Applicability Of Cryo-Electron Microscopy In The Study Of Kidney Disease

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Abstract

The main structural and functional element of the kidneys is the nephron, which consists of a glomerulus (branching of renal capillaries) located inside the double-walled Shumliansky-Bowman capsule (capsula glomeruli), which extends into a system of renal tubules flowing into the urinary tubes. Nephrons are divided into superficial (surface), intracortical and juxtamedullary.

Keywords: Urology, cryo-electron microscopy, kidney disease

Introduction

There are about 1 million nephrons in each kidney. While ageing about half of the nephrons become sclerotic and die, leaving about 1 million for both kidneys. The kidneys are the most vascularised organ in the human body [1]. Blood flow in the nephrons is regulated by sympathetic nerve fibres, penetrating the kidney from the celiac trunk in the renal artery wall, the rennin-angiotensin (pressor) and kallikrein-kinin (depressor) systems. Renin is produced by dense spot cells (macula compacta renalis) located in the triangle formed by the accessory artery, the excretory artery and the second order convoluted tubule, and regulates blood flow and urine production in each separate nephron. Renin, synthesized by cells of the juxtaglomerular complex by breaking down angiotensinogen (blood glycoprotein), leads to decapeptide angiotensin I, which under the influence of tissue kininase II (angiotensin converting enzyme - ACE) turns into a powerful vasopressor – angiotensin II (octapeptide), which regulates the tone of the glomerular accessory artery and determines blood flow and urine formation in each individual nephron. In addition kininase II destroys kinine (callidine, bradykinine), reducing arterial tone and causing vasodilation [2].

According to various epidemiological research studies, renal impairment occurs in 5.5…8% of the population [1] and is associated with further progression of renal disease into terminal renal failure, requiring costly renal replacement therapy (RRT) and high mortality due to cardiovascular complications, the risk in patients with renal failure is ten times more than in the overall population [2,3].

Light microscopy (magnification: x 50, x 200 and x 400) is most commonly used to study morphological changes in the renal glomeruli, periglomerular space, tubules (peritubular space) and renal interstitium, having the following slice coloring techniques: haematoxylin-eosin (H+E) – as a review coloring to study the structure of normal or altered renal tissue fragment presented by a slice through the thickness of cortical and medullary substance (coloring result: nuclei - blue, cytoplasm and intercellular substance - pink), haematoxylin-picrofuxine (by Van Gizon's) – for more detailed coloring of connective tissue (result of coloring: connective tissue has bright red-pink color - sclerosis, fibrosis; all other tissues have brownish-yellow color), aniline blue (by Masson's) – also for more reliable visualization of renal connective tissue (result of coloring: nuclei – red-purple, connective tissue – blue) and PAS reaction to glycogen coloring and detecting the preservation and continuity of basal membrances of tubules and glomeruli (coloring result: PAS positive substance (containing a large number of mucopolysaccharides)) are colored in bright red. There are also used histochemical methods for coloring oil red for fat, pycro-syrius red for collagen, alcian blue for glycosaminoglycans, silver metenamine by Jones, etc.

It's necessary to note that intrarenal vessels have no adventitia, and its role is fulfilled by an interstitial tissue through which the pulse wave from these vessels promotes the itrerrenal movement of urine [5].

For a deeper understanding of pathological changes in renal tissue cells it is advisable to use electron microscopy, which theory is based on the work of eminent physicists, Nobel Prize winners: Thomson, who discovered the electron in 1898; E. Retherford,

who created the planetary model of the atom; and Louis de Broglie, who found the wave nature of the electron beam in the early 1920s.

An electron microscope is an electro-optical device, where electrons with megavolt energy from beams give a magnified image of a microscopic object onto a fluorescent screen. A transparent electron microscope acts much like a light microscope to illuminate samples, but unlike a light microscope, it does not use light, i.e. a flow of photons, but electrons flows and electromagnetic lenses instead of glass lenses. The electron image is formed by electric and magnetic fields in the same way as light is formed by optical lenses. The wave length of an accelerated electron is determined by the magnitude of the accelerating voltage (De Broglie formula). And in modern devices the resolution limit is up to 0.1 nm, which is sufficient for investigating atomic and molecular structures [7; 8].

Electron microscopy, unlike optics, does not use glass lenses but electromagnetic lenses, which are multilayered coils through which a direct current is passed. By creating a certain geometry field with an electromagnetic lens, electrons can be gathered into a single point (focus) [8].

In biology and medicine electron microscopic analysis is used in toxicology, haematology, diagnostics, detection of the elemental composition of tissues, revealing foreign substances, in pathomorphology, and for pharmaceutical quality control, etc. [8].

An important role in electron microscopy is dedicated to test preparation – the preparation of samples for examination. There are a large number of methods that can be used to visualize successfully a wide variety of samples under an electron microscope. In order for a sample to be viewed under a transparent electron microscope, it must meet the following criteria: be solid, contrasting, clear to electron passage, dry, resistant to high vacuum and electromagnetic radiation. In addition the sample must be very thin (no more than 100 nm) so that the electron beam can pass through it. In order for this condition to be met, the sample must either be a small particle or be pre-poured in primer resin and cut into ultrathin slices [8].

Electron microscopy predominantly gives a magnification of 6000 to 16500. Thus, for electron microscopic research, pieces of renal tissue were fixed with 1% buffered osmium tetrahydroxide solution (pH=7.2). The material was embedded in araldite. Cutting was done on an ultrathome LKB-4800. The ultrathin sections were viewed in a JEM-100B electron microscope [8].

As an example of electron microscopy results of the bloodstream is given the following: imaging of abdominal aortic wall in rats allowed to identify successfully endothelial cell nuclei, that have classical spindle (elongated) shape, electron-solid elastic fibers (almost black color) delimiting layers of the middle vessel membrane (medium) and delimiting it from inner membrane (intima) and outer membrane (adventitia), and smooth muscle cell nuclei of oblong or rounded shape. The structure of the collagen fibers of the adventitia was easily distinguishable from the elastic fibres of the media and perivascular fat tissue. The different structure of the medial sheath of arteries and veins (the latter were characterized by the presence of only external and internal elastic membrane without multiple layers of elastic fibers between them and clearly colored plasma membranes of smooth muscle cells of polygonic shape) was clearly visualized and allowed to differentiate vessels of arterial and venous type.

High quality imaging of the overall microanatomy of the vascular wall allowed to analyze it at the level of individual anatomical structures within the arteries and veins themselves and the surrounding perivascular fat tissue – the blood supplying vasa vasorum and the lymphoid follicles responsible for the inflammatory response to the vascular wall damage. Vasa vasorum in adventitia and perivascular fat tissue were detectable and characterized in detail with magnification up to 5000 times. Due to their small caliber they may not contain elastic fibres and consist only of a monolayer of endothelial cells and a basal membrane; besides, they can also be distinguished by erythrocytes in the vascular lumen [4].

The adaptive role of the kidneys under such extreme conditions as acute massive blood loss has been investigated. Acute massive blood loss leads to disruption of basic renal functions such as glomerular filtration, tubular reabsorption and secretion of various organic and non-organic substances. The aim of these research studies was to investigate the ultrastructural changes of renal nephrons during acute massive blood loss in high altitude conditions in non-adapted and adapted animals. The author found out that the ultrastructure of renal nephrons undergoes a series of sequential changes aimed at restoring the test condition. Acute massive blood loss caused immediate mobilization of secretory resources of myoepithelial cells, spasm leading to dilation of afferent arterioles, blood capillaries drop of renal vascular glomeruli, sharp decrease in glomerular filtration, reabsorption reinforcement in nephron tubules. The obtained data indicated a significant involvement of structural and functional renal reactions in the adaptation to acute massive blood loss at the high altitude level [5].

In 2017 the Nobel Prize in Chemistry was awarded to Jacques Dubochet from the University of Lausanne (Switzerland), Joachim Frank from Columbia University (USA) and Richard Henderson from the Molecular Biology Laboratory of the Medical Research Council (UK) for their innovative work in cryo-electron microscopy (cryoEM) field and image analysis for structural analysis of biological macromolecules such as proteins and nucleic acids [9]. The space structure of biomolecules is fundamental and very important information required not only in biology but also in medicine and medicinal drug production. Moreover, cryo-EM has proven to be a powerful device for structural analysis, complementing the capabilities of methods such as nuclear magnetic resonance (NMR) and X-ray structural analysis. CryoEM has drawn the attention of researches because it

permits to reach atomic resolution in structural analysis of very small amounts of sample solution, measured in tens of micrograms without the need of its crystallization. The aforementioned Nobel winners are considered to be the founders of this method [10]. Thus, cryo-EM is a unique and innovative method of examining samples at the cellular and molecular level.

In addition to chemical fixation method of the sample at room temperature, cryo-electron microscopy techniques are used. The cryofixation method consists of only three steps: 1) cryoprotection and instant freezing (in liquid nitrogen); 2) lyophilisation; 3) obtaining frozen slices on an ultramicrotome with a cryorefrigerator [13].

Under common freezing conditions water forms ice crystals which damage the sample. There are several approaches to avoid crystallization. The first approach is the use of substances that prevent freezing at low temperatures, such as sucrose, as in the Tokuyasu method. The second approach is instant cooling [11].

In the vitrification process the sample is placed in a thin layer of amorphous ice, for which FEI offers an automated programmable approach. A water suspension containing a protein complex is immersed in a solution of liquid ethane, where it instantly freezes at -180°C. The process goes so far that water molecules have no time to crystallize and the ice remains amorphous, and thus transparent to the electron beam. Though, the structure of the sample is preserved as much as possible [12].

The advantages of cryofixation are faster preparation of samples, availability of reagents, cost-effectiveness, greater preservation of cellular structures due to the absence of chemical exposure and maximum availability of antigens. On the other hand, there are problems of sample storage, compression of slices and problems of deep cells freezing without damage [14]. In order to avoid cell rupture due to crystallization of intracellular fluid, a vitrification procedure must be carried out. Vitrification is the process where liquid is cooled so rapidly that water molecules harden without crystallization, forming amorphous (or vitreous) ice. Such a sample is said to have been cryoimmobilised and solidified in vivo state [15]. This rapid freezing is only possible under high pressure conditions. There are special devices for high-pressure freezing, which allow to freeze the sample to a depth of several hundred micrometers in hundredths of a second timing without forming ice crystals [16]. Cryofixation is most effective on small objects, so it is best suited for viruses, cell suspensions, small tissue fragments and purified macromolecules. The main methods of cryo-electron microscopy are freeze-substitution cryo-slice method, replica method and cryo-scanning electron microscopy [17].

Cryo-slices preparation, i.e. slices of vitrified material are carried out on ultramicrotomes equipped with a cryorefrigerator in a liquid nitrogen ambiance. The cryoslices are stored on sieves at a temperature below -160°C and placed in electron microscope with a cryo extension (console) at the same temperature to prevent devitrification [18]. This method allows direct visualization of the native structure without any chemical action. However, this method is costly and complicated and requires expensive equipment and, besides, up to 50% of slices are lost due to compression (in the case of routine ultratomies, this problem is solved by putting them in a bath, where slices are spread out on the water surface) [19].

An additional abrupt increase in efficiency was provided by enhancing the sample temperature from 4 K to about 50 K as a result of stopping the liquid helium supply from the tank in the column to the top-loaded sample holder. Although lowering the temperature to 4K helps to reduce the radiation damage to the sample, the quality of most images was degraded due to local blurring [20]. The latter was caused by electrostatic charging under the action of the electron beam, due to the extremely low electrical conductivity of the thin ice film of the sample at the extremely low temperature. As a result, only a few percent of the obtained images were suitable for subsequent analysis. The author solved this problem by raising the temperature of the sample to about 50 K, after the quality of most obtained images improved and they became suitable for analysis [21]. These technological improvements have reduced project time from several years to a few weeks, in obtaining the primary images and creating a three-dimensional reconstruction of them. The authors were able to decode the structures of such biomolecular systems as bacterial flagellar hooks, the actin filament of muscle tissue, ParM-protein microfilaments that ensure the movement of plasmids for bacterial cell division, the thin tubular needle of the type III secretion system that ensures virulence of pathogenic bacteria and the actomyosin complex [22; 23]. Three-dimensional images of the above structures were obtained with a resolution of 5 … 7 Å. The atomic 3D models explaining the functions of molecular systems were built on the basis of crystal structures images [24; 25]. With 3.8 Å resolution, a cylindrical structure formed by protein of tobacco mosaic virus membrane was imaged in about two weeks, which allowed to visualize most side chains.

To improve the speed of data acquisition for structural analysis of single particle images, a side-loading sample holder developed by JEOL was proposed to use in 2011; that enables to computerize data acquisition and processing. This upgraded version of cryo-transparent electron microscopy (cryoTEM) was designated as G6N [26; 27].

The use of the new sample holder in combination with the modified objective lens pole tip not only has improved the operational comfort, but also has increased the resolution of images, which happened to be above any expectations. One of the standard methods for checking the maximum achievable resolution of a cryoTEM is to take an image of a reference sample, such as a thin film of an amorphous platinum-iridium alloy, under defocus conditions of about 1micron [28]. Light and dark rings (Tone rings) alternate in the example of the obtained image. A similar method has often been used by the authors to test EM images of biomolecules frozen in the ice, and the high defocus value is necessary to increase the contrast of low resolution images to

make the analysis of these biomolecules images possible [29]. Using an alloy thin film as a test object allows to be exposed to high-energy electron beams without the risk of damaging the sample in order to evaluate a wide range of signals and reproduce Tone rings corresponding to the highest possible resolution. A resolution test, performed after completing the G6N cryoTEM setup, showed a resolution above 2.0 Å. It should be noted that in order to display rings corresponding to the highest possible resolution under highly defocused conditions, the cryoTEM electron-optical system must provide a parallel beam of electrons to illuminate the sample. Fortunately, the pole tip of the objective lens of this cryoTEM has been designed so that the above requirement is met under any imaging conditions, almost regardless of user settings [23].

A major milestone in the development of cryoEM has been the installation of a digital camera on a microscope with direct electron registration based on CMOS-kenno matrix. David Agard's group from the University of California, San Francisco in the collaboration with a company GATAN, one of the largest manufactures of digital cameras for electron microscopy, has developed such a device based on CMOS-kenno matrix for registration of X-ray images that previously were developed by colleagues at the University of California, Berkeley. GATAN completed the development of this system and began mass production in 2013 under the name of K2 Summit. This CMOS camera matrix had excellent features: a matrix size of 4000x4000 pixels, resistance to direct radiation caused by high energy electrons, accelerated to 300 eV and higher, minimal blurring of image when electrons scatter in a very thin detecting semiconductor layer, and, as the final completion, the 16-megapixel matrix data capture rate of 400 fps, allowing individual electrons to be counted. Since the typical total electron dose for cryo-EM is 20…30 e-/Å per second of exposure, the number of electrons hitting the CMOS-matrix sensor surface in taking a single image is limited, and it becomes possible to count individual electrons at tenfold dose reduction and corresponding increase of exposure time [24]. Counting individual electrons has the great advantage of reducing the noise level in the image. It should be noted, that cryo-EM is characterized by a high level of statistical noise in the useful signal, since the number of electrons forming the image is low due to the operation with low radiation doses in order to minimize radiation damage to the samples [25]. It is impossible to avoid this [26]. However, counting individual electrons makes it possible to minimize Landau noise - an internal detector noise, caused by large variation in signal amplitude, which is peculiar to all types of image detectors with energy accumulation, such as CCD detectors designed to record individual electrons.

CryoEM is used in the standardization and quality control of drugs, in particular in the visualization and characterization of the physical parameters of liposomal drug forms [28]. The method allows to study liposomes in their native state without the use of additional fixation or coloring procedures. Depending on the sample thickness, a resolution of up to 5 nm can be achieved [29]. This allows to trace the influence of the molar ratio of the active molecule to lipids on the morphology of the vesicles. Thus, [30] at molar ratio of 0.05, liposomes expectedly resemble coffee beans with a doxorubicin nanocrystal inside, but at larger molar ratios (0.37) even triangular structures were unexpectedly found, in which three doxorubicin nanocrystals formed a regular triangle within a single liposome. In the case where the loaded drug does not make a crystalline form in the liposome, the cryo-EM method may also be useful. Having compared images of control unloaded vesicles, the incorporation of vincristine, vinblastine and vinorelbine into the inner liposome cavity was assessed during the work [31] on the increased electron density of the liposomes inner cavity. In Prof. Barenholz's laboratory, an approach has recently been proposed to reconstruct the 3D structure of liposomes to 2D cryo-electron microimages, which provides additional information about the distribution of the drug in the vesicles. This approach can be extremely useful when investigating the interaction of liposomes for example with lipophilic-modified drugs, which can distort the membranes structure [32].

In structural biology CryoEM is widely used to build three-dimensional models of the studied particles with a resolution of a few angstroms Å. The most common techniques for obtaining such high-resolution models are single-particle cryo-electron microscopy and cryo-electron tomography. In both techniques the key point of algorithms in the data processing is to combine images of two-dimensional for single-particle and three-dimensional for cryo-electron tomography. There are a number of image matching methods for combining 2D and 3D cryo-images. However, it is not possible to assess the quality of such methods on real data due to the lack of 'ground truth'. Also, quality assessment of the matching methods based on real data is complicated by the high-level of noise. Subsequently, methods have been proposed to create sets of two- and three- dimensional realistic data of single-particle cryoEM and cryo-electron tomography [34].

The basic principle of cryoEM operation is the imaging of radiation-sensitive samples in a transmission electron microscope under cryogenic conditions. There are various cryoEM techniques: cryo-electron tomography, single particle cryoEM [35] and electron crystallography, all of them have been successfully used to analyse biological structures in various situations. One widely used approach is single-particle cryoEM [36]. In this method, a large number of high-resolution (<4Å) images are taken, each containing hundreds of identical but randomly oriented particles. Then, all projections of the particles are localized in each of the obtained images. For this purpose the projections are classified into several classes, containing projections and corresponding to the same orientations in three-dimensional space. Within each class, projection images can have different orientation in two-dimensional projection surface, as well as a small displacement [37]. Using image matching methods, the projections of particles within each class are transformed to the same orientation in the two-dimensional projection surface, and then iterative averaging is performed. In this way the projections of particles whose signal-to-noise ratio is much higher than in the original objects, are obtained [38].

In cryoelectron tomography in order to obtain data, samples are frozen same as in cryoEM, to protect them from damage resulting from radiation. The frozen sample plate containing a large number of studied particles is turned at the different angles with respect to the electron beam in increments of 1…2º in the range from -60◦ to +60◦ and the sample is projected through an electron microscope, obtaining a two-dimensional projection of the sample for each angle. The resulting image series is used for three-dimensional reconstruction, which is usually performed using weighted reversed projection [39]. At the result there is a three- dimensional tomographic image containing dozens of identical particles oriented in different ways. In order to obtain a three-dimensional particle image where the signal-to-noise ratio is significantly higher, all particles are localized in the resulting three-dimensional tomographic image [40]. Then, using image matching methods of the subtomogram, the found particles are converted into the same orientate on in space and then iteratively averaged. In order to assess the quality of the image matching method, synthetic data is needed, where the orientation of the objects is specified in a controlled manner. At the same time such data should be as similar as possible to the real cryoEM and tomography images. Sets of two- and three-dimensional synthetic cryoEM and tomography images have been created [41]. For this purpose, an algorithm is proposed for generating a three-dimensional model of an object similar to the real molecular complexes studied by means of the above methods. Then, there is a modeling of the processes for the synthesized images, that take place on receiving data by means of electron microscope, specifically simulation of noise and contrast transfer function, as well as the construction of two-dimensional projections for single particle cryoEM and simulation of the missing wedge effect for cryo-electron tomography. The data obtained by the proposed method is used to test a matching algorithm of three-dimensional biomedical image. The structure of proteins has been studied using cryoEM by a number of scientists [42; 43; 44; 45]. New data on the structure of protein molecules have been obtained.

Nowadays, the world's most important scientific database includes studies on the pharmacodynamics of drugs [50 - 53], the structure of microbial cells [54 - 57], ion channel structures [58 - 61], viruses [62; 63], chromosomes [64], bacteriophages [65], haemoglobin and myoglobin [66; 67], vitreous [68] and kidneys [69-71].

Thus, the application of the cryo-electron microscopy method opens new opportunities and perspectives in the study of pathological changes in the kidneys, as well as the drugs production and their forms for the treatment of kidney diseases.

References

1. Hill, N.R. Global Prevalence of Chronic Kidney Disease. / N.R.Hill, S.T. Fatoba, J.L.Oke et al.// A Systematic Review and Meta-Analysis. PloS one. 2016 - 11. - e0158765.
2. Liu, M. Cardiovascular disease and its relationship with chro nic kidney disease. /M. Liu, X.C. Li, L.Lu et al.// European review for medical and pharmacological sciences. - 2014. - 18. - P.2918-2926.
3. Fan, J, Salameh H. Impact of Chronic Kidney Disease on Risk for Vascular Events. /J. Fan, H. Salameh// Current vascular pharmacology. – 2016. – 14. – P.409 - 414.
4. Mukhamadiyarov, R.A. Study of normal and pathological microscopic anatomy of blood vessels using scanning electron microscopy in backscattered electrons / R.A. Mukhamadiyarov, A.G. Kutikhin // Fundamental and Clinical Medicine. – 2019. – V.4-№1. – P.6 – 14.
5. Aidarbekova, Z.M. Changes in electrcton microscopy of renal nephrons, their mitotic activity in acute massive blood loss in high altitude conditions in non-adapted and adapted animals / Z.M. Aidarbekova, Zh.A. Makhmudov, A.Z. Aidarbekova, Kalugina O.P.//
6. Aidarbekova, Z.M. Ultrastructure of kidneys and its morphometric parameters at extreme situation in conditions of mountains// Bulletin of KSMA named after I.K. Akhunbaev, Bishkek-2010-№1 P.57-61.
7. Aidarbekova, Z.M. Electron microscopy of kidneys in acute massive blood loss in non-adapted animals in high mountain conditions // Central Asian Medical Journal. – 2005-№3, P.250-255.
8. Morozova, K.N. Electron microscopy in cytological studies: manual / K.N. Morozova // Novosibirsk. – Novosibirsk State University, 2013. – 85p.
9. Vagina, F.V. Nobel Prize in Chemistry 2017: cryo-electron microscopy / F.V. Vagina, S.K. Dmitriuk, I.V. Vasiltsova // In the collection: Chemistry and Life. Collection of XVII International Scientific and Practical Student Conference, 2018. – P.10-17
10. Ryabchikova, E.I. Development of cryo-electron microscopy / E.I. Ryabchikova // Science at first source. – 2017.- №5-6 (67). – P.15-17.
11. Namba, K. Cryo-electron microscopy: evolution of equipment and methods. /K. Namba, T. Kato//Laboratory and Production. - 2019. - № 3 (7). - P. 92-102.
12. Cressey, D. Cryo-electron microscopy wins chemistry Nobel. /D.Cressey, E. Callaway//Nature. – 2017. – 4. - 550(7675). – P.167.
13. Milne, J. L. Cryo-electron microscopy – a primer for the non-microscopist. /J.L. Milne, M.J. Borgnia et al. // FEBS Journal. – 2013. – 280. – 1. – P. 28-45.
14. Schmid, M. F. Single-particle electron cryotomography (cryoet). /M.F. Schmid// Adv. Protein. Chem. Struct. Biol.. – 2011. – 82. – P. 37–65.
15. Li X., Mooney P., Zheng S., et al. Electron counting and beam induced motion correction enable near-atomic-resolution single-particle cryo-EM /X. Li, P. Mooney, S. Zheng et al.// Nature Methods. - 2013. - №10. - P. 584-590.
16. Dodd, T. Simulation-Based Methods for Model Building and Refinement in Cryoelectron Microscopy. / T.Dodd, C.Yan, I. Ivanov//J. Chem. Inf. Model. - 2020 - 60(5). – P. 2470-2483.
17. Danev, R. Cryo-Electron Microscopy Methodology: Current Aspects and Future Directions. /R. Danev, H.Yanagisawa, M. Kikkawa// Trends. Biochem. Sci. 2019 - 44(10). – P. 837-848.
18. Lyumkis, D. Likelihood-based classification of cryo-em images using frealign. /D. Lyumkis, A. Brilot et al.//Journal of structural biology. – 2013. - 183. – 3. – P. 377-388.
19. Sigworth, F. J. Principles of cryo-em single-particle image processing. /F. J. Sigworth// Microscopy. 2016. – 65. – 1. – P. 57- 67.
20. Fujii, T. Direct visualization of secondary structures of F-actin by electron cryomicroscopy /T. Fujii, A.H. Iwane, K. Namba// Nature. - 2010. - №467. - P. 724-728.
21. Nakane, T. Single-particle cryo-EM at atomic resolution. /T. Nakane, A. Kotecha, A. Sente et al./Nature. – 2020. - 587(7832). – P. 152-156.
22. Fujiyoshi, Y. Observation of membrane proteins through an electron beam /Y. Fujiyoshi// JEOL News. - 2009. - №44. - P. 23-31.

23. White, H.E. Structural Study of Heterogeneous Biological Samples by Cryoelectron Microscopy and Image Processing. /H.E. White, A. Ignatiou et al.// Biomed. Res. Int. – 2017. – P.1032432.
24. Drulyte, I. Approaches to altering particle distributions in cryo-electron microscopy sample preparation./I. Drulyte, R.M. Johnson, E.L. Hesketh et al.// Acta Crystallogr. D. Struct. Biol. - 2018. - 1. - 74(Pt 6). – P. 560-571.
25. Herzik, M.A. Jr. Cryo-electron microscopy reaches atomic resolution. /M.A.Jr. Herzik//Nature. – 2020. - 587(7832). – P. 39-40.
26. Baxa, U. Imaging of Liposomes by Transmission Electron Microscopy. /U. Baxa// Methods Mol. Biol. – 2018. – 1682. – P. 73-88.
27. Le – Deigen, I.M. Experimental methods to study interaction mechanism of lipid membranes with low molecular drugs /I.M. Le – Deigen, A.A. Skuredina, E.V. Kudryashova // Bioorganic Chemistry. – 2020. – Vol.46. - №4. P. 340 – 359.
28. Cizmar, P. Detection and Characterization of Extracellular Vesicles by Transmission and Cryo-Transmission Electron Microscopy. /P. Cizmar, Y. Yuana//
29. Methods Mol. Biol. – 2017. – 1660. – P. 221-232.
30. Thonghin, N. Cryo-electron microscopy of membrane proteins. / N. Thonghin, V. Kargas et al.//Methods. – 2018. – 1. – 147. – P.176-186.
31. Renaud, J.P. Cryo-EM in drug discovery: achievements, limitations and prospects. / J.P. Renaud, A. Chari, C. Ciferri. et al.// Nat. Rev. Drug Discov. - 2018 -17(7). – P. 471-492.
32. Tocheva, E. I. Electron cryotomography. / E. I. Tocheva, Z. Li, G. J. Jensen// Cold Spring Harbor perspectives in biology – 2010 – 2. – 6. - a. 003442.
33. Miyazawa, A. Cryo-Electron Microscopy. /A. Miyazawa// Brain Nerve. -2018. - 70(6). – P. 639-649.
34. Wang, H.W. Biological cryo-electron microscopy in China./H.W.Wang, J. Lei, Y. Shi// Protein Sci. – 2017. - 26(1). – P.16-31.
35. Nogales, E Cryo-EM. /E. Nogales// Curr. Biol. – 2018. – 8. -28(19). - R1127-1128.
36. Frank, J. J. Time-resolved cryo-electron microscopy: Recent progress./ J. J. Frank// Struct. Biol. – 2017. - 200(3). P. 303-306.
37. Benjin, X. Developments, applications and prospects of cryo-electron microscopy. Benjin, L.Ling// Protein Sci. – 2020. - 29(4). – P. 872-882.
38. Hoenger, A. Cellular tomography./ A. Hoenger, C. Bouchet-Marquis// Adv Protein Chem. Struct. Biol. – 2011. - 82. – P. 67–90.
39. Yonekura, K. Complete atomic model of the bacterial flagellar filament by electron cryomicroscopy /K. Yonekura, S. Maki-Yonekura, K. Namba// Nature. - 2003. - №424. - P.643-650.
40. Yeates, T.O. Development of imaging scaffolds for cryo-electron microscopy. Yeates, M.P. Agdanowski, Y. Liu//Curr. Opin. Struct. Biol. – 2020. – 60. – P.142-149.
41. Yonekura, K. Complete atomic model of the bacterial flagellar filament by electron cryomicroscopy /K. Yonekura, S. Maki-Yonekura, K. Namba// Nature. - 2003. - №424. - P.643-650.
42. Shigematsu, H. Electron cryo-microscopy for elucidating the dynamic nature of live-protein complexes./H. Shigematsu//Biochim. Biophys. Acta Gen. Subj. – 2020. - 1864(2). P.129436.
43. Abriata, L.A. Will Cryo-Electron Microscopy Shift the Current Paradigm in Protein Structure Prediction? /L.A.Abriata, M. Dal Peraro//J. Chem. Inf. Model. – 2020. – 26. - 60(5). - P.2443-2447.
44. Trellet, M. Protein-Protein Modeling Using Cryo-EM Restraints./M. Trellet, G. van Zundert, A.M. J.J.Bonvin//Methods Mol. Biol. - 2020. – 2112. – P. 145-162.
45. Yang, G. Cryo-EM structures of human γ-secretase. /G.Yang, R. Zhou, Y. Shi//Curr. Opin. Struct. Biol. – 2017. – 46. – P. 55-64.
46. Schmidt, C. Combining cryo-electron microscopy (cryo-EM) and cross-linking mass spectrometry (CX-MS) for structural elucidation of large protein assemblies. Schmidt, H. Urlaub// Curr. Opin. Struct. Biol. – 2017. – 46. – P. 157-168.
47. Toro-Nahuelpan, M. Tailoring cryo-electron microscopy grids by photo-micropatterning for in-cell structural studies. /M. Toro-Nahuelpan, I. Zagoriy, F. Senger et al.// F. Nat. Methods. – 2020. - 17(1). - P. 50-54.
48. Baker, M. Cryo-electron microscopy shapes up. /Baker M.//Nature. – 2018. - 561(7724). – P.565-567.
49. Dillard, R.S. Biological Applications at the Cutting Edge of Cryo-Electron Microscopy./R.S.Dillard, C.M. Hampton, J.D. Strauss et al.// Microsc. Microanal. - 2018 - 24(4). – P.406-419.
50. Weis, F. Combining high throughput and high quality for cryo-electron microscopy data collection./F.Weis, W.J.H. Hagen//Acta Crystallogr. D Struct .Biol. – 2020. – 1. - 76(Pt 8). P. 724-728.
51. Shimada, I. GPCR drug discovery: integrating solution NMR data with crystal and cryo-EM structures. /I.Shimada, T. Ueda, Y.Kofuku et al.// Nat. Rev. Drug Discov. - 2019 - 18(1). – P. 59-82.
52. Boland, A. The potential of cryo-electron microscopy for structure-based drug design. /A. Boland, L. Chang, D. Barford// Essays Biochem. – 2017. - 8. - 61(5). – P. 543-560.
53. Vénien-Bryan, C. Cryo-electron microscopy and X-ray crystallography: complementary approaches to structural biology and drug discovery./C. Vénien-Bryan, Z. Li et al.// Acta Crystallogr. F. Struct. Biol. Commun. – 2017. – 1. -73(Pt 4). – P.174-183.
54. Scapin, G. Cryo-EM for Small Molecules Discovery, Design, Understanding, and Application. /G. Scapin, C.S. Potter, B. Carragher//Cell. Chem. Biol. – 2018. – 15. - 25(11). – P.1318-1325.
55. Fujii, T. Identical folds used for distinct mechanical functions of the bacterial flagellar rod and hook ./T. Fujii, T. Kato, K.D. Hiraoka et al.// Nature Communications. – 2017. - №8. Article number: 14276.
56. Kawamoto, A. Common and distinct structural features of Salmonella injectisome and flagellar basal body /A. Kawamoto, Y.V. Morimoto, T. Miyata et al.// Scientific Reports. - 2013. - №3. - Article number: 3369.
57. Kaelber, J.T. Structure of the AAVhu.37 capsid by cryoelectron microscopy./J.T. Kaelber, S.A. Yost, E. Firlar et al.//Acta Crystallogr F Struct Biol Commun. – 2020. – 1. - 76(Pt 2). – P. 58-64
58. Kooger, R. CryoEM of bacterial secretion systems./R. Kooger, P. Szwedziak et al.// Curr. Opin Struct. Biol. – 2018. – 52. – P. 64-70.
59. Liao, M. Structure of the TRPV1 ion channel determined by electron cryo-microscopy /M. Liao, E. Cao, D. Julius// Nature. - 2013. - №504. - P. 107-112.
60. Cao, E. TRPV1 structures in distinct conformations reveal activation mechanisms /E. Cao, M. Liao, D. Julius// Nature. - 2013. - №504. - P. 113 - 118.
61. Samanta, A. Cryo Electron Microscopy of TRP Channels. Hughes, V.Y. Moiseenkova-Bell//Methods Mol. Biol. – 2019. – 1987. – P. 39-50.
62. Lau, C. Never at rest: insights into the conformational dynamics of ion channels from cryo-electron microscopy. /C. Lau, M.J. Hunter, A. Stewart et al.// J. Physiol. – 2018. – 1. - 596(7). – P.1107-1119.
63. Luque, D. Cryo-electron microscopy for the study of virus assembly. /D. Luque, J.R.Caston//Nat. Chem. Biol. – 2020. - 16(3). – P. 231-239.
64. Mak, J. Recent advances in retroviruses via cryo-electron microscopy. Mak, A. de Marco//Retrovirology. – 2018. – 23/ - 15(1). – P. 23.
65. Zhou, B.R. Distinct Structures and Dynamics of Chromatosomes with Different Human Linker Histone Isoforms /B.R.Zhou, H. Feng, S. Kale et al.// Mol. Cell. – 2021. – 7. - 81(1). P. 166-182.
66. Cuervo, A. Observation of Bacteriophage Ultrastructure by Cryo-electron Microscopy /A. Cuervo, J.L. Carrascosa// Methods Mol. Biol. – 2018. – 1693. – P. 43-55.
67. Khoshouei, M. Revisiting the Structure of Hemoglobin and Myoglobin with Cryo-Electron Microscopy. Khoshouei, R. Danev et al.// J. Mol. Biol. -

2017. – 18. - 429(17). – P. 2611-2618.
68. Atanasova, M. Structural glycobiology in the age of electron cryo-microscopy. /M. Atanasova, H. Bagdonas, J. Agirre//Curr. Opin. Struct. Biol. – 2020. – 62. – P. 70-78.
69. Al-Amoudi, A. Cryo-electron microscopy of vitreous sections./A. Al-Amoudi, J.-J. Chang, A. Leforestier et al./The EMBO journal – 2004. – 23. - 18 -P. 3583–3588.
70. Van der Wijst, J. TRPV5 in renal tubular calcium handling and its potential relevance for nephrolithiasis /J. van der Wijst, M.K. van Goor et al.//.Kidney Int. – 2019. - 96(6). - P.1283-1291.
71. Verrier, C. Topography, Composition and Structure of Incipient Randall Plaque at the Nanoscale Level./C. Verrier, D. Bazin, L. Huguet et al.//J. Urol. – 2016. - 196(5). P.1566-1574.
72. Tomas, N.M. Across scales: novel insights into kidney health and disease by structural biology./N.M. Tomas, S.A. Mortensen et al.//Kidney Int. – 2021. - 100(2). – P. 281-288.