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# Nanopatterned Surfaces for Biomedical Applications

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## 1. Introduction

### 1.1 Nanotopography and stem cells

Topography was first identified to influence cell behaviour as early as 1911 when R.G. Harrison observed the guidance of cells along the fibres of a spider's web (Harrison 1911). Since this discovery, investigations into the topographical effect on cell behaviour identified that topography can have not only a strong affect on cell morphology, but it can also influence cell adhesion, proliferation and gene expression.

The development of biomaterials has lead to the generation of tissue engineering, whereby a combinatorial approach is utilized, merging elements of biology and engineering with the overall aim to develop functional tissues. The first generation of biomaterials were developed to be bioinert and provide mechanical support, the next generation were designed to be bioactive (elicit a desired cell response) and third generation biomaterials will need to provide reproducible influence of cells at the molecular level (Hench and Polak 2002). The inclusion of factors such as topography may allow this reproducible level of molecular influence to be incorporated into materials that are e.g. biodegradable and/or load-bearing without sacrificing their engineering role.

It is interesting that nanotopography appears to have as strong an influence on cells as microtopography (Dalby, Riehle et al. 2002; Teixeira, Abrams et al. 2003; Curtis, Gadegaard et al. 2004) as it is on the same size scale as the cell receptors rather than the whole cell. It is this rather bottom-up organisational approach with cells being e.g. aligned by nanogrooves one adhesion (containing integrin receptors) at a time rather than the top-down alignment of microgrooves where the whole cell has to conform to the feature and the adhesions will then follow. Importantly, current research has gone on to show that nanotopography has the ability to elicit specific cues and promote the controlled differentiation of stem cells *in vitro*. The use of stem cells to potentially generate patient-specific tissues using biomaterials provides huge scope for their use in regenerative medicine. A pioneering and historic perspective has been published by Curtis (Curtis 2004).

The ability to produce such topographical substrates has largely come from fabrication techniques that are routinely used within the electronics industry. These techniques include photolithography and electron beam lithography, to produce precise, reproducible nanoscale topographies. As technology has advanced within this field, it has allowed the production of increasingly smaller feature sizes; currently electron beam lithography enables the production of feature sizes down to approximately 5 nm (Vieu 2000). Injection

moulding further provides a viable platform for the fast, relatively inexpensive polymer replication of many identical topographical substrates produced by such techniques. Other techniques for producing nanotopographical substrates include, polymer phase separation and electro-spinning; these, however, produce more random topographies.

These nanoscale topographies have gained more prominence in terms of biomimetic comparison as *in vivo* nanotopographical patterns of tissues and their biological implications has become more widely acknowledged. It is the potential to replicate these features and thereby their biological properties *in vitro* that holds great potential. Typically within a tissue there is a hierarchy of features, for instance in bone, the bone tissue itself is in the macro scale, with fibrillar structures at the micro scale and then nanometer scale interactions such as protein: protein. This interaction of proteins and cells is hugely important; binding of integrin receptors to the extracellular matrix (ECM) form what are known as focal complexes, points of attachment between a cell and the extracellular matrix. The disruption or alteration of these focal complexes may have a two-fold effect altering cell signalling, gene expression and ultimately differentiation. This can be either indirectly influenced via intracellular signalling of focal adhesion kinase and activation of downstream molecules in a signal cascade (McBeath, Pirone et al. 2004; Kilian, Bugarija et al. 2010), or directly influenced via changes in the cytoskeleton and nucleoskeleton leading to alterations in gene expression via changes in chromosomal packing and positioning (Dalby, Biggs et al. 2007).

## 2. Micro- and nanofabrication technology

A major leap in investigating cell response to topographic features was made possible by the continuous development of semiconductor technologies such as lithography and etching techniques. With these new techniques in hand it was now possible to design and manufacture various patterns with very specific dimensions. It quickly became clear that cells responded to features in the micron range and thus sparked the question of how small dimensions cells can detect. At that time, the technology required to fabricate patterns in the sub-micron range was immature and it was not till the 1990s that electron beam lithography (EBL) successfully was deployed to make such patterns. The strength of EBL is its maskless properties where any micron and sub-micron shape can be realised, in contrast to all other lithography techniques. The results from the EBL patterns clearly showed that cells can respond to features as small as 15-30 nm which is comparable to the size of individual proteins or cell receptor ligands. In the early days of cell engineering, most of the results were realised by optical and electron microscopy. This provided important information on a single cell level. In turn this meant that the requirements for samples could be limited to 1mm<sup>2</sup>-1cm<sup>2</sup>, thus each sample could be directly produced from the lithography and etching processes. Typical materials at the time were quartz for its optical properties and silicon because the fabrication flow was “borrowed” from the semiconductor industry. With the aim to gain a deeper understanding of the molecular mechanisms controlling the cell behaviour, biochemical and genetic methods were later being applied. Such techniques required larger patterns or more samples to gather sufficient material for the assays. Replication techniques such as hot embossing and later injection moulding have enabled the fabrication side to supply the biological demand. Modern lithographic and replication techniques will be presented and discussed in the next sections.

Today, most of the topographies investigated have been fabricated to produce more or less specific patterns for the cells to be exposed to. There are several good text books describing

the lithographic process in more detail (Franssila 2004; Madou 2011) as well as advanced fabrication technologies are available (Wang 2010). In most cases the fabrication flow requires three distinct steps to make the samples for the biological experiments:

1. Pattern definition
2. Pattern transfer
3. Pattern replication

## 2.1 Clean rooms

With a continuously decreasing size of features fabricated it is necessary to operate in dust-free conditions, since the features fabricated are comparable or smaller than dust particles. Such clean conditions are obtained in a clean room where the air is constantly filtered and when entering the room its temperature and humidity is also very carefully controlled. The most common measure for the quality of a clean room is its operational class, where a class 1000 clean room has less than 1000 particles ( $0.5\ \mu\text{m}$  or smaller) in a cubic foot. Most academic labs operate between class 1000-10000, whereas semiconductor industry is 1-10. Other important factors inside a clean room are stable temperature and humidity. The stability of the temperature is important as the chemical processes carried out are sensitive to variations in the temperature and an increase in temperature will lead to an increase in the rate of a chemical reaction. Some of the polymers (resists) being used in the clean room are sensitive to moisture and will change their properties depending on the humidity, hence the important to keep that stable too. Finally, because the resists are sensitive to light, the lighting in a clean room is yellow which prevents inadvertent exposure of the resists.



Fig. 1. Preparing for entering the clean room involves dressing in a clean room suit. The yellow light of the clean room area is visible in the background. Image courtesy of the James Watt Nanofabrication Centre @ Glasgow

## 2.2 Patterning techniques

The first step in producing substrates with a given surface topography, a lithographic process is carried out. There are several ways to generate surface topographies depending on the length scale and degree of control one is aiming at, Fig. 2. One of the first techniques to generate precise and well-defined topographies at a length scale comparable to a single cell (5-100 micrometer), was photolithography (Brunette 1986; Clark, Connolly et al. 1987; Clark, Connolly et al. 1990; Oakley and Brunette 1993; Curtis and Wilkinson 1997; Walboomers, Monaghan et al. 1999) and is still a frequently used technology. However, with an increasing interest in smaller length scales, alternative methods have been developed to

meet these demands. As this is primarily driven by the biomaterials community access to clean room facilities is often limited. A relatively simple method to generate micro- and nanotopographies with a certain degree of control is by phase separation of polymers. This can either be polymer blends (Affrossman, Henn et al. 1996; Affrossman, Jerome et al. 2000) or block copolymers (Olayo-Valles, Lund et al. 2004; Krishnamoorthy, Pugin et al. 2006). Here the polymers are dissolved in a common solvent and spin coated on relevant substrates (often glass). During the evaporation of the solvent the incompatibility of the polymers drives the phase separation leading to a topographical landscape with features of varying lateral dimensions but with identical height. By carefully controlling the topographical parameters, it is possible to tune the cellular response to the generated topography (Dalby, Giannaras et al. 2004). It is noteworthy that the samples can be used directly and does not require further processing unlike most other techniques.

A step up in controlling the degree of topographical order, is the use of colloids. Here colloidal particles ranging from 30 nm to several microns (Denis, Hanarp et al. 2002; Hanarp, Sutherland et al. 2003) are suspended in an ionic solution and cast on the substrate. Depending on the strength of the ionic solution, the distance between the colloids can be controlled (Hanarp, Sutherland et al. 2003). The deposited colloids then acts as a mask in further processing steps to obtain a master substrate. The resulting substrate has features of identical lateral and vertical dimensions but their geometric arrangement is poorly controlled.

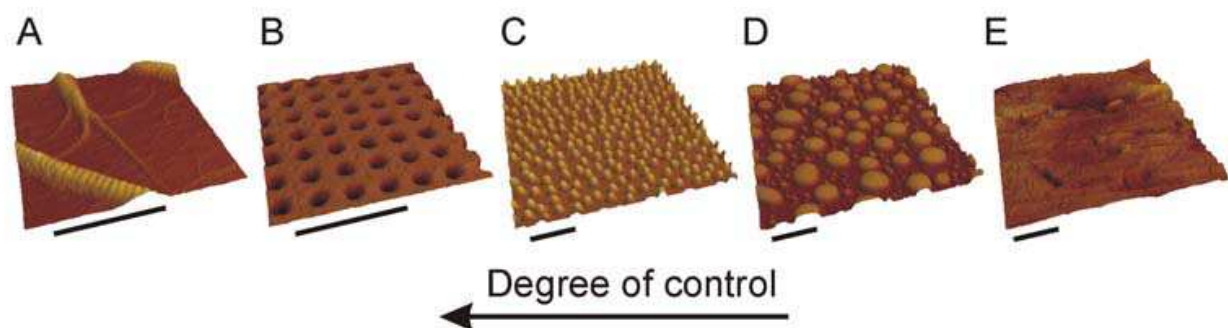


Fig. 2. AFM micrographs of various nano topographies used to control cell behavior. The topographies are arranged by the degree of control. A) Collagen is a naturally occurring protein which forms fibrils with a characteristic 67 nm cross-banding topography. B) Electron beam lithography pattern of highly ordered nanopits. C) Colloidal lithography. D) Polymer phase separation. E) Titanium surface. Scale bar is equal to 1  $\mu\text{m}$  (Gadegaard, Dalby et al. 2006).

Besides the lithographic techniques described above, some attempts have been made to replicate the structure of the natural environment the cells are surrounded by. Flemming et al prepared PMMA replicas of decellularised blood vessels containing the 3-dimensional structure of the extra cellular matrix (Flemming, Murphy et al. 1999). Gadegaard et al demonstrated the ability to replicate the nanometric structure of collagen fibrils and fibres (Gadegaard, Mosler et al. 2003).

Although, there is a range of alternative technologies available for patterning surfaces for biological applications, the majority of the research is still applying semiconductor techniques such as photolithography and electron beam lithography. We will discuss these techniques in more details in the next sections.



### 2.3 Photolithography

Although the main aim of this chapter is to describe the impact of nanotopography for cell and tissue engineering applications, microlithography started this field in the early 1980's and the processing step involved are similar to the ones used in for modern nanolithography. Moreover, there is still a large activity on micropatterned materials in stem cell research (Kilian, Bugarija et al. 2010). Photolithography was the first semiconductor technology applied to make artificial patterns for cell engineering research (Curtis and Wilkinson 1997). An excellent historical overview covering both optical and electron beam lithography has been given by Wilkinson (Wilkinson 2004). At the time photolithography was the only technology capable to preparing precise patterns with dimensions comparable to the size of a single cell.

The first step in the lithographic process is to choose a relevant substrate material for the fabrication process. The choice is typically between quartz (or glass) and silicon. Both substrate materials are available with very low surface roughness, typically below 1 nm, which is crucial for the fabrication process. The most notable differences between the two materials is that quartz is optically transparent and non-conduction whereas the opposite is the case for silicon. The next step in the process is to apply a light sensitive polymer coating to the substrate called resist. This is applied by spin coating where the substrate quickly is rotated (2000-6000 rpm) leaving a very reproducible and uniform coating of the resist. To remove remaining solvent from the resist, a soft bake step is carried out before exposure. The resist is then patterned through a mask which is a quartz substrate with a chrome pattern preventing light to pass through. This step is typically carried out using a mask aligner which enables precise illumination time and the possibility to register the mask to the sample if required. The exposure time for a complete wafer 4-8 inches in diameter is typically 1-30 seconds depending on the pattern and resist.



Fig. 3. The photolithographic patterning is done on a mask aligner where accurate exposure can be controlled. Image courtesy of the James Watt Nanofabrication Centre @ Glasgow

There are two types of resists to choose from, coined positive and negative tone. Positive tone resists are the most commonly used in the fabrication process and exposed areas are dissolved during the development process, whereas negative tone resist become insoluble in the exposed areas. A notable negative resist commonly used in microfabrication for biological devices is SU-8 (Campo and Greiner 2007). Two main factors play a role in the

popularity of SU8-8 in this field. One is that the polymer is biocompatible and cells interact positively with the polymer which means that it can be a part of the final device. The other factor is that it is possible to make thick layers (20-100 micron, or more) which is ideally suited for microfluidic systems (Delamarche, Bernard et al. 1997). These steps complete the lithographic process.

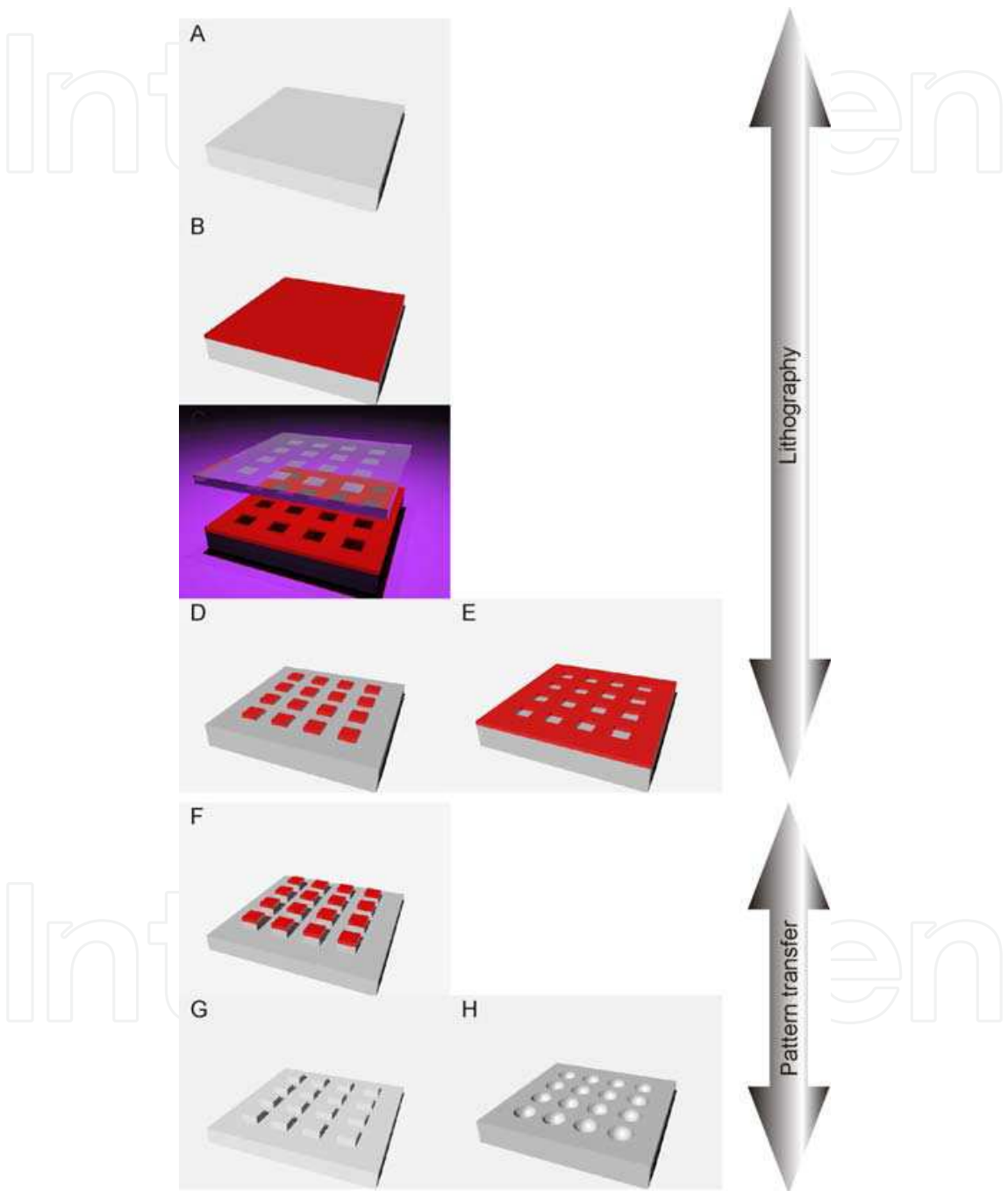


Fig. 4. Lithographic fabrication flow. (A) a substrate is cleaned and prepared for use. (B) A light sensitive polymer is coated on the substrate. (C) the sample is exposed through a quartz mask with the desired pattern. After development, the final pattern is realised in (D) positive or (E) negative resist. (F) The patterned is then transferred into the substrate through an etching process. (G-H) Finally the resist is removed completing the process.

The achievable resolution by photolithography can be estimated by the Rayleigh criterion where the wavelength used ( $\lambda$ ) can be related to the smallest feature obtainable,  $R$ .

$$R = \frac{0.61\lambda}{NA} \quad (1)$$

In most academic research facilities i-line (365 nm) mask aligners are used which results in 250 nm ( $\lambda=365\text{nm}$ ,  $NA=0.9$ ). However, in reality the best obtainable resolution is typically about 1  $\mu\text{m}$ . So with the exception of the complicated “tricks” played by the semiconductor industry on highly specialized equipment where features below 30 nm are obtainable, the only possibility is to reduce the wavelength.

## 2.4 Electron beam lithography

Electron beam lithography (EBL) is *the* technology of choice for full control of pattern arrangement and lateral dimensions in the sub-micron range. Dimensions as small as 3-5 nm are possible (Vieu, Carcenac et al. 2000). It is based on the principle of a scanning (transmission) electron microscope where electrons are accelerated from an electron source. The beam of electron are focused to a narrow spot, typically about 2-5 nm, through a set of electrostatic lenses, Fig. 5.

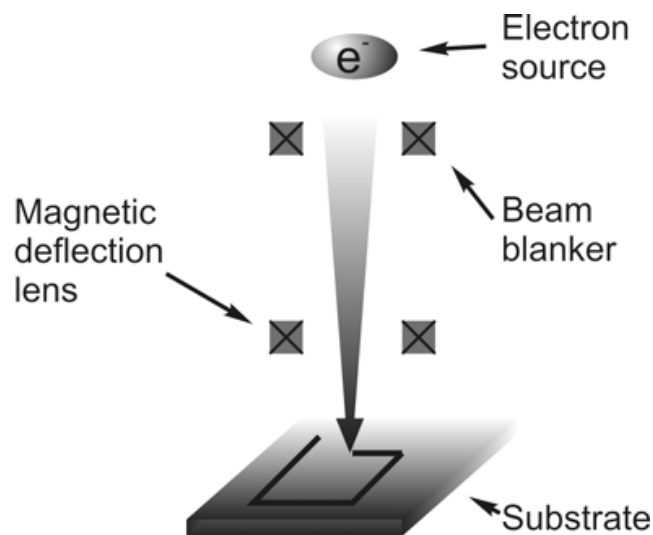


Fig. 5. Cartoon of an electron beam lithography set-up

Deflection coils are then used to control the position of the electron beam on the sample surface, much in the same way as a TV screen, and so make is possible to raster scan the surface. One of the main reasons that electron beam lithography is able to make patterns down to just 5 nm is the fact that the wavelength of the electrons is much shorter than for photolithography. The wavelength of an electron accelerated in an electrical field can be calculated from the equation below

$$\lambda = \frac{h}{\sqrt{2m_0eU}} \quad (2)$$

where  $h$  is Planck's constant,  $m_0$  is the rest mass of the electron,  $e$  is the charge of an electron and  $U$  is the acceleration potential. Most electron beam lithography systems operate at 100



kV which gives a wavelength of  $\lambda = 0.003$  nm. This is also known as the de Broglie wavelength.

Where photolithography is a parallel process (a whole wafer can be exposed at the same time), electron beam lithography is a serial technology. For example with a pixel size of  $10 \times 10$  nm<sup>2</sup> and a patterning rate of 5 million pixels per second (typical values for general patterns) it will take nearly 6 hours to pattern a  $1 \times 1$  cm<sup>2</sup> area with 10% pattern density. This time exclude the stage movement, calibration and settle time during the exposure which easily can double the actual lithography time. To overcome this time constraint we have developed a method that dramatically reduces the exposure time (Gadegaard 2003). This will be described in more detail in the following section.

The fabrication procedure is similar to photolithography, where a substrate is coated with a resist sensitive to radiation. In contrast to photolithography which uses light, EBL uses an electron sensitive polymer which either breaks down during exposure (positive tone) or cross-links (negative tone). After exposure the sample is developed to reveal the exposed pattern. One major difference between the two lithographic techniques is that EBL requires a conducting sample or the surface will build charge as a result of the electron bombardment. Here either a conducting substrate is used (typically silicon) or a metallic film can be deposited on non-conducting substrates.

## 2.5 A fast and flexible EBL nanopatterning model system

To gain the ultimate degree of pattern control at the nanometre length scale Gadegaard has for a decade used electron beam lithography (EBL). EBL is found at the heart of semiconductor production in the generation of the photolithographic masks for exactly this ultimate performance. Its nature of serial patterning means that it is generally regarded a slow technique. However, over the years we have developed technologies to overcome this limitation. A first endeavour has been to develop a highly flexible model system able to prepare areas of at least  $1 \times 1$  cm<sup>2</sup>.

When designing patterns for EBL suitable CAD software is used to generate the relevant data files for the tool. When exposing the patterns the features are made up from several smaller exposures, Fig. 6A. This is very similar to the operation of a printer, however, this is a lengthy process. Thus we have increased the size of the exposure to match the feature size desired and only using a single exposure, Fig. 6B. This accelerates the process by nearly two orders of magnitude.

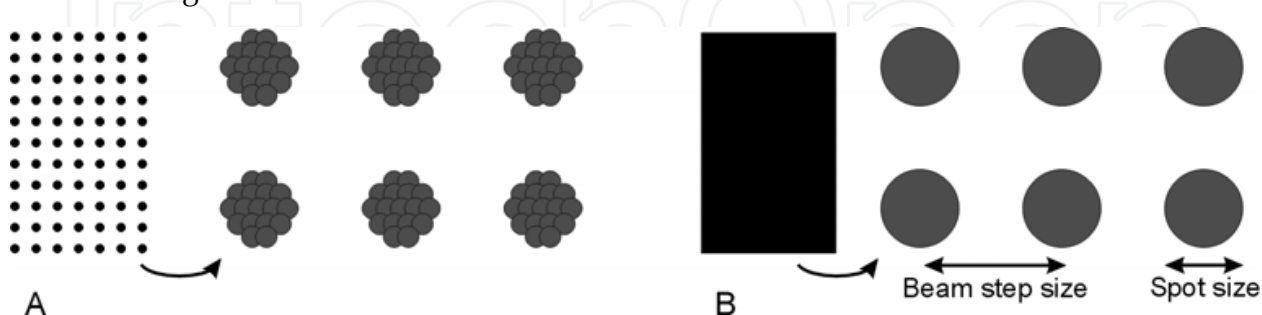


Fig. 6. (A) In a traditional design and exposure process, the features are designed in a CAD software and exposed on the EBL tool using multiple exposure for each features. (B) In our fast EBL patterning, a rectangle is drawn covering the areas for exposure. The diameter of the feature is controlled by the spot size (larger than traditionally) and the pitch by the beam step size.

With the fast EBL technique it is also possible to exactly control (see Fig. 7.):

- Feature size (Gadegaard 2003; Gadegaard, Dalby et al. 2008)
- Surface coverage (pitch) (Gadegaard 2003; Gadegaard, Dalby et al. 2008)
- Geometric arrangement of the features (Curtis, Gadegaard et al. 2004; Dalby, Gadegaard et al. 2007; Gadegaard, Dalby et al. 2008)
- Polarity (holes or pillars) (Gadegaard, Thoms et al. 2003; Martines, Seunarine et al. 2005; Martines, Seunarine et al. 2005)
- Height/depth (Martines, Seunarine et al. 2005; Martines, Seunarine et al. 2006)

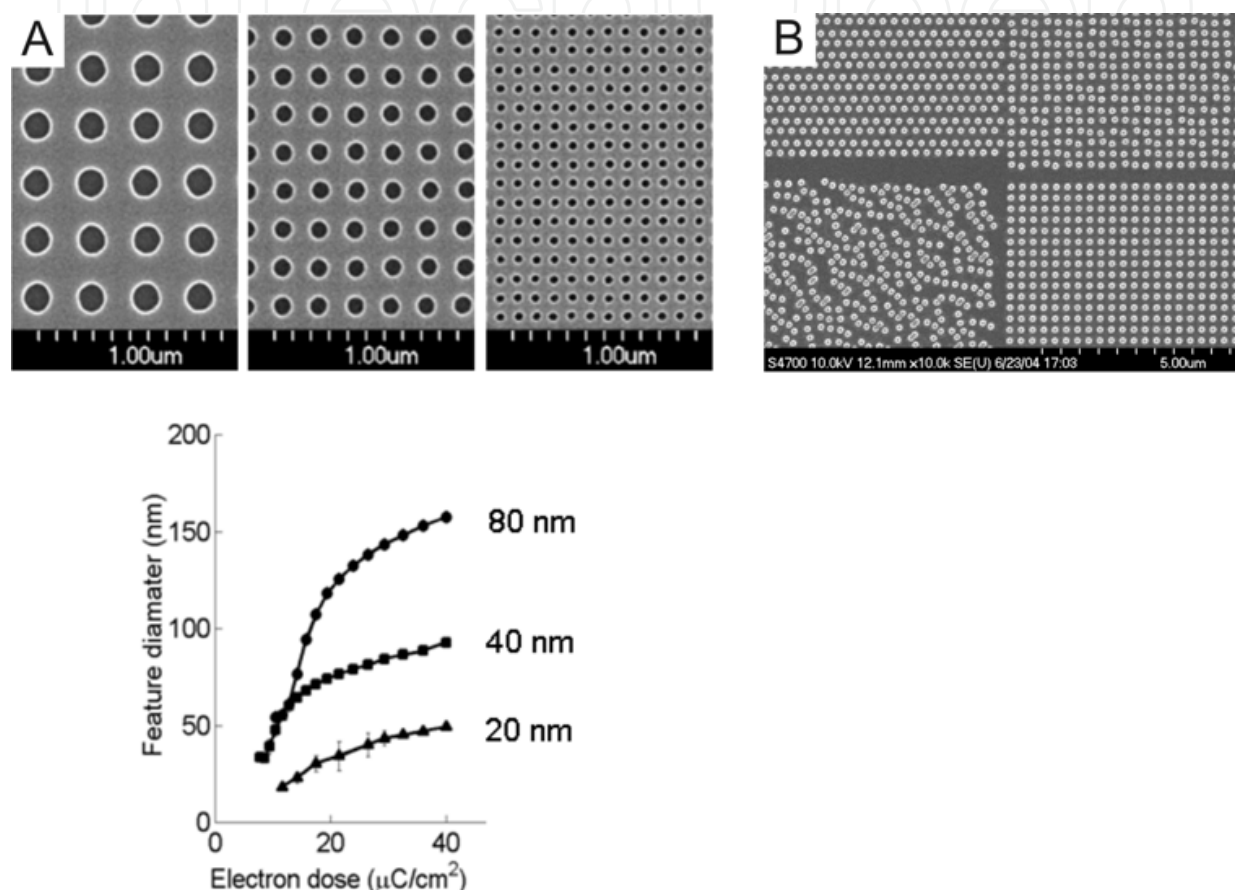


Fig. 7. (A) The dot diameter is controlled by a combination of spot size and the electron dose. (B) SEM image of 100 nm diameters dots arranged in different geometries illustrating the flexibility of the fast EBL patterning platform.

## 2.6 Pattern transfer

Once the pattern has been lithographically established it is in most cases necessary to transfer the patterns into the supporting substrate. This step is typically carried out using an etch process which can be more or less selective to the substrate. The patterned resist will act as a mask during the etching process. Depending on the substrate material and the type of etch, two etch geometries are possible, Fig. 8. During anisotropic etching the etch rate is different in different directions of the samples. Most typically such anisotropic etching is obtained in a reactive ion etching equipment where the reactive gas is directed towards the sample. For isotropic etching, the etch rate is the same in all direction of the sample resulting in half-pipe or hemispherical shapes in the substrate. Such etching is typical for wet etching.

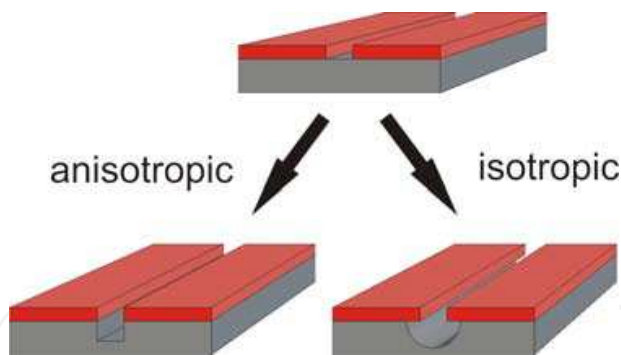


Fig. 8. The patterned resist will act as a mask during etching. There are different types of etching depending on the substrate and type of etch yielding either anisotropic or isotropic profile.

2.7 Replication

As the fabrication process often is lengthy and expensive it is rarely feasible to use the fabricated samples directly for biological experiments. Hence, the lithographically prepared master sample can be replicated either by hot embossing or injection moulding, Fig. 9.

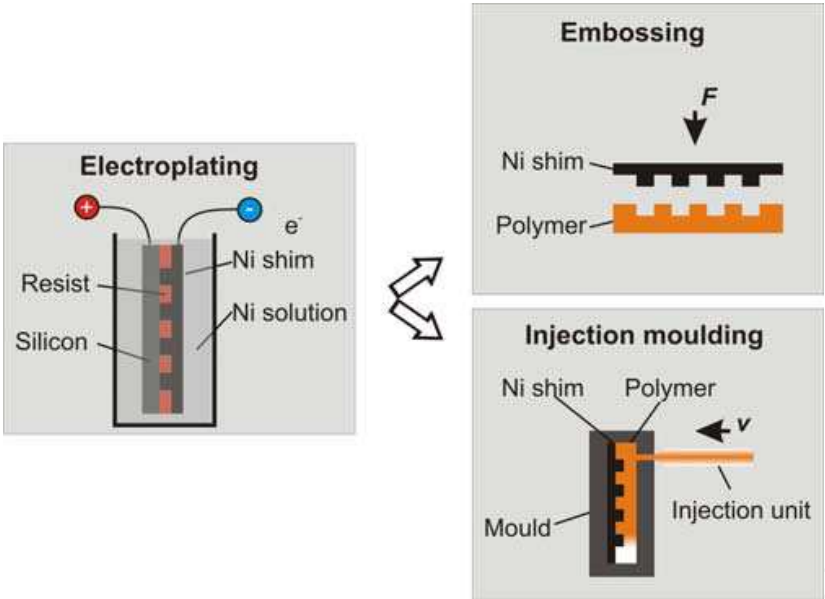


Fig. 9. Replication techniques. From the lithographically prepared master it is possible to make nickel shims used for either hot embossing or injection moulding.

The most commonly used materials used for *in vitro* cell experiments are polymeric materials for a number of different reasons. An important feature is that many polymers do not pose toxic properties to the cells and can support cell adhesion. Another important feature is that the original topographical pattern fabricated by lithography and pattern transfer can easily be replicated in a polymer in a very simple and fast manner by heating and cooling the polymer.

For injection moulding, a nickel shim is prepared through a galvanic process originally developed by the CD and DVD industry. The lithographically defined master is first sputter coated with a thin metal layer which acts as an electrode during the galvanic plating. The sample is inserted into a tank with nickel ions and when drawing a current a layer of nickel

can be deposited in the master substrate. This shim will then be fixed in the cavity of the injection moulding tool (Gadegaard, Mosler et al. 2003).

## 2.8 Hot embossing

On an academic scale, hot embossing is the most common technique by which samples can be prepared (Gadegaard, Thoms et al. 2003; Mills, Martinez et al. 2005). Here a thermoplastic polymer is heated above its glass transition temperature where the polymer becomes soft enough to deform if a pressure is applied. Once melted a master substrate is pressed into the polymer and then left to cool down before the polymer replica is released from the master. A particularly simple setup can be as simple as a hot plate, Fig. 10. Typically it takes 5-20 min to make a single replica.



Fig. 10. A simple setup for hot embossing using a hotplate.

## 2.9 Injection moulding

On an industrial scale, injection moulding is the preferred technology platform for producing thousands of polymeric replicas. Currently, the most demanding injection moulding process for replicating surface topographies is that of optical storage media such as CDs, DVDs and Blu-ray discs.

The injection unit consists of a hopper which feeds the polymer granulates to the screw, Fig. 11. The screw has a number of functions. It transports the polymer from the hopper to the melting zone, where it is plasticized, homogenised, and degassed. The plasticization is a

combination of heating from the heating bands and mechanical friction. The mechanical friction can to some extent be controlled by the backpressure. The backpressure prevents the screw from moving back during rotation thus forcing the polymer melt to flow over the thread leading to friction and as a result extra heat is supplied to the melt. Controlling the backpressure may be critical because the temperature at the core of the polymer melt may be higher than what is read out at the thermocouples near the heating bands. The effect is amplified due to the low thermal conductivity of polymers.

The extra heating as a result of an applied backpressure results in a more homogenous temperature of the melt. However, by applying too high a backpressure the polymer could be degraded caused by an excess in temperature. Finally the screw acts as a piston during the reciprocating motion. The cavity in front of the screw is normally filled with slightly more (<10%) polymer material than is needed to fill the object cavity. This is to prevent degradation of the polymer during extended time in the screw chamber.

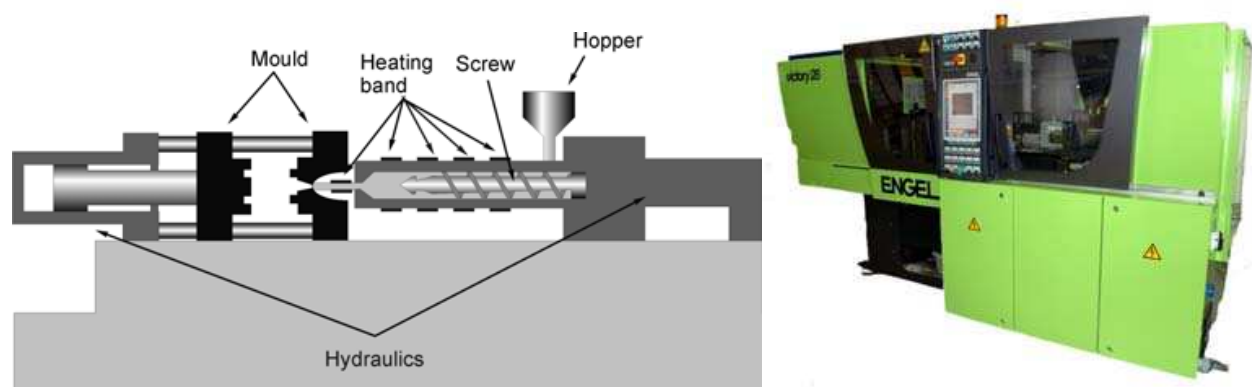


Fig. 11. Left, cartoon of an injection moulding machine illustrating key components. Right, photo of an industrial injection moulding machine.

The melt is injected into the mould cavity that is kept at a temperature below the glass transition temperature,  $T_g$ . This means that once the polymer is introduced into the mould it very quickly cools and the injection moulded part can be removed from the cavity without losing its shape at the end of the injection moulding cycle. This means that the polymer will solidify at the walls during injection. This thin *skin layer* will build up behind the polymer melt front. There is no evidence that under normal moulding behaviour that the melt slides along the walls of the cavity (Rosato and Rosato). The polymer melt is injected at a specified pressure which, after the cavity has been filled, is changed to the packing pressure. The packing pressure minimises the shrinkage of the part during cooling. A high packing pressure results in good part dimensions but may also lead to difficulties in separating the part from the mould. A low packing pressure gives less residual stress in the part.

The filling speed is important to control properly. A high filling speed minimises the thickness of the frozen skin layer before packing pressure is applied. This is of paramount importance in this work where nanostructures are attempted to be replicated to the surface of the polymer part. However, a high injection velocity also leads to heating of the polymer melt near the mould walls caused by shear. In worst case this could lead to degradation of the polymers leaving it unusable for surface replication. Finally, high filling speed also results in an increased residual stress which could be important in certain applications, e.g. optical applications (Pranov, Rasmussen et al. 2006).



### 3. Stem cells

Stem cells can be categorized into two groups; pluripotent (embryonic) and multipotent (adult or tissue specific), and they share two properties which separate them from other somatic cells; firstly, the ability to self-renew, and secondly, to undergo differentiation into a specific cell type given sufficient cues. Pluripotent stem cells however have a multi-lineage potential, and have been identified as having the ability to differentiate into all cell types of the body. Multipotent, on the other hand, are lineage-restricted in their differentiating potential, and this is usually determined by their tissue of origin, e.g. bone marrow-derived mesenchymal stem cells have the ability to differentiate into bone, fat, cartilage etc. Controversially these stem cells are also thought to have the ability to trans-differentiate into neuronal cells, a phenomena which may point towards the potential for these stem cells having a more pluripotent phenotype. Following recent advances in stem cell development, there are now two main types of pluripotent stem cells, the first of which, embryonic stem cells, are derived from the blastocyst of an embryo and the second, are known as induced-pluripotent stem (iPS) cells. These were first developed by reprogramming an adult somatic cell, typically a fibroblast, using viral transfection of four key genes including oct3/4, sox2, klf4 and c-Myc. Recent studies however have also shown that somatic cells can be reprogrammed without the need for viral vectors, a necessary requirement if iPS cells were ever to be feasibly used for stem cell therapy in humans.

Embryonic stem cells therefore have a distinct advantage over adult stem cells in their differentiation potential, but this can become overshadowed by difficult cell culture requirements (ES cells require complicated cell culture techniques involving mouse embryonic fibroblasts (MEFs)), and the many ethical issues surrounding their use. With the development of iPS cells at least some of these issues have the potential to be overcome. Adult stem cells, although only being multipotent have their own advantages. They require lower-level ethical consent for use and are relatively easy to culture. However, one drawback arises under long-term culture conditions when adult stem cells are prone to undergo spontaneous differentiation (asymmetric cell division as opposed to symmetrical) resulting in a loss of the stem cell population.

With regards to stem cells, there are two requirements for which biomaterials may serve a purpose. Firstly, there is a need to maintain an undifferentiated, proliferating cell population; the ability to promote symmetrical cell division in adult stem cells and in the case of ES cells, feeder-free maintenance is desirable. Secondly, the ability to direct differentiation down a specific cell lineage in a non-invasive manner without the need for chemical supplements, which may either be toxic or contain animal products, and therefore unable to be used, or with only restricted use, within the body. In response to these requirements, researchers have been working to develop material strategies to overcome these problems.

#### 3.1 Embryonic stem cells

Currently, the *in vitro* maintenance of embryonic stem cells (ESCs) requires the use of feeder layers. This requirement makes investigations into the effect of nanotopography on pluripotent stem cells often difficult to undertake due to possible masking of the nanotopography by the feeder layer. As a result there is a lack of scientific papers exploring the effect of nanotopography on embryonic stem cell self-renewal. In one key study however, Nur-E-Kamal et al were able to investigate the effect of a three-dimensional

polyamide nanofibre substrate, designed to mimic the *in vivo* extracellular matrix/basement membrane, on the self-renewal of mouse embryonic stem cells (mES) (Nur, Ahmed et al. 2006). This study was conducted largely in the absence of mouse embryonic fibroblast (any MEFs used were carried over from passaging (~5%)). By culturing mES cells on 3D nanofibrillar substrates an increase in colony size of undifferentiated stem cells was noted when compared to culture on a glass coverslip. Interestingly, when cultured on flat polyamide alone cells were unable to attach indicating that it is in fact the 3D nanotopography that was influencing cell proliferation.

Not only is it necessary to identify biomaterials with properties favourable to controlling stem cell self-renewal and differentiation, but it is also important to decipher the mechanisms behind their effect in an attempt to gain further insight into stem cell biology. In light of this, the authors went on to further elucidate the mechanism behind the response of mES cells to the 3D nanofibrillar structure. By identifying the levels of Rac, a protein of the Rho family of GTPases involved in cell growth, proliferation and cellular signalling, in mES cells culture on flat and 3D nanofibrillar substrates it was shown that increased Rac activity occurs in cells on the 3D nanofibrillar substrates, and plays an essential part in the increased levels of proliferation seen only in cells cultured on the 3D nanofibrillar substrates. The authors then went on to identify upregulation of Nanog, an essential protein required for maintaining the stem cell pluripotency, in response to the 3D nanofibrillar substrates via the PI3K pathway; a pathway linked to Rac.

By showing that pluripotent stem cells can be induced to undergo self renewal and proliferation in response to a 3D system culture system, where the only distinction between a flat control is the topographical mimicry of an *in vivo* ECM/basement membrane identifies the extent that geometry alone can influence stem cell fate, and further provides an exciting platform for feeder-free culture.

In contrast to maintenance of self-renewal and proliferation, the main goal of tissue engineering is to produce functional tissues. In the case of embryonic stem cells, their use is of critical importance when it comes to replacement of diseased or injured tissues, where an affected site is too large for an autologous graft or the patients' own stem cells are defective. This is of particular necessity when a disease is hereditary or in the case of neural degeneration from diseases such as Alzheimer's and Parkinson's disease. It is therefore no surprise that the main areas of research where nanoscale topography have been applied are in the development of neurogenic (Xie, Willerth et al. 2009; Lee, Kwon et al. 2010) and bone tissue (Smith, Liu et al. 2009; Smith, Liu et al. 2009; Smith, Liu et al. 2010). Several material strategies have been employed including nanofibres (Smith, Liu et al. 2009; Smith, Liu et al. 2009; Xie, Willerth et al. 2009; Smith, Liu et al. 2010), grooves (Lee, Kwon et al. 2010) and carbon nanotubes (Chao, Xiang et al. 2009).

By developing 2D and 3D nanofibre substrates that are designed to mimic the topographical pattern of *in vivo* type I collagen the authors were able to show that both mES and hES cells undergo osteogenic differentiation. Conversely, Xie et al showed that in the presence of neurogenic media mES cells when cultured on nanofibres particularly in an aligned geometry, the nanotopography acts to enhance the differentiation of mES cells into mature neural cells. Human ES cells were also shown by Lee et al to undergo neural differentiation, in the absence of any differentiation supplements, this time using nanogrooved substrates. A similar result was also seen when hES cells were cultured on the carbon nanotubes coated with poly (acrylic acid).

### 3.2 Skeletal stem cells

Skeletal stem cells (SSCs) as mentioned previously, have been found to undergo differentiation into various cell lineages including bone, fat, cartilage (Owen and Friedenstein 1988; Pittenger, Mackay et al. 1999) and neurons (Song and Tuan 2004; Shih, Fu et al. 2008) using chemically defined media. It is now becoming clear however that topography alone or in conjunction with standard differentiation protocols may provide a more efficient means for directing stem cell differentiation. The use of nanotopography to direct skeletal stem cell differentiation has two areas of application, i) implant surface patterning to promote bone encapsulation of an implant; currently implant failure occurs due to soft tissue formation, and ii) in vitro growth/differentiation of autologous stem cells for implantation back into the patient.

Results from several key studies have generated compelling evidence on the effect that substrate topography, especially at the nanoscale, can have on skeletal stem cells. It has been found that by changing only a few parameters, this can have a dramatic effect on stem cell differentiation. In a study by Dalby et al, it was shown that osteogenic differentiation of SSCs can be initiated by alterations in the geometry and degree of disorder of nanopits embossed into the polymer polymethylmethacrylate (PMMA), Fig. 12. By creating a nanopitted topographical pattern having a fundamentally square geometry, but with a controlled level of disorder has the ability to promote the differentiation of SSCs down an osteoblastic lineage (Dalby, McCloy et al. 2006; Dalby, Gadegaard et al. 2007).

In a similar study undertaken by Oh et al. SSCs were shown to differentiate down an osteoblast lineage, this time in response to carbon nanotubes with a diameter of 100 nm (Oh, Brammer et al. 2009). In this case, the diameter of the nanotubes was identified as a crucial factor in promoting differentiation, with SSCs cultured on nanotubes of less than 50 nm producing negligible amounts of osteogenic markers.

Other studies have included investigation the effect of nanotopography on metal surfaces, as a pre-emptive step towards orthopaedic clinical applications (Popat, Chatvanichkul et al. 2007; Sjöström, Dalby et al. 2009).

In addition, the transdifferentiation of SSCs down a lineage of endodermal origins into neuronal-like cells has been shown to occur in response to nanogratings (Yim, Pang et al. 2007). Yim et al identified the upregulation of mature neuronal markers when SSCs were cultured on nanogratings in the absence of differentiation media. Interestingly, the authors went on to report higher levels of neuronal marker expression in response to the nanograting topography without differentiation media than chemical induction alone.

It is therefore evident that nanotopography can have a huge effect on skeletal stem cell differentiation but the mechanisms which underlie this topographical regulation, such as those described above are only recently beginning to be deciphered. It is hypothesized that the distinct topographical profile of a substrate primarily affects focal adhesion formation via altered protein adsorption to the surface as indicated by Oh et al who hypothesized that protein adsorption decreased with increasing nanotube diameter altering the sites for initial cell attachment (Yamamoto, Tanaka et al. 2006; Oh, Brammer et al. 2009; Scopelliti, Borgonovo et al. 2010) or the disruption of the cells ability to form focal complexes. In 2007, Dalby et al demonstrated that nanotopography could lead to changes in gene expression and later identified differences in gene expression patterns between topographically and chemically differentiated SSCs (Dalby, Gadegaard et al. 2007; Dalby, Andar et al. 2008) which indicates that topography may work via a distinct mechanism. Biggs et al went on to



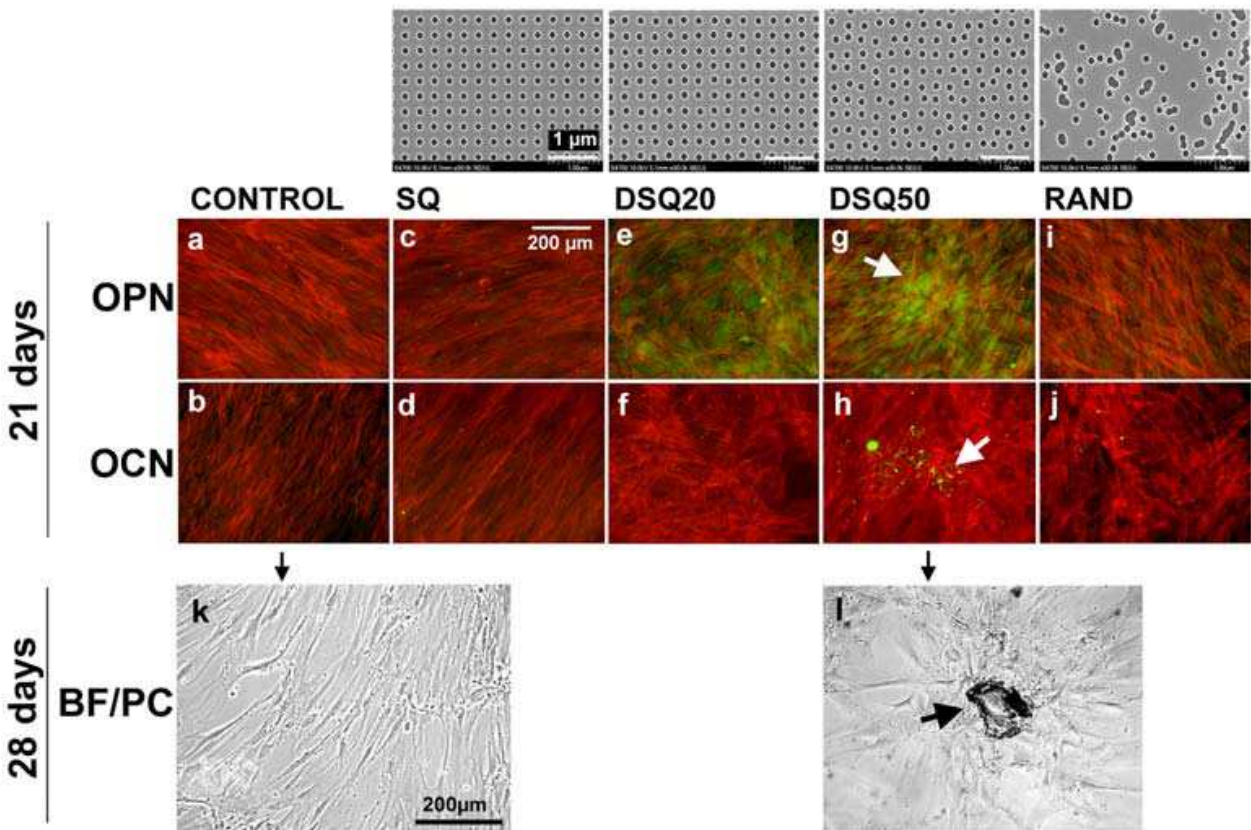


Fig. 12. OPN and OCN staining of MSC cells after 21 days and phase-contrast/bright-field images of alizarin-red-stained cells after 28 days. The top row shows images of nanopatterns fabricated by EBL. All have 120-nm-diameter pits (100nm deep, absolute or average 300nm centre-centre spacing) with square, displaced square 20 ( $\pm 20$ nm from true centre), displaced square 50 ( $\pm 50$ nm from true centre) and random placements. a-j, MSCs on the control (a,f), note the fibroblastic appearance and no OPN/OCN positive cells; on SQ (b,g), note the fibroblastic appearance and no OPN/OCN positive cells; on DSQ20 (c,h), note OPN positive cells; on DSQ50 (d,i), note OPN and OCN positive cells and nodule formation (arrows); on RAND (e,j), note the osteoblast morphology, but no OPN/OCN positive cells. k,l, Phase-contrast/bright-field images showing that MSCs on the control (k) had a fibroblastic morphology after 28 days, whereas on DSQ50 (l), mature bone nodules containing mineral were noted, (Dalby, Gadegaard et al. 2007)

further correlate these changes in gene expression with differences in focal adhesion formation on various nanopatterned substrates (Biggs, Richards et al. 2009; Biggs, Richards et al. 2009). In a later study Yim et al identified that the disruption of focal adhesion formation results in changes in the mechanical properties of cells, and also identified changes in gene expression (Yim, Darling et al. 2010).

### 3.3 Neural stem cells

The identification of neural stem cells (NSCs) in the adult mammalian brain has lead to renewed hope for cures for debilitating diseases such as multiple sclerosis and other degenerative diseases of the nervous system, as well as replacement of tissues caused by injury e.g. spinal cord damage. Currently nerve repair is limited due to scar tissue formation, and in many cases once destroyed nerve cells are usually not replaced leading to

permanent loss. It is therefore of critical importance to develop substrates which induce the differentiation of neural stem cells for replacement of tissues or that guide nerve repair with minimal scar formation.

Nanotopographical effects on NSCs have largely been investigated in response to nanofibers, a topography that mimics natural collagen. Studies conducted have investigated NSC response with respect to fiber diameter, orientation as well as 2D and 3D matrices. It has been found that fiber diameter plays an important part in both proliferation and differentiation of NSCs, with a smaller fiber diameter increasing both proliferation (Christopherson, Song et al. 2009) and differentiation (Yang, Murugan et al. 2005). In a comprehensive study, Lim et al identified a correlation between fiber diameter and orientation on the morphology and subsequent differentiation of NSCs (Lim, Liu et al. 2010). In this instance the alignment of fibers was found to promote elongation of the cells leading to changes in the cell cytoskeleton and subsequent intracellular signalling, specifically the Wnt/ $\beta$ -catenin pathway. The authors proposed  $\beta$ -catenins dual role as a cytoskeletal/cellular signalling component in linking changes in morphology caused by the aligned nanofibers with increased Wnt/ $\beta$ -catenin activity, a pathway involved in neurogenesis.

It has been demonstrated that even the slightest alteration in geometry, width, depth, orientation or pattern can affect the differentiation of stem cells. The use of nanotopographical substrates therefore provides a highly tuneable non-invasive platform for the control of stem cell differentiation; a highly valuable tool with many application for use in regenerative medicine.

#### 4. Outlook

A real step change is needed from the current curiosity driven research to meet the future demands from clinical applications. Nanotechnological solutions for clinical applications are very promising, however, there are still many hurdles to overcome before this becomes precedence rather than exception. One of the grand challenges is the use of a broader range of clinical relevant materials than is currently deployed at the research level. This would include metals/alloys, composites and (biodegradable) polymers. Although many examples of nanopatterning of such materials with the associated differential biological response have been demonstrated, they are more often special cases of a specific treatment of a given material rather than engineered solutions. Most studies have focused on a specific cell response, and in the case of adult stem cells specific lineage differentiation. Such a single lineage differentiation is limiting for the broader use of such materials in regenerative medicine. In reality it is much more likely that clinical applications will demand the use of mix and match patterning to elicit several different lineage specific differentiations in specific positions.

Area specific patterning can be met through various lithographic processes, however, as has been demonstrated high precision will be needed. This means, as it has been the case so many times in the past, we should be looking at the future of semiconductor manufacturing. As always, there is a continuous increase in the complexity of the designs accompanied by a constant decrease in feature dimensions. The latter may although prove not to be so important for the regenerative medicine in the future, whereas precise pattern control and placement seems critical. Such requirements are readily met by for example electron beam lithography (EBL), which offers the high resolution and pattern flexibility as described above. Another important aspect to be met, is the demand of scalability from the current



research level of relatively small areas of  $0.2 \times 0.2 - 1 \times 1 \text{ cm}^2$  to what is needed in a clinical device which easily could extend to tens of  $\text{cm}^2$ . Here, EBL may fall short to deliver due to the serial manner the patterns are produced. As already is in place, this can be overcome through a replication process. Finally, the majority of the materials produced so far are two dimensional as a result of the fabrication technologies. This is particularly true for semiconductor lithographic processes, whereas a biomedical implant inherently will require 3D patterning. This patterning may range from non-planar surfaces to truly 3D interconnected materials. This is a complexity level not yet tackled by the semiconductor industry and new innovations from other fields can be expected. The dual requirement of scalability and 3D may be met by technologies such as injection moulding or imprint technologies, e.g. nanoimprint lithography and flash imprinting (Seunarine, Gadegaard et al. 2006).

As the first products may start to hit the market the next trends to be expected will be a more predictive system from which multiple tissues can be targeted. This is currently dealt with through a comprehensive library of patterns and materials reported in the literature but produced in many different ways. The interplay between material design and biological response directly aimed at regenerative medicine will need a commitment from engineering, biological and computing disciplines.

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