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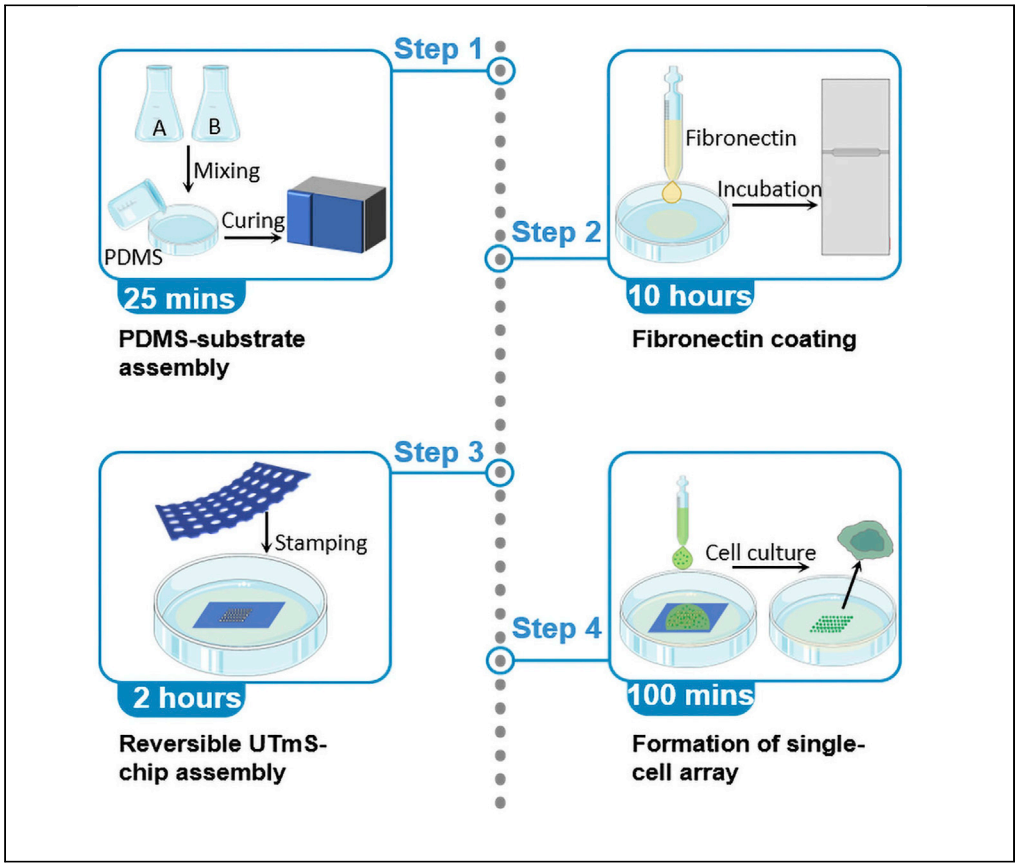
Protocol for high-throughput single-cell patterning using a reusable ultrathin metal microstencil



Exploiting convenient strategies for single-cell preparation while maintaining a high throughput remains challenging. This protocol describes a simple workflow for high-throughput single-cell patterning using a reusable ultrathin metal microstencil (UTmS). We describe UTmS-chip design, fabrication, and quality characterization. We then detail the preparation of flat substrates and chip assembly for single-cell patterning, followed by culturing of cells on a chip. Finally, we describe the evaluation of single-cell patterning and the downstream applications for studying single-cell calcium release and apoptosis.

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

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Highlights

Protocol for efficient single-cell patterning on various flat substrates using UTmS-chip

Procedures for fabricating the chip with laser drilling and cleaning for reuse

Steps for preparing flat substrates, chip assembly, and single-cell patterning

User-friendly and can be used in different fields and cell-related studies

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Protocol for high-throughput single-cell patterning using a reusable ultrathin metal microstencil

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SUMMARY

Exploiting convenient strategies for single-cell preparation while maintaining a high throughput remains challenging. This protocol describes a simple workflow for high-throughput single-cell patterning using a reusable ultrathin metal microstencil (UTmS). We describe UTmS-chip design, fabrication, and quality characterization. We then detail the preparation of flat substrates and chip assembly for single-cell patterning, followed by culturing of cells on a chip. Finally, we describe the evaluation of single-cell patterning and the downstream applications for studying single-cell calcium release and apoptosis.

For complete details on the use and execution of this protocol, please refer to Song et al. (2021).[1]

BEFORE YOU BEGIN

Many efforts have been expended in developing efficient and reproducible strategies for large-scale single-cell isolation and manipulation.[2–4] In particular, the microstencil method, which employs a similar lift-off lithography concept for creating cell patterns, has gained popularity as an alternative method owing to its suitability and easy-assembly for single-cell patterning on substrates. However, practical applications of microstencil-based devices in general biological laboratories are limited, owing to three major draw-backs. First, most of these devices are micropatterned via standard photolithography to fabricate microsized through-holes.[5,6] This technology requires sophisticated facilities and procedures, including standard photolithography, and lift-off techniques, to fabricate the micromold, thus limiting biologists and chemists to general biochemical laboratories. Second, some stencil platforms based on gravity-induced cell sedimentation have simple structures, but only show low single-cell resolution with a time-consuming isolation process.[7] Moreover, some platforms apply centrifugation to achieve cells sedimentation into microstencils in several minutes.[8] However, these require auxiliary facilities and fixtures for sealing and hence, induce extra obstacles for operation. Finally, polydimethylsiloxane (PDMS) or SU8 microstencils with only tens of microns thickness are fragile freestanding thin membranes that require delicate handling for chip assembly and releasing; thus, they are disposable microstencils and cannot be reused.[9] Therefore, it still remains challenging to achieve high-efficiency single-cell patterning on desired locations, serving as a practical alternative for various researchers in common laboratories.

This protocol describes the detailed procedure for the design, fabrication, assembly, and use of the UTmS-based platform. The UTmS-based chip (Figure 1A) is developed as a simple and rapid

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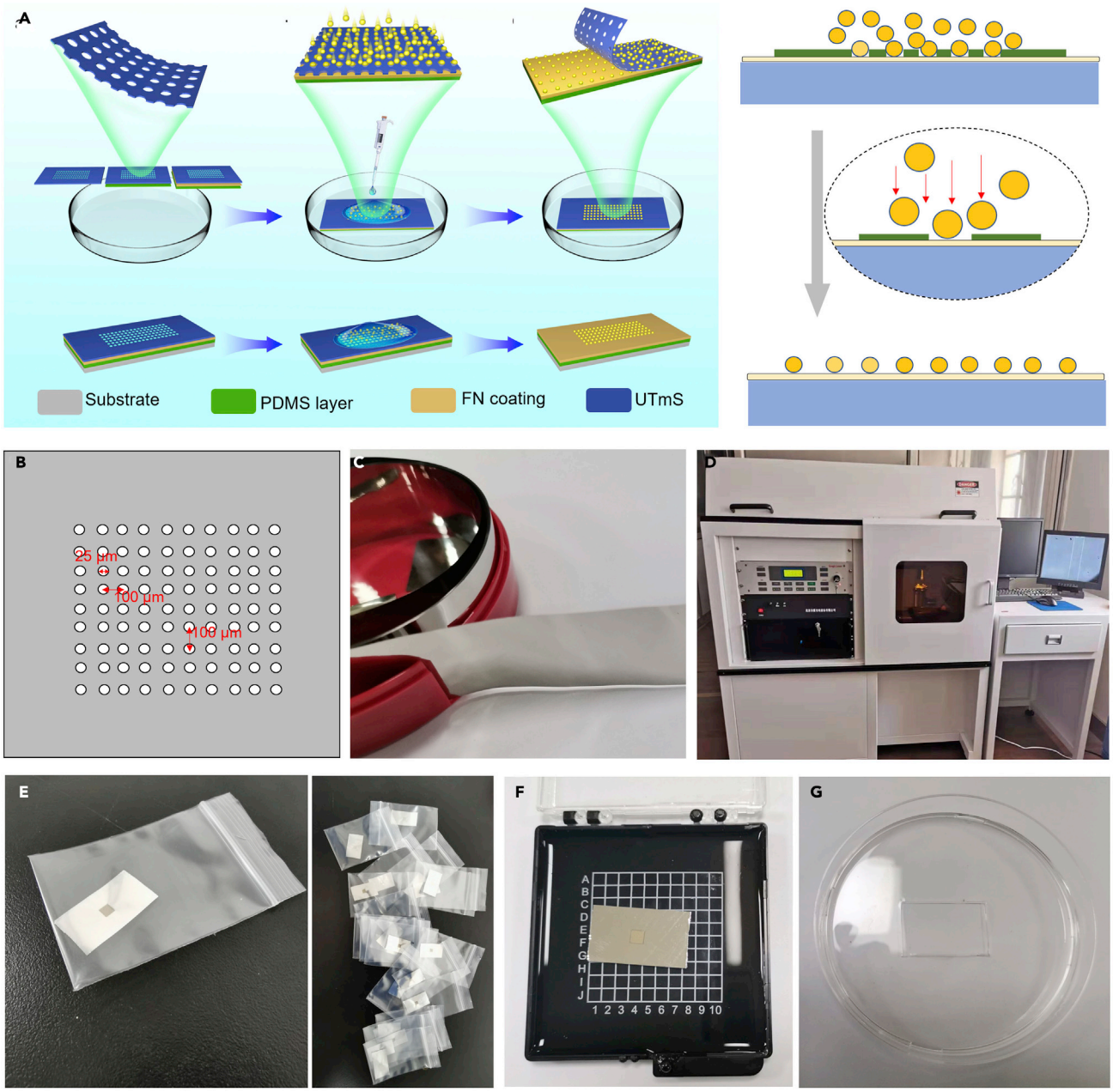

Figure 1. Design and fabrication of the UTmS chip

(A) Schematic diagram of the UTmS-based platform for single-cell patterning.

(B) Chip design.

(C) Stainless steel sheet with 5 μm thickness.

(D) Laser driller.

(E) Fabricated UTmS before cleaning.

(F) UTmS after cleaning.

(G) Polydimethylsiloxane (PDMS) layer after cutting. Schematic and images in (A and E) reproduced from Song et al. with permission from the Royal Society of Chemistry.[1]

approach to achieve rapid and high-efficiency single-cell patterning for the real-time and heterogeneous study of calcium release and apoptosis behaviors.[1] The protocol can also be successfully applied to various adherent cells and planar substrates with the adoption of various metal stencils.

Consequently, the timing and design parameters are expected to differ depending on the adherent cell lines. The following protocol describes the specific steps for using HeLa cells. However, this protocol can also be used for MCF-7 and A549 cells. Moreover, it is important to wear appropriate personal protective equipment (gloves and lab coats) at all times during the experiments to ensure safety, especially when handling chemicals and cell samples.

A 5 μm-thick ultrathin steel microstencil is first fabricated using a one-time process on laser drilling equipment, which has the advantages of easy operation and quick preparation at a significantly low cost and complexity. Next, the stencil is attached directly to the modified substrate without the need for additional fixation or chemical bonding, and with no leakage of the solution. Then, a small amount of cell suspension can sediment into the UTmS under gravity-induced sedimentation without the need for reaction pools or centrifugation procedures. Finally, rapid and efficient single-cell prototyping can be achieved by gently washing and peeling off the UTmS. Moreover, real-time and heterogeneous studies of calcium release and apoptosis behaviors of single cells can be performed on patterned substrates. Notably, this method is suitable for the fast and efficient single-cell patterning of different cellular types on substrates of different materials with metal stencils. This provides excellent flexibility for applying the UTmS-based strategy to an arbitrary planar substrate such as glass, silicon, metal, plastic, or polymer.

In summary, the proposed method presents several advantages. First, the laser-drilling approach circumvents the lithographic process for the preparation of high-throughput through-holes on ultrathin metal foil, which are easily and cost-effectively fabricated. Second, auxiliary fixation and centrifuge equipment are not necessary,[4,10] but direct attachment and natural sedimentation are required. Third, the 5 μm-thick metal stencil can be easily handled with tweezers owing to its good rigidity and superior toughness, and can be reused after a general cleaning procedure. This is the first time that an ultrathin 5 μm-thick is used to fabricate high-resolution single-cell patterns without the need for lithographic processes.[6,7] All combined, the UTmS-based chip provides efficient and reproducible single-cell patterning for real-time and heterogeneous studies of individual cells on various flat substrates.

UTmS-chip design

Timing: 1 h

1. Use computer-aided design software (AutoCAD or L-Edit) to draw high-throughput through-holes with array-arranged patterns and different diameters.

   Note: For example, UTmS is designed as a 50 × 50 array with a diameter of approximately 25 μm diameter and a spot-to-spot spacing of 100 μm, obtaining a rectangular array (Figure 1B).

   Note: The timing for this step is excluded from the overall timing of this section. The time will vary depending on the complexity of the patterns and the existing templates in the database can be used as reference during the design process to minimize the timing.

UTmS fabrication

Timing: 1 h

⚠ CRITICAL: Laser drilling should be performed using personal protective equipment. It is typical to follow the standard operating procedures of the instruments used.

2. Prepare a 5 μm-thick flat metal strip, such as a stainless-steel strip, that is larger than the area of the pattern.

⚠ CRITICAL: During the cutting process, always maintain keep the metal sheet flat without bending, indentation, or deformation.

3. Use a homemade fixture, such as a pair of ring magnets or plate clips, to fix the edges of the metal sheet.

Note: Use a screw clamp to further clamp the assembly onto the x-y translation stage of the laser drilling device while ensuring that the central area is aligned with the laser head.

⚠ CRITICAL: A suitable homemade fixture, which can effectively prevent the deformation and displacement of the ultrathin sheets caused by laser heating, is critical for uniform fabrication.

4. Import the pattern design into the laser driller (for example, MDS-10B, Beijing Mars Optics).
5. Use the equipped laser source (for example, a femto-second diode-pumped solid-state laser, DPL) to automatically generate through-holes at the center of a metal sheet (Figure 1D).

Note: The optimum laser drilling parameters (laser device, laser power, intensity, and pulse duration) are set depending on the instrument used. For example, the commercial femto-second DPL laser drilling instrument (MDS-10B, Beijing Mars Optics) used in our experiments is set to a wavelength of 532 nm, laser power of 4 W, and pulse duration of 20 fs for stainless-steel material.

6. Verify that the microstencils conform to the design requirements, including the regularity of the circle and uniformity of the through-hole array.

⚠ CRITICAL: An upright lens is used on the device to visually verify that no defects are present in the generated patterns. The optical lens on the drilling instrument can only roughly confirm the regularity and uniformity of the microarrays. Further detailed characterization should be performed using scanning electron microscope (SEM).

Note: Owing to the extremely high laser power and extreme laser pulses, all the 5 μm-thick metal sheets are rapidly perforated while maintaining the edges regular without major defects.

7. Hold the prepared UTmS directly with tweezers or hands while maintaining the original flat conformation of UTmS.

Note: Although the thickness is only 5 μm, the UTmS-chip still has good mechanical properties because of the rigidity of the metal material (such as stainless steel).

⚠ CRITICAL: No significant mechanical change should be visible in the UTmS after the through-holes are punched on the same metal sheet.

8. Before cleaning, place the UTmS-chips in ziplock bags (Figure 1E) or small dishes to prevent bending.

UTmS cleaning and storage

Timing: 45 min

⚠ CRITICAL: Handle all chemicals in a fume hood.

Note: All cleaning steps for the UTmS-chips must be performed in clean glass-crystallizing dishes. The UTmS does not need to be fixed but remains free in the glass dish. Tweezers are required to pick up and place the stencils during cleaning and storage. The following step uses ultrasonic cleaning as an example.

9. Cleaning procedures for newly fabricated UTmS.
   a. Place the newly prepared stencil in glassware and ultrasonically clean it in acetone for 3 min to remove organic impurities, such as oil stains.

⏸ Pause point: The acetone step can be skipped if the chip is not dirty.

   b. Ultrasonically clean it in ethanol for 5 min to remove residual acetone.

Note: Cover the glass ware with aluminum foil during cleaning because acetone and ethanol are volatile.

⏸ Pause point: Use a water bath at 65°C as an aid for ultrasonic cleaning if the chip is dirty.

   c. Place the stencil in another glass ware filled with deionized (DI) water, rinse thoroughly twice to remove ethanol residues.
   d. Dry at 80°C for 2 h in a drying oven.

Optional: Bake the UTmS at 200°C for 10 min on a hot plate to remove water residue from its surface. Although this is a very common approach for chip drying, it is not recommended because airborne dust and other impurities may land on the chip surfaces.

⚠ CRITICAL: Do not use tweezers to blow dry the UTmS with nitrogen gas to avoid repeated bending and subsequent indentation.

   e. Sterilize the glass slides using UV-ozone disinfector (BIOBASE, BJPX-SVO60) for 30 min.

Optional: Vacuum vapor sterilization is also a reliable chip sterilization method.

Note: Because the UTmS is made of metal material, the chip is suitable for a variety of cleaning methods and reagents, including ultrasonic, organic, heating, and plasma cleaning, without any observable change in chip characteristics. Therefore, the chip is suitable for reuse multiple times after cleaning.

10. Store the UTmS-chips in culture dishes.
   a. Place the cleaned UTmS-chips in a 30-mm sterile cell culture dish without any fixation.
   b. Seal the Petri dish with parafilm and store the UTmS-chips in a vacuum desiccator for extended periods.

Optional: Storing the UTmS-chips in a gel-box with a polymer silicone layer is also recommended. The appropriate viscidity of the UTmS-chips ensures that the chip tile is fixed at the bottom (Figure 1F).

Preparation of PDMS

⏱ Timing: 40 min

11. Use a plastic or paper cup to completely mix the PDMS base elastomer (8 g) and curing agent (Dow Corning, Sylgard 184) at a 10:1 (w/w) ratio.

Note: Stir the mixture for 3 min using a disposable pipette to achieve complete mixing.

⚠ CRITICAL: Incomplete mixing or inadequate catalyst quantity results in the PDMS not curing completely.

12. Slowly pour the 8.8 g PDMS mixture over a disposable Petri dish (100 mm) to form a PDMS layer with an approximate thickness of 1 mm.

⚠ CRITICAL: A PDMS layer with this thickness has a strong adsorption capacity without additional bonding, including one side of the PDMS layer on the planar substrate and the other side on the UTmS-chip.

13. Place the dish in a vacuum desiccator for 3 min to remove air bubbles from the PDMS mixture.
14. Cure the PDMS layer in a dry oven at 80°C for 50 min and then remove the dish from the oven until cool to room temperature (10°C–30°C).

Note: The PDMS mixture will be completely solidified when it is fully cured.

⚠ CRITICAL: Place the dish horizontally during the PDMS curing process to ensure that the shaped PDMS layer is flat.

15. Use a scalpel to cut the PDMS layer into 30 mm × 30 mm rectangles while ensuring that the cutting position is in the center of the dish where the top surface is flatter than the edges (Figure 1G).

Note: It is worth noting that the area of the rectangle should be larger than that of the chip. Use minimal pressure to prevent the Petri dish from breaking.

⚠ CRITICAL: Do not peel off the PDMS layer after curing. Cut and peel off when required because freshly peeled PDMS membranes can adhere more firmly to a clean substrate via self-adsorption without bonding or adhesion assistance.

Preparation of the substrate

Timing: 25 min

16. A wide variety of substrate materials with planar top surfaces, including silicon, glass, metal, or plastic, can be adopted for single-cell patterning using a PDMS spacer layer (Figure 2C).

Note: UTmS can be applied to a planar substrate using a PDMS spacer layer. As shown in Figure 1A, apply freshly prepared PDMS layer on the substrate and then press the 5 μm-thick UTmS onto the PDMS surface.

17. Clean and sterilize all the substrates before use.

Note: Common procedures include ultrasonic cleaning in ethanol, followed by thorough rinsing with DI water. Thereafter, blow dry the samples with nitrogen gas.

18. Store the samples in a clean dish and seal it for storage.

Figure 2. Characterization of the UTmS and its assembly

(A) SEM characterization of the UTmS patterns, scale bar: 100 µm. Inset: enlarged view, scale bar: 25 µm.

(B) Characterization of the mechanical properties of UTmS and PDMS membrane.

(C) PDMS-substrate assembly.

(D) Fibronectin coating.

(E) Fibronectin coating characterization with AFM.

(F) UTmS-Chip assembly.

(G) Contact angle measurement.

Figure 2. *Continued*
(H) Chip assemble characterization for the adhesion. The optical and fluorescence images of the UTmS assembly were obtained from the back. Scale bar: 200 μm. The expanded fluorescence view was obtained 3 h after Cy3 solution loading, scale bar: 50 μm.
(I) Chip assemble characterization for the leakproofness. The inset shows the vertical section of the through-hole area on the PDMS layer. Scale bar: 50 μm. Images and plot in (A and D–I) reproduced from Song et al. with permission from the Royal Society of Chemistry.[1]

Note: Aseptically-packaged petri dishes or aseptic packaging can be used for this purpose.

⚠ CRITICAL: Regardless of the type of substrate, the flat surface of the substrate must be clean and dust-free as it will affect the adhesion between the substrate and PDMS layer.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Sylgard 184 silicone elastomer kit	Dow Corning	Cat#1064291
Cy3	Invitrogen	Cat#SA1010
Phosphate buffered solution (PBS)	Solarbio	Cat#P1020
Dulbecco's modified eagle medium (DMEM)	Hyclone	Cat#SH30243
Trypsin	Hyclone	Cat#SH30042
Fetal bovine serum (FBS)	BBI	Cat#04-001
Penicillin/streptomycin (P/S)	Gibco	Cat#15140-122
Fluo-8 No Wash Calcium Assay Kit	Abcam	Cat#ab112129
Fibronectin	Solarbio	Cat# F8180
Tetramethylrhodamine methyl ester (TMRM)	Invitrogen	Cat#T668
Staurosporine (STS)	Sigma	Cat# 569396
Hoechst 33342	Invitrogen	Cat#H3570
Fluorescein diacetate (FDA)	Sigma	Cat#F7378
Dimethyl sulfoxide (DMSO)	Sigma	Cat# D8418
Acetone	Hushi	Cat#10000418
Ethanol absolute	Hushi	Cat#10009218
Experimental models: Cell lines		
Human: HeLa cells (Passage number: 4–14)	Shanghai Xiangf Biotech	N/A
Human: A549 cells (Passage number: 4–14)	Shanghai Xiangf Biotech	N/A
Software and algorithms		
ImageJ	Schindelin et al.[11]	https://imagej.nih.gov/ij/download.html
CAD software (AutoCAD)	Autodesk	https://www.autodesk.com
Other		
Cell culture dish 35 mm × 12 mm Style	NEST	Cat#706001
15 mL Centrifuge tube	NEST	Cat#601051
50 mL Centrifuge tube	NEST	Cat#602051
Tweezer	VETUS	ST-13, ST-16, ESD-15
Commercial laser drilling instrument	Beijing Mars Optics	Cat# MDS-10B
Ultrasonic cleaning	Kunshan Ultrasonic Instruments Co. Ltd	Cat#KQ218
CO_2 incubator	Thermo Scientific	3111
Laminar flow hood	Suzhou Zhijing Purification Equipment Co., Ltd	SW-CJ-1FD
Vertical Heating Pressure Steam Sterilizer	SHENAN	LDZX-50KBS
Ultraviolet disinfection cabinet	BIOBASE	BJPX-SV200
Electric heating constant temperature blast drying box	Keelrein Instrument Co., Ltd	DHG-9070A
Stainless steel strip (12.7 × 0.005 mm with ±0.002 tolerance)	HASBERG (1.4310C INOX)	N/A
Scanning electron microscope (SEM)	Nova, NanoSEM, FEI, USA	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Atomic force microscope (AFM)	Bruker Multimode V, Germany	N/A
DSA100 instrument	Krüss, Germany	N/A
Inverted fluorescence microscope	Olympus, Japan	N/A
CCD camera	Olympus	Cat#DP73

MATERIALS AND EQUIPMENT

Preparation of the cell culture medium

The cell culture medium contains DMEM, FBS, and P/S. Place 50 mL FBS and 5 mL P/S (−20°C) in a 37°C water bath to preheat the mixture. Mix 500 mL DMEM, 50 mL FBS and 5 mL P/S and shake well in a laminar flow hood.

Cell culture medium

Reagent	Final concentration	Amount
DMEM	89%	445 mL
FBS	10%	50 mL
Penicillin/streptomycin (P/S)	1%	5 mL
Total	**N/A**	**500 mL**

The mixture should be stored at 4°C and the maximum time for storage is 3 weeks. The entire process is performed in a laminar flow hood, and the bottle is sealed with a sealing film.

Preparation of the Hoechst 33342 solution

Dissolve the dyes in DMSO to obtain concentrated stock solutions up to 10 mg/mL. Add 1 μL Hoechst 33342 (10 mg/mL) to 999 μL PBS and mix thoroughly.

Hoechst 33342 solution

Reagent	Final concentration	Amount
Hoechst 33342 (10 mg/mL)	10 μg/mL	1 μL
1×PBS	N/A	999 μL
Total	**N/A**	**1 mL**

Hoechst dye solutions should be stored at 4°C and be protected from light. Stock solutions are stable for at least 6 months in dry, cool (2°C–8°C), and well-ventilated conditions.

⚠ CRITICAL: Hoechst dyes are known mutagens that should be handled carefully. Wear gloves during the procedure.

Preparation of the fluorescein diacetate (FDA) solution

Dissolve FDA dyes in DMSO to obtain concentrated stock solutions of up to 10 mg/mL. Add 1μL FDA (10 mg/mL) to 999 μL PBS and mix thoroughly.

Prepare FDA solution

Reagent	Final concentration	Amount
FDA (10 mg/mL)	10 μg/mL	1 μL
1×PBS	N/A	999 μL
Total	**N/A**	**1 mL**

FDA dye solutions can be stored at 4°C, protected from light. The stock solutions are stable for at least six months when refrigerated at −20°C.

⚠ CRITICAL: Wear gloves during the procedure.

Preparation of the Cy3 solution

Add 50μL Cy3 (0.1 mg/mL) to 450 μL PBS and mix thoroughly.

Prepare Cy3 solution		
Reagent	Final concentration	Amount
Cy3 (0.1 mg/mL)	10 μg/mL	50 μL
1×PBS	N/A	450 μL
Total	**N/A**	**0.5 mL**

Cy3 dye solutions can be stored at 4°C, protected from light. The stock solutions are stable for six months at 2°C–8°C.

⚠ CRITICAL: Wear gloves during the procedure.

Preparation of the fibronectin solution

Thaw fibronectin (0.2 mg/mL) at room temperature (10°C–30°C), and then pipette 1 mL fibronectin into a 15 mL centrifuge tube containing 7 mL PBS. Mix gently to prepare stock solutions of 25 μg/mL concentration. Next, add 200 μL of a stock solution of fibronectin (25 μg/mL) to 800 μL PBS to prepare a 5 μg/mL working solution and mix thoroughly.

Prepare fibronectin solution		
Reagent	Final concentration	Amount
Fibronectin (25 μg/mL)	5 μg/mL	200 μL
1×PBS	N/A	800 μL
Total	**N/A**	**1 mL**

Working solutions of fibronectin (5 μg/mL) can be stored at 4°C for 2–4 weeks. For long-term storage, the stock solution can be aliquoted and stored at −20°C for 12 months.

⚠ CRITICAL: Handle fibronectin gently to avoid damaging the protein activity.

Preparation of the staurosporine (STS) and tetramethylrhodamine methyl ester (TMRM) solutions

Fill an original STS reagent vial (100 μg) with 241 μL DMSO to obtain a 1 mM STS stock solution, and gently mix using a pipette. Divide the stock solution into 20 μL per tube and store at −20°C in the dark.

Dissolve solid TMRM dyes (5 mg) in 1 mL DMSO to prepare concentrated stock solutions of 10 mM. Add 10 μL TMRM (10 mM) to 990 μL DMSO to obtain a 100 μM TMRM solution. Divide the stock solution into 8 μL per tube and store at −20°C in the dark.

STS and TMRM solution		
Reagent	Final concentration	Amount
STS (1 mM)	10 μM	20 μL
TMRM (100 μM)	400 nM	8 μL
Cell culture medium	N/A	1,972 μL
Total	**N/A**	**2 mL**

The solution can be stored at 4°C for 1 day. Therefore, the solution should be used immediately and protected from light.

Preparation of the Fluo-8 dye-loading solution

Thaw all kit components at room temperature (10°C–30°C) before use. Add DMSO (20 μL) to the Fluo-8 vial (Component A) and mix thoroughly to obtain Fluo-8 stock solution. Then, add Hanks' Balanced Salt Solution (HHBS, Component C) (9 mL) to 1 mL 10 × Pluronic F127 Plus (Component B) and mix thoroughly to obtain a 1× assay buffer.

Fluo-8 dye-loading solution

Reagent	Final concentration	Amount
Fluo-8 stock solution	N/A	2 μL
1 × assay buffer	N/A	998 μL
Total	N/A	1 mL

The working solution is stable for at least 2 h at room temperature (10°C–30°C). The Fluo-8 stock solution and 1× assay buffer can be stored at 4°C for 6 months.

STEP-BY-STEP METHOD DETAILS

Characterization of UTmS

Timing: 40 min

This step verifies that the prepared UTmS-chips maintain good rigidity, superior toughness, and easy handling with tweezers.

1. Fix and load the UTmS chip for SEM imaging.
   a. Fix the UTmS-chip on a metal tray with conductive tape.
   b. Bring the SEM chamber to atmospheric pressure to open the sample door.
   c. Open the door and place the metal tray into the holder.
   d. Close the door back into the chamber and place it back under vacuum.
   e. Zoom in to the desired magnification and adjust the sample height to focus.
   f. Capture the SEM image once the sample is in focus.
2. Verify that the through-hole conforms to the design requirements, including the regularity of the circle, smoothness of the edges, and uniformity of the through-hole array.

   Note: The 5 μm-thick UTmS should be perforated with smooth and regular edges without burning residues (Figure 2A).

3. The chip remains upright and flat when lifted with tweezers, thereby verifying its good rigidity.

   ⚠ CRITICAL: An approximately 10 μm-thick PDMS layer is difficult to handle with tweezers because it easily bends, collapses or conglutinates. The UTmS-chips can be maintained upright with tweezers, rather than bent or collapsed as is the case with the PDMS layer (Figure 2B). Therefore, this is one of the significant advantages of metal stencils over PDMS stencils.[6]

4. The UTmS can be bent without fracturing or creasing by bending the chip with fingers, verifying its superior toughness (Figure 2B).
5. Place the cleaned and sterilized UTmS chip in a Petri dish and co-culture it with MCF-7 cells suspension for 3 days in an incubator.

   Note: Given that the action time of the UTmS in this experiment is only 20–30 min, the effect of the UTmS on the cell activity is not a concern in the single-cell patterning. The adhesion and proliferation of cells in a Petri dish verifies the biocompatibility of the UTmS chip.

PDMS-substrate assembly

Timing: 10–25 min

This step covers the top surface of different substrates with PDMS membranes, to apply the UTmS-based strategy to an arbitrary planar substrate (such as glass, silicon, metal, plastic, or polymer) for subsequent cell patterning, owing to the biological compatibility of PDMS.

6. Prepare substrates with clean and planar top surfaces.

⚠ CRITICAL: A clean and planar surface is very important for PDMS attachment.

7. Peel off a freshly-prepared PDMS layer without patterns pre-cut in step 15 (Before beginning) with tweezers.

⚠ CRITICAL: Be careful not to touch the PDMS surface to avoid conglutination.

8. Past the uncovered fresh side of the PDMS towards the top surface of the substrate by gradually releasing the film from one end of contact to full adhesion to remove air bubbles.

⚠ CRITICAL: Avoid tiny bubbles that could affect the PDMS surface smoothness and adhesion.

Note: In the event that bubbles accidentally occur, use tweezers to slowly peel off from one end and refilm. Alternatively, place the PDMS-substrates in a vacuum desiccator for 15 min to allow the trapped gas bubbles present between the surfaces to passively escape.

9. Gently press on the assembled device to ensure that the surfaces of the PDMS-substrate are well-bonded (Figure 2C).

⚠ CRITICAL: Avoid pressing with small-headed tools, such as tweezers, to prevent rugged surfaces.

Note: An exception to this step is that if the substrate is originally a cell culture dish (Petri dish) suitable for cell culture, thus eliminating the need for PDMS coating modification.

Fibronectin modification

Timing: 10 h

Since fibronectin has been demonstrated to promote the adhesion and proliferation of cells,[12,13] this step decorates the Petri dishes or PDMS surfaces with fibronectin at a high concentration before UTmS attachment to accelerate the adhesion of cells from the suspension onto the substrates.

⚠ CRITICAL: Handle all chemicals in a fume hood.

Optional: In addition to fibronectin and fibrinogen, a wide variety of surface functionalization methods with adhesion promoting agents can be adopted to accelerate the single-cell trapping process without compromising performance, including collagen, (aminopropyl)-triethoxysilane, or poly-L-lysine.[12,14]

10. Prepare a fibronectin coating solution with a final concentration of 5 μg/mL using PBS in Eppendorf tubes with sufficient mixing in a biosafety cabinet.

Note: The fibronectin solution is expected to be stable when stored in PBS (pH 7.2–7.6) for long storage (several weeks). Therefore, the excess fibronectin solutions can be stored at 4°C.

Pause point: Fibronectin is not completely dissolved in PBS because of its non-polar nature. The pipetting time should be appropriately extended to achieve maximum dissolution and mixing.

Note: The high concentration of fibronectin solution (5 μg/mL) aims to ensure full and high-density coverage on the Petri dish or PDMS surface by physical adsorption through non-specific interactions, thus increasing the protein adsorption probability, and the single-cell capture yield. However, it should be noted that the proper fibronectin concentration is subject to biological testing.

Optional: We recommend the preparation a mixed coating solution, including human fibronectin and fibrinogen with final concentrations of 4 and 1.5 μg/mL using PBS, and then mixing the solution using a vortex mixer for 5 s. The coating efficiency of the mixed coating solution is higher than that of the fibronectin only solution.

11. Use a custom-made PDMS gasket as the liquid chamber to hold the coating solution on the planar substrate.

Note: If the required coating area is small, a droplet of fibronectin solution can be directly dispensed onto a Petri dish or PDMS surface to cover the required coating area. This operation eliminates the need for a gasket because the fibronectin solution remains in a droplet shape rather than spreading out owing to the hydrophobicity of the Petri dish or fresh PDMS surface.

12. Pipette the fibronectin solution into the PDMS gasket to cover the entire bottom and allow it to settle to the bottom (Figure 2D).
13. Incubate the surfaces for a minimum of 3 h at 36°C or 10 h at 4°C, which allows the fibronectin to settle until it is physically adsorbed at the bottom.

⚠ CRITICAL: Cover the dish with aluminum foil to prevent liquid evaporation from the dish during incubation, especially at a temperature of 36°C.

14. After incubation, remove the soaking solution and gently wash the Petri dish with PBS using a pipette to remove unbound fibronectin.

⚠ CRITICAL: Do not pipette back and forth to prevent fibronectin detachment.

⚠ CRITICAL: Do not allow the pipette tip to touch the base when aspirating the fibronectin solution or washing with PBS to avoid damaging the protein layer.

15. Blow-dry the residues with nitrogen gas until a white crystalline layer appears (Figure 2D).

⚠ CRITICAL: Nitrogen gas should be applied at a distance of at least 30 cm from the substrate to prevent the rapid airflow damaging the protein layer.

⚠ CRITICAL: During the blowing process, carefully check the protein layer on the surface, and stop blowing as soon as it becomes white to prevent overdrying of the fibronectin layer.

16. Store the fibronectin-modified chips at 4°C for up to one week until the subsequent device assembly.

Fibronectin coverage characterization

Timing: 2 h

This step verifies that the immobilized fibronectin maintains a high coverage efficiency on the surface, including both the thickness and uniformity.

⚠ CRITICAL: Handle all chemicals in a fume hood.

Pause point: This step is optional. However, considering the limited depth of the holes after tight compaction with the stencil, the fibronectin coating thickness, which is closely related to the fibronectin concentration and incubation time, must be optimized. Therefore, characterization of the fibronectin coverage is recommended to verify physisorption to the substrate.

17. Perform AFM measurements in dry mode using an AFM (Bruker Multimode V, Germany).
    a. Attach the fibronectin-coated PDMS membrane on a glass slide.
    b. Place the prepared glass slide in the AFM sample stage.
    c. Place a probe in the AFM and activate the laser and photodetector.
    d. Use a cantilever with Bruker DNP10 (silicon nitride with elastic coefficient of 0.6N/m).
    e. Scan the boundary of the fibronectin area in the Scanasyst air mode.
    f. Create an AFM image of the surface line by line.

Note: Our results showed that the optimal thickness of the fibronectin layer was approximately about 550 nm (Figure 2E).

Reversible UTmS-chip assembly

Timing: 1 h

This step produces a reversible UTmS-chip assembly for further cell sedimentation on the precoated Petri dish or PDMS surface.

18. Place the clean UTmS-chips using tweezers at the center of the freshly prepared culture dish or PDMS layer at ambient temperatures and under atmospheric pressure.

Note: The adhesion between PDMS and UTmS-chip enables reversible UTmS-chip assembly without bonding or clamping fixation (Figure 2F). See also the Methods video S1 (Operation procedure of UTmS-chip assembly and single-cell array preparation.) for this step.

⚠ CRITICAL: Avoid tiny bubbles or wrinkles that can affect the leak-proofness between the UTmS and substrate.

19. Gently press on the UTmS with the finger to ensure tight contact with the substrate surface, then load with sustaining liquid without floating and leakage.

⚠ CRITICAL: Ensure the use of clean and sterile gloves before pressing the UTmS with the finger.

⚠ CRITICAL: Press the central area first and then extend it to the edges because the through-hole array is located in the central area of the UTmS chip.

Note: Because the bonding between the UTmS and substrate is reversible, the UTmS-Chip can be easily separated by peeling off the metal stencil.

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Chip assembly characterization

Timing: 2 h

This step verifies whether the physical adsorption between the UTmS and wet substrate is sufficient to achieve a good leak-proofness without additional clamps. Additionally, this step also verifies whether the UTmS is sufficiently hydrophobic to meet the sample loading requirements without the use of a reaction chamber.

Pause point: This step is optional. However, contact angle measurements are recommended to verify the hydrophobicity of the UTmS surface, whereas backside fluorescence characterization is recommended to verify the leak-proofness of the UTmS-chip.

20. Dispense a drop of DI water on the center area of the UTmS and measure the static contact angle (SCA) using a drop shape analyzer (DSA100, Krüss, Germany).

Note: The static contact angle measured on our through-hole-patterned area was 92° (Figure 2G).

⚠ CRITICAL: No obvious static contact angle change should be observed after the routine cleaning process. Therefore, the UTmS can be reused at least nine times for single-cell patterning.

21. Dispense Cy3 solution on the center of the UTmS surface, which remains in a droplet shape rather than spreading out owning to the hydrophobicity of the metal surface.

⚠ CRITICAL: The hydrophobicity of the metal surface enables that the assembled UTmS-chip can be directly sampled without requiring an additional reaction chamber.

22. Observe the green fluorescence of the Cy3 droplet from the backside of the assembled UTmS-chip using an inverted fluorescence microscope (Olympus, Japan) equipped with 10× and 20× objective lenses and a camera (DP73, Olympus).

Note: The fluorescence intensity should be evenly distributed in the hole array, and no liquid leakage should be visible under static conditions for 3 h (Figure 2H).

23. Use a scalpel to cut the UTmS-Chip in half to observe the longitudinal section of the through-holes area.

Note: Figure 2I shows that there was no gap between the UTmS and substrate, and the bottom of the through-holes was well sealed.

⚠ CRITICAL: The fibronectin-modified surface or contact side of the UTmS must be maintained in a moist state to ensure tight adsorption between them, thus avoiding leakage of the liquid.

Culture cells on a chip

Timing: 100 min

This step will demonstrate single-cell patterning with high-density cell suspensions based on the UTmS-chip. See also the Methods video S1 (Operation procedure of UTmS-chip assembly and single-cell array preparation.) for this step.

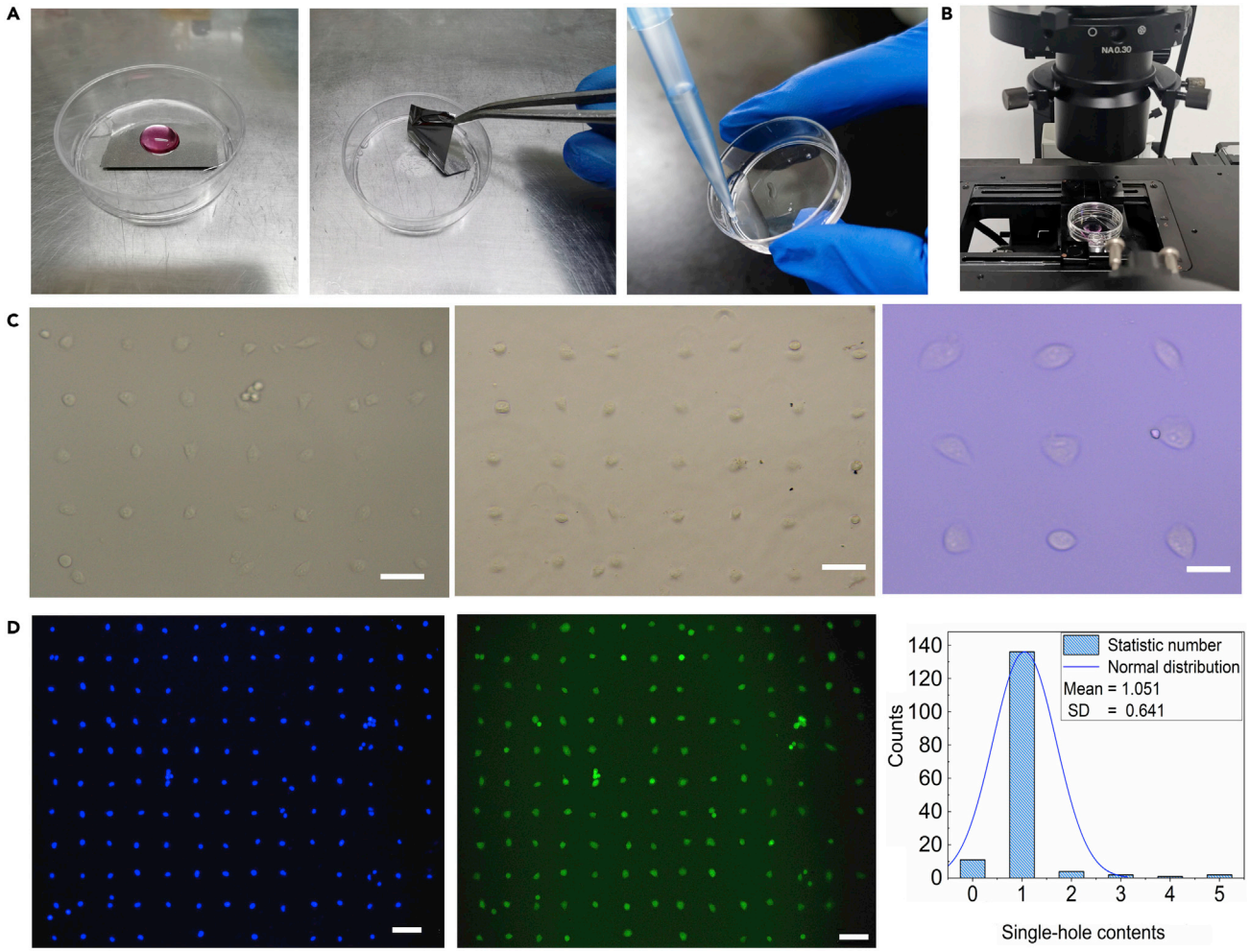

Figure 3. Evaluation of the UTmS-based single-cell platform

(A) The assembled UTmS chip on a 35 mm Petri dish was loaded with a cell suspension droplet for single-cell preparation.

(B) The UTmS is peeled off from one corner like tape.

(C) The bright fluorescence images of HeLa and A549 cell arrays under the lower magnification (scale bars: 100 μm) and the higher magnification (scale bar: 50 μm).

(D) The blue and green fluorescence images of the MCF-7 cell array. Scale bar: 100 μm. Images and plot in (A–D) reproduced from Song et al. with permission from the Royal Society of Chemistry.[1]

24. Prepare cell suspensions at a density of 1×10^6–3×10^6 cells per mL and mix thoroughly for subsequent cell loading.
25. After assembling the device, pipette a drop of the cell-suspended medium onto the stencil region to cover the stencil array (Figure 3A).
26. Transfer the chip to a CO_2 incubator at 37°C for 20 min to allow the cells to attach to the fibronectin-modified substrate under gravity-induced natural sedimentation.

Note: The incubation time may be increased to 25–30 min depending on the cell line and their ability to attach.

Note: Cancer cells, such as MCF and A549, require approximately 2 h to attach to a bare substrate without a fibronectin coating.

27. After short-term cultivation, gently aspirate the cells suspension before peeling the UTmS layer and then gently rinse with a warm medium to remove the unattached cells.

⚠ CRITICAL: Ensure that the tip aspirates all the cell suspensions from the edge of the droplet at a very low rate to avoid damaging the weak adhesion between the cells and substrate surface through the holes.

28. Use tweezer to gently peel off the UTmS from the substrate without disturbing the cell array, starting from one corner (Figure 3A).

⚠ CRITICAL: Gradual tape-like peeling is beneficial for protecting newly formed single-cell arrays.

29. Gently rinse with a warm medium to remove the extra cells attached to a single-point cell before further incubation (Figure 3A).

⚠ CRITICAL: The suction and injection of liquid manipulated with a pipette must be gentle and slow to avoid detachment of cells from the array patterns.

30. Recover the single-cell array with fresh culture medium after the peel-off process and incubate it in an incubator for another 20 min.

⚠ CRITICAL: Incubation for another 20 min is beneficial to strengthen cell adhesion for further downstream experiments and analyses.

31. Immerse the single-cell array in PBS to maintain stable adhesion when stored for several hours.

Single-cell patterning evaluation

Timing: 40 min

This step verifies the viability of individual cells on the fibronectin-coated Petri dish or PDMS layer, and demonstrates the high throughput of the single-cell array.

32. Prepare the staining solution of FDA and Hoechst33342.
    a. Add 4μL FDA (5 mg/mL) and 2 μL Hoechst solution(10 mg/mL) to 2 mL of the complete culture medium.
    b. Mix thoroughly and maintain in dark conditions.

Note: The final working concentration of FDA and Hoechst33342 is 10 and 10 μg/mL, respectively.

33. Gently aspirate the PBS in the assembled dish, and slowly add 2 mL staining solution into the Petri dish to immerse the cells.
34. Transfer the device into a CO_2 incubator at 37°C for 10 min.
35. Remove the staining solution after incubation, and rinse the dish twice with PBS.
36. Image the single-cell array using an inverted fluorescence microscope (Olympus) in the dark using a DAPI and FITC filter cube with a 10× and 20× air objective (Figure 3B).

Note: The bright-field (Figure 3C) and blue fluorescence images (Figure 3D) demonstrate the integrity of the nucleus of the single cells, whereas the clear green fluorescence indicates that the viability of the MCF-7 single cells on the substrate is approximately 96 ± 1.7% (Figure 3D).

Note: We conducted single-cell patterning on three different cancer cells using the UTmS strategy and found that each could form single-cell arrays. Our chip is suitable for single-cell array preparation of different types of cells, and there is no obvious difference in the survival rate of different single cells prepared simultaneously.

Single-cell apoptosis observation

Timing: 4.5 h

This process validates the concept of the UTmS strategy for single-cell capture, culture, and real-time monitoring and demonstrates the heterogeneous activity of cells.

37. Prepare the mixed reagent for cell apoptosis characterization.
    a. Add 8μL TMRM (100 μM) and 20 μL of the STS apoptosis inducer (1 mM) to 2 mL of the complete culture medium.
    b. Mix thoroughly and maintain the solution in the dark.

Note: The final working concentrations of TMRM and STS are 400 nM and10 μM, respectively.

38. Image a single-cell array using a Cy3 filter cube and 10× objective on an inverted fluorescence microscope equipped with temperature, humidity, and CO_2 control for apoptosis assays (measurement of fluorescence intensity).
    a. Turn on the microscope, camera, CO_2 supply, water pump, and heating unit at least 1 h before use.
    b. Set the heating unit temperature to 37°C and set the 10× air objective.
    c. Place the assembled dish in the plate holder.
    d. Slowly immerse the single-cell array in PBS and add 2 mL of the mixed reagent to 10 μM STS in a 3.5-cm Petri dish.
    e. Prepare a control group without STS.
    f. Select the positions to record using the software interface and adjust the focus, exposure time, light power, time length of the acquisition, and acquisition rate, and start imaging.

Note: We used an Olympus microscope with dedicated software in our experiments. Any microscope equipped with temperature and CO_2 control, air objectives, TRITC filter cube, or dedicated software can be used. We typically imaged the single-cell array in one image every 10 min for 4 h in the corresponding fluorescent channel.

39. Use ImageJ to measure the gray values of the red fluorescence images of individual cells acquired at 10 min intervals (Figures 4A and 4B).
40. Use Origin (2018 version) to acquire real-time plots of the normalized fluorescence intensity of individual cells as a function of the culture time (Figure 4C).

Single-cell calcium release

Timing: 3 h

This step further emphasizes the versatility of single-cell patterning using our strategy. Here, we selected MCF cells and a Fluo-8-based calcium assay kit to conduct single-cell calcium release monitoring under the stimulation of KCl agonists, which could induce intracellular calcium release through membrane depolarization.

41. Prepare 1× loading solution of Ca^{2+} indicator dye (Fluo-8 no wash calcium assay kit) with Fluo-8 stock solution and 1× assay buffer.

Note: Mix thoroughly and maintain the solution in the dark.

42. Construct three single MCF-7 cell arrays in a 10 × 20 array with the UTmS-chip and independently incubate for 1 h with 1 mL Fluo-8 dye-loading solution.

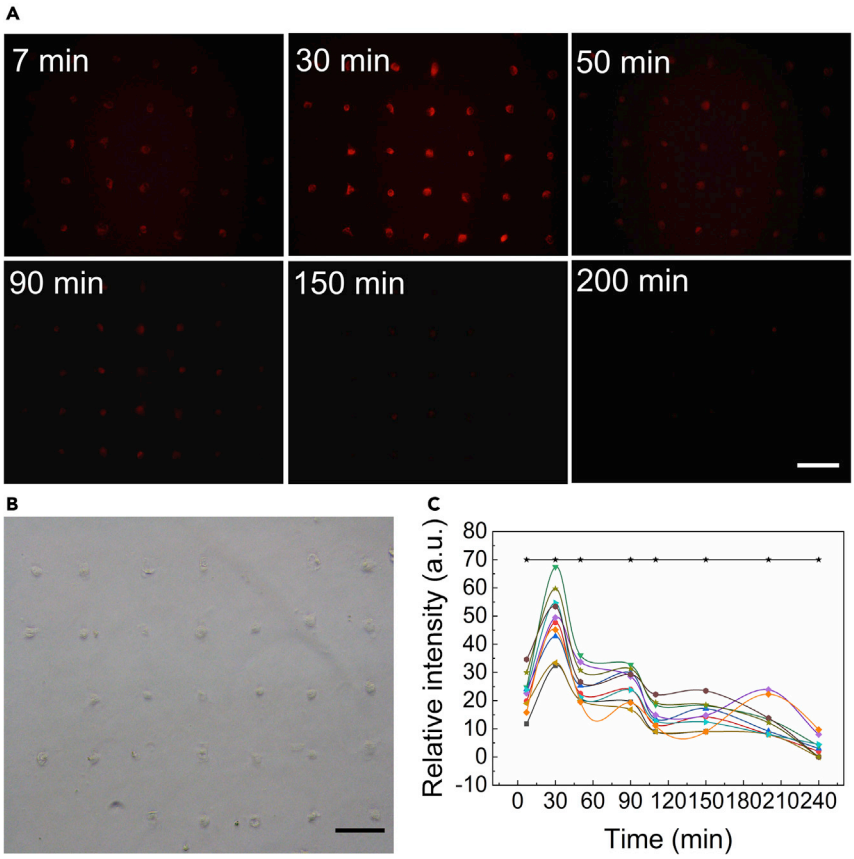

Figure 4. Single-cell apoptosis observation

(A) Real-time fluorescence images showing the cell apoptotic process of the MCF-7 cells, scale bar: 100 μm.

(B) The bright-field image of the MCF-7 cells after STS treatment for 240 min. Scale bar: 100 μm.

(C) Real-time measurements of the normalized fluorescence intensity of 10 individual cells and one control group as a function of culture time. Images and plot in (A–C) reproduced from Song et al. with permission from the Royal Society of Chemistry.[1]

Note: Protect the entire process from light.

43. Add 100 μL KCl solutions of 0, 100, and 200 mM to stimulate the individual cells in the three assembled devices.
44. Image a single-cell array using an FITC filter cube and a 10× objective on an invert fluorescence microscope equipped with temperature, humidity and CO_2 control for the calcium release assays (measurement of fluorescence intensity).
    a. Turn on the microscope, camera, CO_2 supply, water pump, and heating unit at least 1 h before use.
    b. Set the heating unit temperature to 37°C and set the 10× air objective.
    c. Place the assembled dish with a single MCF-7 cell arrays in a 10 × 20 array in the plate holder.
    d. Slowly aspirate the PBS covering the single-cell array and add Fluo-8 dye-loading solution (1×) for a 3.5-cm Petri dish.
    e. Incubate for 1 h in dark conditions.
    f. Slowly add 100 μL KCl solution to stimulate the single cells in the assembled device.

⚠ CRITICAL: In this experiment, three concentrations of KCL solutions (0, 100, and 200 mM) were added to the assembled device. Thereinto, 0 mM KCl was administered with DI water as the control group.

g. Using the software interface, select the positions to record, adjust the focus, exposure time, light power, time length of the acquisition, and acquisition rate, and start imaging.

⚠ CRITICAL: Keep the parameters constant throughout the observation period.

Note: An Olympus fluorescence microscope was used in this step. Any microscope equipped with temperature and CO_2 control, air objectives, and filter cube can be used. We typically imaged the single-cell array in one image every 5 min for 60 min in the FITC fluorescent channel.

45. Use ImageJ to measure the gray values of the green fluorescence images of individual cells taken at 5-min intervals (Figure 5A).
46. Use Origin (2018 version) to acquire boxplots of the single-cell assay data (Figure 5B) and real-time plots of the normalized fluorescence intensity of individual cells as a function of the culture time (Figure 5C).

UTmS cleaning for reuse

Timing: 30 min

⚠ CRITICAL: Handle all chemicals in a fume hood.

⚠ CRITICAL: All UTmS cleaning steps can be performed in clean glass dishes or disposable tube. Stencils must be handled using tweezers.

47. Cleaning procedure for used UTmS-chips.
    a. Peel off the UTmS after single-cell patterning and immerse it in a dish or tube with trypsin at 37°C for 10 min.

Note: Trypsinization is performed to ensure that no residual cells remain on the metal stencils.

    b. Remove the UTmS with tweezers and rinse with DI water.
    c. Place the UTmS in a clean dish or tube with ethanol and then ultrasonically clean for 10 min.

⚠ CRITICAL: If the chip is dirty, a water bath at 65°C can be used as an aid for ultrasonic cleaning.

    d. Hold the UTmS chips using tweezers and wash thoroughly with DI water to remove ethanol residues, and dry using nitrogen gas.
    e. Sterilize the stencil using UV-ozone disinfector for 30 min.

Optional: Vacuum vapor sterilization is also a reliable method for chip sterilization.

48. Place the UTmS under backlight observation to verify whether the cleaned UTmS has clean through holes.

⚠ CRITICAL: The UTmS is considered clean if there are no obstacles in the through-hole. Therefore, use an inverted microscope to visually verify that no defects are present in the through-hole array of the cleaned stencils.

49. Place the cleaned UTmS-chips in a 35-mm sterilized Petri dish without fixation (Figure 2F).

Note: Seal the Petri dish with parafilm and store the UTmS-chips in a vacuum desiccator for extended periods.

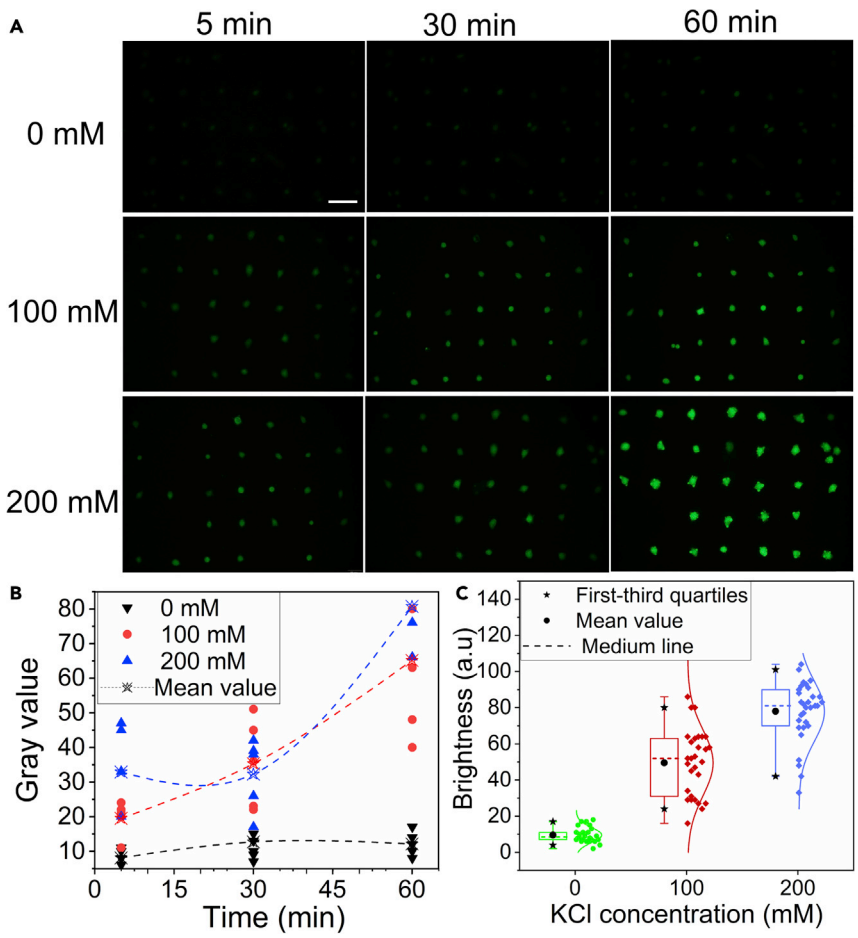

Figure 5. Intracellular calcium release assay

(A) Assay results with the addition of 0, 100, and 200 mM KCl to induce calcium release, scale bar: 100 μm.

(B) Boxplots of single-cell assay data. The boxes represent first–third quartiles, and the whiskers indicate 5%–95% values.

(C) Real-time measurements of the normalized gray values of four individual cells in different concentrations of KCl. Images and plots in (A–C) reproduced from Song et al. with permission from the Royal Society of Chemistry.[1]

Optional: As mentioned earlier, it is also recommended to store UTmS-chips in a Gel-box with a polymer silicone layer to ensure that the chip tile is fixed on the bottom.

EXPECTED OUTCOMES

A 5 μm-thick reusable UTmS with a high-throughput through-hole array using laser drilling can be routinely fabricated using the above protocol. A single-cell occupancy of approximately 88% could can be easily achieved on fibronectin-modified substrates under gravity-induced natural sedimentation within 30 min without significantly affecting the cells. As expected, a heterogeneous apoptosis and significant cell-to-cell variations in intracellular calcium release between these single cells can be observed. The UTmS-based single-cell patterning strategy is suitable for different cellular types and arbitrary planar substrates, as well as for the real-time study of single-cell behaviors using high-throughput arrays and common tools available in any laboratory. It is also expected that the reported UTmS method will offer a simple, efficient, user-friendly, and low-cost approach for constructing single-cell patterns without requiring additional fixation, reaction pools, and centrifugation procedures without the need for single-cell preparation, which is especially valuable for researchers in different fields.

LIMITATIONS

For the UTmS preparation, the pulse parameters of laser drilling are critical for obtaining consistent and regular microstencils with high throughput. Insufficient laser power or an excessively long drilling time results in unsmooth edges and irregular through-holes. Second, prolonged incubation of cells on the UTmS-chip causes excessive cell aggregation at the same site. This protocol is optimized for fibronectin coatings. The coating of Petri dishes or PDMS layers with other adhesion promoting agents will require individual optimization. The coverage of the fibronectin coating based on physical adsorption is random and uncertain. Therefore, repeated attempts are encouraged during the preparation of the fibronectin coating. Third, the adhesion strength of UTmS to the substrate largely depends on the moisture of the two surfaces in contact. It is recommended that the fibronectin-coated substrates stored at 4°C should be placed in a laminar flow hood for several minutes to allow the surface to condense before use. The specific resting time also depends on the ambient temperature and humidity. Fourthly, inconsistent conditions during cell sedimentation and attachment can provide less than optimal efficiency and throughput of single-cell array patterning. Cell viability and density must be confirmed using flow cytometry before each assay. A further limitation of single-cell patterning is the state of growth in various cell lines before trypsinization into a cell suspension, which must be considered before cell loading. Finally, when the sediment is finished after incubation and the UTmS peels off, manual washing to detach extra cells must be performed carefully. At the same time, there is no specific flow rate. Researchers must repeat the experiment several times to determine the optimal washing operation.

TROUBLESHOOTING

Problem 1

Approximately > 10% of the through-hole arrays possess two or more cells owing to intercellular adhesion (step 28).

Potential solution

After digestion, mix the sample with a pipette for 20 s, and use repeated pipetting to thoroughly homogenize the cell suspension, which ensures that the cells are sufficiently dispersed without aggregation. Additionally, gently rinse the surface of the UTmS-chip multiple times before the peeling step and observe under a microscope while rinsing.

Problem 2

Approximately > 8% of the through-hole arrays have no cells owing to the PBS washing operation (step 28).

Potential solution

Although adherent cells can adhere to the substrate owing to the fibronectin coating, the anchoring strength remains weak at the initial stage of cell adhesion. If apoptotic reagents or fluorescent dye solutions are added directly, the cells forming the array are washed away and a complete matrix cannot be formed. Operations such as UTmS peeling or PBS rinsing can lead to cell detachment and vacancies at the through-holes. Ensure that manual washing to detach extra cells is performed gently and carefully. Therefore, repeated attempts to gently rinse the cells are encouraged to detach them from the substrate.

Problem 3

Liquid leakage at the UTmS-chip interface during cell loading and incubation (step 25).

Potential solution

Even if the assembled chip fits tightly and does not leak before use, there is still a low chance of liquid leakage during cell loading and incubation. In this case, the chip cannot be reused, and must be replaced.

Problem 4

Even if the rinsing with a warm medium is very gently, it still causes lightly attached single cells to fall off along with the extra cells attached to a single-point cell before further incubation (step 27).

Potential solution

Try to inject the warm medium gently from the edge of the petri dish to recover the bottom of the dish, and then tilt the dish slightly to suck the solution away from the corner using a pipette.

Problem 5

After the second-round incubation in an incubator for another 20 min, there are still many cells detaching from the substrate, resulting in low efficiency of single cell formation (step 30).

Potential solution

The growth state of the cells in the dish before digestion to prepare the cell suspension is very important for the success rate of single-cell pattering. Ensure that the cell suspension must be prepared after all the steps of UTmS-chip assembly have been completed. If it is confident that all experimental operations are in accordance with the protocol, try to repeat the experiment with the best growing cells.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ying-Nan Sun (syingnan@126.com).

Materials availability

The study did not generate new/unique reagents.

Data and code availability

This study did not generate new data sets/codes.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102115.

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AUTHOR CONTRIBUTIONS

Y.-N.S. helped with conception, design, methodology, investigation, and writing; Q.T. performed the experiments; Q.T., K.X., and Y.-N.S. carried out the data analyses and wrote the manuscript. Y.L., Q.W., and H.S. helped with data collection and draft proofreading; Y.-N.S. and S.Z. were responsible for supervision and resources.

DECLARATION OF INTERESTS

The authors have a Chinese patent related to this work: Y.-N.S., Yuhan Song, Q.T., Jianhong Liu, Wenting Guo, S.Z. Patent authorization data: April 8, 2022. The invention relates to a preparation method of a single-cell array. Patent number: ZL202011190716.4.

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