Microstructure array made of hydrogel, for the formation, growth and analysis of multicellular 3D cancer spheroids.

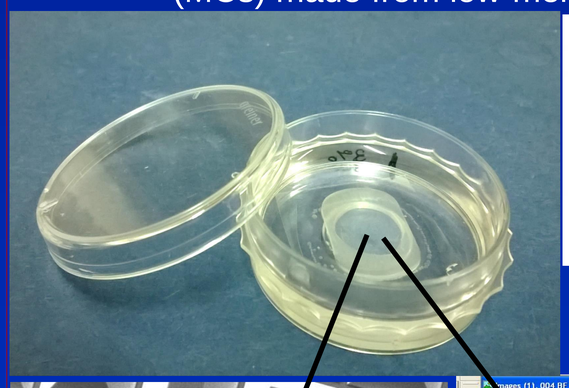
Elena Afrimzon*, Naomi Zurgil, Yana Shafran, Maria Sobolev and Mordechai Deutsch

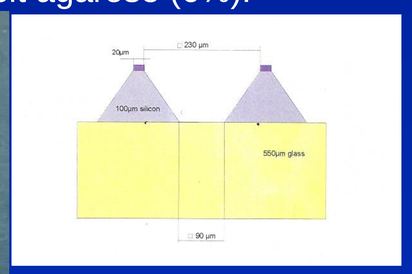
The Biophysical Interdisciplinary Schottenstein Center for the Research and Technology of the Cellome, Bar-Ilan University, Ramat Gan, Israel

Background. Tumor microenvironment is a key component in cancer progression which involves cell proliferation and invasion. One of the factors which play an important role, is the local biomechanical forces generated in the tumor environments. The effect of local pressure on tumorigenesis in primary tumors, as well as in tumor metastasis, remains poorly understood. During the last decade, research in 3D modeling has been recognized as superior to 2D monolayer culturing in developing a prediction model for tumor progression and drug screening.

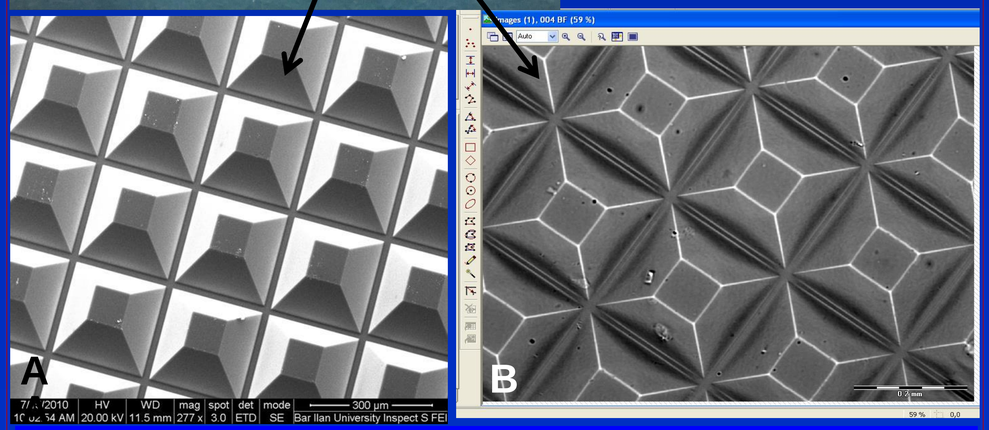
Methods:. This study proposes a new methodology designed to culture 3D cancer spheroids *in vitro* under the conditions with different mechanical properties. This approach is based on the cell retainer methodology [Deutsch M. et al, 2006] and is utilized for real-time investigation of cancer spheroids' formation, growth and functional analysis. Human breast cancer MCF-7 cells' suspension was loaded onto the agarose-made picowells and then, set aside to allow initial cell aggregation for overnight. Next, the cellular conglomerates were overlaid by melted agarose at various concentrations (1%, 2% or 3%) and allowed to grow for 6 days as individual spheroids.

The new culturing device is comprised of a 35 mm glass bottom Petri dish equipped with an array of microchambers (MCs) made from low-melt agarose (6%).



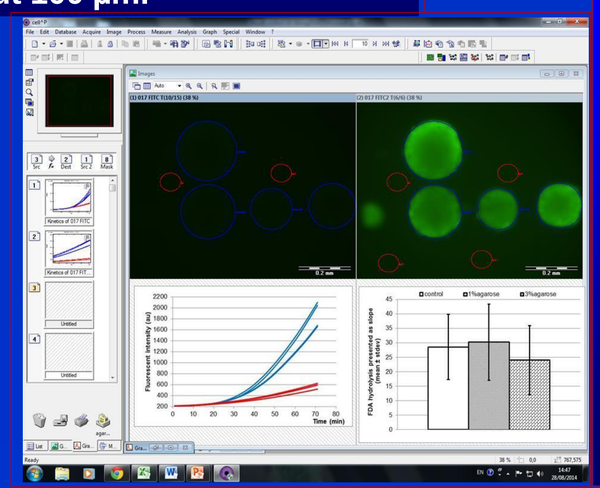


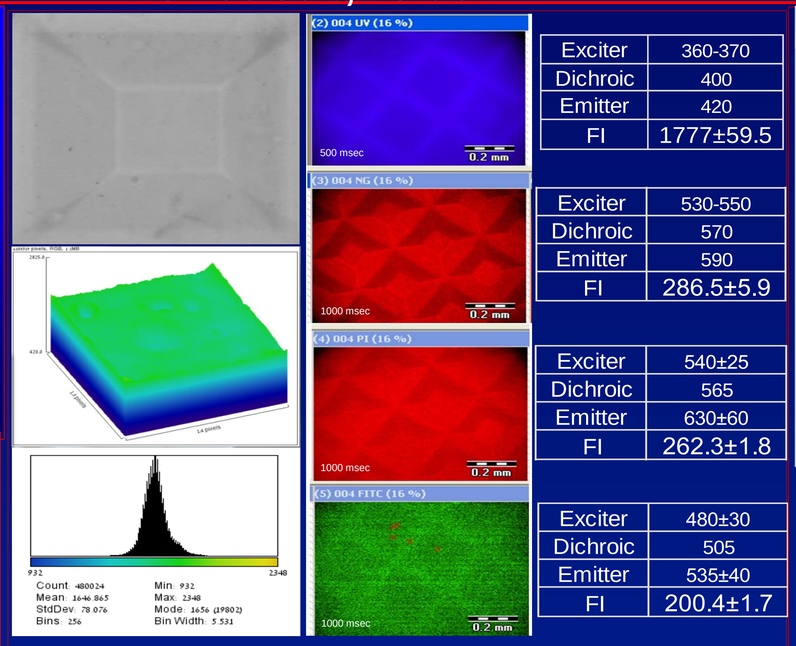
Scheme of the single MC



SEM image of the PDMS master (A) used for MCs embossing in agarose. Each MC (B) is designed to accommodate a single 3D spheroid (magnification ×10). MCs' pitch is 250 µm and depth is about 100 µm.

The system facilitates simultaneous creation, culturing and monitoring of large numbers of spheroids/"quasi-tissues" (about 160 in each Petri dish), as well as screening their response to mechanical stress, while the location of each spheroid is preserved in the same MC throughout the process.





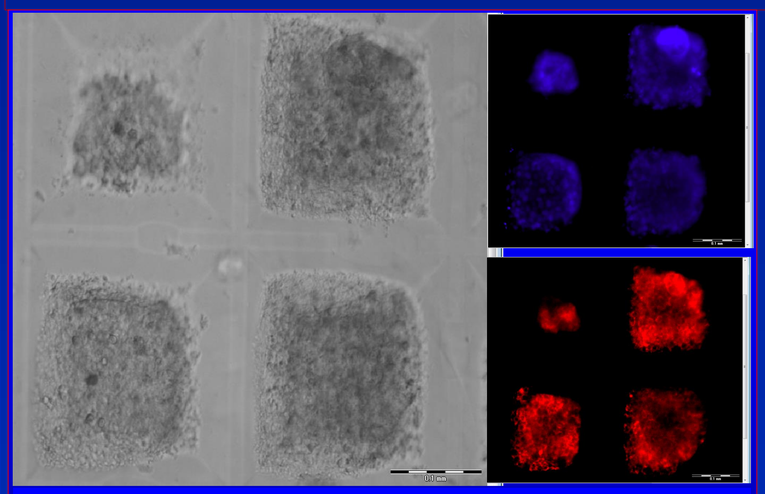
Exciter	360-370
Dichroic	400
Emitter	420
FI	1777±59.5

Exciter	530-550
Dichroic	570
Emitter	590
FI	286.5±5.9

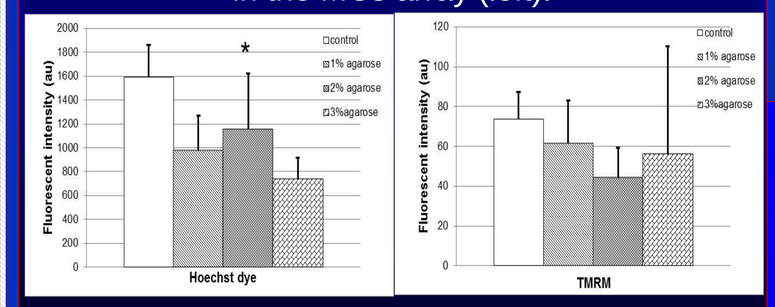
Exciter	540±25
Dichroic	565
Emitter	630±60
FI	262.3±1.8

Exciter	480±30
Dichroic	505
Emitter	535±40
FI	200.4±1.7

Excellent optical properties of the agarose hydrogel (refractive index 1.33) facilitates minimal light transmission fluctuations (<3%) and improves live cell imaging. Low agarose background signals were observed in each wavelength used for fluorescent measurements.

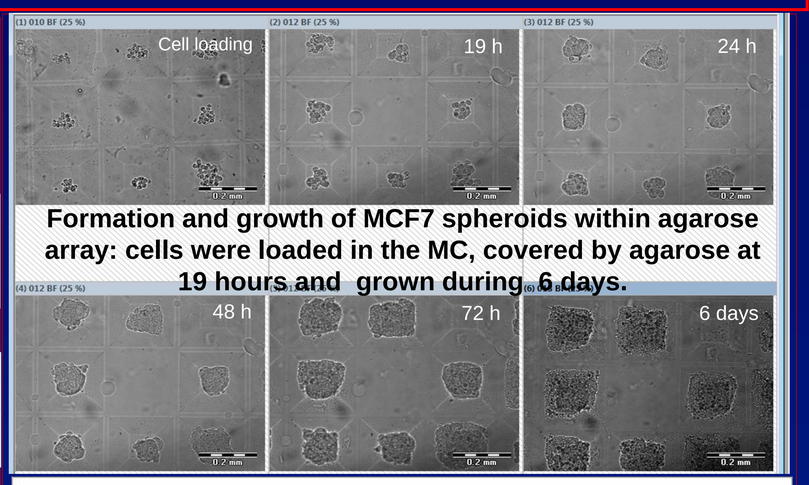


Fluorescence images (right panels) of Hoechst 33342 (upper) and TMRM (lower) stained 3D multicellular structures (BF) coated by 2% agarose in the MCs array (left).

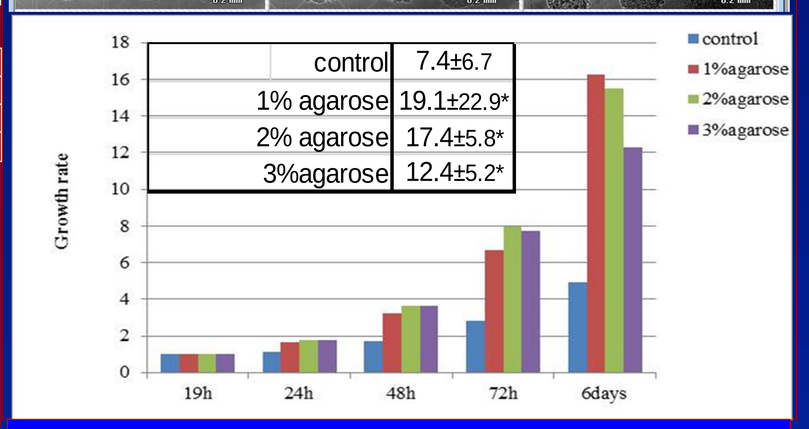


Averaged FIs in the 3D multicellular objects grown under the agarose layers

Spheroids' viability and plasma membrane integrity were assessed by measuring the enzymatic rate of fluorescein diacetate (FDA) hydrolysis. Enzymatic rate was calculated as linear slope from repeated FI measurements in each single 3D multicellular spheroid.
Time dependent FI change in individual spheroids, stained with FDA, is presented in left panel. Averaged slopes of FDA hydrolysis are exhibited in the inset (lower right panel).



Formation and growth of MCF7 spheroids within agarose array: cells were loaded in the MC, covered by agarose at 19 hours and grown during 6 days.



control	7.4±6.7
1% agarose	19.1±22.9*
2% agarose	17.4±5.8*
3%agarose	12.4±5.2*

Spheroid growth rate is presented as ratio of spheroid volumes at indicated time points to the volume of cell conglomerate before agarose coating. Averaged growth rates are presented in the inset.

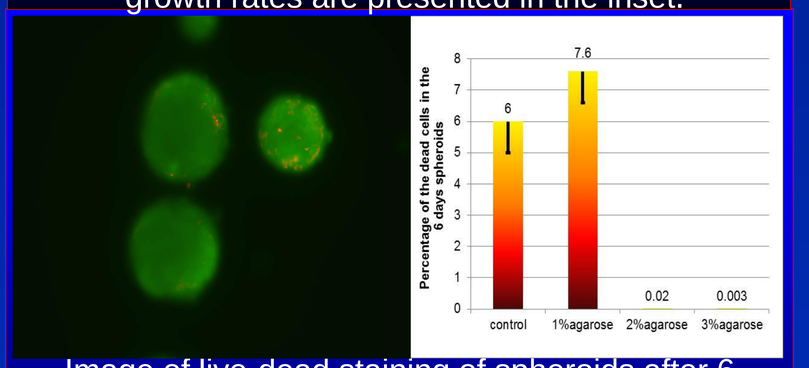


Image of live-dead staining of spheroids after 6 days (left) : green staining (FDA) indicates live cells while red staining (PI) indicates dead cells which are rarely seen. Percentage of dead cells is presented in the graph.

Results. Spheroid' growth under the agarose layer was significantly more rapid in comparison to control spheroids. However, increase in the agarose concentration resulted in the slowdown of spheroid growth at later time points.
Enzymatic rate demonstrates the decrease in ability to utilize FDA in 3D multicellular objects grown under a layer of 3% agarose. On the other hand, agarose coated spheroids do not differ significantly from control ones with respect to mitochondrial membrane potential.

Conclusion. Study of morphological and functional heterogeneity in breast cancer 3D multicellular spheroids under conditions of mechanical stress at the single object level, may provide sufficiently detailed information which can contribute to understanding the diversity of individual tumors, and potentially, to individualization of anti-cancer therapy.