The investigation of spheroids' formation in designated microstructures: Tracing, characterization, simulation and prediction.

Multi-cellular spheroids of cancer cells are considered improved cell based in vitro models for the research of biological characteristics of the micro-metastasis resulting from cancerous tumors. Presently, the conventional means for growing cells for anticancer drug testing is a two-dimensional monolayer. This method of cell growth has been proven effective for many studies including the study of cellular mechanisms of action, functions of various intracellular proteins, signal transduction and even simulation of simple tissue responses to treatment. The need arises for a three-dimensional model that better imitates the physiological status of the tumor and its reaction to drugs, as we simulate the solid cancerous tumor and especially its metastases. Since spheroids preserve the shape and physiological features of the original cancerous tumors, the rate of differentiation and metabolism and their resistance to radiotherapy and chemotherapy treatment is similar to that of cancerous tumors in the body.
The use of spheroids in cancer research requires a quick and easy method for the controlled growth and real-time measurement of spheroid populations, accurate analysis of drug treatment which requires a measurement system that allows simple intra- and extra-cellular measurements, as well as, simultaneous monitoring of spheroid populations before, after and during treatment with various medications in differing dosages. Despite the increasing use of multi-cellular spheroid models in research in general, and particularly in cancer research, and the development of special techniques for harvesting uniformly sized spheroids, the models presently in use do not meet these needs primarily due to technological limitations.

In this study tools were developed for simultaneous creation and culture of large numbers of spheroids which maintain their position in microscopic wells (micro-wells). A theoretical model was developed as well, to describe the process of spheroid formation from scattered individual cells. The system is suitable for automatic screening of anticancer drugs on spheroids. Spheroid volume is easily controlled by seeding density. The location of each spheroid is preserved in the same micro-well, throughout stages of cell seedig, spheroid growth, treatment with soluble agents and imaging, for more than 72 hours (time varying among the various structures). The system permits the continuous tracking of spheroid formation, growth, and analysis of the impact of treatments such as chemotherapy drugs on any individual spheroid and as part of the population as well. Measurement systems and methods used in this study will allow adjustment of personal drug therapy for breast cancer patients in the future.

Another disadvantage to the currently used spheroid creation methods is that they are methods of long-term growth in culture, and the spheroids created are nonuniform in size, shape and number of cells per spheroid. The systems do not enable continuous monitoring of a single spheroid. Methods exist for creating individual spheroids, (single spheroid per well of 96 wells as compared with hundreds of

spheroids in our method), such as the method used in the first part of our experimental work. However, this method has been tested in the past only for large spheroids (1000-20000 cells per spheroid) and it is clear that this size does not reflect micro metastases, thus complicating the distinction of individual cells within the spheroid.

In our initial experiments, using protocols based on the above mentioned method, we have found that spheroids can be created with a high rate of success (>90%) from HEK 293 cells (embryonic ephithelial kidney cells) and from MCF-7 cells (breast cancer cells), which are nearly uniform in size, from initial numbers of at least 500 to 5000 cells. It is possible to create spheroids nearly uniform in size, which have lower success rates, from initial minimal numbers of 100 cells. From a lower initial number, the success rate for spheroid creation is quite low, probably due to the physical distance between the cells, which significantly reduces the chance of contact and prevents “communication” (transferring adhesion molecules such as caderin). Differences exist, which arise from the type of cell and its function, with regard to the ability of living cells to create three dimensional structures. We found that changes in external conditions (surface geometry and initial number of cells), as well as differences in cell type (MCF-7 or HEK 293) significantly affect the ability of cells to form spheroids, as well as the quality and quantity of said spheroids.

Capabilities acquired in measuring spheroid size, and thus calculating its component cells, were thereafter used as tools for comparing chemotherapy treated and non treated spheroids.
The ability to disaggregate spheroids and count their component cells allows quantitative measurement of apoptotic or necrotic cells within large spheroids and their comparison to cells grown as monolayers. However, for small spheroids disaggregation is unnecessary, since observation, imaging and measurement can be done under a microscope and a clear distinction is possible between cells dyed as apoptic, necrotic or live.

In order to characterize spheroid creation and growth from a small number of cells, and in order to allow chemotherapy drug testing on spheroids derived from small samples of cancer cells with a limited initial number of cells, a need has emerged to develop tools suitable for growing spheroids uniform in size from an initial small number of cells.

In the current study, five types of micro–structures for simultaneous creation and monitoring of large spheroid populations were examined. Spheroid formation was recorded and analyzed, and simulation models were developed and fitted to the experimental results in order to facilitate analytical investigation of the process of spheroid formation, as well as the factors which affect it. The five types of micro-structures are briefly described below:
1) Smooth flat glass or polymer surface.

2) A glass honeycomb shaped surface made of a dense array of close fitting micro-wells, hexagonally shaped (top view) with a concave cross-section,100 microns in diameter with a maximum depth of 20 microns.
3) As in (2) above, a glass honeycomb shaped surface made of a dense array of close fitting micro-wells, hexagonally shaped (top view) with a concave cross-section, but in this case 250 microns in diameter with a maximum depth of 35 microns.. These structures were pre-coated with liquid silicon.
4) A polymer honeycomb structure of micro-wells made of UV adhesive polymerized on glass, hexagonally shaped (top view), with a rectangular (flat-bottomed) cross-section , 250 microns in diameter with a depth of 170 microns.
5) A polymer honeycomb comprising of close fitting micro wells, square shaped (top view), with their cross-section being a trimmed pagoda shape (flat bottomed with straight walls to a height of 60.3 microns, then sloped to the bottom).

Experiments were conducted to create spheroids from MCF-7 cells on the various surfaces. Impressive success was achieved for all structures with variations in rate of spheroid formation and the optimal time frame for their growth in the structures (time in which a single spheroid grows in each well without leaving the well or fusing with a neighboring spheroid).

Results were obtained which show the resistance to chemotherapy drugs of breast cancer cells grown as spheroids, compared with the same cells grown in monolayers. Comparison was made between spheroids created in micro-structure (type 2) (100 micron concave wells with a maximum depth 20 micron) and the monolayer which was grown in the same structure, under the same conditions, but lacking the coating which prevents cell adherence to surface.

Dynamics of spheroid formation as a base for the model was observed and characterized from the stage of cell seeding on an open surface (lacking wells) as opposed to their movement on polymer surfaces (types 4,5).

In addition, the dependence of spheroid size on seeding concentration was discovered (experiments were conducted on polymer structure (type 4)), and the percentage of dead versus live cells was analyzed in spheroids formed within this micro-structure, before and following chemotherapy treatment.

Within the framework of the current research is the development of a theoretical model which describes the movement of cells in the process of spheroid formation. This model can predict the mode of spheroid formation on the different microstructures, based on predefined initial conditions, (e.g with or without micro-wells, varying well sizes). With regard to theoretical analysis of spheroid formation; the following findings have been reached: the formation process can be abstractly described by the Brownian motion of cells, which, with certain probability, clump together to form clusters, which also move according to the

same laws. From the theoretical work which relies on prior experimental study, we discovered the parameters required to describe the optimal simulated dynamics of spheroid formation. Comparison between model expectations and experimental results subsequent to creating the model, given identical initial conditions, showed good correlation between simulation and experimental results. It seems that the chosen parameters agree with the experiments.

According to simulation results, it appears that spheroid formation dynamics has a strong non-linear dependence on cell density.