SUPPLEMENTAL MATERIAL

Planar AFM macro-probe to study the biomechanical properties of large cells and 3D cell spheroids

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1. Biological samples

1.1 3D cell spheroids

Cells of the T47D human breast cancer cell line (ECACC, Salisbury, UK) were cultured at 37 ºC in a humidified 5% CO_2 atmosphere, in RPMI 1640 (Biochrom AG, Berlin, Germany) supplemented with 2 mM glutamine (Sigma-Aldrich, St.Louis, MO, USA), 40 mg/l gentamycin (Biochrom AG) and 10% heat-inactivated fetal bovine serum (Biochrom AG). Cells were routinely tested and shown to be negative for mycoplasma contamination using MycoAlertTM PLUS Mycoplasma Detection Kit (Lonza Walkersville Inc., MD, USA). T47D spheroids were obtained by the liquid overlay method as described previously [1, 2]. Briefly, cells (500 cells/well in 150 µl of standard culture medium) were plated in 96 round-bottomed well plates (Cellstar®, Greiner Bio-One GmbH, Kremsmünster, Austria) previously coated with an agar gel layer (50 µl of a 0.7% w/v solution of agar in complete medium) to initially prevent cell attachment. The cells, unable to adhere on the petri dish, grew as a three-dimensional spheroid. After 7-10 days from inoculation in each single well a single spheroid with a diameter in the range of 120-200 µm were observed. An Evos (AMG, Life Technology) digital inverted microscope was used to routinely monitor spheroid size.

1.*2 Human oocytes*

Women undergoing IVF at Assisted Reproduction Unit of the Institute for Maternal and Child Health IRCCS (Burlo Garofolo, Trieste, Italy) donated their supernumerary oocytes for this research. These patients were subjected to the assisted reproduction procedures according to the Italian law n. 40/2004 and the Italian Guidelines applied for assisted reproductive technology in clinical practice (2015). Informed written consents were obtained from all patients who donated their supernumerary oocytes for this research. This research project was approved by the internal review board of the Institute for Maternal and Child Health IRCCS Burlo Garofolo, Trieste, (Italy) and the Italian Ministry of Health (RF-2011-02351812). Oocytes were retrieved transvaginally 36 hours after ovarian stimulation.

Before analysis, oocytes were denuded of their surrounding cumulus cells both enzymatically and mechanically between 0 and 2 h after retrieval. The denuded oocytes were then classified according to their maturation stage and identified as dysmorphic for the presence of anomalous cellular characteristics. These latter oocytes are generally rejected for intracitoplasmatic sperm injection (ICSI) procedure, because with high probability they fail in embryo development. All these oocytes, 3-4h after retrieval, were used for stress-relaxation measurements by AFM.

2. The generalized Maxwell model

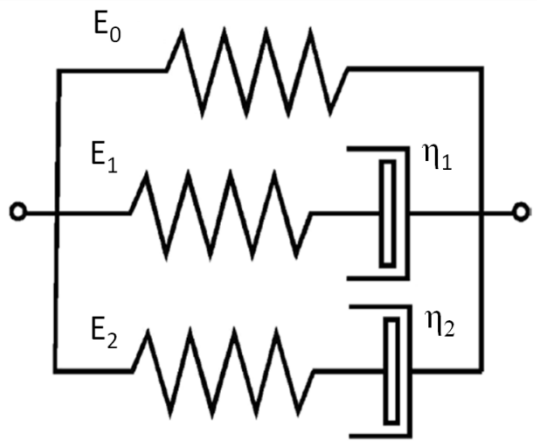

Figure S1: Illustration of the generalized Maxwell model (one elastic and two Maxwell elements arranged in parallel) used to analyze the relaxation process of both oocytes and tumor cell spheroids.

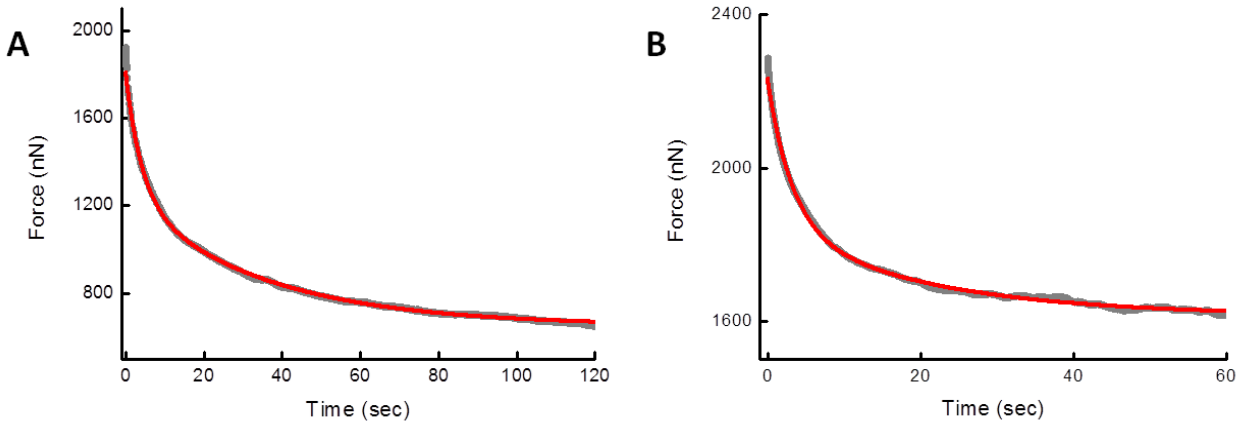

Figure S2: Example of experimental data and fitting curve as obtained by the generalized Maxwell model for a tumor spheroid (200μm) (A) and oocyte (B): experimental data (*gray line*) and fitting curve (*red line*). In the fitting procedure: *t0* was set as a constant and $\tau 2 > \tau 1$. The discrepancy between the first points (less 1 sec) of

experimental data and fitted curve may be due to fast rearrangement of feedback loop to keep the position constant.

3. Analysis of the biological object deformation

The vertical deformation was evaluated by considering the height of the object at the initial stage and in the compressed stage. The image contrast of side-view images was maximized to better identify the object contour and the height at initial stage (A and C) and at the compressed stage (B and D) of the spheroid and the oocyte (green line); initial and compressed stage of ooplasm (yellow line).

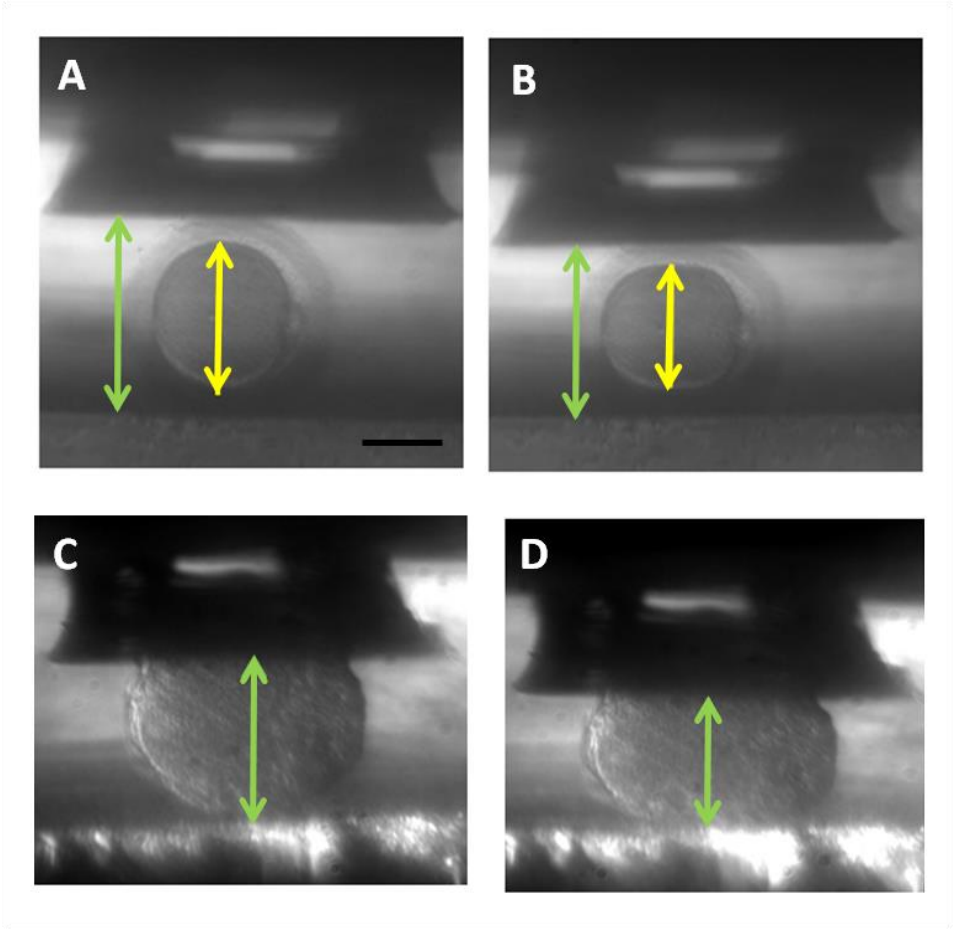

Figure S3: Side view images acquired in stress relaxation measurements: the oocyte, the ooplasm and the spheroid before compression (A and C) and in the compressed stage (B and D). Bar 50µm.

4. TEM imaging

Several tumor spheroids were collected into a single tube a fixed in 4% paraformaldehyde (Electron Microscopy Sciences) containing 5 mM CaCl2 and 0,1% Tannic acid in 0.1 M cacodylate buffer, pH 7.4, for 2 h at room temperature. They were rinsed twice for 15 min with the buffer and postfixed with 1% osmium tetroxide and 1,5% potassium ferricyanide in the same buffer for 2 h at 4 °C. The spheroids were then dehydrated in ascending alcohols, treated with propylene oxide and embedded in Araldite (Electron Microscopy Sciences). Ultrathin sections of the samples (less than 100 nm thickness) were cut on a Top Ultra 150 ultramicrotome (Pabish) and collected on 300-mesh copper grids. The grids were stained with uranyl acetate and lead citrate and examined at 80 kV using a Jeol Jem 100S transmission electron microscopy. The 3D tumor spheroids were divided into two groups according to their growing schedule time and diameter (below and above 200-300µm).

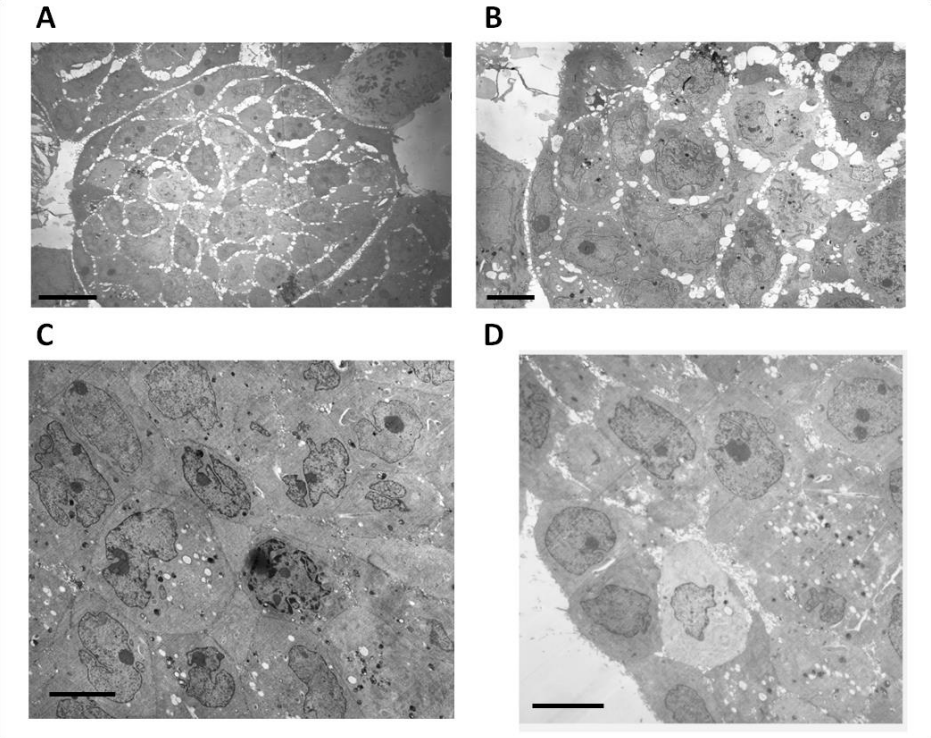

Fig. S4: TEM images of 3D tumor spheroids having diameter between 130 and 325 µm. Tumor spheroids with less than 300 µm diameter: (A) central inner region (bar 20 µm) and (B) zoomed image on the superficial layer (bar 10 µm). 3D tumor spheroids with diameter higher than 250 µm: (C) the inner area (bar 10 µm) and (D) zoomed image on the superficial layer (bar 10 µm).

5. Evaluation of oocyte mechanical parameters according to the reduced model

Starting from Hertz's contact model, Shen et al. proposed a "reduced model" in which the contributions of the ZP and the ooplasm can be extracted, exploiting different loading conditions (i.e. width of the indenter and indentation depth) while measuring the mechanical properties of the whole oocyte [33]. In particular they introduced a weight parameter (β) to quantify the importance of the two contributions. They provided that the mechanical features of the ZP and ooplasm can be decoupled by controlling the β parameters via the indenter's size. With small indenter it is possible to evaluate the ZP elasticity and viscosity, while the use of a large indenter enables to extrapolate the ooplasm contribution. Since the macro-probe is infinitely large as indenter we use the model to evaluate the elastic modulus and viscosity of the ooplasm by the relations they provided.

$$\beta = (1-h/R)^3 \exp\left[-(w/h)^{-0,9}\right]$$

For large indenter ($w \gg h$), being w the size of the indenter and h the thickness of the ZP, the value of β value converges to $(1-h/R_0)^3$ where R_0 is the oocyte radius. As result the elastic modulus of the ooplasm (E_c) can be extrapolated according to the following formula:

$$Ec = (9F_{L2}/16\beta\sqrt{R})\, \delta_L^{-3/2} - 1-\beta/\beta\, (Ezs+Ezd)$$

F_{L2} is the total force registered at the compression δ_L; R the distance between the indenter and the half height object; Ezs is the static elastic modulus and Ezd the dynamic elastic modulus of the ZP. In this estimation we used E1 from our previous work [12] as the (Ezs + Ezd) sum. This choice is motivated by the fact that the E1 value has been associated with the external layer of the ZP and E2 with the internal layer and the rest of the oocyte (see [12] for further details).

The experimental value of F_{L2} can be calculated from the fit parameters and corresponds to the sum of A0, A1, A2, which represent the amplitudes of the exponential functions and their sum is the total force.

We then estimated the ooplasm viscosity (η_c) by using the following relation:

$$\eta_c = \tau_c E_c$$

Considering τ_c as the fast relaxation time constant, as proposed by the reduced model [33].

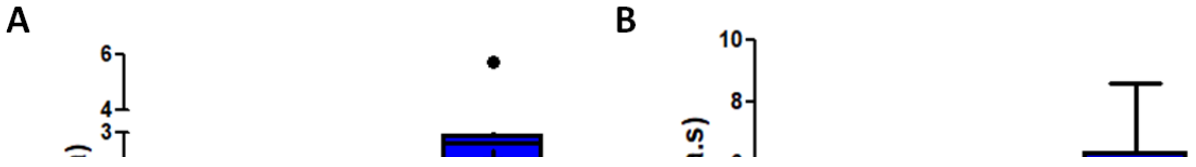

Figure 5S: Box plot representation of all Ec (A) and ηc (B) values as obtained by using the reduced model: bar is the median and (+) mean value. Ec values of dysmorphic MII were found to be significantly different from those of MII (p=0.023); η_c values of dysmorphic MII are different from MII and MI η_c values (p=0,0014).

References

[1] A. Baruzzi, S. Remelli, E. Lorenzetto, M. Sega, M., R. Chignola, G. Berton, Sos1 regulates macrophage podosome assembly and macrophage invasive capacity, J. Immunol. 195 (2015) 4900-4912.

[2] E. Milotti, V. Vyshemirsky, M. Sega, R. Chignola, Interplay between distribution of live cells and growth dynamics of solid tumours, Sci. Rep. 2 (2012) 990.