TOP-DOWN APPROACH TO NANOTECHNOLOGY FOR CELL-ON-CHIP APPLICATIONS

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Abstract:

Standard cell culture techniques have remained basically unchanged for almost a century; today, thanks to advances in micro and nanotechnology related techniques, the toolbox available to biologists is rapidly growing, enabling them to increase their experimental capabilities up to the level of manipulating single cells and controlling cells environment. The transition from macro to microscale gives unprecedented possibilities to generate gradients and patterns that cannot be captured in Petri dishes and well plates. Micro and nanofabrication integrating micropatterning techniques with advanced surface chemistry makes it possible to reproducibly tailor the cell microenvironment at cellular resolution. Nanopatterned surfaces that investigate the extracellular matrix with microfluidic networks for soluble factor signaling delivery have the potential to modulate spatially and temporally differentiated cellular phenotypes. Moreover, properly engineered micro and nanopatterns allow a great enhancement in the detection techniques both from the spatial and spectroscopic point of view. Future perspective will lead to develop platforms for cocolture of different populations on a single chip, in order to mimic cellular communication systems in vivo. In this paper we explore the importance of reducing the observation scale of the cellular environment and provide an essential overview of the micro and nanofabrication potentials and capabilities which are today available.

KEYWORDS : Nanodevices; Microfluidics; Photolitography

ABBREVIATIONS AND ACRONYMS USED IN THE TEXT:

μCP microcontact printing
μFTIR Fourier Transform Infrared microspectroscopy
μTAS micro-total analysis system
μTM microtransfer molding
bioMEMS biomedical microelectromechanical systems
AC alternating current
CE capillary electrophoresis
DC direct current
DUV deep ultraviolet
EBL electron-beam lithography
ECM extracellular matrix
EUV extreme ultraviolet
EUVL extreme-ultraviolet lithography
FTIR Fourier Transform Infrared Spectroscopy
IR infrared
LIC lab in a cell
LOC lab on chip
LSP localized surface plasmons
MEA Multi Electrodes Arrays
MEMS microelectromechanical system
MIMIC micromolding in capillaries
MIR Mid-Infrared
MIRS Mid-Infrared Spectrum
MST micro system technology
nCP nanocontact printing
NIL nanoimprint Lithography
PC polycarbonate
PCL poly(caprolactone)
PEG poly(ethylene glycol)
PDMS polydimethylsiloxane
PGA poly(glycolic acid)
PLA poly(L-lactic acid)
PLGA poly(L-lactic-co-glycolic acid)
PMMA poly(methyl methacrylate)
PS polystyrene
QCL Quantum Cascade Laser
SAM self-assembled monolayer
SAMIM solvent-assisted micromolding
SEM scanning electron microscopy
SERS surface enhanced Raman scattering
SPP surface plasmon polaritons
SPR surface plasmon resonance
SR Sinchrotron Radiation
XRL X - ray Lithography

1 INTRODUCTION

In our experiences of everyday life we are accostumed to the omnipresence and importance of gravitational forces and inertia. However, when going down in size equilibria that take place are easily dominated by surface effects negligible at the macroscopic scale. In fact, at microscale volume the above forces become irrilevant because of the huge increase in surface volume ratio, often by several orders of magnitude.

For example in fluid dynamics, phenomena such as diffusion, capillarity, surface tension and viscosity become ever more important. This leads us to modify our way of thinking in a world that operates very differently from the one we perceive and live in[1]. In practice, it is necessary to determine first the new balance of forces active in microsystems, in order to decide whether or not miniaturizing will provide any advantage. Controlling or overcoming such forces is a basic goal of microfluidic systems. Microfluidic devices employ fluids with Reynolds numbers that are small enough for inertial effects to be irrelevant. Due to their small dimensions, microfluidic systems have flow patterns that tend to be dominated by viscous forces, thus allowing precise control and use of laminar flows. In a pioneering work Purcell provided an excellent description of this counterintuitive world [2] showing, among other things, that bacteria travel more slowly than diffusing nutrients and waste. This implies, as an example, that *E. coli* does not need to actively search for food since it can efficiently gather nutrients by simply waiting for them to diffuse nearby.

Exploiting available micro and nano scale engineering and scale dependence of several physical phenomena[1] is revolutionizing our ability to precisely tailor matter properties to our needs with application spanning from materials processing and analytical chemistry to biology and medicine. Just to provide a few glimpses, limited to the scope of this paper, of the possible fields of applications, we cite here: i) the possibility to control the chemical micro-environment of a tissue, or even of a single cell, by microfabricated fluidic channels [3], ii) the modification of single cell substrates interface by changing the topography and/or the chemistry at the nanoscale by nanopatterning techniques, iii) the improved capabilities of some spectroscopic or optical techniques both from the spatial resolution and the sensitivity point of view (in some cases up to 14 orders of magnitudes, allowing the detection even of single molecules). Such items, to be discussed in detail hereafter, will illustrate how, by means of nanodevices we can realize in-vitro environments more closely mimicking the in-vivo situation. By controlling physical or chemical cues with resolution comparable to the location of proteins on the cellular membrane we can, in principle, talk even with single cells.

In order to get the highest possible benefit from this fascinating technology, however, the main difficulty remains the set up of a common language among biologists, physicists, chemists and engineers.

2 CELLS ON CHIPS: MICRO AND NANOENGINEERING CELL MICROENVIRONMENT

A main challenge of modern biology is to explore how we can finely manipulate cells and tissue and direct the resulting behavior of individual cells and multicellular biological systems. In vivo cells respond to spatially and temporally organized signals in their microenvironment. In addition, cellular processes such as adhesion, migration, growth, secretion, and gene expression are triggered, controlled, or influenced by the biomolecular three-dimensional organization of neighboring surfaces. In a Petri dish, however, cells experience a quite different environment from their natural milieu. Nanotechnology [2], in the end is a multidisciplinar approach involving engineers, physical scientists and biologists devoted to the fabrication of devices with precise control of the nanometric and micrometric characteristics. It demonstrates how one can mimic more closely the chemistry and physics of biological systems in ways that reveal hitherto unknown information (see for example [6,7]).

A wide range of micro-nanofabrication techniques has been developed to produce miniature components and devices with micrometer and nanometer-scale resolution. Nanoscale materials and devices can be fabricated using either bottom-up or top-down approaches and strategies that have elements of both. Bottom-up approaches

[1] A complete description of scaling laws is beyond the scope of this paper; however some comprehensive and quantitative reviews dealing with the topic may be found in [4,5].

[2] According to the definiton given by the U.S National Nanotechnoloy Initiative, Nanotechnology includes: *Nanometer scale science* (Systematized knowledge of nature and physical world); *Engineering* (Application of science to the need of humanity accomplished through knowledge), *Mathematics*, and practical experience applied to the design of useful objects or processes); *Technology* (Knowledge of using tools and machines to do tasks efficiently.

start from certain processes that result in a higher-ordered and -organized structure. Examples of bottom-up approaches include systems that self-assemble, a process that is triggered by a local change in a chemical or physical condition. Related techniques include templating and scaffolding methods, such as biomineralization, which rely on backbone structures to support and guide the nucleation and growth of a nanomaterial. In a top-down approach complex structures are built up by patterning layers upon layers from the surface of a planar substrate[78]. The realization of the single layer is based on the *lithographic process*, which in order to define the whole fabrication process can be rougly summarized in three steps: patterning, etching and depositing (Figure 1).

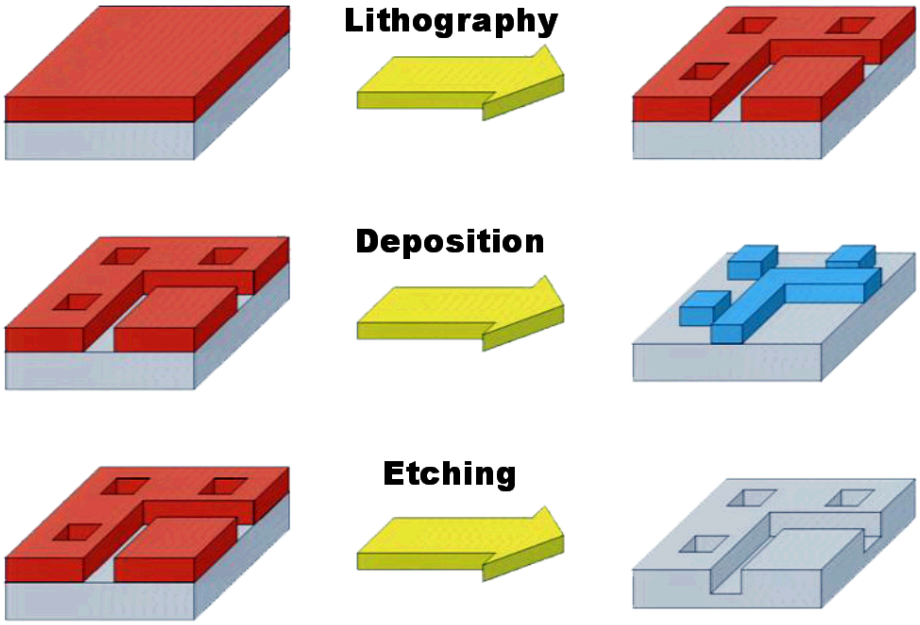

Figure 1: *A typical lithographic process.* In a lithographic process a flat substrate is covered by a photosensitive polymer, called resist, which, after exposure to a certain radiation (electrons, photons or ions) become soluble and can be removed using the appropriate developer (normally a weak base) . The obtained pattern can be considered the final device, for example a microfluidic channel, or can be used to add or remove material. In the first case we talk of deposition process and we fill the obtained holes with some other material, for example a metal by an electrolytic process. In the second case, called etching, we use the resist as a mask to remove material from the substrate using some chemical attack.

Culturing cells in a nanotechnological devices requires understanding fundamental principles that span multiple disciplines, including biology, biochemistry, physics and engineering. Several groups have developed platforms to create microenvironments of greater physiological relevance for high-throughput culturing and analysis of cells under a large number of conditions. Micro and nano fabrication integrating patterning techniques with advanced surface chemistry makes it possible to reproducibly engineer cell microenvironment at cellular resolution and below, allowing a better understanding of the response of a cell towards outer stimuli. These features include the chemical nature of the surface adhesive molecules, their precise spatial distribution at nanometer and micrometre levels, and the physical properties of the surface, such as its topography, stiffness and dimensionality. This understanding leads to more reproducible and meaningful assays.

Since cells normally live in a wet environment, in the following sections we focus mainly on the physics of microfluidics as well as on applications addressing leading questions in biology, and discuss how microfluidics can contribute to control cellular microenvironments. We will also discuss how micro and nanofabrication can help in allowing or enhancing our detection ability. In Appendix 1 we highlight the key elements of the cellular microenvironment helpful to design in vitro models more closely mimicking the *in vivo* conditions.

2.1 Fluids at microscale: the peculiar physics of the micro-environment.

The main advantage of microfluidics is utilizing scaling laws for new effects and better performance[37]. In order to manipulate and work with microfluidic flows, one must understand the physical phenomena that govern at the microscale. High surface-to-volume ratios are key in defining fluid-flow characteristics at the microscale. The passage from a bulk-dominated flow to a surface-dominated flow gradually occurs when approaching and going below the millimeter scale. As a system shrinks, surface tension can dominate gravity and viscosity can dominate inertia [4]. The ratio of inertial to viscous forces on fluids is characterized by the Reynolds number (Re), one of the most mentioned dimensionless numbers in studying fluids flowing in microsystems. The dominance of viscosity determines flow to be laminar, providing predictable streamlines through microchannels. In fact, in this regime of low Re, fluids do not mix convectively: two adjacent streams flowing side-by-side down a single channel maintain a well-defined interface without eddies or turbulences [2,4,17,30,32]. Therefore, diffusion is the only effective mechanism to mix solutes that are present at different locations in the channel cross-section. Since diffusion is a slow process, microfluidic channels can be used to create cross-sectional gradients in solute concentration to study the effect of these gradients on distinct areas of cells within this channel, or to pattern cell adhesives and repellents on the substrate prior to introduction of cells[38]. By creating networks of these diffusive contacts, this controlled mixing may be used to generate complex and temporally stable gradients; this can be applied in the study of chemotaxis. Strategies for accelerating mixing in microfluidic devices rely on reducing the characteristic length of the laminar streams by repetitive folding of the fluid onto itself, by induced chaotic advection or by hydrodynamic focusing[37].

The relative importance of diffusion and convective bulk flow for transporting solute and solvent molecules is given by the Peclet number, Pe, and can be readily adjusted through the choice of flow velocity and the dimensions of the system used. The dominance of viscous forces makes microfluidic systems also ideally suited for realizing purely diffusive (Pe = 0), convection-free environments that cannot be obtained on macroscale. Only under such conditions solute released from a source diffuse in all directions with equal probability, its concentration decreasing with increasing distance from the source. The extent of this diffusive layer, a secondary interface, depends on the rate of solute release (or production) at the source and on the solutes diffusivity. If microchannel walls are close, solute will accumulate in a predictable way[3]. Surface tension forces at the microscale are also significant. They are the result of cohesion between liquid molecules at the liquid/gas interface. As an example, consider that a water spider can easily walk on the surface of water, whereas a human cannot . The effect allows for more efficient mass and heat transfer in microsystems. If, for example, surface tension varies along a surface or interface as a result of thermal or concentration gradients the so-called Marangoni flows can arise and effectively homogenize the thermal or concentration gradients. Since relatively more interface is available for transfer to occur, and less total mass or energy needs to be transferred to reach the final state. Therefore, both the creation and the homogenization of solute and temperature gradients are faster as system size is reduced[32,34]. So exothermic and/or high temperature reactions are performed in an efficient and controllable (isothermal) manner and high specificity of chemical and physical properties (concentration, pH, temperature) can also be ensured.

Surface tension on the microscale can be also used for pumping fluid through channels. According to the YoungLaplace equation, droplets with smaller radii of curvature have a higher internal pressure than those of larger curvature of the same fluid. The resulting pressure difference is adequate to move microliters in 1s, even when working against gravity, and has been referred to as passive-pumping. Similarly, capillarity forces can play a significant role in micro-device behavior[39]. Surface properties can be selected to influence the competition between viscous forces and capillary forces. This makes it possible to adjust the competition (which is quantified by the capillary number Ca). Finally, the large surface-to-volume ratio achieved in microfabricated flow structures may be exploited to enhance detector sensitivity by greatly increasing the interaction of a sensing element with a large sample.

2.2 Macroscale vs microscale cell cultures

The significant differences between several physical phenomena at the micro- and macroscale have been exploited to provide new types of cellular assays [3] impossible to achieve by conventional laboratory techniques [14].

[3] A cell assay is defined as the measure of cellular response to chemical and/or physical stimuli. Diverse responses characterize the cell phenotype, such as alterations of intracellular and extracellular biochemical pathways, cell morphology, motility, and growth properties. When culturing cells in vitro, a number of variables can affect cell phenotype (e.g. contamination,

The ability to culture cells *in vitro* is one of the cornerstones of modern biology and has revolutionized hypothesis testing in basic cell research, becoming a standard methodology for massive production of proteins and vaccines, drug screening and toxicology assays. It essentially consists of growing cells on a large surface (polystyrene or glass dishes or multiwells) able to stimulate cell binding and immersed in a homogeneous medium [8,16]. In macroscale cultures, large volumes of medium are available to ensure the access to necessary metabolites. Continuous secretion of signal molecules by cells causes spatial variability in the concentration of solutes in the culture media. Convection by stirring or shaking generates a rapid homogenous distribution of metabolites within the entire cell culture medium with minimized waste accumulation. As a result, autocrine and paracrine signaling is, at least temporarily, impaired[17]. Therefore, many of the factors that induce or stabilize differentiated phenotypes are poorly understood and difficult to mimic by *in vitro* standard 2D culture: soluble growth factors are present at abnormally high concentrations, 3D cues are largely absent, oxygen tension is too high, long distances between other cells and cell volume/medium volume ratios less than one[12,16].

It is worth stressing that culturing cells at microscales allows control over microenvironmental cues, such as cell-cell and cell-matrix interactions; the use of small culture volumes; and the ability to integrate with microsystem technologies for on-chip experimentation. Moreover, by better control of fluid flow and mass transport mechanisms, microfluidic techniques can provide temporal and spatial patterning of soluble factors or cells not otherwise possible. Microfabrication technologies have enabled researchers to design, with nanometer control, the biochemical composition and topology of the substrate, the medium composition, as well as the type of neighboring cells surrounding the microenvironment of the cell. They also can be used to link cell culture with integrated analytical devices that can probe the biochemical processes that govern cell behaviour.

Some cell-based microsystems simply represent miniaturized versions of conventional laboratory techniques, whereas others exploit the advantages of small length scales and low Reynolds numbers, such as favourable scaling of electrical fields and the ability to create well-controlled laminar flows. Studies are emerging that examine the combinatorial effects of soluble factor signaling and cellmatrix interactions on cell behavior. They may have a great impact especially for drug screening tests and for optimization of microenvironments in tissue engineering applications where long-term performance of tissue constructs are dependent upon many factors working synergistically[11,18].

Nowadays, analysis of the cellular microenvironment in microfluidic platforms remains an issue to be addressed, and general progress has been somehow hindered by the lack of a complete understanding of why living cells behave differently when moved from macroscale culture to confined microscale geometries[11]. Although microfluidics holds enormous potential to provide a platform for relevant cellular assays, in depth investigation of the biological influence of the engineered microenvironments are still required for this potential to be fully realized [19].

2.3 Miniaturization of cell assays in microfluidics

Introduced in the early 90s by activities in microfabrication techniques as a spin-off from Micro-Electro-Mechanical Systems (MEMS) or Micro System Technology (MST) research, the concept of microfluidics was dedicated on analytical chemistry applications, with a major role for capillary electrophoresis (CE) on a chip[20,21]. Since then, a large variety of microfluidic phenomena has been explored based upon fast thermal and species diffusion, large surface-to-volume ratio or large field gradients (electrical, acoustic, optical), resulting from the small structural dimensions. Besides these new phenomena, perhaps the most important driving force for microfluidics is the ability to handle minute amounts (sub-nanoliter) of liquid in a controlled and reproducible manner.

One may state that microfluidics devices prepared by soft-lithographic procedures really entered life science after the introduction of polydimethylsiloxane (PDMS)-based (a biocompatible, easy to carve silicone rubber)[22,23]. PDMS devices can be easily replicated from standard photolithographically fabricated molds (see Appendix 2), and enable a wide variety of applications. Initially, two approaches were followed in this field: one aiming at combining microsensors with fluidic components (pumps, flow sensors) into systems; the other, which had a much greater impact, focused on miniaturization of analytical chemical methods, in particular separations, with a lot of emphasis on DNA analysis[24,25]. As genetic analysis has now become a more or less routine method, the new focus has been for some time on using micro Total Analysis Systems (μTAS) for proteomics[17]. In addition, in the past few years, the analysis of even more complex biological systems such as

degree of confluence, presence of cell-cell adhesion, and seeding density) [15].

living cells with the use of microfabricated structures has attracted increased attention[26,27,28]. From a biological standpoint, microfluidics seems especially relevant considering that most biological processes involve small-scale fluidic transport at some point. Examples stem from molecular transfer across cellular membranes, to oxygen diffusivity through the lungs, to blood flow through microscale arterial networks[17].

Microscale techniques for cell biology have been applied from single cell analyses and flow cytometry-like techniques, to treating fields of cells in gradient generating devices, patterned 3-dimensional cultures, to microscale versions of more traditional assay types such as cell culture (via perfusion, or static cultures)[14] . The toolbox for biologists is rapidly growing, enabling them to carry out different kinds of sophisticated experiments on the cellular level. An example of such potentiality is the concept of Lab-in-a-Cell, LIC, [24,29], where a sophisticated cell-environment interface transform a single cell in a complete chemical and biological laboratory. In the next section the components of a microfluidic platform are briefly discussed.

2.4 Components of a microfluidic device.

An operative definition of microfluidics is necessarily based on the ability to process or manipulate small (10^{-9} to 10^{-18} litres) amounts of fluids, using a series channels and chambers ranging from one to a few hundred microns in size [30]. Microfluidics is today the core technology to realize smart cell culturing chambers with fine spatial and temporal chemical control of cellular microenvironment. Inlet and outlet ports of the system serve as points of interaction between the culture region and the external world. The size of loading reservoirs with one end open to the ambient environment and the other end connected to the microchannel network is typically in the millimeter scale to facilitate manual or automatic liquid loading by means of syringe needles or pipettes. The liquid loaded in the reservoir can then be drawn into the microchannels by capillary force, differential pressure, or electrokinetic force.

Howewer, a microfluidic platform is not a simply network of channels. One must add components to increase functionality of the system suited for the specific application. So the design of a microfluidic device typically consists of a combination of fluidic control (method of introducing reagents and samples and for moving these fluids around on the chip) and sensing components for combining and mixing them(e.g mixers, heaters, and sensors).Therefore, besides components for on chip handling of nano and pico liter volumes, a complete system includes others tools interacting with fluids such as micropneumatic systems, i.e. microsystems for the handling of off-chip fluids (pumps,valves,etc) and sensors(electrodes, phodetectors, chemical sensors) [31,32,33,34]. While selective integration of these components is largely application dependent, all devices require an interface between the microdevice and the macro world.

Microfluidic devices designed specifically for cell culture have certain requirements that distinguish them from microscale systems used for other applications in chemistry or physics[11]. To optimize the system and the experimental design according to the particular application the engineer and the biologist have to evaluate: (1) the choice of material for device fabrication; (2) the geometry and dimensions of the culture region (lithographic techniques allow extreme flexibility but not infinite possibilities to modify the geometry of the platform); (3) the method of pumping and controlling fluid flow[35]; (4) the detection techniques which will be used to study biological systems. We will discuss point (4) more deeply in the next, but we want to spend some more words on point (3) which is often extremely critical: it deals with how the microfluidic device is connected to external components of the overall system. A system typically require multiple interconnects and in many cases the packaging components are much larger than the microsystems they interface[17,36]. A successful microfluidic interface must be reliable, mechanically robust, and leak free, allowing to introduce samples into or out of system without contamination. In addition, structural mismatches between the interface and microchannel, create easily dead volumes reducing the efficiency of the microfluidic platform. The difficulty derives from the fact that samples and reagents are typically loaded in quantities of microliters to milliliters, whereas microfluidic devices consume only nanoliters or picoliters of samples/ reagents due to the micrometric size of reaction chambers and channels. But the primary requirement is the creation of a sealing channels network to form flow conduits[22]. The fundamental trade-off here is between cell accessibility for downstream assays, which requires reversibly sealed or puncturable channels, and channel robustness, which implies a permanently sealed channel. Though numerous passive and active microfluidic components have been realized, the microfluidic interface remains an area that is relatively overlooked and under-studied. As a matter of fact, a reliable robust packaging is crucial for the success of microfluidic perfusion culture systems and has been identified as one of the great limiters on acceptability of mycrosystems into the broad world market[17,22].

2.5 Active and passive pumping.

Transporting fluid volumes in microfluidic cell culture devices can be done by active pumping, using valves and pumps that are either externally linked up to the device, or directly built into the system. Many perfusion systems employ external pumps connected to the access ports via tubing to pump constant fluid flow for nutrients supply and waste products removal (e.g., syringe pumps for non-recirculatory flow, and peristaltic roller pumps for recirculatory flow) [11]. Multilayer soft lithography, with multiple layers of structures in soft materials, make possible to assemble pneumatic valves into the system to produce fully automated, high-throughput culture systems[40].The large number of connections required and the potential for leakages at those connections are the major issues with these integrated components. A microfluidic plaform that utilizes programmed movement of arrays of pins of a Braille display as integrated valves and pumps was developed by the Takayama group [11,17,41]. Valving action is generated by movement of a Braille pin up and down to deform thin PDMS sheet into microchannels in specific sequences to generate peristaltic flow. A new method of fluid replacement was introduced by the Beebe group in the early 2000s [42].The surface tension of different-sized droplets placed at the inlet and outlet ports is exploited to drive fluid from one port to the other[42].The differential pressure between ports due to the difference in droplet volumes generates flow in the microchannel. Passive pumping can be performed simply by pipetting the appropriate volumes of droplets at the ports, without need for interconnections to external interface.

The ability to design experiments in a simple way with ”tubeless” microfluidics may attract an increasing number of biologists but it is limited to low volume flows and low pressures. Steady continuous perfusion of microchannels is better controlled by external pumps[11]. Alternatively, electrokinetics is now studied in a variety of forms for controlling microflows. Electro-osmosis, where the fluid moves relative to stationary charged boundaries, dielectrophoresis, which moves an interface (often a particle) in a gradient of electric field, and electrowetting, where the electric field modifies wetting properties, have all been exploited. AC and DC fields can be considered and the system response then depends on frequency and amplitude of the field [17,35].Other means can be used to control flows. In particular, external fields can be used to induce motion of objects embedded in the fluid, or the channel walls can be systematically distorted: magnetic fields can influence flows directly or manipulate dispersed magnetic particles, sound fields can produce acoustic streaming motions,cyclic deformation of a wall can induce peristaltic pumping, etc. For each manner of driving a fluid motion, the surface characteristics of the device can also be exploited to provide additional control for optimal mixing, reacting, detecting, analyzing, and separating.

3 Control of the *in vitro* microenvironment

The most influential benefit of using microfluidics for biological applications is the ability to engineer the cellular microenvironment. Cues of the microenvironment are integrated by the cell and affect cellular phenotype (i.e.,gene expression and cell behavior), while at the same time the cell can also affect its microenvironment. The balance and interplay between the microenvironment and cellular phenotype is therefore an important and complex problem in cell biology[39]. Microfluidics can be exploited as a platform for microenvironmental screening to study cellular response to the microenvironment by helping to create a map between environmental conditions and cellular phenotype. Some applications search for ways to produce phenotypes of interest. For example, it could be useful to develop optimal conditions for stem cell self-renewal in order to expand cell resources for use in cell based therapies. Other approaches aim to identify the mechanisms responsible for the produced phenotypes. Using the same stem cell analogy, they can evaluate signaling pathways by self-renewal of cells induced by extracellular cues for insight into development and disease. Moreover, microenvironmental screening could give a contribute in the identification of biochemical targets for therapy development, by exploring which in vivo environmental conditions are related to disease (e.g in tumor microenvironments). Other types of screening have been applied to observe the effect of a large number of parameters one-at-a-time (e.g., the use of chemical libraries on particular cell phenotypes in drug screening) or control a single parameter on many different readouts (e.g., micro-arrays for gene expression).The simultaneous combination of multiple microenvironmental factors such as soluble molecules, extracellular matrix (ECM) composition, and cellcell contact have been relatively less explored. Therefore the core of spatial and temporal modulation of the cell growth and stimulation is to integrate nanopatterned surfaces mimicking the complex features of the extracellular matrix, with microfluidic channels

regulating transport of fluids and signaling by soluble factors [12].

3.1 Microfluidcs for precise spatio-temporal chemical concentration gradients: chemotaxis study

Gradients of soluble signaling molecules play an important role in mediating a number of physiological processes in vivo such as chemotaxis, proliferation and differentiation, axon guidance and embryogenesis. Insight into the interplay between a chemical gradient treatment and the corresponding cellular response may help to determine the cues responsible for regulating specific cellular activities. Traditional macroscale devices (e.g, Boyden chamber, Zigmond chamber, Dunn chamber, under-agarose assay and micro-pipette assays) generally lack the ability to maintain and manipulate stable gradients, have poor reproducibility and require a large number of cells [17,38,43]. Additionally the incompatibility with real-time optical imaging methods further limit the application in modern biological experiments. Understanding the importance of these chemical cues may help researchers develop controlled microenvironments where the desired cellular response is produced by combining the effects of exogenous controlled gradient treatments with ongoing endogenous cell-cell signaling.

Microfluidic technology has brought about a broad range of methods for exposing cells to engineered gradients (time invariant gradients, subcellular resolution gradients, continuos or discrete gradients, fast response dynamic gradients), each method having a unique set of attributes and disadvantages. In general, gradient-generating microfluidic platform can be divided into two classes: laminar flow and flow-free based systems. The first class create chemical gradients by taking advantage of diffusional mixing across the interface of adjacently multiple laminar flows in a single channel or microchannels network. Diffusion between the stream produce a gradient across the channel, perpendicular to the flow direction having a diffusing profile that is stable at every fixed point in the channel but evolved along the channel. The laminar nature of fluid flow through microconduits permits many streams containing not only different substances but also different concentrations of the same substance to flow side by side. With these devices, it is possible to treat a cell population with a controlled chemical gradient and observe the biochemical and morphological responses in vitro. The continuously flowing streams of fluid provide precise control over the stability, gradient profile, concentration range and slope of a chemical gradient. The stimulus of interest can be changed on the fly to create a sequential chemical gradient treatment scheme. Gradient generation typically yields close agreement with theoretical predictions and allows gradients with defined simple or complex shapes to be produced and manipulated. The second type generates time evolving or stable gradients by free-diffusion of chemicals in a free-flow environment such as membrane-based and hydrogel-based assay. In cell motility and migration studies, special attention has been paid to chemotaxis studies, in many areas of biology, including microbiology and immunology[15]. Many cell types exhibit the ability to sense certain chemicals and move, or taxis, toward higher concentrations of chemoattractants known as chemokines. As an example, leukocytes respond to chemokines released from damaged cells.

Chemotaxis studies require a way to deliver chemicals to cells in a controlled gradient because cells are responsive to concentration differences down to 2% across their diameter to direct their motion. Spatial complexity and localization in intracellular systems is important in chemotaxis and polarized signaling and could thus benefit greatly from the high-specificity of microfluidics. Modeling approaches to chemotaxis signaling have demonstrated robustness and adaptation as properties of the bacterial chemotaxis signaling system. Jeon et al. [44] developed a microfluidic device capable of creating a single cell-resolution concentration gradient by feeding a small number of input fluid streams with initial concentrations of diffusible substances . The system consists of an embedded network of microchannels with two main regions: the gradient generation region that is a pyramidal branched microchannel structure to split, mix and combine fluid streams as they flow through and the observation region in which cells are placed and analyzed. Initially, a network of serpentine channels allowed adjacent laminar streams to flow together long enough for full diffusive mixing. As the streams travel down the network, they are repeatedly split, and some combined with neighbouring streams and allowed to mix by diffusion within a channel before being split and combined again. At the end of the network, many individual streams of different concentrations were flowed adjacently into a single outlet that will have a complex concentration profile perpendicular to its flow direction. If the bottom level output channels are not converged into a single outlet, the device could be used to create and isolate several differently concentrated mixtures of the input streams. The gradient shape was adjustable in width and shifted from side to side by changing the individual input stream flow rates, which can be performed dynamically. With little modification, both linear and nonlinear concentration gradients were realized.

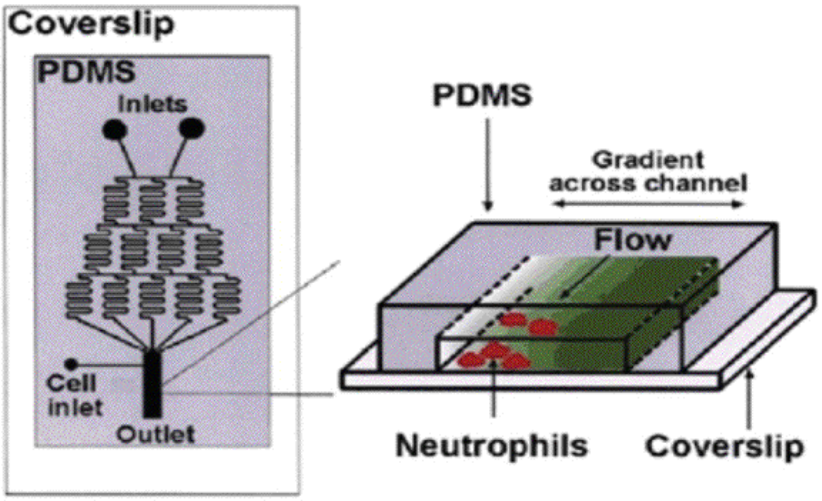

Figure 2: *Schematics of the chemotaxis assay.* Left: Top view of the embedded network of microchannels with two main regions: the gradient generation region that is a pyramidal branched array of microchannels to split, mix and combine fluid streams as they flow through and the observation region in which cells are placed. Right: 3D exploded view of the gradient generation through the PDMS microfluidic platform bonded to a glass coverslip. Courtesy of [43.]

Chemotaxis of human neutrophils (immune cells that migrate to sites of infection or injury) in interleukin-8 (IL-8) gradients and breast cancer cell chemotaxis was investigated using the gradient generator (Figure 1).

The continuously flowing streams that are necessary to maintain chemical gradients make the type of devices in Figure 1 unsuitable for addressing biological questions where soluble factors are important in regulating cell behavior. One way that cells respond to chemical cues in their environment is by secreting signaling factors that either affect the secreting cell itself (autocrine), or affect other types of cells (paracrine). In flow based systems, autocrine/paracrine factors cannot accumulate because flowing fluid streams immediately carry away secreted factors. In situations where cellcell communication (via soluble factors) plays a critical role in regulating biochemical activity, the removal or accumulation of secreted factors may lead to distinctly different cellular behavior. Beebe and coworkers [45] monitored neutrophil chemotaxis under concentration gradients formed statically, i. e.,without flowing fluid . The characterization of this system is an essential step that lays the foundation for quantitative use of this device to determine when cellcell communication (via soluble factors) is an important consideration. The chemical gradient is maintained by using a source/sink construct that includes a high fluidic resistance membrane. Chemical species diffused into a cell microchamber through a membrane which imposed high fluid resistance to minimize convective flows. In this manner, soluble cues including autocrine factors and paracrine factors were not washed away with a laminar flow but rather accumulated in the microenvironment. This design allows for examining the influence of cellcell communication on chemotaxis or on other cell responses to chemical stimuli (Figure 2).

Flow-free microfluidic systems also offer intriguing opportunities for the study of processes such as cell division and migration, intercellular communication and the emergence of cell polarity during development (where molecular gradients are known to play an important role). For instance, cell proliferation studies on a number of different cell types have revealed that the proliferation characteristics are markedly different when using microchannels instead of traditional mass culture systems. To understand this difference in behaviour, consider the rather different environments experienced by the cells: in the constrained medium within a microchannel, signalling molecules produced by a given cell (autocrine signals) or surrounding cells (paracrine signals) can accumulate, whereas such signaling molecules will be diluted or even removed by the convective flows that inevitably arise in mass culture systems or flowing microfluidic systems. Culturing in microchannels in the absence of flow, where transport is purely by diffusion and the size of the system prevents extensive dilution, appears to increase the sensitivity of proliferating cells to the effects of soluble factors. Similar effects may explain why the efficiency of embryo development improves in microchannels under no-flow conditions. So microfluidics should open new opportunities for studying cell signaling, where convection-free culture conditions allow signalling molecules secreted by a cell to form diffusive layers and influence the secreting and surrounding cells [3].

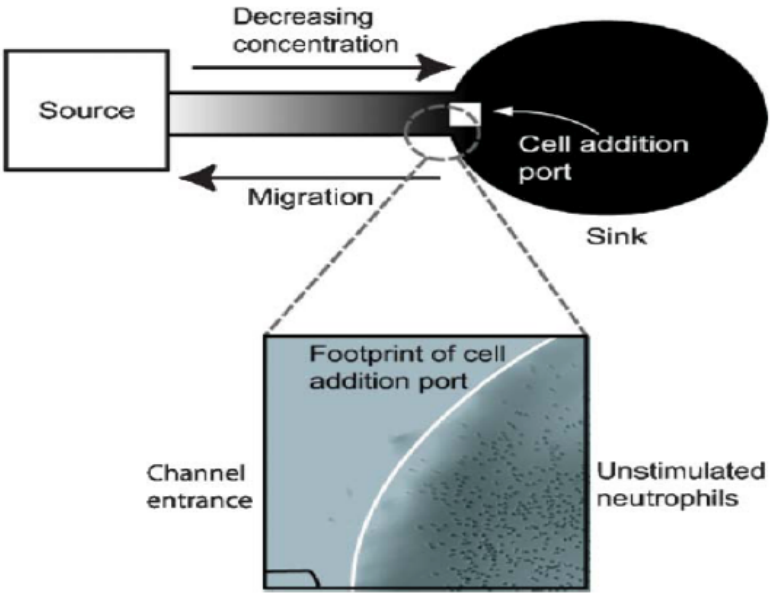

Figure 3: *Schematics of gradient-generation microdevice.* The chemical stimulus of interest is added over the membrane in the source region. The molecules diffuse through the membrane and into the channel creating a chemical gradient along the axial (x) direction of the channel. Reproduced by permission of The Royal Society of Chemistry [45].

3.2 Engineering chemically and nanotopographic patterned substrates.

In standard cultures of adherent cells, cells are randomly seeded on the surface of the culture substrate. Chemical and topographic substrate surface patterning can control the size, shape and spatial position of cells anchored to a surface. Spatial control of cell adhesive substrates allow us to mimic signaling tracks which are naturally present in vivo on the ECM. For example, gradients of surface properties are used to study the behavior of motile cells or neuronal path findings. Moreover forcing cells to follow a given adhesion pattern can be exploited to study interplay between the geometrical constrains and cell behavior such as changes in cell polarity. Elastomeric microchannels can also be used as microfluidic templates to chemically pattern surfaces generated by delivering adhesion promoting proteins or cell suspension to desired regions of a substrate. In most devices, cells adhere on glass surfaces via incubation of the microchannels with ionic polypeptides like poly-lysine or proteins like fibronectin .In some systems cell binding domain are patterned on proteinresistant ("non-fouling") surfaces. Poly(ethylene glycol) (PEG) immobilized onto surfaces is widely used to confer high resistance to protein adsorption and cell adhesion[46]. Nanotopographic cues are a subset of substrate engineering, representing signaling modality to direct complex cellular processes including the specificity and efficiency of lineage-specific stem cell differentiation and tissue organization. Cells interact with native structures on the micron and sub-micron scale native ECM structures in many ways, often through a phenomenon known as contact guidance. This mechanism is an essential component in regulating cell migration, such as axonal guidance and growth cone motility. Synthetically nanofabricated topography can also influence cell morphology, alignment, adhesion, migration, proliferation, and cytoskeleton organization. Cell-nanotopography interactions also vary across cell type, feature size, and feature geometry as well. There are virtually an infinite number of potential combinations of cell types, biomaterial composition, and topographic feature arrangements.

Three basic nanotopographic geometries are nanogratings, nanopost and nanopit arrays. The nanofabrication of substrates with a long-range order across a wide range of feature sizes and geometries has been realized using a variety of methods including traditional photolithography, e-beam photolithography, and interference lithography. Nanotopography could be utilized in addition to other types of cell-biomaterial surface conditions including microcontact printed chemistries and bulk mechanical properties of the substrate. Bulk materials processing and nanofabrication strategies for many nanotopographic surfaces are typically fine-tuned for silicon, silicon oxide, polycrystalline silicon, and other inorganic material systems such as titanium. These substrates can be used directly or serve as masters for replica-molding of organic polymers such as PDMS, polystyrene (PS),

poly(methyl methacrylate) (PMMA), polycarbonate (PC), and poly(ethylene glycol) (PEG) for in vitro applications or biodegradable polymers such as poly(caprolactone) (PCL), poly(L-lactic acid) (PLA), poly(glycolic acid) (PGA), and poly(L-lactic-co-glycolic acid) (PLGA) for potential use in vivo. Advanced fabrication techniques and compatible materials must eventually be synergized as a means to integrate cell-nanotopography interactions into advanced tissue e engineering scaffolds[47]. An overview of these techniques can be found in Appendix 2.

4 Detection of biological processes in living cells

Nowadays micro and nanofabrication potentialities have been extensively used to improve our ability to measure biological quantities. The production of standard micro arrays for genomics and, more recently, proteomics, is based or take advantage from one or more of the lithographic processes described in Appendix 2. Probing the chemistry and physics in a living cell is one of the basic goals in modern cellular biophysics and an important prerequisite for progress in both molecular and nanomedicine, thus it has been one of the major research subject of the past few years. When we focus on the detection of living cells process, the most mature applications of nanotechnology, and probably the first ones coming to mind, deal with reading electrical signals from neurons, as in the case of Multi Electrodes Arrays (MEA)[49,50], or, more recently, Multi Transistor Array[51-53]). This is due to the enormous amount of expertise developed in electrodes and electrical amplifiers fabrication during the past forty years by microelectronic industry.

When dealing with cells other than neurons or when looking for biological processes not associated with electrical signals, optical and spectroscopic techniques are more general and record more complete information being able to detect changes at the molecular level. In particular, in the past decade there has been a significant increase in the use of vibrational spectroscopies (Raman and Mid-Infrared) as tools for single cell analysis thanks to their being minimally invasive and label free. These properties are useful particularly when looking at unknown processes activated by external agents as, for example, in drug-cells interaction studies or toxicology, or if fast cell screening is needed as in a biopsy where diseased cells must be discriminated from the healthy ones. However, Raman or Mid-Infrared (MIR) investigations of living cells are not complication-free because probe volumes are small and concentrations of intrinsic cellular molecules low. Moreover, just to make some examples, Raman scattering has very low cross section and, hence, signals. Thus, a trade off between the cell damage and applicable maximum intensity of the excitation laser, must be made. Moreover, MIR suffer from the lack of transparency of the most common materials used in biology (plastics, glass, water). Thus, as already stated, the choice of detection technique which will be used in our experiments is one of the four keystones for the design and fabrication of the devices to be used, and will determine many of its properties. Just to make some examples, the detection techniques will contribute to define the material to be used as a substrate (for example taking in account the transparency to certain electromagnetic wavelength), physical constraints of our measurements apparatus (parameters like the depth of focus or numerical aperture of our microscope, or the overall sizes allowed by the microscope stage) will define the final geometrical setup of our measurement tools). In the next two sections we will briefly introduce some applications of micro and nanotechnologies to enhance or permit the detection of cellular processes in in-vitro experiments.

4.1 Enhancing signals for vibrational spectroscopies: plasmonics[4]

Surface plasmon polaritons (SPP) are electromagnetic excitations propagating at the interface between a dielectric and a conductor, evanescently confined in the perpendicular direction. These electromagnetic surface waves arise via the coupling of the electromagnetic fields to oscillations of the conductors electron plasma. This means that if we send a laser beam onto a dielectric plate (glass, plastic, etc.) over which we have deposited a thin (few nanometers) metallic fim, at a certain angle between the laser beam and the plate surface, we can induce an oscillation of the metal electrons at a very specific frequency. Obviously, this phenomena subtract part of energy to the laser beam, and, if we look at the reflected laser beam, we will observe a diminution in the reflected intensity. Since the electron oscillation frequency is linearly connected to the laser/plate angle we are able to measure any change in the frequency oscillations just measuring at which angle we observe the minimum in the

[4]The interested reader can find detailed information about theory, fabrication and applications of plasmonic structures as biosensors in may textbooks and scientific paper. Most of the material of this section is derived from [54-56]

laser beam reflected intensity. In figure 4 a surface plasmon polariton excitation is schematized in the case of the dielectric plate substituted by a prism.

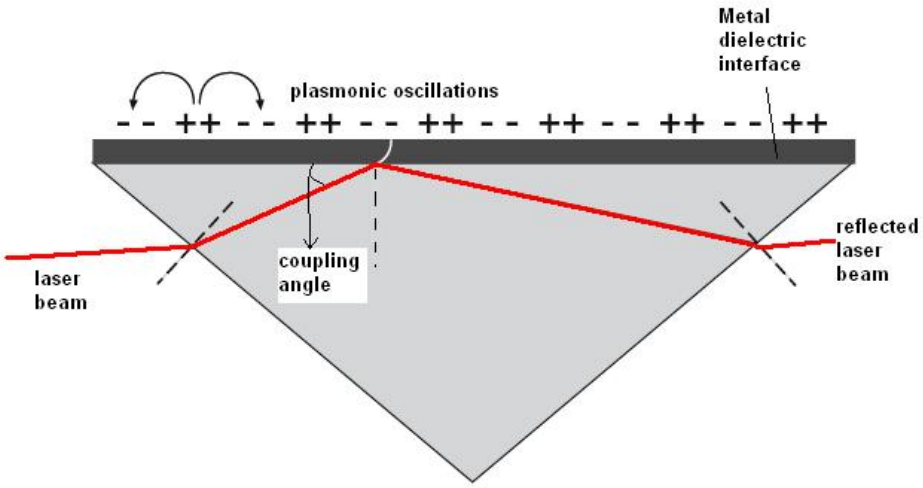

Figure 4: *Scheme of excitation of a metal electrons plasmonic resonance.* The laser beam make the electron oscillates, varying the electric field on the metallic surfaces indicated by the + and - symbols. Oscillation frequency is strongly related to the refraction index of the material in contact with the metallic film, thus the presence of a molecular film on the metallic surface can be detected monitoring the reflected laser beam intensity.

SPP are today widely used to realize commercial and laboratory biosensors (see for example [57-59]). In fact, most of surface plasmon sensing work carried out so far has been based on the interrogation of propagating SPP waves at a metal/air interface. Using surface functionalization, agent-specific binding can be achieved, changing the refractive index of the metal surface superstrate and thus the dispersion relation of the propagating SPPs. Binding events can then be monitored by studying the changing phase-matching condition via either wavelengthor angular interrogation. Many reviews of these techniques in a sensing context can be found in literature (see for example [60]).

Micro and nanofabrication plays a fundamental role in engineering plasmonic based biosensors, both in enhancing the coupling between the excitation laser and the metal plasma elctrons, by ad hoc structures (gratings) structures, and in enhancing the electrical fields on certain points of the surface. The second point is necessary to work with the second fundamental excitation of plasmonics: localized surface plasmons (LSP). Localized surface plasmons are non-propagating excitations of the conduction electrons of metallic nanostructures coupled to the electromagnetic field. These modes arise naturally from the scattering problem of a small, sub-wavelength conductive nanoparticle in an oscillating electromagnetic field. The curved surface of the particle excerts an effective restoring force on the driven electrons, so that a resonance can arise, leading to field amplification both inside and in the near-field zone outside the particle. This resonance is called the *localized surface plasmon* or *short localized plasmon resonance*. Another consequence of the curved surface is that plasmon resonances can be excited by direct light illumination, in contrast to propagating SPPs. One of the most spectacular applications of LSP to date is surface enhanced Raman scattering (SERS), which exploits the generation of highly localized light fields in the near-field of metallic nanostructures for enhancing spontaneous Raman scattering of suitable molecules. Using chemically roughened silver surfaces, Raman scattering events of single molecules have been recorded [61,62], with estimated enhancements of the scattering cross section by factors up to 10^{14}. The majority of this enhancement is believed to arise from the highly enhanced fields in metal nanoparticle junctions due to localized surface plasmon resonances. Termed hot spots, these highly confined fields also enable an increase of fluorescent emission, albeit with more modest enhancement factors. A proper understanding and control over the generation of these hot spots, for example in the form of nanoscale plasmonic cavities, is currently one of the major driving forces behind the design of nanoparticle ensembles with tuned optical properties both with nanofabrication and

chemical (top-down and bottom-up) techniques.

5 Mid Infrared Spectroscopy of Bio-systems

Infrared spectroscopy, and more specifically Fourier Transform Infrared Spectroscopy (FTIR) is widely used for probing the chemical composition and molecular structures of complex bio-systems such as tissues, body fluids or cells. A Mid-IR spectrum (MIRS) provides the vibrational fingerprints of the all constituents of a bio-system, namely proteins, carbohydrates, nucleic acids and lipids, in one single experiment. On the base of the specific biochemistry of the system under investigation, specific group vibration can be assigned primarily to one cellular constituent as detailed in many scientific reports and books [63,64] and schematized in Figure 5, referred to an eukaryotic cell. MIRS is a fast, label-free, non destructive analytical technique whose sensitivity have been exploited for diagnostic purposes, such as characterization of amyloid plaques in brain tissues of Alzheimers disease patients [65] and prion infected tissues [66], for monitoring cancer progression [67] and the drug-response of tumoral cells [68, 69], for the characterization of cell-cycle stages [70] just to give a few examples.

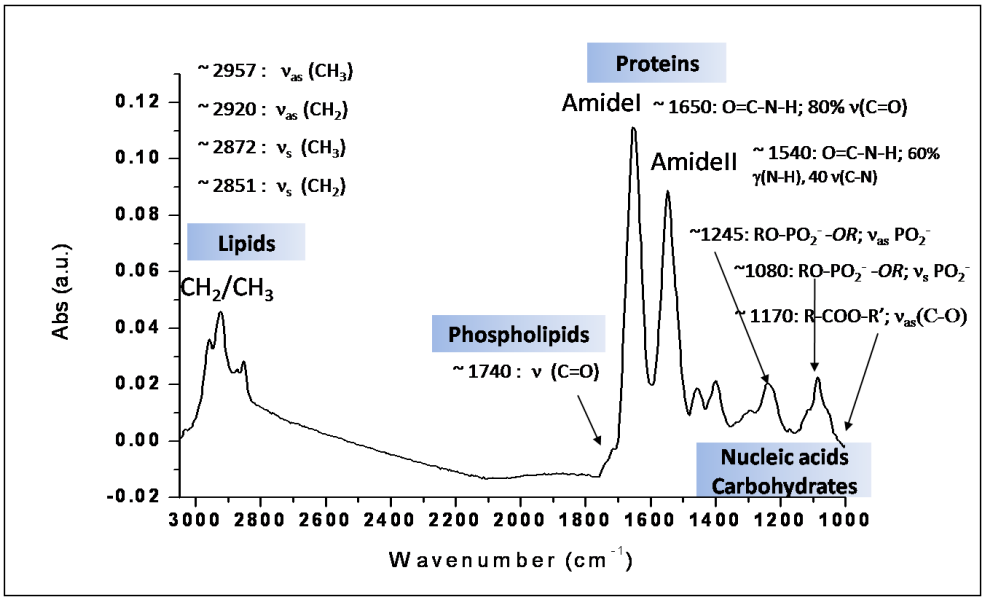

Figure 5: *Representative IR spectrum of an eukaryotic cell.* Bands of major interest for diagnostic purposes are shown: lipids, phospholipids, proteins, nucleic acids and carbohydrates. Band assignment of major functional bio-groups is also reported (ν=stretching; γ=bending; s.=symmetric; as.= asymmetric). During cellular processes the band structure changes revealing that something in the cell is changing. For example, during a differentiation process we first observe a strong change in the nucleic acid band (change in the chromatine structure, then increase the RNA band indicating a transcription process, then a change in the protein band)

However, the possibility to work at single cell and sub-cellular level at diffraction limit, maintaining an acceptable data quality in terms of signal to noise ratio, is nowadays guaranteed only by the brightness advantage of synchrotron radiation (SR) sources, defined as the photon flux per source area and solid angle [72,71]. The increasing number of infrared beamlines dedicated to biological applications is evidencing the great interest of the bio-medical community for this technique: at the beginning of the 90s, only two IR beamlines where operative in Europe while their number is today 10. Despite this, their number is still limited and the restricted access to SR facilities can not satisfying the huge community of interested scientists. In the next future broad band source based in mid-infrared quantum cascade lasers (QCL) [73,74] could transfor FTIR microspectroscopy (μ-FTIR) at the single cell level a competitive techniques to be used at the single biolab level. Nowadays, commonly measured samples by μ-FTIR are fixed or dried, which allow only acquiring *still frames* of the phenomenon under investigation. This is the feasible way to avoid the strong absorbance of water-based cellular media, since the compensation of the buffer contribution from collected spectra, for disclosing the Amide I band, limits the path-length of measurements cells to 10 μ m or less [75]. Due to these technical difficulties associated with living

cells measurements in physiological environment there are still only a few pioneering reports [76,77] where the fluidic chambers are realized by spacing apart 2 optical windows by using few microns thick plastic spacers. This approach cannot avoid seal problems and inaccurate pathlength control and also imposes limitations on the device design flexibility. The application of micropatterning and microfluidic concepts to IR transparent materials opens new opportunities for μ-FTIR spectroscopy of living cells. The implementation of both IR and visible transparent 3D microfluidic devices will allow the real time observation by μ-FTIR of biochemical rearrangements undergone by living cells upon chemical and/or mechanical stimulations. Moreover, by exploiting the brightness of Synchrotron Radiation (SR), diffraction limited spatial resolution can be achieved to collect individual cell spectra and chemical maps at diffraction-limited spatial resolution.

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Appendix 1
The cellular microenvironment

Cell reside in a complex milieu with 3D features instead of the 2D environment experienced in standard culture dishes. Although cell culture has been performed for over a century, many of the present tissue systems dont mimick the native microenvironment[8]. Thus, the ability to understand and recreate the local conditions for a cell is of great interest in areas such basic bilogy, drug discovery and tissue engineering. In general, cell behaviour and phenotype are modulated by intrinsic and extrinsic factors. The intrinsic factors are the internal genetic makeup defined by transcription factors and gene regulatory networks. Although intracellular events sometimes regulate cell behavior, much of the related signals are extrinsic and derived from the surrounding microenvironment [9]. Inside the body, cells are exposed to a controlled microenvironment with specific physicochemical properties (pH, oxygen tension, temperature, and osmolality) that is tightly regulated with respect to interactions with the surrounding cells, soluble factors, and ECM (extracellular matrix) molecules as schematized in figure 6. The spatial and temporal distribution of these chemical, electrical and mechanical signals constitutes the cell microenvironment.

The set of extracellular cues, integrated with intracellular signaling pathways, rules cell structure, function and physiology, and, ultimately, influences growth, development, and tissue regeneration. For stem cells, the local microenvironment, or stem cell niche, holds the key for regulating stem cell survival,self-renewal, and differentiation. In cancer biology, tumor and organ microenvironments can give rise to cancer cells that are conditioned for metastasis at ectopic locations[11]. With respect to biochemical components, cell signal each other through small molecules like hormones as well as larger molecules such as signaling proteins, including cytokines, growth factors and chemokines. Specific binding of these soluble signalling factors to cell surface receptors induces a signal transduction cascade transferring information to the nucleus and influencing gene expression.

Soluble factor signaling occurs mainly via autocrine (produced by a signal-releasing cell) and paracrine (by the surrounding cells) processes. Endocrine signaling relies more on convective transport of hormonal signals from distant locations in the body to the local microenvironment[12]. The effects of soluble factors on cell regulation depend on the concentration, half life, and receptor binding affinities of the ligand of interest. The complex fibrillar architecture of proteins on the outside of cells form the Extracellular matrix (ECM), which provides

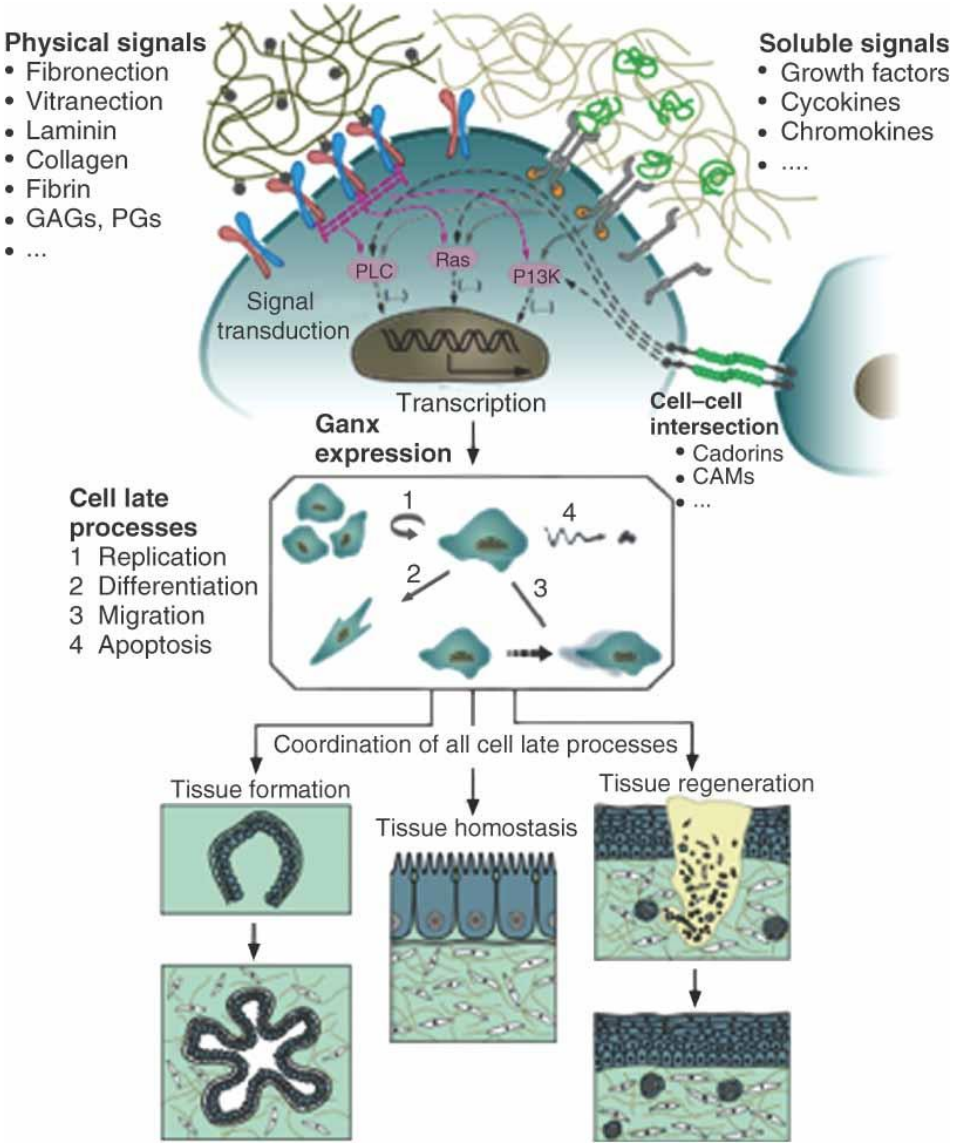

Figure 6: *The cellular microenvironment* Cellular processes are regulated by intracellular signaling pathways and by the extracellular microenvironment. Cells are exposed to spatial and temporal variations in these local extracellular cues,such as: soluble signaling molecules and dissolved gases, the chemistry and mechanics of the extracellular matrix, and the proximity and behavior of neighboring cells. Cells are continually faced with the complex tasks of sensing these inputs, processing the signals through complex signal transduction and gene regulation networks. Reproduced with permission from Ref 9. Copyright 2005 Nature Biotechnology) [10].

a structural scaffold to which cell can anchor and generate tissue. ECM components are organized in nanotopographic structures presenting mechanotransductive cues that contribute to local migration, cell polarization, and other functions. Cells sense ECM features via cell-surface receptors transducing mechanical signals into chemicals ones from focal adhesion sites to the cytoskeletal machinery [13].

Direct cell-cell interaction is another important mechanism of cellmicroenvironment communication. Neighbouring cells of similar (homotypic events) or different type (heterotypic) can realize long-lasting mechanical and biochemical connections to each other through adhesion proteins that interact with the cellular cytoskeleton. Homotypic interactions stabilize epithelia, which typically lie upon a sheet of specialized ECM called the basement membrane. Heterotypic interactions govern many normal cellular phenomena including embryouterine implantation, immune surveillance, cell migration during embryogenesis, and neurotransmission. They also characterize

the pathological states of cancer metastasis, rejection of transplanted tissues and organs, and inflammations[9].

Appendix 2
Top-down micro and nano scale technologies for biochemical and cell biology research

The typical tools of a nanofabrication facility can be classified into two categories, those that precisely engineer matter (etching and depositing) and those that define the shape of the elements to be engineered (patterning, mainly lithographic techniques). Although most of the top-down techniques were initially developed for the semiconductor industry to fabricate integrated circuits, they have been adopted and modified to manufacture a large variety of tools and materials for biological research. Integration of glass and silicon technology with lithographic approaches for processing soft materials is at the heart of many labs-on chips and has been a driving force for the development of new types of microsystems combining electrical and mechanical functions with microfluidic functions (valves, pumps, microwells, microchannels). The use of plastics and polymers is now a strong component of micromanifacturing and opens new prospects in terms of cost, flexibility and performance gain by making a complete new range of materials with a wide variety of properties accessible. The following is a brief overview of the state-of-the-art of the different technologies used for fluidics, biochemical patterning and biomedical applications, intended for the purpose of introducing terminology. For more detailed coverage on traditional microfabrication methods see [79].

Microfabrication methods: Photolithography

Photolithography is historically the most widely used micropatterning technique; the size of the features can be precisely controlled (depending on the photomask resolution) down to micrometer dimensions, a size domain comparable or smaller than a single cell. The working principle is basically an extension of photography. The photolithographic process consists of a number of steps in which a desired pattern is generated on the surface of a substrate through the selective exposure of regions of a material sensitive to ultraviolet (UV) light. Figure 6 summarizes the main steps followed in photolithography[78-80].

The resulting photoresist patterns can be used as lift-off masks for patterning of biomoleculese.g., to create cell-repellent and/or cell-adhesive substrates. The material of interest is deposited on the photoresist pattern, and the photoresist is lifted off (often by sonication in acetone) leaving the desired biomolecule pattern. This approach has the drawbacks that exposure to organic solvent (if the photoresist is to be removed) leads to denaturation of most biomolecules, which may or may not be a concern, and makes it incompatible with many polymers widely used in cell culture (such as polystyrene, which dissolves in acetone). Although water-soluble photoresists exist, there is still a concern that photoresist residues may be left on the surface if the surface cannot be cleaned thoroughly after processing, because it is partially covered by biomolecules.

Nanofabrication methods:Electron Beam Lithograpy

The heart of the top-down approach of miniaturization processing is the nanolithography technique, such as Electron Beam Lithography (EBL), Nanoimprint Lithography (NIL), X-ray Lithography (XRL), and Extreme Ultraviolet Lithography (EUVL). Among them, the EBL approach is the front-runner in the quest for ultimate nanostructures due to its ability to precisely focus and control electron beams onto various substrates.

EBL is the principal nanofabrication technique used to create features at the nanoscale level on a material by exposing electrically sensitive resists to an electron beam. It utilizes the fact that certain chemicals change their properties when irradiated with electrons just as photographic film changes its properties when irradiated with light. Additionally, its precision and nanolithographic capabilities generally make it the tool of choice for making masks for other advanced lithography methods[80,81]. An EBL instrument is a result of working a scanning electron microscope (SEM) in reverse, that is, using it for writing instead of reading. Its view field and throughput are, therefore, limited by the nature of this working principle. Similar as in the SEM, an EBL instrument consists of three essential parts: an electron gun, a vacuum system and a control system. Compared

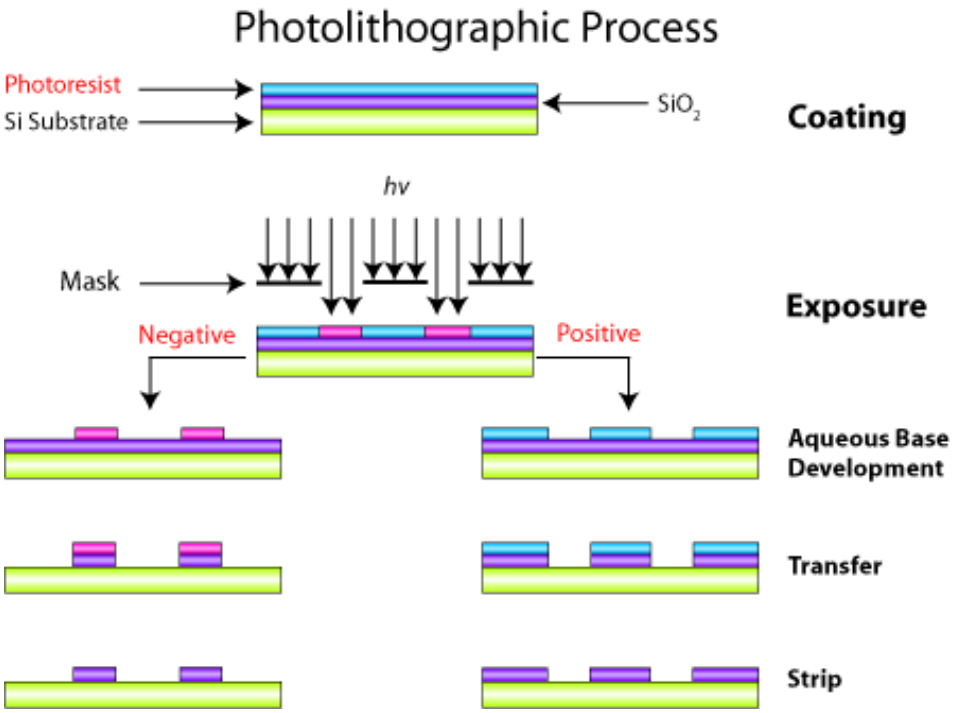

Figure 7: *Photolithographic process.* Generally, photoresist solution is dispensed onto a flat substrate, usually a silicon or glass wafer, spun into a thin film, and dried. When this photosensitive layer is exposed to UV light through a photomask, a transparent plate with the desired opaque metallic film (usually chrome) pattern on its surface, the regions of the photoresist exposed to the light undergo a chemical modification. Depending on the type of photoresist utilized, the photoresist polymer will undergo one of two possible transformations upon exposure to light. In the case of a positive photoresist (by defi nition), the irradiated polymer molecules break down and become much more soluble in a specific developer solution than the unexposed regions. On the other hand, in a negative photoresist (e.g., the widely used SU-8 photoresist developed by IBM to produce tall structures), light induces photochemical crosslinking of the photoresist, which renders the exposed regions virtually insoluble in the developer.

with other lithographic instruments, the use of electron guns to scan a material and form the desired pattern is the core characteristic of the electron beam lithography technique.

The principles of electron guns can be understood by analogy with light rays optics. The electron optical elements act simply as their optical counterparts. Based on the physical laws of electron emission and the desired energy conversion at the work point, almost all guns are of similar design, although they might differ widely with respect to beam power, acceleration voltage, and electron current. In the gun, free electrons are first generated from emitters, or cathodes, and are then shaped into a well-defined beam, which is ultimately projected onto the work point. Magnetic lenses are used to focus the beam. Commonly used electron sources are thermoionic emitters and thermal field emitters which have outputs in the range of 1 to 200 keV, but are most commonly used in the range of 50100 keV[82].The resolution obtained through this type of lithography is greatly influenced by the beam spot size. Specimen position and beam characteristics are electronically controlled to achieve the desired nanoscale resolution. With computer control of the position of the electron beam it is possible to write arbitrary structures onto a surface, thereby allowing the original digital image to be transferred directly to the substrate of interest. Various researchers have demonstrated resolutions on the order of less than 10 nm.

Soft lithography

The patterning on nanoscale dimensions using conventional techniques means higher equipment cost, and going to smaller dimensions requires scanning beam serial techniques, which have a low throughput. The unconventional methods provide an alternative way to fabricate micro and nanostructures because instead of radiation exposure they use physical deforming or material transfer to replicate the patterns. One of these alternative lithographic techniques is called soft lithography. It was pioneered at Harvard University by Prof. George White-

sides to enable replication and pattern transfer on multiple length scales (from nanometers to centimeters). The term broadly refers to a suite of sister techniques based on using a patterned elastomeric polymer as a mask, stamp or mold, to pattern soft materials(for example, polymers, gels and organic monolayers, rather than silicon and glass)[83]. Soft lithography offers overcomes to many of the shortcomings of photolithography and other conventional microfabrication techniques for applications in which patterning of nonplanar substrates, unusual materials, or large area patterning are the major concerns. In particular, as a technique for fabricating microstructures for biological applications, it offers the ability to control the molecular structure of surfaces and to pattern the complex molecules relevant to biology, to fabricate channel structures appropriate for microfluidics and to pattern and manipulate cells.

For the relatively large feature sizes used in biology, parallel production of prototype patterns and structures is convenient, inexpensive and rapid. The fabrication of complex functional nanostructures using soft lithography techniques will, however, require a further understanding and control of several issues. They include (a) distortion or deformation of polymer nanostructures, (b) optimization of conditions for pattern transfer and replication of nanoscale features and c) registration of nanoscale features in soft materials for multilayered patterning. The microanalysis systems that can be fabricated using soft lithographic techniques are broadly applicable to (a) rapid and low-cost manufacturing of patterned arrays of the structure needed for high-throughput screening, (b) proteomics (when combined with surface engineering), (c) studies of cell biology (e.g. the cell cycle and stem cells) and (d) studies of cocultures for tissue engineering.

Usually the first step in soft lithography is the master fabrication and it needs the use of conventional lithography, so is not independent of cleanroom facilities and high-cost equipment. A silicon wafer with polymeric structures patterned on its surface is typically referred to as the master copy, or simply the master and is used as the negative for replication. It can be fabricated by photolithography, or if features below 1 μm are needed, by EBL . High-cost, high resolution, slow-throughput equipment can be used in the master fabrication, and low-cost soft lithography in replication of the features of the master. So the strength of Soft lithographic techniques is rather in replication :you can produce numerous molds and replicas from the same master and thus provide nanofabrication capabilities not commonly available in academic research laboratories. The core set of soft-lithographic techniques, organized as proposed by Gates et al. [83] are the following: replica molding, embossing and printing to clarify the differences among them and to describe the characteristics that make them useful in cell biology. Replica molding is any process that transfers topographical features from a rigid or elastomeric mold into another material by solidifying a liquid in contact with the original pattern.

The tools of soft lithography

Replica molding

Replica molding is a technique for duplicating the shape, size and pattern of three-dimensional features in a single step on a master and provides a method of patterning materials than are not possible to pattern using photolithography. The most frequently used replication technique in microfluidics at the moment is undoubtedly the casting of poly(dimethylsiloxane) (PDMS), a transparent silicone rubber (see Figure 8).

An excellent combination of properties have made PDMS one of the most prominent materials in microfluidics[84]. It has a Youngs modulus that makes it a moderately stiff elastomer (1 MPa). In the visible and near-UV (below 280 nm) regions, transparent PDMS is suitable for optical visualization and spectroscopy to monitor the interior of PDMS device. Its low surface free energy enables easy release from many surfaces. PDMS can replicate submicron features with high fidelity, can form a reversible seal with smooth surfaces (PDMS, glass, silicon, polystyrene, polyethylene, and silicon nitride). From the biological point of view, PDMS is non-toxic and can thus be suited for cell cultivation and in-vivo devices. PDMS is also permeable to gases, which is enough to supply cell cultures with the necessary oxygen and to fill dead-end channels. PDMS contains surface-exposed methyl groups (-CH3) and so is intrinsically hydrophobic, but its surface can be rendered hydrophilic by brief exposure to a oxygen plasma to generate reactive silanol groups (-SiOH) at the surface. Channels formed by conformal contact contain a weak physical seal between the two layers of material (e.g the PDMS and a glass slide) and liquid can escape from the channels if the fluid is put under pressure. To obtain an irreversible bonding freshly oxidized PDMS is brought into contact with clean, oxidized glass (or oxidized PDMS) that produce Si-O-Si bonds between the layers. The bonding of PDMS to glass (or PDMS) makes it possible to form sealed microfluidic devices in which fluids can be pumped at pressures as high as 50 psi (350 kPa) without failure.

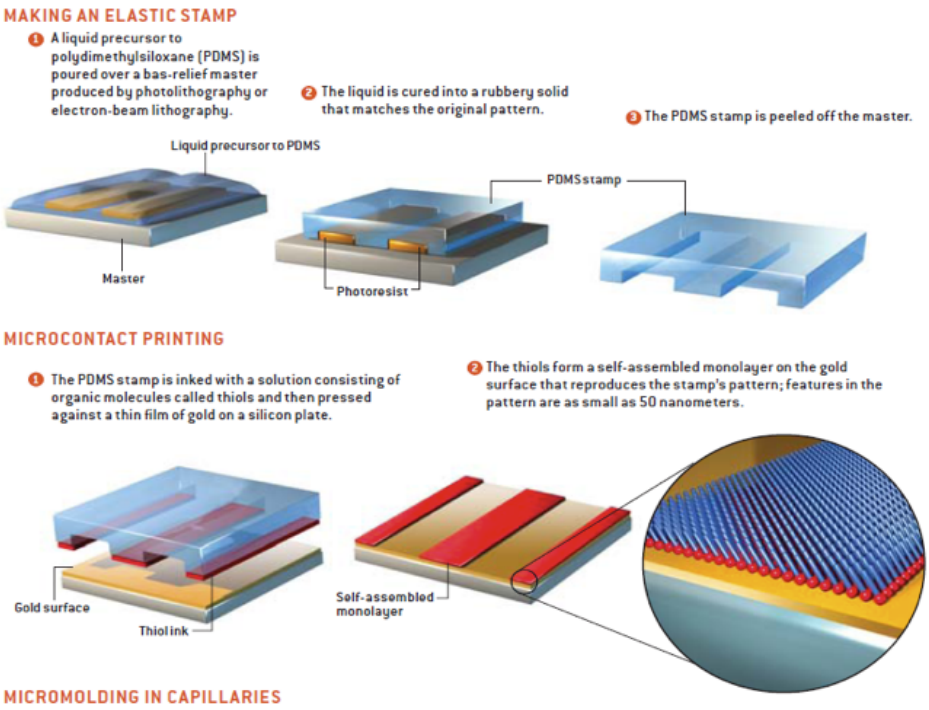

Figure 8: *Printing and molding process of soft lithography [46]* Upper panel: The process begins by manufacturing of the mold ,frequently by bulk machining of silicon or by thick SU-8 photolithography. A two-part mixture containing PDMS pre-polymer and its curing agent (suggested ratio of 10:1(v/v) is poured over the positive relief master and allowed to cure turning the liquid into rubber. For all microfabrication purposes it is important to degas PDMS in a dessicator to remove air bubbles before use. After cross-linking,the elastomer is carefully peeled off the substrate , access ports are then bonded to a flat substrate, enclosing microchannel structures. The master mold can be reused multiple times to replicate devices. Thus, this method is especially desirable due to its low cost, flexibility, and rapid prototyping. Bottom panel: The transferred molecules, or inks, are coated onto the stamp before contact with the substrate and deposited in a pattern defined by the raised surfaces of the stamp. The stamp is then brought into conformal contact with the surface for a period ranging from 30 s to several minutes, depending on the application. A large variety of types of molecules (including small biomolecules, DNA[85], proteins[86], polyelectrolytes and suspensions of cells) can act as inks and have been patterned directly on a number of different substrate using CP .

Microcontact printing

Microcontact printing (CP) is a soft-lithography technique used to transfer chemical and biological molecules onto a surface using a topographically patterned elastomeric mold or stamp, which is typically fabricated from PDMS (see Figure 7).

The CP process is attractive because of the ease and low cost of generating the stamp with commercially available precursors. However,some of the disadvantages include the blurring of the pattern by lateral diffusion of the ink, the distortion in the pattern that reflect deformations in the stamp and the substantial number of defects in the film [87-91]. The best-known and extensively studied system for this technique is the printing of self assembled monolayer (SAM) of alkanethiolates (SH-(CH2)n-X) onto the areas of noble and near-noble metal films (e.g gold, silver, palladium, platinum)[92]. SAMs are structures generated by the spontaneous chemisorptions and self-organization of alkanethiols. Alkanethiols with long alkyl chains (n = 16 or 18) form hydrophobic monolayers, whereas those with different terminal functional groups (X) can form hydrophilic, hydrophobic or charged SAMs.

Many synthetic methods are available for attaching ligands to functional groups (X) on SAMs. If required by the intended application, the substrate can be dipped into a solution of a second alkanethiol to form a different SAM on bare regions of the metal surface that were not in contact with the stamp. This technique has been used to make islands of SAMs that adsorb proteins and cells, and which are surrounded by SAMs that resist the adsorption of biomaterial. Other examples of applications of CP are silanes on oxides or alkyl phosphates on

metal oxides and proteins on silicon, glass or polystirene. The ability to engineer the properties of surfaces with spatially defined pattern with SAMs has made it possible to study biological processes, such as cellular responses to various adhesive environments, that occur at interfaces as well as to conduct miniaturized and high-throughput assays [93]. Most current biological uses of micropatterning require features with sizes below 100 nm.

Nanoimprint lithography

Nanometer surface topologies can be fabricated by imprint lithography (about 5 nm minimum feature, 30 nm resolution)[94]; in addition, it is possible to control the spatial distribution of chemical species on the structured surface. Nanoimprint lithography (NIL), also called hot embossing, is the term coined for a procedure that transfers a pattern using a hard rigid master to physically deform a solid polymer film coated on a rigid surface. The material, typically a polymer, is heated above its glass-transition temperature (e.g., 90200C), while the master is pressed against it (e.g., 50130 bar). The polymer is deformed filling the voids in the master, then the mold is removed after cooling the substrate to below this glass-transition temperature (Tg) revealing a pattern that is the inverse of the master). Imprinting can replicate structures with aspect ratios up to 10:1 and as small as 10 nm. Pattern transfer takes about 5 to 10 min (or sometimes longer) depending on the efficiency of heat transfer within the system. Photolithography is typically used to generate the surface structure but is limited in applications where bio-specific adsorption and functionalities are required to mimic the microenvironments for cellular development. Thus, imprint methods are useful because they permit direct construction of a variety of shapes with varying physicochemical properties and are accessible methods for academic research.