

Soft Lithography, a Tool to Address Single-Objects Investigations

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1. Introduction

Nowadays, research is following a new trend where nanotechnology meets biology and vice-versa. In fact, nanotechnology offers remarkable tools to improve our understanding of biology's mechanisms but at the same time, biology shields precious clues that could inspire nanotechnology to answer to today's and tomorrow's issues and needs.

The study of cells and molecules at the single-object level has become essential in this complementary exchange and multidisciplinary effort. But, to track single proteins at work, watch nanoscale biomachines or control cell adhesion and differentiation, one of the main challenges is still the integration of these bioentities onto desired locations on a substrate. In other terms, one has to be able to single-out the object of interest from a given media, organize it and fix it on a surface while preserving its functionality. This integration of objects further implies being efficient and having a high-throughput capacity in order to conciliate single event recording with statistical analysis. Thus, efforts on creating defect-free and well-ordered assemblies are crucial for the study of single objects.

To define the number, position, and distances of the objects on a surface, it is often necessary to direct their assembly by means of suitable prepatterned substrates or stamps. Nanopatterned surfaces have been fabricated by nanoimprinting (Wang et al., 1999), contact printing (Kumar & Whitesides, 1993; Xu et al., 2007), microtransfer molding (Zhao et al., 1996), pattern replication induced by an electric field (Morariu et al., 2003), lithographically induced self-construction (Chou et al., 1999), grid-assisted self-organization (Cavallini et al., 2002), and self-assembly (Chou & Zhuang, 1999) among others. Most of these techniques rely on a soft mould or stamp (Quake & Sherer, 2000).

Soft-lithography emerged in the early 90's as a technique based on the use of an elastomer material named polydimethylsiloxane (PDMS). This polymer can be poured onto a silicon-based master with micro- or nanostructures made by photolithography or electron beam lithography respectively, to obtain a PDMS replication of the master's structures. The corresponding PDMS stamp can then be inked with a molecule and put in contact with a target surface to transfer the molecules into a patterned layer. This is what we call microcontact printing (Kumar & Whitesides, 1993; Xia & Whitesides, 1998). The major advantages of microcontact printing are that the contact with the surface is made in a very gentle manner, so the bioentities are transferred without damaging them. The flexibility of the

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PDMS stamp and the ability to achieve conformal contact between the stamp and the surface enables high-resolution patterning on both planar and non-planar surfaces. Areas greater than 50 cm² have been patterned by microcontact printing (Xia et al., 1996).

So this technique is suitable for large-area micro- or nanopatterning, and is attractive because of its simplicity, biocompatibility, speed, and low cost.

Directed Capillary Assembly is another interesting technique that has received constant interest throughout the years. This technique was first introduced by Denkov et al. in the 90's. At that time, their objective was to produce photonic band gap materials based on the compact assembly of dielectric colloids (silica beads). The method relied on the direct evaporation of a droplet of a colloidal solution on a surface and was called Capillary Force Assembly (CFA). The nanoparticles dragged by the convective flux in the droplet were forced to assemble on the surface close to the liquid meniscus. A few years later, the group of Alivisatos (Klein et al., 1996) showed how this method could be combined with topographic patterns in order to control the position of the assembly as well as the shape of the nanoparticles' patterns and this was called "Lithography Guided Capillary Force Assembly". Kraus et al. further demonstrated that this high-precision inking could be performed by dragging the meniscus of the objects in suspension over a structured stamp with a controlled speed. As a result, the objects are trapped and assembled inside each cavity and the obtained assembly can be subsequently transferred onto a target surface by simple contact (Malaquin et al., 2007; Kraus et al., 2007).

Soft-lithography and directed capillary assembly thus remain very attractive and straightforward techniques capable of patterning and nano-ordering over large areas at low cost. Therefore, to study single objects, the methodology we developed is declined following two strategies: either we combine soft-lithography with a simple incubation technique, or we combine soft-lithography with directed capillary assembly. Both approaches allow us to tightly control the assembly process to direct the precise ordering of the objects of interest. With these two strategies, we have been able for example to generate arrays of single living bacteria for fundamental studies and regular arrays of single stretched DNA molecules for genetic analysis or medical diagnostics. We have also been able to produce periodic bi dimensional matrixes of gold nanoparticles for different applications in the field of plasmonics.

2. First strategy: chemical patterning

In the case of cells, we developed a fast, simple and reproducible procedure for isolating and depositing individual living cells onto a surface along well-defined and precisely registered micrometric patterns. During the process, the species are not extracted from their natural media so there is no risk of damaging them. The approach consists in chemically engineering the surface with hydrophobic/hydrophilic contrast to induce and localize single cell immobilization events in a deterministic manner. The principle of the methodology we perform for assembling cells is depicted in Figure 1. It is based in the combination of conventional microcontact printing with a simple incubation technique. In practice, the PDMS stamp with topographic structures is used to print a first ink, in this case a hydrophobic molecule, on the surface. Then, by incubating a second ink, this time a hydrophilic molecule, the hydrophilic molecules fill out the empty patterns. After incubating a droplet of cells in suspension on this engineered surface, we obtain a selective adsorption of cells on the local chemical patterns producing highly ordered arrays of single

living cells with a success rate close to 100% and over a 1cm² area. By tuning the chemistry of the surface we are able to modulate the binding strength of the cells onto the patterns. This allows the technique to be flexible as concerning the choice of the molecules used to graft the cells by using untreated or chemically treated cells. The molecule used mainly influences the strength of cell's binding onto the surface rather than their localization. In fact, only the surface microstructuration is responsible for the cells trapping, so this trapping occurs whatever the affinity of the cells with the surface is. This methodology has allowed us to realize more fundamental studies on single cells with the possibility to perform statistics on them as we obtain chips with high-throughput. By correlating AFM spectroscopy with staining techniques we have further proved that the grafted cells remain alive after patterning (Cerf et al., 2008; Cerf et al., 2009).

By extension, this methodology could be a basic building block for interfacing living cells with artificial microsystems or for generating arrays of any kind of microorganisms by simply using the appropriate pattern functionalization.

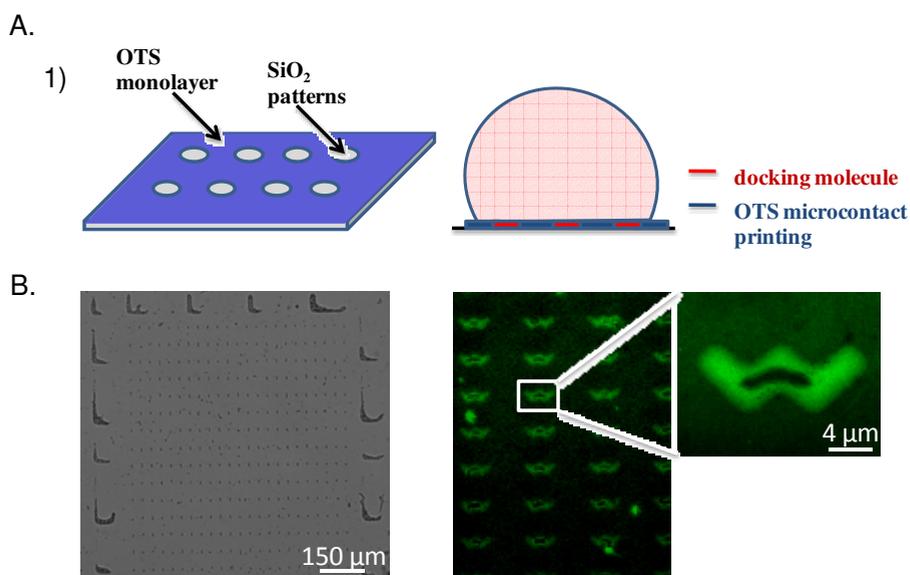


Fig. 1. Chemically engineered surfaces used to trap individual bacterial cells along the chemical patterns so-defined (biopatterning). A) OTS microcontact printing (1) followed by a functionalization by incubation of the bare patterns with docking molecules (2). B) On the left, bright field image of highly ordered arrays of single living bacteria and on the right, fluorescence image of the same array. Here the patterns are functionalized with streptavidin molecules and bacteria are biotinylated so that they strongly bind to the surface.

3. Second strategy: use of topographical structures

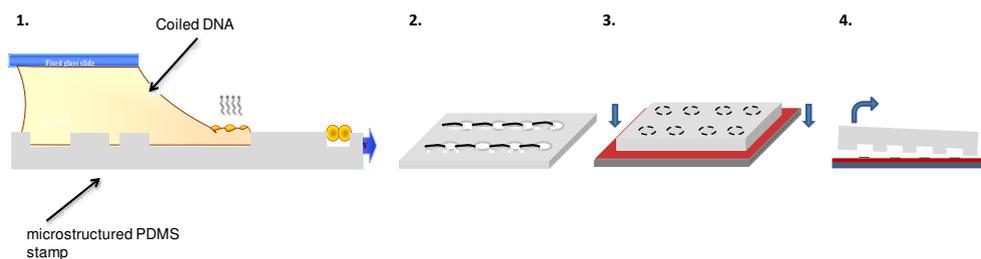
If we travel now from the micrometric scale down to the nanometric scale, we find other interesting objects to study such as DNA molecules. Our purpose in this study was to single out individual DNA molecules and stretch them into controlled arrays for fundamental investigations but also medical applications. This time, we did not use chemically

engineered surfaces but structured PDMS stamps as templates with cavities that would allow the trapping of the objects of interest inside each cavity. In fact, in this case, we combine two techniques: soft-lithography with directed capillary assembly.

The technique of directed capillary assembly that we perform is carried out using a dedicated setup. The so-called directed assembly setup consists in a motorized translation stage where we put a PDMS stamp with micro- or nanostructures. Above this stamp we place a fixed glass slide at a distance of 1mm and we put in between a certain volume of the colloidal suspension of the object to deposit. The liquid meniscus is then moved over the structured PDMS stamp at a controlled velocity (Scheme 1). As the meniscus encounters the stamp's topographic structures, it gets pinned during a certain time. Meanwhile, the objects undergo a convective flux and are directed towards the liquid front line. There, they are trapped by the capillary forces exerted inside each topographic cavity or well and when the elastic energy stored exceeds the pinning energy, the meniscus disrupts and releases the objects.

By adjusting the displacement speed of the stage, the concentration of the colloidal suspension and the temperature of the PDMS substrate thus the evaporation rate of the solvent, we are able to control the number of objects to be trapped inside each micro- or nanowell of the structured PDMS stamp with high placement accuracy. Once the objects are properly assembled under optimized experimental conditions, the objects can be transferred onto a target surface by simple contact printing.

In the case of DNA molecules, we use this particular methodology: the assembly of coiled DNA molecules is performed on a microstructured PDMS stamp at a relatively high velocity of 1mm/sec inducing almost simultaneously the selective trapping of single molecules at each well of the stamp and their stretching by the meniscus disrupting. The resulting assembly of single stretched molecules is then transferred by contact printing onto a freshly cleaved mica sheet or onto a glass slide coated by vapor deposition of 3-aminopropyltriethoxysilane (APTES) molecules (Scheme 1).



Scheme 1. Generation of single stretched DNA molecules arrays by directed assembly. 1) Capillary assembly of DNA molecules on a microstructured PDMS stamp. 2) Microstructured PDMS stamp with assembled and stretched DNA molecules. 3) The assembly is then transfer-printed on an APTES-coated surface. 4) The PDMS stamp is finally peeled away.

Figure 2A depicts the nice and regular arrays of single stretched DNA molecules we obtain with a success rate close to 100% (Cerf et al., 2009). We observe through AFM cross-section measurements that the stretched DNA molecules are 1.7 nm high which corresponds to the theoretical height of a single DNA molecule. So the generation of these chips with single DNA molecules could be interesting for genome mapping or medical diagnostics through a

fast and more efficient optical readout. Another advantage of this methodology is that the experimenter is able to tune the size of the PDMS wells and their periodicity so as to obtain continuous lines of DNA molecules or much more complex DNA patterns. In fact, the size, organization and center-to-center distance between the wells also play an important role in the number of molecules to be anchored and in the arrangement we want to give to the stretched DNA molecules. In Figure 2B, we notice that with a shorter center-to-center distance between the wells, the combing of the molecule can continue over the next well, where the formation of another stretched molecule is initiated. As a result, the two consecutive stretched molecules bundle together in the knot that appears brighter in the fluorescence micrograph. Figure 2C shows the influence of the wells' arrangement and the moving direction of the meniscus on the DNA molecules' stretching. In the case of wells in a quincunx arrangement, the molecules tend to cling to the shape of the next microwell while stretching when the meniscus breaks. The orientation of the DNA molecule will be the same as the moving direction of the receding meniscus. Thus, by controlling the size, period and arrangement of the PDMS wells as well as the moving direction of the meniscus, we can further create more complex DNA templates. Thus, in a broader sense, this methodology could also be very useful for microelectronics as by metalizing these robust 1D nanostructures or by functionalizing them with nanoparticles (Braun et al., 1998; Keren et al., 2002; Deng & Mao, 2003; Monson & Woolley, 2003; Nguyen et al., 2008; Köhler et al., 2001), one is able to generate very appealing conducting wires and complex networks over a large area and at low cost.

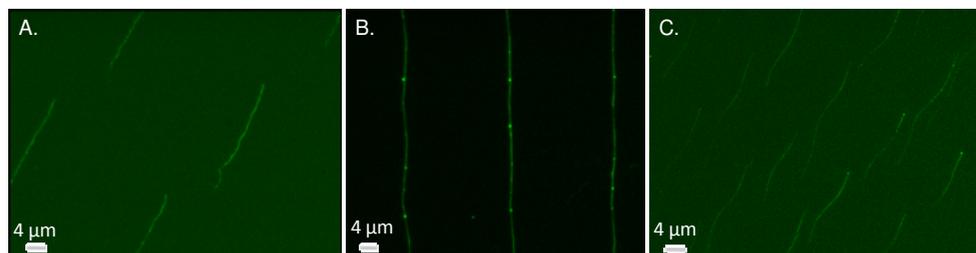


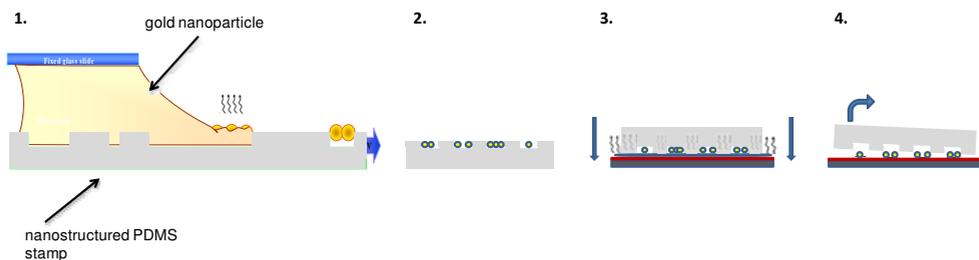
Fig. 2. Highly organized chips of stretched DNA molecules (A and C) and continuous lines of stretched DNA molecules (B) on APTES-coated surfaces.

Another example of direct application is the integration of nanoparticles onto a target surface.

Recently, we explored the possibility to generate bi dimensional matrixes of periodic sub-100-nm gold nanoparticles patterns by directed assembly. Small nanoparticles are the target of intense research due to their astonishing properties and their large window of applications in biology, nanoelectronics, spintronics, optics, chemical sensing, etc. Assembling nanoparticles into well-ordered structures provides new nanostructures with remarkable and very distinct collective properties. In fact, by controlling their type, number, and precise arrangement, one can benefit from the unique properties they offer at that scale in the field of plasmonics in particular (Surface Enhanced Raman Scattering, Metal Enhanced Fluorescence).

By using the same technique as previously described, but this time by using a nanostructured PDMS stamp and by placing between the fixed glass slide and the mobile stamp a colloidal suspension of gold nanoparticles, we are able to generate matrixes of gold nanoparticles with different numbers of particles and different arrangements. In this case,

the speed of the translation stage needs to be very low ($1\mu\text{m}/\text{sec}$). This time, accounting from the size of the nanoparticles, their irregular shape and the fact that the resulting assembly is dry, the formed nanoparticles arrays were extremely difficult to transfer onto a solid surface. So we investigated different strategies to transfer print the assembled nanoparticles without the need for an adhesion layer. Generally what is commonly used in the literature is an intermediate polymer layer to emboss so to speak the particles onto the target surface (Kraus et al., 2007; Yan et al., 2004). But for further technological steps, this polymer layer has to be removed from the surface by hydrogen plasma and this additional processing is not always feasible depending on the object or the application sought. Thus we explored other possibilities and we found an alternative approach to transfer print an assembly from a PDMS stamp onto a surface by performing the contact through a thin liquid film. In fact we deposit a droplet of solvent (deionized water or ethanol) on the target surface coated by vapour deposition of 3-aminopropyltriethoxysilane (APTES) molecules and we put the PDMS stamp with the assembled nanoparticles in contact. We place the overall in an oven at 100°C for a few minutes to evaporate all the solvent and we peel the stamp off (Scheme 2). Figures 3A and 3B show the results obtained after directed assembly of gold nanoparticles on a nanostructured PDMS stamp and transfer printing onto a silicon surface.



Scheme 2. Generation of 100nm gold nanoparticles arrays by directed assembly. 1) Capillary assembly of gold nanoparticles on a nanostructured PDMS stamp. 2) Nanostructured PDMS stamp with assembled gold nanoparticles. 3) The assembly is then transfer-printed with solvent mediation. 4) The PDMS stamp is finally peeled away.

We obtain nice and regular nanoparticle patterns and the totality of the particles is transferred. Furthermore, we can observe from the dark field micrographs that the assembled particles retain their optical activity (figure 3A), so in a broader sense, we could say that the assembly process does not alter the intrinsic properties of the objects (Cerf & Vieu, 2009).

These encouraging results allowed us to investigate the possibility to detect single objects or molecules at low concentrations via optical techniques. Optical techniques are very interesting indeed because they have the advantage of being non-invasive, non-destructive and non-contact, but are in principle not sensitive enough to study a single nano-object or isolated molecule. Different approaches have been explored in our group among which, those relying on diffractive sensing or more recently on surface enhanced Raman scattering. In this last case, a way to enhance the signal is to position the objects of interest in the close vicinity of noble metal nanocrystals (silver, gold). Such nanocrystals offer remarkable properties due to the localized surface plasmon resonances (SPR) that induce, under the

effect of an optical excitation with a frequency close to the SPR, a very intense local electric field enhancement nearby the surface. The interest of surface-enhanced Raman scattering (SERS) effect is that the enhancement can reach 10^{14} , sufficient enough to detect a single molecule (Nie & Emory, 1997). The conception of ordered nanometer-sized metallic structures that allow controlling the position of these “hot spots” where the electromagnetic field is locally amplified is the heart of intense research, both theoretical and experimental (Käll et al., 2005). What is known, is that some of these properties only appear with very precise arrangements such as regularly spaced coupled particles with short interdistance between them for instance (Haynes et al., 2005a). In this perspective, we set the experimental conditions of our directed assembly setup to generate gold nanoparticle arrays as previously described but aiming at investigating the exact number of particles giving the maximum local electric field enhancement. We deposited a Raman label onto the substrate and we performed some Raman measurements. We evidenced that, effectively, the electromagnetic field is locally amplified at the sites corresponding to the nanoparticle patterns. As we can further observe in figure 3D and 3E, the intensity varies among the patterns so the intensity of the enhanced Raman signal depends on several parameters, namely the number of particles, their arrangement, their size, and their orientation... This substrate showed its great efficiency as a SERS but also as a metal-enhanced fluorescence (MEF) substrate. In fact, figure 3C shows that when a fluorophore is deposited at low concentrations onto the substrate, fluorescence is locally amplified at the sites corresponding to the nanoparticles patterns only (Cerf et al., 2009). So we could later imagine including these imaging-enhancing structures collocated with DNA tethers for single molecule fluorescence.

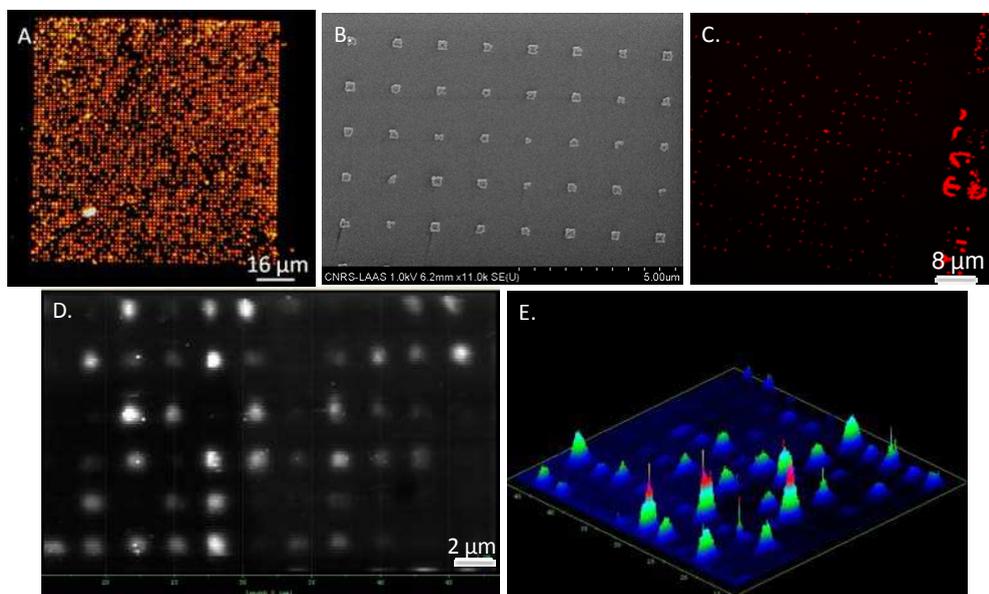


Fig. 3. A) Dark field image of a periodic bi dimensional matrix of 100nm gold nanoparticles. B) Scanning Electron Microscope image of a nanoparticle matrix. C) Confocal image of a controlled array of gold nanoparticles revealing a metal- enhanced fluorescence effect. D) Raman mapping of a controlled array of gold nanoparticles and E) its equivalent in intensity levels revealing a SERS effect.

So we could later imagine including these imaging-enhancing structures collocated with DNA tethers for single molecule fluorescence.

4. Conclusion

Therefore, soft-lithography has become an invaluable technique at the service of single objects studies. This process is essential in the creation of chips with single cell, molecule or particle resolution. We have set a versatile toolbox based on soft-lithography combined with capillary assembly and incubation techniques to generate controlled arrays of single objects at the micrometer and the nanometer scale for different applications. We have shown that the strategies developed are very efficient to generate arrays of all kinds of objects ranging from cells, molecules to metallic nanoparticles with a high success rate in a precise, large-scaled and cost-efficient manner. We have demonstrated that our methodology is capable of high-throughput, and is fully compatible with different types of supports including glass or silicon. The objects assembled preserve their functionality and intrinsic properties and exhibit remarkable potential for a great number of applications in the fields of medical analysis, gas sensing, optics, but also in the construction of tomorrow's nanoscale devices.

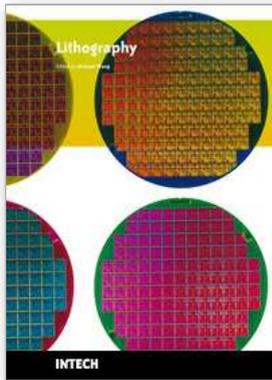
The next step in this development would be to couple these techniques and results to build more complex scaffolds and monitor specific interactions at the single object level. For instance, one should later imagine evolving towards a label-free biochip with optical readout by coupling SERS substrates with biological molecules or cells.

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Lithography, the fundamental fabrication process of semiconductor devices, plays a critical role in micro- and nano-fabrications and the revolution in high density integrated circuits. This book is the result of inspirations and contributions from many researchers worldwide. Although the inclusion of the book chapters may not be a complete representation of all lithographic arts, it does represent a good collection of contributions in this field. We hope readers will enjoy reading the book as much as we have enjoyed bringing it together. We would like to thank all contributors and authors of this book.

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