for 10 min, then immersed with Sulfo-SANPAH under UV light (320-350 nm) for 2 hours to functionalize binding surfaces. Then we wash the assays with 1mL of PBS 1× and DDI water. After drying the samples, we transfer the 3D cell suspension solution to the assay using 100 um pipet tips through the inner channel port (center port).
Then, using the rotary stage, we place the treated blade at the center of the assay and rotate it for a given time with a defined RPM (128RPM for 4 min as in the main text). Following this process, we polymerize the solution for 21 minutes at room temperature and then for 25 min in a 5% CO_2 incubator at 37°C. Then we add 1 mL of a serum-free growth medium to each reservoir of the assays and keep it in the incubator for 6 hours (serum starvation period). Rotation with zero RPM results in radially aligned ECM.

After transferring the assays to the microscope, we replace the center reservoirs growth medium with a 20% serum-rich and the outer one with a serum-free L15 medium to generate a serum gradient across the chemotaxis channel. After 6 hours, the sample is ready for imaging.

FITC-labeled type I collagen ECM
850 μL of 10 mg/ml Collagen Type I, Rat Tail (purchased from ibidi GmbH, Grafelfing, Germany) mixed with 150 μL FITC conjugated water-soluble Collagen (Type I) (obtained from AnaSpec, Fremont, CA). We store the solution at 4°C and in the dark. We gently mix it once a day for about 30 seconds for ten days prior to experiments. The final concentration of the collagen solution is 1.5 mg/mL after adding cell suspension.

Microscopy
We use a Leica TCS SPE confocal microscope equipped with a stage-top incubator. We capture the pictures at a rate of 1 frame per hour over 18 hours using a 10× air objective. The raw images are grayscale with a resolution of 1024×1024 pixel^2 (1.1×1.1 mm^2) along the x-y plane and with two slices at a step of 100 μm along the z-direction. We simultaneously use the multichannel capability to image collagen fibers and MDA-MB-231 cells.

Image processing
Images are analyzed using custom scripts compiled using MathWorks MATLAB and Python 3. First, cell images are binarized to obtain cell objects. Connected components are found and manually screened to represent single cells. We calculate cell centroids and morphology from binarized images.

To calculate the local collagen orientation and coherence, we use the ImageJ plugin OrientationJ. This software package computes the orientational order of an image based on its gradient matrix. With a sliding window, one can obtain the field of local principal direction and coherence $c$. The principle direction indicates the direction along which the image intensity vary minimally, while the coherence $c$ measures the level of alignment in the local structure. When all fibers are in parallel, $c$ reaches a maximum value of 1. When the fibers are randomly aligned $c$ approaches its minimal value of 0. Note that the imaging noise generally suppresses the calculated coherence. And the intrinsic disorder of biopolymer networks forbids perfect alignment. Therefore the theoretical upper bound of 1 can not be reached. By comparing calculated coherence with visual inspection and cellular responses, we consider collagen fibers to be well aligned when $c > 0.2$.

Results
MC³A consists of a rotary stage that is controlled through computer interface, and a disposable culture insert (Figure 1, see also extended data section S1-S2). The rotary stage incorporates translation motors to move a spinning head. The spinning head consists of a blade coupled with a rotary motor. When making samples, these motors are manually controlled or follow pre-programmed protocols to dip the blade through the blade port of culture inserts. After facilitating the ECM self-assembly, user uses the z-motor to lift up the blade and the culture insert can be manipulated or imaged in ways similar to a standard tissue culture petri dish.

To produce desirable ECM microstructure, MC³A takes advantage of a blade with experimentally optimized tip shape to guide the gelation of ECM polymer solution. Additionally, spinning of the blade creates a flow in the ECM solution. The flow is both driven by the rotational motion of the blade, as well as constriction walls built inside cell culture insert. After setting the initial flow, the blade exits from the solution and the polymer solution solidifies into a biopolymer network whose microstructure is templated by the initial flow. The shape of the blade, its rotation protocol, and the geometry of constriction walls of the culture insert work synergistically to make tissue mimicking biopolymer networks.

Above the engineered ECM microstructure, we place a pair of open channel reservoirs to deliver soluble factors. The reservoirs can be filled with the same or different chemicals to control the biochemical microenvironment of the cells. As an example, we would fill the inner reservoir with growth medium supplemented with 20% volume concentration of serum while the outer reservoir with growth medium without serum. The passive diffusion between the reservoir sets up a serum gradient, which drives chemotactic motion of MDA-MB-231 breast cancer cells.
To characterize the microenvironment of cells created by MC\textsuperscript{3}A, we first examined the profile of diffusive factors. To this end, we constructed the ECM with 2 mg/mL type-I collagen matrices (see Methods). We then filled the inner reservoir with rhodamine B in Phosphate-buffered saline (PBS) solution, and filled the outer reservoir with pure PBS. We measured the fluorescence intensity of rhodamine B, which provided a proxy of a diffusive factor’s concentration profile.

As shown in Figure 2, the fluorescence intensity uniformly expands from the inner reservoir in the radial direction towards the outer reservoir. Eight hours after filling the reservoirs, an approximate linear gradient has established along the radial direction in the ECM outside the inner reservoir. The intensity profile continues to stabilize. After 12 hours of passive diffusion, the intensity gradient in the radial direction reaches a steady state that can last for more than 12 hours. In the current design, the inner and outer reservoirs each have a capacity of 1.5 mL. Increasing the dimensions of the reservoirs, such as by raising the height of the retaining walls, will further elongate the duration of stable chemical gradient.

Figure 1. Schematic design of Modular Control of Microenvironment for Cell Migration and Culture Assay (MC\textsuperscript{3}A). (A) The rotary stage of MC\textsuperscript{3}A harbors multi-axis translation and rotation motors to manipulate a blade. These motors are interfaced with a computer that instructs the self-assembly of extracellular matrix (ECM) proteins into fibrous architectures. (B) The exploded view of a culture insert, which contains ECM for 3D cell culture and motility assay. The geometry of the culture insert can be altered to produce various types of microenvironments for cells, therefore offering an expansion of functions. The design presented here allows simultaneous chemical gradient and mechanical cues from ECM fibers with programmable alignment.

Figure 2. The spatial-temporal profile of diffusive biochemical factors in the culture insert of Modular Control of Microenvironment for Cell Migration and Culture Assay (MC\textsuperscript{3}A) simulated by rhodamine B solution. Left: the relative fluorescence intensity of rhodamine B at varying distances to the center of the inner reservoir, and at different time points. Right: raw fluorescent images at different time points. The fluorescence intensity is symmetric around the center of the inner reservoir. See also Underlying data.29
To characterize the mechanical microenvironment of cells created by MC³A, we employed FITC-labeled type-I collagen so the ECM microstructure can be accessed through fluorescent confocal imaging (see Methods). As representative examples, we examined two distinct configurations produced by executing two simple rotational protocols of MC³A.

When the blade is held still before lifting up from the solidifying collagen solution, the blade combines with the restriction walls in the culture insert to form a boundary condition that facilitates the nucleation of collagen fibers along the radial direction. Confocal images show the expected ECM microstructure (Figure 3A). To further quantify the local ECM geometry, we calculated the principal direction (Figure 3B-C) and coherence $c$ (Figure 3C) of collagen fiber alignment as reported previously $^{17,21,22}$ (see Methods). Figure 3B shows the spatial distribution of principal fiber direction. Despite of the fluctuations expected from the disordered nature of biopolymer networks, collagen fibers evidently show alignment in the radial direction.

In addition to the principal direction, coherence $c$ measures the level of alignment in the local structure. When all fibers are in parallel, $c$ reaches a maximum value of 1. When the fibers are randomly aligned $c$ approaches its minimal value of zero. Note that the imaging noise generally suppresses the calculated coherence. And the intrinsic disorder of biopolymer networks forbids perfect alignment. Therefore the theoretical upper bound of one cannot be reached. By comparing calculated coherence with visual inspection and cellular responses, we consider collagen fibers to be well aligned when $c > 0.2$. $^{22}$

As shown in Figure 3C, the value of coherence starts from around 0.30 near the inner reservoir, and gradually decreases to approximately 0.25 at 2 mm from the device center. The change is well within the range of fluctuations resulted from ECM structural disorder. Therefore the ECM within the culture insert of MC³A demonstrates relatively uniform microstructure.

When MDA-MB-231 cancer cells are embedded in the culture insert, cells experience both the chemotactic serum gradient, and 3D contact guidance from the local fiber alignment. We set the chemical gradient by filling the inner reservoir with 20% volume concentration of serum, and filling the outer reservoir with serum free growth medium. To characterize the resulted cellular dynamics, we performed confocal live cell imaging for 18 hours after chemical gradient

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**Figure 3.** The microstructure of radially aligned extracellular matrix. (A) Confocal images showing the fluorescently labeled collagen fibers. The images are further processed to calculate the principal direction and level of alignment (quantified as coherence) of collagen fibers. Center of the image is at the center of the inner reservoir, similar to Figure 2. (B) A spatial map showing the principal direction of collagen fibers. (C) The coherence $c$ at varying distances from the center of the inner reservoir. The data points are color coded by the cosine angle $\Delta \theta$ between local fiber direction (principal direction) and radial direction. See also Underlying data.$^{30}$
stabilized. On the left panel of Figure 4A we show a temporal projection of the cells where each trace represents a single cell from the beginning of recording (blue, zero hour) to the end of recording (red, 18 hours). Because the chemical and mechanical cues are in parallel, cells move in the radial direction with little excursions.

To further characterize the cell motility, we tracked the radial and tangential displacements of a random subset of cells as shown in Figure 4B. Consistent with the temporal projection, cell displacement in the tangential direction ($\theta$) is very small. The trajectories in Figure 4B also reveal that the random walk of a cell often observed in 3D ECM now occurs with reduced dimension. Cells constantly make 180 degree turns, while still showing net displacements towards the center of the device, where serum concentration is higher. Within a frame interval of one hour, cells travel at an average instantaneous velocity of 9.5 $\mu$m/hr. To quantify the efficiency of cells tracing chemoattractant gradient, we also calculated the mean chemotaxis index $\overline{CI}$:

$$\overline{CI} = \overline{\vec{v}} \cdot (-\overline{\vec{r}}),$$

where $\overline{\vec{v}}$ is a unit vector along the direction of velocity, and $(-\overline{\vec{r}})$ the direction of chemotaxis. $\overline{CI}$ ranges between 1 and -1, with greater values indicating more efficient migration seeking higher chemoattractant concentration. For the cell trajectories shown in Figure 4B, we find the mean chemotaxis index equals 0.3. The tendency of moving along the

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**Figure 4.** 3D Cell motility is modulated by the chemoattractant gradient, which is in parallel to the extracellular matrix (ECM) fiber alignment. (A) Left: temporal projected recording of MDA-MB-231 embedded in the culture insert of Modular Control of Microenvironment for Cell Migration and Culture Assay (MC3A) for 18 hours. Right: a snapshot at five hours. (B) Cell trajectories in the radial ($\Delta r$) and azimuthal ($\theta$) coordinates. (C) A scattered plot showing cell aspect ratio and circularity. The black dashed line indicates the circularity of an ellipse at a given aspect ratio. The blue dashed line indicates 80% of circularity corresponding to an ellipse at a given aspect ratio. We empirically consider data points below the blue dashed line as strongly protrusive cells. In (B-C) 70 cells are tracked. See also Underlying data. 30
serum gradient accumulates as cells navigate their microenvironment, such that over 18 hours, the average net radial displacement of cells is 85 μm towards the device center.

In addition to cell motility, we have also characterized cell morphology. In particular, we calculated the circularity and aspect ratio of binarized single cell images (Figure 4C). Here circularity is defined as $4\pi \frac{\text{area}}{\text{perimeter}^2}$, which equals to one for a circle and generally becomes smaller when a cell generates protrusions such as invadopodia.

We find strong ECM contact guidance coupled with a chemical cue in the parallel direction promotes cell elongation. About half of times a cell sampled in Figure 4C have aspect ratio greater than three, and in over 27% cases cells are highly elongated to have aspect ratios greater than six.

Despite of the elongation, most cells do not deviate from elliptical shape (Figure 4C, black dashed line) to demonstrate significant surface fluctuation. This is expected as the parallel mechnochemical cues provide consistent polarizing signals for cell morphology. To better quantify the morphology of cells, we empirically classify a cell to be strongly protrusive if its circularity is less than 80% of the circularity of an ellipse with the same aspect ratio (Figure 4C, blue dashed line). We find that 19% of cells fall into this category. Together, these results show that cell morphology can be characterized as approximately elongated ellipses.

While the parallel chemical and mechanical cues lead to strong cell polarization and dimensional reduction of motility, we notice that in physiological condition the two cues may vary independently and form different angles. MC3A allows us to conveniently investigate cellular dynamics in such microenvironment configurations. As a demonstration, we rotated the blade of MC3A at a constant speed of 128 RPM, which drives the flow of collagen solution that directs the nucleation and growth of collagen fibers primarily in the tangential direction. After four minutes of rotation, we lifted the blade out of the solidifying collagen solution and the ECM self-assembly was continued for the next 40 minutes. Confocal images in Figure 5A and computed local principal direction in Figure 5B show the expected fiber alignment. Note that because the global flow field resembles a vortex pattern, a radial components of fiber alignment is still observed (see also extended data Figure S7 for other rotational protocols). In most locations sampled in the device, the angle between chemical gradient (along radial direction) and contact guidance (ECM principal direction) is between 45 to 90 degrees (Figure 5C). As a result, cells in this configuration experience chemical and mechanical cues in diverging directions. Here as in the

![Figure 5](image_url)

**Figure 5. The microstructure of the vortex flow aligned extracellular matrix.** (A) Confocal images showing the fluorescently labeled collagen fibers. The images are further processed to calculate the principal direction and level of alignment of collagen fibers. The center of the image is at the center of the inner reservoir, similar to Figure 3A. (B) A spatial map shows the principal direction of collagen fibers. (C) The coherence $c$ at varying distances from the center of the inner reservoir. The data points are color coded by the angle $\Delta \theta$ between local fiber direction and radial direction. See also Underlying data.
prior case, the strength of chemical gradient was set by filling the inner reservoir with 20% volume concentration of serum, and the outer reservoir with serum free growth medium.

As the serum gradient drives the MDA-MB-231 cells radially inward, ECM fibers present contact guidance that steers the cells in the tangential direction. The temporal projected confocal recording demonstrates the joined effects of mechanical and chemical cues to cell motility (Figure 6A). We track a random subset of 70 cells as shown in Figure 6B. Compared with the previous configuration where collagen fibers align radially, cells in the current configuration exhibit pronounced migration that vary their azimuthal angles. Indeed, instantaneous velocity (approximated by the displacement between one hour frame intervals) shows a mean chemotaxis index of 0.14, less than half of the value for radially aligned ECM. Over the course of 18 hours, the net radial displacement averaged over all tracked cells is 35 μm towards the device center, which is again less than half of the value for radially aligned ECM.

The diverging mechanochemical cues also modulate the cell morphology. In particular, only 17% cells sampled have aspect ratios greater than six, compared with 27% in the presence of parallel mechanochemical cues. Additionally, of all the cells sampled 26% show characteristics of strongly protrusive cells, compared with 19% in the case of radially aligned ECM. Together, these results show that when contact guidance and chemotaxis are along diverging directions, cells show significant reduction in their migration along the chemical gradient. At the same time, cells tend to demonstrate small aspect ratio shapes while featuring strong surface fluctuations.

**Figure 6.** 3D Cell motility is modulated by the chemoattractant gradient, which makes large angles with respect to the extracellular matrix (ECM) fiber alignment. (A) Left: temporal projected recording of MDA-MB-231 embedded in the culture insert of Modular Control of Microenvironment for Cell Migration and Culture Assay (MC³A) for 18 hours. Right: a snapshot at four hours. (B) Cell trajectories in the radial (Δr) and azimuthal (θ) coordinates. (C) A scattered plot showing cell aspect ratio and circularity. The black dashed line indicates the circularity of an ellipse at a given aspect ratio. The blue dashed line indicates 80% of circularity corresponding to an ellipse at a given aspect ratio. We empirically consider data points below the blue dashed line as strongly protrusive cells. In (B-C) 70 cells are tracked. See also Underlying data.31
Conclusions

Many biological processes involve cells to navigate 3D ECM, which contains multiplexed environmental cues. It is conceivable that modeling the cross-talk of biochemical and biophysical factors will improve the physiological relevance of in vitro cell-based assays. Here we present a modular platform that allows one to conveniently pattern the microstructure of 3D ECM, so that contact guidance from the ECM fiber alignment and spatial gradient of soluble factors can be independently controlled to jointly modulate the cellular dynamics.

Our platform, MC3A, generates sustained chemical gradient over more than 18 hours under passive diffusion. MC3A features an open channel design, which makes it easy to deliver soluble factors to the cells, and to extract samples for downstream analysis such as sequencing. This is in contrast to other microfluidics culture and chemotaxis platforms, where the cells in the closed channels are often difficult to be retrieved, especially when they are embedded in solidified matrices. Additionally, the reservoirs are easily accessible so that additional solutions can be brought in externally to generate time-dependent chemical environment or to simply extend the duration of stable gradient.

In MC3A, cells are cultured in thick layer of ECM (>500 μm) that provide true 3D support. This is in contrast to many microfluidics-based chemotaxis device where the limited channel depth, often less than 100 μm, can not sufficiently screen the mechanical effects of rigid boundaries.

In MC3A we optimize the geometric design of the boundaries of both spinning blade and dish insert such that the ECM microstructure can be easily controlled through the programmable rotational protocols of the blade. This approach avoids pre-loaded mechanical stress in the matrix when fiber alignment are induced by external stress. Compared with other flow-based ECM aligning methods, MC3A produce uniform ECM alignment over a much larger spatial range (three times more effective area than in, and five times more area than in22). MC3A is also compatible with most tissue-derived proteins (such as collagen, matrigel and fibronectin) or synthetic hydrogels. Therefore the user can take full advantage of current and future progress in tissue-mimicking biomaterials.

MC3A utilizes a modular design such that the shape of the blade, and the geometry of the culture insert can be altered for expanded functionalities. As an example, we have constructed a device to fit two separate sets of ECM with their inner reservoirs connected (see also extended data Figure S6). With different combinations of medium in each reservoir, we can use this dish insert to run replicating experiments or to make side-by-side comparison between distinct microenvironments. Because we make dish inserts through standard 3D printing, MC3A allows rapid prototyping to explore expanded functionality.

In summary, MC3A provides a simple and reliable platform to program complex 3D tissue-mimicking microenvironment. Given the importance of multiplexed chemical and mechanical cues, we think MC3A will advance the state-of-the-art of in vitro models in drug screening, regenerative medicine, and many areas of fundamental research.

Data availability

Underlying data

- Figshare: Chemical gradient. https://doi.org/10.6084/m9.figshare.2104998

This project contains the following underlying data:

- chemical gradient.zip. This file contains raw microscopy images of showing the diffusion of a tracer dye (fluorescein) in the collagen ECM of a MC3A device.

- Figshare: Radially aligned sample. https://doi.org/10.6084/m9.figshare.2011295

This project contains the following underlying data:

- ECM microstructure snapshot.zip. This file contains raw microscopy images of collagen ECM aligned radially in a MC3A device.

- Cell 3D cultured snapshot.zip. This file contains raw microscopy images of MDA-MB-231 cells cultured in collagen ECM that is aligned radially in a MC3A device.

- Cell migration timelapse.zip. This file contains time lapse recording images of MDA-MB-231 cells migrating in collagen ECM that is aligned radially in a MC3A device.
This project contains the following underlying data:

- ECM microstructure snapshot.zip. This file contains raw microscopy images of collagen ECM aligned tangentially in a MC3A device.
- Cell 3D cultured snapshot.zip. This file contains raw microscopy images of MDA-MB-231 cells cultured in collagen ECM that is aligned tangentially in a MC3A device.
- Cell migration timelapse.zip. This file contains time lapse recording images of MDA-MB-231 cells migrating in collagen ECM that is aligned tangentially in a MC3A device.

Extended data

- Figs: Supplementary Materials. https://doi.org/10.6084/m9.figshare.20915452

This project contains the following underlying data:

- SI_final.pdf. This file includes additional information regarding the device manufacture and additional experimental details.

Data are available under the terms of the Creative Commons Attribution 4.0 International license (CC-BY 4.0).
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The manuscript entitled, “Patterning ECM microstructure to investigate 3D cellular dynamics under multiplexed mechanochemical guidance”, outlines the development of a platform for 3D cell culture that can modulate chemical and mechanical cues respectively. This novel device can be used to study the relative contribution of chemotaxis and contact guidance on cell morphology and migration. The ability to independently control fiber alignment vs chemotaxis provides opportunities to address unanswered questions about how these cues impact cell morphology and migration. The utility of the platform is of interest to researchers in this field; however, the manuscript lacks detail in the description of methods and lacks experimental evidence of reproducibility. Addressing these shortcomings is required prior to publication.

1. For a manuscript that defines the development of a novel platform the methodology is extremely vague.
   - As written, it is very unclear how radial vs tangential fiber architecture is achieved. Some aspects of the mechanical perturbations (rotation of the blade) are provided in the methods section, while some are in the results text. The descriptions of each condition seem to overlap. Please define both radial and tangential fiber alignment protocols in the methods section. (blade rotation speed, duration of spinning, whether collagen is unpolymerized at the time of spinning, the polymerization conditions, and the exact timing of serum addition vs initiation of image acquisition)

   - Collagen labeling methods are not clear. The FITC-labeled collagen from AnaSpec is a quenched collagen, which fluoresces upon enzymatic cleavage (MMP activity). Presumably diluting this collagen into high-concentration Collagen Type I alleviates the quenched fluorescence so that a subset of fibers are fluorescent. These details should be added to the methods section.

   - The cell concentration is not defined. “…we suspend cells at low density in neutralized collagen solutions.” For reproducibility, a cell concentration should be defined.
2. It is not clear that the authors have reproduced the alignment conditions. The experimental N is not reported. How reproducible is the fiber alignment? Chemical gradients? The tangential alignment appears quite variable (45-90 degrees), which is a large margin. The analysis of chemical gradients and alignment should be repeated in at least 3 experiments. Furthermore, were all cell metrics acquired from one device for tangential and one device for radial alignment? Similarly, the migration speeds/distances for radial vs tangential fiber alignment should be repeated across a minimum of 3 independent experiments.

3. The authors state that cellular protrusions are altered between alignment/chemical gradient conditions. However, images were collected with a 10x objective every hour for 18hrs. Protrusion dynamics in 3D have been reported to occur on the scale of minutes. Higher magnification and shorter imaging intervals are necessary to ensure that cellular protrusion dynamics are accurately quantified.

4. Finally, if the method is interpreted correctly by this reviewer, the cells are subjected to blade rotation for either 0 or 4 minutes. The authors do not account for the application of direct mechanical stress on the cells in their response to tangential alignment. This is an important factor that needs to be controlled or minimally discussed.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Mechanotransduction, 3D collagen cell culture, image analysis, cell migration and invasion, cancer cell biology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.
In this manuscript, Esfahani and Sun described a method to create an experimental platform where cell response to gradient in chemical factors and to the organization of extracellular environment can be decoupled. This is interesting as cells are known to sense and respond to both factors, but the interplay between them is difficult to study systematically. Thus, the approach and the insights obtained from it can be very useful to the community. The work and analyses are solid and were performed nicely, and the manuscript is a pleasure to read. My only suggestions are to include some pertinent missing information, which are crucial for the readers to understand the study.

1. The working mechanism of the device and how it can be used to tune collagen microstructure can be better explained. It is not clear how the blade and boundary condition of the device alone (apparently even without rotation) can already create fiber alignment.

2. The authors state that the value of coherence $c$ is highly affected by noise in the image and therefore can only reach a max value of 0.2 rather than the theoretical 1.0. Some examples of images with different levels of noise and the corresponding $c$ value will be nice (perhaps as a supplementary figure).

3. The authors stated that the tip shape of the blade was optimized to guide the collagen gelation. Since the blade shape appears to be critical to the whole mechanism of the approach, description of the different tip shapes that were tried and the final tip shape and dimensions used will be very useful.

4. It will be great if dimensions are added to the schematic in Figure 1.

5. Since the goal of the study is to study the individual and combined effects of chemotaxis and collagen orientation, it will be most interesting to compare the anisotropic (Figure 3,4) vs. isotropic condition (not included yet) and chemotaxis (Figure 5,6) vs. no chemotaxis (not included yet). Basically including the outcomes of the control conditions.

6. The chemical gradient experiment was done using rhodamine B as a proxy for diffusive factor’s concentration profile. It will be informative to mention the diffusivity and molecular size of rhodamine B as compared to those of chemoattractants normally present in the context of cancer.

7. The chemical gradient experiment was performed only for one condition (isotropic gel),
which is different from the conditions used in the rest of the paper (anisotropic gel). It will be more convincing to include results from the anisotropic gels too (those in Figure 3 and in Figure 5).

8. Information about how the chemical gradient experiment was performed is missing in Methods. Also, at what height were the images in Figure 2 taken to analyze the fluorescence intensity?

9. Regarding the experiments described in Figure 4 and 6, as far as I understand the chemical gradient is positive (serum is at the center of the device). So why did the cells move outwards (away from serum) and not inwards (towards serum)? What is the zero position in Figure 4B and 6B?

10. Serum has been previously reported to affect collagen gelation, resulting in different collagen fiber thickness and microstructure. In the presence of serum concentration gradient, could it be that there is also a gradient in collagen fiber formation and microstructure, which could potentially also affect cancer cell migration? If the authors have characterized the collagen at different radial positions, this will be nice to add. Otherwise, at least the authors could comment on this.

11. I think it will be more precise if the authors indicate that the approach specifically allows radial-circumferential patterning of collagen and radial chemical gradient (and not some other axis).

Is the work clearly and accurately presented and does it cite the current literature?  
Yes

Is the study design appropriate and is the work technically sound?  
Partly

Are sufficient details of methods and analysis provided to allow replication by others?  
Partly

If applicable, is the statistical analysis and its interpretation appropriate?  
Yes

Are all the source data underlying the results available to ensure full reproducibility?  
Yes

Are the conclusions drawn adequately supported by the results?  
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: biomedical engineering

I confirm that I have read this submission and believe that I have an appropriate level of
expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 04 November 2022

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The authors described a novel 3D cell culture system and characterized its application to control independently ECM fiber alignment and spatial gradient of soluble factors, which is an important contribution to the field. The manuscript is well written and clearly demonstrates the advantages of the new platform.

One minor comment: it will be helpful to show representative images of cell morphology when there is a diverge between the directions of ECM alignment and serum gradient.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Microfluidic

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.
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