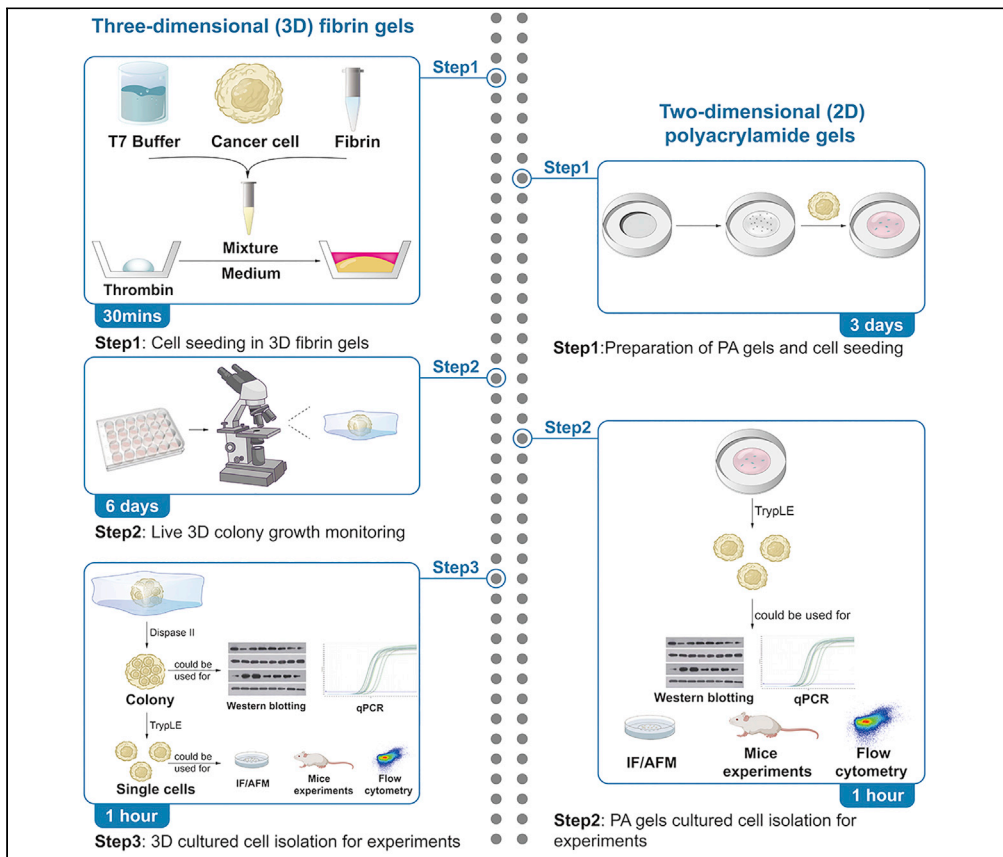


Protocol

Tumorigenic cell selection and substrate rigidity manipulation using 2D and 3D extracellular matrices



Juan Zhang, Wentao Ha, Yuhan Chang, Yutong Ge, Xiaowei Wei, Youhua Tan, Qiong Jia

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Highlights

Protocol of tumorigenic cell selection and substrate rigidity manipulation

Fabricate and maintain soft 3D fibrin gels with tumorigenic cell selection capacity

Tune substrate rigidity by 2D polyacrylamide (PA) gels

Application of 3D fibrin and 2D PA gels in tumor mechanotransductive research

Soft fibrin gels are used to select tumorigenic cells and regulate the stemness and metastasis of colorectal cancer cells via mechanotransduction. This protocol details steps to produce two-dimensional (2D) and three-dimensional (3D) extracellular matrices for substrate rigidity manipulation and tumorigenic cell selection. We also describe how it can be applied to tumor mechanotransductive research by colony growth monitoring and cell isolation.

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

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Protocol

Tumorigenic cell selection and substrate rigidity manipulation using 2D and 3D extracellular matrices

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<https://doi.org/10.1016/j.xpro.2022.101577>

SUMMARY

Soft fibrin gels are used to select tumorigenic cells and regulate the stemness and metastasis of colorectal cancer cells via mechanotransduction. This protocol details steps to produce two-dimensional (2D) and three-dimensional (3D) extracellular matrices for substrate rigidity manipulation and tumorigenic cell selection. We also describe how it can be applied to tumor mechanotransductive research by colony growth monitoring and cell isolation.

For complete details on the use and execution of this protocol, please refer to Chang et al. (2022).

BEFORE YOU BEGIN

Extracellular mechanical force defines the fate of tumor cells. Various extracellular matrix conditions, including physiological physical microenvironment, can be simulated by altering biomechanical conditions. This protocol described the method of rapid construction of 2D and 3D extracellular matrix with tumor malignant regulation capacity. We have used this protocol to select colorectal tumorigenic cells with high stemness and metastatic capacity, which are regulated by extracellular substrate rigidity (Chang et al., 2022).

Institutional permissions

Mice used in this protocol were purchased from Institute of Comparative Medicine of Yangzhou University for experiments and maintained under specific pathogen-free conditions. Approval by the Experimental Animal Ethics Committee of Nanjing First Hospital, Nanjing Medical University were obtained (No. SYXK2021-0007).

The protocol below describes the specific steps for using colorectal tumor cells (HCT116). However, we have also used this protocol in melanoma (B16), lung (A549), and prostate (PC-3) carcinoma cells.

Prepare materials and cells

⌚ Timing: 3 days

1. Start a HCT-116 cell culture. Resuscitate uncontaminated HCT116 cells and seed the vigorously proliferating cells into a 6-well cell culture plate to ensure that the cells reach about 70% confluence within 2 days. Cells have to be subcultured at least twice before cell seeding.



2. Prepare materials for fibrin gels
 - a. T7 buffer (50 mM Tris, 150 mM NaCl, pH 7.4): Prepare T7 buffer by dissolving Tris and NaCl in double-distilled water (ddH₂O). Sterilize the buffer with a 0.22 μm membrane filter and store the buffer at 4°C for up to three months.
 - b. Fibrinogen stock (20 mg/mL): Dissolve 100 mg Salmon fibrinogen powder in 5 mL T7 buffer on ice and store at -80°C in 500 μL aliquots for up to 3 months.
 - c. Thrombin stock (0.1 U/μL): Prepare Salmon thrombin by dissolving 1000 U thrombin powder in 10 mL T7 buffer and store at -80°C in 150 μL aliquots for up to 3 months.
 - d. Dispase II stock: Dilute 500 mg Dispase II in 5 mL sterile PBS and store at -20°C in 500 μL aliquots for up to 3 months.
3. Prepare materials for PA gels
 - a. 0.5% glutaraldehyde solution: Dilute 25% glutaraldehyde to 0.5% with sterile PBS, and dilute it before use.
 - b. Ammonium Persulfate (APS) solution: Prepare 10% APS by dissolving 0.1g APS in 1 mL ddH₂O and store at 4°C in dark. It is not recommended to use 10% APS stored for more than one week.
 - c. Sulfo-SANPAH stock: Prepare 5 mg/mL Sulfo-SANPAH stock solution by dissolving 50mg Sulfo-SANPAH in 10 mL dimethyl sulfoxide (DMSO) and store at -20°C in dark in 500 μL aliquots for up to 6 months.

Note: 0.5% glutaraldehyde solution has to be prepared fresh and used on the same day. APS is harmful to health. Wear protective gear and operate safely. All the materials and their preparation processes should be kept strictly sterile. The formula for the preparation of the materials above is listed in the [materials and equipment](#).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
E-Cadherin (24E10) Rabbit mAb #3195(1:100)	Cell Signaling Technology	Cat# 3195S; RRID: AB_2291471
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (1:1000)	Abcam	Cat# ab150077; RRID: AB_2630356
Chemicals, peptides, and recombinant proteins		
Salmon Fibrinogen	Sea Run Holdings	Cat# SEA-133
Salmon Thrombin	Sea Run Holdings	Cat# SEA-135
Dispase II	Sigma-Aldrich	Cat# D4693
Collagen Type I, Rat Tail	Corning	Cat# 354236
3-Aminopropyltrimethoxysilane	Sigma-Aldrich	Cat# 281778
Acrylamide solution	Sigma-Aldrich	Cat# A4058
N, N'-Methylenebisacrylamide solution	Sigma-Aldrich	Cat# M1533
Tetramethylethylenediamine	Sigma-Aldrich	Cat# T22500
Ammonium persulfate	Sigma-Aldrich	Cat# 248614
0.25w/v% Trypsin-EDTA	Gibco	Cat# 25200-072
TrypLE™ Express	Gibco	Cat# 12605010
Fetal bovine serum	Sigma-Aldrich	Cat# F8687
Normal Donkey Serum	Solarbio	Cat# SL050
Triton-X 100	Sigma-Aldrich	Car# 93443
Lipofectamine™ 2000	Invitrogen	Cat# 11668019
Phalloidin-Atto 594	Sigma-Aldrich	Cat# 51927
DAPI	Sigma-Aldrich	Cat# D9542
Tris base	Sigma-Aldrich	Cat# 93362
NaCl	Sigma-Aldrich	Cat# NIST3530

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
PBS	Gibco	Cat# 10010023
Sulfo-SANPAH	Sigma-Aldrich	Cat# 803332
dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat# D2438

Experimental models: Cell lines

Human: HCT116	National Collection of Authenticated Cell Cultures	TCHu 99
Mouse: B16	National Collection of Authenticated Cell Cultures	TCM 2
Human: A549	Gift from Professor Dongping Wei's laboratory	Cat# CCL-185, RRID: CVCL_0023
Human: PC-3	Gift from Professor Yong Xu's laboratory	Cat# CRL-1435, RRID: CVCL_0035

Software and algorithms

Image J	NIH	https://imagej.net/ ; RRID: SCR_003070
GraphPad Prism (version 8.0)	GraphPad Software Inc.	https://www.graphpad.com/scientific-software/prism/ ; RRID: SCR_002798

Other

6-well plate	Corning	Cat# 3506
24-well plate	Corning	Cat# 3526
96-well plate	Corning	Cat# 3596
Glass bottom dish (35mm dish with 14mm bottom well)	Cellvis	Cat# D35-14-0-N
Glass bottom dish (35mm dish with 20mm bottom well)	Cellvis	Cat# D35-20-0-N
Slide glass	NEST	Cat# 801011
Kimwipe	Nippon Paper Creca	Cat# 62011
Microscope	Olympus	IX71
Membrane filters (0.22 μm)	Merck	Cat# GVWP04700

MATERIALS AND EQUIPMENT

T7 buffer

Reagent	Final concentration	Amount
Tris base	50 mM	3.03 g
NaCl	150 mM	4.38 g
ddH ₂ O	N/A	n/a
Total	N/A	500 mL

Store at 4°C for up to three months.

Fibrinogen stock

Reagent	Final concentration	Amount
Fibrinogen	20 mg/mL	100 mg
T7 buffer	N/A	5 mL
Total	N/A	5 mL

Store at -80°C for up to three months.

Note: It may take more than 5 h to dissolve Salmon Fibrinogen in T7 buffer. Keep shaking on a shaker and stay cold during dissolution to prevent protein degradation.

Thrombin stock

Reagent	Final concentration	Amount
Thrombin	0.1U/ μ L	1000U
T7 buffer	N/A	10 mL
Total	N/A	10 mL

Store at -80°C for up to three months.

Dispase II stock

Reagent	Final concentration	Amount
Dispase II	100 mg/mL	500 mg
PBS	N/A	5 mL
Total	N/A	5 mL

Store at -20°C for up to three months.

0.5% glutaraldehyde solution

Reagent	Final concentration	Amount
25% glutaraldehyde	0.5%	1 mL
ddH ₂ O	N/A	49 mL
Total	N/A	50 mL

The solution was prepared just before use.

Ammonium Persulfate (APS) solution

Reagent	Final concentration	Amount
Ammonium Persulfate	10%	0.1 g
ddH ₂ O	N/A	1 mL
Total	N/A	1 mL

Store at 4°C for up to one week.

Sulfo-SANPAH stock

Reagent	Final concentration	Amount
Sulfo-SANPAH	5 mg/mL	50 mg
DMSO	N/A	10 mL
Total	N/A	10 mL

Store at -20°C in dark for up to six months.

STEP-BY-STEP METHOD DETAILS

Fabrications of fibrin gels

⌚ Timing: 2 h

1. Preparation of fibrinogen and cell solution mixture.
 - a. Trypsinize cells with TrypLE or trypsin to dissociate and suspend in medium and count cell concentration.
 - b. Add appropriate amounts of fibrinogen, cell solution, cell medium, and T7 buffer to get a cells/fibrinogen mixture. Keep the mixture on ice. Different concentrations of fibrinogen form matrices with different stiffness (Liu et al., 2012; Tan et al., 2014). The formula of some frequently-used and stiffness calibrated fibrin gels is shown in Table 1.

Table 1. Fibrin gel stiffness is controlled by varying amounts of fibrinogen

Gel stiffness (Pa)	Fibrinogen (μL)	T7 buffer (μL)	Cells mixture / culture medium (μL)	Thrombin (μL)	Total (μL)
90	12.5	112.5	125	5	255
180	25	100	125	5	255
420	50	75	125	5	255
1050	100	25	125	5	255

2. Cell seeding in 3D fibrin gels.
 - a. Add 5 μL thrombin (0.1 U/ μL) to the center of each well (24-well plate).
 - b. 250 μL mixture of the fibrinogen and cell solution is seeded immediately into each well.
 - c. Put the 24-well cell culture plate for 30 min in a 37°C incubator with 5% CO₂ until fibrin gel polymerization. The visual workflow of fibrin gels generation is shown in [Figure 1. Troubleshooting 1](#).
 - d. Add 1 mL medium to each well, and put it back into the 5% CO₂ incubator.
 - e. Carefully remove the old culture medium every two days, and add 1 mL of fresh medium into each well. Tilt the plate slightly during the liquid change to prevent the pipette from disturbing the gels.

Note: Do not allow the gel to wrinkle when mixing fibrinogen and cell solution mixture with thrombin. It is recommended to place the culture plates on ice throughout the process on the premise of ensuring an aseptic operation. [Troubleshooting 2](#).

Note: HCT116 cells form smooth and proliferating colonies in 90-Pa 3D fibrin gels ([Figure 5](#)). With the increase of gel stiffness, the colony shape becomes smoother, but the growth rate decreases gradually. [Troubleshooting 3](#).

Live 3D colony growth monitoring

⌚ Timing: 6 days

3. Colony size and circularity analysis.
 - a. Seed 5000 cells into 3D fibrin gels per well in a 24-well plate. Two repeats for each condition.
 - b. Capture at least 15 colony images for each condition every two days with a regular widefield microscope.
 - c. Analyze Colony size or circularity quantitatively by image J software. Draw the growth curve of colonies based on the measured size or circularity across time.
4. Colony numbers analysis.
 - a. Culture 500 cells into 3D fibrin gels per well in a 96-well plate for each condition. Three repeats for each condition. Count the numbers of round colonies in the wells under the microscope every two days.
 - b. Draw the growth curve based on the colony numbers across time.

Note: Culture plates of 3D fibrin gels should be selected according to the specific needs of experiments. The cell numbers seeded in 3D fibrin gels are variable. Make sure that there is still space between colonies after 6 days of culturing without touching each other. The formula of reagents and cell concentrations in different plates is shown in [Table 2](#).

Note: For the experiments that need to detect the colony growth after cell transfection, direct transfection in a 3D fibrin gel system is not recommended, as it is difficult for Lipofectamine to reach cells or colonies through fibrin gel pores. It is recommended to transfect cells before 3D fibrin gels generation.

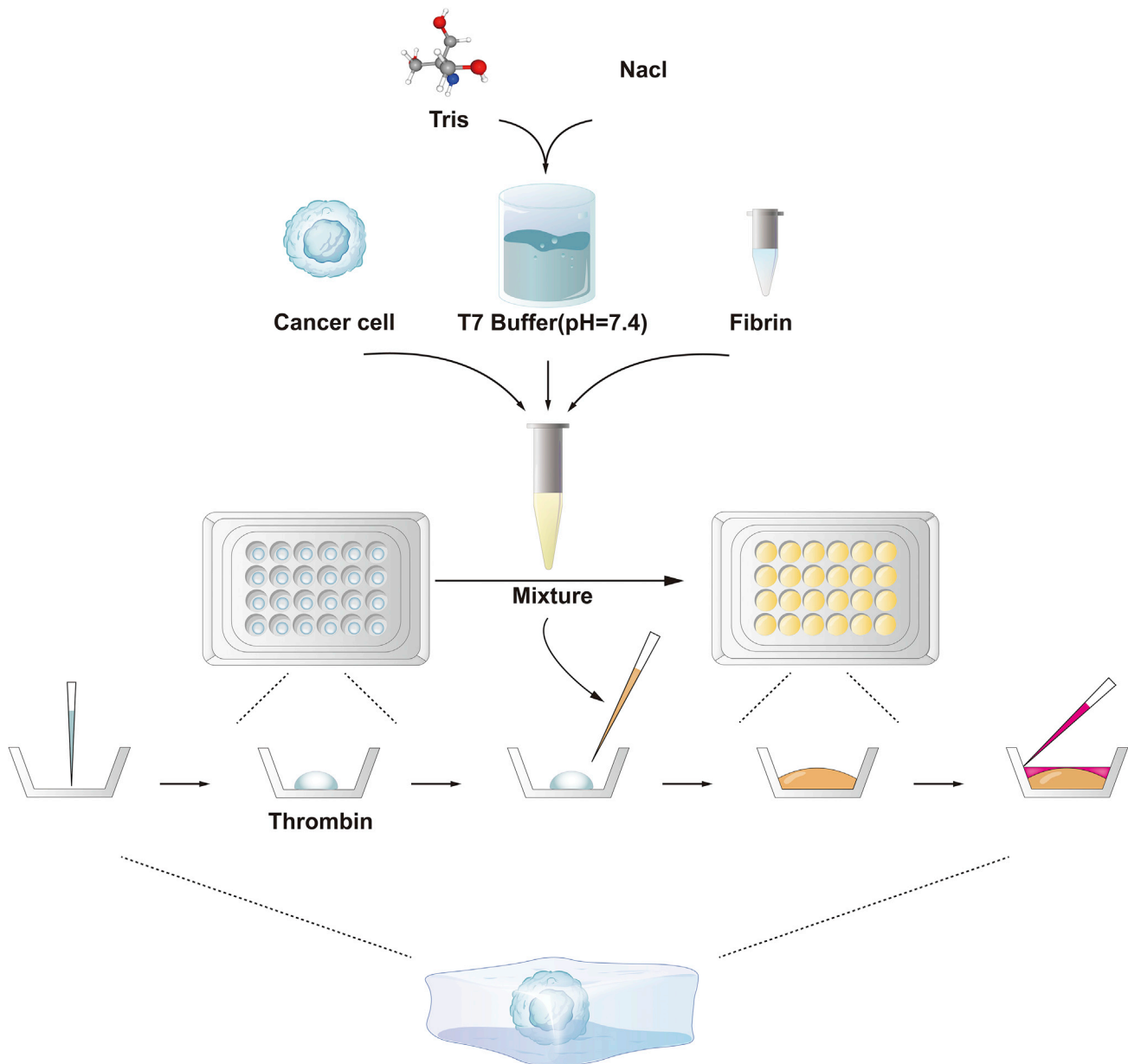


Figure 1. The operation flow of fibrin gels generation in a 24-well plate

Note: Capture colony images randomly rather than selectively, and note that the completely spreading tumor cells tend to be differentiated which may not have tumorigenic potential.

3D cultured cell isolation

⌚ Timing: 1h

3D cultured colonies or single cells need to be extracted from fibrin gels for subsequent mechano-transductive study. 3D cultured colonies can be used for qPCR and western blotting, while 3D cultured single cells can be used for immunofluorescence staining (IF), atomic force microscope (AFM) measurement, and flow cytometry.

Table 2. Reagent amounts of 3D fibrin gels (90-Pa) for each well in different plates

Plates	Fibrinogen (μL)	T7 buffer (μL)	Cells mixture / medium (μL)	Cell concentration range	Thrombin (μL)	Total (μL)
24-well	12.5	112.5	125	40,000–240,000/mL	5	255
96-well	2.5	22.5	25	1,600–8,000/mL	1	51
Glass bottom dish (14mm bottom)	10	90	100	4,000–16,000/mL	4	204

5. Fibrin gel degradation.
 - a. Take out a Dispase II stock (100 mg/mL) and melt it on the ice. Dilute it to 2mg/mL with PBS.
 - b. Take a 24-well plate with fibrin gels and colonies out from the incubator. Remove culture medium and wash with PBS once.
 - c. Discard PBS and add 200 μL of Dispase II solution into each well.
 - d. Incubate the matrix at 37°C with 5% CO₂ for 5–15 min.
 - e. After gel degradation, collect the solution to a 15 mL centrifugal tube and centrifuge at 200 × g for 5 min to obtain the colonies, which could be used for qPCR, western blotting, and other experiments that do not require single cells.
6. Colony dissociation.
 - a. Disperse the colony pellet in step 5.d with 1 mL of TrypLE. Place the tube in a 37°C water bath.
 - b. Disperse the cell mass every 5 min and observe the degree of colony dissociation under the microscope.
 - c. When it is observed that the colonies are completely dissociated into single cells, add the culture medium to terminate the digestion. Harvest single cells after centrifugation at 200 × g for 5 min. [Troubleshooting 4](#).
 - d. Single cells can be counted for injection of mice experiments or flow cytometry, or replated onto dishes for high-resolution analysis including single-cell imaging or AFM measurement.

Note: It usually takes 15–20 min to dissociate colorectal colonies into single cells. The whole colonies or single cells harvest process should be controlled within 1 hour, as the physical environment has been altered once fibrin gels degrade. The properties of 3D fibrin gels selected colonies or cells may change due to their plasticity when placed in a tube or rigid plastic well for a long time.

3D cultured cell fixation for fluorescence imaging

⌚ **Timing:** 3 days

7. Paraformaldehyde fixation.
 - a. Seed 5000 cells into 3D fibrin gels on a glass-bottom dish according to the reagent amounts shown in [Table 2](#).
 - b. After 2–6 days of culturing, carefully aspirate the culture medium and wash with PBS once.
 - c. Add 1 mL 4% Paraformaldehyde to each dish and shake at 25°C–27°C for 10 min.
 - d. Wash with PBST (0.05% Triton-X in PBS) on a shaker for 20 min three times.

⏸ **Pause point:** After fixation, the glass bottom dishes with gels and colonies covered with PBS can be stored at 4°C for two weeks.

8. Immunofluorescence.
 - a. Remove PBST and add 500 μL Normal Donkey Serum (blocking serum) to each dish. Block for at least 5 h at 4°C.
 - b. Prepare 100–200 μL of primary antibody solution diluted with blocking serum for each glass-bottom dish, and the dilution ratio of primary antibody is about 1:50 to 1:100.

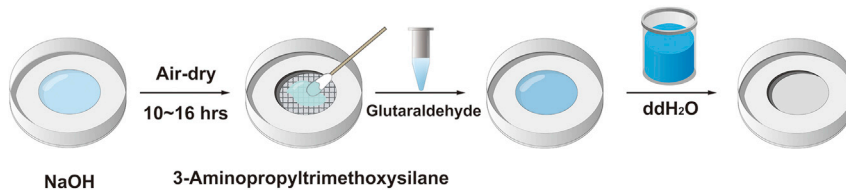


Figure 2. Glass-bottom dishes activation

- c. After blocking, remove the blocking buffer and add 100–200 μL primary antibody solution to the center of the glass-bottom dish. Incubate the antibody at 4°C for 5–12 h.
- d. Remove primary antibody and wash with PBST on a shaker for 20 min three times.
- e. Repeat step 8. a and prepare 500 μL of second antibody solution diluted with blocking serum for each glass-bottom dish, and the dilution ratio of the second antibody is 1:1000.
- f. After blocking, remove the blocking buffer and add 500 μL s antibody solution to the center of the glass-bottom dish. Incubate at 25–27°C and protect from light for 2 h.
- g. Remove the second antibody and wash with PBST on a shaker for 20 min three times. The dishes are ready for imaging.

Alternatives: When using dyes such as fluorescent labeled phalloidin or DAPI for fluorescent staining, dye solution can be directly added for staining (skip 8a to g). After 30 min staining, imaging can be carried out after PBST washing for 20 min three times.

Note: During fluorescence imaging, try to focus on the colonies in the middle of the gels instead of those on the bottom. Because colonies on the bottom of gels directly contact the rigid glass of the dish, its properties are different from the tumorigenic cells.

Preparation of PA gels and cell seeding

⌚ **Timing:** 3 days

9. Preparation of activated glass-bottom dishes.
 - a. Add 200 μL 0.1 N NaOH with a pipette to the glass bottom dishes (35 mm with 20 mm glass-bottom well) and air-dry for 10–16 h.
 - b. Smear 100–200 μL of 3-aminopropyltrimethoxysilane evenly on the glass bottom dishes with a sterile q-tip for 6 min.
 - c. Rinse with ddH₂O on a shaker for 15 min twice with gentle agitation.
 - d. Cover the glass with 0.5% glutaraldehyde and incubate at 25°C–27°C for 1h.
 - e. Remove the glutaraldehyde, rinse with ddH₂O for 15 min twice and remove the ddH₂O by aspiration and UV treated Kimwipe paper. The activation steps of glass-bottom dishes have been completed (Figure 2).

⚠ **CRITICAL:** NaOH, 3-aminopropyltrimethoxysilane, and glutaraldehyde are harmful to health. Wear protective gear and operate safely.

⏸ **Pause point:** Activated glass bottom dishes can be stored for 48 h at 25°C–27°C or 2 weeks at 4°C.

10. Preparation of polyacrylamide Solution.
 - a. The gel precursor solution is mixed from 40% acrylamide solution and 2% bis-acrylamide solution in ddH₂O, and gels can be polymerized by adding 1/200 volume of 10% APS and 1/2000 volume of TEMED to the precursor solution. PA gels stiffness is controlled by varying amounts of 40% acrylamide and 2% bis-acrylamide solutions (Engler et al., 2004; Yeung

Table 3. PA gel stiffness is controlled by varying amounts of reagents

Gel stiffness (kPa)	40% acrylamide solution (μL)	2% bis-acrylamide solution (μL)	ddH ₂ O (μL)	10% APS (μL)	TEMED (μL)
0.15	75	20	905	5	0.5
1	75	40	885	5	0.5
5	125	75	800	5	0.5
8	125	150	725	5	0.5

et al., 2005; Wang and Pelham, 1998). Table 3 shows the formula of PA gels with different stiffness.

- Immediately add 40 μL of the precursor solution onto the preactivated glass-bottom dishes and cover with an 18 mm coverslip.
- Put inverted dishes 30–40 min in a 37°C incubator for acrylamide polymerizing. The time can be adjusted according to the gel stiffness to make sure the polymerization is complete.
- Add 2 mL 100 mM HEPES to dishes and remove the coverslip gently using forceps.
- Wash the gel with 100 mM HEPES on a shaker for 15 min twice.

△ CRITICAL: Acrylamide, bis-acrylamide, and APS are harmful to health. Wear protective gear and operate safely.

Note: When preparing the PA gel precursor solution, it is recommended to add reagents in this order: ddH₂O → 40% acrylamide solution → 2% bis-acrylamide solution → 10% APS → TEMED. The process of PA gels preparation is shown schematically in Figure 3. Troubleshooting 5.

▣ Pause point: The gels submerged in HEPES could be stored for 2 weeks at 4°C.

Note: HCT116 cells show a tendency of spheroidization on the softer PA gels (Figure 7), which is consistent with our previous finding that soft matrix contributes to the stemness of colorectal cancer cells (Chang et al., 2022).

- Collagen type I coating on PA gel surface.
 - Prepare 700 μL Sulfo-SANPAH working solution for each dish by diluting 1 part of 5 mg/mL stock solution with 99 parts of HEPES.
 - Remove HEPES from the dishes containing gels and add 350 μL Sulfo-SANPAH working solution. Place gels under UV light for 6 min. Ensure that the distance between UV light and PA gels does not exceed 6 cm.
 - Remove the darkened Sulfo-SANPAH solution and wash with 100 mM HEPES on a shaker for 10 min twice in dark.
 - Repeat step 11. b and c.
 - Prepare 350 μL of collagen type I (200 $\mu\text{g}/\text{mL}$) for each dish by diluting the appropriate amount of collagen type I in PBS.
 - Remove HEPES and incubate with 200 μL 200 $\mu\text{g}/\text{mL}$ of collagen type I or fibrinogen at 4°C for 10–16 h.
 - Wash the gels with PBS carefully. The PA gels coated with Collagen type I are ready for cell seeding and can be stored for 2–3 weeks at 4°C submerged in PBS. The process of gel coating is shown schematically in Figure 4.

Note: Prepare fresh Sulfo-SANPAH working solution before use. Make sure the gels are fully covered with Sulfo-SANPAH. Remove liquids carefully and avoid touching the gels. The gels submerged in PBS can be stored at 4°C for 2 weeks.

- Cell seeding.

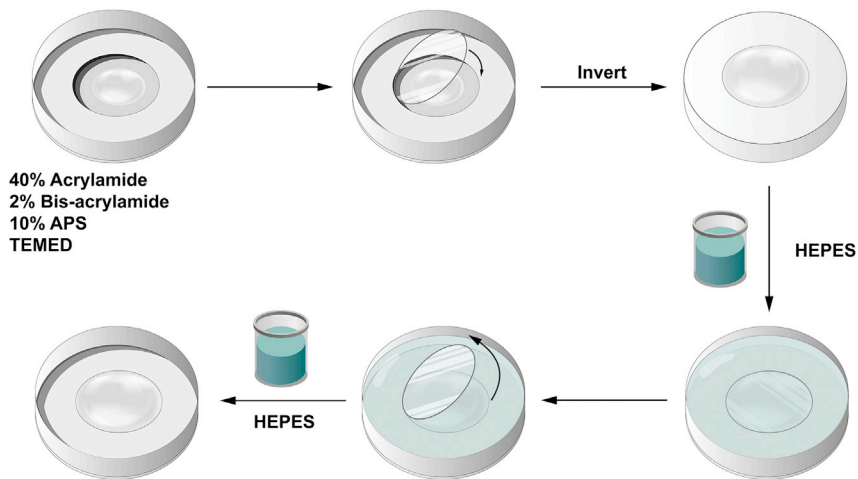


Figure 3. PA gels generation

- a. Sterilize the gels under UV light for at least 30 min. Remove PBS and add cell culture medium to gels. Place gels in a 37°C incubator with 5% CO₂ for 35–45 min.
- b. Seed cancer cells onto the PA gels with an appropriate density. Generally, the cells should reach 70% confluence within 2 days. [Troubleshooting 6](#).

PA gels cultured cell harvesting

⌚ Timing: 1h

13. Cell digestion.
 - a. Culture cells on PA gels for 24–72 h. Remove the culture medium. [Troubleshooting 7](#).
 - b. Wash the dishes with PBS and digest the cells from PA gels with 500 μ L TrypLE for each dish.
 - c. Collect PA gels cultured cells by centrifugation at 200 \times g for 3 min.

Note: The cells digested from PA gels can be used for most molecular biology experiments. For the single-cell experiment of measuring cell rigidity by atomic force microscope (AFM), it

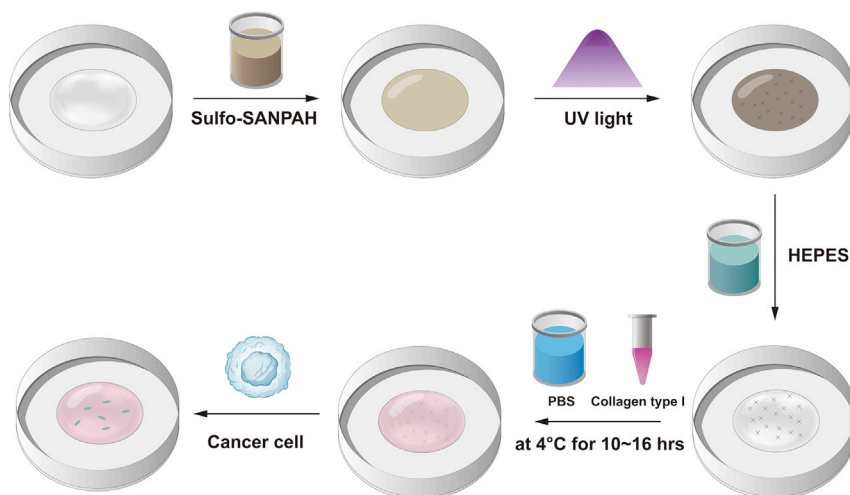


Figure 4. Collagen type I coating and cell seeding on PA gel

is recommended to replant the single cell on the surface of the glass bottom dish without gels. And the AFM measurement should be finished within 30 min for each dish in case of changes of cells seeded on glass.

EXPECTED OUTCOMES

At the end of performing the protocol, the user will make two kinds of extracellular matrix gels, obtain the cells or colonies cultured with 3D fibrin and 2D PA gels respectively, and understand the steps and precautions required for their use in the subsequent mechanotransductive study.

Generally, the user can master the manufacturing methods of the gel generation through training within two weeks, and master the basic process of mechanotransductive research using these two kinds of gels within 1–2 months.

Figure 5 shows the growth of colonies in 3D fibrin gels with different stiffness and the process of colony size or circularity analysis by Image J. Input the colony images to ImageJ software, set measurements (area and perimeter) and scales according to the different parameters of the lens, and click “Freehand selections” to trace the outline of the colony. Calculate colony size (volume) or circularity according to the measured numbers.

Figure 6 shows the results of immunofluorescence staining of colonies in 3D fibrin gels. It can be seen that immunofluorescence staining in fibrin gels does not affect the specificity of the fluorescence signal, and fibrin gels do not retain fluorescent dyes to produce background interference. Note the lower F-actin distribution in the 3D colony and the specific expression of E-cadherin at the cell junction.

Figure 7 shows the PA gels before crosslinking and the growth of cells on PA gels with different stiffness.

QUANTIFICATION AND STATISTICAL ANALYSIS

The colony size (volume) is calculated using the following formula: $\frac{4}{3}\pi \left(\frac{\text{Area}}{\pi}\right)^{3/2}$, assuming the colonies always maintain the shape of the sphere. The colony morphology is prone to heterogeneity. Thus, it is recommended to calculate colony circularity through the formula: $\frac{4\pi \text{Area}}{(\text{Perimeter})^2}$. Surface area and perimeter numbers are obtained from colony images by ImageJ software.

Student t-test is used for statistical analysis by GraphPad prism 8 software. Data are presented as Mean \pm SEM. Error bars represent SEM.

LIMITATIONS

Our protocol provides the specific steps of constructing 2D and 3D extracellular matrices *in vitro*, as well as the methods of cell extraction and fixation to serve the subsequent mechanotransductive research. These methods are summarized based on the published methods and our previous research which got high operability and repeatability.

However, these two kinds of gels have their own limitations. As the stiffness of 3D fibrin gels depends on the concentration of fibrinogen, the variable range is narrow. 20 mg/mL seems to be the upper limit of the concentration of fibrinogen. Moreover, the structure of 3D fibrin gels is unstable and degraded after 6–8 days. Therefore, it cannot be well applied to the *in vitro* culture experiment for a long time. On the other hand, PA gels are not suitable for the 3D matrices model, because their components are toxic to cells. We generally use these two methods simultaneously to carry out experiments to ensure the preciseness of our research. Looking for an *in vitro* organoid model with more stable mechanical properties could be the focus of our future work.

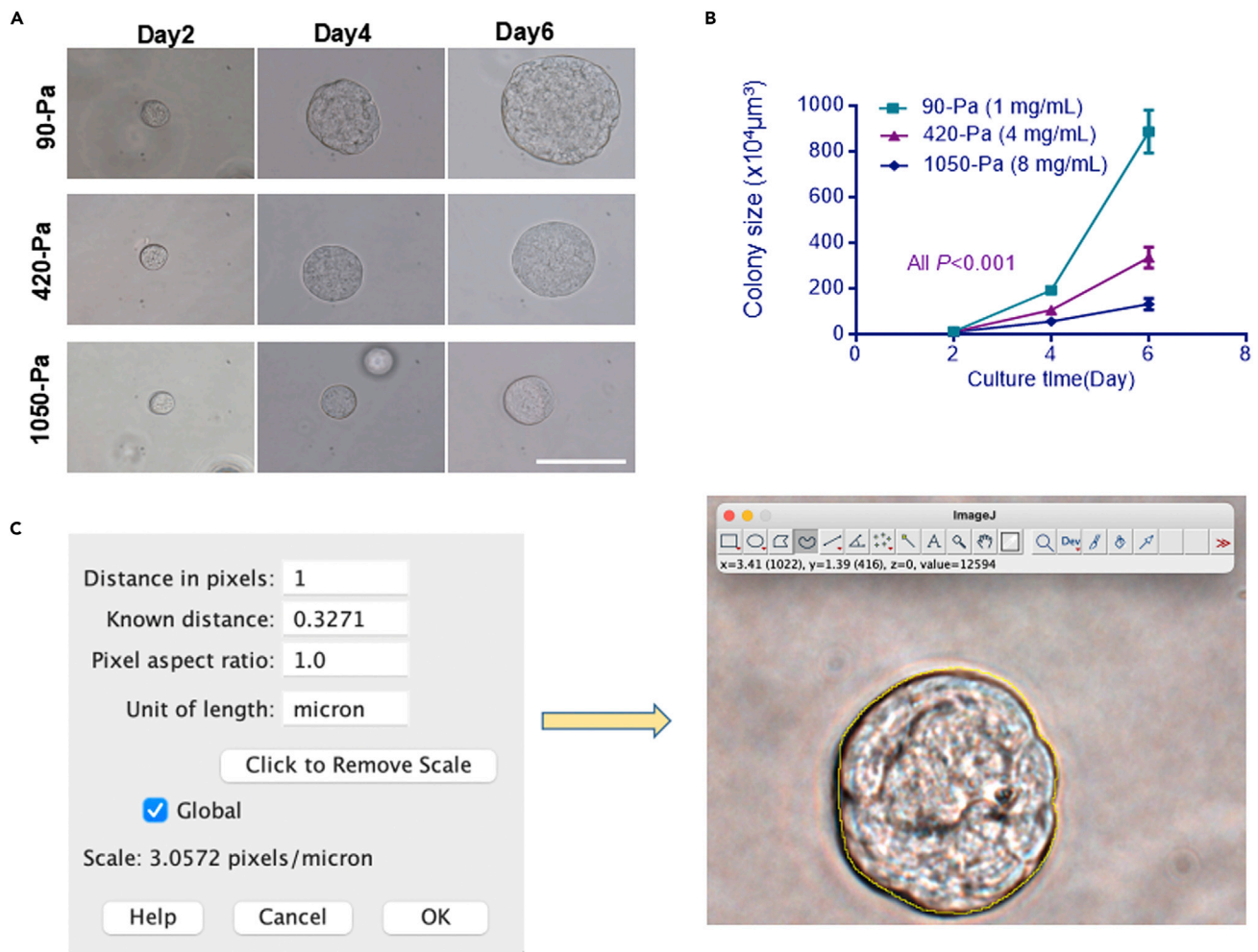


Figure 5. Colony growth in 3D fibrin gels

(A and B) HCT116 cells were cultured in 3D fibrin gels with different stiffness for 6 days. Images of colonies were captured by Olympus IX71 microscope and analyzed by ImageJ software. Scale bar, 200 μm . Mean \pm SEM; $n = 15$ colonies (All $P < 0.001$).

(C) Analyze Colony size or circularity quantitatively by ImageJ software.

TROUBLESHOOTING

Problem 1

The fibrin gels do not solidify or are easy to be dissolved.

Potential solution

The quality of thrombin and the pH of T7 buffer will affect the polymerization of fibrin gels. Please make sure that the activity of thrombin is normal and that the pH of T7 buffer has not changed. In particular, pay attention not to freeze-thaw thrombin repeatedly to prevent its inactivation.

On the other hand, the fibrin gels encapsulating cells may easy to be dissolved after long-term culturing, especially the very soft ones. Try not to use fibrinogen less than 1mg/mL for gel fabrication. Please finish the 3D culture within 6 days. If a longer culture period is needed, try to extract colonies or single cells and replant them into fresh fibrin gels.

Problem 2

The fibrin gels are uneven with obvious folds inside.

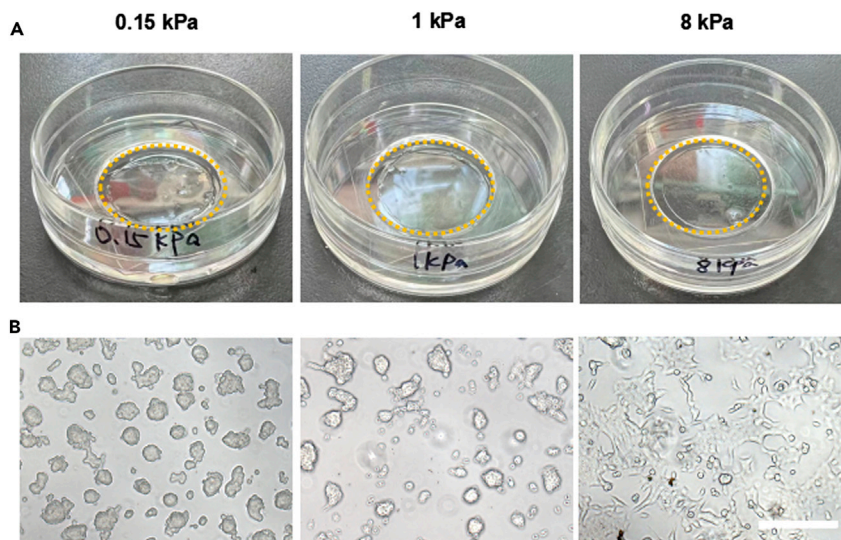


Figure 6. Representative images of 3D fibrin gel cultured colony staining
3D fibrin gel cultured colonies were fixed and stained with phalloidin, E-cadherin antibody, or DAPI. Scale bars, 50 μm .

Potential solution

Most of these kinds of problems are due to insufficient cooling of the gel solution. When mixing the cells/fibrinogen mixture with thrombin, if it is not cooled enough, the gels will solidify too fast, resulting in folds. Therefore, please put all the prepared solutions on ice for precooling for at least 5 min.

Problem 3

Cells do not form round colonies in 3D fibrin gels.

Potential solution

There are several possible reasons for this problem. First of all, the stiffness required for different kinds of cells to form round colonies in 3D fibrin gels is not exactly the same. We have known that colon, melanoma, and lung cancer cells can form colonies in 90-Pa 3D fibrin gels, while gastric cancer cells only form colonies in gels with a stiffness of more than 180-Pa. Some kinds of cells, such as

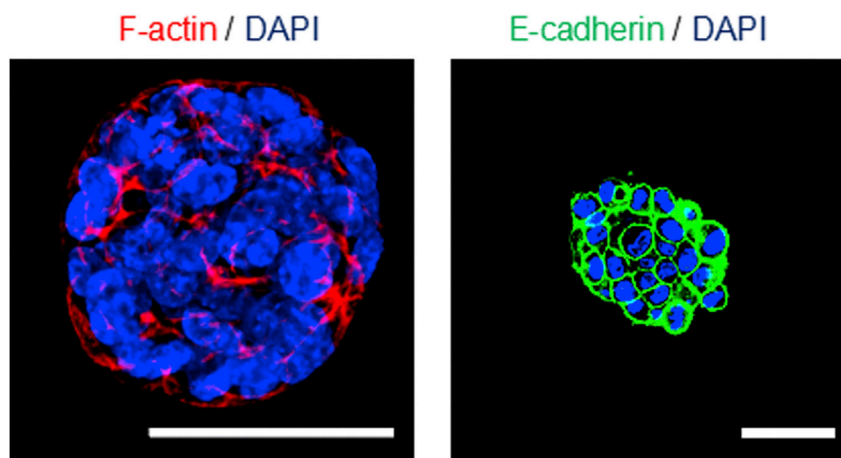


Figure 7. PA gels with different stiffness
(A) The orange circles indicate the PA gels with different stiffness coating on the glass bottom dishes.
(B) HCT116 cells were cultured onto PA gels with different stiffness for 72 h. Scale bar, 500 μm .

muscle cells, hardly form colonies in 3D fibrin gels. Therefore, before using the 3D fibrin gel model, it is necessary to explore the stiffness required for cell spheroidization.

In addition, we found that cells spread near the folds of gels. The quality or smoothness of fibrin gels also had a great impact on cell spheroidization.

Problem 4

Fail to isolate single cells from 3D fibrin gels.

Potential solution

The isolation of single cells from fibrin gels contains two steps. The first step is to degrade fibrin gels, which is usually achieved in 5–10 min. The next step is to dissociate the obtained colonies into single cells, and the time required for this step is cell-specific. For melanoma cells, TrypLE treatment for 5–10 min is sufficient to get single cells, while it takes about 30 min for colorectal cancer cells. For colonies that are difficult to dissociate, it is suggested to increase the dosage or intensity of trypsin, or place them on a constant temperature shaker for slowly shaking. In addition, the trypsin should be neutralized quickly after dissociation to prevent excessive digestion from causing cell damage.

Problem 5

PA gels cannot polymerize.

Potential solution

If PA gels cannot polymerize, all reagents need to be carefully tested. 10% APS and TEMED are the key components for PA polymerization. Note that the amounts of TEMED required to configure PA gels are very small. Only 0.5 μ L needs to be added per mL of PA gel solution. Please ensure that TEMED is added. In addition, 10% APS is easy to denature. Once 10% APS is denatured, PA gels cannot polymerize. So, we recommend preparing fresh 10% APS before use.

Problem 6

Cells are not well attached to PA gels.

Potential solution

Make sure reagents are properly prepared and collagen type I is not over-diluted. Also, the gels should be fully submerged in HEPES or PBS throughout the preparation, to prevent PA gels or collagen type I from drying out.

Problem 7

Cells grown on PA gels are contaminated.

Potential solution

Cells cultured on PA gels can be contaminated due to contamination during cell preparation or PA gels preparation. To test whether the contamination is due to the cell preparation when seeding cells on the gels, seeding cells on wells without gels at the same time. If cells grown on wells without gels are not contaminating then the contamination should be caused by PA gels preparation. Sterilize the gels under UV light for at least 30 min. Remove old PBS and add fresh PBS, repeat sterilizing the gels under UV light to avoid contamination before cell seeding.

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. Qiong Jia (jiaqiong2020@njmu.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique dataset or code.

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AUTHOR CONTRIBUTIONS

Q.J. conceived the project. Q.J., J.Z., and W.H. designed the experiments. Q.J., J.Z., W.H., Y.C., and Y.G. carried out experiments and performed analyses. W.H. drew all the sketch maps. Q.J., J.Z., W.H., Y.C., Y.G., X.W., and Y.T. all participated in writing the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Chang, Y., Zhang, J., Huo, X., Qu, X., Xia, C., Huang, K., Xie, F., Wang, N., Wei, X., and Jia, Q. (2022). Substrate rigidity dictates colorectal tumorigenic cell stemness and metastasis via CRAD-dependent mechanotransduction. *Cell Rep.* 38, 110390. <https://doi.org/10.1016/j.celrep.2022.110390>.

Engler, A.J., Griffin, M.A., Sen, S., Bönnemann, C.G., Sweeney, H.L., and Discher, D.E. (2004). Myotubes differentiate optimally on substrates with tissue like stiffness: pathological implications for soft or stiff microenvironments. *J. Cell Biol.* 166, 877–887. <https://doi.org/10.1083/jcb.200405004>.

Liu, J., Tan, Y., Zhang, H., Zhang, Y., Xu, P., Chen, J., Poh, Y.C., Tang, K., Wang, N., and Huang, B. (2012). Soft fibrin gels promote selection and growth of tumorigenic cells. *Nat. Mater.* 11, 734–741. <https://doi.org/10.1038/nmat3361>.

Tan, Y., Tajik, A., Chen, J., Jia, Q., Chowdhury, F., Wang, L., Chen, J., Zhang, S., Hong, Y., Yi, H., et al. (2014). Matrix softness regulates plasticity of tumour-repopulating cells via H3K9 demethylation and Sox2 expression. *Nat. Commun.* 5, 4619. <https://doi.org/10.1038/ncomms5619>.

Wang, Y.L., and Pelham, R.J., Jr. (1998). Preparation of a flexible, porous polyacrylamide substrate for mechanical studies of cultured cells. *Methods Enzymol.* 298, 489–496. [https://doi.org/10.1016/s0076-6879\(98\)98041-7](https://doi.org/10.1016/s0076-6879(98)98041-7).

Yeung, T., Georges, P.C., Flanagan, L.A., Marg, B., Ortiz, M., Funaki, M., Zahir, N., Ming, W., Weaver, V., and Janmey, P.A. (2005). Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil Cytoskeleton* 60, 24–34. <https://doi.org/10.1002/cm.20041>.