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A PILOT STUDY OF METHODS ABOUT DETECTION OF INTER-MOLECULAR INTERACTIONS AS INDIVIDUAL UNITS OF INTRA-CELLULAR STRUCTURES, INTRA- AND EXTRA-CELLULAR CASCADE REGULATORY PATHWAYS

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ABSTRACT

Different methods for investigation on the mechanisms of participation of cell proteins in direct and/or indirect intra- and extra-cellular inter-molecular (protein-lipid, protein-protein, protein-RNA, protein-DNA interactions, etc.), by cascade regulatory pathways were developed and tested. These bio-molecules (in particular microtubule proteins and cyclins) have been found as parts from different complex structures from the nuclear, cytoskeleton and membrane cell fractions, as mitotic spindle, endocytosis vesicles, cell organelles, membrane structures, which are important for basic cell functions. The techniques, developed in the current study, could be useful for identification of the proteins/peptides, participating in the composition of these structures, but also of the interactions between them, on molecular and intra-molecular levels. For confirmation of the results obtained and the reliability of the applied methods, routine techniques as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) assays were used. In all cases, the main goal is directed to search of molecules, participating in the control of cell growth and proliferation, as well as of such, which determine the further cell fate during cell differentiation and maturation.

Keywords: Inter-molecular interactions, Cell interactions, Molecular complexes, Laser irradiation, 3D-imaging.

Contribution/ Originality

The current study contributes in the existing literature with confirmation of the inter-molecular interactions for the cell differentiation direction. The current paper underlines the relationship between the changes in three different levels: cell morphology; intra-cellular organelles structure, and bio-molecules, by application of novel detection methods.

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1. INTRODUCTION

Interactions between the different proteins, as well as of proteins with nucleic acids have been proved to be important in cascade regulatory pathways, underlining processes as cell growth, proliferation, differentiation, transformation, as well as of their control [1, 2]. On the other hand, signaling strength, kinetics and specificity of these pathways have been shown to be modulated at many levels by distinct regulatory proteins, which could result in the differentiation of cells in concrete lineages and/or sub-lineages, including in different immune cell types, which depends of the respective extracellular stimuli [3]. According many literature data, a lot of proteins with tumor-suppressor action function are members of cascade regulatory pathways in processes as *trans*-Golgy-transport, cell division, cell-cell interactions, by connection with cytoskeleton components of the cell cycle as microtubule proteins [4] and cyclins [5, 6]. In this way, those molecules have been proved as able to connect with histones and histone-like nuclear proteins [7-9]. In this connection, the importance of development methods and techniques for identification of unknown proteins, protein-protein- and protein-DNA-/RNA-interactions, has been found to be necessary [10-15]. On the other hand, these bio-molecules have been found as parts of different complex structures in the nuclear, cytoskeleton and membrane cell fractions, as mitotic spindle, cell organelles, endocytosis vesicles, which are important for basic cell functions, as cell division, growth and intra-cellular contacts [4]. For a better understanding the role of bio-macromolecules in the functional activity of the complex structures, a lot of scientific messages about development and application of different methods for identification of the proteins/peptides, participating in their composition, but also of the interactions between them, on both molecular [16, 17] and ultra-structural levels [18-22] have been received. The objective of the current study is connected with development of different methods for identification of different types of intra- and extra-cellular interactions, but also of target biological macromolecules, as participants in biological structures on the one hand, and of cascade mechanisms, on the other.

2. MATERIALS AND METHODS

2.1. Cell Cultures

Normal fibroblasts from embryonic mouse Balb/c 3T3 line, malignant mouse myeloma cells, as well as mixed cultures from both cell types, were prepared. All cell cultures (1 x 10^6 cells/ml), were incubated at 37^{0}C in the incubator with 5% CO_2 and 95% air humidification, in RPMI 1640, Dulbecco's Modified Minimal Essential Medium (DMEM) (high glucose) or combinations of both media, supplemented with 10% Fetal Calf Serum (FCS), 100 UI/ml Penicillin, 0.25 mg/ml Streptomycin and 0.25 mg/ml Amphotericin-B, in 24-well plaques. When the formation of confluent cell monolayer was observed, the cells were trypsinised (by treatment with trypsin/EDTA solution), and tested for viability by Trypan Blue Dye Exclusion Test. Cell sub-populations were then pre-cultured in the presence of supplemented cultural fluid, obtained by centrifugation and filtration after previous cultivation of myeloma cells. All cell cultures were

observed as native light-microscopy preparations by inverted light microscope, supplied with mega-pixel CCD-camera.

2.2. Preparation of Fixed Light-Microscopy Slides

Fixed preparations from normal mouse embryonic 3T3 fibroblasts, as well as from cultures of the same type normal embryonic cells, co-cultivated with malignant mouse myeloma cells, were prepared by fixation with 95% ethanol, washing with Phosphate Buffered Solution (PBS), after which they were treated with Giemsa dye or by Hematoxillin/Eosin technique, respectively, washed with tap water, and dried at room temperature.

2.3. Laser Irradiation

Fixed light microscopic slides from normal mouse embryonic cells 3T3 and from mixed cultures, prepared as described above, were subjected on laser light irradiation with the next parameters described: Q-switched Nd^{3+} YAG laser at 1.06 μm; 950 KW/cm^2 - power density. The illuminated spot (intensity distribution) was practically homogenous (transversal multimode emission). Smears from human and mouse peripheral blood on glass slides were prepared and similarly proceeded.

2.4. 3D-Imaging

Optical recording of cells and their 3D presentations are performed by digital holographic microscopy (DHM). In difference to the usual optical microscopy, in DHM not only the amplitude, but also the phase information of the objects could be recorded and reconstructed as 3D images. These properties, as well as the non-invasive nature of the method, makes it applicable to many scientific fields. Recently, DHM has attracted more attention with its applications in the biomedical field, where the quantitative phase detection can be used for imaging and morphological measurements of the cells [23]. The principle is based on recording of interference patterns, produced by summation of the objects and the non-modulated reference beams. The most essential for digital processing of the recorded interferograms are the techniques for retrieval of the phase information and filtering in the frequency domain. The most widely used algorithms are based on the fast Fourier transform (FFT) and the phase-stepping methods. In our case, five steps algorithm is used, which provides better accuracy and resolution in comparison with the FFT methods. Holographic recording is performed by CW generating diode laser, emitting in the red spectral region - linearly polarized single mode ML520G5 – 110mW, 638nm. Magnification of the optical system is x200, obtained by the microscope objective (panchromatic x100, NA 0.86), coupled with projective and TV lens, 25mm, 1:1.4 for projection of the images onto CCD target (1/2 interline progressive scan target 780x582 pxls, sized 8.3x8.3μm with scanning zone 6.47x4.83 mm, product of the firm Baumer Optronics).

2.5. Preparation of Fixed Slides for Scanning Electron Microscopy (SEM) Assay

For confirmation of changes in the cells, indicated by 3D-imagination, laser irradiation and combination of both methods, but also on the reliability of these techniques, scanning electronic microscopy (SEM) analysis of the same probes were necessary. For this goal, after turning-off of the cultural fluids, the cells were washed twice with PBS and 2.5% glutaraldehyde. The so prepared probes were observed by microscope Philips 515.

2.6. Preparation of Fixed Slides for Transmission Electron Microscopy (TEM) Assay

After turning-off the cultural fluids, the cells were washed twice with PBS and fixed in 10% Fomaline. The so prepared probes were put on netts on drops from the obtained liquid suspensions. For ultrastructural assay, TEM – JEOL JEM2100 microscope, with maximal tension of 200 kV and magnification 200÷1 500 000x was used.

3. RESULTS AND DISCUSSION

In co-cultivation with malignant cell antibodies (Figure 1 - B), normal embryonic cells increase in size, acquire rounded form, but also centrally-located dark-stained nuclei and changed nuclei/cytoplasm ratio could be observed, in comparison with the control 3T3 cultures (Figure 1 - A). The data obtained were in agreement with the cited literature sources, concerning similar cultivation conditions of immature normal cells [24]. In this way, the noticed morphological changes in the normal cells could be accepted as primary signs of early myeloid differentiation, probably in particular of embryonic stem cell sub-populations from the entire 3T3 cell line, which confirms the literature findings in this aspect [25]. These results confirmed many literature findings, connected with a better understanding of underlying important intra-cellular processes inter-molecular interactions, based on differences in the electron density of macro-molecules with different chemical structures and physical properties [26].

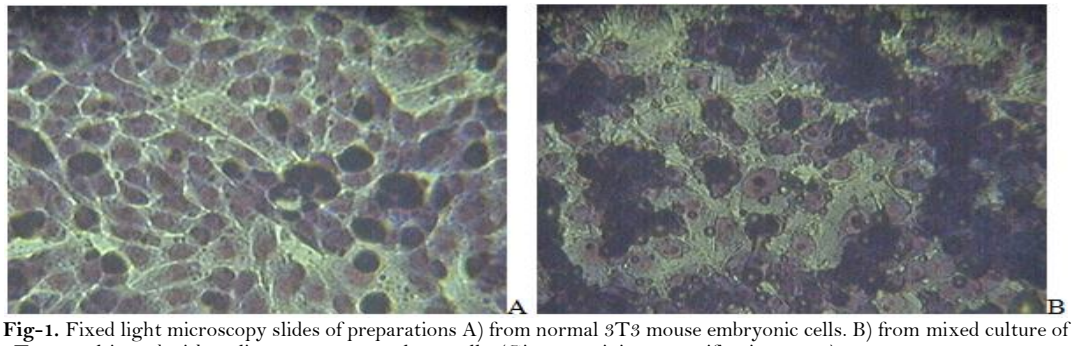

Fig-1. Fixed light microscopy slides of preparations A) from normal 3T3 mouse embryonic cells. B) from mixed culture of 3T3, co-cultivated with malignant mouse myeloma cells. (Giemsa staining, magnification: x100)

For better understanding the mechanisms of cell growth, proliferation and differentiation/transformation on the influence of respective factors, but also of the control mechanisms of these processes, investigations are necessary. These investigations could give

more information about the intracellular changes in the cell, in particular of the separated cell structures and hence, about the processes in the cells. For this goal, observation of the tested cell probes as 3D-imaginations, as well as after their subjection on laser irradiation, but also by a combination of both techniques, was used in the current study. In observation of cells as 3D-imaginations, differences due to the different density in different intracellular parts could be seen (Figure 2). These features were established both in cultures from normal mouse embryonic fibroblasts (Figure 2 – A, B), as well as in the mixed cultures (Figure 2 – B, D).

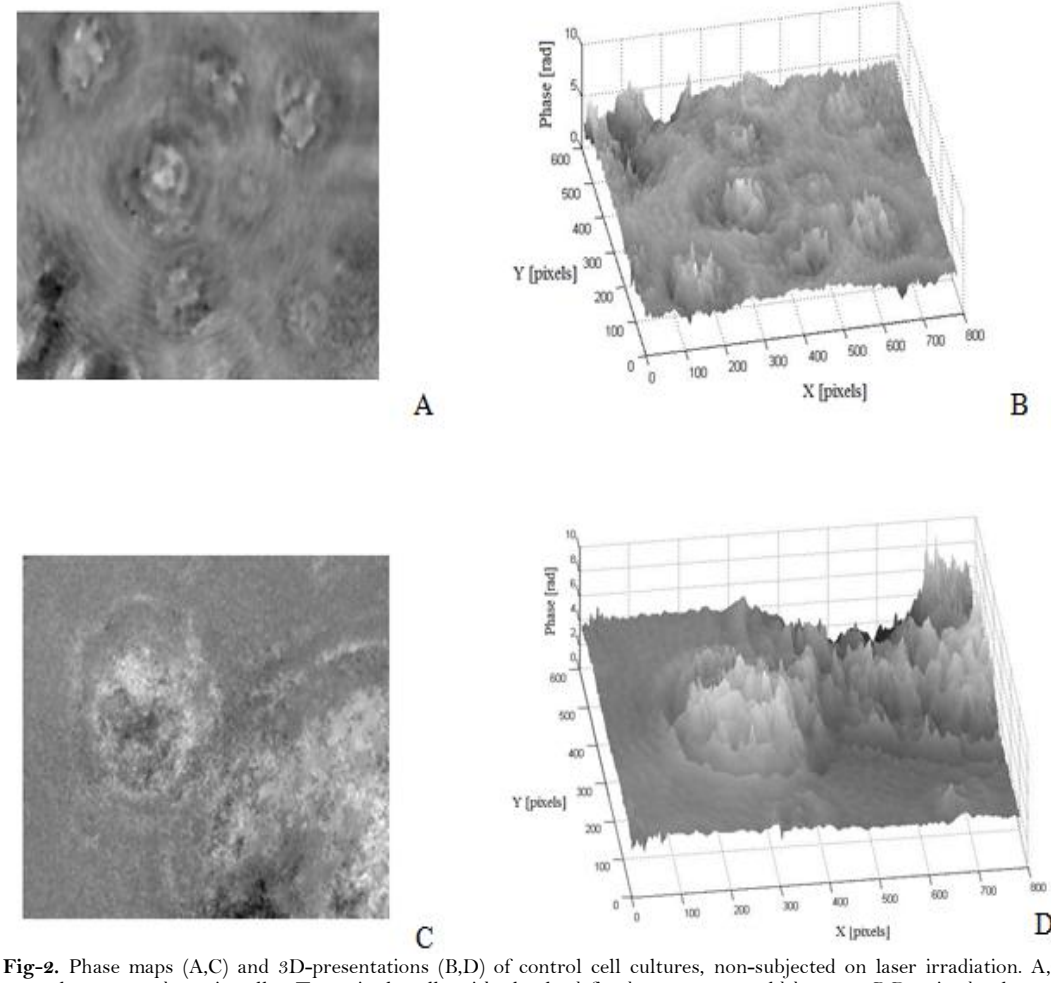

Fig-2. Phase maps (A,C) and 3D-presentations (B,D) of control cell cultures, non-subjected on laser irradiation. A,C: normal mouse embryonic cells 3T3 – single cells with clearly defined structures could be seen; B,D: mixed culture of normal mouse embryonic 3T3 fibroblasts, co-cultivated with malignant mouse myeloma cells – cell-cell interactions could be seen, in which the components of the interacting structures could be clearly observed (magnification x200)

In subjection of the cells on laser irradiation, changes in large parts of the irradiated cells, but also debris of cells, destroyed on the influence of laser light treatment, could be seen, both in the preparations from normal mouse embryonic fibroblasts (Figure 3 - B) and from the mixed cultures (Figure 3 - D), in comparison with the respective control non-irradiated cultures (Figure

3 - A, Figure 3 - C). Variations from slight density decrease in the affected structures till their full ablation could be indicated.

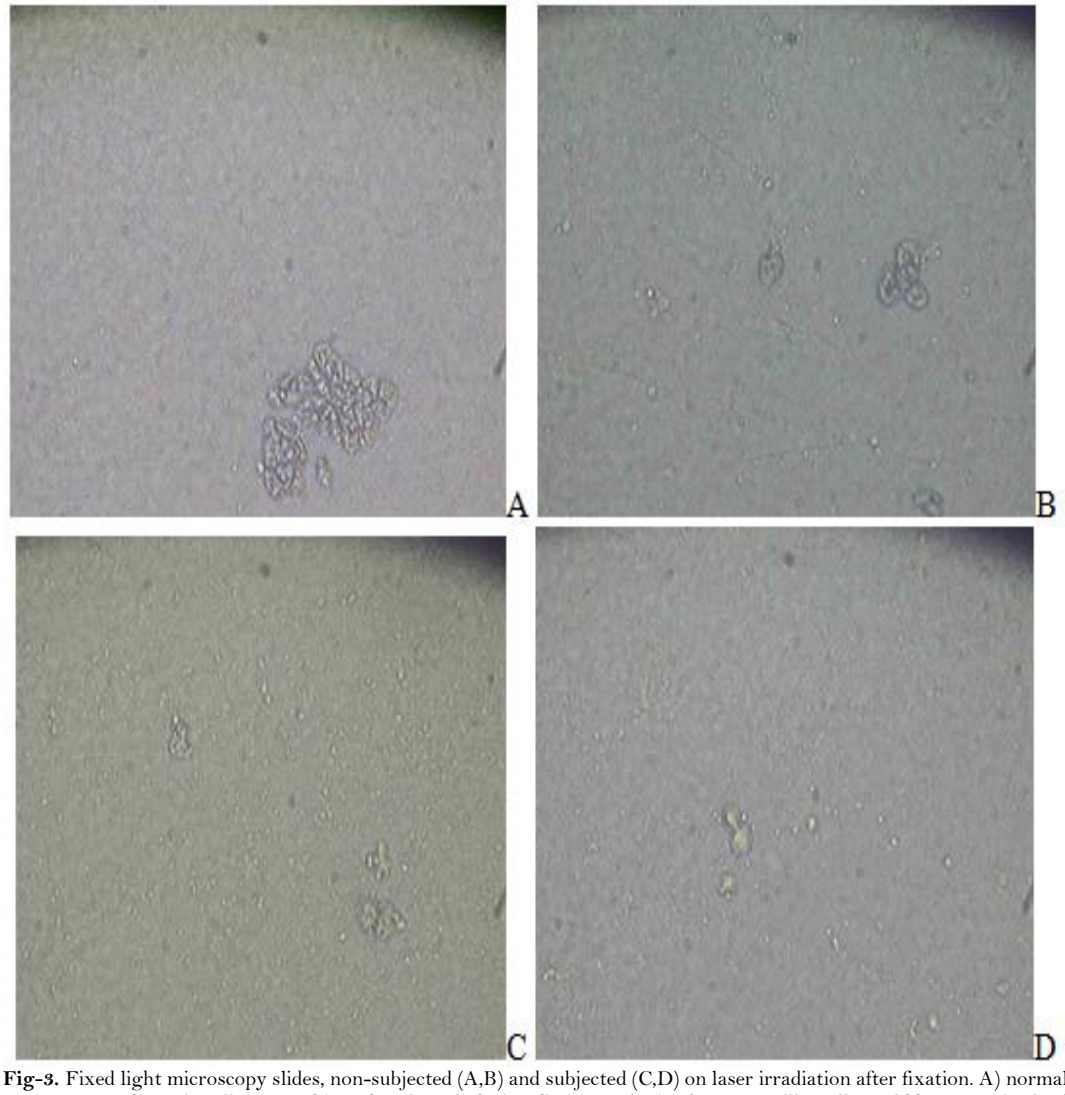

Fig-3. Fixed light microscopy slides, non-subjected (A,B) and subjected (C,D) on laser irradiation after fixation. A) normal 3T3 mouse embryonic cells, non-subjected on laser light irradiation. Colonies from stem-like cells could be seen. B) mixed culture of 3T3 and mouse malignant myeloma cells. Different interactions between separated partially-differentiated cells in different sub-phases of early myeloid lineage could be noted. C) normal 3T3 mouse embryonic cells, non-subjected on laser light irradiation. (a, c) and– (b, d) (magnification: x200)

The noted changes could be strongly confirmed by the observed differences between subjected and non-subjected on laser irradiation different types of normal cells on smears from peripheral blood of human and mouse, respectively (Figure 4). Interesting is also the noted equivalence in the changes of the pictures from human (Figure 4 – A, B) and mouse (Figure 4 – C, D) origin. In all cases, strong deformation and swelled form of the irradiated cells was observed. In both types of irradiated cultures, pale regions of destructed membrane, cytoplasm and nucleus components were noted, probably according to the exact hit of the laser ray in the process of

irradiation. These changes, on the other hand, were different, depending of the cell content differences according to both cell type and differentiation/maturation stage. In this way, the results obtained also suggested some of the intra-molecular mechanisms of laser irradiation in therapeutic procedures. On the untreated fixed smears of both mammalian species, different cell types could be noted, the nuclei could be seen, possessing respective shape and features depending of the respective cell type (Figure 4 – A, B). After treatment of the fixed blood smears from both species with laser light, only spots/shadows of cells are seen, no cell structures and features could be noted (Figure 4 – B, D), as a result of destroyed biological structures in the cell. Strongly affected were the round or segmented nuclei and cytoplasmic granules of cells from the myeloid lineage (Figure 4 – B, D), as well as those, which were partially differentiated in that direction (Figure 3 – B), unlike on the same components on the non-irradiate cell probes, where the same structures were noted as intact, differed those cells from the other cell types in the preparation (Figure 1 – B; Figure 4 – A, C), as well as from these in the culture of mouse embryonic cells (Figure 1 - A). In all cases, injures of biological structures as mitotic spindle (Figure 5 – B, C, F, G), as well as inter-cellular contacts (Figure 5 – D, H), could be seen. With those differences could be explained the deformed shape of the cells, subjected influence of laser irradiation (Figure 3; Figure 4 – B, D).

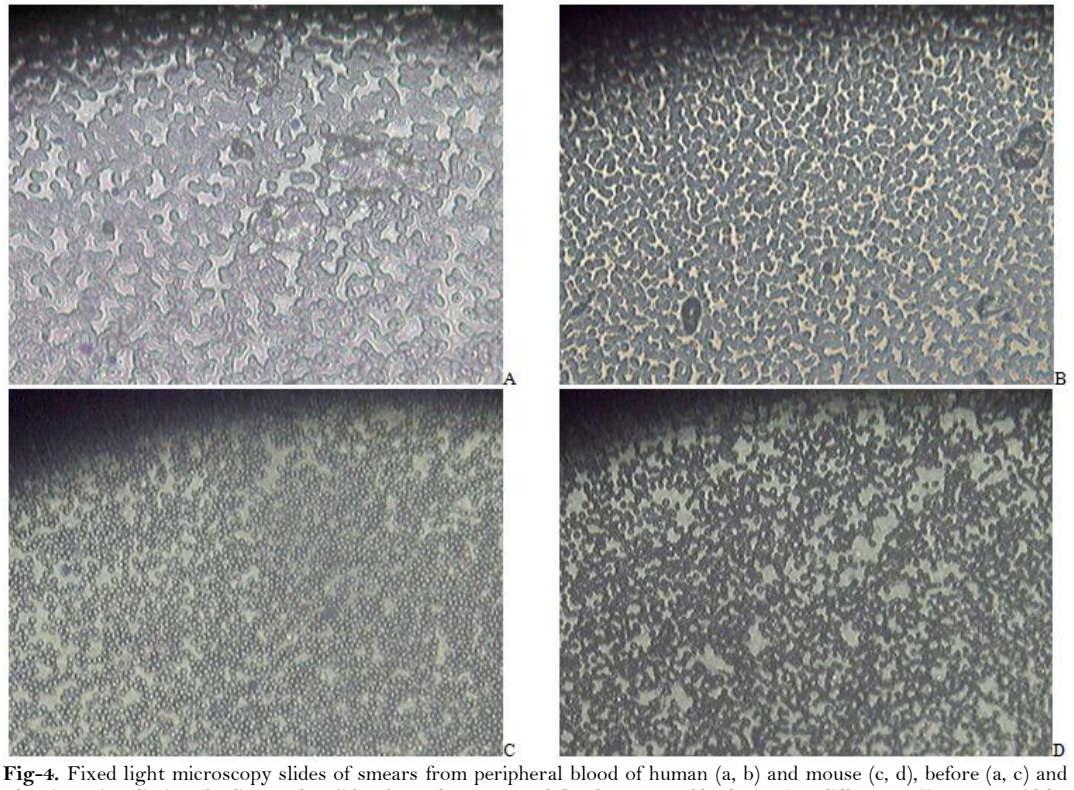

Fig-4. Fixed light microscopy slides of smears from peripheral blood of human (a, b) and mouse (c, d), before (a, c) and after laser irradiation (b, d). On the slides from the untreated fixed smears of both species, different cell types could be noted, the nuclei could be seen, which possess respective biological characteristics according to the cell type (a, b). After treatment of the same fixed smears with laser light, only shadows of destroyed cells are seen, no cell structures and features could be noted (b, d) (Hematoxyllin/Eosin staining, magnification: x100)

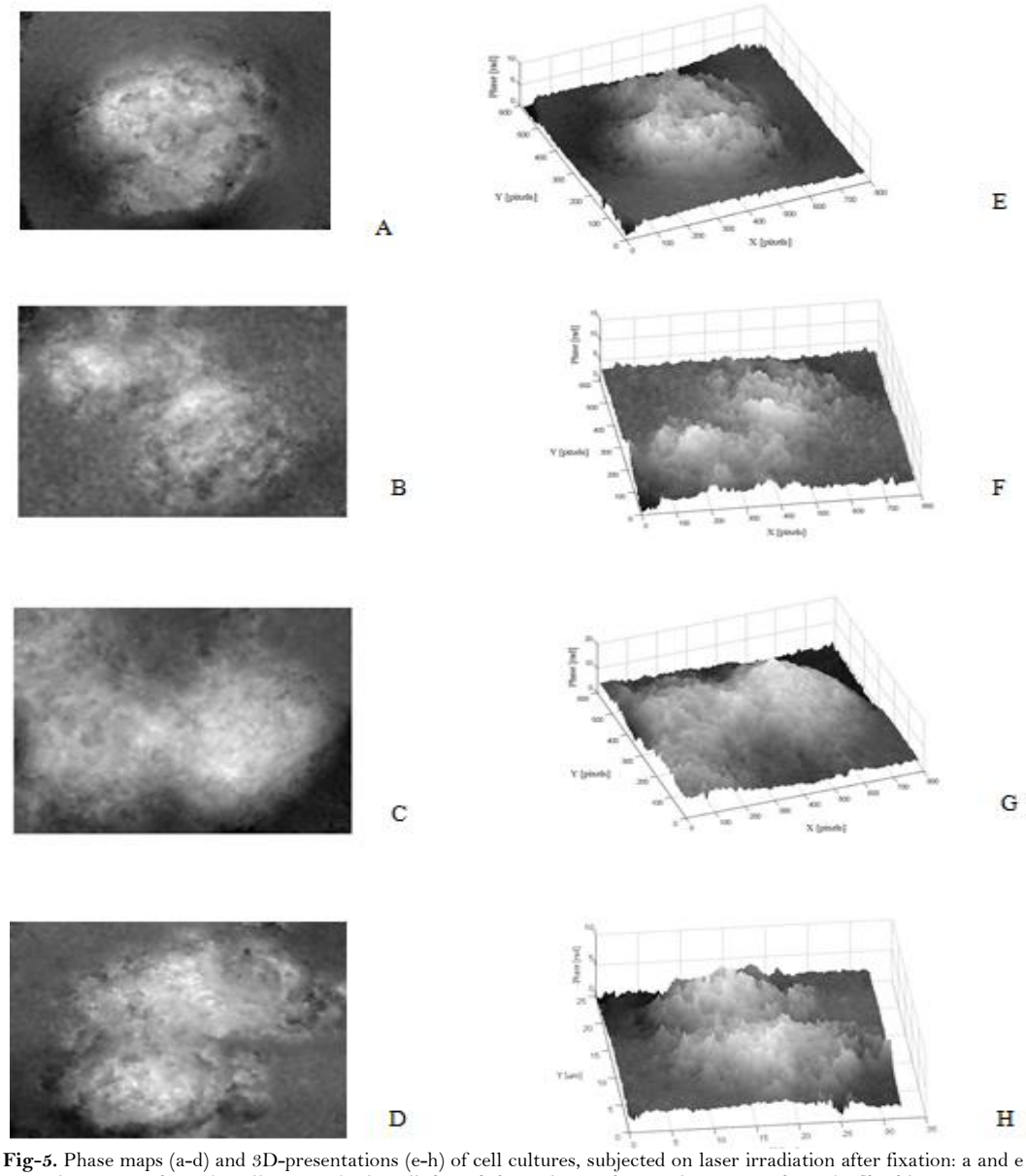

Fig-5. Phase maps (a-d) and 3D-presentations (e-h) of cell cultures, subjected on laser irradiation after fixation: a and e - normal mouse embryonic cells 3T3, single cell; b and f – culture of normal mouse embryonic fibroblasts 3T3, two daughter cells after division; c and g - mixed cultures of normal mouse embryonic 3T3 fibroblasts, co-cultivated with malignant mouse myeloma cells, dividing cell; d and h - mixed cultures of normal mouse embryonic 3T3 fibroblasts - cell division (d) and cell-cell interaction (h) (magnification x200)

Unlike 2D photos, 3D-imaging has also been proved to indicate phase differences between the separated probes [23]. This provides information about of the optical path differences, as a result of different optical density (e. g. refractive indexes differences) in separated regions of the cell. In this way, initial information about the nature of bio-macromolecules, participation in the composition of complex structures, important about basic cell functions and processes, could be received. The observed contacts between the cells, as well as the similarities in the structures with those, shown on Figure 2 and Figure 5, strongly supported the suggestion about the role of bio-molecules, building these structures, in important biological processes both in the individual cell,

but also between the cells [27]. Also, the data from the current study confirmed our previous results, received by application of label-free spectrophotometry and mass spectrometry assay [28]. On the other hand, the results obtained are in agreement with literature findings, obtained by application of other methods for identification of biological macromolecules, as well as of interactions between them [29]. These data confirmed the proved analogy of mouse cell with human cell as a convenient experimental model [21, 30]. By taking in consideration the proved possibility of different types intra-molecular changes on the influence of laser light, these data supported the usefulness of the applied method for precise establishment of novel interactions between the biological macro-molecular [14, 27]. On the other hand, these results could give initial information about the therapeutic effect of the laser light in cases of laser ablation treatment [15]. The results from the current study, but also the reliability of the applied and tested methods were confirmed from the data of routine techniques as scanning electron microscopy (SEM) and transmission electron microscopy (TEM) analyses. In this way, the observed changes, becoming on the influence of laser irradiation, once more confirmed the proposed participation of different cell structures in specific biological processes, but also the importance of building bio-molecules for the respective needs. The data, obtained from SEM and TEM assays of the same probes, where extra-cellular cell surface protrusions and contacts between the cells could be noted (Figure 6, Figure 7), both in the colonies of embryonic cells (Figure 6 – A), as well as in the mixed culture (Figure 6 – B), but also their ultra-structural characteristics (Figure 7 – A, B), supported the noted differences between the cells from one or different types, in different phases of proliferation and maturation/differentiation, both non-subjected (Figure 2) and subjected (Figure 5) on laser irradiation, as well as the reliability of the methods applied. These suggestions were additionally supported by the data, obtained in application of 3D imaging of fixed preparations after their subjection on laser irradiation. On the other hand, these results proposed some of the intra-molecular mechanisms of laser irradiation in therapeutic procedures.

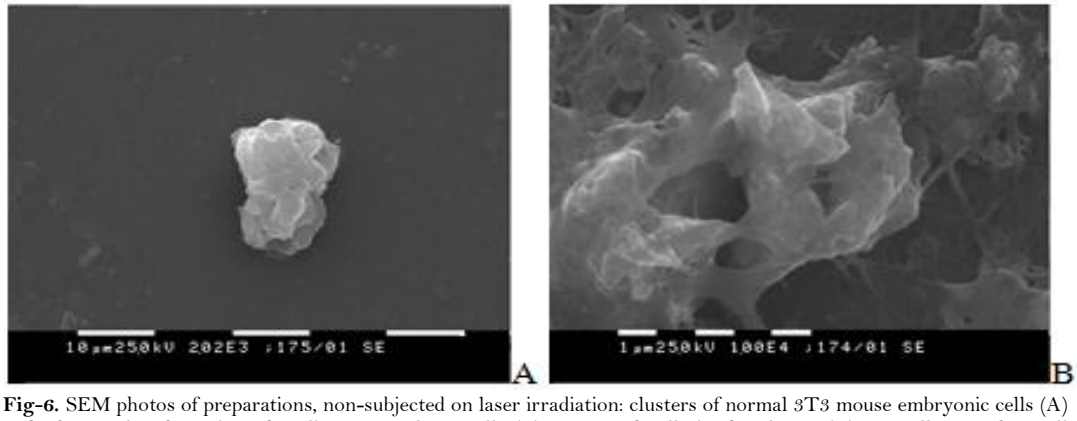

Fig-6. SEM photos of preparations, non-subjected on laser irradiation: clusters of normal 3T3 mouse embryonic cells (A) and of normal embryonic and malignant myeloma cells (B). Separated cells in the cluster (A), as well as surface cell protrusions and inter-cellular contacts (B), respectively, could be noted

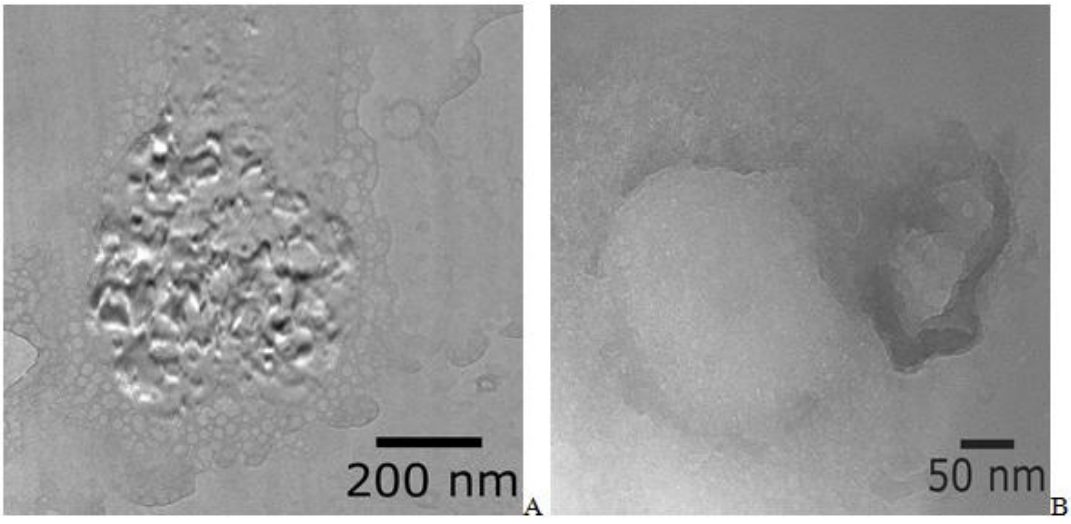

Fig-7. TEM photos of preparations, non-subjected on laser irradiation: cell, belonging to culture from normal 3T3 mouse embryonic fibroblast line, possessing stem/progenitor features (A); cell, possessing ultra-structure characteristics of early myeloid differentiation, belonging to mixed culture of normal embryonic and malignant myeloma cells, both with mouse origin (B) (magnification: x40000 and x100000)

The data obtained were in agreement with the cited literature sources, concerning similar cultivation conditions of immature normal cells [24]. In this way, the noticed morphological changes in the normal cells could be accepted as primary signs of early myeloid differentiation, probably in particular of embryonic stem cell sub-populations from the entire 3T3 cell line, which confirms the literature findings in this aspect [25]. These results confirmed many literature findings, connected with a better understanding of underlying important intra-cellular processes inter-molecular interactions, based on differences in the electron density of macro-molecules with different chemical structure and physical properties [26]. 3D-imaging of both normal mouse embryonic fibroblasts and cells in the mixed cultures clearly indicated differences in the density of different intracellular parts. This could help for a better understanding of intra-cellular processes inter-molecular interactions, based on differences in the electron density of macro-molecules with different chemical structure and physical characteristics. This explanation could be confirmed by the changes in large parts of the irradiated cells, observed on the influence of laser irradiation on both types of cultures, as well as on smears from blood of mouse and human. The influence of the laser light was clarified by 3D-imaging of cells, subjected on laser irradiation, where injures of biological structures as mitotic spindle and inter-cellular contacts could be noted. The data observed were additionally supported by results from widely used routine techniques as SEM and TEM assays.

3. CONCLUSION

Additional investigations on different types of macromolecules and inter-molecular interactions in the living cell during different phases in its life, proliferation and differentiation are necessary. Furthermore, concrete, specific protein and peptide molecules, which participate in the

structure of cell organelles, on the one hand, as well as in different steps from cascade regulatory pathways, should be characterized and identified. In this way, the results presented suggested some of the intra-molecular mechanisms of laser irradiation in therapeutic procedures, for which detailed investigation is also one of the subjects of future studies.

4. ACKNOWLEDGEMENTS

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