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SPECIAL ISSUE: 3D CELL BIOLOGY

COMMENTARY

How cells respond to environmental cues – insights from bio-functionalized substrates

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ABSTRACT

Biomimetic materials have long been the (he)art of bioengineering. They usually aim at mimicking *in vivo* conditions to allow *in vitro* culture, differentiation and expansion of cells. The past decade has witnessed a considerable amount of progress in soft lithography, bio-inspired micro-fabrication and biochemistry, allowing the design of sophisticated and physiologically relevant micro- and nano-environments. These systems now provide an exquisite toolbox with which we can control a large set of physicochemical environmental parameters that determine cell behavior. Bio-functionalized surfaces have evolved from simple protein-coated solid surfaces or cellular extracts into nano-textured 3D surfaces with controlled rheological and topographical properties. The mechanobiological molecular processes by which cells interact and sense their environment can now be unambiguously understood down to the single-molecule level. This Commentary highlights recent successful examples where bio-functionalized substrates have contributed in raising and answering new questions in the area of extracellular matrix sensing by cells, cell–cell adhesion and cell migration. The use, the availability, the impact and the challenges of such approaches in the field of biology are discussed.

KEY WORDS: Biomimetic interface, Environmental sensing, *In vitro* culture, Mechanobiology, Mechanosensing, Microniches

Introduction

Environment sensing and signaling is increasingly recognized as a set of fundamental pathways that influence cell behavior, cell fate and pathologies. These mechanobiological principles contextualize the gene-expression-centric views that are more traditionally observed in cell biology. Soluble factors, xenobiotic factors, nutrients, oxygen and the chemical nature of the extracellular matrix (ECM) have long been recognized as essential signaling components of the local cellular microniches. However, cells are equally sensitive to some biophysical aspects of the environment, such as the density and mechanical properties of the ECM, physical confinement and mechanical tension, all of which can elicit, inhibit or synchronize cell responses. *In vivo*, all these parameters are largely intertwined. Cell–cell interactions, cell–matrix interactions and paracrine signaling epitomize a triad of intertwined environmental cues that, at the same time, elicit a cellular response and are modified by the cells. In the past decade, combining molecular biology tools with *in vitro* reductionist approaches involving bio-functionalized micro-fabricated substrates has enabled scientists to identify and understand a growing number of cellular processes that are governed by mechanobiological cues (Fig. 1).

This Commentary reviews recent examples of important processes in the fields of matrix sensing, cell–cell interactions and cell migration that have been elucidated using bio-functionalized surfaces. Many fundamental studies have been carried out in these fields using exclusively molecular and cell biology tools, such as gene editing and gene sequencing. We do not intend to minimize the importance of these approaches. However, this review focuses on the additional insights that were obtained when *in vitro* controls of the cellular micro-environment are applied. For an exhaustive view of each field the reader can refer to more specific reviews (e.g. Bonnans et al., 2014; Cavey and Lecuit, 2009; Kramer et al., 2013).

Brief overview over bio-functionalized substrates

Bio-functionalized surfaces are typically substrates with controllable biophysical properties that can elicit specific interactions with cells in a close-to-physiological way. The canonical examples are Petri dishes coated with ECM that has been simply adsorbed on their surface by incubation from a solution. Covalent binding can also be achieved by specific surface chemistry, which ensures proper mechanical coupling with the underlying substrate and reduces any matrix restructuring. The next level of complexity consists of patterning in two-dimensional (2D) adhesive areas. Many techniques can be used to lay down a pattern, with most being micron-scale approaches usually used in the textile industry. These include micro-serigraphy or micro-stenciling, which uses removable membrane with holes of different shapes to mask the exposed area of the surface (Masters et al., 2012; Ostuni et al., 2000), and micro-stamping, which uses soft textured material to 'ink' the region of contact with proteins (Piel and Thery, 2014b). Another approach is dip-pen lithography, which uses deposition of protein with a sharp tip (Salaita et al., 2007). Alternatively, deep UV patterning uses polymer degradation under intense light to reveal surfaces with adhesive properties (Azioune et al., 2009). A resolution down to a few hundreds of nanometers can be achieved (Fig. 2A).

Multi-protein printing (Fig. 2A) is enabled by repeating these processes in sequence (Strale et al., 2016). These protein deposition techniques can be used on substrates of various rigidities (Fig. 2B) that range from soft hydrogel (1–10 kPa), elastomers (100 kPa–1 MPa) to glassy materials (GPa) (Piel and Thery, 2014b). However, a caveat should be drawn here. The rheological properties felt by the

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cells might differ from that of the substrate owing to the adsorbtion and deformability of the ECM proteins (Smith et al., 2007). The rheological properties of the substrates can also be controlled by adsorbing patterned or unpatterned functionalized supported lipid bilayers with various fluidity (Fig. 2B).

Furthermore, another layer of complexity can be added by imprinting soft or rigid topographic features (e.g. pillars, groves or pits) onto the substrates (Fig. 2C). Most of these substrates are 2D or quasi 2D, although three-dimensional (3D) matrix surrogate hydrogels with controllable properties can also be devised. Inert biocompatible polymeric backbones can be functionalized with proteinaceous residues of a given nature and density. The density of crosslinking sites can also be tailored using different types of molecular reaction (e.g. click chemistry, acrylate based or hydrogen bonds). 3D microstructured rigid substrates can also be fabricated and coated with proteins (Klein et al., 2011) to create fibrillar environments (Fig. 2B). Moreover, microwells can be used to structure the 3D spatial arrangement of cellular adhesion and to create bona fide cellular microniches (Fig. 2A) (Charnley et al., 2012; Li et al., 2016). Substrates with gradients of adhesive properties (Fig. 2D) or properties that vary in time, such as hydrogel with controlled aging rheological properties (DeForest and Tirrell, 2015; Young and Engler, 2011), or patterns with on-demand adhesion (Rolli et al., 2012; Vignaud et al., 2012) have also been designed (Fig. 2D). Table 1 summarizes these approaches and the commercial availability of any such devices.

The principal advantage of using bio-functionalized surfaces is that they provide minimal, standardized and reproducible conditions with defined biochemical and biophysical characteristics for long-term, high-resolution observations of cell behavior. Therefore, their use allows a precise spatio-temporal control of individual environmental parameters that are not easily accessible *in vivo*. Thus, they constitute a tool of choice to precisely investigate the impact of mechanotransduction pathways through which cells feel the surrounding biophysical cues, such as extracellular mechanical properties, mechanical tension, shear flow and geometrical constraints. In addition, they are also instrumental in studying environment-dependent cell responses to soluble factors, such as growth factors, drugs and hormones. Finally, a controlled geometry allows the precise mechanical modeling of cellular functions (Albert and Schwarz, 2016). Specific recent examples are discussed in detail below.

ECM sensing

Among the different environmental-sensing processes, how cells perceive the molecular and biophysical properties of the ECM surrounding them is by far the most appreciated. Matrix properties can be exquisitely recapitulated *in vitro*. Many different types of secreted matrix can be purified and used for cell culture (Caliari and Burdick, 2016), or even assembled into microarrays to create a micro-screen of matrix-induced responses (Reticker-Flynn et al., 2012). For instance, the signaling from the 24 types of integrins

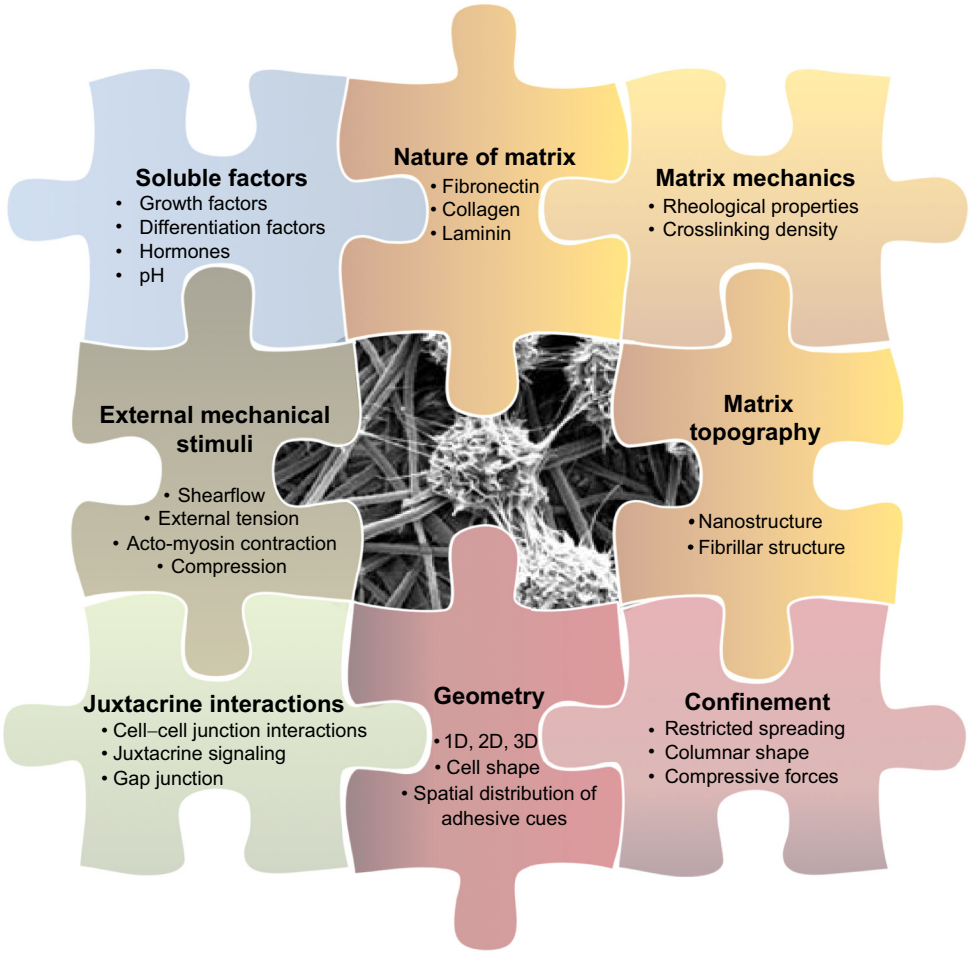

Fig. 1. Schematic representation of the various biophysical parameters that comprise the local microniche surrounding a single cell. *In vivo* these parameters are largely intertwined and their contribution to cell response can rarely be unambiguously evaluated. Recapitulating and varying one or a combination of each of these parameters using biomimetic interfaces allows us to decipher how cells perceive environmental cues and respond to them. The cell image is courtesy of Professor Hai-Quan Mao, Johns Hopkins University.

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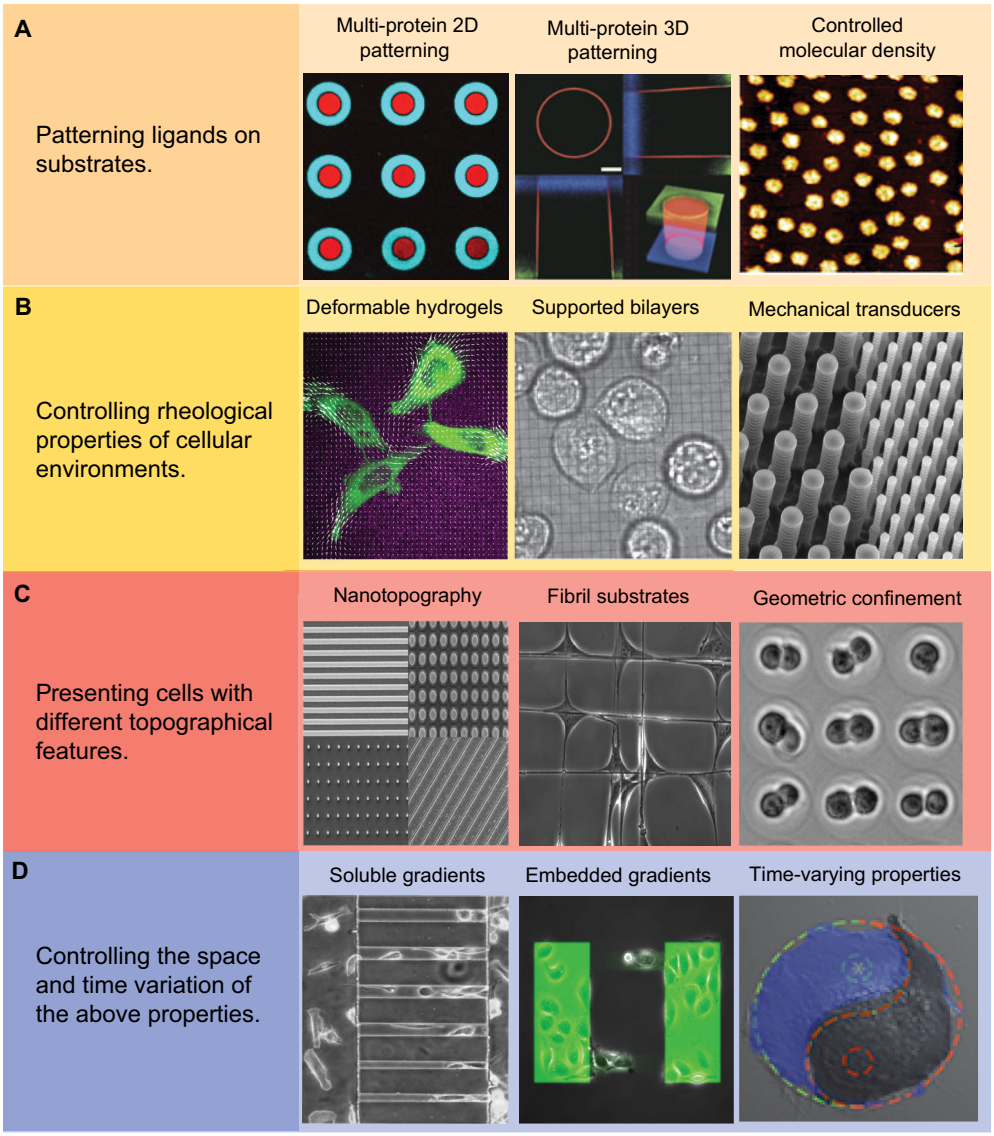

Fig. 2. Examples of technological solutions to control the cellular environment surrounding cells *in vitro*. (A) Patterning of different ligands. Left, intricate multi-protein patterns (E-cad in blue, fibronectin in red) made using deep UV patterning; middle, 3D differential protein coating on 20 micron microwells; right, an evenly spaced array of nanogold dots with controlled density of grafted RGD. (B) Examples for controlling the rheological properties of cellular environments. Left, fibroblasts plated on soft polyacrylamide gels; middle, MCF10 cells placed on a biofunctionalized fluid lipid bilayer; right, arrays of deformable micropillars that allow cellular traction measurement. (C) Illustration of how cells can be presented with different topographical features. Left, nanotextured polystyrene substrates with different topography that can enhance stem cell differentiation into a specific lineage; middle, fibroblasts cells migrating in 3D fibril environment; right, cells confined in micropits with precise geometrical properties such as curvature, size and shape. (D) Means to control the properties of the *in vitro* environment in time and space. Left, chemokine gradients generated across microfluidic channels; middle, gradients of fluorescent fibronectin density between two adhesive compartments; right, cell spreading on adhesive pattern before (dark) and after (blue) addition of biotinylated fibronectin, which selectively binds to the upper half of the pattern.

(reviewed in Campbell and Humphries, 2011) has been precisely studied using this approach (Roca-Cusachs et al., 2009).

The simplest substrate is a plastic or glass dish coated with adsorbed matrix proteins. Such a dish has been long used for cell culture, but with the advent of super-resolution microscopy, the ability to image focal adhesions at the single-molecule level led to the discovery of their layered structure and fresh insight as to how this spatial organization enables their function as mechanosensitive signaling hubs (Kanchanawong et al., 2010; Patla et al., 2010).

Single-particle tracking has revealed the dynamic properties of the constitutive integrins dimers involved in focal adhesions. Their successive periods of immobilization and dissociation from the underlying actin cytoskeleton proved to be an important part of their adhesive and signaling role (Rossier et al., 2012). Integrins also require assembling into nanoclusters to be functional. The existence of a maximum distance of 55 nm between two activated integrins prior to triggering of adhesion was shown using RGD-peptide-coated gold nanodots arrays (Arnold et al., 2004; Cavalcanti-Adam and Spatz, 2015; Huang et al., 2009; Liu et al., 2014; Selhuber-Unkel et al., 2008), or nanopatterns of defined spacing and grafting densities (Coyer et al., 2012).

Beyond adhesion, integrin clusters are also essential signaling hubs for mechanosensation. Cells do not only exert mechanical deformation on the matrix but also sense the response of the matrix and react to it. Imaging substrate deformation of continuous gels with fiducial tracers (Oakes et al., 2012; Plotnikov et al., 2014; Soiné et al., 2015) or of flexible pillars (Rahmouni et al., 2013) enables quantitative measurements of cellular traction and cellular response. These approaches have unambiguously revealed that mechanical tension is crucial for the maturation of the focal adhesion (Ghibaudo et al., 2008; Schiller et al., 2013) and for matrix rigidity sensing (Humphrey et al., 2014; Vogel and Sheetz, 2006). Recent studies performed on pillars of 500 nm in diameter have unraveled a mechanism by which cells ‘pinch’ the matrix, thereby assembling a contractile molecular complex (Ghassemi et al., 2012; Meacci et al., 2016; Wolfenson et al., 2016). The signaling downstream of these pinching events is dependent on the mechanical tension that is necessary to contract the functional unit. This provides a mechanism by which cells can sense substrate rigidity. A striking demonstration of this principle was observed by comparing cell spreading on glass substrates, nano-corralled lipid bilayers and fully fluid bilayers bio-functionalized with identical

Table 1. Overview of the available biofunctionalized substrates

Type	Approach	Reviews	Commercial availability	Biological applications
Substrates with structured protein coating				
2D protein printing	Stamping (SQ) Stenciling (SQ) Light induced (Q)	Piel and Thery, 2014a,b D'Arcangelo and McGuigan, 2015 Ricoult et al., 2015	Innopsys Alveole Cytoo	2D confinement, forcing cell size and shape Forcing cytoskeleton organization Inducing of front/rear polarization Restricting migration Inducing nuclear stress Spatially organizing cell culture Quantitative interactions with ligand High- or super-resolution imaging
3D protein patterning	On microstructures (SQ) Two photons (Q)	No relevant reviews available	Nanoscribe	
Nanoprinting	Nanodots (Q) Dip pen lithography (NQ) Nanostenciling (SQ)	Ru et al., 2014 Salaita et al., 2007 Ross et al., 2012 Custódio et al., 2014	Innopsys	
Substrates with controlled rheological properties				
Hydrogels or soft elastomers	Biomimetic/extract of ECM (SQ), control of ligand density and growth factors Functionalizable hydrogels of various rigidity (e.g. PEG, PAA, haluric acid) (Q) Particle-filled hydrogels (Q)	Piel and Thery, 2014a,b Ross et al., 2012 Sant et al., 2010	In Sphero Qgel IBIDI Advanced BioMatrix	3D vs 2D cell culture Adhesion on soft substrates Traction force microscopy Spatial clustering of surface receptors Cooperative signaling from receptor clusters Rigidity sensing Migration in absence of adhesion Matrix-dependent cellular response
Functionalized lipid bilayers	Supported lipid bilayers functionalized with ligand (Q) Nanostructured bilayers (Q) (e.g. nanopatterned, nanofences) Multilayered lipid substrate with various rigidity (SQ)	Castellana and Cremer, 2006 Yu and Groves, 2010 Groves, 2007 Tanaka and Sackmann, 2005	eDAQ	
Micro-textured substrates	Micropillars/nanopillars of various length, diameters and densities (Q)	No relevant reviews available	NCA	
Substrates with topographic features				
Micro-textured substrates	Micro-fabricated topographic features on rigid substances (Q) Flexible pillars for force transduction (Q) Micro-cavities or micro-containers (Q)	Bettinger et al., 2009 Nikkhah et al., 2012	Mechanobiology Institute Nunc Microsurfaces Aggrewell	Curvature or topography sensing Enhanced stem cell differentiation Mechanical stress measurements (single-cell analysis or organoids) Induction of front–rear polarization 3D migration Confined migration Chemotaxis Effect of fluidic shear
Nano-textured substrates	Planar substrates with nanofeatures (Q) Electrospun nanofibers Nano silicon/graphene fibers	Lim and Donahue, 2007 Kim et al., 2012 Baharvand, 2015	NCA	
Microfluidics	3D channels for migration assays Flow chambers Soluble factor gradient generators	Dupin et al., 2013	Cellasic ONIX Millipore IBIDI Gradientech Fluxion BellBrook Labs Hurel Kirkstall Mimetas	

Abbreviations: NCA, no current commercial availability; NQ, non quantitative; PAA, polyacrylic acid; PEG, polyethylene glycol; Q, quantitative; SQ, semi-quantitative.

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densities of RGD peptides (Yu et al., 2011). As the substrate became more fluid, cells were unable to exert any mechanical load on the engaged integrin clusters, preventing the maturation of focal adhesions; this caused the cells to round up. A combination of these approaches has also revealed that talin, a multidomain protein localized to focal adhesion, sequentially unfolds under various mechanical loads, thus serving as a 'mechanical ruler' (del Rio et al., 2009; Hu et al., 2016; Margadant et al., 2011; Yao et al., 2014, 2016). This constitutes the best-understood mechanism (although it is not unique) by which cells can sense the level of force they exert.

By using stenciling membranes to constrain the extent of cell spreading, a seminal study demonstrated that a minimal value for cell spreading is required to avoid cell death (anoikis) (Chen et al., 1997).

It is now increasingly clear that rapid events (over a timeframe of seconds to minutes) for rigidity sensing directly influence the activity of transcription factors (Fourel et al., 2016; Petropoulos et al., 2016; Renz et al., 2015), and ultimately cell behavior and fate (Inman et al., 2015). As an example, apical polarization has been shown to be highly dependent on matrix organization (Akhtar and Streuli, 2013; Rodríguez-Fraticelli et al., 2012). In this context, the mechanotransduction role of β1 and β3 integrins was singled out (Fourel et al., 2016; Schiller et al., 2013). In addition, 2D and 3D protein printing has demonstrated how the spatial structuration of the adhesive environment influences the localization (Rodríguez-Fraticelli et al., 2012) and shapes (Li et al., 2016) of apical lumens. Epithelial morphogenesis has also been found to depend on the biophysical properties of surrogate matrix gels (Enemchukwu et al., 2016). The 2D confinement of cells on ECM patterns was also shown to elicit the translocation of the co-transcription factors YAP and TAZ from the nucleus to the cytoplasm. This provides a molecular basis for signaling pathways that regulate cell proliferation in a manner that depends on cell confinement and confluence (Dupont et al., 2011).

In addition, the curvature of ECM micropits has been found to control the branching morphogenesis of epithelium, and this demonstrates the importance of the geometry of cell confinement (Nelson et al., 2006). Last but not least, the rheological properties of the matrix, cellular confinement and geometrical constraints have all been found to have a crucial role in stem cell differentiation and cell fate reprogramming (Engler et al., 2006). Paradigm-shifting experiments have demonstrated that biophysical cues interfere with cell differentiation programs and contribute to the cell lineage commitment (Engler et al., 2006; Gilbert et al., 2010; Wen et al., 2014). Stem cell differentiation has emerged as being a combination of a response to soluble factors and an integrated response to environmental factors, including geometrical, rheological and topographical cues (Discher et al., 2009; Griffin et al., 2015; Murphy et al., 2014). Consequently, a fundamental understanding of cell differentiation, as well as technical solutions to enhance cell differentiation, has been drawn from these observations. In this perspective, how mechanical properties of the substrate are transduced to the nucleus to trigger mechanosensitive control over genomic programs is being intensely scrutinized. A possibility emerges that geometrical constrains impinge on nuclear morphologies (Li et al., 2014; Oakes et al., 2014; Versaevel et al., 2012), chromatin compaction states (Makhija et al., 2016) and chromosome territories (Thomas et al., 2002). Here, the cytoskeletal rearrangements that are induced by the spatial structuration and confinement of the adhesive area result in nuclear reorganization and genome reprogramming. Taken together, all these studies illustrate the long-term downstream consequences of rapid environmental sensing process. The biggest challenges in this area are to unravel the routes by which early pathways of environmental-sensing signal to transcriptional, genomic and epigenomic programs downstream. As outlined above, the tools required to answer these questions are ready and await use by the different communities of biologists.

Cell–cell interactions

ECM factors are crucial for microniche signaling. However, cell–cell interactions need to be considered to an equal extent as an environment signaling cue. Technological developments required to unravel downstream consequences of sensing at cell–cell junctions are lagging behind those used for cell–ECM adhesion. Understanding the mechanobiology of intercellular contacts is intrinsically a multi-component, interconnected problem (as compared to the interactions between a single cell and ECM or soluble factors). A cell both responds to and serves as a 'substrate' for its neighbors. Bio-functionalized materials thus could be instrumental in decoupling both aspects. Indeed, new methodologies are being developed to achieve a degree of control similar to that obtained for cell–ECM interactions as outlined below.

For instance, the type of measurements described for ECM substrates can be utilized for substrates that have been functionalized with cell–cell adhesion proteins, such as E- or N-cadherins (Plestant et al., 2014; Vega et al., 2014) or antigens (Plestant et al., 2014). Studies with E-cadherin (E-cad)-coated substrates have revealed the existence of E-cad nanoclusters as fundamental units for cell–cell adhesion, and their existence has been confirmed by super-resolution imaging of mammalian cell–cell contacts (Wu et al., 2015; Strale et al., 2015), as well as in *Drosophila* (Truong Quang et al., 2013). In addition, both E- and N-cad-coated deformable pillars allow the measurement of the traction forces that are exerted by a single cell across cadherin bonds. Mechanical traction was found to be in the order of 5 to 10 nN per square micron (Ganz et al., 2006; Ladoux et al., 2010). An alternative approach consists of fluorescence resonance energy transfer (FRET)-based force sensors coupled to E-cad to measure the mechanical load that is placed on the adhesion molecule; it amounts to be ∼2 pN for each E-cad molecule that is engaged in a real cell–cell contact (Borghi et al., 2012). Taken together, these measurements point towards a mechanical tension exerted at the adherens junction that is of the order of 100 nN (for a junction of ∼10 µm in length). These approaches have also revealed that there are strong structural similarities in cortical organization between the mechanical responses of the ECM (i.e. integrin-based) and those of cadherins. Similarly, the spreading mechanisms of macrophages on an antigen-presenting glass (Vega et al., 2014) has been shown to be dynamically and structurally similar to that of fibroblasts spreading on fibronectin. Taken together, these results indicate that there is a universal cytoskeleton organization for the development of force, which is based on cluster adhesion, force-mediated reinforcement and the development of traction fibers. This also raises questions as to whether these organizing principles arise primarily owing to the way the adhesive ligands are presented to the cells (i.e. soluble, on a fluid substrate, or immobilized) and if the nature of the receptors plays a role. This issue has been illustrated by the different cellular responses elicited by identical growth factors depending on whether they are soluble or attached to the surrounding matrix (Crouzier et al., 2011).

The use of bio-functionalized lipid bilayers is also well suited to address these types of questions. Adhesive ligands can be coupled to phospholipids and incorporated into the supported lipid bilayer

at controlled densities. Furthermore, the mobility of the ligands in the bilayer can be varied from fluid to static. The cells are thus left free to reorganize spatially the ligand–receptor pairs. E-cad cluster formation was found to be highly dependent on the viscous drag felt by aggregated ligands on the bilayer. On fluid bilayers, the clusters were unstable and transient (Biswas et al., 2015; Perez et al., 2005). In contrast, reduced diffusion of E-cad (by varying the bilayer fluidity or binding to the cytoskeleton) stabilized the cluster, strengthened the adhesion and redistributed the adhesive zones along the edge of the surrogate contact into a morphology that is reminiscent of the apical actin belt (Biswas et al., 2015). This observed morphology shares many similarities with real cell–cell contacts that are established between two suspended cells (Engl et al., 2014; Maitre et al., 2012). To our knowledge, similar observations have not been reported for integrin-mediated adhesion. Taken together, these observations indicate that the reorganization of the actin cytoskeleton to which adhesive clusters transiently bind is both responsible for and dependent on the biophysical properties and spatio-temporal distribution of these adhesive ligands. The ability of actin flows to organize and segregate ligand–receptor pairs at cell–cell contacts has been best demonstrated by using bio-functionalized lipid bilayers as a surrogate for immunological synapses. Imaging of these surrogate substrates has revealed the segregation of different immune receptor pairs into concentric regions called central supramolecular activation clusters (cSMACs), peripheral SMACs (pSMACs) and distal SMACs (dSMACs). This demonstrates that the actin-based spatial segregation of the different receptor signals is key to eliciting the immunological response of the T-cell (Dustin and Groves, 2012; Groves, 2007; Pageon et al., 2016; Tanaka and Sackmann, 2005; Yu and Groves, 2010).

The supported bilayer approach has also been used with other classes of intercellular receptors. For example, it has been shown that the signaling downstream of the binding of EPHA2, a receptor tyrosine kinase involved in cell motility and organ boundary formation, to an ephrin-A1-functionalized bilayer largely depends on the size and spatial structuration of the ligand receptors cluster (Greene et al., 2014; Salaita et al., 2010). The misregulation of this force-activated pathway has also been found to be a hallmark of the metastatic potential of breast cancer cells (Salaita et al., 2010).

One can devise a third approach for using bio-functionalized substrates to study cell–cell adhesion. ECM patterning can be used to indirectly force cells to interact with each other in a controlled manner. For example, 2D bow-shape patterns have been used to measure the traction force that two cells exert on each other as the imbalance of the mechanical tension they exert on the substrate (Liu et al., 2010; Maruthamuthu et al., 2011; Ng et al., 2014). 2D ECM geometrical patterns have also been used to show that adherens junctions orient away from ECM adhesion (Mertz et al., 2013; Sim et al., 2015; Tseng et al., 2012). This idea was recently extended in 3D to differential protein printing in small pits. When coated with an anti-fouling treatment, they provide ideal substrates to perform en-face imaging of junction formation between two stacked cells (Engl et al., 2014) or during mitosis (Wollrab et al., 2016). Coating pits with different proteins on their top, sides and bottom allows exquisite control over cell–cell interactions that are induced by matrix adhesion in 3D. This approach has been recently used to reveal how intercellular tension guides the luminogenesis in hepatocytes (Li et al., 2016). Embedding micromirrors in close vicinity to these micropits enables 3D super-resolution imaging of cells in their environment with a sectioning capability of up to tens of microns above the coverslip (Galland et al., 2015).

Taken together, we believe that the recent technological developments and the growing number of studies focusing on mechanotransduction at cell–cell contacts will bring this field to the same level of understanding as has been obtained for cell–ECM adhesion. We anticipate that this will also result in a better understanding of how environmental factors act as spatially structured triggers for cell polarization and organogenesis.

Cell migration

The substrates described so far are characterized by a structured but homogenous bio-functionalization. Spatial gradients or properties that vary over time can also be built in to study directed cell migration (taxis). For instance, patterning with light-induced release of adhesive constraints (Rolli et al., 2012; Vignaud et al., 2012) has been used to study the transition from static to migratory behaviors. Our understanding of migration along gradients of soluble factors (chemotaxis) has benefited from microfluidics, where gradients of soluble factors that are transverse to the direction of microfluidic flow can be created (Chung and Choo, 2010; King et al., 2016; Li Jeon et al., 2002; Toh et al., 2014). Passive diffusion of a chemoattractant across a porous membrane has also been used to create local gradients in the absence of flow (Dupin et al., 2013). Furthermore, imprinting of protein gradients on rigid substrates (Ricoult et al., 2015; Wu et al., 2011) or controlled stenciling and/or photo-immobilization techniques (Bélisle et al., 2009, 2012; Strale et al., 2016) has revealed the molecular basis for haptotaxis, the migration of cells along an ECM gradient. In addition, the use of 3D matrix hydrogels containing smooth gradients of crosslinking density (i.e. to create a ECM with differing concentrations of crosslinking components) (Millon-Frémillon et al., 2008) has provided insights into how macrophages migrate along gradients of differing matrix stiffnesses (i.e. durotaxis) (Nemir and West, 2010). Finally, a recent study employed traction force microscopy on hydrogels with an embedded stiffness gradient to demonstrate that cells can collectively sense large-scale matrix density gradients resulting in collective durotaxis (Sunyer et al., 2016).

In absence of directional cues, spontaneous modes of collective cell migration are also found to largely correlate with the physical constraints of the environment. Removal of a physical obstacle from an ECM-coated substrate allows monolayers of epithelial cells to suddenly access a surface where cells are free to migrate. This migration assay with controlled boundary conditions results in a large-scale, swirling collective motion within the monolayer, as well as in the appearance of ‘leader cells’ with distinct migratory characteristics (Poujade et al., 2007). Cell swirls largely depend on the lateral confinement of the monolayer (Deforet et al., 2014; Doxzen et al., 2013; Rørth, 2012; Tanner et al., 2012; Vedula et al., 2013). The emergence of this synchronized collective cell motion can be correlated with contractile waves that involve multiple cells as measured by traction force microscopy (Angelini et al., 2010; Serra-Picamal et al., 2012). The existence of leader cells is also an indication that cells can migrate by using different modes of motility. Indeed, it has been shown that switching between different modes of migration can be elicited by environmental cues (te Boekhorst et al., 2016).

The various modes of cell migration can be recapitulated by *in vitro* reconstitution of idealized migration conditions (Charras and Sahai, 2014; Friedl and Wolf, 2010; Rao et al., 2014). Here, bio-functionalized substrates provide an excellent platform to understand how differences in spatio-temporal coordination of an identical pool of regulators (e.g. Rho GTPases) and cytoskeleton effectors (e.g. myosin II, Arp2/3, formins and filamin A) trigger the

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Box 1. Co-culture of cells organized in 3D

In most of the studies described here, the controlled microenvironment is applied to a single cell type or to the study of homotypic interactions. However, an increasing number of studies now aim to co-culture different cell types in a spatially structured manner in order to better recapitulate cell–cell interactions within tissues or organs. To that end, 3D microchannels (~200-µm wide) created in an ECM hydrogel can be used to culture endothelial cells; this mimics blood vessels, and interactions of endothelial cells with cancer or stromal cells can be investigated (Jeon et al., 2015; Miller et al., 2012; Shin et al., 2011). In addition, 2D protein patterns have been used to localize hepatocyte islands amidst an interacting fibroblast layer, which has been shown to result in enhanced bile production (Bhatia and Ingber, 2014). Finally, a fast developing method for cell culture in 3D is to create organoids, with cells being grown either in hanging drops or in an ECM surrogate (Clevers, 2016; Fatehullah et al., 2016; Yin et al., 2016). The development of tissue-specific organoids relies on the provision of soluble factors and on the self-organization of the cells therein as they grow. We anticipate that extending our control over cell culture in 3D to being able to induce the interactions between different cell types in 3D-structured organoids will advance our capability to culture tissue *in vitro*, as well as increase our knowledge of how the spatial structuration of the environment influences cell fate.

activation of distinct cytoskeleton protrusions, which ultimately lead to different migration modes. The ‘world cell race’ (Maiuri et al., 2012) is an iconic example of how migration conditions can be standardized over an extensive roster of cell types, and migration speed and migration persistence of 50 cell types has been probed on one-dimensional (1D) ECM-coated lines. Extending these findings to 2D and 3D *in vitro* and *in vivo* environments has led to a comprehensive understanding of how actin flows mediate a universal (1D, 2D and 3D) coupling between these parameters (Maiuri et al., 2015). 1D migration has been further shown to favor filopodia-driven migration of fibroblasts (Guo and Wang, 2012), with cells adopting elongated spindle-like shapes (Chang et al., 2013; Doyle et al., 2012; Guo and Wang, 2012; Monzo et al., 2016). When various mammalian cells, such as transformed or untransformed fibroblasts and cancer cell lines, including glioma and carcinoma of various origin, migrate along ECM-printed lines, their motility mode becomes intermittent, or saltatory, similar to that described for neuronal motility (Guo and Wang, 2012; Irimia and Toner, 2009; Monzo et al., 2016; Pathak and Kumar, 2012). This contrasts with the ‘classic’ gliding migration of fibroblasts or keratinocytes on 2D surfaces, which results from the dynamic protrusion of a lamellipodium at the front of the polarized cell and a contractile actomyosin network at its rear (Verkhovsky et al., 1999). Formins play a crucial role in generating and organizing the long actin cables that are necessary to support the elongated shape of the cells during 1D migration (Monzo et al., 2016; Vargas et al., 2016; Wilson et al., 2013), whereas Arp2/3 is more crucial for migration in 2D. Furthermore, if cell migration takes place on suspended electrospun nanofibers coated with ECM (Johnson et al., 2009), free actin waves propagate from the cell body to the tip of the cellular protrusion. This occurs in an asymmetric manner to polarize the movement of long spindle-shaped cells (Guetta-Terrier et al., 2015). These thin actin protrusions differ in nature from the bulky actin protrusions, which are used to crawl across the matrix pores and termed lobopodia, that appear when cells move in a dense 3D matrix (Petrie et al., 2012). The use of a 3D matrix with controlled pore sizes has also enabled the investigation of mechanical nuclear deformation during migration and its consequences for DNA damage (Petrie et al., 2014; Raab et al., 2016; Thiam et al., 2016).

Control over the cellular microenvironment has not only revealed the existence of different modes of migration, but has also provided insights into how exactly cells switch from one mode of motility to another (Friedl and Alexander, 2011). Non-adherent confining substrates, which can be created by microchannels (Bergert et al., 2015) or with a double layer of inert hydrogel (Ruprecht et al., 2015; Liu et al., 2015), have been shown to induce a myosin-II-dependent switch to an amoeboid migration mode that involves the formation of a stable, bleb-like and actin-depleted protrusion at the cell front (Bergert et al., 2015; Liu et al., 2015; Ruprecht et al., 2015). These studies have also determined that the driving force for this amoeboid migration mode is due to a reverse actin flow mediated by non-specific friction between the cell and its substrate.

Taken together, as outlined above, by being able to control the cellular environment *in vitro*, many of the environmental parameters and migration modes that are observed in different *in vivo* contexts, such as embryogenesis, wound healing, metastasis, neuron development or the inflammatory response, can be recapitulated and further investigated in detail.

Conclusions and perspectives

The primary advantage of using bio-functionalized surfaces is that they provide the ability to isolate and vary a single environmental parameter in order to unambiguously decipher its contribution to a given cellular process. Combining these interfaces with imaging at the nanometer scale and the genetic alteration of cells is key to understanding the molecular and cellular processes by which cells sense their environment. It is increasingly clear that cells do not exist in a single state, but rather are able to switch between different programs that dictate their behavior. However, the decision to engage in a certain state is not only dictated by secreted or soluble factors, but is made in conjunction with probing of the microenvironment. In that sense, environmental cues can be seen as external triggers of autonomous cell programs. Testing the extent to which an individual environmental cue triggers a specific program is a promising approach to being able to understand the molecular pathways by which cells engage into such programs, or how their behavior (such as drug resistance and differentiation) is conditioned by their microniche. We would like to argue here that there is already a technical solution to recreate most of the environmental parameters individually *in vitro*. Developing a single approach that allows for a combinatorial control over all environmental factors will be crucial to building platforms that will allow cells to be able to sense the entire cellular environment (Dolatshahi-Pirouz et al., 2014; Gobaa et al., 2011). In particular, precisely combining two or more ligands within an engineered environment has already helped to unravel the combinatorial interplay of different adhesive pathways. For example, an increasing number of studies scrutinize the crosstalk between integrins and growth factors (Fourel et al., 2016; Lee et al., 2011), as well as between integrins and cadherins (Borghi et al., 2010; Stapleton et al., 2014), by incorporating one factor at a time in order to generate *in vitro* the essential aspects of *in vivo* complexity. Because the downstream consequences of environmental sensing most likely originate from the integrated signaling of combined environmental cues, it is important to reconstruct the complexity of the microniche from individually controlled cues. The combination of the minimal number of external cues needed to trigger an emergent cellular property or differentiation or polarization program could thus be deciphered. Another challenge that awaits the field is to extend the technological know-how and biological knowledge that has been acquired at the single cell level to extend to co-cultures of cells in 3D. These systems are expected to provide insights into the environmental

cues required for the formation of multi-cell type organoids. These concepts are being developed in so-called 'organ-on-a-chip' approaches (see Box 1).

The examples described above illustrate the popularity and usefulness of bio-functionalized surfaces in cell biology over the last decade. Their development and use is clearly an area where biology, physics and engineering overlap. However, the need for multidisciplinary expertise still impedes access of many laboratories to these techniques. Although the commercial availability of these technologies is growing (see Table 1), the particular tools offered will always be based on economical profits and thus most likely limit the extent and capability of the devices that can be purchased. As many of the substrates described above can be realized with a combination of soft lithography techniques and protein and/or lipid adsorption, the scientific community would benefit greatly from the creation of an open-source global repository of these devices, similar to that Addgene provides for plasmids. To that end, the Mechanobiology Institute in Singapore is committed to offering such a tool to the community. It actively seeks academic partners to complete an online declaration of interest to raise starting funds for the initiative (http://www.mechanobio.info/resources/pdms-survey/).

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Competing interests
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