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Authors

Zhang, Yan

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Zhu, Zijie

et al.

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Yan Zhang, Rachel M. Lee, Zijie Zhu, ..., Tingrui Pan, Wolfgang Losert, Min Zhao

yanzhang@hznu.edu.cn (Y.Z.)
minzhao@ucdavis.edu (M.Z.)

Highlights

An increased throughput system for electrotaxis of large epithelium cell sheets

Step-by-step guide for efficient patterning of epithelium cell sheets

Quantification of collective cell migration with PIV

Here, we present a protocol for electrotaxis of large epithelial cell sheets without compromising the integrity of cell epithelia in a high-throughput customized directed current electrotaxis chamber. We describe the fabrication and use of polydimethylsiloxane stencils to control the size and shape of human keratinocyte cell sheets. We detail cell tracking, cell sheet contour assay, and particle image velocimetry to reveal the spatial and temporal motility dynamics of cell sheets. This approach is applicable to other collective cell migration studies.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

STAR Protocols

Protocol

Protocol for electrotaxis of large epithelial cell sheets

Yan Zhang,[1,2,3,5,12,*] Rachel M. Lee,[4] Zijie Zhu,[5] Yaohui Sun,[1] Kan Zhu,[1] Zhengping Xu,[3] Francis Lin,[10] Tingrui Pan,[5,7,8,9] Wolfgang Losert,[4,6] and Min Zhao[1,11,13,*]

[1]Department of Ophthalmology & Vision Science, University of California, California, Davis, CA 95616, USA

[2]School of Public Health, Hangzhou Normal University, Hangzhou 311121, China

[3]Institute of Environmental Medicine, Zhejiang University School of Medicine, Hangzhou 310058, China

[4]Institute for Physical Science and Technology, University of Maryland, College Park, MD 20742, USA

[5]Micro-Nano Innovations (MiNI) Laboratory, Department of Biomedical Engineering, University of California, California, Davis, CA 95616, USA

[6]Department of Physics, University of Maryland, College Park, MD 20742, USA

[7]Institute of Biomedical and Health Engineering, Shenzhen Institute of Advanced Technology, Shenzhen 518055, China

[8]Shenzhen Engineering Laboratory of Single-molecule Detection and Instrument Development, Shenzhen, Guangdong 518055, China

[9]Suzhou Institute for Advanced Research, University of Science and Technology of China, Suzhou 215123, China

[10]Department of Physics and Astronomy, University of Manitoba, Winnipeg, MB R3T 2N2, Canada

[11]Department of Dermatology, University of California, California, Davis, CA 95616, USA

[12]Technical contact

[13]Lead contact

*Correspondence: yanzhang@hznu.edu.cn (Y.Z.), minzhao@ucdavis.edu (M.Z.)
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SUMMARY

Here, we present a protocol for electrotaxis of large epithelial cell sheets without compromising the integrity of cell epithelia in a high-throughput customized directed current electrotaxis chamber. We describe the fabrication and use of polydimethylsiloxane stencils to control the size and shape of human keratinocyte cell sheets. We detail cell tracking, cell sheet contour assay, and particle image velocimetry to reveal the spatial and temporal motility dynamics of cell sheets. This approach is applicable to other collective cell migration studies. For complete details on the use and execution of this protocol, please refer to Zhang et al. (2022).[1]

BEFORE YOU BEGIN

Protocol overview

Cells migrate collectively during wound healing, development, and regeneration *in vivo*. This kind of collective migration is also found in monolayer wound healing assays, cell sheet migration of keratinocytes, and MDCK cells *in vitro*. Cells in collective migration keep physical, chemical, and biological cell-cell connections during migration, and behave differently compared with cells that migrate individually. A qualitative and quantitative understanding of the cell motility dynamic at spatial and temporal dimensions under various conditions is critical in studying cell collective migration. Some very elegant studies have demonstrated characteristics of collective cell migration in electric fields.[2–4] We have studied collective cell migration in organ culture and cell culture.[1,5–8] Here, we describe our specific step-by-step guide on how to direct and analyze a set of large epithelium cell sheets migrating in an electric field using well-patterned human keratinocyte cell sheets as the model. In principle, this protocol can be adapted for cell collective migration under other treatments.

Fabrication of polydimethylsiloxane (PDMS) cell stencils and strips
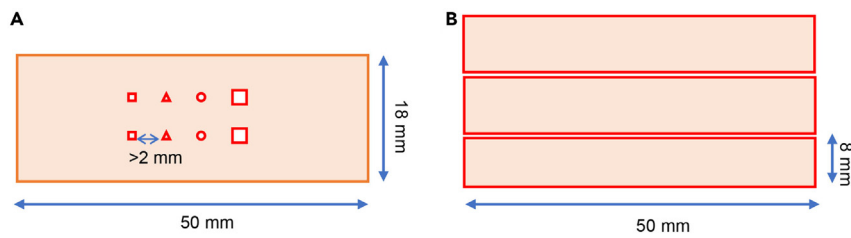
© Timing: 2 h

Figure 1. Design for PDMS Cell stencil and strips
(A and B) Top view of the cell stencil (A) and strips (B).

1. Fabricate PDMS stencils for cell patterning and strips for the electric field (EF) stimulation chamber.
   a. Precisely cut the PDMS pieces (200 μm in thickness) as designed (Figure 1) using a cut-through mode of a computer-controlled CO_2 laser cutter (VLS 2.30).
   b. Gently remove the undesired debris (highlighted in white in Figure 1A) with tweezers.
   c. Thoroughly clean PDMS stencils in a 95% ethanol ultrasonic bath for 5 min.
   d. Air dry the PDMS stencils.
   e. Store the PDMS stencils in a sterile petri dish.
   f. Sterilize the PDMS stencils and strips with UV lights in the biosafety cabinet for 30 min right before use.

Note: The size and shape of the patterning in the PDMS stencil can be adjusted at your desire. In Figure 1A, the size of the small square, triangle, and circle presented are 1 mm^2 and the size of the larger square is 4 mm^2. Square cell sheets ranging from 0.5 × 0.5 mm^2 to 4.0 × 4.0 mm^2 in size have been tested and proved feasible in this system. To form a firm attachment between PDMS and cell culture dish, the minimal distance between two cell sheets in the PDMS stencil should be more than 2.0 mm, as it is illustrated in Figure 1A. At this distance, the adjacent cell sheets will not migrate into others, or over the surface used to be occupied by other cell sheets in a 12-h experiment, thus avoiding complicating factors such as interaction with neighbor cell sheets. This distance can be adjusted depending on the purpose of experiments, and whether the interaction of neighbor cell sheets is going to be investigated.

⚠ CRITICAL: To keep PDMS stencils clean and dry, they should be handled with clean tweezers throughout the experiment.

Fabrication of the EF stimulation chamber

⏱ Timing: 1 h

2. Fabricate the EF stimulation chamber.
   a. Gently attach the sterilized PDMS strips in parallel to the 100 mm culture dish (Figure 2A). The gap between PDMS strips should be ∼20 mm.
   b. Add four high-vacuum silicone grease blockers to form the side walls of the EF stimulation chamber (Figure 2A).

⚠ CRITICAL: Air bubbles should be avoided between PDMS strips and the cell culture dish to make a firm attachment of PDMS stencils to the cell culture dish. The silicon grease blocker together with PDMS strips should be sealed and waterproof and are supposed to build up two isolated reservoirs (on the left and right side of the PDMS strip located in the center of the chamber) in the electrotactic chamber. High-vacuum silicone grease is soft,
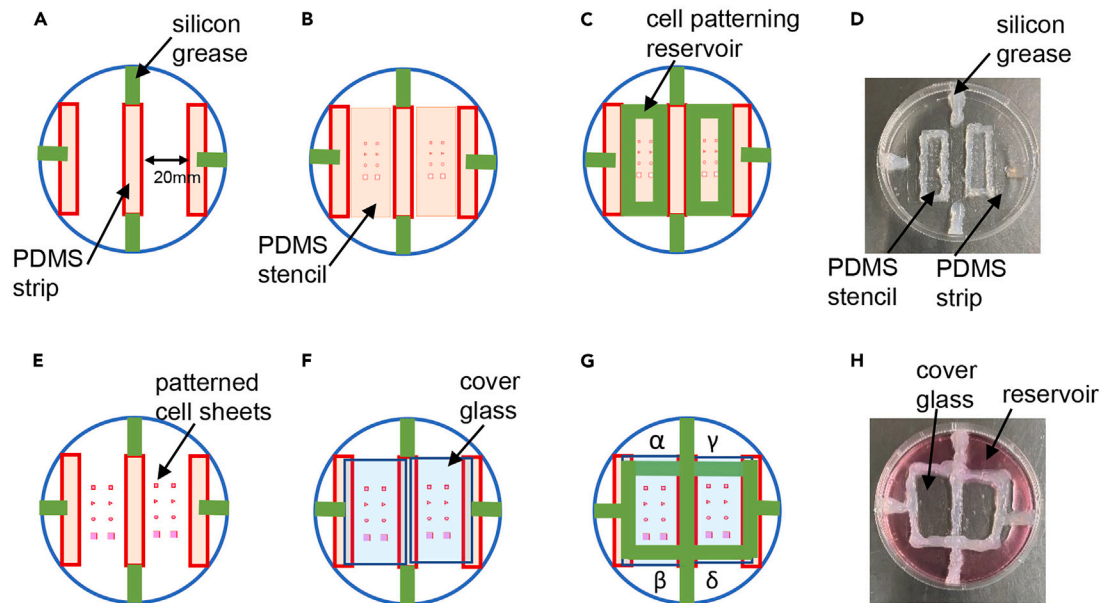
Figure 2. Design of EF stimulation chamber
(A–H) Top view of the EF stimulation chamber in each stage. Green lines indicate the high-vacuum silicone grease. (D and H) are the photos of the chamber in the stage (C and G).

non-melting silicone designed for sealing and lubrication in vacuum and pressure applications. No elapsed time is required before proceeding to the next step.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
FNC Coating Mix ® (a specially formulated reagent that increases the attachment of cells to the plastic substratum used for culturing mammalian cell lines, According to the vendor, it is a specially formulated mixture of fibronectin, collagen, and albumin.)	Athena Environmental Sciences	Cat#0407
DMEM	Gibco	Cat#C11965500BT
FBS	Gibco	Cat#10099-141
Penicillin-streptomycin	Gibco	Cat#15140-122
$Ca(NO_3).4H_2O$	Sigma	Cat#C2786
$MgSO_4.7H_2O$	Sigma	Cat#63138
Tris	Sigma	Cat#10708976001
Agar	Sigma	Cat#A7002
Deposited data		
MATLAB code for PIV	This paper	Mendeley Data: https://doi.org/10.17632/d4ytd5n98f.1
Experimental models: Cell lines		
HaCat	Cell Lines Service	CVCL_0038
Software and algorithms		
MATLAB	MathWorks	MATLAB R2021b https://www.mathworks.com/products/MATLAB.html
ImageJ/FIJI	Schindelin et al.[9]	https://imagej.net/Fiji
MTrackJ	Meijering et al.[10]	https://imagescience.org/meijering/software/mtrackj/
COMSOL Multiphysics	COMSOL Inc	v5.0

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Metamorph NX	Molecular Device	MetaMorph NX 2.5, https://www.molecular.devices.com/products/cellular-imagingsystems/acquisition-and-analysissoftware/metamorph-microscopy
MatPIV1.6.1	Sveen et al.[11]	https://www.duo.uio.no/handle/10852/10196
Other		
Cover glass (50 × 24 mm^2)	Fisher Scientific	Cat#12-548-5MP
PDMS pieces/Silicone rubber (200 μm in thickness)	B&J Rubber Products	N/A
high-vacuum silicone grease	Dow Corning	Cat#Z273554
Silver electrode	Advent Research Materials Ltd	Cat#AG549109
U-shaped glass tube	Donghai Xinyou Quartz Products Ltd.	N/A
True-RMS digital multimeter	RadioShack	Cat#2200087
Laser System	Engraver's Network	VLS2.30,
Soldering Iron Kit (30 W)	Marksman	N/A
Zeiss Axiovert 200M Inverted Fluorescence Microscope	Carl Zeiss	N/A
XL Incubation for Inverted Research Microscopes	Carl Zeiss	N/A
DC power supply	Beijing Liuyi Ltd.	DYY-6C

Note: Depending on the hardware used, users may require alternative imaging software. FNC coating mix is optional depending on the cell line you adopt.

MATERIALS AND EQUIPMENT

Cell growth medium formulation		
Reagent	Final concentration	Amount
DMEM	1×	44.5 mL
FBS	1×	5 mL
Penicillin-Streptomycin	1×, 100 U/mL	0.5 mL
Total	N/A	50 mL

Keep at 4°C for up to 3 months.

Steinberg's solution (10×)		
Reagent	Final concentration	Amount
NaCl	580 mM	34 g
KCl	6.7 mM	0.5 g
Ca(NO$_3$).4H$_2$O	4.4 mM	1.0 g
MgSO$_4$.7H$_2$O	13 mM	3.13 g
Tris	46 mM	5.52 g
ddH$_2$O	N/A	~1 L
Total	N/A	1 L

Keep at room temperature (16°C–25°C) for up to 3 months.

Agar gel formulation		
Reagent	Final concentration	Amount
Agar	N/A	1.5 g
Steinberg's solution (1×)	1×	100 mL
Total	1.5% wt/vol	~100 mL

Dissolve it in a microwave, and keep it at room temperature (16°C–25°C) for up to 1 week.

DC power supply

A direct current supply that is normally used in the lab for electrophoresis can be used in this experiment. The voltage of the supply should range from 0 to 150 V.

U-shaped glass tube

The diameter of the glass tube should be in the range of 0.5 cm–0.8 cm. The length and shape of the U-shape glass tube should be adjusted according to the height of the beakers you use. See Figures 4A and 4B.

Lid of the EF stimulation chamber

Two holes are punched in the lid of a 100 mm cell culture dish with a heated soldering iron, as shown in Figure 4B. The distance between the two holes is ~50 cm. The diameter of the holes should be ~ 1 mm larger than that of the U-shaped glass tubes. Oversized holes on the lid should be avoided to prevent excessive evaporation of the cell culture medium during the long-term experiment.

Microscope

An inverted microscope (Carl Zeiss, Oberkochen, Germany) equipped with an XY motorized stage, time-lapse imaging software (Metamorph NX; Molecular Device, Sunnyvale, USA), and a Carl Zeiss incubation system was utilized. The microscope system was able to capture images of multiple locations.

STEP-BY-STEP METHOD DETAILS
Plating cells for imaging

⏲ Timing: 18 h

Seeding of cells inside the EF stimulation chamber reservoirs and patterning of cell sheets in the designed size and shape of the PDMS stencil.

1. Preparation for cell patterning.
   a. Precoat the area between each strip with 0.5 mL FNC at room temperature for 5 min.
   b. Remove the FNC coating mix with a pipette.
   c. Wash the area with PBS one time.
   d. Air-dry the coating area.
   e. Gently attach the sterilized PDMS stencils between the strips in the cell culture dish (Figure 2B).
   f. Add silicone grease around the PDMS stencils (Figure 2C) to make two reservoirs to store cell suspension for cell patterning. Figure 2D is the photo of chamber at this stage.

   Note: For each electrotaxis chamber, two PDMS stencils are applied, one for the electrical stimulation group and the other for the control (without electrical stimulation).

   ⚠ CRITICAL: Air bubbles should be avoided between the PDMS and the cell culture dish to make a firm attachment of the PDMS stencils to the cell culture dish. The device's reservoirs are designed to be sealed and leak-proof.

2. Seeding cells for cell patterning (Figure 3).
   a. Prepare a cell suspension at a concentration of 8×10^6 cells/mL.
   b. Add 1 mL of cell suspension for each cell patterning reservoir.
   c. Gently move the dish to an incubator and allow cells to attach for 15 min.
   d. Observe the cells with a microscope to ensure the desired cell density (troubleshooting 1).
   e. Gently remove the medium containing any unattached cells with a pipette.
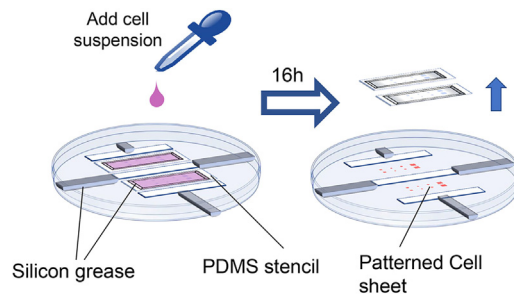   f. Add fresh cell culture medium and incubate cells overnight (~16 h).

Figure 3. Cell patterning workflow
Reproduced with permission from iScience.[1]

g. Observe the pattern of cell sheets with a microscope and confirm whether a confluent mono-layer is formed.
h. Using a pipette, remove the cell culture medium from the reservoirs.
i. Gently peel off the stencil with tweezers.
j. Gently wash the patterned cell sheet with the culture medium.
k. Using a microscope, examine the pattern of the cell sheets and confirm that they are in the desired size and shape (troubleshooting 2) (Figure 2E).

⚠ CRITICAL: For the HaCat cells, we recommend to keep the passage number lower than 30. Move the dish gently to avoid disturbing the settlement and adhesion of cells after seeding. The time required for cell attachment is cell type-dependent. When peeling off the stencil, try to peel it off from corners with tweezers and avoid contact with grease. In case the tweezers are contaminated by the grease, clean the tweezers with 75% ethanol and tissue paper. PDMS stencils will remain intact after peeling off. Discard the used PDMS stencils.

Assembling the EF stimulation system

⏱ Timing: 1 h

This section describes the assembly of the EF stimulation chamber, preparation of salt bridge and set up the electrotaxis system.

3. Assembling the EF stimulation chamber
   a. Add 0.5 mL cell culture medium in the area between PDMS strips.
   b. Gently put cover glasses over the stimulation area of the chamber (Figures 2E and 2F).
   c. Seal the stimulation chamber into two reservoirs (on the left and right) by placing silicone grease on the top of the cover glasses (Figures 2F–2G). Figure 2H is the photo of the chamber at this stage.
   d. Add 6 mL fresh cell culture medium in each reservoir.

Note: Since the distance between PDMS strips is less than 20 mm, thus the cover glass (50 × 24 mm) can be laid on the PDMS strips. In Figure 2G, areas α and β, γ and δ are connected by the intervals between the cell culture dish and cover glasses, respectively.

⚠ CRITICAL: Air bubbles should be avoided between the cover glass and the cell culture dish. The device's reservoirs are designed to be sealed and leak-proof. Make sure the medium can go through the space between the cell culture dish and the cover glass. To store 6 mL of cell culture medium in each reservoir, we have to build up walls of the reservoir with silicon grease, as shown in the Figure 2G.
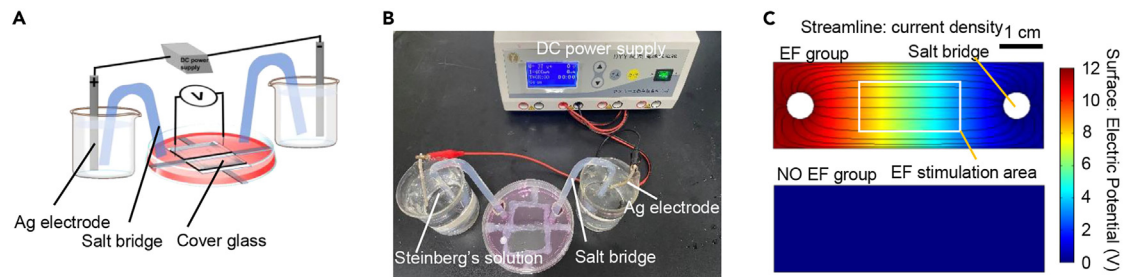
Figure 4. Electrotaxis system setup
(A and B) electrotaxis experiment setup.
(C) Electric potential and current density of the electrotaxis chamber simulated by COMOSL Multiphysics. Scale bar = 1 cm. Reproduced with permission from iScience.[1]

4. Preparation of salt bridge.
   a. Melt the 1.5% agar in 1× Steinberg solution by microwave.
   b. Slowly fill the agar in the glass bridge.
   c. Leave the salt bridge at room temperature to solidify.

   ⚠ CRITICAL: The agar solution should be fully melted before filling the glass bridge. Air bubbles should be avoided in the glass bridge.

5. Set up the electrotaxis system.
   a. Preheat the incubator of the living cell imaging system (37°C, 5% CO_2).
   b. Move the electrotaxis chamber into the incubator of the living cell imaging system.
   c. Full-fill two beakers (100 mL) with 1× Steinberg's solution (Figures 4A and 4B).
   d. Connect the electrotaxis system including the power supply, electrodes, salt bridges, beakers with saline, electrotaxis chamber, and wires (Figures 4A and 4B).
   e. Switch on the DC power supply.
   f. Monitor the voltage by inserting electrodes of a multimeter at the two ends of the coverslip in the EF stimulation chamber, as indicated in Figure 4A.
   g. Adjust the total voltage of the current supply to achieve the desired voltage between the two ends of the cover glass.
   h. Switch off the DC power supply.
   i. Cover the cell culture dish with a sterilized lid with holes in it.
   j. Align the electrotaxis chamber with the motorized loading stage of the microscope and make sure that the applied electric field is parallel to one edge of the stage.
   k. Note the relative position of the cathode and anode in the electrotaxis system.

   Note: For our experiment, the length of the cover glass is 50 mm, and to obtain an electric field of 200 mV/mm, the voltage measured should be 10.0 V.

   ⚠ CRITICAL: Wear insulating gloves and take caution against electric shock. The desired voltage between the two ends of the cover glass depends on the targeted strength of electric fields and the size of the cover glass. Electric fields distribute uniformly in the EF stimulation area, as indicated in Figure 4C, and cell sheets should be patterned in this field. Note the relative position of the electrodes.

Monitoring collective cell migration using time-lapse microscopy during EF stimulation

⏲ Timing: 9 h

This section describes a step-by-step walkthrough of live cell imaging of collective cell migration in a large cell sheet.

6. Preparation for imaging.
   a. Start the image acquisition software and set up the parameters required for the time-lapse.
   b. Select the objective according to the experimental demand. Here we use a 10× phase contrast objectives lens for imaging.
   c. Bring the imaging window of the stimulation chamber into focus.
   d. Navigate to a well-patterned cell sheet.
   e. Navigate an XY position to find a corner of the cell sheet and save the position in the image acquisition system.
   f. Navigate a series of XY positions to cover the whole cell sheet and make sure each adjacent region of interest (ROI) has a 20% overlap along the x and y axes.
   g. Navigate to the other cell sheets in the chamber and save the position in the imaging acquisition system. Cell sheets from both the EF-stimulation group and the NO EF group can be acquired in order.
   h. Give it a dry run by clicking the acquisition button.
   i. Stop image recording after finishing the first frame of all ROIs.
   j. Check the quality of the images to confirm that the focus and exposure of the image are set correctly and that there is no shift during multi-position acquisition.

   Note: In this study, the time interval was set as 5 min. The duration was set as 7 h. In our setup, 9 ROI with 20% overlap are required for a square cell sheet up to 1 mm × 1 mm in size.

   ⚠ CRITICAL: Cell sheets keep expanding after we peel off the PDMS stencil, thus to have a well-controlled experiment, the time spent on setting up the electrotaxis system and setting path of the imaging should be controlled within a certain time (in our experiment, it is about 1 h), thus making each independent round of experiments comparable.

7. Perform real-time imaging with precisely controlled EF stimulation.
   a. Start multi-position time-lapse recording by clicking the acquisition button again.
   b. Switch on the DC power supply at the desired time.
   c. Monitor the voltage during stimulation and adjust it if needed.
   d. Check that all stage positions are still in focus at later points.
   e. Stop image acquisition at a defined time point.
   f. Switch off the computer and microscope system.

   Note: In our study, EF is applied 1 h later after the time-lapse recording, and the EF stimulation duration is 6 h.

   ⚠ CRITICAL: Stage drift is a common complication when performing time-lapse recordings that span multiple hours. If images need to be refocused, pause imaging recording at an appropriate time point (at the end of the last round acquisition), adjust the focus and resume imaging within a few seconds, up to a couple of minutes. EF stimulation will not need to be stopped while adjusting the focus.

8. Generation of time-lapse movies of integrated cell sheets using ImageJ software.
   a. Launch the ImageJ program and import the imaging file using ''File>Import>Image sequence''.
   b. Adjust the brightness and/or contrast to attain a better view of the images by clicking ''Image>Adjust>Brightness/Contrast''.
   c. Images are then stitched together using Grid/Collection Stitching plugin in the ImageJ.
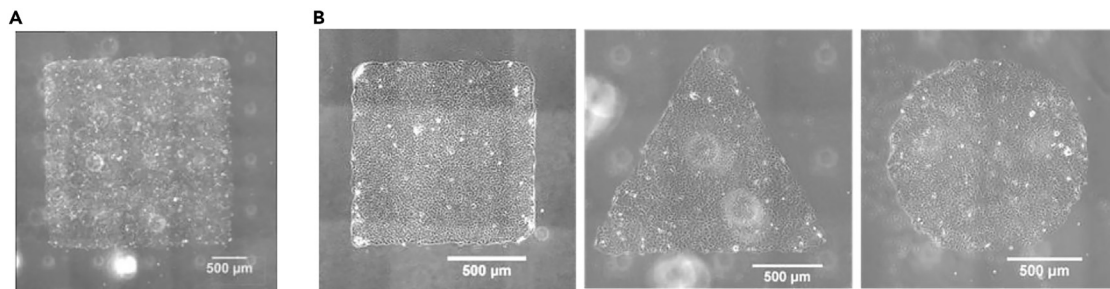
Figure 5. Engineering of the cell sheets
(A and B) Cell sheets of defined size and shape were engineered using a PDMS stencil. Scale bar = 500 μm. Reproduced with permission from iScience.[1]

d. Slight shifts of the images in the video are corrected by using Registration-Register Virtual Stack plugin in ImageJ software by clicking "Plugins>Registration>Register Virtual Stack".
e. Save the video as single ''.tiff'' files (troubleshooting 3) (Figure 5).

⚠ CRITICAL: Video must be saved as an image sequence in the form of TIF to meet the requirement of further analysis in ImageJ and MATLAB.

EXPECTED OUTCOMES

At the end of the protocol, the stitched time-lapse video will form a full image of the well-patterned cell sheet (Figure 5) which can be used to visualize the response of collective cell migration to an electric field. Results from the individual cell tracking in the different regions of the cell sheet can reveal the region-dependent directionality and speed dynamics of collective cell migration of the cell sheet (Figure 8) on the scale of individual cells. Individual cell tracking is limited in throughput, and thus the PIV analysis complements the tracking by allowing the measurement of motility dynamics across entire cell sheets (Figures 9 and 10).

QUANTIFICATION AND STATISTICAL ANALYSIS

⏱ Timing: 3 h

This section describes a step-by-step walkthrough of the collective cell migration assay with ImageJ and custom-made MATLAB code based on MatPIV1.6.1.[11]

1. Cell migration trajectory analysis with ImageJ.
   a. Launch the ImageJ program and import the imaging file using File>Open.
   b. Set the scale of the image by using" Analyze>Set Scale". In our study, the scale is 0.61 pixels /μm.
   c. Individual cells were tracked using ImageJ's MTrackJ plugin (Plugins>MTrackJ > Add).[10]
   d. To analyze region-dependent cell migration in the cell sheet, we define cells in the leading region, rear region, and lateral region (right) as the 10 rows of cells away from the free edge (Figures 6 and 8A).
   e. Measure cell migration trajectories by clicking "measure" in the toolbox of the MTrackJ.
   f. By clicking "save" in the toolbox of the MTrackJ, export the coordinates of each cell at each time point from ImageJ.
   g. Use the directionality to quantify the directedness of cell migration, which is defined as the cosine of the angle between the cell motion and the y-axis (θi) (Equation 1) (Figure 7);

Cell migration speed is defined as the accumulated migration distance (D_i) divided by the total time (Equation 2) (Figures 7 and 8D).
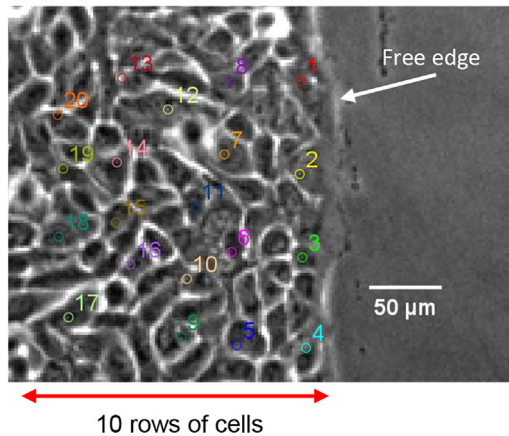
Figure 6. Illustration of selection of cells at the right region of the cell sheet for cell migration trajectory analysis

Cells in the cell sheet are not arranged strictly in rows, we can only roughly count the rows. Scale bar: 50 μm.

Note: In this study, the position of a cell is defined by its nucleus. For each region, 20 unconnected cells are tracked at 10-min frame intervals. In our experiment, the electrical field was applied along the y-axis, and directed down the image (cathode at the top and anode at the bottom). A cell migrating directly toward the anode would have cosine = 1 and sine = 0 (Equation 1) (Figures 7, 8B, 8C, 8E, and 8F).

$$\text{Directionality} = \frac{1}{n} \sum_{i=1}^{n} \cos \theta_i \qquad \text{Equation 1}$$

$$\text{Speed} = \frac{1}{n} \sum_{i=1}^{n} \frac{D_i}{\text{duration}} \qquad \text{Equation 2}$$

2. Cell collective cell migration analysis with PIV in MATLAB (Methods video S1).
   a. Download the "PIV" folder from the Mendeley Data repository and save the folder on your desktop.
   b. Install MATLAB 2016.
   c. Set the "PIV" folder as the current folder in MATLAB.
   d. Open the file *PIV_batch_script.m* in MATLAB.
   e. Change the directories listed at the top of the script. For example, to run the code on the test images, use directories = {'C:\Users\...\Desktop\PIV\test\'}.
   f. Run the PIV_batch.m script file by clicking "EDITOR> Run".
   g. Find the newly generated files in the directories set in step f: *200 mvdot_piv.mat* and *200mvdot_piv_filtered.mat*. For further analysis, we will use *200mvdot_piv_filtered.mat*.
   h. Generate the migration speed heatmaps of the cell sheet (Figures 9A and 9B).
      i. Load *200mvdot_piv_filtered.mat* into the workspace.
      ii. Input *plot_piv_pcolor_speed (dot_piv)* in the command window in MATLAB and click Enter on the keyboard.
      iii. Find the newly generated files in the image folder under a subfolder with the image name, e.g., 200 mv. This folder includes a set of speed heat maps of the cell sheet.
      iv. You can open and edit the figures in the format of .fig with MATLAB.
   i. Generate the directionality heatmaps of the cell sheet (Figures 9A and 9B).
      i. Load 200mvdot_piv_filtered.mat into the workspace.
      ii. Input plot_piv_pcolor_sin (dot_piv) in the command window in MATLAB and click Enter on the keyboard.
      iii. Find the newly generated files in the image folder under a subfolder with the image name, e.g., 200 mv. This folder includes a set of directionality heat maps of the cell sheet.
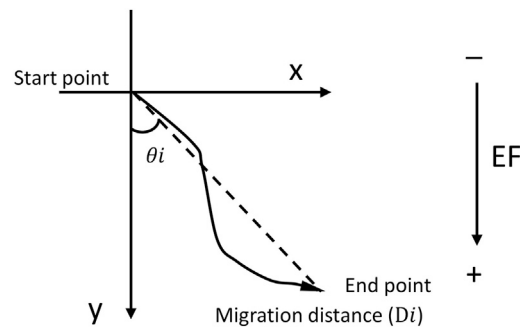   j. Generation of kymographs (Figures 9C and 9D) and rose plot.

Figure 7. Schematic illustration of analysis of cell migration

  i. Open the *piv_custom _analystics.m* script in MATLAB.

  ii. Change the directories to the location of the image folder, e.g., directories = {'C:\Users\...\Desktop\PIV\test\'}.

  iii. Run *piv_custom _analystics.m* by clicking "EDITOR> Run".

  iv. Find the newly generated files in the image under a subfolder, *Analytics Data.* This folder includes the directionality kymograph, speed kymograph, and rose plot for the cell sheet collective migration. You can open and edit the figures in the format of .fig with MATLAB.

 k. Generate the contour dynamics of the cell sheet (Figure 10).

  i. Open *outline_batch_script.m.*

  ii. Change the directories to the location of the image, e.g., directories = {'C:\Users\...\Desktop\PIV\test\'}.

  iii. Find the newly generated files in the image folder under a subfolder for outline images combined with the image name, e.g., *OutlineImages_200mv.* This folder includes a set of images of the cell sheet outline.

⚠ CRITICAL: Operators are assumed to be familiar with MATLAB and coding. For a quick test of these codes, don't change the name of any document and test the code on the provided test tif. The computer operating system we used is Windows 10. We used MATLAB R2016a, and the code has also been tested in MATLAB 2021b and MATLAB 2022b. After a successful test, you can change the parameters in the codes and run it for your experiment. If you save all the videos generated in one experiment (with the same resolution, duration, etc.) in one folder, *PIV_batch.m*, *piv_custom _analystics.m*, and *outline_batch_script.m* will analyze all the images in that folder sequentially. A step-by-step operating video of cell collective cell migration analysis with PIV in MATLAB was recorded, see Methods video S1.

Note: Note that these files are named with the prefix 200mv as this is the name of the provided test tif file. The diagnostic window size in PIV is 64 × 64 pixels, the secondary window size is 32 × 32 pixels, and there is a 50% overlap of adjacent windows; the interval of each image is set by the imaging parameters (in this case, 5 min). The distinction between cellular and cell-free areas is made by detecting changes in grayscale intensity using a Sobel filter, and the program automatically removes the unnecessary signal in the cell-free area. The contour and geometric center of the cell clusters are calculated by MATLAB's built-in programs. The edge effect in the PIV analysis is minimized by removing 8 pixels from the edges of the cell area. u stands for the velocity in the horizontal direction and v stands for the velocity in the vertical direction. For each 16 × 16 pixel window, PIV calculates a v and u for each time interval. Cell migration speed was calculated as Speed = $\sqrt{u^2 + v^2}$. Directionality was calculated as $\cos \theta = u/ \sqrt{u^2 + v^2}$. The formulas for calculating speed and directionality here in MATLAB are different from that in ImageJ, because the parameters generated by ImageJ
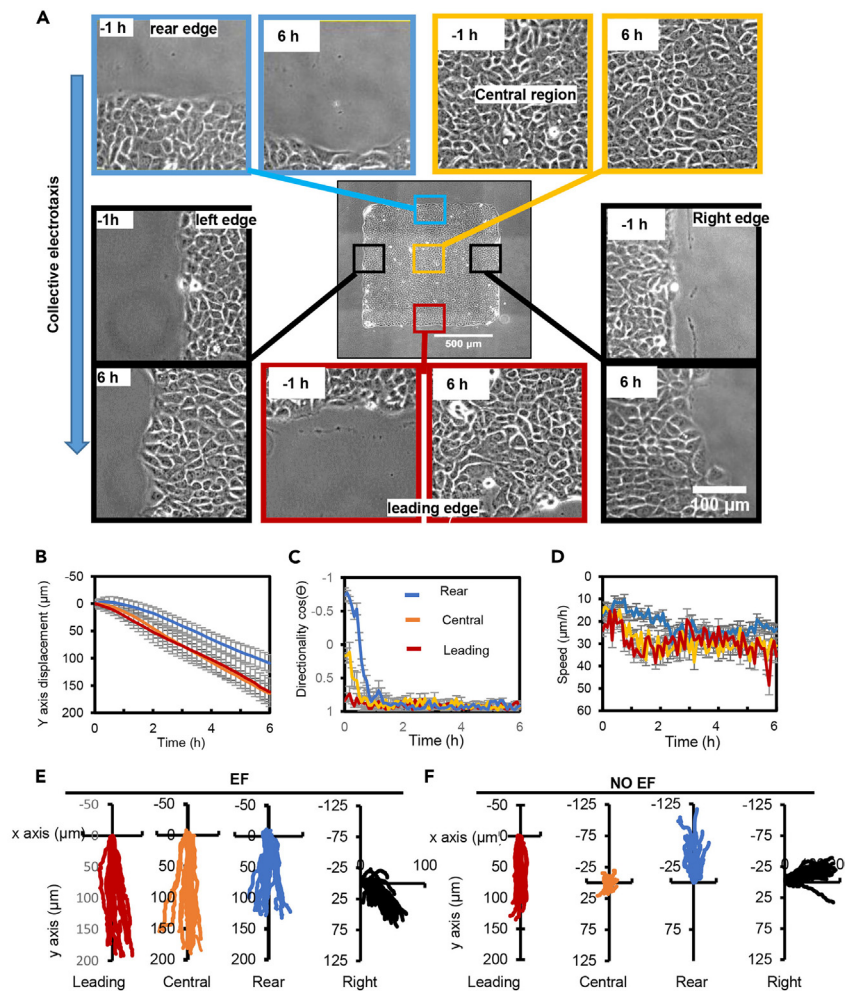
Figure 8. Leading cells lead collective electrotaxis with suppressed migration sideways

(A) Snapshot of regions of a cell sheet 1 h before the onset of EF and 6 h in the EFs. Scale bar: 100 μm.

(B–D) Cells in the leading (L), Central (C), and Rear (R) regions were tracked, for Y-axis displacement (B), directionality (C), and speed (D) as a function of time during EF application. Data are presented as mean +/− SEM from 20 cell migration trajectories from three independent experiments.

(E and F) Migration trajectories of cells in the leading region, central region, right region, and rear region of the cell sheet in an EF and NO EF group. Data are representative of three independent experiments (n = 20 for each group). EF = 200 mV/mm. Reproduced with permission from iScience.[1]

and MATLAB are distinct. However, the principles of the quantification are the same, and, if desired, the PIV equations could also be applied to the ImageJ tracking data. The u, v, speed, and directionality can be color-coded to indicate their corresponding values and projected on the corresponding positions of the heatmap.

Kymographs are used to quantify and visualize the spatiotemporal dynamics of directionality and speed from the PIV measurements. For each data matrix from the PIV analysis, we computed the average value for each column or row parallel to the edge and then derived a one-dimensional segment for each time point.

The contour of the cell sheets was segmented by custom MATLAB code that uses Sobel filtering to find edges in the images. Small, segmented regions such as those from individual scattered cells are excluded. The centroid of the cell sheet was computed using MATLAB's regionprops function.
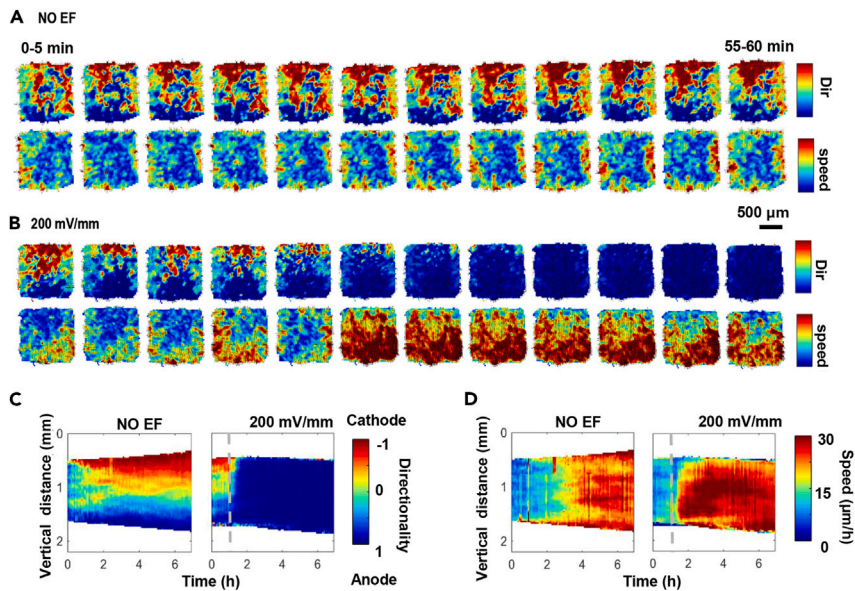
Figure 9. EFs, overriding default directional cues at the free edge, guide and mobilize cell sheets by inducing the propagation of increased directionality and speed across the whole cell sheet

(A and B) Snapshots of directionality (Dir) and speed heatmaps of a cell sheet in the first hour after EF application. Directionality and speed of movement of the cell sheet are shown as heatmaps from PIV analysis from two adjacent images with a time interval of 5 min. Scale bar = 500 μm.

(C and D) Kymographs of directionality and speed as a function of time along the x-axis (C) and y-axis (D), respectively. Kymographs in the no EF group show that directionality and speed propagate from the free edge to the center of the cell sheet and then deflect back when they meet. Kymographs in an EF (200 mV/mm) show a dramatic increase in directionality and speed propagating from the leading edge to the rear edge. Dashed lines indicate the onset of the field. The experiment duration is 7 h. The kymographs presented are drawn from one experiment and confirmed in three independent experiments. Reproduced with permission from iScience.[1]

LIMITATIONS

The electrotaxis system setup and preparation for imaging need a considerable period (~1 h) and as a result, the response of the cell sheet right after peeling off the PDMS stencil can't be recorded in our system. Long-term active electrochemical reactions between silver electrodes and Steinberg's solution can result in a significant resistance variation of the system and pH change in the cell culture medium and Steinberg's solution. Thus this system is not designed for long-term observation of EF stimulation beyond 12 h.

TROUBLESHOOTING

Problem 1

There are air bubbles at the corner of the area where you desired to pattern cells and this results in incomplete patterned cell sheets. This issue is more frequent when your desired cell patterns are very small. Related to step 2.

Potential solution

The following steps can be used to avoid air bubbles before cell plating.

- Add 0.5 mL of 75% alcohol to the cell patterning reservoirs before adding the cell suspension.
- Observe the cell patterning reservoirs in the microscope and there are no air bubbles in the corners of the desired pattern areas.
- Remove the remaining alcohol by rinsing the patterning reservoirs with 1 mL of cell culture medium 10 times.
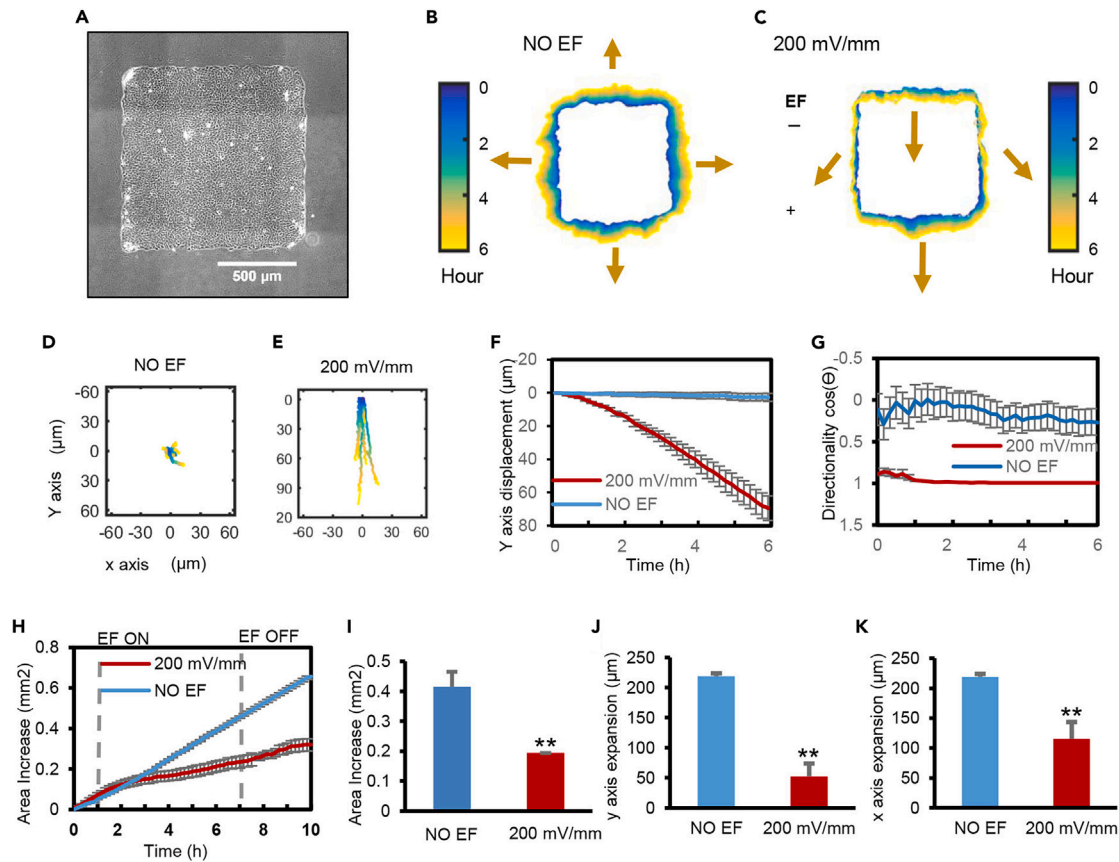- Add cell suspension and continue the experiment.

Figure 10. EFs induce collective electrotaxis of large epithelial sheets that maintain cell-cell junctions
(A) Phase contrast image of a square cell sheet. Scale bar = 500 μm.
(B and C) Collective electrotaxis is demonstrated by a contour assay showing the color-coded edge positions of cell sheets. Color coding from blue (0 h) to yellow (6 h). Arrows indicate overall edge migration direction and distance. An EF guided directional migration of the cell sheet downward (C), whereas a control cell sheet without EF expanded in all directions (B). The polarity and strength of the field are as shown.
(D–G) Collective electrotaxis of whole cell sheets is shown by the trajectories of the geometric center (centroid) of cell sheets (n = 6) (D and E). Y-axis displacement (F), directionality along the field line (G), as a function of time. Directionality is calculated as cosθ, where θ is the angle between each centroid trajectory and the electric field line. Data are represented as mean +/- SEM from 6 independent experiments. A positive value of cosθ indicates migration to the anode; a negative value indicates migration to the cathode, and zero indicates a random direction.
(H–K) EFs maintained cell sheet geometry by suppressing the dispersal of cells. Quantification of area increase (H–I), y axis expansion (J), and x axis expansion (K). (**$p < 0.01$).
Data are represented as mean +/− SEM from 6 independent experiments. Reproduced with permission from iScience.[1]

Problem 2
Cell sheets are damaged and incomplete after the peeling of the PDMS stencil. Related to step 2.

Potential solution
Shorten the co-culture time of cells and the PDMS stencil. Peel off the PDMS stencil right after cells form a confluent cell monolayer.

Problem 3
The edges of the cell sheet don't migrate forward after peeling off the PDMS stencil. Related to step 3.

Potential solution
Make sure that you have precoated the cell culture dish with the FNC coating mix and that the PDMS stencil you used doesn't leave any smears or tracks on the cell culture dish after being peeled off.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Min Zhao (minzhao@ucdavis.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The dataset and code used during this study are deposited at Mendeley Data and are publicly available as of the date of publication. The DOI is listed in the key resources table. A previously published article[1] includes all the datasets analyzed in this study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102288.

AUTHOR CONTRIBUTIONS

Y.Z. and M.Z. designed the experiments. W.L., Z.X., T.P., F.L., and M.Z. supervised the study. Y.Z. performed the cell experiment and analysis. R.M.L. wrote scripts for analysis. Z.Z. designed and fabricated the stencil. Z.Y. and M.Z. wrote the manuscript. All authors commented and/or edited the manuscript and figures.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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