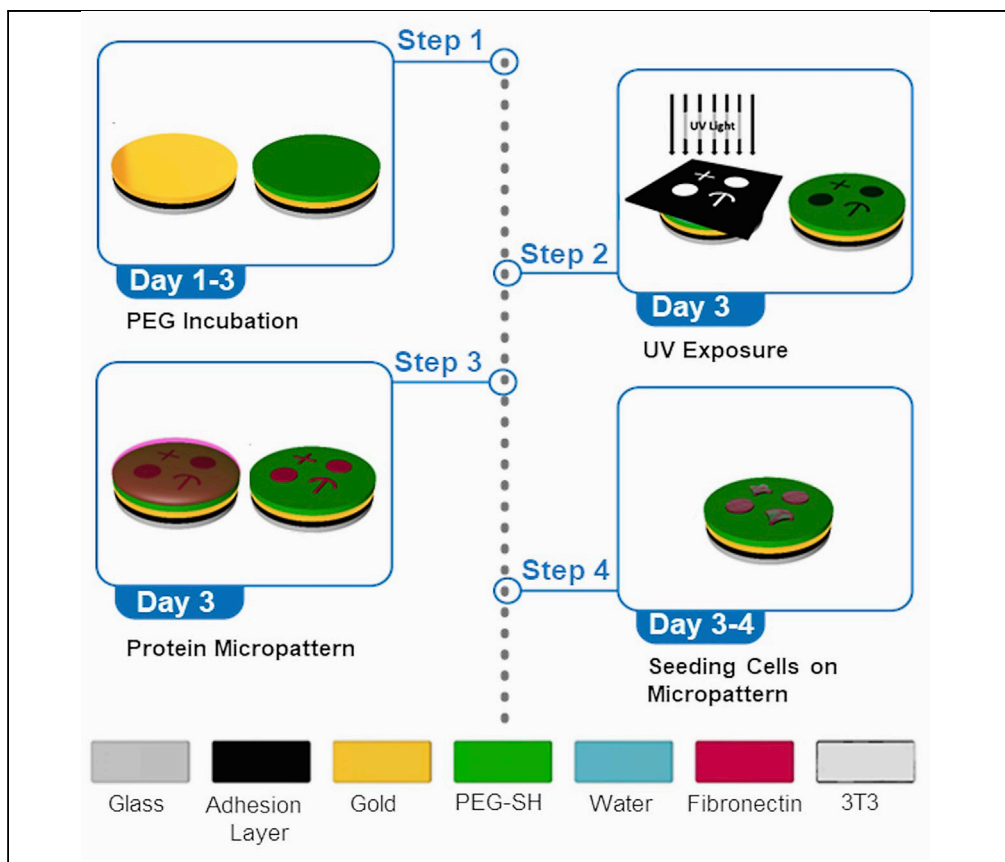


Protocol

Micropatterning of Cells on Gold Surfaces for Biophysical Applications



We developed a reproducible micropatterning method to manipulate and normalize cell shape and cell-cell separation on gold. We used methoxy polyethylene glycol thiol (PEG-SH) to create a self-assembled monolayer that can be oxidized at desired shapes through a photomask with deep UV light. The oxidized PEG can be coated with extracellular matrix proteins and seeded with cells adopting the pre-defined shape. The developed and analyzed surfaces can be used in a wide range of biophysical applications.

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HIGHLIGHTS

Micropatterning protocol on a thin gold layer for various biophysical methods

Investigation of isolated cells with pre-defined normalized shape

Varying matrix proteins allows investigation of biochemical cues in addition to shape

Micropatterning protocol enables insights into different cellular mechanisms

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Protocol

Micropatterning of Cells on Gold Surfaces for Biophysical Applications

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SUMMARY

We developed a reproducible micropatterning method to manipulate and normalize cell shape and cell-cell separation on gold. We used methoxy polyethylene glycol thiol (PEG-SH) to create a self-assembled monolayer that can be oxidized at desired shapes through a photomask with deep UV light. The oxidized PEG can be coated with extracellular matrix proteins and seeded with cells adopting the pre-defined shape. The developed and analyzed surfaces can be used in a wide range of biophysical applications.

BEFORE YOU BEGIN

Note: The gold coating of coverslips (Figure 1A) and the developing of a quartz mask can be outsourced to several commercial providers if the necessary equipment is not available in house.

Note: The flow chart in Figure 2 assists in following the protocol.

Gold Coating

1. Cleaning

Put coverslips in an ultrasonic bath (400 W).

- In a beaker with acetone for 5 min.
- In a beaker with isopropanol for 5 min.
- Dry the cleaned coverslips in a stream of dry nitrogen.

△ **CRITICAL:** Use a “Wash-N-Dry Coverslip Rack” to clean your coverslips in a beaker. This allows contaminants to drain off better and prevents them from accumulating on your coverslip. Do this in every cleaning step up to the protein coating step.

△ **CRITICAL:** Use a reverse tweezer while blowing nitrogen gently from the side. Otherwise your coverslips may break. This applies to the whole protocol.

2. Coating

Coat coverslips first with a 3 nm titanium layer as an adhesion layer and a 20 nm gold layer in an electron beam evaporation system (Varian 3117). with a deposition rate for gold and titanium of 2 Å/s



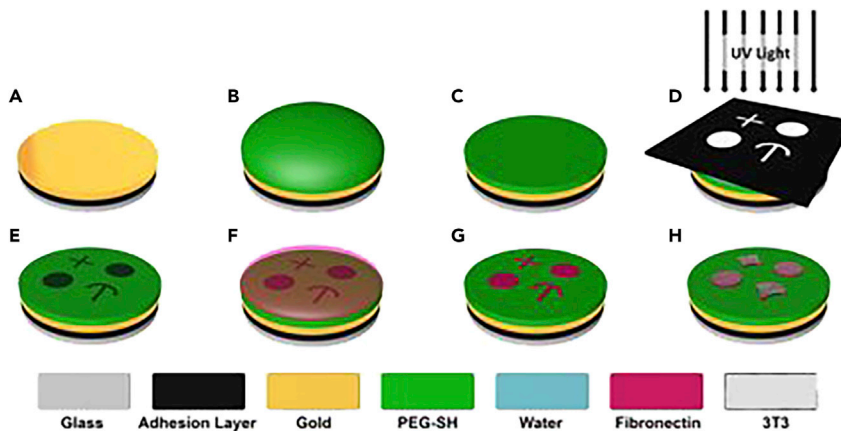


Figure 1. Schematic Diagram of the Micropatterning Procedure

(A) Coverslips are cleaned and coated with a titanium adhesion layer and a 20 nm gold layer with an electron beam evaporation system.
 (B) The cleaned and surface pre-treated coverslips are incubated in a PEG-SH water solution.
 (C) After 48 h a SAM was built, the coverslips are drip dried and cleaned in ethanol.
 (D) To transfer the patterns the samples are exposed to UV radiation through a chromium mask.
 (E) The samples with the oxidized patterns are cleaned in a mixture of acetone, ethanol, and water.
 (F) The pegylated and exposed coverslips are incubated in a fibronectin solution.
 (G) The fibronectin binds to the oxidized PEG-SH and create the desired micropatterned substrate.
 (H) The protein micropattern were incubated with 3T3 mice fibroblast cells, which adhered to the pattern and change their shape due to the underlying pattern shape.

and a chamber pressure of 10^{-6} mbar. To control layer thickness use a quartz crystal microbalance (Maxtek TM-100).

Quartz Mask

3. Preparation of Quartz mask

Design the photomask for the desired pattern (for example with the free software KLayout). For our aim we used different patterns (cross, crossbow, circle) with a diameter of $35\ \mu\text{m}$ and a distance between neighboring single patterns of $100\ \mu\text{m}$ (Figure 3), large enough so that cells cannot bridge the gap between individual adhesive islands.

Note: Test different diameters for your cell line. The size of a single pattern has to be optimized to the desired experiment, for example to allow cells to spread enough to be under stress, but small enough to inhibit multiple cells from adhering to a single pattern. The steps size for optimizing the pattern size is cell type dependent. For 3T3 fibroblasts, $5\ \mu\text{m}$ steps are reasonable. The diameter can be estimated from the approximate projected cell area of the cell without pattern. For the 3T3 fibroblast cell line, $35\ \mu\text{m}$ diameter was well-suited: the cells adhere to the patterns, form stress fibers, and closely follow their shape. For larger patterns ($40\ \mu\text{m}$ diameter) the cells do not adopt the pre-designed geometry, while for smaller patterns ($30\ \mu\text{m}$ diameter) it relaxes even for high-stress patterns like the crossbow.

- Expose the commercial quartz mask (with a chromium and a poly(methyl methacrylate) (PMMA) layer (g-materials) as resist on top) in an electron beam lithography system (Leica EBPG 5 HR). Expose with 20 kV and 200 nÅ.
- Afterwards, develop the resist in a mixture of methyl isobutyl ketone (MIBK) in isopropanol (3:1) for 20 s and after in a mixture of MIBK and isopropanol (1:1) for 10 s.

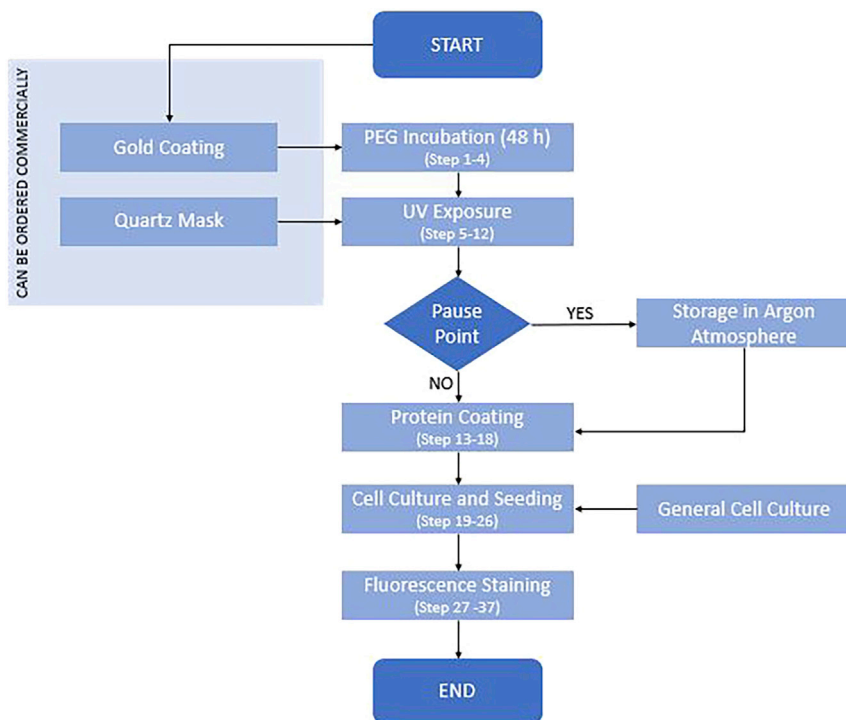


Figure 2. Flow Chart of the Protocol Provides an Overview of the Individual Steps

The gold coating and the quartz mask can be ordered from commercial providers. Then follow the steps PEG incubation, UV exposure, protein coating, cell culture, and seeding as well as fluorescence staining.

6. Chromium is etched with chromium etchant $(\text{NH}_4)_2[\text{Ce}(\text{NO}_3)_6]/\text{HClO}_4$ (MicroChemicals) for 45 s.
7. The remaining (unexposed) resist is finally stripped in N-methyl-2-pyrrolidone (NEP) at 100°C for 5 min.

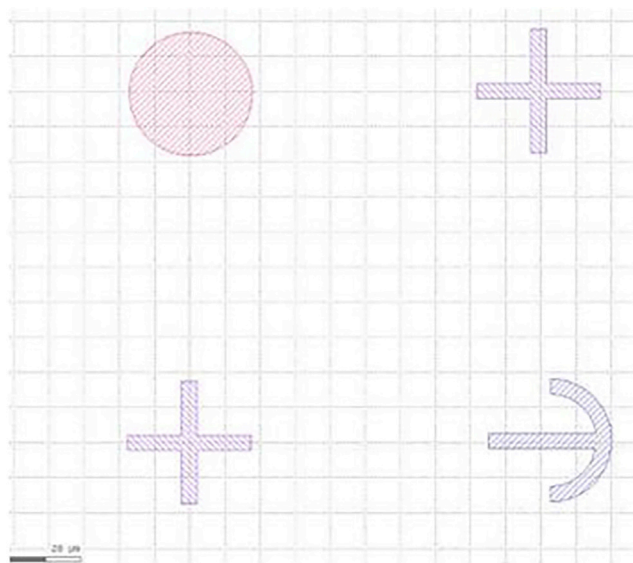


Figure 3. Mask Design in the KLayout Software

The patterns cross, crossbow, and circle are arranged in an array. The distance from center to center is 100 μm and the linewidth is 5 μm.

Cell Culture

8. Split your 3T3 cell line every three days. Keep them in a Dulbecco's Modified Eagle Medium (DMEM) medium consisting of phenol red, 10% fetal bovine serum and 1% antibiotic/mycotic solution.

Note: Here we describe the use of NIH 3T3 cells, but have also employed this protocol with the A549 and R3/1 cell line.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Alexa Fluor 568 phalloidin	Thermo-Fisher-Scientific	CAT#A12380
Cy3 Amersham Mono-Reactive Dye Pack	GE Healthcare	CAT#PA23001
Biological Samples		
Fibronectin from human plasma	Sigma-Aldrich	CAT#F2006-2MG
Chemicals, Peptides, and Recombinant Proteins		
Acetone	VWR	CAT#20063.365
Antibiotic/Antimycotic Solution	GE Healthcare Hyclone	CAT#SV30079.01
Bovine serum albumin (BSA)	Sigma-Aldrich	CAT#A8806
Chromium Etchant ((NH ₄) ₂ [Ce(NO ₃) ₆]/HClO ₄)	MicroChemicals	N/A
Dulbecco's Modified Eagle Medium (DMEM), high glucose	Gibco by live technologies	CAT#41965-039
Gibco Dulbecco's phosphate-buffered saline (DPBS), without calcium and magnesium	Gibco by live technologies	CAT#14190-144
Ethanol	Merck	CAT#1009832511
Fetal Bovine Serum (FBS) Gold Plus	Bio&Sell	CAT#FBS.GP.0500
Formaldehyde 16%	Polysciences	CAT#18814
Gold, 99.999%	Kurt J. Lesker	CAT# EVMAU50QXQ
Isopropanol	VWR	CAT#20922.364
Methyl isobutyl ketone (MIBK)	Technic	CAT#108-10-1
NEP (N-ethyl-2-pyrrolidone)	AllResist	CAT#AR300-72
Polyethylene glycol thiol (PEG-SH)	Sigma-Aldrich	CAT#729140-1G
Titanium, 99.995%	Kurt J. Lesker	CAT# EVMTI45QXQB
Triton X-100	Sigma-Aldrich	CAT#T8787
Trypsin 0.25%	Biowest	CAT#L0910-100
Ultrapure Water	Q-Pod mit Millipak Express 40	MPGP04001
Experimental Models: Cell Lines		
NIH/3T3 mice fibroblasts	ATCC	ATCC® CRL-1658™
Software and Algorithms		
KLayout Software	N/A	https://www.klayout.de/
Other		
Air plasma Femto	Diener electronic	N/A
Cantilever	Olympus	CAT#OMCL-AC240TSA-R3E
Coverslips, borosilicate glass, 24 mm	Paul-Mariefeld	CAT#0111640
Dry Nitrogen	N/A	N/A
Electron beam evaporation system	Varian	3117

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Electron beam lithography	Leica	EBPG 5 HR
Lifting platform (Swiss Boy 115)	Rudolf Grauer AG	N/A
MicroTime200	PicoQuant	N/A
Nanowizard 4	JPK	N/A
Olympus IX83 microscope	Olympus	N/A
Quartz crystal microbalance	Maxtek	TM-100
Quartz mask with chromium and poly(methyl methacrylate) (PMMA) layer	g-materials	N/A
Ultrasonic bath (400 W)	Haver&Boecker	USC 200-90
UV lamp, OSRAM 15 W, 185 nm and 254 nm	UV Technik Speziallampen	CAT#HNS15WG13
Wash-N-Dry Coverslip Rack	Sigma-Aldrich	CAT#Z688568-1EA

MATERIALS AND EQUIPMENT

△ CRITICAL:

Use a quartz mask, since other mask materials may absorb UV light.

△ CRITICAL:

NEP is toxic and causes skin irritation, eye irritation, and may irritate the respiratory tract.

MIBK is toxic and inflammatory, may irritate the eyes, skin, and respiratory system.

Chromium Etchant is a very strong oxidant and causes severe skin burns and eye damage.

16% formaldehyde is toxic and harmful to health. Working in a fume cupboard is necessary.

Alternatives:

Gold covered glass slides can be ordered commercially.

Also, quartz masks can be ordered from several commercial providers. If the mask is cleaned regularly and stored properly, it can be used virtually eternally. Masks can also be written using a UV laser. However, electronic beam lithography has the advantage that it can be used when high resolution of smaller feature sizes is required or line edge roughness is very important.

Due to the possibility to outsource many critical steps in mask and slide production, the protocol can be carried out in any laboratory, since few materials are needed.

If you do not have an extra UV lamp in the laboratory, a UV lamp for may also be suitable for exposure via a quartz mask. Due to the different intensity compared to the lamp used in the protocol, the exposure time has to be adjusted.

STEP-BY-STEP METHOD DETAILS

PEG Incubation

⌚ Timing: 45 min, day 1, 48 h incubation

The gold cover glasses are incubated with PEG-SH (Figure 1B). The thiol groups covalently bonds with gold to form a self-assembled monolayer (SAM), which is protein repellent.

1. Prepare a solution of 1 mg/mL PEG-SH in ultrapure water (Solution 1).

⚠ **CRITICAL:** Store the solution at 4°C.

Note: The solution remains stable for several months at 4°C and does not have to be freshly prepared before each incubation.

2. Wash gold coated coverslips in ethanol in an ultrasonic bath (400 W) for 2 min and dry them in a stream of dry nitrogen.
3. Do surface pre-treatment to clean gold coated coverslips in an air plasma (100 W) for 40 s.
4. Incubate the coverslips with 200 µL of solution 1 at room temperature for 48 h in argon atmosphere.

⚠ **CRITICAL:** Store the coverslips in argon atmosphere. During incubation, cover slips should not be exposed to large room temperature fluctuations.

Note: Use a weighing bottle. This can be filled with argon and sealed tightly with parafilm.

Note: Atomic force microscopy (AFM) tapping measurements were performed to check the change in surface chemistry, which indicates a PEGylation on the surface on the cover glass. This step is useful during development of the protocol. However, it is not critical. Checking the end result using fluorescently labeled protein coating suffices. As shown in Figure 4 both gold and PEG-layered gold form a smooth surface on the cover glass with different surface roughness. Smooth surfaces are ideal for passivation. The distribution of phase values of the PEG surface is broader than the gold, indicating changes in surface chemistry/composition.

UV Exposure

⌚ **Timing:** 45 min, day 3

In the protein-repellent SAM (Figure 1C), oxidized, protein adhesive patterns are generated by deep UV irradiation through the pre-designed photomask (Figure 1D).

5. After 48 h drip dry the remaining solution on coverslips. Then clean them in ethanol in an ultrasonic bath (400 W) for 5 min and afterwards dry them in a stream of dry nitrogen.
6. Clean the photomask by rinsing in isopropanol, water, and ethanol. Then dry the photomask in a stream of dry nitrogen.
7. Clean the photomask in air plasma (100 W) for 40 s.
8. Place the mask on a paper tissue on the laboratory bench so that the chrome side is facing upwards. As an adhesion layer between the chromium side of the mask and the pegylated coverslips use ultrapure water (MilliQ). Pipette 5 µL onto the mask (Figure 5A). Turn your cover glass so that the non-pegylated side is facing up and the pegylated side is in contact with the water (Figure 5B).

Note: Before exposure, make sure that there are no air bubbles between coverslip and photomask. For this, it is best to use the back of a plastic pipette tip (100–1,000 µL volume) to carefully press the cover glass to the mask. Apply pressure with pipette tip carefully and directly onto the cover slips. Be careful that they do not break and the mask is not damaged. Otherwise, even exposure is not guaranteed, and the patterns will show defects.

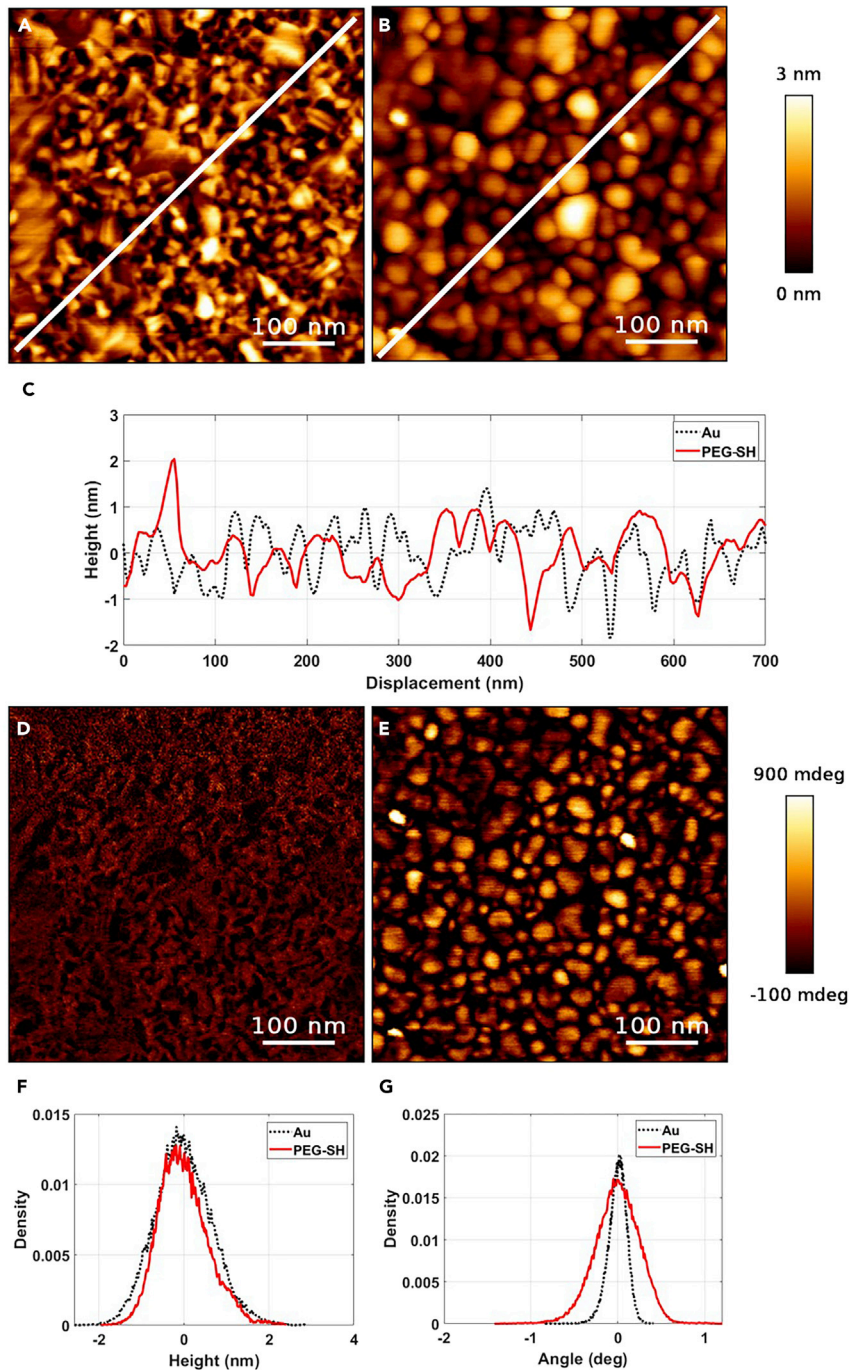


Figure 4. AFM Tapping Measurements of Gold and PEG-SH Layer

(A and B) (A) Height image of gold and (B) PEG-layered gold.

(C) Cross-section of height at line indicated in A, B.

(D and E) (D) Phase image of gold and (E) PEG-layered gold.

(F and G) (F) Height histogram and (G) phase histogram.

Gold is shown in gray and PEG-layered gold in red.

9. Turn the mask over so that the side of the mask with the coverslips is facing down on the lifting platform. A distance of 0.5 cm has proven to be effective for producing accurate patterns. With lifting platform the distance between mask and UV lamp can easily be adjusted (Figure 5C). The mask with the cover glasses can now be exposed.

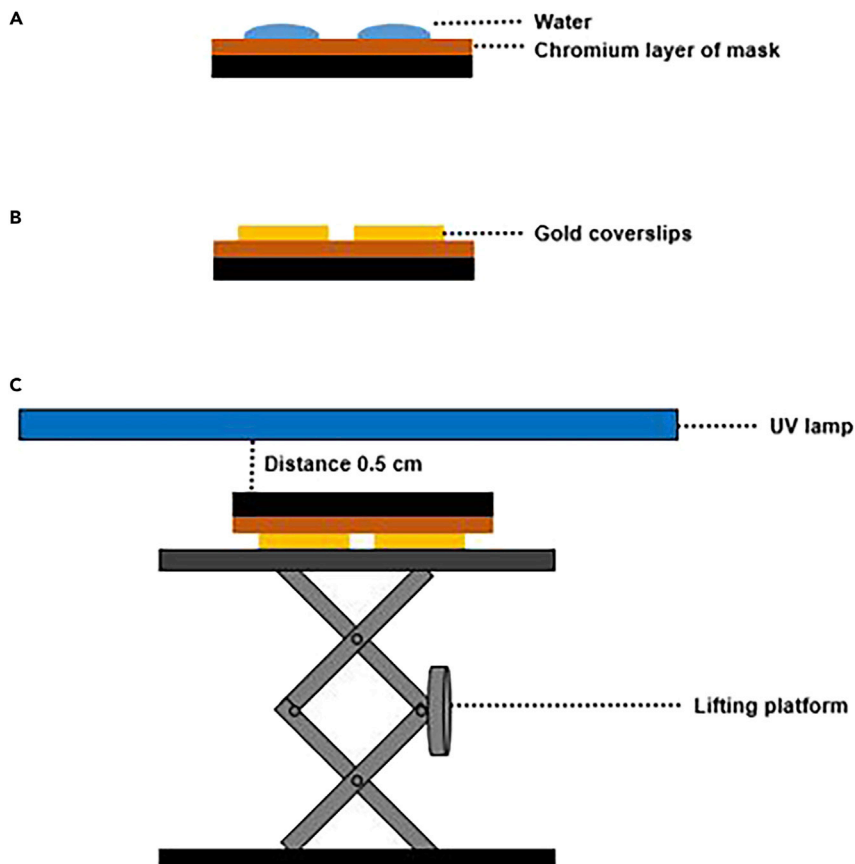


Figure 5. Scheme of UV Exposure

- (A) The distance of 0.5 cm between mask with cover glasses and UV lamp is adjusted by a lifting platform.
 (B) The gold side of the coverslips is placed on the water drop.
 (C) The mask is turned upside down. The chromium side with the coverslips points down and is placed on a lifting platform. With this platform the distance of 0.5 cm to the UV lamp is regulated.

Note: The even exposure depends on the intensity distribution of the used UV lamp.

10. Irradiate the coverslips with UV light for 5 min at an energy dose density of the lamp is 70 mW/cm². The irradiation time need to be optimized for different lamps and intensity distributions.

Note: It is essential that the step is carried out in a fume cupboard or in a flow. The deep UV light produces ozone during the reaction. Due to the UV radiation it is important to wear protective goggles and not to expose your skin during the reaction and to make sure that no people enter the laboratory.

11. Rinse the mask carefully with water and remove the cover glasses.

Note: Using the back of a plastic pipette tip (100–1,000 μ L volume) while rinsing with water, the cover slips can be carefully pushed off the mask to avoid scratches.

12. Clean the irradiated coverslips in a mixture of acetone, ethanol, and ultrapure water (1:1:1) in an ultrasonic bath for 1 min. Afterwards dry them in a stream of dry nitrogen.

Pause Point: Before incubating with protein, the cover slips can be stored for several weeks.

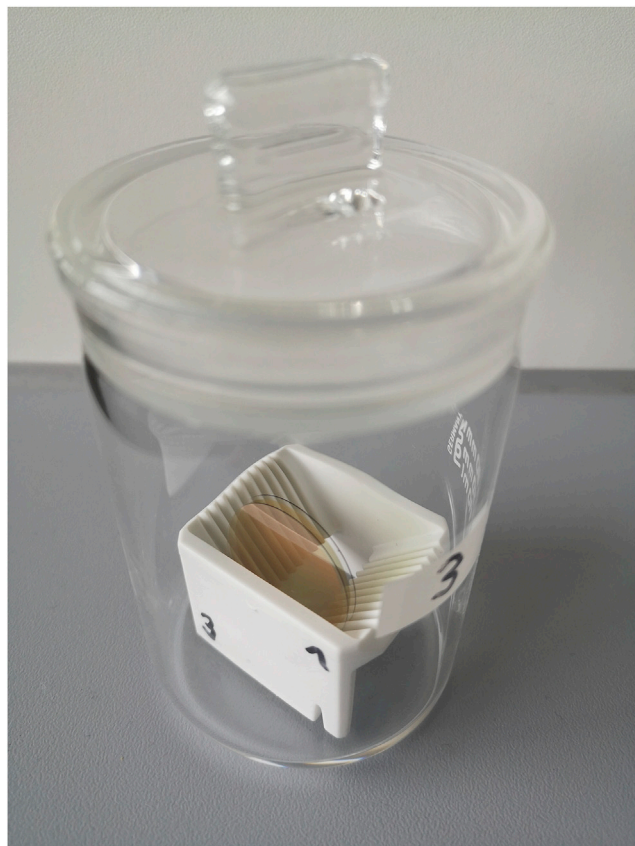


Figure 6. Coverslips Stored in a Rack in a Weighing Bottle with PDMS on Bottom and Filled with Argon

The PDMS prevents the holder from slipping. A storage for several weeks is possible, cause the coverslips are stored vertically and it is more difficult for agglomerates to adhere.

Note: It is recommended to store the cover slips in a weighing bottle filled with argon and sealed with parafilm. The cover slips are placed vertically in the Wash-N-Dry rack in the weighing bottle, so that no agglomerates can develop on the surface. A thin layer of PDMS has been poured into the weighing bottle to prevent the holder from slipping. (Figure 6) The rack can be used to assign numbers to the individual samples, so that it is possible to determine which sample will be used later.

Note: In order to achieve a reliable transfer of the micropatterned array from the mask to the PEG-SH layer, the UV exposure time was adjusted stepwise. Different exposure times were tested and the transferred oxidized patterns were examined in the AFM for their shape and size. The quality of transfer was determined by the comparison between the single patterns in the mask and in the PEG-SH layer. AFM images of the three different patterns in the mask (Figures 7A–7C) show the expected size of the whole patterns and a linewidth of approximately 5 μm (Figure 7G). After 5 min exposure the PEG-SH layer shows the successful transfer of the patterns (Figures 7D–7F). The exposure left an around 1 nm deep oxidized pattern in the layer (Figure 7H). The AFM images confirm the highly accurate, high-fidelity transfer of the patterns onto PEG-SH with virtually unchanged linewidth. In contrast, an oxidized pattern with a too long exposure time of 6 min (Figure 7I) is larger than the mask pattern and appears less sharp. This can also be seen in the cross-section (Figure 7J). In the AFM height image, agglomerates and additional layers can also be seen, because at this time of protocol development no washing step with rack for vertical storage of the cover glass was performed after the

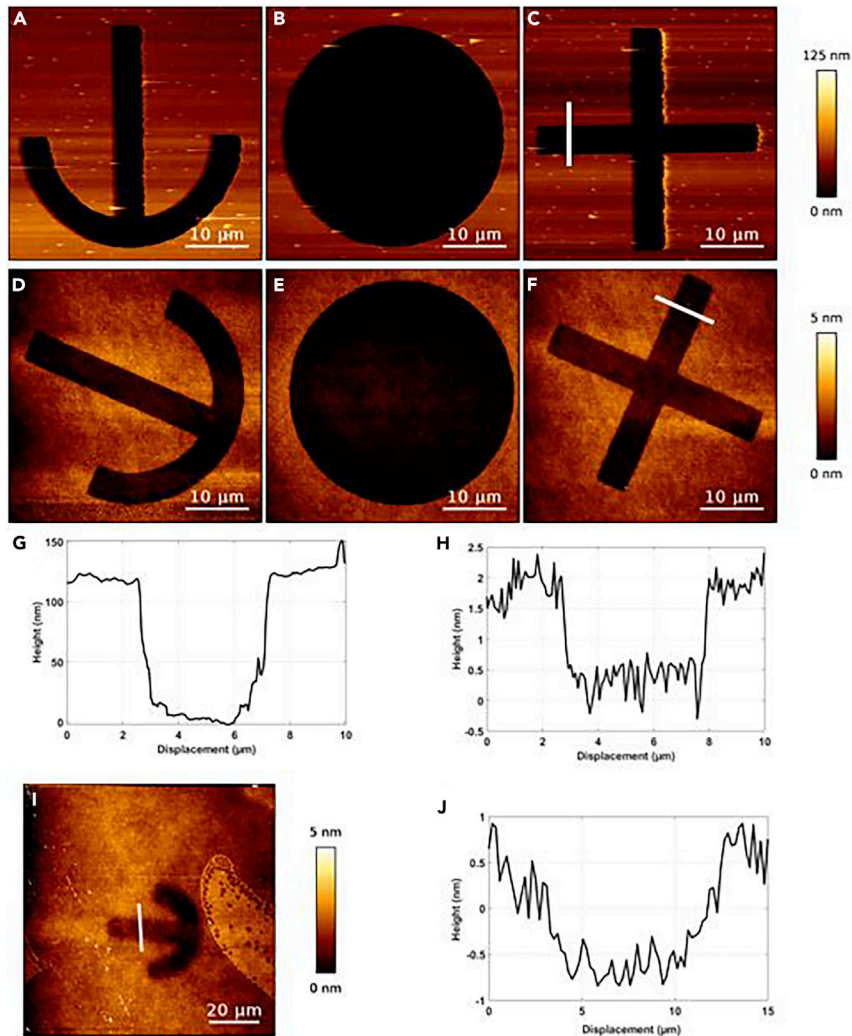


Figure 7. AFM Images of Micropatterns

(A–C) AFM tapping images showing the etched crossbow, circle, and cross pattern in the chromium mask.

(D–F) AFM tapping images showing the oxidized crossbow, circle, and cross pattern in the PEG layer.

(G and H) Cross-sections of cross patterns from the mask (G) and the oxidized PEG layer (H).

(I) AFM tapping image of an oxidized crossbow pattern with an exposure time of 6 min and without a washing step after PEG incubation.

(J) Cross-section of the oxidized crossbow pattern (K).

PEG incubation and before the UV exposure. The measurement with the AFM is not critical. It was used to develop the protocol and to demonstrate the fidelity of the pattern transfer.

Protein Coating

⌚ Timing: 2.5 h, day 3

The pegylated and exposed coverslips (Figure 1E) are incubated in a fibronectin solution (Figure 1F) to create the desired fibronectin pattern (Figure 1G). Cells can later attach to this extracellular matrix protein using the RGD motif.

13. Prepare a sterile solution of 1 mg/mL fibronectin in phosphate-buffered saline (PBS) (solution 2).

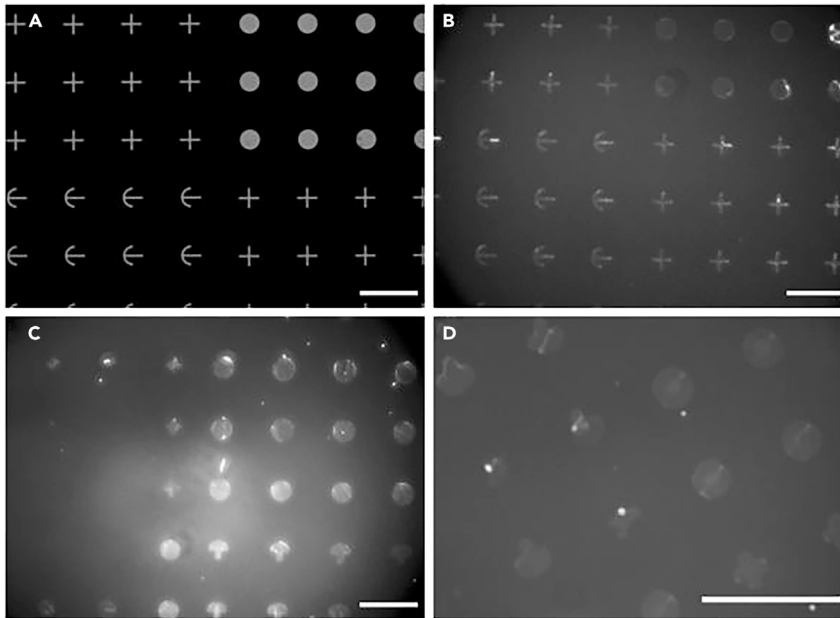


Figure 8. Microscopic Images of Micropatterns

- (A) Arrays of the cross, crossbow, and circle pattern in the chromium mask. Scale bar, 100 μm .
 (B) Fluorescence image of Cy3 stained fibronectin showing the transferred cross, crossbow, and circle pattern.
 (C) Example for bad pattern with exposure time of 8 min.
 (D) Example for bad pattern with an exposure time of 10 min. Scale bar, 100 μm .

- Mixing solution 2 with Cy3 as described in the Cy3 Amersham Mono-Reactive Dye Pack yields solution 3.

△ CRITICAL: The dye Cy3 is only used to check the pattern transfer of the photomask (Figure 8A) to the protein pattern of the PEG layer (Figure 8B) in the fluorescence microscope. If the cells are stained later, this Cy3 staining may be counterproductive and overlapping spectra must be avoided. The fibronectin solution without the dye is also diluted to a concentration of 10 $\mu\text{g}/\text{mL}$ like in step 16. Figures 8C and 8D shows inadequate fibronectin-Cy3 patterns. The patterns are arranged in an incomplete array. They are blurred, wider than desired, and additional fibronectin is visible on the surface.

Note: Protect the dye from light with aluminum foil and switch off the light or darken the room.

- Rinse the pegylated coverslips in ethanol in the biosafety cabinet to get them sterile.
- Dilute solution 3 to 10 $\mu\text{g}/\text{mL}$ in PBS (solution 4).
- Pipette 500 μL of solution 4 on top and incubate for 2 h.

Note: Protect the coverslip from light with aluminum foil and switch off the light or darken the room.

- Wash carefully three times with PBS after 2 h.

Cell Culture and Seeding

⌚ Timing: 24 h, day 3

The protein micropattern were incubated with 3T3 mice fibroblasts, which adhered to the pattern and change their shape due to the underlying pattern shape (Figure 1H).

Note: All values refer to a T25 cell culture flask.

19. Check the general health state of the 3T3 cell line with the microscope. Aspirate the old medium.
20. Wash gently with 5 mL PBS and aspirate it.
21. Detach your cells with 0.5 mL 0.25% trypsin and store them for 3 min in an incubator 5% CO₂ 37°C.
22. Tap gently against the cell culture flask and look under the microscope to see if the cells have detached.
23. Stop the reaction with 4.5 mL medium and suspend well to obtain evenly distributed single cells.
24. Determine the cell count.
25. Seed the cells in 3 mL medium per coverslip and petri dish. For 3T3, a total of 60 000 cells has proven to be a good value.
26. Keep the cells incubated for 8–10 h in an incubator 5% CO₂ 37°C.

Fluorescence Staining of Cells on Pattern

⌚ Timing: 24 h, day 4 + 5

To observe, analyze, and evaluate the effect of the shape on the cytoskeleton of the cell, the cells were fixed, and actin fibers were stained.

Note: All values refer to a T25 cell culture flask.

27. Look at the cells after 8 h in the microscope and judge whether you have single cells and notice changes in shape.

⚠ **CRITICAL:** If that worked, follow protocol. If you notice that you have single cells that have not adhered enough, incubate longer. [Figure 9](#) shows seeded 3T3 mice fibroblasts on fibronectin cross micropatterns adhere to the pattern which leads to separated single cells. They adjust their cell shape accordingly to the underlying pattern. If you have a confluent cell layer, start the protocol at the beginning with PEG incubation.

Safety Warning: 16% formaldehyde is toxic and harmful to health. Working in a fume cupboard is necessary.

28. Prepare a solution of 4% formaldehydes in PBS.

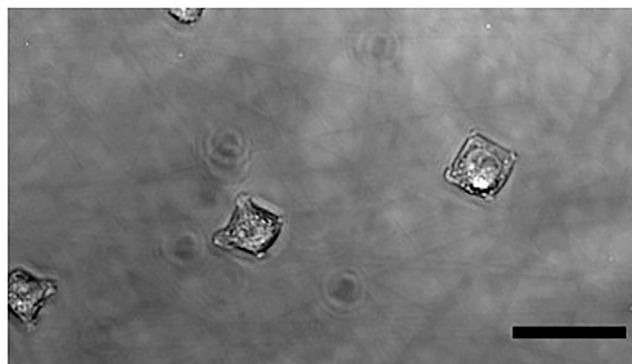


Figure 9. Optical Image of 3T3 Mice Fibroblasts Adhering to the Fibronectin Cross Micropattern
Scale bar, 50 μ m.

Note: Always prepare the formaldehyde solution fresh. It can polymerize with oxygen.

29. Remove the medium and carefully wash your cells with PBS.

Note: It is very important to wash carefully at this step and do not pipette directly onto the cover glass. The cells can adhere less to the protein patterns and are not as relaxed as on a normal protein layer. If not washed carefully enough, the cells may be detached.

30. Incubate the coverslips for 10 min with 2 mL 4% formaldehyde.

31. Wash 3 times with PBS.

32. Prepare a solution of 0.5% Triton X-100 in PBS.

Note: Triton X-100 must be pipetted very slowly and very carefully. It is highly viscous. It is best to use reverse pipetting. Do not cut off the tip of the pipette, this will change the volume. After pipetting in PBS, it is vortexed for a few minutes. A strong foaming will occur. Make sure that a homogeneous solution has formed in the falcon by holding it against the light. If not vortex further.

33. Incubate the cover glasses for permeabilization with 2 mL of the 0.5% Triton X-100 solution for 10 min. Do not be surprised about the foam.

34. Wash 3 times with PBS.

35. Incubate the coverslips for at least 30 min with 2 mL solution of 3% bovine serum albumin (BSA) in MilliQ water.

36. Remove the BSA. Pipette 2 mL of Alexa 568 phalloidin diluted in PBS (1:100) into the petri dish.

Note: Depending on the research interest, other cell components may be of interest, for which another dye can be used.

37. Incubate 10 h at 4°C. Then remove the dye and wash three times with PBS.

Note: The fluorophore must be protected against light.

38. Store the slide with the cells upwards in a petri dish in PBS for imaging. Additional mounting depends on the application.

EXPECTED OUTCOMES

Cells are highly sensitive to geometrical and mechanical factors and react to changes of those. The geometry of the cell influences many cellular processes such as cell growth, apoptosis, mitosis, cytoskeleton-reorganization, gene expression, nuclear deformation, epigenetic profile, chromatin contraction, and 3D chromosome organization (Chen et al., 1997; Jean et al., 2004; Vergani et al., 2004; Thery and Bornens, 2006; Thery et al., 2006; Le Beyec et al., 2007; Versaevel et al., 2012) and the differentiation of stem cells (Lee et al., 2013; Driscoll et al., 2015; Heo et al., 2015; Chaudhuri et al., 2016; Heo et al., 2016). Choosing a suitable geometry enables the control of cell proliferation and physiological conditions can be mimicked. (Singhvi et al., 1994; Thery, 2010) While restraining cell geometry on microscope slides is well established, a method for micropatterning on metal surfaces is lacking, but urgently needed. Different biophysical methods use gold surfaces, e.g., surface-enhanced Raman scattering (Kneipp et al., 2002), protein biochips or biochip interfaces (Pavlickova et al., 2003; Rusmini et al., 2007), applications including microelectronics in cell environments (Xing et al., 2006), microelectrode arrays (Nam et al., 2004), surface plasmon resonance (Couture et al., 2013), and metal induced energy transfer (MIET) (Enderlein, 1999; Chizhik et al., 2014). These studies can tremendously profit from rationally manipulating cell shape. This underlines the need for a protocol creating protein-coated micropatterns on metal surfaces for advanced cell studies.

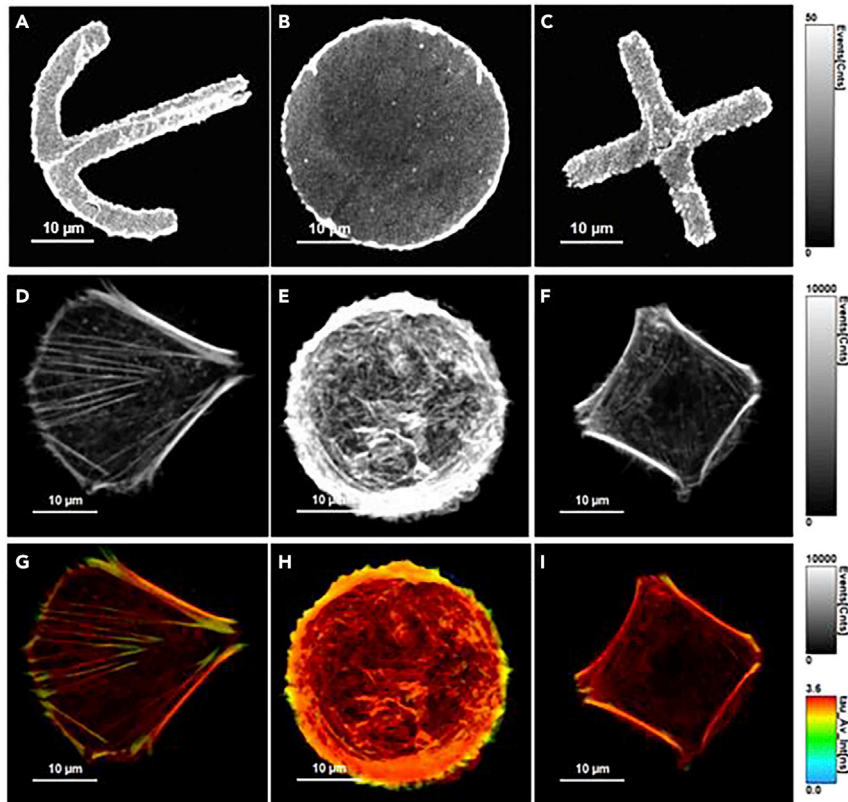


Figure 10. Confocal Fluorescence Images of Micropatterns and Cells

(A–C) Confocal fluorescence images of Cy3 stained fibronectin patterns of a crossbow, circle, and cross.
(D–F) Confocal fluorescence image of fixed 3T3 mice fibroblasts stained with Alexa 568 phalloidin on crossbow, circle, and cross micropatterns showing how the cells adapted their shape to the underlying pattern.
(G–I) Confocal fluorescence lifetime images of fixed 3T3 mice fibroblasts stained with Alexa 568 phalloidin showing the lifetime quenching of the underlying gold layer.

We expect the cells to adjust their shape to the pre-defined geometry (Théry and Bornens, 2006; Théry et al., 2006; Théry, 2010). Investigation of single patterns using confocal fluorescence imaging of Cy3 labeled fibronectin was conducted. Fibronectin adheres to all three shapes and the non-oxidized PEG layer repels fibronectin around the patterns (Figures 10A–10C). The gold layer thickness still allows fluorescent microscopy with appropriate photon yields. Depending on the pattern shape the cells adjust their cytoskeleton. On the circle no stress fibers are visible (Figure 10E). On the cross shapes stress fibers are formed between the tips spanning over the non-oxidized PEG layer (Figure 10F). Cells on the crossbow patterns form stress fibers from the front edge of the crossbow to the tip on the other end (Figure 10D). These results are consistent with the literature. Since the underlying surface is a metal, a quenching of the fluorescence lifetime is expected (Enderlein, 1999; Sönnichsen et al., 2005; Chizhik et al., 2014; Chizhik et al., 2017; Karedla et al., 2018). Therefore, we performed fluorescent lifetime imaging (Becker, 2012). The quenching effect of the underlying gold layer on the fluorophores is observed by the shortened lifetime of actin filaments in close proximity to the surface. (Figures 10G–10I).

In this protocol we presented a method for micropatterning on gold surfaces. It allows the control of cell shape and the investigation of isolated cells with pre-defined shape on an ultra-thin metal layer, allowing the usage of a variety of biophysical methods. As surfactant we used a thiol-functionalized polyethylene glycol (PEG-SH). The thiol head group of the PEG-SH binds in an oxidative addition with a strong metal thiolate bond to the gold, followed by a reductive elimination of the hydrogen

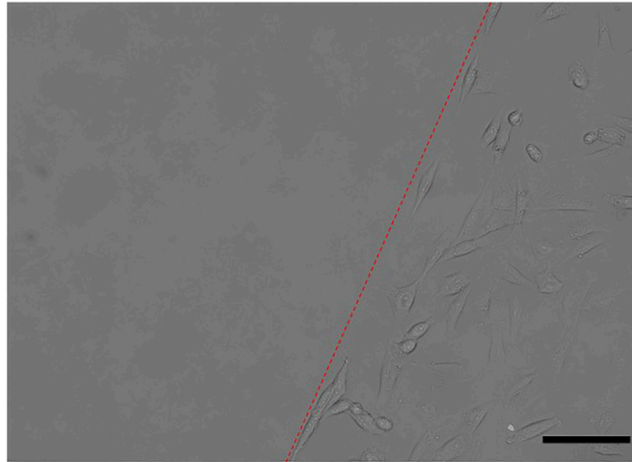


Figure 11. Microscopic Image of 3T3 Mice Fibroblasts Sitting on the Edge of the Pegylated Area Showing the Fibronectin Repellent Property of the PEG Layer

The red dotted line indicates the transition (Scale bar, 100 μm).

(Nuzzo et al, 1986; Ulman, 1991). PEG is protein repellent, but perfectly biocompatible. (Jeon et al., 1991; Harris, 1992) Using a photomask and deep UV radiation allows tailored oxidation of pre-defined areas of the SAM, which then can be incubated with a variety of proteins, for example extracellular matrix proteins like fibronectin. The amino groups of the proteins react with the carboxy group of the oxidized pattern and form a stable peptide bond. In case of fibronectin, cells can now bind via the RGD sequence to the patterned surface. (Théry, 2010) Alternating the matrix protein allows to investigate biochemical cues in addition to shape. The conductive nm-thin metal base additionally allows the cells to be electrically addressed and manipulated as well as to be examined spectroscopically and microscopically at the same time, properties that glass as a support layer do not have. It is an elegant method with only non-toxic, biocompatible, and cheap chemicals and a small number of easy steps. No photo-sensitive ligands or photo-activatable substrates are needed. Give the ease of the method and its high fidelity, we are confident that additional targeted studies with this method will allow novel insight into the mechanisms of cell function, proliferation, apoptosis, and differentiation.

LIMITATIONS

During the production of the micropatterns it has been noticed that the quality of the patterns varies during the summer and winter period due to the humidity. This problem has been described in other self-assembled monolayer studies, also with PEG. (Gidi et al., 2018)

The protocol is also feasible with other cell lines. However, it should be an adherent cell line. Due to the different size of cells, a different mask design with a different diameter must be used.

The exposure time for the UV lamp, when using the same intensity, does not need to be adjusted. Fluorescent labeling of proteins provides sufficient information about the transfer if the same power of the UV lamp as in the protocol has been used. Therefore, the patterns do not necessarily have to be examined with the AFM. The measurement with the AFM was used for protocol development and validation. But the AFM is helpful if the UV lamp has a different output and the exposure time has to be adjusted.

The PEG is protein repellent, but not toxic for cells and therefore perfectly biocompatible. Figure 11 shows the difference between PEG and non-PEG at an edge of the cover glass. The difference to microcontact printing, which has already been done on gold and not uses PEG on the surface, is

that you can still better control where cells grow, cause with our method a protein-repellent layer is created. Further, once a mask is written, the method is easier and allows for smaller feature sizes. You can separate cells and get single cells that adapt to the patterns. The protein-repellent property of PEG is abolished by the oxidation and allows fibronectin to adhere to the pattern.

When working with biological systems, even this micropatterning method will not achieve a 100% occupation of cells on patterns that adhere and deform. Nevertheless, enough cells can be found on patterns that adapt their shape to be used for various biophysical measurements.

TROUBLESHOOTING

Problem

There are no patterns visible under the fluorescence microscope.

Potential Solution

There are many possibilities why no protein patterns are visible.

There is no PEG on the gold surface if a closed protein layer is observed in the epifluorescence image.

In contrast, if there is no protein on the surface, it is a closed PEG layer, but the exposure is not appropriate. Go through all steps of the UV exposure again. It is important to note that the gold side and not the glass side was used for the incubation, because thiol groups can only bind to gold. The UV light must pass through the mask to transfer the pattern into the underlying pegylated gold cover glasses. This is only possible if the mask material is quartz. Soda lime cannot be used for deep UV radiation.

Problem

In the fluorescence microscope the fibronectin patterns look smeared. Also, they may not have a distinct shape when analyzing them with the AFM.

Potential Solution

The cause of inaccurate, smeared protein patterns is usually due to exposure (step UV exposure). The first thing to check is whether the same UV lamp intensity is used as in the protocol. A lower or higher intensity affects the exposure time. If the intensity is the same, it is necessary to keep an exact exposure time of 5 min and a distance of 0.5 cm from the quartz mask to the UV lamp ([Figure 5](#)).

It is important to ensure that no air bubbles are formed when fixing the cover slip on the mask. If air bubbles are formed, the cover glass will not be close enough to the mask and the exposure will be affected. This problem can be avoided by applying pressure using the pipette tip.

Problem

Cells do not adapt their shape properly.

Potential Solution

There are several possibilities why the cells do not adapt their shape to the protein patterns. To exclude that it is due to UV exposure, it is advisable to perform the protein coating step with Cy3. With this the protein patterns can be examined under the fluorescence microscope. It should also be noted if all cleaning steps have been carried out, because only then can a protein-repellent monolayer be formed.

There is also the possibility that the cell line is not in good condition. For the micropatterning procedure it is necessary that the cells are not stressed, because they do not prefer isolation and prefer to form cell-cell contacts.

Problem

There are no single, pattern shape-adapting cells on the gold coverslips but a confluent layer with cells that look typical for the cell line.

Potential Solution

A confluent layer of cells on an otherwise protein-repellent layer indicates that the PEGylation did not succeed. It is best to restart the protocol at the PEG incubation step. First check the critical steps like storage in argon and check for influencing factors like humidity. It is also important that the PEG solution for the formation of a protein repellent monolayer has the necessary concentration and that the incubation time of 48 h was kept.

In order to avoid wasting time during problem solving, it is best to omit the UV exposure and instead verify how the cells behave with the fibronectin on the cover glass.

Another cause for insufficient PEG coating can be that a closed gold layer has not formed during gold evaporation and thiol groups cannot bind everywhere. Review the gold coating step and make sure that the cover glasses are thoroughly cleaned before evaporation.

Problem

Before fixation there were adherent, shape-adapting cells on the cover glass. After fixation, there are less cells on the cover glass.

Potential Solution

Cells have much less surface area on fibronectin patterns to adhere to than on a closed fibronectin layer. Therefore, the fixation has to be done very carefully. Pipetting should therefore always be performed at the edge of the petri dish at the lowest speed of the electric pipetting assist. Following fixation, the petri dish with PBS is carefully swiveled.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Kay-Eberhard Gottschalk (kay.gottschalk@uni-ulm.de).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

Data available on request from the authors.

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AUTHOR CONTRIBUTIONS

C.G. performed the research, evaluated the data, and wrote the paper, P.K. evaluated the data and wrote the paper, F.P. evaluated the data, K.E.G. designed the research, evaluated the data, and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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