Summary

This work focuses on quantitative evaluation of Reactive Oxygen Species (ROS) levels in monocytic and lymphocytic cells and the involvement of these reactive species in the apoptotic process.

This study mainly concerns the monitoring of kinetic changes in cell populations and individual cells resulting from exposure to various substances that affect (in most cases increase) the intracellular ROS concentrations.

The motivation for this work stemmed from the crucial importance of ROS due to their involvement in diverse physiological and pathological processes and the difficulty in their quantitative assessment due to the chemical and physical properties of these molecules.

The uniqueness and novelty of this work lie in creating a new technology, developed in the course of this work, which addresses the requirement for repetitive measurements of the same individual non-adherent cells, such as blood cells.

This work consists of four chapters:

- The first chapter is an introduction, surveying the relevant biological backgrounds and methods for ROS measurements. Also, it presents a general survey of the state of knowledge and the technological achievements in the field of repetitive (kinetic) measurements of non-adherent cells.

- The second chapter deals with experimental materials and methods used in this research.

- The third chapter extensively discusses the development and calibration of the novel system for kinetic measurements at an individual cell resolution. In order to address this challenge, our laboratory has developed the Live Cell

Array (LCA) technology, having a cell carrier as its essential element (M. Deutsch, et. al., Lab Chip, 2006).

The use of the LCA allows to preserve the uniqueness and vitality of the cells under investigation during prolonged periods, under diverse measurements and biological manipulations. Measurements of non-adherent cells are done using an optical imaging system, when the cells are “trapped” inside pico-liter-sized wells of the LCA made of transparent glass, which preserve the information on the spatial location of each cell, according to the “cell per well” principle.

- The fourth chapter describes and discusses the experimental results. In order to estimate ROS, the indirect approach of measuring changes in fluorescence intensity of fluorescent probes (indicators) which rises as a result of probes oxidation by intracellular ROS was used. For these measurements, I used the following fluorescent probes: 2,7-dichlorofluorescein diacetate (DCFDA), Dihydrorhodamine123 (DHR), and Dihydroethidium (DHE). Analysis of experimental results includes the average behavior of cell populations in time, and cell distribution within a population. The latter option is only feasible when the monitoring of the kinetic changes in fluorescence intensity is enabled from individual cells. This capability was provided by the Live Cell Array technology. Concomitantly, additional parameters were examined (such as changes in mitochondrial membrane potential, phosphatidylserine exposure on the cell membrane surface, changes in the expression of Bcl2 and Bax proteins) in order to evaluate the apoptotic processes in these cells.

The present biological research includes two principal parts.

In the first part of the research, I used model cells from cell lines, mainly U937 cells (the human promonocytic cell line).

The following goals have been accomplished:

- Development, calibration and characterization of the fluorescent techniques for kinetic measurements of changes in ROS levels in cell populations and individual cells as a result of exposure to hydrogen peroxide.
- Estimation of the involvement of superoxide in the intracellular oxidation of fluorescent ROS probes using SOD enzyme.
- Simultaneous measurements of intracellular ROS levels and mitochondrial membrane potential in the same cells.

In the second part of the research, I used techniques that were developed in the first part to measure changes of the above parameters in U937 cells and lymphocytes, either resting or activated by phytohemagglutinin (PHA) from Unstable Angina patients and healthy donors with no signs of heart disease, before and after cells exposure to lysophosphatidylcholine (LPC). LPC is a phospholipid which has many important effects in the regulation of various stages of atherosclerotic plaque formation. In addition, a series of experiments to determine the apoptotic status of these cells were conducted.

Briefly, the obtained results demonstrate the following:

- Unique techniques have been developed to repetitively and specifically measure Reactive Oxygen Species in non-adherent cells.
- High heterogeneity was observed in basal ROS levels in individual U937 cells in the population, and with regard to ROS changes due to cells exposure to H_2O_2 and LPC.

- The measurements of intracellular oxidation of the different fluorescent probes under effect of H_2O_2 and in the presence of SOD, emphasize the differences in the specificity of these ROS probes.
- There is a correlation between a rise in ROS levels and a decrease in mitochondrial membrane potential in the same measured U937 cells.
- SOD is in the inhibition of the apoptotic process induced by H_2O_2 in U937 cells.
- LPC causes an increase of ROS levels in lymphocytes activated by PHA in healthy donors with no signs of heart disease. In contrast, no significant change was observed in ROS levels of lymphocytes after the same treatment in unstable angina patients.

There was found a correlation between ROS levels in lymphocytes treated with LPC and the apoptotic status of the same cells. There is an apparent connection between the resistance reaction of the patient's cells to LPC and the disruption of the apoptotic process in these cells.

In conclusion, in the course of this work, numerous technological and scientific objectives have been accomplished. First, there was developed the exclusive LCA technology allowing to conduct repetitive measurements of individual non-adherent cells, such as blood cells. The use of the LCA opens new applicative possibilities in cell biology. It's most important novel trait is that it changes the investigation approach from measuring dynamic biological processes occurring in the entire cell population toward the study of individual cells in the population. This work was highly received and a LCA image was published as the cover page in August 2006 issue of the journal Lab on a Chip (the cover image is shown in the appendix to this work).

In addition, ROS measurements, according to the mode presented in this work, can have great scientific and applicative significance. These kinetic measurement modes, developed in the course of this study, enable the simultaneous real-time monitoring of physiological and pathological processes at the resolution of a single cell.

In the course of this work, the following scientific papers have been published:

1. Concomitant real time monitoring of intracellular reactive oxygen species and mitochondrial membrane potential in individual living promonocytic cells. N. Zurgil, **Y. Shafran**, E. Afrimzon, D. Fixler, A. Shainberg, M. Deutsch**.** Journal of Immunological Methods 2006 Aug 23.
2. Lymphocyte resistance to lysophosphatidylcholine mediated apoptosis in atherosclerosis. N. Zurgil, E. Afrimzon, **Y. Shafran**, O. Shovman, B. Gilburg, H. Brikman, Y. Shoenfeld, M. Deutsch. Atherosclerosis. 2006 Mar 23.
3. A novel miniature cell retainer for correlative high-content analysis of individual untethered non-adherent cells. M. Deutsch, A. Deutsch, O. Shirihai, I. Hurevich, E. Afrimzon, **Y. Shafran**, N. Zurgil. Lab Chip. 2006; 6: 995-1000.
4. Microplate cell-retaining methodology for high-content analysis of individual non-adherent unanchored cells in a population. Deutsch A, Zurgil N, Hurevich I, **Shafran Y**, Afrimzon E, Lebovich P, Deutsch M. Biomed Microdevices. 2006 Jun 27.

5. A cluster pattern algorithm for the analysis of multiparametric cell assays. Kaufman M, Bloch D, Zurgil N, **Shafran Y**, Deutsch M. J Comput Biol. 2005 Sep;12: 1014-28.
6. Monitoring the apoptotic process induced by oxidized low-density lipoprotein inJurkat T-lymphoblast and U937 monocytic human cell lines. Zurgil N, Solodeev I, Gilburd B, **Shafran Y**, Afrimzon E, Avtalion R, Shoenfeld Y, Deutsch M. Cell Biochem Biophys. 2004;40: 97-113.
7. Monitoring of intracellular enzyme kinetic characteristics of peripheral
   mononuclear cells in breast cancer patients. Afrimzon E, Zurgil N, **Shafran Y**, Sandbank J, Orda R, Lalchuk S, Deutsch M. Cancer Epidemiol Biomarkers Prev. 2004 Feb;13: 235-41.

The different stages of this study have been presented in the following international conferences:

1. ISAC 1st International Cytomics Conference, Wales. 2 poster presentations: “Lysosomal involvement in oxidative stress induced apoptosis”. Zurgil N, **Shafran Y**, Afrimzon E, Deutsch M.; and “Real time monitoring of intracellular reactive oxygen and nitrogen species levels in individual live cells,” Zurgil N, **Shafran Y**, Afrimzon E, Deutsch M.
2. ISAC XXII International Congress France, May 2004. Poster presentation: “Measuring intracellular levels of reactive oxygen and nitrogen species in individual living cells”. Zurgil N, Afrimzon E, **Shafran Y**, Deutsch M.

3. 4th International Congress on Autoimmunity, Budapest, Hungary. Nov. 2004. Oral presentation: “Lymphocyte Apoptosis Induced by Lysophosphatidylcholine,” by Afrimzon E, Zurgil N, **Shafran Y**, Shovman O, Gilburd B, Brikman H, Shoenfeld Y, Deutsch M.
4. ISAC XXIII International Congress. Quebec, Canada. May 2006. 2 oral presentation: "Enabling Repetitive Prolonged Measurements Of Non-Tethered Non-Adherent Individual Cells In a Microtiter Plate." Deutsch M, Zurgil N, Afrimzon E, **Shafran Y**, Deutsch A.; and "The involvement of Lysophosphatidylecholine (LPC) in lymphocyte apoptosis in atherosclerosis." Afrimzon E, Zurgil N, **Shafran Y**, Deutsch M.