Supplementary information for the manuscript "Probing molecular crowding in compressed tissues with Brillouin light scattering"

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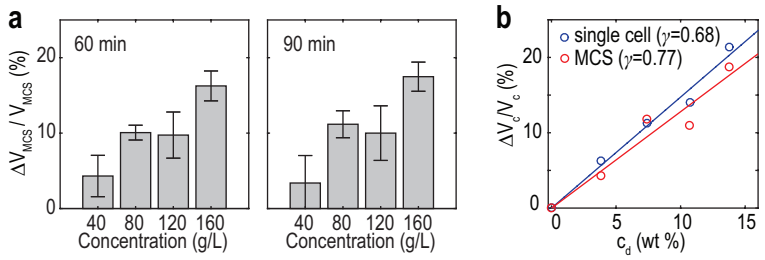

FIG. S1. **a.** Volume variation of MCS at times 60 and 90 min after the osmotic shocks, determined from the white light images of the BLS microscope. **b.** Variation of single-cell volume vs Dextran concentration measured by FXm on single cells (blue markers and line fit) and determined from MCS volume variations.

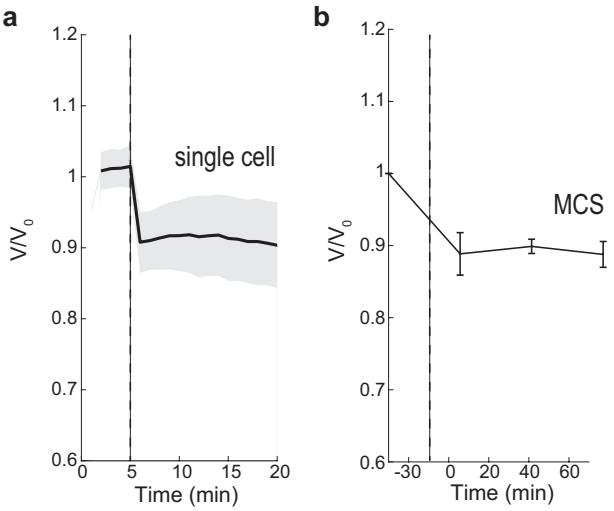

FIG. S2. **a.** Volume of single cells obtained with FXm during osmotic compression (80g/L, $n = 36$). **b.** MCS volume obtained from bright field images during osmotic compression (80g/L, n=10). Vertical lines indicate the time of the shock.

Supplementary note 1: Cell volume increment

To estimate cell volume in MCS, we measured MCS area from the white light images taken with the BLS microscope during the shocks. From this area we determine the volume, V, assuming that the MCS is a sphere. We plot in Fig. S1a the normalized MCS volume variation $\Delta V_{MCS}/V_{MCS}$ at times 60 and 90 min after shock with 6kDa Dx. We see that it increases with Dx concentration, but remains constant over time, demonstrating the stability of the volume after the shock. To confirm this observation, we plot the volume variation in single cells and in MCS over time see

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Fig. S2). We see that in both cases the volume decreases rapidly and remains constant. This data demonstrate that single cells or aggregates reach a steady-state volume after Dx-generated osmotic pressure is applied.

We then assume that the totality of MCS volume variation originates from cell volume variation, as is the case with small Dx molecules: we can thus extract cell volume as $\Delta V_c/V_c = \Delta V_{MCS}/\phi V_{MCS}$, where $\phi = 0.9$ is the cell volume fraction. We plot $\Delta V_c/V_c$ obtained from MCS volume (red circles) in Fig. S1b, and compare it to single cell volume (blue markers). We see that both approaches give similar results. The linear fits allow determining the γ coefficient in each case.

Supplementary note 2: Mixing law

We describe the physical properties of the multicellular tumor spheroid (MCS), refractive index n, density ρ and elastic modulus M, using a mixing law [1–4]:

$$n_t = (1-\phi)n_i + \phi n_c, \tag{S2.1}$$

$$\rho_t = (1-\phi)\rho_i + \phi \rho_c, \tag{S2.2}$$

$$\frac{1}{M_t} = \frac{1-\phi}{M_i} + \frac{\phi}{M_c}, \tag{S2.3}$$

where ϕ is the volume fraction of cells of in the MCS. The subscripts 't', 'i' and 'c' of each parameter stand for 'total equivalent', 'inter-cellular' and 'intra-cellular', respectively. We have also the relation $M = \rho u^2$ [5, 6], with u the acoustic velocity. Equation (S2.3) can then be rewritten as:

$$\frac{1}{\rho_t u_t^2} = \frac{1-\phi}{\rho_i u_i^2} + \frac{\phi}{\rho_c u_c^2}. \tag{S2.4}$$

We remind the formula of Brillouin frequency shift:[1, 5–7]:

$$\nu = \frac{2n_t}{\lambda}\sqrt{\frac{M_t}{\rho_t}} = \frac{2n_t u_t}{\lambda}. \tag{S2.5}$$

The Brillouin frequency shift for the MCS after the osmotic shocks can then be predicted by using Eq. (S2.5). In the following we calculate ν as a function of the concentration of the added Dextran solution, c_d. We start in the next section by discussing the relationship between the ratio of the inter- to intra-cellular concentrations (c_i/c_c) and c_d.

Supplementary note 3: Inter-/intra-cellular concentration *vs* c_d

3.1. Inter-cellular concentration c_i

For the inter-cellular concentration c_i, we consider that the 6 kDa Dextran penetrates into the MCS. The initial ICS is assumed to behave as isotonic water before the shock, and is replaced by Dx after the shock so that $c_i = c_d$.[8]

3.2. Intra-cellular concentration c_c

For the intra-cellular concentration c_c, we start with the definition of the concentration of solutions:

$$c_c = \frac{m_s}{m_c}, \tag{S3.1}$$

where $m_c = m_s + m_w$ is the total mass of the total mixture (i.e. the cell), m_s is the mass of the solute and m_w is the mass of the solvent (i.e. water). We then express the intra-cellular concentration as a function of the variation of the total mass, Δm_c, after the osmotic shocks:

$$c_c = \frac{m_s}{m_c + \Delta m_c} = \frac{c_c^0}{1 + \frac{\Delta m_c}{m_c}}, \tag{S3.2}$$

with c_c^0 the initial concentration in the cells before. Assuming that only the solvent is flowing out of the cell (i.e. a constant dry mass m_s), we then have

$$\frac{\Delta m_c}{m_c} = -\frac{\Delta V_c}{V_c}, \tag{S3.3}$$

with V_c the volume of a single cell and ΔV_c the variation of volume that we measured. We observed a linear relationship between the Dextran concentarion (c_d) and the variation of the cell volume:

$$c_d = \gamma \delta V_c. \tag{S3.4}$$

with $\delta V_c = \Delta V_c / V_c$ (unit of [%]). Coefficient γ is estimated from linear fit to the data.

Using the relationship between cell volume and Dx concentration, we plot the δV_c *vs* c_d curve in Fig. S3. We observe largely linear increase that we fit to a line. Thus, c_c could be expressed as a function of c_d

$$c_c = c_c^0 / \left(1 - c_d/\gamma\right), \tag{S3.5}$$

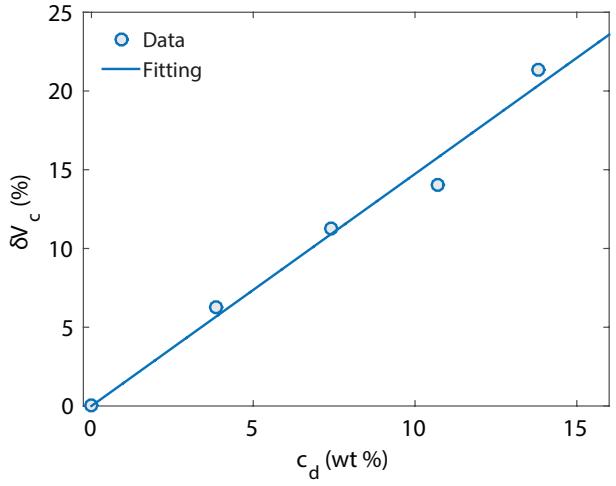

FIG. S3. Measurement of the Dextran concentrations (c_d) *vs* variations of the cellular volume (δV_c), fitted by a linear relation.

with $\gamma = 0.679$ obtained from the fit. We use the initial cell concentration $c_c^0 = 10$ wt % measured in a similar cell line,[9] also consistent with other measurements.[10, 11]

For illustration, the evolutions of c_i and c_c as a function of c_d, calculated by using Eq. (S3.5) and $c_i = c_d$, are shown in Fig. S4. We observe a maximum increase of the intracellular concentration of $\sim 4\%$.

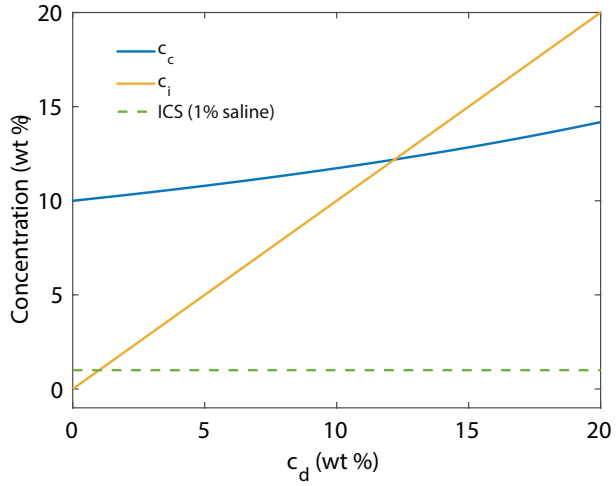

FIG. S4. Evolutions of inter-/intra-cellular concentrations as a function of osmolyte concentration, c_d.

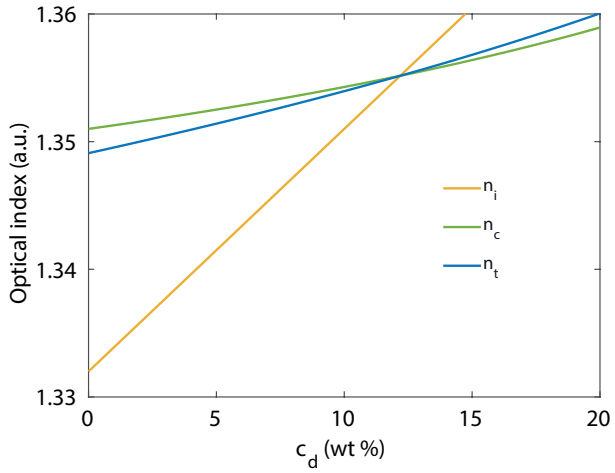

FIG. S5. Evolutions of inter-/intra-cellular and total optical index as a function of osmolyte concentration (c_d).

Supplementary note 4: Refractive index *vs* c_d

We consider the empirical relation between the variations of refractive index and of the solute concentrations:

$$\Delta n = \alpha \Delta c, \tag{S4.1}$$

where $\alpha = 0.0019$ is the proportionality factor taken from Ref. 12. The inter- and intra-cellular refractive indices are written as:

$$n_i = n_w + \alpha_i c_i, \tag{S4.2}$$

$$n_c = n_w + \alpha_c c_c, \tag{S4.3}$$

where $n_w = 1.332$ is the refractive index of water [13] at 37 °C. Since α is largely independent of the type of molecule,[12, 14] we take $\alpha_i = \alpha_c = \alpha$. With the help of Eq. (S3.5), and after taking Eq. (S4.2) and Eq. (S4.3) into Eq. (S2.1), we can calculate the evolutions of n_i, n_c and n_t as a function c_d (see Fig. S5).

Supplementary note 5: Mass density *vs* c_d

5.1. Inter-cellular mass density ρ_i

We have the equation for inter-cellular mass density:

$$\rho_i = \rho_w + \xi_i c_i, \tag{S5.1}$$

where $\rho_w = 1$ g/cm^3 is the density of pure water. $\xi = 0.00375$ g/cm^3 is estimated by fitting the measured Dextran mass density found in Ref. 15 *vs* concentration, as shown in Fig. S6.

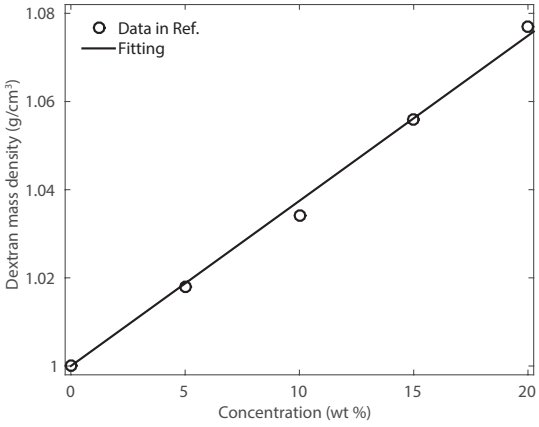

FIG. S6. Measured Dextran mass density [15] with linear fitting.

5.2. Intra-cellular mass density ρ_c

For the intra-cellular density ρ_c, we suppose that the cellular volume, V_c, is equal to the volume of solvent (i.e. water), V_w, and we can calculate ρ_c by using the definition of mass density:

$$\rho_c = \frac{m_c}{V_c} = \frac{m_c}{V_w} = \frac{m_c}{m_w/\rho_w} = \rho_w \frac{m_c}{m_c - m_s} = \rho_w \left(\frac{1}{1-c_c}\right). \tag{S5.2}$$

Combining Eqs. (S5.1), (S5.2) and (S2.2), we obtain the relations for ρ_i, ρ_c and ρ_t *vs* c_d. For illustration, the calculated evolutions of ρ_i, ρ_c and ρ_t as a function c_d are shown in Fig. S7.

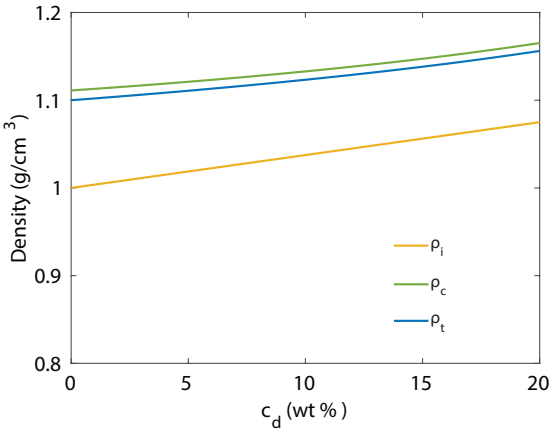

FIG. S7. Evolutions of inter-/intra-cellular and total mass densities as a function of osmolyte concentration (c_d).

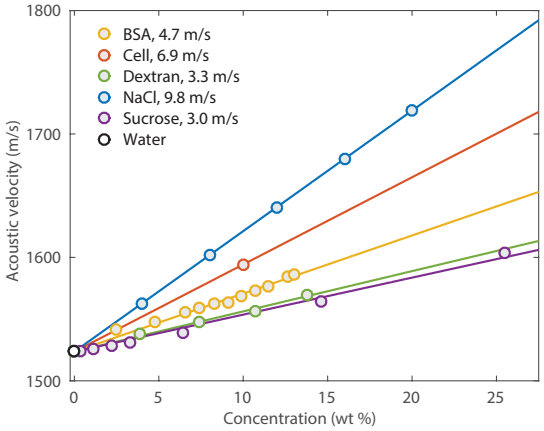

FIG. S8. Acoustic velocity as a function of solute concentrations for difference materials: BSA solution (our data); Dextran (our data); HCT-116 cell (our data); NaCl [16]; Sucrose [17]; pure water [18].

Supplementary note 6: Acoustic velocity *vs* c_d

6.1. Velocity increment of biorelevant solutions

We initially consider a linear relationship between the acoustic velocity variation and solute concentration:

$$\Delta u = \beta \Delta c, \tag{S6.1}$$

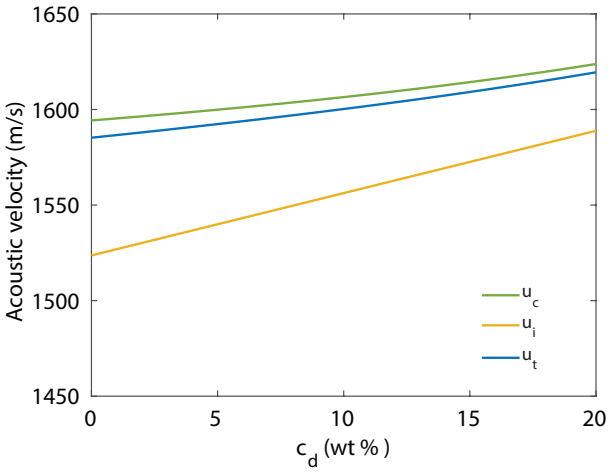

FIG. S9. Evolutions of inter-/intra-cellular and total acoustic velocity as a function of osmolyte concentration (c_d).

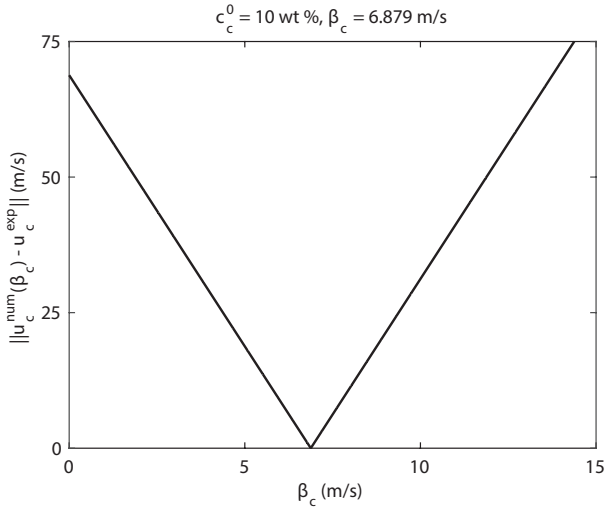

FIG. S10. Absolute difference between the numerically calculated values and the experimental value of acoustic velocity in cells as a function of β_c.

where β is the proportionality factor. The inter- and intra-cellular acoustic velocity are written as:

$$u_i = u_w + \beta_i c_i, \tag{S6.2}$$

$$u_c = u_w + \beta_c c_c, \tag{S6.3}$$

with $u_w = 1524$ m/s the velocity of pure water at 37 °C [18]. $\beta_i = 3.3$ m/s is the proportionality factor of Dextran solutions estimated by fitting our BLS data (see Fig. S8); $\beta_c = 6.9$ m/s is the proportionality factor of cells determinated using BLS frequencies in MCT before osmotic shocks

TABLE S1. Physical properties.

Parameter	Symbol	Value	Refs.
Optical index proportionality factor	α	0.0019	[12]
Velocity proportionality factor of Dextran	β_i	3.3 m/s	[Exp.]
Velocity proportionality factor of cells	β_c^0	6.9 m/s	[Exp.]
Initial cell concentration	c_c^0	10 wt %	[9]
Refractive index of pure water	n_w	1.332	[13]
Acoustic velocity of pure water	u_w	1524 m/s	[18]
Density proportionality factor of Dextran solution	ξ_i	0.00375 g/cm^3	[15]
Density of pure water	ρ_w	1 g/cm^3	[19]
Dx concentration / cell volume ratio (small Dx)	γ	0.679	[Exp.]
Dx concentration / cell volume ratio (large Dx)	γ	1.360	[Exp.]

(see Fig. S8). As a comparison, we plot acoustic velocities and corresponding β-values for several materials: Bovine serum albumin (BSA) solution (our BLS data), HCT-116 cells (our data obtained on MCS), Dextran solution (our data), NaCl [16], sucrose[17] and pure water [18].

6.2. Determination of velocity increment in cells, β_c^0

We first calculate u_c^{exp} from our data before osmotic shocks by using Eq. (S2.4):

$$u_c^{\mathrm{exp}} = \sqrt{\frac{\phi}{\frac{\rho_c}{u_t^2\rho_t} - \frac{\rho_c(1-\phi)}{u_i^2\rho_i}}}. \tag{S6.4}$$

We obtain $u_c^{exp} = 1594$ m/s, a value similar to that measured on MCF7 cells by acoustic microscopy.[20] Using Eq. (S6.3), we calculate the value of acoustic velocity as a function of β_c, u_c^{num}:

$$u_c^{\mathrm{num}}(\beta_c) = u_w + \beta_c c_c^0. \tag{S6.5}$$

We then minimize the cost function:

$$f_{\mathrm{cost}}(\beta_c) = \|u_c^{\mathrm{num}}(\beta_c) - u_c^{\mathrm{exp}}\|. \tag{S6.6}$$

The result of the cost function is shown in Fig. S10, leading to $\beta_c^0 = 6.9$ m/s.

Utilizing Eqs. (S6.2), (S6.3) and (S2.4), the evolutions of u_i, u_c and u_t, as is shown in Fig. S9.

Supplementary note 7: List of physical properties

A list of acoustical and optical properties used in this work is give in Tab. S1.

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