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Current Opinion in Cell Biology

# Novel tools to study cell-ECM interactions, cell adhesion dynamics and migration

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**Abstract**

Integrin-mediated cell adhesion is essential for cell migration, mechanotransduction and tissue integrity. *In vivo*, these processes are regulated by complex physicochemical signals from the extracellular matrix (ECM). These nuanced cues, including molecular composition, rigidity and topology, call for sophisticated systems to faithfully explore cell behaviour. Here, we discuss recent methodological advances in cell-ECM adhesion research and compile a toolbox of techniques that we expect to shape this field in future. We outline methodological breakthroughs facilitating the transition from rigid 2D substrates to more complex and dynamic 3D systems, as well as advances in super-resolution imaging for an in-depth understanding of adhesion nanostructure. Selected methods are exemplified with relevant biological findings to underscore their applicability in cell adhesion research. We expect this new "toolbox" of methods will allow for a closer approximation of *in vitro* experimental setups to *in vivo* conditions, providing deeper insights into physiological and pathophysiological processes associated with cell-ECM adhesion.

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**Current Opinion in Cell Biology** 2024, **88:**102355

This review comes from a themed issue on **Cell Architecture 2024**

Edited by **Carsten Janke** and **Ohad Medalia**

For a complete overview see the Issue and the Editorial

Available online xxx

**https://doi.org/10.1016/j.ceb.2024.102355**



## Introduction

Integrin adhesion complexes (IACs) are essential in orchestrating various processes, from development and cell fate decisions to maintenance of tissue architecture, cell proliferation, migration and invasion. IAC dysregulation underlies the development of pathologies such as fibrosis or cancer [1]. Integrins, the primary components of IACs, represent a family of 24 transmembrane receptors that facilitate cell interaction with the extracellular matrix (ECM) [2]. IACs are one of the key mechanical sensors in cells and they function as bidirectional signalling hubs by clustering active integrins into multiprotein complexes that translate biochemical and mechanical signals in the cell, while simultaneously exerting forces on the ECM to remodel and guide ECM fibre assembly [ [3,4], reviewed in Ref. [5]]. In IACs, integrins link the ECM with the actin cytoskeleton via force-sensitive proteins such as talin and vinculin. Talin unfolding, in response to tensile stress, reveals vinculin binding sites and triggers IAC reinforcement and stress fibre formation transducing signals from the ECM to the nucleus [reviewed in Ref. [6]].

The complexity of ECM within tissues and organs *in vivo* is characterized by a diverse array of biochemical and biophysical features, encompassing ligand composition and density, elasticity, stiffness and a variety of topological cues [reviewed in Ref. [7]]. Given the high number of possible integrin-heterodimer pairs exhibiting a broad range of ligand specificity and mechanical sensitivity, IACs have the propensity to translate a multitude of ECM cues into biochemical responses within the cell. Much of our understanding of cell-ECM interactions stems from research on individual cells on rigid and static single ECM surfaces. While this approach has provided in-depth understanding of adhesion composition and regulation [8], it is essential to study cell-ECM crosstalk dynamically and within a complex environment. Accordingly, the development of more versatile systems and sensitive tools to investigate all the aspects of cell interaction with its environment represents one of the major challenges in current research.

In this brief review, we describe recent technological and methodological advances in IAC research, focusing on methods to study how cells sense their local environment

and respond to complex ECM cues, and how constantly improving imaging techniques propel forward our knowledge of IAC regulation in the nanoscale.

## Sensing the cell environment

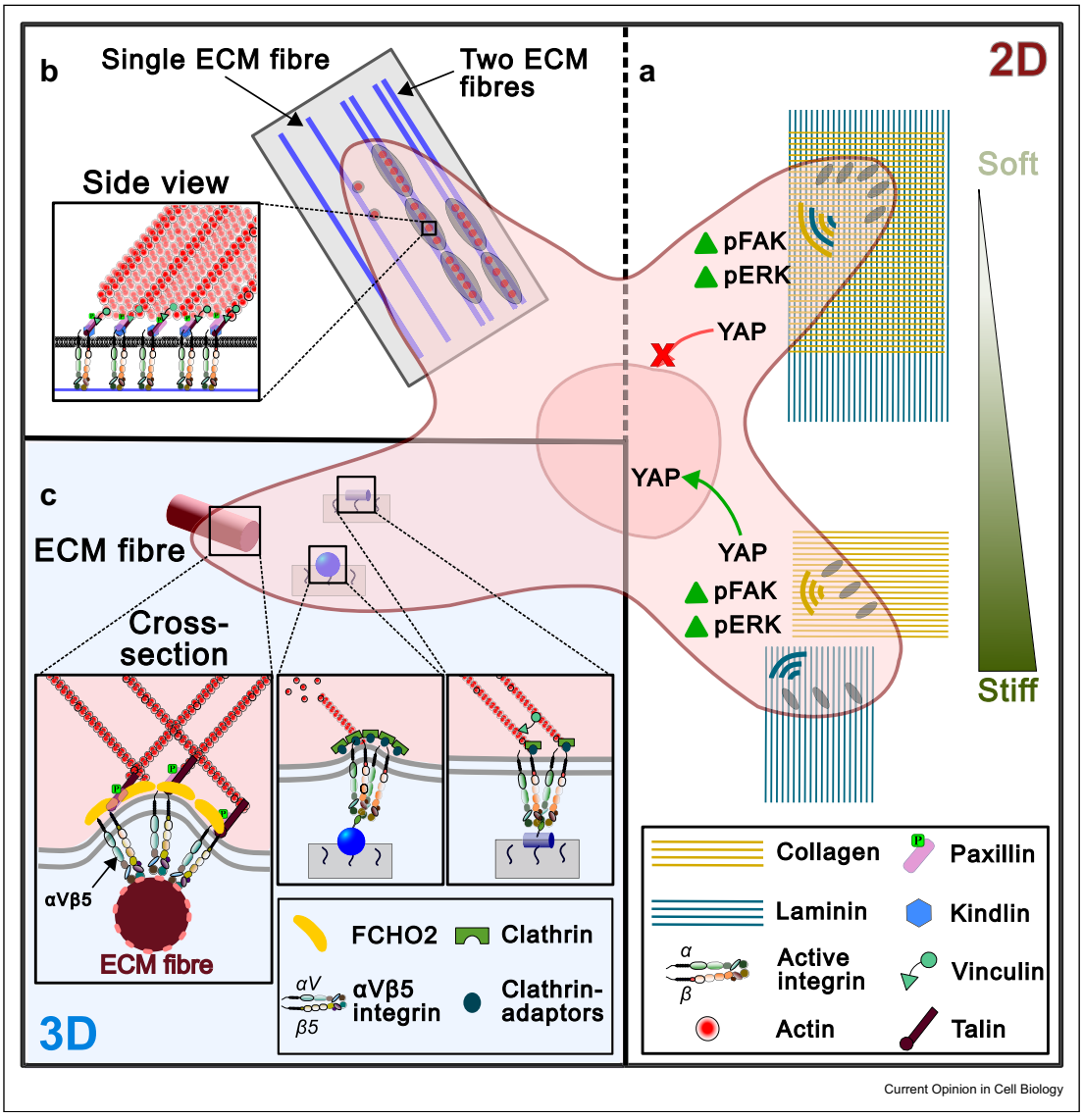
*In vivo*, the ECM is extremely complex, consisting of different soluble and structural subunits and varying geometrical and topological features that define the tissue. Furthermore, ECM rigidity ranges from as low as 1−3 kPa in the brain to more than 5 MPa in bone and cartilage [9]. The cellular response to these intricate extracellular cues (in 2D and 3D) remains largely understudied. Dynamic micropatterning is an innovative approach to simultaneously release cells from one ECM component to another to mimic cells encountering different ECM landscapes (see Toolbox) [10]. This approach has highlighted the impact of distinct integrin-ECM ligand interactions and cell geometry on cell polarity and cell migration, and has the potential to be used for analysing not only cell-ECM, but also cell−cell adhesion crosstalk. To study cell responsiveness to more complex ECM compositions over different rigidities, an ECM spot array system was recently developed [11]. This system, allowing cells to adhere to distinct ECM protein mixtures (integrin ligands) printed on soft or stiff hydrogels, revealed that a specific combination of integrin ligands can induce 'stiff-like' cell spreading on soft substrates [11]. These data are in contrast to the generally accepted view that cells spread more efficiently on stiffer substrates and demonstrated that engagement of specific combinations of integrins can uncouple cell spreading from stiffness and YAP nuclear translocation (Figure 1a).

To investigate the mechanical properties of the cells and quantify the forces they exert on their surrounding substrate, traction-force microscopy (TFM) has been commonly used. To overcome the limitations of traditional TFM in terms of resolution and sensitivity, novel methods such as fluctuation-based super-resolution (FBSR) TFM [12] or highly sensitive DNA-based molecular tension sensors have been developed [13]. Unlike conventional TFM, FBSR-TFM combines gels with smaller, more densely packed fluorescent beads (40 nm, vs. 200 nm in TFM) with super-resolution microscopy through fluorophore intensity fluctuation analysis to improve the accuracy of bead tracking and the spatial resolution of force-exerting cell regions down to individual IACs [12]. In contrast, molecular tension sensors generally rely on the mechanical properties of DNA molecules and enable more sensitive and precise measurement of forces exerted by the cell on individual integrin ligands such as RGD-peptides. The recent integration of the DNA-based molecular tension sensors into soft hydrogels (see Toolbox) opens up new ways to study cellular mechano-responsiveness to ECM properties in a more physiological context [14,15]. Notably, the use of hydrogels of different stiffness showed that fibroblasts respond to increasing substrate stiffness by recruiting new integrins to the adhesion sites rather than reinforcing already existing adhesions [14], thus highlighting the importance of integrin dynamics in rigidity sensing during processes such as spreading [11] or durotaxis [16]. In addition, methods such as 3D TFM have provided insights on the importance of dynamic forces across adhesions in tissue during development [reviewed in Ref. [17]].

Additional approaches are generally used to evaluate forces at the onset of adhesion formation and during adhesion disruption. For instance, bead-based rheology, using ligand-coated beads and magnetic tweezers, has provided key parameters for molecular-clutch modelling of adhesions [18]. Similarly, atomic-force microscopy-based single-cell force spectroscopy (SCFS), which involves the attachment of cells to a probe on the AFM cantilever to measure forces with high spatial resolution, has been used to determine force loading of different integrins [19] or integrin and syndecan crosstalk supportive of adhesion to fibronectin fibrils [20]. SCFS can also determine adhesion strength between receptor−ligand pairs or between neighbouring cells in processes such as mitosis [21]. Moreover, development of approaches that enable active monitoring of cell-ECM and cell−cell adhesions, such as a novel platform with tuneable presentation of peptide ligands to integrin (RGD) or N-cadherin (HAVDI) [22] (see Toolbox), will be invaluable to dissect the balance between these two adhesion types in tissues.

It is becoming increasingly evident that ligand geometry and spacing play an essential role in orchestrating force-dependent IAC maturation [23,24]. Using nanopatterned substrates mimicking thin ECM fibres revealed that, while adhesion maturation is impaired on single fibres, it is supported on adjacent fibre pairs, implicating the role of integrin nanoclustering in bridging thin ECM fibres on 2D surfaces (Figure 1b) [24]. While many current nanopatterning approaches rely on materials not compatible with super-resolution imaging, such as gold, this limitation has recently been overcome by functionalizing titanium oxide for use in nanopatterns [25]. Advances in nanosubstrate engineering have also helped to increase our understanding of how cells respond to changes in 3D substrate topology (see Toolbox). A novel type of αvβ5-containing IACs, called 'curved adhesions' (CAs), has recently been characterized in cells cultured on 3D nanobars/nanopillars designed to mimic their interaction with protein fibres in soft ECM [26] (Figure 1c). The CAs lack several canonical components of classical IACs in 2D, such as vinculin or pFAK [26], emphasizing their distinct molecular composition [recently reviewed in Ref. [27]]. Moreover, topology cues in 3D have been shown to regulate the balance between clathrin-mediated endocytosis and integrin β3-mediated adhesion of human mesenchymal stem cells (Figure 1c) [28].

**Figure 1**




**Sensing the cell environment in 2D and 3D**

**(a)** Schematic representation of "stiff-like" cell spreading on soft where cells on specific combination of ECM ligands (such as collagen and laminin) spread on soft 2D substrates, engage an increasing number of integrins and spread as effectively as cells plated on single ligands on stiffer hydrogels. While spreading is supported by mixed ECM on soft, these conditions are not supportive of YAP nuclear translocation. **(b)** Role of integrin nanoclustering in bridging two adjacent thin ECM fibres was demonstrated using nanopatterning. In contrast, single fibres do not support IAC maturation. **(c)** 3D ECM topology cues promote the formation of 'curved adhesions' (CAs) on thick ECM fibres. Mechanistically, formation of the CAs is dependent on integrin β5 interaction with a curvature-sensing protein FCHo2. Moreover, ECM topology influences the balance between clathrin-mediated endocytosis and formation of IACs.

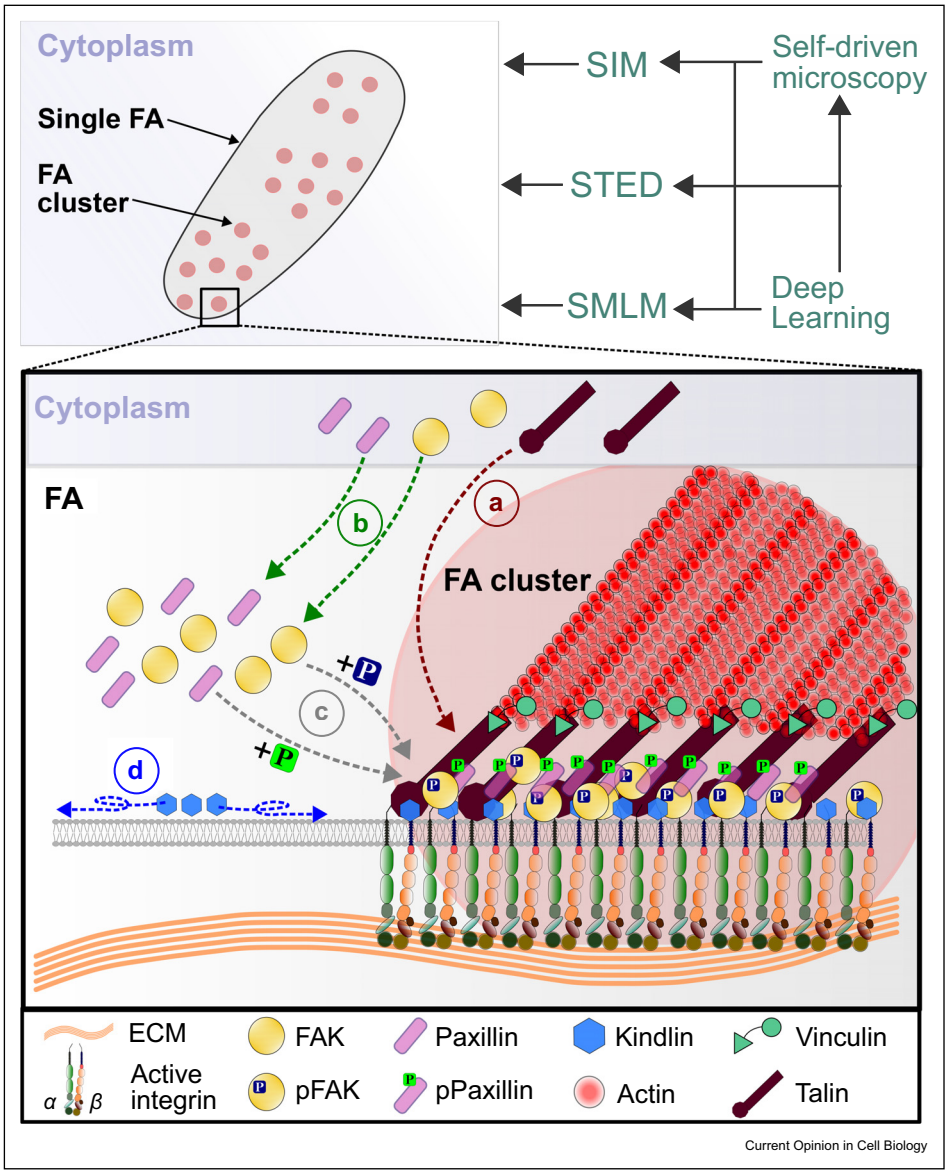
## Delving deeper into the adhesion nanostructure

The continuous improvements in imaging probes, methods, analysis pipelines and even hardware [29,30] have paved the way towards routinely breaking diffraction-limited barriers, resolving molecular complexes and visualizing their dynamics. In the context of integrin-mediated cell-ECM adhesions, this has opened gates to probe deeper into the lateral and axial ultrastructure of focal adhesions (FAs), as well as other IAC structures like nascent and fibrillar adhesions [2]. The nanoarchitecture of FAs as distinct axial layers was reported more than a decade ago using a super-resolution microscopy (SRM) technique called interferometric photoactivated localization microscopy (iPALM) [31]. Axially, starting proximal to the plasma membrane, FAs are divided into an integrin signalling layer, an intermediate force transduction layer and a topmost actin regulatory layer. Improvements in SRM methods, namely structured illumination microscopy (SIM), stimulated emission depletion (STED), and single-molecular localization microscopy (SMLM) have allowed a deeper understanding of both the lateral and

axial organization of FAs with their distinct molecular components (Figure 2) [32].

Application of SRM methods has facilitated probing dynamic protein changes, such as post-translational modifications, in proteins residing within FAs. For example, SIM imaging revealed that phosphorylated forms of paxillin and FAK localize to spatially defined lateral clusters within adhesions which, in the case of phosphorylated FAK, were dependent on the ability of FAK to adopt an open conformation on the membrane (Figure 2b, 2c) [33]. Furthermore, live imaging revealed that, within adhesions, the discrete FAK clusters were more dynamic compared to the relatively stable paxillin ones. Similarly, the spatiotemporal dynamics of Src clusters has been recently profiled using a novel biosensor called "binder/tag". This approach relies on the high−affinity interaction between a fluorescent reporter (binder) and a short tag peptide fused to the studied protein, that is only exposed after the protein

**Figure 2**




**Delving deeper into the cell-ECM adhesion nanostructure**

Integrin-driven cell-ECM adhesion is mediated by the formation of IAC structures like focal adhesions (FAs). Each FA shows further axial and lateral organization of the active adhesome components, and this can be studied in nanoscale and/or single molecule resolution using various SRM methods. These methods have provided insights into the mechanisms and dynamics of essential FA regulators. **(a)** Molecules like talin are recruited to FAs directly from their cytoplasmic pool; **(b)** Other important regulators like FAK and paxillin seem to form oligomers and undergo post-translational modifications before being recruited to active FA clusters; **(c)** Such post-translational modifications are critical for the recruitment of these molecules to the active clusters; **(d)** Kindlin, on the other hand, diffuses freely along the plasma membrane and this is necessary for its immobilization in active FA clusters.

changes conformation. This method enabled the identification of active Src "islands" within Src clusters and showed that they move slower than the inactive, fast-diffusing Src [34]. However, such conformational regulation, although a common feature, may not be necessary among all adhesion components. Single-particle tracking PALM (sptPALM), a type of SMLM technique, showed kindlin-*2* to undergo free diffusion proximal to the plasma membrane to support IACs and integrin activation [35]. Unlike FAK, mere targeting of kindlin to the membrane, via a non-specific tag, was sufficient to support IACs (Figure 2d). Quantitative single-molecule colocalization analysis (qSMCL), another SRM framework combining SMLM, cluster detection and theoretical simulations has allowed visualization and quantification of absolute numbers of proteins within large complexes in crowded environments [36]. qSMCL indicated active integrin β1, talin-1 and kindlin-*2* to form a molecular complex. Thus, although many of the adhesome proteins, both within and across the IAC axial layers display a cytoplasmic distribution and may function together, their mechanisms of localization and action might still be distinct (Figure 2). Development of a better camera system to enable fast live-cell SMLM, such as ultrafast direct stochastic reconstruction microscopy (dSTORM) and PALM has identified laterally "enriched-protein islands" within FAs [29,37]. These FA-protein islands have a mean diameter of 13−100 nm, but strikingly, show variable protein stoichiometries within individual islands.

The pairing of SRM with DNA-based tension sensors has allowed the visualization and quantification of forces in the order of piconewtons applied by integrin-based adhesions [38,39]. These studies have reinforced the findings that FA components display a non-random lateral and axial organization during their formation and maturation. In addition to SRM, cleverly designed probes and imaging approaches have helped to decipher the spatiotemporal dynamics of IAC proteins. For instance, a FAK activity probe, termed FAK-SPARK, uncovered polarized FAK activity at the distal tip of incipient IACs during cell migration [40]. Strikingly, in addition to their organization, the orientational order of IAC components is essential in fine-tuning cellular responses to ECM properties [41]. By applying a combination of emission anisotropy total internal reflection microscopy (EA-TIRFM) and computational modelling, the orientational order of IAC proteins and actin was shown to be responsive to changes in ECM density.

The development of SRM methods over the past few years has been supported by improved image processing and analysis pipelines. Particularly, the increasing application of deep learning networks in image analysis has shown immense potential. Various aspects of image processing including drift correction, segmentation, denoising, artificial labelling, etc. [reviewed in Refs. [42,43]], can now be covered by deep learning. Deep learning has permitted gentler live-cell imaging with a limited photon budget, and substantial improvements post acquisition. Moreover, adaptive microscopy approaches such as data-driven microscopy [44] and event-driven acquisition [45] show potential to bridge the gap between throughput and resolution (see Toolbox). Recently, re-applying the principles of SMLM in a method called resolution enhancement by sequential imaging (RESI) [46], researchers have reached unprecedented Ångstrom-resolution, and have shown proof-of-principle demonstrations of resolving extremely fine cellular structures.

## Future perspectives

A substantial part of our comprehension of IAC biology arises from studies involving fixed cells cultured on rigid substrates, where individual adhesion components are often overexpressed, thus inevitably disregarding or influencing IAC dynamics. Although these approaches have been instrumental in shaping the current knowledge in adhesion composition, regulation and nanostructure, we are convinced that pushing the limits towards more complex experimental systems is essential on multiple levels. Firstly, there is a need to study how regulation of IACs dynamics shapes the live cell response to changes in substrate composition, stiffness and underlying geometry cues. This includes a gradual transition from 2D models to 3D environments, evolving from single ECM ligands on rigid supports to intricate ECM compositions with alternating biophysical properties that would more closely mimic *in vivo* conditions. Secondly, with the advent and progress of microscopy methods and pipelines facilitating live-cell microscopy, we are now at a stage where visualization of cell behaviour is possible not only under simplistic conditions, but also in dynamic and complex cellular environments. Moreover, the application of SRM methods on live cells offers significant potential for gaining deeper insights into the spatiotemporal and structural dynamics of the IACs and should be capitalized on in future research. Last but not least, although not discussed in this review, the constant improvement and accessibility of gene engineering tools [reviewed in Ref. [47]] should encourage researchers to validate their experimental findings using model systems with endogenously tagged proteins, whenever feasible. As such, this should become a gold standard not only for investigating IAC dynamics, but also for cell biology research in general.

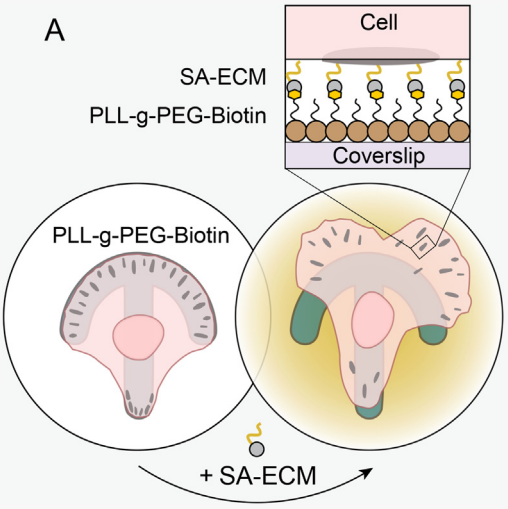
Within this brief review, we have highlighted a number of novel tools for investigating cell-ECM adhesions, as well as improvements of already well-established methods. We expect that these emerging new toolboxes will enable researchers to simulate scenarios closely resembling *in vivo* conditions to answer relevant biological questions, thereby resulting in better understanding of physiological and pathophysiological processes related to IACs.

## Groundbreaking toolbox

While it is not possible to cover all pertinent methods within the confines of this brief review, in this toolbox, we will highlight recent methodological advances that we believe will play a significant role in shaping the field of adhesion biology in the near future.

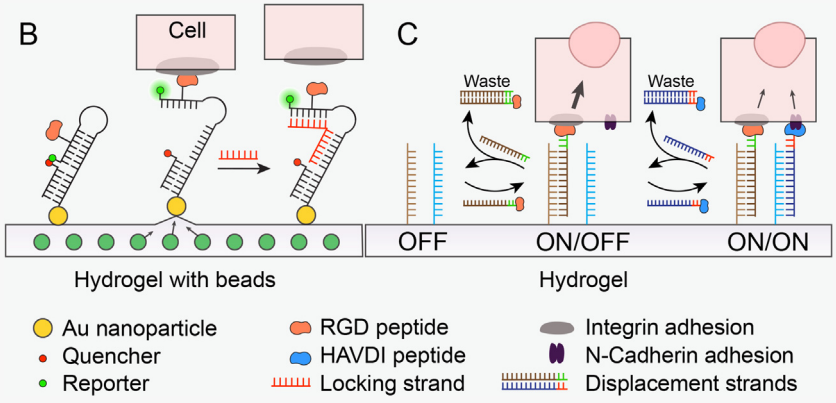
## Dynamic micropatterning

Micropatterning represents a well-established array of methods to study biological processes in cells within controlled, spatially defined environments with precise biochemical cues [48]. Typically, micropatterns are generated by photopatterning coverslips coated with a non-adhesive polymer, such as PLL-g-PEG (Poly-L-Lysine-grafted Polyethylene glycol), using a photomask of the desired shape. Irradiated coverslips are then coated with ECM ligands allowing the cells to adhere and spread only on the ECM-coated shape. Alternatively, micropatterns can be prepared by microcontact printing using ECM-inked stamps. In contrast to traditional micropatterning techniques, **dynamic micropatterns** can be generated, for example, by using PLL-g-PEG-Biotin as the non-adhesive polymer. Application of a secondary Streptavidin-conjugated ECM ligand (SA-ECM) thus enables a rapid conversion of the non-adhesive area to support specific cell-matrix interactions and release of the cells from confinement to study cell response to changes in ECM cues (panel A) [10].




## DNA-based tools

Over the span of more than a decade, the continuous development of DNA-based molecular probes and sensors has played a pivotal role in significantly advancing our understanding of cell mechanosensing [summarized in 13]. Recently, DNA-based **molecular tension probes** have been integrated with soft hydrogels to approximate physiological ECM conditions [14,15]. In a method developed by Wang et al., DNA-tension sensors are adsorbed to golden nanoparticles (AuNP) and covalently attached to the surface of hydrogels of varying stiffness (panel B). Integrin-mediated traction causes the sensor to "open", displacing the reporter from the quencher. Moreover, embedding fluorescent beads into the hydrogels enables compatibility with conventional traction force microscopy (TFM), providing additional information including net cell traction and force orientation. The use of a locking strand complementary to the "opened" sensor hinders probe refolding, allowing characterization of integrin-mediated mechanical sampling of the ECM [14]. Recently, the DNA-tension probes have been employed in the development of **tension-activated cell tagging (TaCT)**, a flow-cytometry-based approach that facilitates high-throughput characterization of mechanically active cells [49].




A DNA-based cell culture platform with **programmable presentation of peptide ligands** has recently been developed to study cell adhesion in mesenchymal development (panel C). Tuneable and gradual presentation of RGD (integrin) or HAVDI (N-Cadherin) peptide ligands through DNA-hybridization and toehold-mediated strand displacement reactions represents a dynamic system to study the crosstalk between cell-cell end cell-ECM adhesions [50].

## Force sensors

Forces across adhesion components such as talin, vinculin, α-catenin and cadherins can be sensitively measured in real time using genetically encoded Förster resonance energy transfer **(FRET)-based tension sensors**. Today, they have been used to measure piconewton-scale forces even in tissue or whole organisms [51]. To assess the importance of individual force-bearing linkages in force transduction, the novel **molecular optomechanics tool** employs fluorescently tagged optical dimers that stably connect the two halves of the studied force-bearing protein and the dimer dissociates upon light stimulation [52]. Moreover, to characterize force-dependent changes in IAC composition, the **fluorescence tension co-localisation (FTC)** method has been developed and validated using vinculin [53]. It couples FRET-based tension sensor measurements with quantitative immunofluorescence detection of endogenous adhesion components. Future application of this method to various adhesion components and sensors could help to elucidate the dynamics of tension-sensitive protein recruitment during IAC maturation.

## Self-driving microscopy

The emerging field of computer-assisted imaging based on deep-learning algorithms has revolutionized live cell imaging. For instance, recent development of **event-driven acquisition (EDA)** enables a switch from slow to fast imaging rate from event onset to progression [45]. Similarly, methods such as **data-driven microscopy (DDM)** [44] or **event-triggered STED (etSTED)** [54] employ real-time object characterization allowing shift in magnification or imaging modality when the phenotype of interest is detected.

## Declaration of competing interest

The authors declare no competing interests.

## Data availability

No data was used for the research described in the article.

## Acknowledgements

We apologize we were not able to cite all the relevant articles given the space limitations of this review and its focus on recent publications. We would like to thank Hellyeh Hamidi for editing and Aleksi Isomursu for comments on the dynamic micropatterning. M.D. was supported by the European Union's Horizon Europe research and innovation programme under Marie Sklodowska-Curie grant agreement n° 101108089. O.J. was supported by the University of Turku Graduate School Doctoral Programme in Technology (DPT) (#2300668) and the Finland Fellowship 2023. J.I. was supported by the Finnish Cancer Institute (K. Albin Johansson Professorship); the Research Council of Finland project grant (# 325464) and Centre of Excellence programme (# 346131); the Cancer Foundation Finland; the Sigrid Juselius Foundation; the Research Council of Finland's Flagship InFLAMES (# 337530 & 357910) and the Jane and Aatos Erkko Foundation.

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