Micro- and nanopatterning by lithographically controlled wetting

Massimiliano Cavallini[1,2], Denis Gentili[1], Pierpaolo Greco[2], Francesco Valle[1] & Fabio Biscarini[1,2]

[1]Consiglio Nazionale delle Ricerche, Istituto per lo Studio dei Materiali Nanostrutturati, Bologna, Italy. [2]Scriba Nanotecnologie, Bologna, Italy. Correspondence should be addressed to M.C. (m.cavallini@bo.ismn.cnr.it).

This protocol describes how to perform lithographically controlled wetting (LCW). LCW enables large-area patterning of microstructures and nanostructures of soluble materials, either organic or inorganic, including biological compounds in buffer solutions or compounds for cell guidance. LCW exploits the capillary forces of menisci established under the protrusions of a stamp placed in contact with a liquid film. In the space confined by each meniscus, the self-organization of the deposited solute yields highly ordered structures that replicate the motif of the stamp protrusions. The method does not require any particular infrastructure and can be accomplished by using simple tools such as compact discs or microscopy grids. Compared with other printing methods, LCW is universal for soluble materials, as it does not require chemical binding or other specific interactions between the solute and the surface. A process cycle takes from 2 to 36 h to be completed, depending on the choice of materials.

INTRODUCTION

Patterning of biological and functional materials is a key step in exploiting their functionality in new applications. LCW is a simple, fast and sustainable wet-patterning process[1] that exploits the self-organization of soluble materials, permitting spatial control provided by the features of a stamp. A schematic overview of the LCW process is shown in **Figure 1**. When a stamp is placed in contact with a liquid thin film spread on a substrate (**Fig. 1a**,**d**), an instability of the fluid layer develops, in which capillary forces pin the solution to the stamp protrusions, giving rise to an array of menisci (**Fig. 1b**,**e**). As the solvent evaporates, the solution forms a subfemtoliter fluid cell under each stamp protrusion (assuming a stamp feature ranging between 100 and 500 nm and a stamp-substrate distance between 50 and 200 nm, sizes commonly used in LCW). When the solution reaches supersaturation, the solute precipitates onto the substrate within the menisci, giving rise to a deposit replicating the protrusion of the stamp (**Fig. 1b**,**c**,**e**,**f**). Intertwined thermodynamic and kinetic parameters such as the nature of the solvent, the capillary flow and evaporation rate, the solute intermolecular interactions and the interactions with the substrate determine the morphology and structure of the printed features[2]. The possible occurrence of different phenomena such as dewetting, self-assembly or crystallization, each one accompanied by its own characteristic length scales, allows one to obtain different patterns[2]; therefore, the motif morphology cannot be generalized, as it will depend on the system (materials of the stamp/substrate, solvents, printable compounds) and it can be substantially changed by a simple change in one parameter of the system.

In the standard configuration, a prefabricated spacer can be used to keep the desired stamp-substrate distance (**Fig. 1a**–**c**). Alternatively, LCW can be carried out using a floating stamp, in which case the system is self-regulating, as the solute itself determines the stamp-surface distance when it precipitates under the stamp protrusion (**Fig. 1d**–**f**). As a liquid is printed, it is not necessary to apply extra pressure. The solvent can evaporate through the stamp, using a stamp made of permeable polymers or a grid, or from the sides of the stamp in stamps that are impermeable to the solvent. LCW also provides the ability to decrease the size of the printed features with respect to the original size of the stamp features[1], allowing printing of nanostructures using micrometric molds. By using a very diluted solution (**Fig. 1c**,**f**) or a calibrated spacer to suspend the stamp, supersaturation is not achieved within the fluid columns. As the solvent evaporates, the residual solution remains pinned to the edges, forming an inverted meniscus under the protrusion. The consequent mass transport to the edge via capillary flow[3] yields a replica of the protrusion edges and not that of the protrusion. In this regime, it is possible to print double features whose size is comparable to the curvature radius of the edges rather than the size of the flat part of the protrusion. Therefore, this regime is suitable to downscale the size of printed features relative to the original size of the stamp features. One order of magnitude improvement was obtained using small molecules[1,4]. LCW can also be used in an additive manner, using a second, orthogonal solvent (i.e., using a solvent that does not dissolve the first material deposited for the deposition of the second pattern)[5]. Furthermore, LCW can be used with electroactive surfaces to spatially control electrochemical reactions[6–8] and to etch reactive surfaces[9].

Applications and limitations

In principle, LCW can be applied to any soluble compound or material. It can be performed on the benchtop without special instrumentation and does not require a clean room. Compared with other lithographic methods (see below), LCW does not rely on specific interactions between the molecules and the surface; therefore, it has a wide range of potential applications, as demonstrated by the large variety of soluble materials reported in the literature[1,4,10–14] to which it could be applied. Although LCW was developed originally for patterning organic semiconductors[5,15,16], its application was extended to many functional materials such as inorganic salts[17], coordination compounds[12,18], coordination polymers[19], molecular magnets[4,20], nanoclusters[13], electroluminescent compounds[11], sensing materials for pH[21] and others[22,23].

Recently, LCW was also applied to biomolecules[10], and in this domain it can have an important role in the fabrication of biomaterial interfaces in biologically inspired applications and in medical devices. Biomolecules in aqueous solutions are often unsuited for conventional fabrication techniques in which extreme environmental

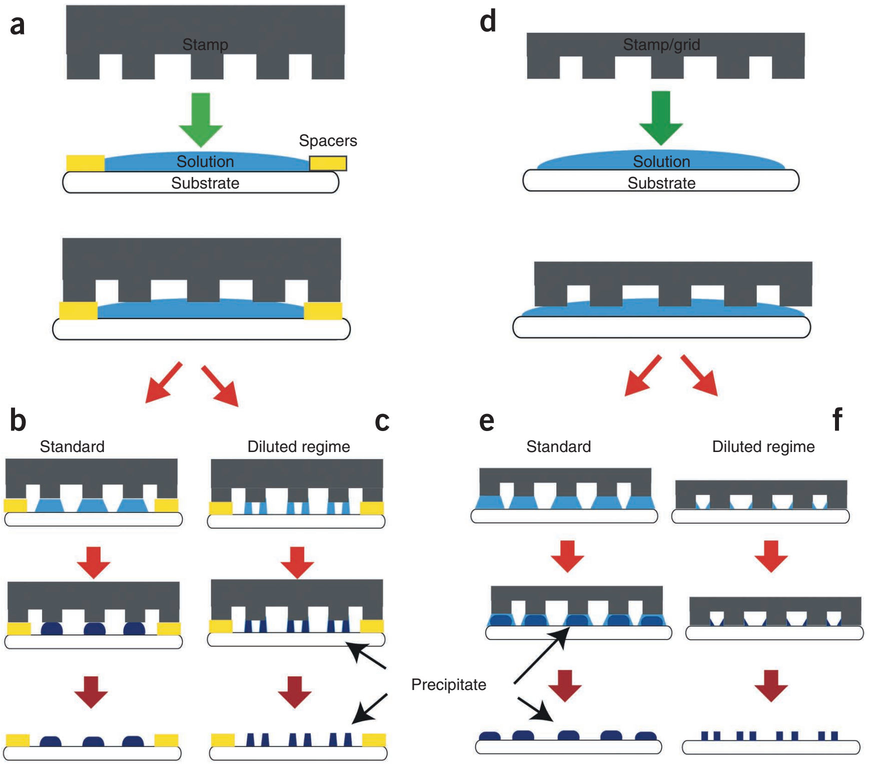

Figure 1 | Scheme of lithographically controlled wetting. (**a**–**c**) Scheme of printing using prefabricated spacers (**a**) in the standard (**b**) and diluted (**c**) regime. (**d**–**f**) Scheme of printing using a floating stamp (**d**) in the standard (**e**) or diluted (**f**) regime. Whereas in standard conditions features with size comparable to the stamp protrusions are printed (**b**,**e**), in the diluted regime a split of the printed features with reduced size takes place (**c**,**f**).

conditions (vacuum, UV light, high temperature and solvents) are required or where specific interactions between molecules and substrate are needed. LCW has been already applied in some experiments for fabricating patterns aimed to guide the adhesion[14], migration and proliferation of cells[24]. Proteins such as laminin have been patterned by LCW onto technologically relevant surfaces with a one-step procedure[14], achieving the fabrication of adhesion-promoting areas embedded into a strongly antifouling material, such as Teflon-AF, widely used in biomedical devices. The relevance of LCW in biological applications stems from its potential impact in engineering the materials to be used as scaffolds in regenerative medicine. Because a chemical modification of the surface could hinder biocompatibility and compromise the use of materials already certified for medical use, a simple LCW patterning with an approved protein preserves the consistency with good manufacturing practice conditions. Similarly, proteins or nucleic acids can be patterned onto sensing surfaces in electronic and optical devices, as well as in lab-on-a-chip applications.

Major limitations of LCW are the solubility of materials that can be used in LCW, as it works only with soluble compounds, and the difficulty of applying LCW to nonplanar surfaces.

Currently available alternative methods

Micro- and nanopatterning of soluble materials cannot be directly achieved by conventional lithographic methods such as photolithography or electron beam lithography. The most commonly used methods to pattern soluble material are ink-jet printing[25] and soft lithography (e.g., microcontact printing[26], micromolding in capillaries (MIMICs)[27] and microtransfer molding (μTM)[26,28]).

In ink-jet printing, a solution droplet is propelled onto the substrate, and then the solvent evaporates leaving the solute on the surface. Although it is a versatile method, it is serial (i.e., droplets are deposited one by one), the ink and solution must be carefully formulated for matching stringent viscosity requirements and aggressive solvents cannot be used.

Microcontact printing is the most famous soft-lithographic method that uses the relief patterns on an elastomeric stamp, typically polydimethylsiloxane (PDMS) to form patterns of self-assembled monolayers (SAMs) of a printable compound (the ink) on the surface of a substrate through conformal contact. Its potential applications range from microelectronics and surface chemistry to cell biology. It requires printable materials chemically grafted to the substrate or able to form SAMs.

In MIMICs, an elastomeric stamp whose motif consists of grooves defining a circuit for liquid flow is sealed to the surface of a substrate. The grooves between the surface-contacted protrusions form microchannels. When a drop of solution is poured in the open end of the stamp, the liquid spontaneously infills the microchannels driven by the Laplace pressure. After the complete evaporation of the solvent, the stamp is gently removed, leaving the nanostructures on the surface. MIMICs can be applied only to fabricate continuous structures. Furthermore, the surface tensions of the substrate, the solution and the stamps must be tuned to allow the infilling. As the metallized stamps cannot be used, MIMICs is restricted to the use of nonaggressive solvents.

In μTM, an elastomeric stamp is filled with a prepolymer or a solution, placed on a substrate and cured. When the stamp is removed, the patterned structure is left on the substrate. This technique can also be used for nonplanar surfaces; furthermore, it can be used to deposit isolated microstructures. As in the case of MIMICs, the major limitation concerns the use of solvents, as μTM cannot be used with aggressive solvents.

LCW is suitable for large-area nanopatterning, requires minimum equipment and can be used with any kind of solvent, from buffer solutions to aggressive solvents, such as chlorinated ones. As in other unconventional fabrication techniques[26,29–32], LCW requires the preparation of some tools (typically the stamp) for printing (see Experimental design for further details). This is similar to other techniques such as soft lithography[32], benchtop nanofabrication[29] or fabrication of patterned membrane[30]. Nevertheless, this step is not strictly required for LCW and can be overcome using some tools that are commercially available and that cover most of the experimental requirements. Low-cost tools with submicrometric features can be obtained from CDs, DVDs and grids for transmission electron microscopy (TEM). A specific protocol for tools fabrication is reported in ref. 33.

Experimental design

Stamp design and fabrication. The choice of the stamp material and design of the motifs depend both on the envisioned experiment and on the chemical compatibility between the materials and the solvent used. The design of the stamp protrusions is strictly linked to the desired patterns of printable material. Under wetting conditions (in which the solution spreads onto a surface and forms a continuous and stable film), the stamp protrusions are faithfully reproduced, whereas in a dewetting regime (i.e., the solution spread onto a surface is unstable, resulting in the film rupturing and forming a pattern of droplets)[34] an ordered pattern of droplets can also be printed using stamps with motifs made of continuous structures (e.g., lines). A wetting regime is obtained using substrates with a hydrophobicity that matches the hydrophobicity of the printable material, whereas a dewetting regime is obtained by printing hydrophobic material on a hydrophilic substrate (or vice versa). Two examples are provided in ANTICIPATED RESULTS.

The choice of the stamp material is related to the solvent used to solubilize the printable material. Polymeric (e.g., PDMS) stamps are used with nonaggressive solvents, such as water and alcohols, and are therefore widely used with biological materials[35]. Metallic or metallized (e.g., gold or gold-coated polymers) and silicon-based stamps are used with aggressive solvents that could swell or damage elastomeric stamps (e.g., chlorinated solvents). The design and fabrication of the master and relative soft (e.g., PDMS) stamps follow well-established procedures described in detail in other protocols[29,30,32,33]. Before use, it is important to note that the stamp must be cleaned and in some cases a functionalization may help the process, as LCW works best when solvent and stamp have comparable surface energies.

Although conventional stamps have no limits in thickness (**Fig. 1a–c**), to use floating stamps (**Fig. 1d–f**) the stamp thickness must be < 25 µm for metallic stamps and <250 µm for polymeric stamps.

Stamp and substrate cleaning (Step 1). Removing organic residues from substrates and appropriate cleaning are key factors in obtaining a high-quality pattern. The specific cleaning procedure is related to the nature of the stamp/substrate. In the absence of any specific requirements, a simple ultrasonic bath cleaning is sufficient (the bath liquid has to be compatible with the materials; for instance, PDMS is well cleaned in a pure ethanol bath). Substrates and stamps made of silicon, SiO_2, glass and gold can be efficiently cleaned by the so-called Piranha solution, which is widely used in microelectronics processes. In the case of silicon, glass and SiO_2, Piranha treatment also activates the surface forming hydroxyl groups that favor a successive chemical functionalization. Plasma treatment is another common method for cleaning and changing the surface tension of materials, in particular polymeric ones (e.g., PDMS). When dealing with polymeric stamps, oxygen plasma removes organic contaminants and activates the surface by exposing different terminal groups on the surface, thus achieving the fine-tuning of surface properties. Some of the surfaces commonly used by high-resolution microscopy, such as Mica (e.g., muscovite) and highly oriented pyrolytic graphite (HOPG), are extremely flat because of their particular preparation procedure based on cleavage. When these materials are used as substrates, their preparation consists of the mechanical removal of a few layers from the surface by cleavage (see Step 1D,E).

Stamp and substrate chemical functionalization (Step 2). To control the surface energy and the wetting properties of stamps and substrates (i.e., the hydrophobicity/hydrophobicity), they can be functionalized by SAMs. Noble metal surfaces (e.g., Ag, Cu and Au) can be functionalized by SAMs of alkanethiols. The molecule for the SAM must be chosen according to the required properties: use fluorinated alkanethiols to yield highly hydrophobic surfaces (contact angle ~110°); use alkanethiols terminated by methyl groups for hydrophobic surfaces (contact angle ~100°); use alkanethiols terminated by amino groups for a mild hydrophilic surface (contact angle ~60°), which can be tuned also with pH; use alkanethiols terminated by ethylene glycol groups if you need mild hydrophilic surfaces (contact angle ~40°); and use alkanethiols terminated by carboxylic acid group if you need hydrophilic surfaces (contact angle ~15°; in addition, here the pH can be used for fine-tuning). It is important to note that alkanethiols with charged terminal groups or short alkyl chains (six or less carbons) form incomplete coverages or defective SAMs.

Glass, silicon and SiO_2 substrates, or other surfaces terminated with hydroxyl groups, can be functionalized by silanes. The molecule for the SAM must be chosen according to the required properties: trichloro(octadecyl)silane (OTS) is useful to yield highly hydrophobic surfaces (contact angle ~105°); alkoxysilanes, such as amino-terminated ((3-aminopropyl)triethoxysilane, APTES), are useful to yield mild hydrophilic surfaces (contact angle ~60°).

Chemical functionalization of complex substrates such as SiO_2 surfaces with gold electrodes, which are often present in devices as interdigitated electrodes of a field effect transistor structure (**Fig. 2**), might need a combined functionalization (e.g., the SiO_2 is functionalized by OTS, which forms a SAM on the substrate but

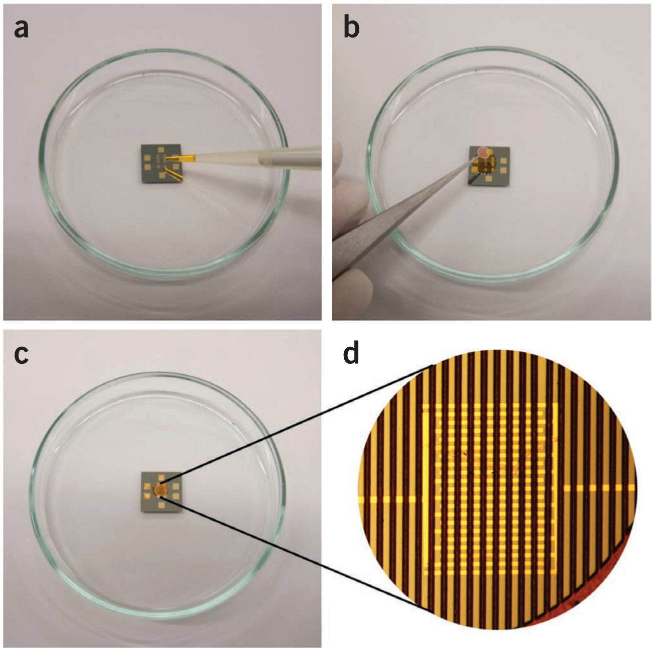

Figure 2 | Principal steps of lithographically controlled wetting. (**a**) Pipetting the solution of fluorescently marked BSA onto the substrate (here the interdigitated electrodes of a field effect transistor working as spacers). (**b**) Placing the stamp (here a grid for TEM with parallel bars 20 µm wide) on the spread solution. (**c**) Waiting for the solvent evaporation. (**d**) Detail of the grid placed on top of interdigitated electrodes after solvent evaporation.

not on the Au electrodes; the electrodes are then functionalized by alkanethiols that form a SAM on Au but not on the substrate).

Spacer fabrication (Steps 3–5). Spacers can be used for achieving a fine-tuning of the LCW patterning by fixing the distance between the stamp protrusions and the substrate. Different materials, such as noble metals or inorganic salts, can be used. To prevent sagging of soft or flexible stamps, the ratio of the distance between two adjacent spacers to the height of the spacers should be $<$5:1 (ref. 2).

LCW printing (Steps 6–10). The main steps involved in LCW printing are spreading the solution onto the surface and tethering it between the substrate and the stamp. These steps involve an accurate choice of the solvent because this will have a crucial role in the wetting/dewetting of the surfaces and may introduce important issues concerning the compatibility with the stamp. As a general guideline, a solvent whose hydrophobicity matches both the hydrophobicity of the substrate and the stamp should be used (e.g., chloroform or alkanes can be used for hydrophobic stamp/substrates, and water or alcohols can be used for hydrophilic stamp/substrates). The LCW-fabricated patterns depend on the solute concentration (C). For example, for the standard regime, a concentration range of $0.1 < C < 10$ mg ml^{-1} should be used (we suggest a starting value of 1 mg ml^{-1}); if you are using the diluted regime, a concentration range of $0.01 < C < 0.1$ mg ml^{-1} should be used (we suggest a starting value of 0.05 mg ml^{-1}).

Some additional issues must be faced when dealing with biological molecules. Proteins or nucleic acids are usually dissolved in buffer with different salt concentrations. It is therefore very important to keep the pattern from being composed mainly of the salt. Moreover, the buffer solution must be chosen so as to prevent unfolding/misfolding and aggregation that may take place upon dehydration because of the locally high concentration achieved within the menisci. In the case of proteins that may undergo unfolding/misfolding and aggregation upon dehydration, a protective agent known to prevent this effect (for instance, osmolytes such as betaine) should be added to the solution. The latter step involves an accurate screening of literature data. **Figure 2** shows the major printing steps using an aqueous solution of fluorescently marked BSA to fabricate micrometric structures on a test pattern of a field effect transistor; here the interdigitated gold electrodes of the devices (150 nm thick) act as spacers. A copper grid for TEM with parallel bars of 30 μm in width is used as the stamp.

MATERIALS

REAGENTS

- Stamp (The specific protocol for stamp fabrication is reported in ref. 33.)
- Substrate (Silicon, SiO$_2$, glass, gold, Mica and HOPG; Ted Pella)
- Ethanol (Sigma-Aldrich, cat. no. 459836) **! CAUTION** It is a highly flammable solution; handle it in a fume hood.
- Toluene (Sigma-Aldrich, cat. no. 244511) **▲ CRITICAL** Ensure that toluene does not contain residue, such as heptachlor epoxide, which can inhibit the process **! CAUTION** Toluene is flammable, toxic and an irritant. Avoid inhalation, ingestion and skin contact. Use gloves, safety glasses and a fume hood.
- Hydrogen peroxide (30 wt%); Sigma-Aldrich, cat. no. 216763) **! CAUTION** Hydrogen peroxide is corrosive and a strong oxidizing agent. Handle it in an acid fume hood and wear proper personal protective equipment (face shield, acid gloves and apron).
- Sulfuric acid (Sigma-Aldrich, cat. no. 320501) **! CAUTION** It is highly corrosive; wear a lab coat, goggles and gloves.
- APTES (Sigma-Aldrich, cat. no. A3648) **! CAUTION** APTES is harmful to skin. Rubber gloves and safety glasses should be used. Store in a light-resistant bottle whose cap is sealed with Parafilm.
- 11-Mercaptoundecanoic acid (MUA; Sigma-Aldrich, cat. no. 450561)
- 11-Aminoundecane-1-thiol hydrochloride (ProChimia, cat. no. FT 004-m11)
- 1-Undecanethiol (UDT; Sigma-Aldrich, cat. no. 510467)
- Trichloro(octadecyl)silane (OTS; Sigma-Aldrich, cat. no. 104817) **! CAUTION** OTS is harmful to skin and eyes. Rubber gloves and safety glasses should be used.
- BSA (Calbiochem)
- Potassium chloride (KCl, molecular biology grade; Sigma-Aldrich, cat. no. P9541)
- Sodium phosphate dibasic (Na$_2$HPO$_4$, molecular biology grade; Sigma-Aldrich, cat. no. S3264)
- Sodium chloride (NaCl, molecular biology grade; Sigma-Aldrich, cat. no. S3041)
- Potassium phosphate monobasic (KH$_2$PO$_4$, molecular biology grade; Sigma-Aldrich, cat. no. P9791)
- Hydrochloric acid (HCl, molecular biology grade; Sigma-Aldrich, cat. no. P1758)
- Sodium hydroxide (NaOH; Sigma-Aldrich, cat. no. 71690)
- Chloroform (Sigma-Aldrich, cat. no. 372978)
- Ultrapurified water

EQUIPMENT

- Hot plates (for surface cleaning, e.g., MR Hei-Standard, Heidolph)
- Nitrogen gas line (for drying stamps and substrates)
- Ultrasonic cleaner (for surface cleaning, e.g., Elmasonic S30H)
- Oxygen and argon plasma cleaner (for surface modification and cleaning, e.g., TePla Technics Plasma 100-E plasma system)
- Optical microscope (for characterizing patterns on masters and stamps at the microscale, e.g., Nikon Eclipse 80i)
- Scanning electron microscope (SEM, for characterizing patterns on masters and stamps at both microscale and nanoscale, e.g., SEM-FEG Hitachi S4000) **▲ CRITICAL** If the operator is not familiar with SEM, the help of a technician is required.
- Atomic force microscope (AFM, for characterizing patterns on masters and stamps at both microscale and nanoscale, e.g., NT-MDT NTEGRA) **▲ CRITICAL** If the operator is not familiar with AFM, the help of a technician is required.
- Contact angle meter (for characterizing substrate, e.g., DGD-DX model, DIGIDROP) **▲ CRITICAL** If the operator is not familiar with contact angle measurements, the help of a technician is required.
- TEM grids (several types, SPI supplies)
- Masks (several types, SPI supplies)
- Parafilm
- Pipette, 20–100 μl and tips (e.g., Gilson P100 Pipetman Classic)
- Vacuum sublimation system

REAGENT SETUP

Piranha solution Piranha solution contains a 3:1 mixture of concentrated H_2SO_4 and H_2O_2 (30%, vol/vol). Add very slowly and mix with a glass rod one volume of H_2O_2 to three volumes of H_2SO_4 in a clean beaker whose volume is at least 10 times the volume of the final solution **▲ CRITICAL** Piranha solution must be freshly prepared and cooled to room temperature (23 °C) before use **! CAUTION** Pay careful attention because there are exothermic processes involved when you add the hydrogen peroxide to the sulfuric acid. Piranha solution reacts violently with organic compounds, is very dangerous to the skin and should be handled with care. It is important to work in a fume hood and wear personal protective clothing (e.g., nitrile or latex gloves, lab coat, safety glasses) when handling solutions; keep solutions away from organic chemicals.

PBS buffer, 1× Dissolve 8 g of NaCl, 0.2 g of KCl, 1.78 g of Na_2HPO_4 and 0.27 g of KH_2PO_4 in distilled water and adjust the volume to 1 liter. Adjust the pH with HCl or NaOH to 7.4. This can be stored for 1 year at room temperature.

Alkanethiol solution Prepare an ~2 mM alkanethiol (for example, UDT) solution in high-purity ethanol. ▲ CRITICAL Keep the solution in a clean glass or polypropylene container. The solution can be acidified (pH ~2, by adding HCl) or basified (pH ~12, adding NaOH) for alkanethiols terminated in acidic or basic groups (for example, MUA or 11-aminoundecane-1-thiol hydrochloride), respectively, to improve the quality of the SAMs.

Alkoxysilane solution Prepare an ~1 mM alkoxysilane (for example, APTES) solution in high-purity ethanol. ▲ CRITICAL Work under nitrogen flux in order to prevent chemical reaction with water from ambient humidity.

OTS solution Prepare an ~1 mM OTS solution in anhydrous toluene. ▲ CRITICAL All glassware should be cleaned and exposed to argon plasma.

PROCEDURE

Stamp and substrate cleaning ● TIMING up to 2 h

1| Use option A (sonication) for any kind of material, option B (plasma treatment) to clean and change the surface energy of materials, option C (Piranha solution) to clean and change the surface energy of nonorganic materials, option D (cleavage) for mica or option E (cleavage) for HOPG (see Experimental design for further details).

(A) Sonication ● TIMING 15 min

(i) Immerse the stamp/substrate in a beaker with the chosen solvent (see Experimental design for further details).
(ii) Put the beaker in an ultrasonic cleaning bath for 10 min.
(iii) Remove the stamp/substrate and dry it in a stream of nitrogen for 60 s.

(B) Plasma ● TIMING 30–60 min

(i) Take the stamp/substrate and place on a glass slide with the motifs exposed on the top.
▲ CRITICAL STEP It is good practice to put a sacrificial piece of the same material together with the substrate to be used for the surface hydrophobicity characterization (in Step 1B(vi)).
(ii) Place the slide into the plasma chamber and start to apply a vacuum until a pressure of ~0.2 Torr is achieved.
▲ CRITICAL STEP A nonadhesive layer must coat the supporting glass slide; otherwise, an irreversible binding of the polymer to the glass could take place.
(iii) Let the oxygen gas enter into the chamber until a pressure of ~0.4 Torr is achieved.
(iv) Select the parameters of the discharge. Usually, for both PDMS and rigid silicon stamps a value between 50 and 100 W, and a time of 1–10 min is used, depending on the final result required. A higher power and a longer time will make the PDMS more hydrophilic (5 min at 50 W produces a contact angle with water of ~10°).
(v) Close the oxygen valve, vent the chamber and remove the sample.
▲ CRITICAL STEP Upon plasma treatment, the surface of the elastomers is highly charged, and thus the elastomers become prone to contamination by any particle present in the local atmosphere; therefore, this step should be performed in a clean room or under a laminar flow hood.
(vi) If required, measure the hydrophobicity by the contact angle (the surface is hydrophilic if the water contact angle is smaller than 90° and hydrophobic if larger than 90°) on an area that is not important for the forthcoming LCW or on a sacrificial piece of material.
? TROUBLESHOOTING
■ PAUSE POINT If a contact angle larger than the one produced by the plasma treatment performed is required, remember that aging produces a hydrophobicity recovery; thus, wait for 1–24 h to recover between 10 and 90% of the contact angle of the untreated material.

(C) Piranha solution ● TIMING 1–2 h

(i) Immerse the stamp/substrate in the solution.
(ii) Heat the solution on a hot plate at 90 °C for 30 min and allow it to cool down to room temperature.
(iii) Remove the stamp/substrate from the Piranha solution and rinse it copiously with ultrapure water, and then dry it in a stream of nitrogen for 60 s.
▲ CRITICAL STEP Use ceramic tip or PTFE-coated tweezers to manipulate the stamp/substrate.

(D) Preparation of thick substrates (typically >0.5 mm) by cleavage ● TIMING 1–5 min

(i) Insert a sharp blade from the side and move it carefully parallel to the surface to divide the substrate into two portions.
(ii) Take one portion with the tweezers and pull it apart.
(iii) Use the face of the substrate that was previously nonexposed for the experiments.

(E) Preparation of thin substrates or those coupled to a support substrate by cleavage ● TIMING 1–5 min

(i) Place the substrate on a clean surface.
(ii) Place a piece of adhesive tape on its top, avoiding the formation of air bubbles.
(iii) Pull it away, thus removing the upper layers.

Stamp and substrate chemical functionalization ● TIMING 1–3 h

2| Use option A to functionalize noble metal surfaces and option B or C for glass, silicon and SiO_2 surfaces (see Experimental design for further details).

(A) Chemical functionalization with alkanethiols ● TIMING 3 h

(i) Immerse the metallized stamp/substrate in 2 mM alkanethiol for at least 2 h, keeping the solution in the dark.
(ii) Rinse the stamp/substrate with ethanol to remove possible multilayers, and then dry it in a stream of nitrogen for 30 s.

(B) Chemical functionalization with alkoxysilane ● TIMING 3 h

(i) Immerse the pretreated stamp/substrate in 1 mM alkoxysilane solution and keep the solution in the dark for at least 2 h.
(ii) Rinse the stamp/substrate thoroughly with pure ethanol, and then dry it in a stream of nitrogen for at least 30 s.

(C) Chemical functionalization with OTS ● TIMING 1 h

(i) Immerse the pretreated stamp/substrate in 1 mM OTS solution for 2 min.
▲ CRITICAL STEP The stamp/substrate should have been pretreated with plasma (Step 1B) or Piranha solution (Step 1C).
(ii) Rinse the stamp/substrate in an ultrasonic cleaner with toluene (10 min), then in chloroform (10 min), then in ethanol (10 min) and then in water (10 min).
(iii) Dry the stamp/substrate in a stream of nitrogen for at least 30 s.
? TROUBLESHOOTING

Spacer fabrication ● TIMING 3–5 h

3| To follow the regime using spacers (**Fig. 1a–c**), fix a metallic mask (use a commercial mask or follow the protocol in ref. 33) onto the cleaned substrate.
▲ CRITICAL STEP All sample holders of commercial vacuum sublimation systems are designed to be able to affix the mask; if the operator is not familiar with the vacuum system, the help of a technician may be necessary.

4| Evaporate through the spacer mask a film of Au or other inert/insoluble material using the vacuum sublimation system, according to the manufacturer's instructions. The thickness of evaporated material determines the thickness of the spacers, and can be monitored by a quartz microbalance that is usually included in the vacuum sublimation system. A good standard thickness is 100 nm.

5| Remove the mask.

LCW printing ● TIMING 2–24 h

6| Pipette the solution of the functional material (see Experimental design for further details) onto the substrate from Step 5 (or from Step 2 if following the regime using a floating stamp rather than spacers; **Fig. 1d–f**). If the solvent has a boiling point <50 °C, pipette 30 $\mu l\ cm^{-2}$ if the solvent has a boiling point >50 °C, pipette 20 $\mu l\ cm^{-2}$.
▲ CRITICAL STEP By using biological materials, dilute the buffer solution to the minimum concentration compatible with protein stability (for example, PBS 0.1× or Tris-HCl 10 mM, pH 7.4).

7| Put the stamp onto the spread solution.
! CAUTION The alignment by mask aligner cannot be done with highly volatile solvents because their vapor can be inhaled by the operator and can damage the instrument.
▲ CRITICAL STEP If stamp alignment is required (for example, when the operator wants to print stripes with a specific orientation and/or in a precise location of the substrate), this operation could be done with the help of a mask aligner.

8| If the solvent has a boiling point <50 °C, deposit an additional volume of 20 μl of pure solvent after the stamp alignment.
? TROUBLESHOOTING

9| Wait for solvent evaporation. Depending on the boiling point (bp), wait for the following durations: ~2 h for solvent with bp <50 °C, ~6 h for solvent with bp between 50 and 70 °C, and >24 h for solvent with boiling point between 70 and 120 °C. For solvent with bp >120 °C, keep the system in a heater at T = bp/3 for >24 h (where T = temperature).

10| Remove the stamp gently from the substrate using plastic tweezers. Do not hesitate in the movements, and take hold of the stamp close to a corner.
▲ CRITICAL STEP This operation must be done very carefully because it can damage the printed features. It is particularly delicate if the printed features do not have a strong interaction with the substrate. In the case of strong adhesion of the stamp to the printed features, it may be necessary to apply a strong force by the tweezers to remove the stamp.
? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1B(vi)	Some small fractures form on the PDMS surface that are reproduced in the pattern	PDMS vitrification due to a too-long/strong plasma treatment	Reduce the timing and/or the power of plasma treatment
2C(iii)	The surface shows some inhomogeneity	Chemical functionalization is not optimized	Increase the treatment time
8	Misalignment of the stamp during solution deposition	The solution moves the stamp during the deposition	Pipette the solution slowly
10	Printed material tends to accumulate at the boundaries of the stamp	The solvent evaporates only from the open end of the stamp	Perform the printing process in an environment saturated by the solvent vapor and wait twice as long for solvent evaporation before removing the stamp

● TIMING

Step 1, stamp and substrate cleaning: up to 2 h
Step 2, stamp and substrate chemical functionalization: 1–3 h
Steps 3–5, spacer fabrication: 3–5 h
Steps 6–10, printing: 2–24 h

ANTICIPATED RESULTS

The experiments described in this protocol should produce different structures depending on the wetting properties of the system as described in the Experimental design. (i) In the wetting regime, continuous or separated microstructures or nanostructures replicating the stamp protrusions are achieved or, working in a diluted regime, the boundaries of the stamps' motifs are replicated. (ii) In the dewetting regime, ordered or spatially correlated droplets are obtained; droplets have sizes comparable to the stamp size and are positioned in correspondence with the stamp protrusions.

Figure 3 shows two typical examples of isolated or continuous microstructures, respectively, fabricated using LCW in the wetting regime. Discotic liquid crystals were printed on a glass surface using a stamp bearing a motif with information coded in binary code (**Fig. 3a**) and continuous lines forming a square grid (**Fig. 3b**).

Figure 4 shows an example of micrometric structures printed in the dewetting regime. In this case, it is not possible to print a continuous structure. Here we used the same kinds of grids as in **Figure 3b**, using amphiphilic catenane molecules[36] deposited on HOPG from acetone. Droplets are

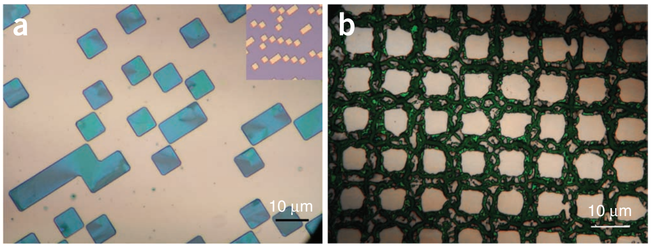

Figure 3 | Printing separated or continuous microstructures in wetting regime. (**a**) Separated microstructures of mesogenic discotic liquid crystals printed on glass using LCW. The inset shows a micrograph of the stamp. (**b**) Continuous microstructures of the same compound are fabricated using a commercial grid for TEM made of square structures as the stamp.

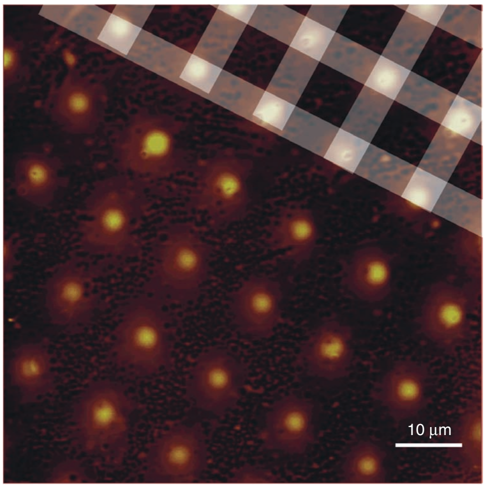

Figure 4 | Printing separated microstructures in the dewetting regime. AFM image of a printed pattern of catenane on HOPG (*z*-scale, 0–60 nm); the printed structures were obtained in the dewetting regime using a commercial grid for TEM made of square structures as the stamp. The transparent lines at the top of the figure show the position of the grid during printing.

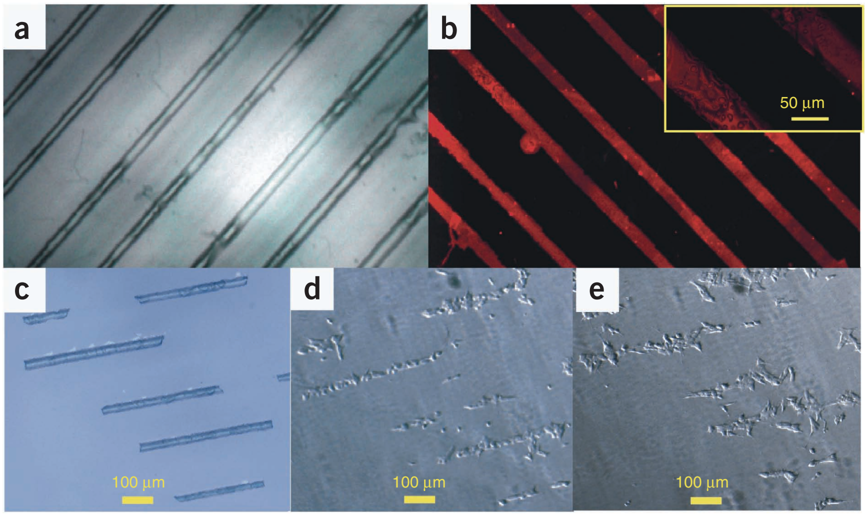

Figure 5 | Printing continuous submicrometric structures of laminin. (**a**,**b**) Optical image of a pattern of laminin with channel width ranging between 17 and 70 µm (**a**) and its relative immunofluorescence staining with anti-laminin upon incubation for 24 h under the conditions used for cell culture (DMEM-F12, 378C; see ref. 15) (**b**); the higher-magnification image in the inset allows appreciation of the strong localization of laminin. (**c**–**e**) Pattern of segments of laminin (**c**,**e**) and the same region after seeding neuroblastoma cells (**d**,**e**). At 24 h, the cells are confined on the laminin pattern (**d**) and they reach confluence only on the patterned regions (**e**) after 48 h. Image reprinted with permission from ref. 14.

positioned to correspond with the cross-points of the grid because of the higher surface tension corresponding to these points.

Figure 5 shows an example of printing of biological materials. In this experiment, micro- and nanopatterning of cell adhesion proteins direct the growth of neural cells, namely, human neuroblastoma SHSY5Y, at precise positions on a strongly antifouling substrate of technological interest (e.g., Teflon-AF). LCW was used to pattern human laminin. The fabrication of substrates with adjacent antifouling and adhesion-promoting regions has allowed us to achieve absolute spatial control in the positioning of neuroblastoma cells on the Teflon-AF films. This patterning approach of a technologically relevant substrate can be of interest in tissue engineering and biosensing[14].

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