Characterization, Monitoring and Analysis of Tumor Cell Spheroid Aggregation Using Image Processing

Spheroids are cell aggregates formed as a result of aggregation processes of cells, usually tumor derived, grown in conditions that encourage them to develop into spherical cell aggregates. Compared to the common cell growing method as monolayer, the 3D spheroid model has proven to better mimic the physiological state of the solid tumor and its metastases and their response to radiotherapy and chemotherapy. In addition, it has proven that the biological processes involved in spheroid aggregation represent physiological processes involved in metastasis generation and spreading. As a result, examining the effects of various compounds on the aggregation process is considered a promising tool for finding novel strategies for drug intervention even in early stages of tumor development. In spite of its clear benefits, the use of the spheroid model in the pharmaceutical industry isn't common due to unsuitability of the common methods for spheroid growing and monitoring to the measurement systems used in the industry, and the lack of simple assays that will characterize the drug effect on spheroids.

This work introduces novel tools for the examination of anti-cancer drugs based on the examined drug effect on spheroids and their aggregation process. These tools provide a complete solution consisting of unique technology for spheroid generation and observation, label free monitoring of the aggregation process using time-lapse transmitted light microscopy, and various image processing based methods for generating clear parameters for testing the drug treatment efficiency. The tools are designed in a way that will be suitable for use in routine automatic drug screening systems. How these tools are used, and the accuracy of results obtained with them are demonstrated on various cell lines through various treatments.

In addition, the work includes a significant theoretical chapter dealing with the characterization of the spheroid aggregation process. The conclusions of this chapter emphasize the need for the developed tools represented in this work.

The work includes five chapters.

The first chapter is an introduction, briefly describing the use of cell based assays for drug screening in the pharmaceutical industry, and emphasizing the urgent need for better assays that will truly predict the success of the examined drugs at clinical trials. The importance of the spheroid model and its potential for improving predictiveness of the parameters measured using common cell growing techniques, as well as for developing novel unique assays (like measuring the change in the

spheroid volume due to chemotherapy) are emphasized. In addition, the importance of spheroid aggregation process monitoring as a tool for improving the understanding of intercellular interaction involved in metastasis generation is pointed out. Finally, the challenges of image processing of transmitted light microscopy images are introduced.

The second chapter includes the theoretical model which investigates the effect of various factors contributing to the spheroid aggregation process on surfaces covered with anti-cell-adhesion material. The chapter is intended to fill the existing gap caused by the lack of theoretical basis that would facilitate drawing accurate conclusions from experiments commonly carried out in these systems to examine intercellular adhesion forces. The effect of these factors is examined using Monte Carlo simulation which mimics cell movement and the forces between them and allows flexibility with the definition of the parameters affecting the aggregation process.

The model reveals that initial cell density, as well as confinement of cells to restricted volumes, has little effect on the number of aggregates formed in a given surface unit. Cell size influences the aggregation process rate but the effect is significant only for large changes in cell size. The main factors contributing to the aggregation rate are cell movement rate and probability of cells merging while they come into contact with each other. As for the last factor, the model reveals that aggregation rate dependence on the probability that a single contact between cells will cause them to merge, is nonlinear. This fact causes the aggregation rate measurement to be unable to quantify cell adhesion ability, but only to qualitatively recognize significant changes. In addition, the model shows that a given kinetic description of the aggregation rate, it can be equally explained for fast moving cells with low merging probability, and for the opposite. This emphasizes the weakness of the parameter to correctly characterize the aggregation process taking place in the open-surface covered with anti-cell-adhesion material experimental system.

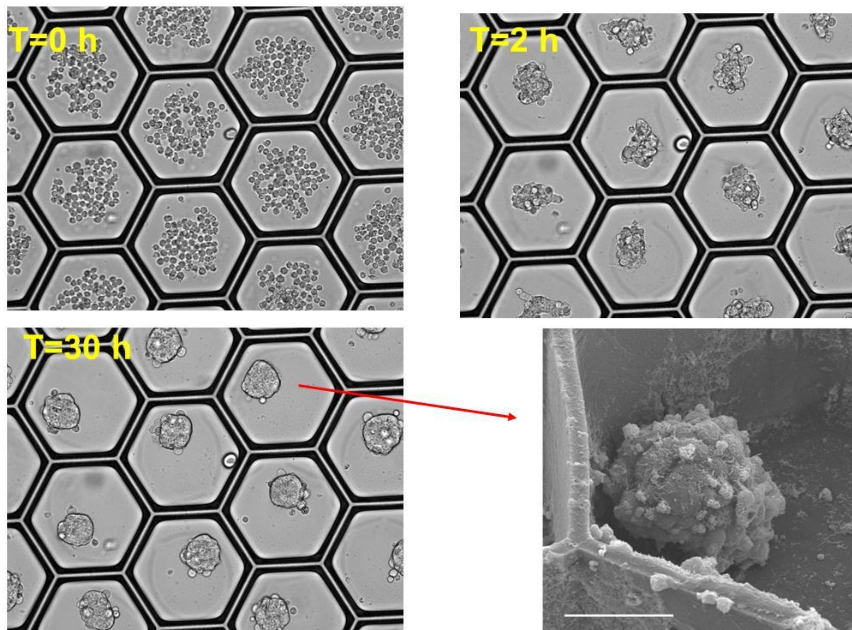
Following previous works which claim that long-term chemotaxis forces that can be described by the existence of a uniform radius of influence around the cell, also affect the aggregation rate, the model examines the effect of such forces on the process rate. Indeed, the model predicts that such forces, if they exist, will have a great effect on the aggregation process rate, and the number of aggregates that will be formed will depend almost entirely on the size of this radius of influence with almost no dependence on other factors. This finding, led me to carefully examine the basic properties of cell movement influenced by intercellular chemotaxis. To my surprise,

also in this case, I found no satisfactory description of this phenomenon in available publications, and was obliged to develop theoretical examination tools on my own.
The theoretical discussion examines several patterns of chemotactic factor secretion, and predicts the spatial-temporal dependence of the concentration of the factors secreted from cells randomly placed in space. Accordingly, the predicted trajectories of "test cells" in the mapped concentration field of each secretion pattern are calculated. It has been found that estimation of the direction of the concentration gradient felt by cells that move under the influence of chemotactic forces requires calculation of their position relative to secreting cells in a few hundred micro-meter vicinity. In addition, it had been found that cells secreting chemotactic factors are not expected to be affected by the secretion of neighboring cells even if the secretion rate is much larger than their own.
These conclusions are totally contradictory to the simplified assumption of the existence of the radius of influence around each one of the cells. In practice, my conclusions lead to the recommendation that cellular systems with strong chemotaxis forces should not be used for the examination of spheroid aggregation.
The final conclusion of the theoretical chapter is that the open-surface covered with anti-cell-adhesion material experimental system is too complicated to be used for examination of intercellular cell adhesion forces and should be replaced with another experimental system.
The third chapter introduces the tools, materials and methods used for the experimental work. Particularly, a unique micro-well array used for spheroid formation, culturing and monitoring, is introduced.
Micro-well array properties encourage self-aggregation of uniform spheroids, one in each of the micro-wells, while the whole aggregation process of each distinct cell group can be fully followed. The array can be fitted to any cell culturing and monitoring device commonly used in the drug screening industry. The unique spheroid-monitoring techniques introduced in this work are based on the advantages of this experimental system.
The fourth chapter deals with monitoring and analysis of the aggregation process within the micro-well arrays using image-processing tools. Algorithms for identifying the micro-well, and the cells and aggregates within it, for monitoring aggregation rate, cell merging and for examining aggregate processes are introduced. Results of these algorithms for monitoring all stages of the aggregation process are described by single-valued parameters that enable derivation of clear conclusions regarding the resulting effect of tested drugs on the aggregation process. A model system

treatment with EGTA demonstrates the usefulness of the developed tools as a means to examine the spheroid aggregation process.

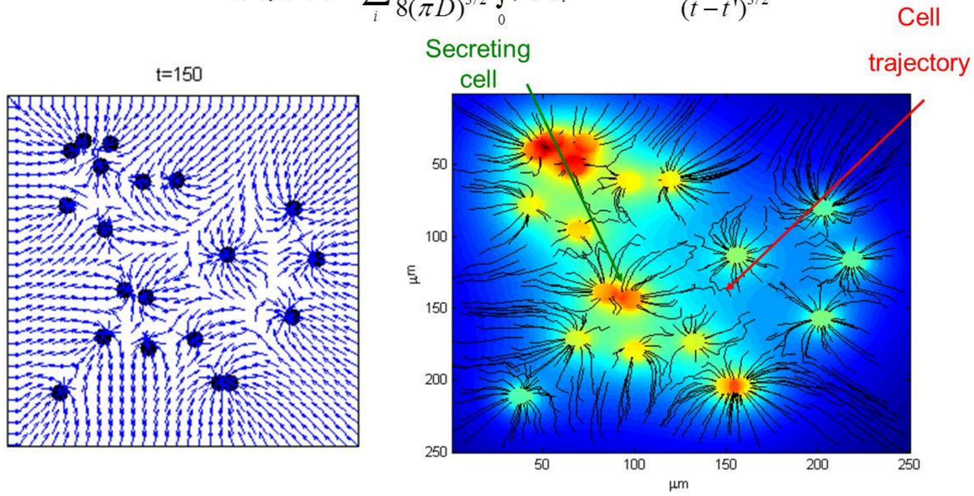
The last chapter deals with developing image-processing based analysis tools for examination of drug effect on spheroids grown in the micro-well array. An Algorithm for spheroid segmentation that allows exact measurement of spheroid volume is introduced. The ratio between the volume of spheroids before and after drug treatment and examination of the distribution of these volume ratios in the spheroid populations are suggested as an efficient tool for evaluating drug treatment success. Results are demonstrated on model systems in which spheroids are exposed to anti-cancer drugs and to Nitric Oxide.

Spheroid development in micro-array

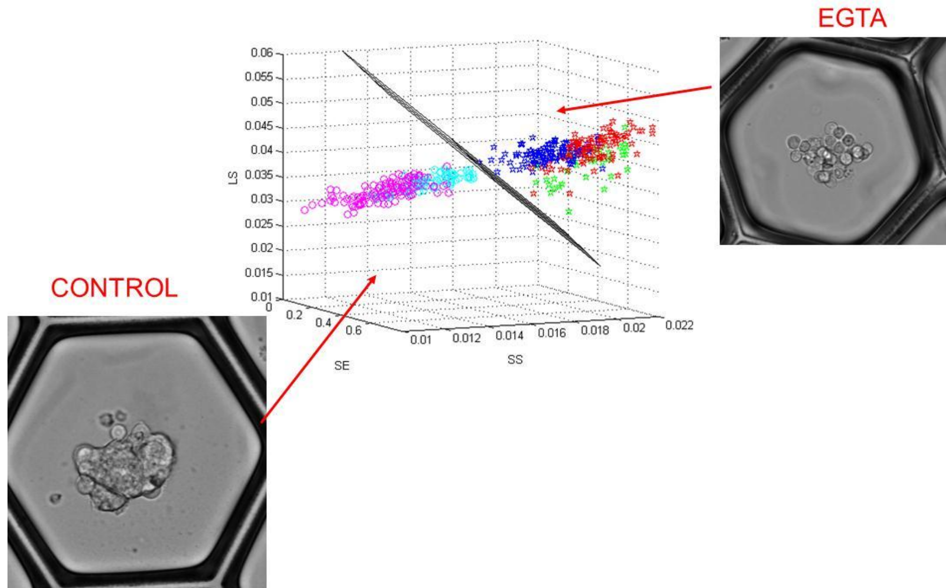


Cells movement during chemotaxis

$$C(x,y,z,t)=\sum_i \frac{1}{8(\pi D)^{3/2}}\int_0^t \varphi(t')_i\, e^{-\vec{r}_i^{\,2}/4D(t-t')}\,\frac{dt'}{(t-t')^{3/2}}$$



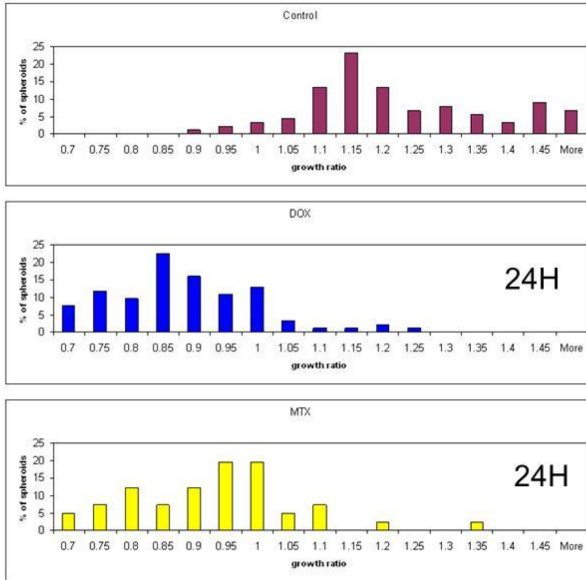
Texture based classification



Spheroid segmentation

$$Growth\ ratio \equiv \frac{V^{late}}{V^{early}}$$

Drug response



272 mOsm/L- 284 mOsm/L (~2Min)