Quantification of substrate and cellular strains in stretchable 3D cell cultures: an experimental and computational framework

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Summary

The mechanical cell environment is a key regulator of biological processes. In living tissues, cells are embedded into the 3D extracellular matrix and permanently exposed to mechanical forces. Quantification of the cellular strain state in a 3D matrix is therefore the first step towards understanding how physical cues determine single cell and multicellular behaviour. The majority of cell assays are, however, based on 2D cell cultures that lack many essential features of the in vivo cellular environment. Furthermore, nondestructive measurement of substrate and cellular mechanics requires appropriate computational tools for microscopic image analysis and interpretation. Here, we present an experimental and computational framework for generation and quantification of the cellular strain state in 3D cell cultures using a combination of 3D substrate stretcher, multichannel microscopic imaging and computational image analysis. The 3D substrate stretcher enables deformation of living cells embedded in bead-labelled 3D collagen hydrogels. Local substrate and cell deformations are determined by tracking displacement of fluorescent beads with subsequent finite element interpolation of cell strains over a tetrahedral tessellation. In this feasibility study, we debate diverse aspects of deformable 3D culture construction, quantification and evaluation, and present an example of its application for quantitative analysis of a cellular model system based on primary mouse hepatocytes undergoing transforming growth factor (TGF-β) induced epithelial-to-mesenchymal transition.

Introduction

The mechanical environment of a cell plays an important role in the determination of its behaviour (Levental et al., 2007; Eyckmans et al., 2011). Cell–matrix interactions regulate crucial biological processes during development (Mammoto & Ingber, 2010), homeostasis (Bissell et al., 1982; Kolahi & Mofrad, 2010) and pathogenesis (Jaalouk & Lammerding, 2010), homeostasis (Bissell et al., 1982; Kolahi & Mofrad, 2010) and pathogenesis (Jaalouk & Lammerding, 2009; Przybyla et al., 2016). To understand how mechanical environment affects behaviour of cells, in vitro cell culture systems are employed.

In comparison to traditional 2D cell culture systems, 3D culture mimics structural, dimensional and mechanical properties of tissue (Page et al., 2013) and allows a more physiological arrangement of cellular structures.

To investigate how the mechanical environment influences cellular processes, the mechanical strain sensed by cells needs to be quantified. This requires a method for induction and measurement of substrate deformations on subcellular spatial scale.

Planar stretchers have been previously used to study force application to cells (Vaziri & Gopinath, 2008; Rodriguez et al., 2013). These exert mechanical forces to cells cultured on flat membranes with subsequent determination of the effects on molecular or biochemical levels (Boerma...
Fig. 1. The 3D substrate stretcher. (A) The 3D substrate stretcher is the experimental tool to induce deformation in 3D culture of living cells embedded in a matrix. The collagen matrix containing the cells and fluorescent beads is biaxially stretched, when the attached silicone membrane is pulled down to the sides upon vacuum induction in the chamber (dashed arrows). A special supporter ring and cover glass lid enable the mounting of the chamber on an inverted confocal line scanning microscopy (CLSM). (B) The multichannel 3D image stack including fluorescent beads, nuclei and cells is acquired via CLSM. Structural information extracted from the different channels via image analysis allows reconstruction of fluorescent beads and the 3D cellular domain (see 'Methods' section). By comparison of consecutive time steps, the displacement of beads and cells is calculated.

et al., 2005; Hornberger et al., 2005), or characterization of cellular (Gavara et al., 2008; Huang et al., 2010) and nuclear (Lammerding et al., 2007) deformations. However, these methods are restricted to applications in 2D cell culture systems.

Although, 3D culture systems provide a more physiological extracellular environment, the majority of current systems is based on embedment of cells into a static extracellular environment. For example, in traction force microscopy the goal is to measure forces exerted by transmigrating cells onto an
Fig. 2. Biocompatibility of the stretcher with primary cells and micromorphological analysis of the collagen matrix. The 3D substrate stretcher chamber allows visualization of living cells, embedded in a 3D cell culture matrix within the working distance of a confocal microscope. (A) Confocal image of mouse hepatocytes in 3D cell culture. GFP expression allows visualization of cell bodies (green) fluorescent tracer beads are shown in blue. Plating of cells between two collagen layers enables the analysis of epithelial sheets. (B) A bile salt secretion assay reveals maintenance of epithelial polarity with functional epithelial cell-cell contacts (bile canaliculi; white arrows). Uniform fibre distribution in the network and high reproducibility of collagen preparation are important prerequisites for 3D substrate stretcher experiments. Backscattered light from collagen fibres and beads was imaged by confocal reflection microscopy (C and D). Maximum projection overlays of representative z-stack images are displayed. (C) Imaging a large microscopic field revealed a dense network of collagen fibres with isotropic distribution. Fibres form a continuous network without matrix discontinuities. No air inclusions or matrix distortions are present. (D) Imaging of single beads with high resolution revealed random inclusion of beads within network pores; no integration of beads into collagen fibres or attachment of collagen fibres to beads was observed. (E) Directionality analysis revealed equal frequencies for fibre directions. Relative frequency distribution histograms of fibre and pore dimension measurements (F–H) and the Young’s moduli measured by atomic force microscopy (I). The corresponding number of measurements (n), mean (M), skewness (S), Kurtosis (K) are listed above each histogram. (J–M) Reproducibility of collagen preparation was evaluated by comparison on the morphological measurements (J–L) and elastic moduli (M) from three individual gels. Scale bars correspond to 10 µm.

otherwise static 3D extracellular matrix (Gjorevski & Nelson, 2012; Koch et al., 2012).

Deformable 3D cell cultures enable application of mechanical forces on living cells in a native 3D environment. Thereby, local substrate deformations can be determined by tracking of naturally occurring inhomogeneities in stained extracellular and intracellular structures (Bell et al., 2012). However, from our experience large and, especially, out-of-focus-plane displacements can substantially alter optical appearance of natural image inhomogeneities and make their automated tracking less accurate (González Avalos et al., 2011).

To overcome the above drawbacks, we extend the planar stretching principle to a 3D substrate stretcher and combine it with advantages of traction force microscopy that rely on the robustness of fluorescently labelled beads. This set-up enables us to perform 3D live cell imaging of cells embedded into bead-labelled extracellular matrix, and to accurately
Fig. 3. Detection, tracking and meshing of beads (from González Avalos, 2013). (A) Bead domains are segmented from raw images. (B) The size of segmented bead domains (here indicated by the standard deviation of image intensity around mass centres) depends on the segmentation threshold. (C) The calculated size of objects serves as an efficient filter for identification of beads suitable for the calculation of substrate deformation. Because of the small variation in size, considerably smaller objects belong to beads out of focus and bigger objects belong to bead clusters. (D) Bead displacements were calculated as difference of the bead coordinates between each two adjacent time points. (E) Finally, the point cloud of beads coordinates was interconnected to a tetrahedral mesh using Matlab to span a network of interpolating elements over the entire 3D substrate and the embedded cell.

determine their local strain using canonical interpolation of continuum deformation over displacements of bead points.

We apply our experimental and computational framework for analysis of primary mouse hepatocytes that undergo epithelial-to-mesenchymal transition (EMT) upon treatment with the transforming growth factor (TGF-β) (Godoy et al., 2009). EMT, the morphologic and molecular transition of differentiated, interconnected epithelial cells to a solitary, mechanically more robust mesenchymal phenotype. It is seen as progression step of malignant transformation in a variety of
Fig. 4. Consistency check of the bead network. In order to quantify substrate inhomogeneities and discontinuities, a consistency check of the bead network was carried out. (A) Surface beads were identified and displacements of internal beads were interpolated. (B) Interpolation was performed over displacements of the neighbouring beads that form an enclosing tetrahedron (see ‘Methods’ section for more details). (C) Beads with high deviation of experimentally observed from interpolated displacement are identified in an iterative manner by evaluating all beads contributing to the deviation. (D) After exclusion of a small fraction of beads with high deviation measure, the accuracy of the displacement interpolation by the network of remaining nodes was improved.

epithelial cancer types (van Zijl et al., 2009; Lim & Thiery, 2012), which is accompanied by characteristic changes in cytoskeletal protein composition and organization including formation of actin stress fibre bundles (Haynes et al., 2011) and overexpression of vimentin intermediate filaments (Willipinski-Stapelfeldt et al., 2005). Our preliminary experiments with untreated (epithelial) and TGF-β treated (mesenchymal) mouse hepatocytes indicate an alteration of the mechanical cell response within a 3D environment in dependence of EMT.

Experimental results

The experimental and computational framework of this study consists of (i) cell and extracellular matrix deformation by the 3D substrate stretcher, (ii) microscopic imaging of fluorescent tracer beads and morphology of cells embedded in 3D fibrous hydrogels and (iii) computational image analysis with subsequent calculation of substrate and cell strain.

3D substrate stretcher

The working principle of the stretcher is schematically displayed in Figure 1(A). A radial stretch is applied to a silicone membrane, placed in a metal chamber on top of a central plastic loading station, similar to previously described planar–stretcher devices (Caille et al., 1998; Wall et al., 2007). A vacuum generated in the air chamber, aspirates the membrane at the periphery, resulting in a homogeneous radial stretch of the membrane on top of the circular plastic loading station (Fig. 1B, arrows).

Several technical adaptations enable acquisition of 3D confocal microscopy images. Chamber height and diameter were adjusted to the confocal microscope stand and sample holder. Total weight was adjusted to the weight limit of the microscope piezoelectric stage motor, necessary for precise z-step control during confocal microscopy. A special lid was designed to allow inversion of the chamber, in order to enable standard inverted confocal line scanning microscopy (CLSM). A supporter ring
Fig. 5. Quantification of local strains. (A) Two examples of substrate deformation within the image fields of around $100 \times 100 \times 30 \mu m^3$ show the distribution of the main strain in the 3D culture matrix. Local strain variations are visualized with the colour map. (B) Surface distribution of the mean strain sensed by the cell is computed using standard linear interpolation over neighbouring beads. Surface distribution of the mean strain values is visualized with the colour map. (C) Staining of the actin cytoskeleton in primary hepatocytes depicts morphological changes upon treatment with TGF-β. Untreated hepatocytes show epithelial polarity with a fine actin mesh below the bile canaliculi at cell–cell contacts, whereas TGF-β treated cells display actin stress fibre bundles and mesenchymal morphology. (D) Stress-induced cell death occurs at high strain rates. (E) Analysis of susceptibility to strain-induced cell death in healthy and TGF-β treated cells. Individual untreated hepatocytes (i.e. epithelial cells) have an elevated frequency of stress-induced cell death events (D) at mid-high strain rates 60–90%, whereas TGF-β treated cells are capable to tolerate significantly higher strain of up to 120%. Interestingly, untreated cells in cell bundles turn out to be also more resistant to high strain than single untreated hepatocytes.

on the lid was designed, in order to mount a cover glass to create a cell culture chamber high enough for 3D cell culture and gel movement, but small enough for image acquisition within the working distance of high-resolution objectives, see Figure 1(A). A syringe stamp system attached to the air valve of the chamber through a rubber tube served as an air-controlling device to fine-tune force generation and its application to gels, and to fix the area of interest during image acquisition.

To study strain application to cells within a 3D environment, 3D collagen cell cultures were cast onto flexible silicone membranes. Precoating silicone membranes with an adhesion mediating layer of rigid, UV-cross-linked collagen allowed anchorage of collagen gels to membranes. Gel thickness was adjusted to enable visualization of cell volume and surrounding matrix within the working range of a $40 \times$ objective (Fig. 2 A). Embedding the cells between two layers of collagen allowed observation and strain application to quasi-planar epithelial cell sheets. In the optimized 3D cell culture, primary hepatocytes showed polarized epithelial morphology with specialized, organotypic cell–cell contacts and formation of functional bile canaliculi (white arrows Fig. 2B) after 48 h of cultivation. Collagen gelation is influenced by many factors, like source and extraction method, concentration, pH value, ionic strength, temperature and CO₂ concentration during polymerization (Wood & Keech, 1960; Christiansen et al., 2000). Further, mechanical agitation during the nucleation phase can affect polymerization (Yang et al., 2009, 2010). Microstructural features of the network structure, such as lateral fibril alignment to fibres, pore size and fibre length in self-assembled collagen depend on these factors (Billiar, 2011). The network microstructure of the collagen matrix is an important determinant of mechanical properties (Raub et al., 2008; Wen et al.,...
Morphology of fibre organization was analysed by confocal reflectance microscopy. The Young’s modulus of membrane anchored 3D substrate stretcher collagen matrices was measured by atomic force microscopy (AFM). Collagen fibres in the 3D substrate stretcher gels form a dense, isotropic network (Fig. 2C). Fluorescent tracer beads incorporated into the matrix prior to gelation are visible as bright dots are not incorporated into fibres (Fig. 2D). The collagen fibres were isotropically distributed in the imaging plane without preferred fibre direction (Fig. 2E). Fibres in the 3D substrate stretcher matrices had a median thickness of 0.27 µm (Fig. 2F). The median fibre length was 5.73 µm (Fig. 2G), and the median pore size was 0.77 µm (Fig. 2H). The collagen gels had a mean Young’s modulus of 26.15 Pa (Fig. 2I). To evaluate whether variation in preparation or other factors cause differences between individually prepared gels, microstructures of three gels were compared. Median differences values of fibre diameter (Fig. 2J), fibre length (Fig. 2K), pore diameter (Fig. 2L) and Young modulus (Fig. 2M) between the three gels were not significantly greater than expected by chance (Kruskal–Wallis test, Dunn’s posttest).

Analysis of 3D substrate deformation

In order to determine matrix deformation, we reconstructed the 3D substrate at each time point by identifying x,y,z coordinates of the fluorescent tracer beads, that were embedded into the substrate prior to gelation (see ‘Experimental’ section). The bright dyes of the fluorescent beads, presenting with a high signal-to-noise ratio, facilitated identification and segmentation of individual beads at micrometer resolution (Fig. 3A). Centre of mass coordinate determination for each bead was very robust in regard to changes of parameters in the segmentation algorithm, despite threshold-dependent object size variations (Fig. 3B). Beads at the edge of the image field as well as larger clusters of beads were excluded by size-threshold selection (Fig. 3C). Displacement of corresponding beads at two adjacent time points was calculated by subtracting x,y,z components. Figure 3(D) shows an example of displacements projected in 2D. The final and complete substrate reconstruction generated a 3D tetrahedral mesh that served as scaffold for strain calculation (Fig. 3E).

Substrate (dis)continuity analysis

In tissues and biopolymeric gels like collagen matrices, forces are transferred along fibres and fibrils, resulting in non-isotropic and nonhomogeneous force propagation throughout the overall nonlinear-elastic properties of the substrate (Billiar, 2011; Wen et al., 2012). To identify local (dis)continuities of the matrix, we constructed a testing procedure, which is based on a comparison of experimentally observed and interpolated displacements of beads. We introduce the deviation-measure (DM) to quantify the relative deviation of the bead displacement from its interpolated value. High values of DM indicate deviation from the displacement in an elastic continuum. The entire procedure is basically iterative. For every bead from the bead network (Fig. 4A), displacement is interpolated over displacements of four neighbouring beads that form the enclosing tetrahedron (Fig. 4B). For details of linear interpolation on tetrahedral elements, we refer to ‘Experimental’ section. Linear interpolation could predict real bead displacements for most of the analysed beads with a mean deviation of around 2% which corresponds to less than 1 µm. For a small fraction of beads (about 5%), a higher deviation from the interpolated displacement (i.e. over 10%) was determined. In a second step, beads with DM> 5% were selected, and it was evaluated whether the selected bead or one of the four interpolating beads caused a high DM. After integrating all DM values, beads that contribute to high DM were identified (Fig. 4C). After exclusion of this rather small fraction of beads, an improved accuracy of displacement interpolation by the network of remaining nodes was achieved (Fig. 4D).

3D cell strain computation and analysis

In order to quantify local substrate and cell deformation in an arbitrary point P, the linearized strain tensor was computed from the displacement gradient between the four beads of the P-enclosing tetrahedron:

$$\epsilon = \frac{1}{2}(\nabla u + \nabla u^T).$$

For characterization of maximum local deformation, the largest eigenvalue of $\epsilon$ was calculated, which describes the maximum rate of local stretching or contraction. Calculations show that maximum strain magnitudes in different experiments reach up to 68% on the cellular surface and up to 180% in the substrate. The median value of strain distribution is 29%. Examples in Figures 5(A) and (B) show spatial distributions of the mean strain in the 3D matrix and on the cell surface for two different experiments.

Hepatocyte EMT on the 3D stretcher

Figure 5(C) shows drastic morphological changes associated with EMT in mouse hepatocytes upon TGF-β treatment, illustrating suitability of this model system. Untreated hepatocytes show rounded epithelial morphology with large cell–cell contact areas, whereas TGF-β treated cells show a dedifferentiated fibroblastoid, mesenchymal morphology. During the stretching procedure, sudden cell death, associated with formation of membrane-bubbles at cell–matrix boundary and damage in the cell nucleus was observed for some cells (Fig. 5D). By estimating the magnitude of cell deformation, we could reveal that rupture events correlated with large substrate
deformations (Fig. 5E). Substrate strain magnitude at which this effect occurs depends on the degree of EMT. Interestingly, untreated hepatocytes started to die at a lower strain threshold than their TGF-β treated, dedifferentiated counterparts. This finding indicates overall enhancement of mechanical properties of TGF-β treated cells. Untreated hepatocytes are capable to survive strains above the critical threshold of 60% relative deformation only when being interconnected to multicellular bundles. This observation supports the assumption that formation of cell bundles provides epithelial cells with higher mechanical resistance, as compared to single cells.

Conclusion

In this work, we present a novel experimental and computational framework for quantitative characterization of the cellular strain state in 3D fibrous hydrogels. By combining planar stretcher with 3D bead-labelled cell culture and computational analysis of microscopic cell images, a detailed picture of mechanical cell behaviour in a deformable 3D matrix is obtained. To overcome shortcomings of previous 2D/3D culturing systems, we combine advantages of traction force microscopy with induction of 3D substrate deformation. Our image analysis indicates that deformation of bead-labelled collagen hydrogels is associated with local substrate discontinuities. Automated discontinuity detection was introduced to improve accuracy of interpolating local substrate and cell displacements. In general, continuous substrate description by the vector field of displaced beads can also be applied for comparative analysis of material behaviour of differently composed hydrogels.

Here, we quantified the spatial strain distribution sensed by primary mouse hepatocytes upon treatment with the EMT-inducing growth factor TGF-β. Our experimental results indicate that interconnected epithelial and single dedifferentiated mesenchymal cells are less sensitive to mechanical stress in comparison to untreated individual epithelial hepatocytes. These findings support the assumption that loss of cell–cell contacts enhances EMT-triggering effects of TGF-β. Further biological experiments are required to verify our exemplary observations of the differential cell response to mechanical stress in 3D environment.

The presented framework for nondistructive analysis of mechanically loaded 3D cell cultures can be applied to other cellular systems and biomedical problems related to the rapidly growing field of 3D cell culture and phenotypic screening.

Experimental methods and protocols

Cell isolation

Primary mouse hepatocytes were freshly isolated by collagenase perfusion with subsequent Percoll gradient purification as described (Klingmüller et al., 2006).

Preparation of deformable 3D collagen cell cultures

For the preparation of membrane anchored deformable 3D cell cultures. 43-mm silicone membranes (Flexcell) were placed in a standard 60-mm cell culture dish, and precoated with an adhesion mediating air dried 1 h UV-cross linked (254 nm, 60 W for 1 h under laminar flow) collagen layer (Fujimori, 1965; Weadock et al., 1995). Soft 3D collagen hydrogels were prepared by gelation of acid solubilized, isotonic rat tail collagen I (8.3 g mL−1 rat tail tendon collagen I, Roche, Germany) through neutralization to pH 7.4 (Klingmüller et al., 2006).

To visualize matrix deformation, the collagen solution was supplemented 1:200 with blue fluorescent beads (diameter: 1.0 μm) prior to gelation. For the first layer of the collagen sandwich system, 250 μL collagen solution was carefully distributed on the precoated membrane and allowed to gel. Cells were plated at a density of 2.5 × 10^4 cells cm−2. After 4 h adhesion, unattached cells were removed and 200 μL of a freshly prepared collagen solution containing beads was added drop wise on top of the cell layer and allowed to polymerize. Gelation steps were carried out for 1 h at 37°C 5% CO2. Cell culture medium was added after gelation of the second collagen layer. The medium contained a recombinant adeno-associated viral (AAV) vector pseudotyped with the chimeric capsid DJ (Grimm et al., 2008) and encoding green fluorescent protein (GFP), which transduced the hepatocytes and allowed us to visualize their cell body. After overnight starvation, medium was changed and cells were treated with the EMT-inducing cytokine TGF-β (5 ng/mL) as indicated.

Confocal reflection microscopy

For imaging of the collagen matrix, the backscattered light from the collagen fibrils was detected by confocal reflection microscopy with 488-nm excitation wave length as described by Artym & Matsumoto (2010) using the Leica SP5 laser scanning confocal microscope in the reflection mode. Brightness and contrast were optimized for visualization in printed formats.

Micromorphological analysis of the collagen matrix

Directionality of the collagen fibres was analysed with the directionality plugin for Fiji (Liu (1991)) and displayed as frequency distribution histogram as fraction of measured values in percent. Due to orientation-dependent light scattering during confocal reflectance microscopy in the z-axis (Jawerth et al., 2010), directionality was analysed from 2D maximum projection overlay images. 3D fibre length was determined from 3D z-stack images using the Simple Neurite Tracer plugin in Fiji (Longair et al., 2011). Fibre diameter was measured by drawing lines perpendicular to the longitudinal axis in maximum intensity z-projection images. Pore size was determined by measuring the shortest inter fibre distance adjacent to a void.
Statistical analysis

Results are displayed as Tukey box and whiskers plot. Top and bottom of the box represent the first and third quartile, the band in the box shows the median. Mean is represented as cross, and outliers are shown as dots. The whisker ends span to the lowest and highest datum still within 1.5 times the interquartile distance or, if shorter, up to the highest or lowest point. The Kruskal–Wallis test with Dunn’s posttest was applied to compare three groups; p values are reported as not significant (ns) for p > 0.05; or significant with * for p ≤ 0.05, ** for p ≤ 0.01 and *** if p ≤ 0.001. Frequency distributions are shown as relative frequencies in percent.

Determining the elastic modulus using AFM

Reviews suggest application of AFM to probe 3D cell culture matrices (Billiar, 2011; Miron-Mendoza et al., 2013). Billiar (2011) report testing AFM for this purpose, but they could not measure differences between polymerized and non-polymerized collagen. A surface tension–induced artefact is mentioned in a comment from Wu et al. (2005) for application of dynamic mechanical analysis of hydrated collagen gels. Our own observations allow the same conclusion. Submerging the collagen gels and cantilever in buffer-enabled measurement of the elastic moduli of collagen hydrogels by AFM. The Young’s moduli of the collagen gels were measured via Nano Wizard I AFM (JPK Instruments AG). The AFM was mounted on an inverse AxioVert 200 microscope (Carl Zeiss Microscopy GmbH) equipped with an additional CellHesion module (JPK Instruments AG) and a custom-made incubator for temperature maintenance at 37°C. To avoid bias by surface tension at the hydrogel to air interface (Wu et al., 2005), all measurements were conducted with the cantilevers submerged in preheated phosphate-buffered saline (PBS). Force spectroscopy measurements were conducted using silicon–nitride cantilevers (CSC38, Mikromasch) with force constants ranging from 0.03 to 0.08 Nm⁻¹ and quasi-conical tips with an opening angle between 34° and 40°. Both parameters were individually determined for each cantilever. The spring constant was measured using the thermal noise method by software provided by the manufacturer. Opening angles were determined by evaluating scanning electron microscopy (SEM) images of the cantilever tips acquired with a Zeiss LEO 1530 scanning electron microscopy. During force spectroscopy z-scan velocity was kept constant at 1 µm s⁻¹ and the relative detection set point of the cantilever was adjusted between 20 and 50 nm. Force curves were evaluated using the software provided by the manufacturer (JPK Instruments AG). Fit ranges were chosen to be in a low indentation regime ranging from 300 to 600 nm. In order to calculate the parameter of interest a quadratic equation is fitted to the force curve. This approach is based on the Hertz model (Hertz, 1881; Sneddon, 1965) and is well described and generally applied to determine the Young’s modulus of soft materials (for further discussion see Lin & Horkay, 2008). At least 12 different force curves at different places were collected and at least eight force curves were evaluated for each individual substrate.

Bile salt secretion assay

Secretion of glutathione-methylfluorescein (GMSF), the fluorescent metabolite of chloromethylfluorescein diacetate (CMFDA) was measured via live cell confocal line scanning microscopy in the substrate stretcher chamber 30 min after addition of the cell tracker (1 µM CellTracker™ Green CMFDA, Invitrogen) and 1:1000 Hoechst33258 to the cell culture medium (Godoy et al., 2009). Images are shown colour inverted. Brightness and contrast were optimized for visualization in printed formats.

Immunofluorescent staining

Immunofluorescent staining of the actin cytoskeleton (Phalloidin–Rhodamin 1:1000, Life Technologies) and the nucleus (Hoechst 33342 1:1000, Thermo Fischer Scientific) was performed as described in Artym & Matsumoto (2010) in fixed cells. Brightness and contrast of the colour-inverted images were optimized for print.

4D live cell imaging and stress induction on the substrate stretcher

A detailed protocol of the experimental pipeline for the 3D substrate stretcher is described in the supplementary material. Briefly, flexible membranes containing living 3D cell culture constructs were transferred to the 3D substrate stretcher chamber and into the environmental chamber of the microscope. A 3D scan of cell body and surrounding fluorescent bead-labelled matrix was recorded prior to stretching. Air was removed from a chamber below the flexible membrane, inducing deflection of the membrane and thereby matrix- and cell deformation. Immediately afterwards, a second 3D scan of the region of interest was taken. Confocal microscopy was performed with a Leica SP5 laser scanning confocal microscope equipped with an environmental control chamber and the LAS software. A 40× oil objective was used: 512 × 512 pixel images were scanned at a speed of 700 mHz and with a line average of two scans, with a z-step size kept constant at 0.3 µm, a pinhole aperture of 1 AU and a working zoom between 2.5 and 3.5. Standard excitation and detection wavelengths were used for the different fluorophores. When combining fluorophores with overlapping emission wavelength ranges, the sequential mode between stacks was used to avoid fluorescent bleed-through.
Calculation of bead displacements

Extraction of the $x\ y\ z$ positions for beads embedded in the matrix was performed with the 3D Object Counter ImageJ plugin (Bolte & Cordelieres, 2006) on each image stack after application of an edge-preserving filtering step. Beads located on the image edges and inside the cell were left out of the calculation to avoid bias. Individual beads displacements were calculated by coordinate subtraction after multiplication with the corresponding voxel values. A first evaluation of strain visualized in 2D was rendered to assess if the degree of substrate deformation was large enough to continue processing the rest of the series and to continue with the mechanical analysis. The coordinates and displacements calculated for the individual beads were analyzed with Matlab; the points were interconnected to an unstructured tetrahedral mesh (Fig. 2E), which was used for continuous interpolation of substrate and cell displacements.

Displacement interpolation on the tetrahedral element

Displacements of the cell surface points are interpolated over the displacements of the neighbour beads. Consider the point $N_0$ of the cellular body enclosed by four neighbour bead points $(N_1, N_2, N_3, N_4)$ that form a tetrahedron. The displacement vector in the point $N_0$ is interpolated over the displacements of the four tetrahedral points using the standard linear interpolation:

$$ u_0 = u_1 + (u_2 - u_1)\alpha + (u_3 - u_1)\beta + (u_4 - u_1)\gamma, $$

where $(\alpha, \beta, \gamma)$ are the three parameters of the linear shape interpolation function that are determined from coordinates of four points:

$$ \begin{bmatrix} \alpha \\ \beta \\ \gamma \end{bmatrix} = \begin{bmatrix} x_2 - x_1 & x_3 - x_1 & x_4 - x_1 \\ y_2 - y_1 & y_3 - y_1 & y_4 - y_1 \\ z_2 - z_1 & z_3 - z_1 & z_4 - z_1 \end{bmatrix} \begin{bmatrix} x_0 - x_1 \\ y_0 - y_1 \\ z_0 - z_1 \end{bmatrix}, $$

where $(x, y, z)$ denote Euclidian coordinates of the points derived from microscopic images. The matrix whose inverse is indicated on the right side of the equation is called the Jacobi matrix of the tetrahedron.

Substrate (dis)continuity analysis

To detect local substrate discontinuities, the following DM was constructed:

$$ DM = 100\% \sqrt{\sum_{i=1..3}(u_i - \bar{u_i})^2 / \sum_{i=1..3}(\bar{u_i})^2}, $$

where $u_i$ denotes the experimentally determined 3D displacement of the bead and $\bar{u_i}$ is the displacement of the same bead obtained from the standard linear interpolation over the nodal displacements of the enclosing tetrahedron (Eq. (2)). Automated generation of tetrahedral meshes and calculation of DM on bead nodes was implemented in Matlab. Beads with the DM exceeding the quality threshold of $DM > 5\%$ were excluded from the network of bead nodes. In the next step, the new tetrahedral grid was recomputed in Matlab for only those beads that match the criterion $DM < 5\%$.

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Competing interests

The authors declare that no competing interests exist.

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