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Effect of an osmotic stress on multicellular aggregates

Sylvain Monnier [a,1], Morgan Delarue [a,1,2], Benjamin Brunel [b], Monika E. Dolega [b], Antoine Delon [b], Giovanni Cappello [b,*]

[a] Institut Curie, Centre National de la Recherche Scientifique, Université Pierre et Marie Curie, Unité Mixte de Recherche 168, 75231 Paris, France
[b] Laboratoire Interdisciplinaire de Physique, Centre National de la Recherche Scientifique, Université Joseph Fourier, Unité Mixte de Recherche 5588, 38402 St. Martin d'Hères, France

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ABSTRACT

There is increasing evidence that multicellular structures respond to mechanical cues, such as the confinement and compression exerted by the surrounding environment. In order to understand the response of tissues to stress, we investigate the effect of an isotropic stress on different biological systems. The stress is generated using the osmotic pressure induced by a biocompatible polymer.

We compare the response of multicellular spheroids, individual cells and matrigel to the same *osmotic* perturbation. Our findings indicate that the osmotic pressure occasioned by polymers acts on these systems like an isotropic mechanical stress. When submitted to this pressure, the volume of multicellular spheroids decreases much more than one could expect from the behavior of individual cells.

1. Introduction

Mammalian cells constantly interact with their mechanical environment [1]. Each cell of connective tissues is embedded in an elastic and remodelable environment, the Extracellular Matrix (ECM). Moreover, the whole tissue is confined and compressed by its surrounding. At the single cell level it is admitted that the mechanical environment can determine cell fate. The stiffness and the geometry of the substrate on which cells grow has indeed a strong impact on cell division [2], migration [3] and differentiation [4]. There are increasing evidences that the mechanical environment also determines the behavior of supra-cellular structures. For instance, tumor multicellular aggregates growth can be inhibited when embedded in an elastic agarose gel [5]. In particular, the stiffer the agarose gel, the stronger the inhibition. Similarly, Alessandri et al. [6] showed that a multicellular spheroid (MCS) confined inside an elastic shell grows more slowly when its peripheral cells touch the shell wall. Both agarose gels and shells are deformed by the growing aggregate. As a consequence, they mechanically react by applying an increasing stress to the spheroid. Above a certain stress, spheroid growth stalls.

Mechanical stress may have a different effect if, like a stationary isotropic pressure, it is kept constant over time. In that case, the growing aggregate can progressively adapt to the external stress on the long timescale (longer than cell division time). In order to explore this possibility and grow multicellular spheroids under a constant compressive stress, we culture them in a medium supplemented with dextran polymer [7]. Unlike more physiological osmolytes, such as glucose or ions (Na^+, Mg^{2+}, etc), dextran macromolecules are not internalized by cells, and do not infiltrate the intercellular space of spheroids [8]. Thus, they exert a persistent osmotic stress onto the whole spheroid and induce a water efflux from the spheroid, similar to what would do a permeable piston squeezing a spongy aggregate. The stress would not persist if physiological osmolytes (e.g. glucose, NaCl or $MgCl_2$) were used instead of dextran. Indeed, their concentration gradients through the plasma membrane would be progressively balanced by ions pumps and channels activity or endocytosis.

In our previous works, we showed that a 5 kPa osmotic stress strongly hampers spheroid growth, by inhibiting cell proliferation in its core. It is worth noticing that 5 kPa is a rather small stress corresponding to the osmotic pressure exerted by 1 mM NaCl. Moreover, it has been observed that such a small osmotic pressure has no long term effect on proliferation or death for cells cultured in 2D (Petri dish). This raises the fundamental question as to why 3D spheroids respond to osmotic pressure while individual cells do not.

In this article we focus on the short timescale response of a spheroid to an osmotic stress, and evaluate the impact of such stress. In particular, we measure the volume decrease of a MCS in the first minutes after the injection of dextran, when the adaptive response is not activated yet and the cell cycle is still

* Corresponding author at: Laboratoire Interdisciplinaire de Physique, 140 rue de la Physique, 38402 St. Martin d'Hères, France.
E-mail address: giovanni.cappello@ujf-grenoble.fr (G. Cappello).
[1] SM and MD equally contributed to this work.
[2] Present address: Physics Dpt, Stanley Hall, UC Berkeley, Berkeley, CA, USA.

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unaffected. Complementarily, we correlate the volume decrease to the change of the cell-to-cell distance, depending on the position inside the spheroids. In order to rationalize the effect of the osmotic stress on a semipermeable system, we submit individual cells on 2D substrate to the same stress and record the corresponding volume change. We also compare the response of living cells and spheroids to the passive contraction of matrigel beads and of polyacrylamide gels, when submitted to similar pressures.

2. Materials and methods

2.1. Osmotic pressure

The osmotic pressure is exerted by supplementing the buffers and culture media with a well-defined amount of dextran. This biocompatible polymer is not internalized by eukaryotic cells and the relationship between its concentration and the osmotic pressure has been previously calibrated [9,10] (see Supplementary Fig. 1). Dextran molecules (Sigma–Aldrich, St. Louis, MO) must be large enough to penetrate neither the polyacrylamide (PA) gel nor the MCS. We observed that dextran molecules with a molecular weight of 100 kDa enter neither the cells nor the MCS [8]. Conversely, their hydrodynamic radius [11] (about 8 nm) is small enough to allow them to penetrate into soft PA gels (1–5 kPa), whose pore size is around 15 nm [12], and Matrigel. In this case, the experiments are performed using larger polymers (2 MDa), with a hydrodynamic diameter of roughly 27 nm.

2.2. Cell culture and multicellular spheroid preparation

Cells are grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 1% antibiotic–antimycotic at 37 °C under 90% air/10% CO_2 atmosphere. We used mouse colon carcinoma cells (CT26 cell line, ATCC CRL-2638). Due to the intestinal transit, colon cells are constantly submitted to shearing and compression and represent a relevant candidate for this study. Nevertheless, similar results are observed on other cell lines, including human carcinoma (HT29), mouse sarcoma (AB6), Mouse schwann (HEI), human breast cancer (BC52), and mouse fibroblasts [14].

Spheroids are assembled for 48–72 h on an agarose cushion [13] (Ultrapure agarose, Invitrogen, Carlsbad, CA) from cell suspensions with roughly 20,000 cells per sample, yielding to an initial radius of about 200 μm.

2.3. Multicellular spheroid size and cellular volume measurement

We previously demonstrated that multicellular spheroids grown on agarose cushions only slightly deviate from the spherical shape [8]. MCS volume is thus inferred by the optical measurement of its radius, with a relative precision of about 1%. In order to evaluate the cellular volume, we perform cryosections, about 3 μm thick, at the level of the equatorial plane. The nuclei of the slices are further stained with DAPI and imaged by confocal microscopy. The relative distance between the nuclei of adjacent cells is measured as a function of their distance from the center of the MCS as described in Ref. [14].

2.4. Polyacrylamide gel preparation

32 mm round glass coverslips are silanized using pure ethanol solution containing 0.36% (v/v) Bind-Silane (GE Healthcare life science) and 3.1% (v/v) 10% acetic acid for 5 min. The coverslips are then gently wiped before being stored in a Petri dish. A high quality quartz mask (Toppan) is first cleaned with iso-propanol and eventually smeared with n-hexane prior to use, in order to maintain a hydrophobic surface [15]. Then, according to the desired stiffnesses [16], pre-mixed acrylamide solutions containing 4–10% acrylamide (Fluka) and 1–2.5% bis-acrylamide (Fluka) are prepared. Drops of 50 μL are polymerized for 45 min between the mask and a silanized coverslip, leading to a typical thickness of 70 μm. Tetramethylethylenediamine (Sigma) is used with ammonium persulfate (Sigma) to catalyze the polymerization. The coverslip, with gel, is carefully removed from the mask and stored in DPBS. PA films are prepared in PBS buffer, with three different stiffnesses. According to the protocol described by Tse and Engler [17], the expected Young's moduli of the PA are respectively 1.1 kPa (3% acrylamide/0.1% bis-acrylamide), 2.0 kPa (4%/0.10%) and 4.5 kPa (5%/0.15%) in water.

2.5. Measurement of gel thickness

The coverslip supporting the gel is mounted in a home-made support and covered with a DPBS solution containing typically 20 nM of Sulforhodamine G (SRG). This low concentration do not perturb the gel and make it possible to perform Fluorescence Correlation Spectroscopy using a 488 laser line, to control the state of the gel (diffusion within the gel is lower than in the solution located above). SRG slightly interacts with the gel which then appears more fluorescent than the surrounding solution (see the confocal signal in Fig. 2A). A motorized focus drive (Marzhauser) is used to vertically scan the gel and determine the gel thickness, from the position of the two transitions (coverslip–gel and gel–solution interfaces). Absolute precision is around 1 μm, which is sufficient to measure a compression of 10% on a gel of 50 μm.

2.6. Measurement of single cells volume

To measure the evolution of the cellular volume in real time, we use the fluorescence exclusion principle recently re-adapted by Bottier et al. [18]. Cells are cultured in a microfluidic device in Polydimethylsiloxane (PDMS), with a constant height of 20.9 μm. The culture medium is supplemented with 1 mM FITC-dextran (10 kDa, LifeTechnologies), a fluorescent dye that is not internalized by the cells, thus cells appear dark while the surrounding medium is fluorescent (see Figs. 4A and 3B). The fluorescence excluded by the cell is then proportional to its volume. Micropillars of 6, 13 and 20 μm high are used to calibrate the measurement (see Supplementary Fig. 2A). Images are acquired with a low numerical aperture objective (10X, NA0.3) in order to get the fluorescence over the height of the chamber. A brass master is fabricated using micromilling machine (MiniMill/3 (Minitech)) with a 100 μm diameter milling cutter to produce the PDMS chambers. This microfabrication technique easily allows producing structures of various heights i.e. the pillars for calibration. Osmotic shocks are generated by simply flowing fluorescent medium (FITC-dextran 10 kDa, LifeTechnologies) as described above, supplemented with the desired amount of 100 kDa dextran to generate osmotic pressure (Sigma–Aldrich, St. Louis, MO). For volume measurements, fluorescence is integrated over a fixed area around the cells of interest. Eventually, the flow arises from the hydrostatic pressure difference originating from the level difference between the inlet and outlet of the chamber.

3. Results

3.1. Multicellular spheroids

We measure the volume change due to the osmotic stress, both at the level of the whole spheroid and at the cellular scale. These

observables are monitored on a timescale of few minutes, i.e. very short compared to the cell doubling time (18 h for this particular cell line). The spheroid size is simply deduced from its diameter, measured by phase contrast microscopy. The MCS diameter is firs measured in the absence of stress then, for the same spheroid 5 min after application of the osmotic pressure.

A displays the relative volume V/V_0 of spheroids as a function of the additional pressure P_D generated by dextran molecules, where V_0 is the initial volume of the MCS. We observe that the volume of MCS monotonically decreases for increasing osmotic pressures. The volume drops by up to 18% for a pressure of 10 kPa. It has to be noticed that at 10 kPa, the relative pressure difference $\delta P = (P - P_0)/P_0$ is around 1.2%, $P_0 \approx 800$ kPa being the osmotic pressure of the isotonic Dulbecco's Modified Eagle Medium (~330 mOsm) [19].

At the cellular level, this global spheroid compression results from a cellular compaction. In an equatorial section of fixed spheroids, the median cell-to-cell distance is measured over rings of constant width but for increasing distances from the center. The experiments are performed on two spheroids of same age and size. The first one is embedded and sliced in the absence of osmotic stress, the second after being kept for 5 min under a stress of 5 kPa. Fig. 1B displays the mean volume occupied by a cell (deduced from the cell-to-cell distance), as a function of its distance from the spheroid center.

In the absence of external pressure (filled circles), the cell-to-cell distance is independent of the position inside the spheroid. Conversely, under a pressure of 5 kPa, we observe a heterogeneous compression inside the MCS (empty squares). Although the cells at the periphery maintain their initial intercellular distance, it decreases considerably in the center of the MCS. Here, the mean volume occupied by the cells drops by 60% in the first 5 min after the shock.

3.2. Osmotic compression of polyacrylamide gels

The large decrease of the cell-to-cell distance observed in the spheroids may be due to the response of single cells and/or the Extracellular Matrix (ECM) to the hypertonic shock. In order to mimic the role of the ECM, we apply solutions of increasing osmolarity to polyacrylamide (PA) hydrogels of different stiffnesses. The polyacrylamide gels are poro-elastic, like an ECM. They are permeable to water, but their pore size is small enough to exclude dextran. We use dextran to compress PA films of about 70 μm of initial thickness, as we did for MCS (see Section 2). The precise thickness of each PA film is measured as described in Section 2, before and after the addition of dextran to the surrounding PBS buffer (Fig. 2A).

According to the protocol described by Tse and Engler [17], the nominal Young's moduli of the PA are 1.1 kPa (red squares in Fig. 2B), 2.0 kPa (blue circles) and 4.5 kPa (green triangles). Being h_0 the film thickness at rest, the relative compression h/h_0 (strain) is larger for softer gels than for stiffer ones. The figure also shows that the strain is proportional to the applied stress, in the limit of low compression (less than 15%). For larger strains, the gels respond to the stress as compressible neo-Hookean solids, as predicted by the Mooney–Rivlin theory of a hyperelastic solid [20,21]. The best fit between the theory (continuous lines) and our experimental data is obtained for a Poisson coefficient of 0.43 ± 0.01. These observations are qualitatively compatible with the response of a PA hydrogel to a mechanical stress [27] and indicate that the osmotic stress practically acts as an isotropic mechanical pressure.

3.3. Matrigel compression

Although polyacrylamide gels are well controlled in terms of stiffness and pore-size, they remain a poor model to describe the behavior of the Extracellular Matrix. Matrigel is a more realistic model to mimic the ECM, even though it suffers of a lack of control in terms of composition, structure and reproducibility. Here we follow the compression of a Matrigel bead, made following the method recently published by Dolega et al. [22] and submitted to the osmotic stress. To facilitate the measurement, the beads are doped with fluorescent nanoparticles. Fig. 3A shows a slow but progressive compression of the bead after exposure to a compression of 2 kPa. The contraction is observed both by fluorescence (top row) and by light absorption (bottom row). In Fig. 3B we plot the compression yield ($\Delta V/V_0$) as a function of the osmotic stresses, between 15 and 500 Pa (filled circles). Error bars correspond to the standard deviation of the statistical sample (more than 8 beads per point). The continuous line represents the best fit to a hyperelastic model developed to the first non-linear term (cubic) [23]. This best fit is obtained for a bulk modulus in the range of 500 Pa, which is compatible with the values measured by Soofi et al. and a Poisson's ratio of 0.25 [24]. Eventually, we notice that the measurements obtained on the whole MCS and on Matrigel fall on the same master curve after rescaling. The empty diamonds represent the data shown in Fig. 1A, with the pressure divided by a factor of 20 and the relative volume V/V_0 multiplied by 4.6.

3.4. Single cells

The mechanical response of individual cells is measured for CT26 cells cultured on a glass slide coated with fibronectin for cell adhesion. Cell volume is monitored every 30 s before, during and

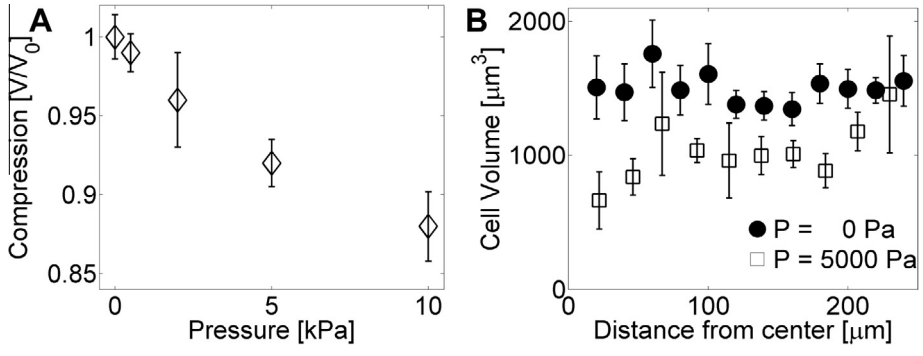

Fig. 1. Pressure-induced volume decrease of spheroids. (A) Relative volume reduction of a whole spheroid for different additional pressures. The spheroid size is measured 5 min after the osmotic shock. The error bars represent the standard deviation ($N = 5$ spheroids per condition). (B) Mean volume occupied by a cell inside the spheroid, as a function of the radial position of the cell (distance from the spheroid center). This volume is constant in the absence of the additional pressure (filled circles). After exposure to $P_D = 5$ kPa (empty squares) we observe a large reduction of the cell-to-cell distance inside the spheroid. The maximal reduction of the mean volume occupied by a cell is observed in the spheroid core. The response of peripheral cells stands within the error bars. Error bars (standard deviation) and mean values are obtained from $N = 3$ spheroids per condition.

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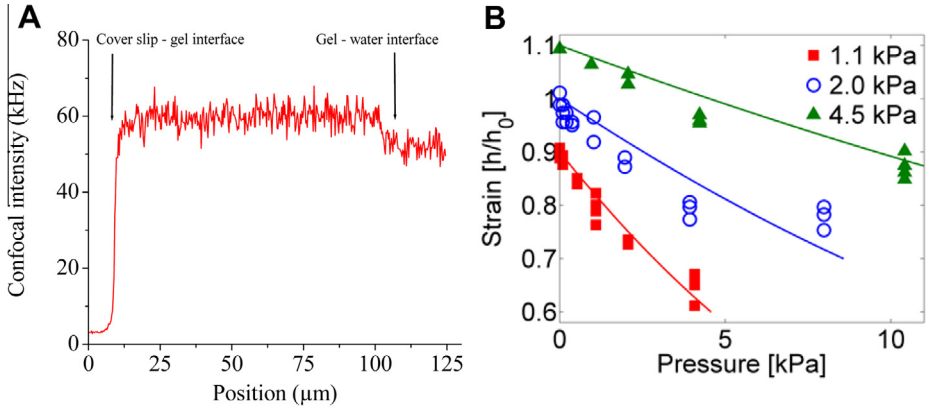

Fig. 2. (A) Gel thickness measured by confocal microscopy. The gel is covered by a solution of PBS containing Sulforhodamine G. This fluorophore being slightly more concentrated in the gel than in solution, a step in the fluorescence intensity is detected at the upper surface of the gel (gel–water interface). (B) Gel compression rate as a function of the osmotic pressure exerted by dextran. Different colors correspond to different nominal stiffnesses of the PA gel (red: 1.1 kPa, blue: 2.0 kPa; green: 4.5 kPa). The data are fitted using a neo-Hookean hyperelastic model (continuous lines), which provides a quantitative measurement of the shear moduli of the gels for a theoretical Poisson coefficient of 0.25. Red and green curves are shifted by −0.1 and +0.1 respectively.

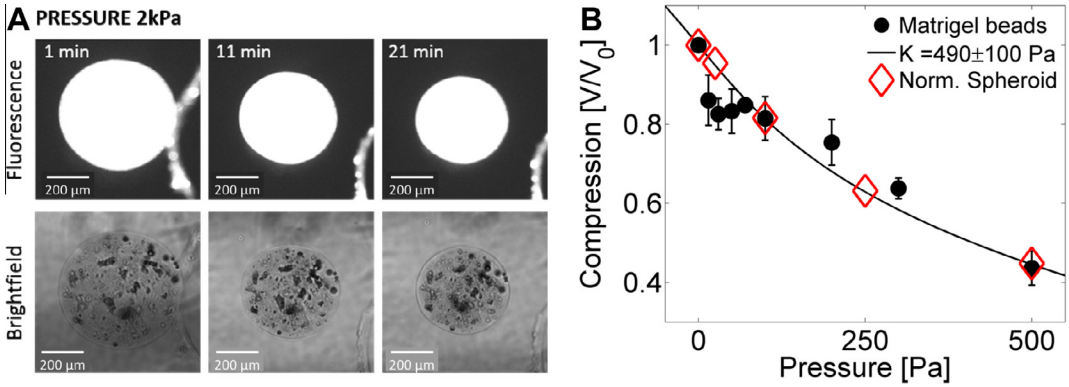

Fig. 3. Time-series of microphotographs of Matrigel microbeads present compression of the gel upon addition of dextran (P = 2 kPa). Scale bar 200 μm. (B) Compression of the Matrigel beads as a function of the osmotic pressure (filled circles). Error bars correspond to the standard deviation computed over more than 8 matrigel beads per point. As a comparison, the compression curve of the spheroid from Fig. 1A is reported here (empty diamonds), after pressure and volume rescaling.

after media exchange. Practically, the whole culture medium in the microfluidic chamber is replaced with new one supplemented with a given amount of dextran. The experiment is performed at 5 and 20 kPa of osmotic pressures.

The results are summarized in the Fig. 4. Cell volume is measured before and after medium exchange. Since during the exchange the concentration of dextran in the chamber is not homogeneous, the volume measurement is not accurate (see Supplementary Fig. 2B and C). In order to evaluate the mean volume response of cells, volume trajectories are averaged to obtain the mean trajectories shown in Fig. 4C and D. The effect of an additional pressure of 5 kPa on the cellular volume is almost not measurable, with a relative contraction of less than 0.2% ± 0.5%, and a large variability between different cells (standard deviation = ± 2.5). Conversely, cells submitted to 20 kPa exhibit a larger contraction which requires 5 min to fully stabilize (see Fig. 3D). In this case, the mean contraction $\Delta V/V_0$ is 2.8% ± 1% (see Fig. 4E).

4. Discussion and conclusion

From a physical point of view, applying a difference of osmolarity between two sides of a semipermeable membrane is equivalent to exerting a mechanical pressure on a membrane. Here, we apply a direct osmotic stress on a multicellular spheroid, in the absence of any semipermeable membrane. Nevertheless, we previously observed that a spheroid responds to this osmotic stress with a rapid reduction of its volume, as it would do if it was actually enveloped by a semipermeable membrane [7]. This is due to the fact that dextran molecules are excluded from the spheroid either by the external layer of cells or by the pore-size of the ECM. Thus, dextran induces the efflux of water and the consequent contraction of the MCS. In this sense, the osmotic stress acts as an isostatic mechanical compression.

In order to mimic the response of the ECM to a hypertonic medium, we apply the osmotic stress to polyacrylamide gels of different stiffnesses, and to matrigel beads. A good agreement between the nominal and the measured shear moduli of polyacrylamide films is obtained for a Poisson coefficient of 0.43 ± 0.01. This value is consistent with the experimental measurements on polyacrylamide gels, whose Poisson coefficient ranges from 0.25 (theoretical value [25]) to 0.5 according to the method and the timescale of the measurements [26,27]. In conclusion, we observed that PA gels respond to the dextran pressure as they would do in case for a pure mechanical pressure. The matrigel beads also respond to the osmotic stress, but exhibit a much larger compliance. We measure a bulk modulus in the order of ∼500 Pa, which seems to be a reasonable estimation of the ECM stiffness.

The most notable result is the large relative compression of the spheroid. The curve in Fig. 1A shows that MCS lose around 14% of their initial volume when submitted to an osmotic stress of 5 kPa. Like PA gels, spheroids show a non-linear response to the compression. From our data, we deduce a bulk modulus of about

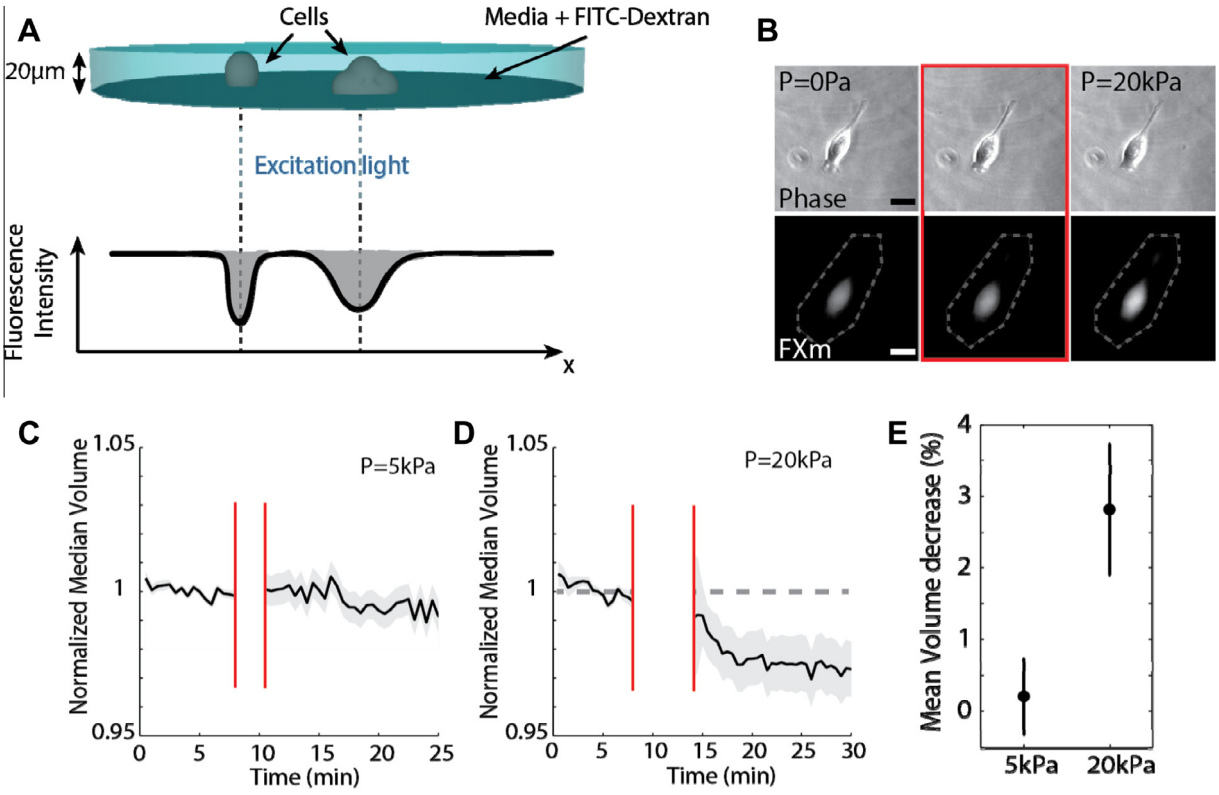

Fig. 4. Impact of osmotic pressure on single cell volume. (A) 3D representation of the volume measurement PDMS chip. (B) Timelapse images of a CT26 cell submitted to a 20 kPa pressure shock. Top: phase contrast images, bottom shows fluorescence exclusion images after background removal (inversed colors). Images where taken respectively before, during (red box) and after dextran injection. Dashed lines display the constant area used for volume calculation. Scale = 10 µm. (C) and (D) Relative volume change after injection of dextran-supplemented culture medium corresponding to 5 kPa and 20 kPa respectively (see Supplementary Fig. 2). Black line shows the volume evolution averaged for 29 cells (5 kPa) and 24 cells (20 kPa), the gray area corresponds to the standard deviation of the statistical sample. Red bars correspond to media exchange. The initial volume of each cell is normalized to 1, to emphasize the relative volume decrease. (E) Mean volume decrease 5 min after pressure shock for 5 kPa and 20 kPa. Error bars show the standard error.

K = 7 kPa for a small compressive stress (less than 500 Pa), which increased for larger strain (the apparent bulk modulus is around 75 kPa for a stress of 5 kPa). One should also notice that whereas the volume occupied by the cells located at the spheroid core shrinks by about 60%, the cell-to-cell distance remains unchanged at the periphery. These values have to be compared to the ones observed for individual cells (Fig. 2E). Under an osmotic pressure of 5 kPa, the volume loss of single cells does not exceed 0.5%. This implies a cellular bulk modulus in the MPa range, i.e. two orders of magnitude greater than what observed in the core of the MCS. Thus, the bulk modulus of spheroids may results from their intrinsic heterogeneity and the large compliance of the Extracellular Matrix, as they are constituted of stiff cells surrounded by soft and compressible ECM.

For single cells, the volume loss can be interpreted as the short time response of the cells to the osmotic shock. In fact, cells are initially adapted to the osmolarity of the culture medium, which is about 330 mOsm in our case. This corresponds to an external osmotic pressure, P_0, of about 800 kPa. Here, we measure the volume change in the first tens of minutes, a timescale much shorter than the cell cycle timescale (~1 day). Adaptation on longer timescales (>1 day) would lead to a more complex cellular response involving pathways regulating cell growth and control of the cell cycle [14,28]. Thus, at short timescale, we only measure the physical response of the cells to the relative increase of the osmotic pressure $\delta P = P_D/P_0$, occasioned by dextran. Under an osmotic stress of P_D = 5 kPa and 20 kPa, the relative pressure increase δP's are respectively 0.57% and 2.27%. These two values are in good agreement with the relative contraction of the cells reported in the Fig. 2E. Everything happens as if the cells passively equilibrated the change in external osmolarity by losing the corresponding amount of water. In such a way, the final chemical potential of water is the same inside and outside the cell. In other words, single cells react on short timescale to an osmotic perturbation as a passive permeable vesicle would do, both in terms of timescale and relative volume loss.

In conclusion, we establish a versatile method to apply an isotropic compression to multicellular aggregates, via an osmotic shock. With several examples, we illustrate the parallel between the osmotic pressure occasioned by dextran macromolecules and a mechanical isotropic stress.

Using this technique, we observe that the spheroids respond in a peculiar manner to an external compressive stress. Spheroids under compression not only shrink much more than expected from the behavior of individual cells, but also present a radial in homogeneity, with the inner layers getting much more compressed than the peripheral ones. This cannot simply be inferred by the response of individual cells that could only account for a 3% compression under a 20 kPa pressure. Part of this compression could be related to the fact that the spheroid is a composite material, made of cells embedded in an elastic Extracellular Matrix more present in the center of the aggregate. As the ECM is rather compliant [29] and highly permeable to water, it is more likely to be compressed than the embedded cells. Actually, neither the over-reaction of the spheroid nor the inhomogeneous response can be totally inferred from the response of single cells or from the presence of ECM in the core, but it might originate from an active response of the cells inside the aggregate as recently suggested [30].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ymeth.2015.07.009.

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