Opinion

on the dissertation of Alexander Sergeev Atemin, a full-time PhD student at the R. Tsanev Institute of Molecular Biology, entitled **"Studying the Dynamics of Processes in Living Cells Using Modern Microscopy Approaches"**, supervised by Assoc. Prof. Dr. Marina Nedelcheva-Veleva and scientific consultant Assoc. Prof. Dr. Stoyno Stoynov, submitted for the award of the educational and scientific degree "Doctor" in the scientific specialty "Molecular Biology" under scientific field 4.3. Biological Sciences.

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The presented dissertation reflects an in-depth study of dynamic processes in living cells conducted using modern microscopy approaches. The methodology serves as the unifying framework for addressing two separate research problems included in the dissertation:

1. The entry of model virus-like particles (VLPs) of SARS-CoV-2.
2. The dynamics of key replication proteins during the cell cycle.

The dissertation by Alexander Atemin spans 128 pages, including appendices and a bibliography. A total of 247 references, all in English and published in reputable journals in the fields of molecular biology and virology, are cited. The work contains 24 figures and one table.

The literature review in Alexander Atemin's dissertation provides a comprehensive and detailed overview of the current scientific knowledge about the SARS-CoV-2 virus, covering both its structure (with detailed descriptions of its proteins and viral genome) and its life cycle. The author thoroughly describes the process by which the virus enters cells via the S protein and its interaction with the ACE2 receptor. The subsequent step—fusion of the viral and cellular membranes—is also thoroughly discussed, highlighting the role of TMPRSS2 protease and other cellular enzymes that mediate proteolytic cleavage and activate the S protein. Therapeutic approaches to treating SARS-CoV-2 infection, as well as vaccines against the virus, are also reviewed.

The theoretical review provides a solid foundation for the research presented in the dissertation. The second part of the literature review is devoted to the process of DNA replication, providing a general description and a detailed analysis of the proteins investigated in the dissertation.

The dissertation outlines two independent objectives. Five specific tasks are defined to achieve the first objective, and one task to achieve the second. The tasks are precisely formulated and focused on attaining each objective.

The "Materials and Methods" chapter of Alexander Atemin's dissertation provides a detailed description of the experimental approaches and techniques used to study dynamic processes in living cells. The main focus is on applying software solutions for analysis, which are critical to successfully achieving the study's objectives.

The primary tool in this research is live-cell microscopy. The author uses high-resolution confocal microscopy performed with the Andor Dragonfly spinning disk confocal system. This high-tech system enables the tracking of labeled molecules in living cells over extended periods, which is crucial for understanding the dynamics of the studied processes.

To study the dynamics of SARS-CoV-2 virus-like particles (VLPs) entering host cells, the author uses specially produced VLPs containing the structural proteins of the virus and lacking essential genetic information to prevent replication. Once the particles bind to the cell membrane, they are tracked at the single-particle level using the SPARTACUSS software package. This enables detailed tracking of the speed, position in 3D space, and fluorescence intensity of the labeled proteins.

To study the dynamics of replication fork proteins, the author creates dual-labeled cell lines in which key proteins such as ORC1, MCM6, CLASPIN, RIF1, and PCNA are fluorescently tagged. This allows detailed monitoring of these proteins' dynamics during different stages of the cell cycle, providing valuable insights into replication processes and their regulation.

For data and image analysis, the author employs custom-developed software solutions specifically designed for the research objectives. A key tool is SPARTACUSS, a software for tracking labeled particles in living cells, allowing detailed analysis of the dynamics of virus-like particles and related cellular processes. The author also applies multiple data normalization steps to ensure high accuracy of the results. Established statistical methods are used to guarantee the reliability of the conclusions.

The "Results and Discussion" chapter in Alexander Atemin's dissertation presents a detailed and systematic analysis of dynamic processes related to SARS-CoV-2 virus-like particles (VLPs) and key proteins involved in cellular replication.

The first major result presented in the dissertation pertains to tracking the process of virus-like particle entry into host cells. The results demonstrate that VLP entry occurs via S protein-mediated binding, followed by either direct fusion with the plasma membrane or endocytosis and fusion with the endosomal membrane.

Experiments show that inhibition of dynamin (a GTPase crucial for endocytosis) significantly reduces the efficiency of VLP entry into cells, highlighting the role of this protein in viral internalization. During entry, an increase in VLP speed and a significant drop in pH below 5 are observed within minutes. This is a key step in the virus's life cycle, necessary for genome release and subsequent replication. An analysis of the kinetics of VLP entry containing various mutations characteristic of the Omicron variant of SARS-CoV-2 is also performed. These results are critical for understanding the virus's evolutionary mechanisms.

In addition to VLP studies, the dissertation examines the dynamics of certain replication proteins during the cell cycle. Using dual-labeled cell lines, the author tracks proteins involved in DNA replication regulation. The results provide new insights into the control of DNA replication.

An essential part of the dissertation is the development of software tailored for the study, enabling precise tracking of fluorescently labeled particles in living cells. The software is used to analyze large datasets, allowing visualization and quantitative measurement of VLP kinetics and movement in host cells.

The results presented in the dissertation make a significant contribution to understanding the mechanisms of SARS-CoV-2 infection and its interactions with host cells. The dynamics of virus-like particles, their dependence on dynamin, and the role of intracellular acidification are key aspects that not only expand knowledge of the viral cycle but also open new avenues for developing therapeutic approaches.

The investigation of replication proteins during the cell cycle provides insights into the regulatory mechanisms and roles of these proteins in replication control. These findings are important for understanding processes related to cell division and genome stability. The conclusions are well-founded based on the presented data, and the contributions clearly highlight the innovations achieved by the author.

Minor language-related remarks are addressed to the author, mainly concerning the extensive use of foreign terms and the introduction of new ones. However, this does not diminish the value of the dissertation, which is overall well-structured, logically organized, and written in an engaging style.

Alexander Atemin is a co-author of six publications with a cumulative impact factor of approximately 36. The total number of citations of his publications on Google Scholar is 170.

Conclusion

The research results of Alexander Atemin provide valuable insights into the dynamic processes of viral infection and cellular replication.

The latest microscopy and experimental techniques and methods were applied. New software solutions for data analysis were developed. These findings expand the knowledge in the fields of study and serve as a foundation for future research and developments, both theoretically and practically (including the newly created software).

Alexander Atemin’s dissertation demonstrates the very high level of preparation of the author. Through its completion, he has established himself as an experienced researcher with a broad range of competencies and the ability to work successfully in various areas of modern biology. His inclination towards developing and applying cutting-edge technological approaches for studying cellular processes is clearly evident. Without any doubt, Alexander Atemin’s dissertation fully meets the requirements for the award of the scientific degree “Doctor” and deserves the highest evaluation, which I recommend the esteemed scientific jury to confer upon him.