JCBPS; Section B; May 2017 – July 2017, Vol. 7, No. 3; 701 -709 E- ISSN: 2249 –1929

[DOI: https:/doi.org/10.24214/jcbps.B.7.3.70109]

Journal of Chemical, Biological and Physical Sciences

An International Peer Review E-3 Journal of Sciences

*Available online at*www.jcbsc.org

Section B: Biological Sciences

CODEN (USA): JCBPAT Research Article

Development of Techniques for Identification of Protein Composition in Mammal Sperm Fluid by Comparison with Other Biological Probes from Mammals, by Combining Different Assay Methods. A Pilot Study

***Iskra V Sainova[1], Ilina Valkova[1], Tzveta Markova, Elena Nikolova, Angel Baldzhiev, Angel Alishev, Marin Nenchev**

[1]Institute of Experimental Pathology and Pathology to Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria;

[2]Medical University, Department of Pharmacology and Toxicology, BG – 1000 Sofia, Bulgaria;

[3]Institute of Optical Materials and Technologies "Acad. J. Malinowski", Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria;

[4]Technical University, Sofia, Bulgaria

Received: 10 April 2017**; Revised:** 27 April 2017; **Accepted:** 01 May 2017

Abstract: Protein composition (types of proteins and eventual protein-protein interactions) in mammal sperm fluid as a biological material, for which is proposed to be supplemented with different molecules, responsible for the control in the life processes of the male germ cells, was isolated and investigated. The protein content was subsequently compared with that of other biological materials, containing previously low-differentiated normal adult stem/progenitor cell types, as well as with protein extracts from anatomic organs from adult mammals, containing normal cell types in different stages of maturation and differentiation. Combinations of various methods for assay were developed and tested. The strongest similarities in the mammal sperm fluid protein

composition with this from biological probe, rich of different normal stem/progenitor cell types, were established. The developed methods for assay, in support of many literature findings, are usable about more precise identification of the composition of the biological structures, but also for identification proteins and protein-protein interactions. As one of the key molecules in these processes was characterized the tri-peptide Glutathione, in particular it's reduced from (GSH). As a proof about that was accepted the established increased levels of this molecule in the process of myeloid cell differentiation. The label-free LC-MS/MS assay was characterized as a usable technique, possessing many advantages in comparison with other proteomics technologies. The combined technique of laser irradiation of biological objects with their 3D-visualization gives a possibility about more precise identification of the changes, occurring in different phases of living processes, on both cellular and organism levels. Future investigations and development of other methods for identification of concrete biological molecules, as well as direct and/or indirect interactions between them, should be performed.

Keywords: biological probes, sperm fluid, protein composition, protein-protein interactions.

INTRODUCTION

Highly complex communication networks, underlining the cellular activities during the spermatogenesis process, have been proved.[1] The results obtained confirmed the usefulness of proteomics assay methods for determination of complete protein profile and protein–protein interactions map in the processes of testicular development and spermatogenesis.[2] Sperm transcriptome and proteome analyses have been similarly used to systematically study of spermatogenesis in mammals,[3,4] but also in some lower chordate species as *Salmon*,[5] as well as in non-chordate species as *Drosophila*,[6] *Caenorhabditis elegans*.[7] HANP1/H1T2 has been characterized as a novel protein, essential for the packaging of protamines and DNA into chromatin in sperm and for the formation of the compact sperm nucleus, which has also been found to be required for natural fertilization.[8] Also, the received data are in support of messages about the role of protein-protein interactions for the structure and functional characteristics of the different cell types.[9] Mutations or disruptions of the *Hanp1/H1T2* gene, coding this protein, have been proposed to underlie some cases of human infertility. Significant changes in chromatin structure, contained mainly with its decondensation, have been proved in the process of fertilization.[10] In this way, possibilities for investigation on eventual properties of the protein molecules to participate in direct and/or indirect intra- and extracellular interactions by cascade regulatory mechanisms, but also with other biological molecules, have been proved.[11,12] The noted similarities in the protein composition of the mammal sperm fluid with that of normal mammalian cells in various phases of differentiation and maturation, is in agreement with the literature data about the importance of the biological molecules and interactions with their participation, about the normal cell differentiation, with prevention of malignant changes, at the same time.[13] Furthermore, in agreement with literature findings, the received data confirmed the role of protein molecules, but also of different interactions between them and with other bio-molecules, by participation in cascade mechanisms, underlining processes as cell growth, proliferation and differentiation.[14-16] These intra- and extra-cellular intra-molecular interactions underline the changes in the process of cell maturation, which is important for determination of the respective cell differentiation direction. Also, the

results obtained confirmed the proved advantages of label-free mass spectrometry in comparison with other proteomics methods.[17]

In this aspect, the main goal of the current pilot study was directed to primary investigation on the protein content (types of proteins and eventual protein-protein interactions) in mammal sperm fluid as a biological material, for which is proposed to be supplemented with different molecules from the living action of the male germ cells, and comparison with the same characteristics in other biological probes, containing different cell types in various stages of maturation and differentiation. Methods, combining several different techniques for protein content assay, were developed and tested.

EXPERIMENTAL DESIGN

Preparation of protein lysates for proof of analogies in the protein composition from different biological probes and determination of key molecules, underlining the cell differentiation direction: Proteins from mammal sperm fluid were assayed, and the data, connected with eventual presence of common or different proteins, were compared with those in tissue probes, containing different normal cell types in early stages of maturation and differentiation, and from adult mammalian brain and pancreas - two anatomic organs, known to be composed of normal cells in different stages of maturation. Also, these organs are known to be rich of proteins, possessing affinity to GSH, which is necessary for the normal cell differentiation[18]. For this goal, both anatomic organs were treated with lysis buffer and put for 2 hours at 4^0C. After centrifugation, the supernatants were turned off, the pellets were resuspended in PBS, and the received suspensions were subjected on separation on GST-Agarose columns for 2 hours with intensive shaking, for receive only of proteins, possessing high affinity to peptide GSH from both probes. All probes were subjected on protein precipitation in cold 100% ethanol overnight, after which the eventually formed precipitates were washed with cold 80% ethanol. After centrifugation, the supernatants were turned-off, and the pellets, eventually containing the proteins from each respective probe, were diluted in SDS- or LDS-buffer. The so prepared probes were subjected on electrophorhesis on Polyacrylamide Gel in SDS-buffer (SDS-PAGE) and consequent Coomasie-blue staining. Subsequently, the gel was prepared for label-free LC-MS/MS assay. For this goal, the gel was washed and sliced with an in-house tool, after which the gel slices were washed with water and acetonitrile, followed by reduction and alkylation of cysteine (Cys) residues by DTT and iodoacetamide. Following overnight trypsin digestion, peptides were extracted by acetonitrile and 5% formic acid, and subsequently concentrated in a speed-vacuum centrifuge. The results from assay the protein composition in mammal sperm fluid was compared with that from mammalian normal cells in different stages of differentiation, on the one hand, as well as with that from malignant mammalian cells, on the other, used as basic biological probes to prove the effectiveness of label-free tandem mass spectrometry, combined with liquid chromatography (LC-MS/MS) assay, for identification of novel proteins and protein-protein interactions, as well as of indirect influences between separate bio-molecules.

Development of novel techniques for protein composition assay laser irradiation by combination of laser irradiation and 3D-imaging: Mouse embryonic fibroblasts from line 3T3 are incubated in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and antibiotic mixture (100 IU/ml Penicilin, 0.25 mg/ml Streptomycin and 0.25 mg/ml Amphothericin), until formation of confluent monolayers, at 37^0C in incubator with 5% CO_2 and 95% humidity. Malignant mouse myeloma cells were also isolated, similarly incubated, and analogically

proceeded. Separated sub-populations from the normal 3T3 mouse cells were pre-cultivated in cultural fluids, obtained by supplementation of the growth medium described with malignant antigens by previous incubation of mouse malignant myeloma cells in it, subsequent centrifugation and filtration. After turning-off of the cultural fluids from the cell cultures and subsequent washing with PBS (Sigma), they were fixed by ethanol and consequently stained by specific anti-mouse GSH antibody (Sigma) plus Giemsa-dye (Sigma) and/or plus Haematoxilin/Eosin (Sigma). Novel techniques about optical recording of the used biological objects and their 3D-presentations by digital holographic microscopy (DHM), combined with laser irradiation, were developed and tested. Unlike the optical microscopy, in DHM not only information for differences in the volume of the tested objects due to absorption, but also in the values of phase of the light, passing through the observed objects, both based on the varying electron density, could be received. These properties, as well as the non- invasive nature of the method, make it applicable to many fields of the science. More attention has been attracted by its applications in the biomedical field, where the quantitative phase detection can be used for imaging and morphological measurements of the cells and sub-cellular components.[19] 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 for cells, but also in red He-Ne- (632nm) and blue/green Ar- laser light (from 488nm to 514nm) for sub-cellular structures/chromosomes, as well as laser light irradiation with the next parameters described: Q-switched Nd^{3+} YAG laser at 1.06 μm; 950 KW/cm^2 - power density.

RESULTS AND DISCUSSION

The strongest similarities in both quality and quantity aspects in the protein composition in mammal sperm fluid, eventually supplemented of metabolites from the living activity of the male germ cells, with that from biological probe with mammalian origin, rich of normal adult stem/progenitor cells, was established (**Figure 1 – lines 2; 3**). The noted nearer protein composition of the mammal sperm fluid with that of normal stem/progenitor mammalian cells was in agreement with the literature findings about the importance of the male germ cells for the stem characters of the embryonic cells, on the one hand, as well as for the prevention of malignant changes in them, on the other.[13,20] In this way, highly complex communication networks, underlining the cellular activities during the spermatogenesis process, were proved.[1]

The protein content of the mammalian sperm fluid also showed a lot of quality and quantity similarities of the protein composition with that of normal cells in different phases of maturation, building the other anatomic tissues and organs from mammals (**Figure 1 – lines 2; 4; 5**). Messages about identification of different types of protein–protein interaction networks in conjunction with expression profiles and cellular co-localization patterns have been made[20]. The importance of different histone variants, as well as of molecules, influencing the functions of histones and genes, coding them, in the regulation of this process, has been indicated. Many histones have been proposed as a key regulators, involved in growth, apoptosis and/or other types of cell death of spermatogenic cells in some disorders, and, in this way, this molecule has been characterized as a key regulator of the spermatogenesis process, both *in vitro* and *in vivo*, as for

example histone HnRNPL.[21] Also, the role of H3.3 in spermatogenesis by regulation of chromatin dynamics has been proved.[22,23] On the other hand, protamines have been found to be responsible for the spermatozoa quality.[24]

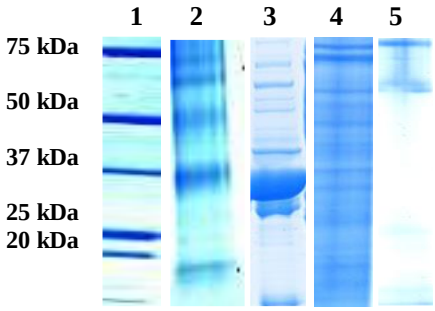

Figure 1: SDS-PAGE of protein extracts: line 1 - marker; line 2 – protein extract from mammal sperm fluid; line 3 – protein extract from composed of normal mammalian stem/progenitor cells in early phases of differentiation and maturation of adult tissue; line 4 – proteins from mammalian brain as anatomic organ, eventually containing normal cells in different phases of maturation and differentiation; line 5 - proteins from mammalian pancreas, as anatomic organ, eventually containing normal cells in different phases of maturation and differentiation (Coomasie-blue staining)

The epigenetic role of the histone-to-protamine transition process has also been proved.[25] The necessity of transcriptomics and proteomics analyses for determination and identification of specific proteins and/or protein-protein interactions during spermatogenesis has been established.[26,27] As a novel method for identification of proteins and protein-protein, in the last years many authors have developed and applied label- free mass-spectrometry technique.[28,29] In this way, possibilities about reveal of direct interactions between the proteins, as well as indirect influences between them in cascade regulatory pathways have been proved.[14-16] In this connection, the characteristic morphogenetic changes in the process of spermatogenesis have been found to be accompanied by profound chemical alterations of the nuclear material, through direct protein interaction and/or post-translational modifications.[30]

In co-cultivation of 3T3 normal mouse embryonic cells with mouse malignant myeloma cells, appearance of myeloid- like characteristics as enlarged cell size, round shape, enlarged round nucleus and increased cytoplasmic content were seen (**Figure 2 - b**), in comparison with the control 3T3 cells (**Figure 2 - a**). Glutathione, in particular its reduced form (GSH) could be noticed as bright cytoplasm regions due to a reaction to anti-mouse GSH-antibody, which was the strongest in differentiated myeloid-like cells (**Figure 2 - b**). These increased amounts in the process of myeloid cell differentiation could be established as a proof about the role of GSH as a key molecule in the cascade regulatory pathways, underlining the life processes.

The observed differences also proved the importance of cytoskeleton components, as well as the inter-molecular interactions with their participation, underlining the respective cell differentiation direction. Glutathione is a thiol-containing tri-peptide (L-γglutamil-L-cysteinyl-glycine), which is ubiquitous in the

cells. The activity of hydrosulfide group determines the biological significance and its activity in antioxidant and detoxifying reactions.[31]

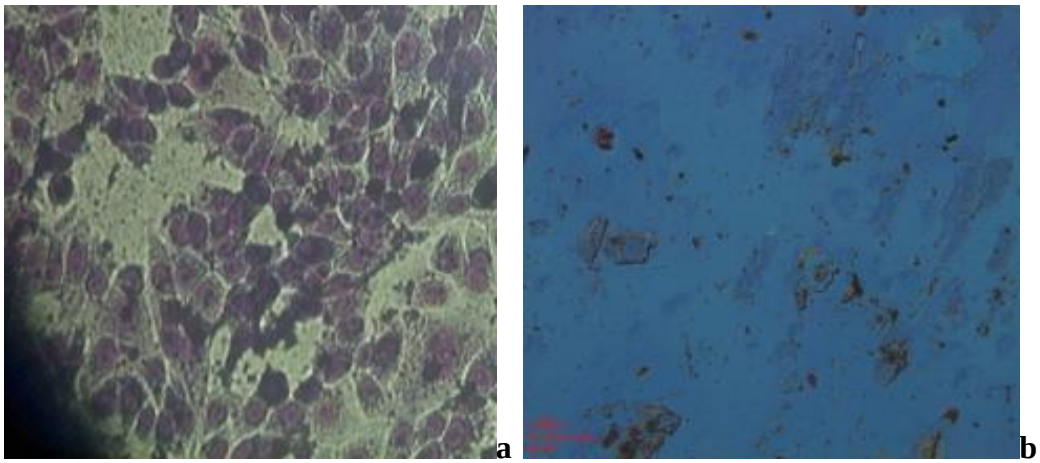

Figure 2: *In vitro*-cultures: normal mouse embryonic 3T3 fibroblasts (control) (double staining by anti-mouse GSH antibody and Giemsa dye, magnification: x100) (a); mixed cell culture of 3T3 normal embryonic fibroblasts and malignant mouse myeloma cells (b) (double staining by anti-mouse GSH antibody and Hematoxilin/Eosin, Magnification: x100)

This substance is responsible for keeping proper thiol-disulfide balance and related redox-potential in the cells. Also, the role of this molecule in the immunomodulatory mechanisms on both cellular and organism levels has been established[32, 33] .The availability of GSH is crucial for antioxidant defense in a biological system. GSH deficit disrupts the redox-status and upsets the physiological balance between pro-oxidants and antioxidants on cellular and organism levels, and lowered cellular GSH is observed in different pathological conditions as pre-malignancies and malignancies, inflammations, Parkinson's disease, AIDS, diabetes and others.[34, 35]. For this goal, combined methods for investigation and understanding the interactions and influence of bio-molecules in the composition of complex inter-molecular structures, were developed and tested.

The 3D-images (**Figure 3 – b, d**) and the respective phase maps (**Figure 3 – a, c**) could give more information about the intra-cellular changes in the cell, in particular of the separated cell structures, and hence, about the processes in the cells. Also, the laser irradiation with a low dose gives a possibility for a better contrast between separated cellular structures (nucleus, cytoplasm and cell membrane). In this way, visualization of features, which could not be noted in the non-irradiated 2D-images, and, hence, a better investigation and understanding the mechanisms of cell growth, proliferation and differentiation/transformation on the influence of respective factors, is possible. The different density in different intra-cellular parts has been proved as the main reasons about the observed changes. So, unlike the 2D-images in white light are due to differences only in the volume of the tested objects due to absorption, the 3D-imaginations,[19,36,37] similarly to other combined methods for microscopy

observation,[38-43] have been proved to also indicate variations in their refractive index, which leads to changed phase of the light, passing through the observed object.

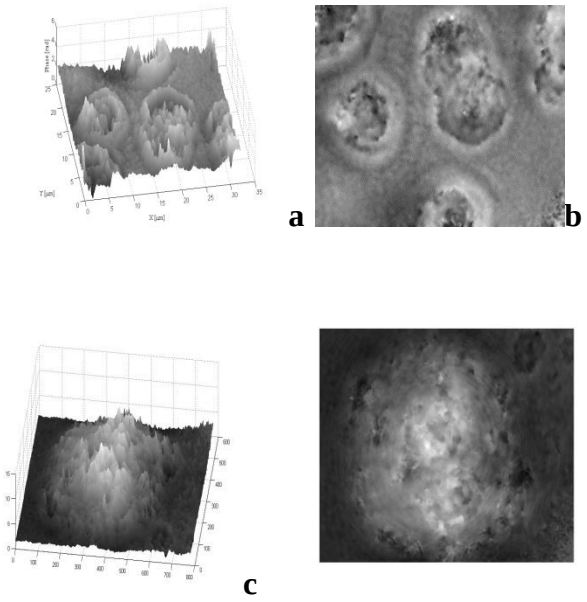

Figure 3: 3D-images (a, c) and phase maps (b, d) of *in vitro*-incubated cells: normal stem-like cells with rounded shape, included two daughter cells after cell division in the center, in culture of mouse embryonic 3T3 fibroblasts (a, b); large round myeloid-like cell with increased cytoplasmic content in mixed culture of normal mouse embryonic 3T3 fibroblasts, co-cultivated with malignant mouse myeloma cells (c, d) (magnification x200)

CONCLUSION

The results obtained revealed possibilities about identification of biological molecules and interactions with their participation, by cascade regulatory pathways, underlining basic processes, supporting the life on both cellular and organism levels. These data are in agreement with many literature findings about the role of inter-molecular interactions for the structure and functional characteristics of the respective cell types and the cell differentiation directions. The applied technologies, combining several methods for assay, as label-free LC-MS/MS assay and 3D-visualization technology, combined with laser irradiation, were developed and proved as usable for these pilot studies, which are initial steps of investigations, allowing the determination of presence and/or localization of respective molecules in the complex structures, by intra- and extra-cellular inter-molecular interactions. Future studies and development of other methods are necessary for identification of concrete bio-molecules and interactions with their participation.

ACKNOWLEDGEMENTS

The current investigations were supported by the European Foundation of Immunology Sciences (EFIS), BG05PO001/3.3-05-001 on scientific program "Development of human resources" to the Bulgarian Ministry of Education and Science, and contract DCOST 01/7-13.12.2012 with National Science Foundation.

REFERENCES

1. S. Chocu, P. Calvel, A.D. Rolland, C. Pineau, *Syst. Biol. Reprod. Med.*; 2012, 58, 179-190.
2. X.-Y. Huang, J.-H. Jia-Hao Sha, *Asian J. Androl.*; 2011, 13, 18-23.
3. R.D. Upadhyay, A.K. Yadav, S. Sonawane, et al., *J. Anal. Bioanal. Tech.*; 2013, 4, 1-9.
4. E.Y. Yu, Y. Zhang, E. Unni, et al., *Proc. Natl. Acad. Sci. U.S.A.*; 2000, 97, 4683-4688.
5. M. Alfert, *J. Biophys. Biochem. Cytol.*; 1956, 2, 109-120.
6. S.J. Raja, R. Renkawitz-Pohl, *Mol. Cell. Biol.*; 2005, 25, 6165-6177.
7. X. Ma, Y. Zhu, C. Li, et al., *BMC Genomics*; 2014, 15, 1-13.
8. H. Tanaka, N. Iguchi, A. Isotani, et al., *Mol. Cell. Biol.*; 2005, 25, 7107-7119.
9. J.Y. Wu, T.J. Ribar, A.R. Means, *Mol. Cell. Biol.*; 2001, 21, 6066-6070.
10. A.O. Zalensky, J.S. Siino, A.A. Gineitis, et al., *J. Biol. Chem.*; 2002, 277, 43474-43480.
11. R. Bortell, J. Moss, R.C., et al., *J. Immunol.*; 2001, 167, 2049-2059.
12. K. Oakata, S. Hisanaga, J.C. Bulinski, et al., *J. Cell. Biol.*; 1995, 128, 849-862.
13. M.D. Westfall, J.A. Pietenpol, *Carcinogenesis*; 2004, 25, 857–864.
14. H. Choi, D. Fermin, Al. Nesvizhskii, *Mol. Cell. Proteomics*; 2008, 7, 2373-2385.
15. T.V. Pham, S.R. Piersma, M. Warmoes, C.R. Jimenez, *Bioinformatics*; 2010, 26, 363-369.
16. B. Zhang, N.C. VerBerkmoes, M.A. Langston, et al., *J. Proteom. Res.*; 2006, 5, 2909-2918.
17. W.M. Old, K. Meyer-Arendt, L. Aveline-Wolf, et al., *Mol. Cell. Proteomics*; 2005, 4, 1487-1502.
18. T. Alfonso, F. Carlos, S. Patricia, et al., J. Biol. Chem.; 2001, 276, 47107-47115.
19. B. Kemper, P. Langehanenberg, G. Von Bally, *Opt. & Photon.*; 2007, 2, 41–44.
20. P. Calvel, A.D. Rolland, B. Je'gou, C. Pineau, *Phil. Trans R. Soc.*; 2010, 365, 1481-1500.
21. J. Li, W. Guo, F. Li, et al., *J. Proteomics*; 2012, 75, 2879-2891.
22. D. Miller, M. Brinkworth, D. Iles, *Soc. Reprod. Fertil.*; 2010, 139, 287-301.
23. B.T.K. Yuen, K.M. Bush, B.L. Barrilleaux, et al., *Development*; 2014, 141, 3483-3494.
24. M. Akmal, A. Aulanni'am, M.A. Widodo, et al., *Asian Pacif. J. Reprod.*; 2016, 5, 357-360.
25. J. Bao, M.T. Bedford, *Reproduction*; 2016, 151, R55-R70.
26. F. Chalmel, A.D. Rolland, *Soc. Reprod. Fertil.*; 2015, 150, R149-R157.
27. C. Rathke, W.M. Baarends, S. Awe, R. Renkawitz-Pohl, *Biochim. Biophys. Acta*; 2014, 1839, 155-168.

28. L. Dicker, X. Lin, A.R. Ivanov, *Mol. Cell. Proteomics*; 2010, 9, 2704-2718.
29. A.S. Haqqani, J.F. Kelly, D.B. Stanimirovic, *Meth. Molec. Biol.*; 2008, 439, 241-256.
30. I. Kasioulis, H.M. Syred, P. Tate, et al., *Molec. Biol. Cell*; 2014, 25, 1216-1233.
31. A. Meister, *Science*; 1983, 220, 472-477.
32. J. El Benna, J. Han, J.W. Park, et al., *Arch. Biochem. Biophys.*; 1986, 334, 395-400.
33. P.P. McDonnald, A. Bald, M.A. Cassatella, *Blood*; 1997, 89, 3421-3433.
34. J. Jahngen-Hodge, M.S. Obin, X. Gong, et al., *J. Biol. Chem.*; 1997, 272, 28218-28226.
35. A.L. Ortega, S. Mena, J.M. Estrela, *Cancers*; 2011, 3, 1285-1310.
36. M. Bail, G. Häusler, J.M. Herrmann, et al., *Opt. Lett.*; 1996, 21, 1087-1089.
37. Y. Hiraoka, J.R. Swedlow, M.R. Paddy, et al., *Semin. Cell. Biol.*; 1991, 2, 153-165.
38. Y. Ding, H. Xie, T. Peng, et al., *Optics Express*; 2012, 20, 14100-14108.
39. B.J. Kopek, G. Shtengel, C.S. Xu, et al., *Proc. Natl. Acad. Sci. U.S.A.*; 2012, 109, 6136-6141.
40. M. Petran, M. Hadavsky, *J. Opt. Soc. Am.*; 1968, 58, 661-664.
41. R.E. Rowland, E.M. Nickless, *Bioscience*; 2000, 26, 3-7.
42. L. Schermelleh, P.M. Carlton, S. Haase, et al., *Science*; 2008, 320, 1332-1336.
43. S.R. Swift, L. Trinkle-Mulcahy, *Proc. Royal Microsc. Soc.*; 2004, 39, 3-10.

Corresponding author: Iskra V Sainova;
Institute of Experimental Pathology and Pathology to Bulgarian Academy of Sciences,
1113 Sofia, Bulgaria**;**
On line publication Date: 1.05.2017