Significance of Nanotopography for \textit{in vitro} Cell-Surface Interactions

L’importanza della nanotopografia per le interazioni \textit{in vitro} cellula-superficie

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E’ costituito dall’accorpamento di diversi dipartimenti, tra i quali quello di Biochimica, ora definita Unità. 
L’Unità di Biochimica, ex dipartimento, fu istituito nel 1982 .

Centro Interdipartimentale di Ingegneria Tissutale (C.I.T)

Si è costituito nel 2006: accorpa almeno 3 dipartimenti che fanno parte della ex Facoltà di Medicina, il Dipartimento di Chimica, la Facoltà di Ingegneria, e il Policlinico San Matteo

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Clinica per la Riabilitazione fondata nel 1965 dal Prof Salvatore Maugeri. Dal 1 Novembre 2011 è stato istituito il Laboratorio di Nanotecnologie a Pavia
• Cell-surface interactions play a crucial role for tissue regeneration, biomaterial interactions and applications

• Cellular adherence, migration, proliferation and differentiation are influenced by:
  a. chemical composition of solid substances
  b. the surface topography in terms of micro- or nanoscale protrusions, pits and grooves.
As materials technology and the field of tissue engineering advance, the role of cellular adhesive mechanisms, becomes more relevant in both research and clinical practice.

In this lesson, we will try to elucidate the principle mechanisms of nanoscale cell-surface interactions *in vitro* through these topics:

- Surfaces, Nanotopography
- Extracellular Matrix (ECM) Components and Cell Integrins
- Mechanism of cell adhesion
- Significance of Nanotopography interactions
- Quantitative determination of cell adhesion by novel physical techniques
Significance of Nano- and Microtopography for in vitro Cell-Surface Interactions

Molecular surface science has greatly contributed to the advancement of nanofabrication technology by providing ideal platforms for engineering surfaces on a molecular level.

- self-assembled monolayers (SAMs)
- bottom–up self-assembly approaches
- redox control,
- conductive AFM,
- field photolithography
- stamping techniques, such as imprinting lithography
- dip pen nano lithography

SEM of the interstitial surface of lamina densa of epidermal basement membrane at high magnification.

The extracellular matrix (ECM) is:

- a. the non cellular component present within all tissues and organs
- b. provides essential physical scaffolding for the cellular constituents
- c. initiates crucial biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation and homeostasis

The extracellular matrix has 3 major components:

1. Highly viscous proteoglycans (heparan sulfate, keratan sulfate, chondroitin sulfate), which cushion cells
2. Insoluble collagen fibers, which provide strength and resilience
3. Soluble multiadhesive extracellular matrix proteins (fibronectin, laminin), which bind proteoglycans and collagen fibers to receptors on the cell surface
- water, proteins and polysaccharides

- each tissue has an ECM with a unique composition and topology that is generated during tissue development through a dynamic and reciprocal, biochemical and biophysical dialogue between the various cellular components and the evolving cellular and protein microenvironment.

- It is also markedly heterogeneous.

- ECM is a highly dynamic structure that is constantly being remodeled, either enzymatically or non-enzymatically, and its molecular components are subjected to a myriad of post-translational modifications.

- ECM generates the biochemical and mechanical properties of each organ, such as its tensile and compressive strength and elasticity, and also mediates protection by a buffering action that maintains extracellular homeostasis and water retention.

- the ECM directs essential morphological organization and physiological function by binding growth factors (GFs) and interacting with cell-surface receptors to elicit signal transduction and regulate gene transcription.
Different combinations of these components tailor the matrix for different functions depending on the amount of strength (for example tendons), cushioning (for example cartilage) and adhesion required.

All extracellular matrix components are synthesized intracellularly and secreted via exocytosis.
PGs fill the majority of the extracellular interstitial space within the tissue in the form of a hydrated gel.

PGs have a wide variety of functions that reflect their unique buffering, hydration, binding and force-resistance properties.

**Proteoglycans (PGs)** are composed of glycosaminoglycan (GAG) chains covalently linked to a specific protein core (with the exception of hyaluronic acid).

PGs have been classified according to their core proteins, localization and GAG composition. The three main families are: small leucine-rich proteoglycans (SLRPs), modular proteoglycans and cell-surface proteoglycans.
Fibrous proteins

Fibrous collagens

Fibronectin

Tenascin

Elastins

Laminin
Collagen is
- the most abundant fibrous protein within the interstitial ECM
- constitutes up to 30% of the total protein mass of a multicellular animal.

To date, 28 types of collagen have been identified in vertebrates

It provides:
tensile strength, regulate cell adhesion, Support chemotaxis and migration, and direct tissue development.

The majority of collagen molecules form a triple-stranded helix that subsequently can assemble into supramolecular complexes, such as fibrils and networks, depending on the type of collagen. Fibrous collagens form the backbone of the collagen fibril bundles within the interstitial tissue stroma, whereas network collagens are incorporated into the basal membrane (BM).
Fibronectin (FN) is intimately involved in directing the organization of the interstitial ECM and, additionally, has a crucial role in mediating cell attachment and function.

FN can be stretched several times over its resting length by cellular traction forces. Such force-dependent unfolding of FN exposes cryptic integrin binding sites within the molecule that result in pleiotrophic changes in cellular behavior and implicate FN as an extracellular mechanoregulator.

FN is secreted as a dimer joined by two C-terminal disulfide bonds and has several binding sites to other FN dimers, to collagen, to heparin and also to cell-surface integrin receptors.

Cell-surface binding of the soluble FN dimer is essential for its assembly into longer fibrils. Moreover, cell contraction through the actomyosin cytoskeleton and the resulting integrin clustering promotes FN–fibril assembly by exposing cryptic binding sites, thus allowing them to bind one another.
**In vitro** studies indicate that endogenous proteins become rapidly adsorbed to a surface in response to surface free energy, providing a structural framework on which cellular adhesion may initiate.

Adherent cells are complex, self-sustaining units that require extracellular matrix (ECM) anchorage to proliferate and undergo differential function. Cells actively probe the physical properties of the ECM; their contractile machinery facilitating the formation of polarized "lamellipodia" (Figure 1, A) and fine hairlike protrusions termed "filopodia" (Figure 1, B), structures which gather spatial, topographical, and chemical information from the ECM and/or material surface.

**Figure 1** - Cell-substrate interactions and focal adhesion formation. (A) Adherent cells form dynamic actin-rich extensions during the process of cellular spreading and migration and (B) probe the underlying (grooved) substratum with fine filopodial extensions (arrows) from the leading and trailing free edge. (C) Adherent cells maintain cellular integrity through a dynamic network of contractile actin stress fibers (red) that terminate in focal adhesion plaques (green), molecular complexes that intimately connect the cytoskeleton with the extracellular matrix.

Two morphological variants of the focal adhesion exist; the ‘dot’ and ‘dash’ variants

‘Dot’, or small initial, contacts are constituted of transmembrane and some linker proteins but are not associated with actin bundles. These are the predominant contact type, with dimensions of 0.2-0.5μm, and are mainly located at the active edge of the cell.

Elongated large mature, or ‘dash’, contacts are also composed of transmembrane and linker proteins but differ due to their association with the cytoskeletal actin bundles. Dash contacts are 2-10μm in length and 0.5μm in width and are located centrally in parts of the lamellae, areas of the endoplasm and under the nucleus.
Filopodia are thin, actin-rich plasma-membrane protrusions that function as antennae for cells to probe their environment. Consequently, filopodia have an important role in cell migration, neurite outgrowth and wound healing and serve as precursors for dendritic spines in neurons.

A lamellipodium is a thin (0.1–0.2 micron) sheet-like protrusion that is filled with a branched network of actin.

By contrast, filopodia are thin (0.1–0.3 micron), finger-like structures that are filled with tight parallel bundles of filamentous (F)-actin.

Non-muscle cells also contain contractile structures called stress fibres, which are composed of actin and myosin bundles, provide contractile forces for cell migration and morphogenesis.

Various cell types express high levels of more than 10 micron long transient filopodia in their spherical state prior spreading, either after trypsinization from cell culture dishes or during mitosis.

On flat surfaces these transient filopodia are known to quickly disappear during spreading in favor of the widely described lamellipodia mediated spreading mechanism.
Time-dependent spreading events of fibroblasts on flat surfaces and in contact with NWs. (a–d) Fibroblasts spreading on fibronectin (Fn) coated flat glass surfaces (2D). Large numbers of ∼10 mm long filopodia cover the entire surface of spherical cells in the first moment of substrate contact. (a arrowheads) Straight and curled filopodia are deposited randomly around the cell periphery. (b arrowheads) Within the first 5 min, most filopodia retracted and only few straight filopodia remained with distal substrate adhesions touching the surfaces under a shallow angle (kink). (c) Cells spread with a circumferential lamellipodium after passing the lag time. (d) The actin-rich lamellipodium replaced the initial surface adherent filopodia contacts (live cell imaging with actin GFP transfected cells). (e) On flat versus fibrillar interfaces, transient filopodia were initially in contact with both, flat surfaces and nanowires. (f) The transient filopodia adhered and aligned with individual nanowires and quickly directed cell spreading towards nanowire adhesion sites, while most filopodia retracted from the Fn coated flat glass surfaces. (g and Suppl. Fig. S1e) The apical membrane that is not facing nanowires was later completely depleted of filopodia, while ruffles appeared after 30 min. (h, i) Initial filopodia formed entangled and centripetally aligned adhesions to individual nanowires when in contact with densely NW decorated substrates. (i and Suppl. Fig. S1d) Aligned filopodia-NW adhesions guided pseudopods/membrane protrusions towards the NW anchorage site. (k) Filopodia-mediated cell spreading lead to dendritic cell shapes in the absence of typical lamellipodia-like membrane protrusions. (l) Confocal 3D reconstruction of phalloidin-alexa 546 stained actin cytoskeleton after 16 hours on 3D nanowires shows dendritic cell protrusions that display numerous filopodia, which are anchored deeply into the nanowire matrix in the absence of lamellipodia. Scale bars 5 mm with the exception of k 5 10 mm and i 90 5 1 mm.
Underpinning mechanisms of topography sensing. (a–c) Schematics of the topography recognizing function of filopodia. (d) Summary of our findings for the different engineered substrates and how differences in cell shape and polarity as well as the spreading dynamics are controlled by filopodia-NW interactions. (d) The first row describes for flat glass (2D) how fibroblasts undergo a rapid phase transition from filopodia-rich initial state towards a lamellipodia-mediated spreading. The second row shows how cells sitting at interfaces between flat surface and NWs contact the NWs, quickly adhere, align and spread towards nanowire adhesions, while filopodia peel-off from the adjacent flat surfaces. After 30 min, delayed lamellae/ruffle formation leads to migration towards the flat surface (Data not shown). This dynamic change in topography preference is reversible during mitotic rounding of fibroblasts. The bottom row shows how fibroblasts on purely nanowire decorated surfaces spread via aligned filopodia-nanowire adhesions into dendritic shapes, without formation of lamellipodia. (e) Schematics showing force distributions on integrins in dependency of the angle $\alpha$ at which a filopodium contacts an object. (f) A simple mechanical zipping model allows to estimate the normal forces acting on individual integrins as function of the contact angle $\alpha$. Since the number of integrins/mm² within the adhesions is unknown, we calculated the normal force per integrin as function of the contact angle for two integrin densities (blue and green symbols) by assuming a filopodia traction force of 2 nN. To estimate the critical angle below which peeling will stall, we next assumed that the integrins within the filopodial shaft can sustain the same force as in focal adhesions (FAs). Cells typically apply traction forces around 5 nN/mm² at focal adhesions (FA). This converts to the force per integrin as marked with red squares and thereby predicts a critical angle of close to 12° below which filopodial adhesions mature rather than peeling off before their tips detach. Supplemental figure S1c displays that only a small fraction of transient filopodia (very long ones with their base close to the flat substrate) formed angles <12°.
Cell adhesion to the ECM is mediated by ECM receptors, such as integrins, discoidin domain receptors and syndecans.
Significance of Nano- and Microtopography for \textit{in vitro} Cell-Surface Interactions
Integrins attach to the ECM and connect indirectly to the actin filaments through protein assemblies of talin-paxillin- vinculin.

These protein assemblies stabilise the focal adhesion structure, as well as relaying signals from the ECM to the nucleus.

Signals from the integrins are relayed to the nucleus by the bridging proteins and the actin fibres

These signals initiate nuclear gene expression that subsequently sends the corresponding response signal.

Diagrammatic representation of the spatial interaction of most of the focal adhesion linker and signalling proteins. Abbreviations $\alpha$-act = $\alpha$-actinin, Pax = paxillin, Vinc = vinculin, Ten = tensin, FAK = focal adhesionkinase, PIP2 = phosphotidyl inositol-4-5 bisphosphate, $\alpha$ & $\beta$ = integrins, FC = focal contact.
Representation of how integrin-mediated activation of FAK/MAPK signal transduction pathway may regulate the cell/substrate interaction. Based on Boudreau and Jones, 1999. (a) Once the cell comes into contact with the substrate it forms focal adhesions. The integrins are thought to relay signals to the nucleus through the MAPKase (Mitogen-activated protein kinase) pathway that is a cascade of proteins. Abbreviated FAK= Focal Adhesion Kinase, P= phosphorylation, TFs= transcription factors, RAS & raf monomeric GTPases, ERKs = extracellular signal related kinases, MEK= MAP kinase kinase. (b) If the signals relayed by the focal adhesions to the nucleus are positive then integrin clustering occurs increasing the area of cell adhesion to the substrate, if the signals are negative then matrix metalloproteinases are released causing integrin substrate detachment decreasing the area of cell attachment. In the extreme, cases of substrate unsuitability cell detachment may occur.
The complex interplay between the mechanical role of cell adhesions and their ‘instructive role’, which is manifested by the activation of a wide variety of signaling networks, is mediated by a group of proteins collectively known as the ‘adhesome’.

The concerted activity of adhesome components affects essentially all cellular functions, including morphogenesis, migration, proliferation, differentiation and viability.

On the cytoplasmic side of the adhesion sites, integrins can interact, via their cytoplasmic tails, with at least 12 different adaptor proteins:

- Tensin (TNS1 gene)
- Filamin (FLNA)
- Talin (TLN1)
- Plectin (PLEC1)
- α-actinin (ACTN1)
- Vimentin (VIM)
- Paxillin (PXN)

Plectin can also directly interact with the intermediate-filament protein vimentin (VIM), and paxillin (PXN) can provide a direct link to microtubules.

Links with the cytoskeleton are further reinforced by a second and third tier of adaptor molecules, which stabilize the adhesome network and connect to the various filament systems of the cell.

The integrin-actin connection is pivotal for the mechanosensory function of the adhesion: forces applied to integrins by contractile actomyosin bundles, for example, play an important role in recruitment to, and reinforcement of, the adhesion site, although the exact mechanism remains unclear.
Presentation of scaffolding network of the integrin adhesome (adhesion receptors, adaptors, actin regulators and the associated cytoskeleton) with its known interactions.

Regulation of each protein is color-coded: phosphorylated at a tyrosine residue (red), a serine or threonine residue (blue), phosphorylated at both tyrosine and serine or threonine residues (purple) and so on.
Nanotopography and focal adhesion formation

Topography and in particular nanoscale features can affect cell behavior and integrin-mediated cell adhesion.

Nanotechnology aims to create and use structures and systems in the size range of about 1–500 nm covering the atomic, molecular, and macromolecular length scales.

The extent to which nanotopography influences cell behavior within an in vitro environment remains unclear, and investigation into this phenomenon is still ongoing.

The processes that mediate the cellular reaction to nanoscale surface structures may be:

- **direct** (a direct result of the influence of the surface topography)
- **indirect** (where the surface structure has affected the composition, orientation, or conformation of the adsorbed ECM components).
Parameters that have already been established as important mediators of mechanotransductive processes and differential gene expression:
1. Temporo-spatial reorganization of the cell cytoskeleton
2. Temporo-spatial reorganization of focal adhesion formation

Adhesive process, is dependent on:
1. Integrin interactions with the substratum
2. The topographical regulation of cell adhesion
   This process seems to be dependent on the symmetry and spacing as well as the x, y, and z dimensions of the topographical nanofeatures

Defined arrays of bound RGD fragments indicate that integrin-substratum interactions are:
   a. disrupted when the integrin spacing is in the range of 70–300 nm
   b. an integrin spacing of less than approximately 60–70 nm is required for protein recruitment to the focal adhesion

In summary:
   decreasing the nanofeature spacing to less than 60–70 nm
   or
   increasing this distance to the submicron range facilitates integrin clustering, thus restoring focal adhesion formation.
Effects of nanoscale protrusions on focal adhesion formation

Parameters: heights, diameter and interspace (density)

Decrease in cellular adhesion with increasing nanoprotrusion height

The restrictive nature of nanofeatures measuring >70 nm in height is the perturbation of focal adhesion that is inhibition of protein reinforcement at the focal adhesion site

Fibroblasts (but also stem cells) adhesion is reduced on 95 nm-high protrusions: cells initially undergo increased cytoskeletal organization and filopodia formation when compared with cells cultured on flat controls but that this initial attachment phase is short-lived and fibroblasts begin to dedifferentiate and undergo anoikis (adhesion-mediated apoptosis) as a result of reduced adhesion and cellular spreading

Reducing the height of nanoprotrusion features to <50 nm numerous cell types to return the frequency of focal adhesion formation to that of cells cultured on planar controls, with accompanying upregulations in proteins critical to cytoskeletal dynamics
**Anoikis** is defined as apoptosis that is induced by inadequate or inappropriate cell–matrix interactions. It is involved in a wide diversity of tissue-homeostatic, developmental and oncogenic processes.

https://www.google.it/search?q=anoikis&source=lnms&tbm=isch&sa=X&ei=W8I4UqejCITitAb92jGADg&
Effects of nanoscale protrusions on focal adhesion formation

Anoikis can be rescued by culturing cells on ECM coated surfaces.
Oligodendrocyte progenitor cells cultured on glass coverslips coated with the natural ECM proteins, fibronectin or laminin, have greater viability compared to cells cultured on PDL coated surfaces.

Blocking the b1 integrin inhibited the survival effect of laminin, suggesting a mechanism of action. Although the mechanism by which anoikis occurs is not fully understood, one proposed mechanism is that following detachment, integrin stabilization of the cytoskeleton via plectin is lost, resulting in Bmf being released from actin. Bmf binds to Bcl-2 in mitochondria and neutralizes its anti-apoptotic effect, which activates caspase-8, releases it from the mitochondria and induces cell death.

https://www.google.it/search?q=anoikis&source=lnms&tbm=isch&sa=X&ei=W8I4UqejCITItAb92IGADg&
The influence of nanoscale protrusions on focal adhesion formation and reinforcement.

A. Integrin clustering and focal adhesion reinforcement is unaffected on nanoscale protrusions with a critical spacing of <70 nm and a nanoprotrusion diameter of >70 nm.

(B) Increasing the interfeature spacing to the submicron scale facilitates cell-basal substratum interactions below a feature height of <70 nm.

(C) Conversely, increasing the feature height restricts integrin binding to the planar basal substrate and restricts focal adhesion formation to the feature apexes.

(D) Integrin clustering and cellular adhesion is greatly perturbed on nanoscale protrusion with a feature diameter of <70 nm and an interfeature distance >70 nm.
Effects of nanoscale pits on cell adhesion

Nanopores are identified as common constituents of tissues in vivo, notably basement membrane of the cornea, the aortic heart valve and the vascular system, and may be implicated in the regulation of cell behavior and function.

**Pit diameter and the spacing and symmetry of pit positioning** produce differing effects on cellular adhesion in vitro.

Introducing a degree of disorder or increasing the interpit area facilitates focal adhesion formation and subsequent cellular spreading.

The conformation of ordered nanopit substrates may also dictate parallel or perpendicular adhesion formation and perturb the radial peripheral focal adhesion formation observed during early cell spreading.
The dorsal (and also probably the ventral) surface of the focal adhesion has a corrugated dorsal surface formed by filamentous structures spaced by an average of 127 nm and protruding by 10 to 40 nm over the interadjacent areas.

These dimensions place a limit on the minimum nanopit depth, which may perturb adhesion formation by direct means.

Arrays possessing an interpit area of <300 nm reduce cellular adhesion

Greater cell adhesion and increased integrin expression occur when topographic features have <10-to 20-nm-scale z-axis dimension (height or depth), and that this occurs despite topographic shapes (island or pit). Also, this effect deteriorates when nanofeatures reach a height or depth of <100 nm.
Effects of nanoscale pits on cell adhesion

The influence of nanoscale pits on focal adhesion formation and reinforcement.
(A) Integrin clustering and focal adhesion reinforcement is unaffected on nanoscale pits with a diameter of <70 nm irrespective of pit depth.
(B) Increasing the pit diameter to >70 nm perturbs integrin clustering when the z dimensions of the pits exceed 100 nm.
(C) Conversely, increasing the pit x-y dimensions and reducing the z dimensions facilitates integrin clustering and focal adhesion formation on the basal planar surface and at the base of the pits.
(D) Integrin clustering and cellular adhesion is greatly perturbed on nanoscale pits with a feature diameter between 70 and 300 nm and an interpit separation of <70 nm.

Nanogrooved topographies consisting of alternating grooves and ridge features differ from both nanoprotrusions and nanopits in that they produce very predictable effects on cellular morphology.

Nanoscale grooves can be considered as a biomimetic ECM design, an attempt to mimic the topographical cues imparted by the fibrous nature of ECM.

ECM components include both individual fibril elements, which have been reported to measure <20–30 nm in diameter in vasculature basement membrane, and fibril bundles, which range from 15 to 400 μm in diameter in tendon tissue.
The interplay between **groove pitch and groove depth** regulates adhesion alignment, yet recent studies indicate that groove depth is the more influential.

The cellular cytoskeletal and adhesion complex alignment is generally more pronounced on patterns with **ridge widths between 1 and 5 μm** than on grooves and ridged topographies with larger lateral dimensions.

Cells cultured on grooves with nanoscale widths produce focal adhesions that are almost exclusively oriented obliquely to the topographic patterns.

Contact guidance is not initiated on groove depths below <35 nm or ridge widths <100 nm.

Similarly, contact guidance or a modulation in focal adhesion formation is not initiated on anisotropic grooved topographies with feature widths significantly greater than that of the cellular diameter.
Micrometer-scale features obtained on substrate surfaces by photolithography can be used to control and affect cell behaviour.

**SEM** micrographs of masters (a) and corresponding polymeric films (b) having 5 μm groove depth and 5 (1), 10 (2), 25 (3), 50 (4) and 100 micron (5) groove widths. Scale bar 50 micron.

**Immunofluorescence micrographs** of C2C12 cells on PLLA–TMC films having 25 μm groove width at different groove depths, 7 days after confluence: 0.5 μm groove depth (a); 1 μm groove depth (b); 2.5 μm groove depth (c); 5 μm groove depth (d); smooth film (e). Staining: blue, nuclei; red, myosin. Arrows indicate the groove direction. Scale bar = 100 μm.

The most encouraging results were observed in the case of microstructured PLLA–TMC films with grooves of 2.5 and 1 μm depth, presenting, in particular, a groove width of 50 and 25 micron.

Cellular mechanotransduction relies on the ability of proteins of the focal adhesion to change chemical activity state when physically distorted, converting mechanical energy into biochemical energy by modulating the kinetics of protein-protein or protein-ligand interactions within the cell.

Little is known about the effects of topographical modification on cellular function or the role of nanoscale features on integrin-mediated activation of adhesion proteins and downstream signaling pathways.

The integrin-dependent signaling pathways are mediated by nonreceptor tyrosine kinases, most notably focal adhesion kinase (FAK), which is constitutively associated with the β-integrin subunit.

FAK localizes at focal adhesions and can influence cellular transcriptional events through adhesion-dependent phosphorylation of downstream signaling molecules, thus controlling essential cellular processes such as growth, survival, migration, and differentiation.

Extensive evidence has shown that FAK is activated in response to both the ECM and soluble signaling factors, suggesting that the FAK family may be at the crossroads of multiple signaling pathways that affect cell and development processes.
The influence of nanoscale features on cellular function. Nanotopographical modification induces functional changes in a wide variety of cell types. Thus far, **nanoscale pits** have shown to upregulate ERK1/ERK2 and FAK signaling in osteoblastic cells. **Nanoprotrusions** enhance the synthesis of osteospecific protein and fibroblast growth factor in osteoprogenitor and endothelial cells, respectively. **Nanogrooves** have been shown to induce neuron differentiation in mesenchymal stem cells and the upregulation of proteins concerned with proliferation in canine kidney cells.

<table>
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<th>Cell type</th>
<th>Chemistry</th>
<th>FBS</th>
<th>Width</th>
<th>Pitch</th>
<th>Depth/Height</th>
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<td>poly(methyl methacrylate)</td>
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<td>500 nm</td>
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<td>350 nm</td>
<td>350 nm</td>
<td>40 nm</td>
<td>Upregulation of cyclin D1 and keratin</td>
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GFAP, glial fibrillary acidic protein; MAP2, mitogen-activated protein 2.
The controlled adhesion of cells to each other and to the extracellular matrix is crucial for tissue development and maintenance.

**Qualitative assays:** CLSM and SEM

Numerous assays have been developed to quantify cell adhesion.

Among these, the use of atomic force microscopy (AFM) for single-cell force spectroscopy (SCFS) has recently been established. This assay permits the adhesion of living cells to be studied in near-physiological conditions.
Three types of single-cell force spectroscopy (SCFS) assays have been developed to measure the strength of cell adhesion down to single-molecule levels.

All three assays use optical microscopes to observe the cell while force measurements are made, but differ in how cells are manipulated and forces are determined.

The oldest method uses micropipettes to grasp and hold cells. The detachment force is measured using a bio-membrane force probe, which can gauge force between \(10^{-2}\) pN (pico-Newton) and 100 pN.

A second method uses a pipette to hold a cell while the strength of interactions between the cell and a functionalized bead are determined using a laser trap. The laser trap allows three dimensional positioning of the bead with nanometer precision and force measurement from \(10^{-2}\) pN to 200 pN.

The third method uses a cell that is attached to a cantilever of an atomic force microscope.
By combining atomic force microscopy (AFM) and optical microscopy, cells can be positioned to assess cellular interactions at a given location on a functionalized surface, tissue or on another cell.

The deflection of the cantilever is used to measure interaction forces.

Among SCFS approaches, the AFM-based technique allows for the widest practical force range, from 10 pN to $10^6$ pN.

We will focus on the use of AFM to measure adhesion strength between a single cell and a substrate that is presented by a functionalized surface or by another cell.
Suspended cells are added to the fluid chamber and allowed to settle. Thereafter, a single cell is captured by gently pressing a functionalized AFM cantilever onto it. This converts the living cell into a probe, which is brought into contact with functionalized surfaces or other cells at a set force and for a specific adhesion time. Subsequently, the cantilever is withdrawn at a constant speed, detaching the cell from its binding place. During this separation process, the cantilever deflection, which is proportional to the vertical force that exists between the cell and substrate, is recorded in a force-distance curve. This curve provides the signature of the cell adhesion.
The de-adhesion of a cell from a substrate that is described by the force-distance retrace curve can be broken into three phases

**During the initial phase (Fig. Ba)**, the retraction of the cantilever inverts the force that is acting on the cell from pushing to pulling. As the overall pulling force increases, the force that is acting at individual cell-substrate adhesion points increases. If many receptors act together, the applied detachment force will be sufficiently high to mechanically deform the cell cortex. The binding strengths of the receptors, as well as their number and geometric placement, determines at what force the cell will start to detach. The largest adhesion force that is recorded, the detachment force ($F_{\text{detach}}$), represents the maximum strength of cells substrate binding. The work that is required to detach the cell can also be used to describe the adhesion strength of the cell. It is calculated from the area that is enclosed by the retraction force–distance curve. Here, it is important to consider that the detachment force include cell elasticity, cortex tension, membrane properties, cell geometry and receptor properties such as binding strength, cooperativity and placement.

After the cell starts to detach from the substrate, individual force steps can be observed during the second phase (Fig. Bb). During this phase, the receptor(s) either detaches from the substrate surface or is pulled away from the cell cortex at the tip of a membrane tether.

During the final phase of detachment (Fig. Bc), the cell body is no longer in contact with the substrate and, thus, attachment is mediated exclusively by tethers.
Current SCFS set-ups do have some limitations.

Adhesion measurements that use single cells are time consuming because only one cell can be characterized at a time. For statistical reasons, many detachment-force–distance curves must be recorded, which limits the length of the contact times that can reasonably be assayed.

Furthermore, the almost unavoidable thermal drift in AFM complicates long-contact-time experiments (>20 minutes) and the tight adhesion of cells after longer contact times (>1 hour) exceeds the capability of the system. Thus, SCFS is currently restricted to short contact times that range from milliseconds to ~20 minutes.

There is also a high cost associated with SCFS

Of particular concern are:
(1) the need to establish controls that demonstrate the specificity of the molecular interaction being studied;

(2) the temptation to over-interpret numerical data that are gleaned from unverified mathematical models;

and (3) the difficulty in appreciating the complexity of both the physics and biology of the systems studied.
Future perspectives

- The exact mechanisms involved in integrin clustering and focal adhesion formation are still being investigated.
- Recent studies indicate that the focal adhesion protein **talin** makes a determining contribution to adhesion disruption through nanotopographical features. This protein provides the link between the transmembrane integrin heterodimer and the contractile apparatus of the cell, and it is the conformation and number of integrin-binding domains of this molecule that dictate the critical spacing of bound integrins required for focal adhesion activation.

Topographical modification of the cell substrate interface is a significant regulator of cellular adhesion and function.

Biodegradable devices may be functionally modified to control cellular interactions, with an aim to enhancing tissue regeneration.

The evolution of biomaterial design may rely on the topographical modification of advanced materials that have been fabricated to include a bioactive component, with an aim to regulating cellular adhesion and differentiation followed by controlled construct resorption.

Development of smart multiphase materials containing discrete surface nanofeatures for a specific regenerative application.
Significance of Nanotopography for *in vitro* Cell-Surface Interactions

Thank you for your attention