ISSN 0006-2979, Biochemistry (Moscow), 2025, Vol. 90, No. 2, pp. 173-187 © Pleiades Publishing, Ltd., 2025.
Published in Russian in Biokhimiya, 2025, Vol. 90, No. 2, pp. 189-206.

REVIEW

Techniques for Selective Labeling of Molecules and Subcellular Structures for Cryo-Electron Tomography

Evgeny P. Kazakov[1,2,a]*, Igor I. Kireev[1,2], and Sergei A. Golyshev[1]

[1]*Belozersky Research Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119991 Moscow, Russia*

[2]*Department of Cell Biology and Histology, Faculty of Biology, Lomonosov Moscow State University, 119991 Moscow, Russia*

[a]*e-mail: kazakov.evgeny.2016@post.bio.msu.ru*

Received November 7, 2024
Revised January 9, 2025
Accepted January 20, 2025

Abstract—Electron microscopy (EM) is one of the most efficient methods for studying the fine structure of cells with a resolution thousands of times higher than that of visible light microscopy. The most advanced implementation of electron microscopy in biology is EM tomography of samples stabilized by freezing without water crystallization (cryoET). By circumventing the drawbacks of chemical fixation and dehydration, this technique allows investigating cellular structures in three dimensions at the molecular level, down to resolving individual proteins and their subdomains. However, the problem of efficient identification and localization of objects of interest has not yet been solved, thus limiting the range of targets to easily recognizable or abundant subcellular components. Labeling techniques provide the only way for locating the subject of investigation in microscopic images. CryoET imposes conflicting demands on the labeling system, including the need to introduce into a living cell the particles composed of substances foreign to the cellular chemistry that have to bind to the molecule of interest without disrupting its vital functions and physiology of the cell. This review examines both established and prospective methods for selective labeling of proteins and subcellular structures aimed to enable their localization in cryoET images.

DOI: 10.1134/S0006297924604015

Keywords: electron microscopy, cryo-electron tomography, correlation microscopy, genetically encoded tags, gold nanoparticles, quantum dots, ferritin, metallothionein, encapsulins

INTRODUCTION

Electron microscopy (EM) has proven to be a reliable and efficient tool for studying the structure of cells with a high resolution unavailable in visible light microscopy. However, preparation of ultrathin sections of cells requires chemical fixation that often greatly changes the fine structure of the sample. Even fixation with aldehydes and osmium tetroxide, which is considered as the “gold standard” of sample preparation, is not completely free of artifacts [1-3].

Cryofixation, i.e., sample stabilization by rapid cooling under conditions preventing formation of ice crystals, has come a long way and become an efficient alternative to chemical fixation. When a sample is rapidly frozen by immersion in a liquid coolant with a high heat capacity [4] or frozen under high pressure, diffusion processes in the cell stop almost instantly [5, 6] and individual protein molecules and macromolecular complexes are stabilized in their native state, the main characteristic of which is preservation of hydrated state of biomolecules [7, 8].

Abbreviations: cryoEM, cryo-electron microscopy; cryoET, cryo-electron tomography; EM, electron microscopy; FKBP, FK506-binding protein; FRB, FKBP-rapamycin binding domain; NP, nanoparticle; QD, quantum dot; GFP, green fluorescent protein.

* To whom correspondence should be addressed.

Similar to visible light microscopy, image resolution in EM depends on the sample thickness. The optimal sample thickness in EM is determined by the mean free path of electron in the sample material [9]. For cryofixed hydrated cells and tissues, this value is 300-400 nm at the accelerating voltage of 300 kV. Consequently, cell biology studies can only be conducted in thin peripheral extensions of cells cultured on substrates transparent to electrons or require advanced techniques, such as obtaining sections of cryostabilized specimens with a cryo-ultramicrotome (CEMOVIS, cryo-electron microscopy of vitrified sections) [10-12] or by milling cryofixed samples in focused ion beam/scanning electron microscope (FIB-SEM) to produce a single lamella plate approximately 200-300 nm thick [13-15]. The sections obtained by these methods are further examined by electron microscopic tomography methods.

Tomography is a reconstruction of a three-dimensional image based on a series of two-dimensional projections of an object obtained at different angles, known as tilt-series [16]. Tomography not only improves the axial resolution, but also significantly increases the contrast of resulting images due to the multiple averaging of brightness values of each volume element (voxel) in the three-dimensional image. Cryo-electron tomography (cryoET) allows to study the structure of cells, down to the three-dimensional structure of individual protein molecules in their native intracellular environment with a resolution of ~3-5 Å, which has been already achieved by using the subtomogram averaging technique [17, 18].

An EM image simultaneously shows lipid, protein, and nucleoprotein structures, aqueous contents of the vacuoles, and polysaccharides of the cell walls, so a researcher often faces the problem of detecting a molecule-sized object of interest against this complex and detail-rich background. This problem is solved relatively easily if this object is abundant (ribosomes), stands out from the background by its geometry (proteasomes and subcellular formations of viral origin), or is associated with certain membrane or cytoskeletal components. At the time of writing this review, according to PubMed, the studies on such objects accounted for 42% (732 out of 1728) of publications using cryoET). However, when the goal is to localize less represented proteins or to detect zones where the processes under study occur, researchers often find themselves in a difficult situation [18-20].

The problem of the object localization in a microscopic image can be solved by using labeling methods. Ongoing progress in the methodology of cell ultrastructure visualization by the cryo-electron microscopy (cryoEM) requires the development of compatible labeling methods [20-22]. Localization of proteins by visible light microscopy has been achieved by using, first, fluorescently labeled antibodies in fixed cells and, then, genetically encoded fluorescent labels in living cells. The problem of protein localization at the ultrastructural level lacks such elegant solutions even for the EM of ultrathin sections. Immunogold techniques, enzyme labels, and selective contrasting by photooxidation are employed in this field with varying success. However, all these methods rely on chemical fixation, which is sometimes extremely destructive for cellular substructures, as it has been shown in the studies that used cryo-methods to investigate the three-dimensional structure of chromatin [23, 24].

The development of efficient labeling tools for EM of cryofixed cells would significantly expand the range of experimental tasks and allow for a more complete implementation of such advantages of cryofixation as the absence of non-specific aggregation of soluble proteins and preservation of the native aqueous environment of subcellular structures. Selecting a proper labeling strategy will also allow to obtain high-resolution data and to fully realize the potential of cryoET.

However, since the main physical mechanisms of contrast generation in the formation of an EM image are elastic electron scattering and phase contrast [9, 25], the main requirement for a labeling particle indicating the location of a studied protein is its ability to efficiently scatter electrons to make it clearly distinguishable against the background of intracellular structures despite the small size (2-10 nm). Therefore, it should be composed of heavy elements, most of which are normally absent in living cells.

A labeling technique compatible with cryofixation should solve a multi-faceted problem: introduction of particles containing heavy elements into a living cell and their binding to the target molecules should not disrupt the functioning of these molecules, minimally affect their structure, and have no negative effects on the cell physiology, which looks like mutually exclusive requirements [26]. At the same time, it is highly desirable to have the ability to pre-identify labeled cells and areas of interest in them by visible light microscopy to implement various variants of cryo-correlated light and electron microscopy (cryoCLEM), which is especially important in the ultrastructural analysis of rare cellular events [27] and production of lamellae using the FIB-SEM method. Obviously, these requirements lead to compromises in the practical implementation of the methods for molecule labeling for cryoET.

This review analyzes implemented and prospective methods for selective labeling of proteins and subcellular structures for their further localization in cell cryosections, as well as the systems that are potentially applicable for solving this problem but have not yet been formalized as protocols.

NANOPARTICLES

For many decades, colloidal gold particles (5-30 nm) conjugated with antibodies have been successfully used as labels in the EM of ultrathin sections with chemical fixation (immunogold labeling method) [28, 29]. Gold particles stand out against the background of cellular components due to their high electron density, while the possibility of detecting proteins on the surface of prepared ultrathin sections eliminates the need for permeabilization of membranes for the delivery of antibodies into the cell [30].

Can gold-conjugated antibodies be used for labeling cellular components in cryoET? Yes, due to the chemical inertness of gold, its low cytotoxicity [31], and high electron density that distinguishes it from the background of vitrified cellular contents. Gold particles conjugated with antibodies can be used in cryoEM/ET for detection of objects on the cell surface, vesicles, and virions [32-36] (Fig. 1a). This approach can be called “live immunogold labeling”.

The question arises of whether it is possible to deliver gold-labeled antibodies into a living cell without significantly disturbing the cellular physiology. Such experiments have been successfully performed, although their results were assessed by the EM of chemically fixed ultrathin sections. Loading antibodies conjugated with an electron-dense label into a living cell can be done in three ways. The first method is microinjection [37]. For example, anti-GFP antibodies conjugated with gold nanoparticles (NPs) were injected into the cell nucleus to visualize and locate gene loci containing artificially introduced repeats of the bacterial *lac* operator labeled with GFP-LacI and replication zones labeled with GFP-PCNA (proliferating cell nuclear antigen).

The second method is loading antibodies conjugated with gold NPs into the cell using lipophilic transfection agents. This approach was used to solve the problem of mapping transcription sites at the ultrastructural level. The authors of [38] used Fab′ fragments of monoclonal antibodies against the C-terminal domain of RNA polymerase II conjugated with 0.8-nm gold NPs [38]. Although only ~10% of available target molecules detected by other methods were labeled, the authors believed it to be a satisfactory result for the experimental task as they were able to demonstrate the absence of RNA polymerase clustering in the nucleus, which, albeit indirectly, favors the mobility of transcription complexes and is against the “transcription factories” concept.

The third method is the delivery of antibodies into the cytoplasm using streptolysin O, a bacterial peptide that forms temporary pores in the cell membrane. The possibility of loading small organic molecules (for example, HaloTag ligands), single-domain antibodies against PCNA, and whole IgG molecules (up to 150 kDa) using this method has been demonstrated in [39, 40]. It should be noted once again that at the time of writing this review, none of these methods for loading antibodies into cells had been used in cryoEM studies; however, there are no fundamental obstacles to adapting these methods for cryofixation.

All the above-described methods for delivering labeled antibodies are to some extent harmful to the cell, but this disadvantage is impossible to avoid. A serious limitation in the use of transfection reagents and streptolysin O is that the label is delivered to the cytoplasm. If the target is located in the nucleus, the labeling method should include an additional mechanism for transporting the label through nuclear pore complexes.

The methods for *in vivo* labeling of intracellular structures, in which an electron-dense label (e.g., an antibody with a bound NP) and a target molecule coexist separately for some time inside the cell generally provide no opportunity to distinguish between the target-bound and unbound labels [41]. This is not a serious problem when the targets are located in the membrane and exposed to the external environment, because the unbound label can be washed off before cryofixation.

Certain species of gold NPs fluoresce [42, 43], which adds to the label versatility and allows its use for the colocalization of labeled cells and structures, although, perhaps, with less efficiency than using specialized low-molecular-weight fluorochromes.

Due to the achievements in synthetic biochemistry, gold NPs can be used as electron-dense labels not only in conjunction with antibodies. Thus, the application of functionalized metal NPs is an interesting approach for labeling proteins *in situ* [22]. The authors of [22] developed a method for synthesizing gold nanoclusters about 2 nm in size, the surface of which was protected by a polyethylene glycol-based surfactant. Cell nucleus was used as a model labeling target, so the particles were additionally conjugated with the NLS (nuclear localization signal) peptide to facilitate the nuclear import. After being delivered to the cell by electroporation, such NPs efficiently relocated to the nucleus, while the control particles without the NLS resided in the cytoplasm.

Another promising approach is functionalization of NP surface with the human immunodeficiency virus TAT (transactivator of transcription) peptide to impart gold NPs with the ability to penetrate cell membranes. Due to the unique mechanism of membrane penetration, the TAT peptide ensures an efficient delivery of NPs into the cell cytoplasm. Various methods for synthesizing such particles and verifying their ability to penetrate into the cell have been recently presented in a number of studies [44-47].

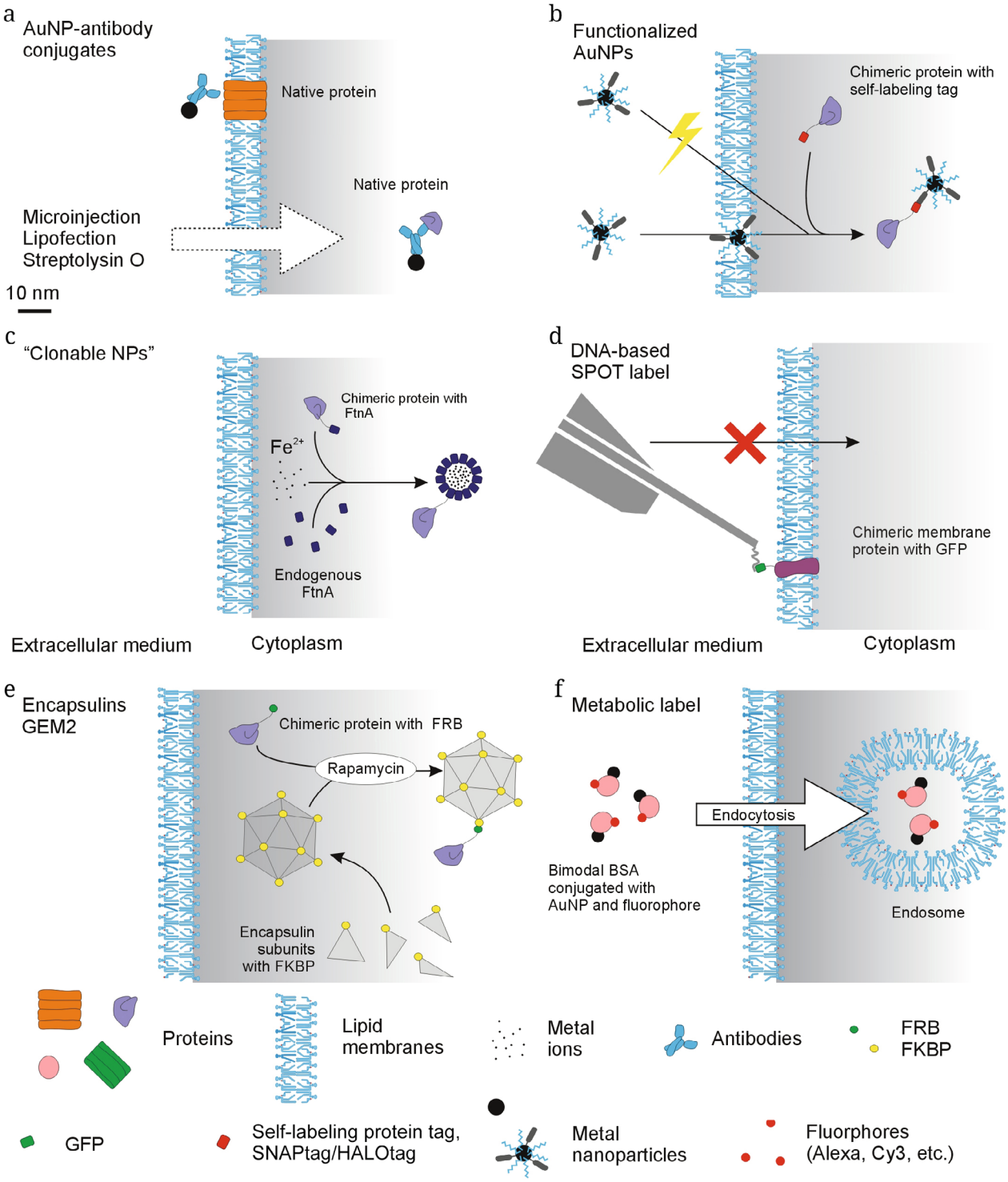

Fig. 1. Comparative sizes of different labels, methods of their delivery into cells, and applicability for labeling biomolecules, subcellular structures, and various cell compartments in cryoET. a) Conjugates of antibodies with gold nanoparticles (AuNPs) or quantum dots label membrane proteins on the cell surface [32-36] and can be delivered to the cytoplasm (tested in combination with chemical fixation and preparation of ultrathin sections [37, 38]). b) Gold nanoparticles (AuNPs) functionalized with substrates of self-labeling tags penetrate the membrane by electroporation or due to a modified surface and bind to chimeric target proteins (partially implemented in [22]). c) Cloneable nanoparticles (NPs) (e.g., bacterial ferritin FtnA). Protein cage that accumulates metal ions is assembled on the chimeric protein [26, 69, 70]. d) DNA origami-based SPOT tag binds via the RNA aptamer to the chimeric protein that exposes GFP to the extracellular environment [73]. e) Encapsulins (e.g., GEM2 system [41]). Cell produces FKBP-conjugated encapsulin subunits which oligomerize into particles, and FRB (FKBP rapamycin binding domain)-containing chimeric target protein. The binding of the particles with the target is induced by rapamycin. f) Metabolic label (BSA or another carrier associated with an electron-dense particle and a fluorescent marker) is absorbed by the cells via endocytosis and accumulated in the endosomes [92].

Another advantage of this method is the ability of the TAT peptide to translocate the associated particle from the cytoplasm to the nucleus [48], i.e., such construct can be used as a basis for developing the systems for labeling nuclear targets, which is a nontrivial task.

The cited works have been mostly focused on the use of produced nanoconstructs as vehicles for the delivery of therapeutic agents into the cell cytoplasm and nucleus. A combination of this rather gentle delivery method with conjugation of functionalized gold NPs with low-molecular-weight ligands potentially allows to create a highly efficient system for labeling intracellular proteins and subcellular structures for cryoET (Fig. 1b). As ligands, this method may use substrates for genetically encoded "self-labeling" protein markers, like SNAP-tag and HaloTag. SNAP-tag is a modified O^6-alkylguanine alkyltransferase (AGT), an enzyme involved in DNA repair [49]; HaloTag is a mutant bacterial haloalkane dehalogenase [50]. Both markers are introduced into cells using plasmids encoding a chimeric protein of interest fused with the marker. The substrate of SNAP-tag is O^6-benzylguanine, while the substrates of HaloTag are various modified chloroalkanes. Both enzymes are modified in a way that their interaction with the substrate results in the formation of a covalent bond that irreversibly binds the substrate to the target carrying the marker. This allows to create a bond between the labeled protein and functionalized particle. However, the development of such a system poses many potential difficulties, including the already mentioned problem of distinguishing between the target-bound and unbound label particles.

Another type of particles potentially suitable for the ultrastructural labeling are quantum dots (QDs). They are fluorescent semiconductor crystals 5-20 nm in size [51]. QDs emit bright and stable fluorescence characterized by a large Stokes shift and narrow emission spectra, which allows simultaneous imaging of a large number of targets.

QDs used in bioimaging have a core containing cadmium and other elements that are significantly heavier than the elements in biomolecules, which permits direct observation of QDs by EM. Therefore, QDs are intrinsically bimodal labels [52]. At the same time, their chemical composition makes them cytotoxic, thus limiting their use in biological research. Various novel types of QDs have been obtained that do not contain cadmium or do not have the metal core at all, but they are not as biologically inert as gold [53].

Even more significant problem is that the delivery of QDs into a living cell is associated with the same difficulties as the delivery of gold NPs. In general, QDs are a potential alternative to gold NPs as labels for cryoET, since they have the advantage of brighter fluorescence but the disadvantage of high cytotoxicity at the same time.

CLONEABLE NANOPARTICLES: METALLOTHIONEINS, FERRITINS, AND OTHERS

The concept of cloneable NPs (by analogy with cloneable or, in the terms of this review, genetically encoded labels) implies the creation of a polypeptide that under certain conditions, forms an electron-dense particle around itself. Research is underway in this field, and significant progress has been made.

Metallothioneins are a family of small (~6 kDa) proteins containing stretches of 20 cysteine residues, which allow them to bind ions of heavy metals (gold, silver, cadmium, etc.) [54, 55]. Metallothioneins have been found in different organisms [56]; they are involved in protection against the toxic effects of heavy metal salts [57] and oxidative stress [58].

The ability of metallothioneins to bind and concentrate metal ions allows their application as genetically encoded labels for identification of protein complexes isolated from cells [59, 60], as well as for labeling proteins inside the cells [61, 62] with subsequent visualization by various EM methods. To enhance the signal, researchers used the systems with tandemly arranged metallothioneins [63], but due to the small size of protein monomers (~6 kDa for metallothionein II), the molecular weight of a tandem of four metallothioneins is comparable to that of GFP. The labeling with heavy metal salts in living cells is usually performed for 30 min to several hours [59], which minimizes the toxic effect of these metals and makes metallothioneins applicable in cryoEM [59].

After successfully testing the metallothionein label with several model bacterial proteins [64], Diestra et al. used this technique to study the location of the Hqf protein in *Escherichia coli* cells by combining metallothionein labelling with cryo-substitution and acrylic resin embedding, as well as with the Tokuyasu technique (which allowed the use of immunogold as a control) [65]. Hqf controls the post-translational fate of mRNAs by organizing their interaction with special small non-coding RNAs involved in the regulation of the synthesis of bacterial membrane proteins [65]. This work demonstrated an unusual near-membrane location of Hqf, which in turn, indicated localized translation of membrane proteins and localized mechanisms for its control, as well as confirmed the versatility of the metallothionein labeling system.

Hirabayashi et al. [66] used metallothionein labeling in combination with the CEMOVIS method to study the organization of protein complexes controlled by PSD95 (postsynaptic density 95) protein on the postsynaptic membranes of neurons [66]. The authors

demonstrated that contrasting the ultrathin sections with heavy metals after chemical fixation did not allow to resolve the structures formed by this protein, just as it was impossible to detect them on the uncontrasted cryosections. Using a chimeric construct consisting of PSD95 and a tandem of three metallothioneins loaded with cadmium ions, the authors were able to show that the individual PSD95-containing small structures, which they termed the "cores", merged into the lamellar complexes under the postsynaptic membrane.

Metallothioneins were also tested in a model system with KRAS GTPase in the cells after cryofixation. This protein was previously shown to preferentially localize to the filopodia by ultrathin sectioning using a protein label incompatible with cryofixation, and then KRAS was detected in the same cell areas using a metallothionein label "developed" by loading with cadmium ions immediately before the plunge-freezing cryofixation [67]. The authors found that the location of KRAS was similar in both cases, noting a significantly better preservation of the intracellular environment during cryofixation, but also pointing out that the labeling efficiency was low. These examples demonstrate a potential versatility of metallothionein labels and their applicability for cryoET imaging.

Metallothionein-based labels have an advantage over some other systems (described in the text above and below) in the fact that the electron-dense particle is generated directly on the labeled molecule, which eliminates the problem of unbound particles, although the background signal from endogenous compounds that bind metal ions cannot be excluded.

Genetically encoded ferritin is another electron-dense label used in EM. Bacterial ferritin is a fairly large (approximately 12 nm in diameter), multimeric protein complex assembled from 24 FtnA monomers. It forms a cage capable of accumulating ~2000 iron atoms, which provides its high electron density and makes this protein detectable in EM images [68] (Fig. 1c). The possibility of using bacterial ferritin for *in situ* labeling of proteins in *E. coli* cells has been demonstrated in [26]. The authors expressed the recombinant genetic constructs encoding ferritin A in tandem with a membrane-targeting peptide and proteins with known localization, such as ZapA (marks the septum in a dividing bacterial cells) and CheY (component of the chemotactic receptor apparatus of *E. coli*, which forms clusters on the plasma membrane inner surface). This work showed that ferritin as a label for cryoET, is suitable for visualization and three-dimensional reconstruction of various functional compartments of bacterial cell. Interestingly, in its native environment, the ferritin label binds free endogenous ferritin molecules to form a cage (Fig. 1c).

However, expression of ferritin subunits with the mitochondrial localization signal in eukaryotic cells has led to undesirable effects, including mitochondrial aggregation and non-specific localization of ferritin particles, which was probably caused by the large size of protein complexes formed by ferritin and involvement of several labeled molecules in the formation of a single ferritin cage. This problem was solved by combining the ferritin label with a rapamycin-inducible system [69]. Ferritin is synthesized as a chimeric protein containing FRB (FKBP-rapamycin binding domain), while the target protein is fused with FKBP (FK506-binding protein). The binding of the ferritin label to the target protein can be induced by incubation with rapamycin for ~15 min only [69], thus minimizing the negative effects of prolonged exposure to rapamycin on the cells. To saturate the ferritin cage with iron atoms, the cells should be cultured in the presence of 1 mM Fe^{2+} for 16 h, which, according to the authors, had no toxic effect on the cells. The system was named FerriTag. Its operation in eukaryotic cells requires additional synthesis of unmodified ferritin molecules as a material for forming the bulk of the particles. A limitation of this technology is the inability to label proteins located inside membrane organelles, since the membranes prevent the interaction of FRB-ferritin with the FKBP-target. Additionally, the FRB-FKBP linkage increases the distance between the target and the label by approximately 5 nm [69].

This system was further optimized for the use in cryoET. The authors of [70] adjusted several parameters of FerriTag, in particular, by constructing a plasmid encoding all the necessary components (target-FKBP, ferritin light chain-FRB, and unmodified ferritin heavy chain). The arrangement of the corresponding genes in the plasmid relative to the IRES (internal ribosome entry site) ensured the correct quantitative ratio of the produced proteins. The system was tested for labeling and localization of the mitochondrial outer membrane proteins TOM20 and Bcl-xL and the membrane protein KRAS located on the cytoplasmic side of the plasma membrane.

In the study of the mechanisms of resistance of the *Pseudomonas moraviensis stanleyae* bacterium to the high concentrations of selenium compounds lethal to other organisms, it was discovered that bacterial cells formed selenium nanoparticles almost uniform in size and with a characteristic non-spherical, but symmetrical shape [71]. The authors identified the enzymes responsible for selenium reduction and reproduced the redox process carried out by the bacterium using glutathione reductase, selenite ion (SeO_3^{2-}), and NADPH. By controlling the reaction through changes in the selenite concentration, they obtained the particles ranging in size from 5 to 50 nm that formed around glutathione reductase molecules, gradually "entombing" the enzyme. The authors suggested that glutathione reductase may be one of the candidates

for the core in a labeling system based on cloneable nanoparticles.

Another option for the creation of cloneable nanoparticles involves the use of proteins labeled with metallothionein-based peptides modified to increase their stability [72]. This method, however, requires cryofixation followed by postfixation via the cryosubstitution procedure followed by the development of the labels through the reduction of gold ions and formation of particles. This method does not solve the problem of obtaining a labeling system that is fully compatible with cryoET, since the redox conditions required for this reaction (e.g., the use of borohydride $NaBH_4$) are unlikely to be realized in a living cell. However, this approach may become an effective replacement for the immunogold labeling, as it combines the advantages of cryofixation with cryosubstitution and ultrastructural localization.

We believe that further attempts in creating cloneable nanoparticles will lead to the emergence of "an EM analogue of GFP" in the future.

SPOT – PHOSPHORUS-BASED LABEL

Gold NPs, QDs, and metallothionein- and ferritin-based labels necessarily contain metal atoms or ions in high concentrations, while the representation of these elements in biomolecules is very low and their potential toxicity cannot be ignored. Among the elements normally present in biomolecules, phosphorus is the most efficient generator of elastically scattered electrons. Phosphorus-based labels have been created using DNA origami and RNA aptamer technologies. The method was named SPOT (signpost origami tag) [73].

DNA origami is a technology for creating nanostructures from a long single-stranded DNA scaffold molecule and short staple molecules complementary to specific segments of the scaffold. Interaction with the staples leads to the reproducible folding of the scaffold into a pre-designed conformation [74, 75]. DNA origami is a promising basis for creating nanodevices and drug delivery systems, although it is generally considered more as an object than a tool of microscopic research. In microscopy, DNA origami has been employed to create support films for EM visualization [76, 77] and to manufacture resolution test samples for sub-diffraction visible light microscopy [78].

Aptamers are oligonucleotides that selectively bind certain chemical groups; they represent nucleotide-based analogs of antibodies [79]. Aptamers are widely used as label carriers and are employed in visible light microscopy, flow cytometry, and various types of computer tomography in medicine and research [80, 81].

SPOT is a wedge-shaped DNA construct of about 7 MDa assembled from a 7.5-kb DNA scaffold and 238 staples. The narrow end of the wedge extends into a rigid stem, at the end of which sits an RNA aptamer that recognizes a fluorescent protein. The density of DNA packing in the SPOT construct ensures a high local concentration of phosphorus, and the asymmetric structure of the wedge, unlike the overwhelming majority of nanoparticle tags that have a spherical or close to spherical shape, indicates the direction to the target, while the long rigid "stem" separates the massive body of the label from the tagged structure, thus minimizing the effect of the label without reducing the accuracy of localization [73] (Fig. 1d). The authors tested the resulting construct using fluorescent constructs based on the herpes simplex virus glycoprotein B located on vesicles and cell membranes and membrane glycoprotein of murine leukemia virus located on viral particles as targets.

The SPOT construct proved to be non-toxic and clearly visible in cryoET images due to its size, shape, and electron density. Since SPOT binds to the target via a fluorescent protein, it is a bimodal label. The main and unique feature of SPOT is that due to its shape, it literally points to the exact location of the target molecule. As the authors of the method claimed, they were able to trace the transition of the SPOT stem into the electron density corresponding to the labeled protein [73].

Despite the listed advantages, the obvious limitation of the SPOT label is its complexity: it is assembled *ex vivo*, undergoes complex purification procedure, and does not penetrate into a living cell. However, the combination of the SPOT properties, as the authors pointed out, makes this system a highly efficient tool for studying proteins on the external surfaces of natural and artificial vesicles, viruses, and cell membranes. At the time of writing this article, we were unable to find published reports on the experimental application of SPOT labeling technique.

ENCAPSULINS – VIRUS-LIKE PROTEIN NANOCOMPARTMENTS

Encapsulins, which are naturally occurring bacterial nanocompartments, offer a yet another basis for developing techniques for labeling subcellular structures for cryoET. These protein complexes were initially identified in the *Brevibacterium* secretions during investigation of their bacteriostatic properties [82]. Encapsulins have a significant biotechnological potential as the vehicles for the delivery of therapeutics into the cells [83].

Encapsulins self-assemble into virus-like icosahedral structures measuring 22 nm or more in diameter [84].

These particles are defined by their triangulation number, such as T = 1 for encapsulins isolated from *Mycobacterium tuberculosis* [85] or T = 3 for nanocompartments from *Myxococcus xanthus*. Currently, the largest known encapsulins, measuring 42 nm in diameter, have T = 4 and are produced by *Quasibacillus thermotolerans* [85-87].

Encapsulins accumulate inside the cells without any significant toxic effect [88], which makes them suitable for the use as genetically encoded labels in EM. While their virus-like shape allows identification in electronograms, their contrast is insufficient for labeling intracellular protein complexes, necessitating signal enhancement. By combining various encapsulin types with differing amounts of inward-facing metallothioneins during nanocompartment assembly, researchers have created a series of large particles capable of binding metal ions. This advancement resulted in the appearance of a novel category of EM labels known as EMcapsulins [89], which can be used not only in *in vitro* cultured cells but also in living model organisms. For instance, researchers employed EMcapsulins to label different cell types in the nervous systems of *Drosophila* and mice, facilitating subsequent connectome reconstruction using FIB-SEM [89].

Finally, it was demonstrated that encapsulin-based tags can be used as selective markers of various cell compartments in the correlation fluorescence microscopy and cryoET. For this purpose, a system called GEM2 [41] was developed based on Srp1 encapsulin from *Synechococcus elongatus*. To prevent aggregation of the protein labeled with large (25-nm) particles formed by encapsulins, the authors designed a system that ensured particle binding to the target molecule only in the presence of the added ligand. To accomplish this, encapsulin and the protein of interest were linked to the above-mentioned FRB and FKBP, respectively, whose binding was initiated by rapamycin (Fig. 1e). The designed system was tested in cryoET with several model targets: mitoGFP located on the outer mitochondrial membrane, mEGFP-Ki-67 located on the surface of chromosomes in a dividing cell, Nup96-eGFP nucleoporin (which faces the cytoplasm), and Seipin-sfGFP, an endoplasmic reticulum protein that pinches off lipid droplets from the membranes. In all these cases, the natural contrast of such large particles was sufficient for localization of labeled proteins and automated analysis of cryotomograms using convolutional neural networks.

The potential of this technology as a selective labeling technique for EM is evident. However, it is crucial to acknowledge that despite advancements in the application of encapsulins, these techniques involve complex and laborious procedures, including genetic engineering and expression of large genetic constructs in the cell. Therefore, meticulous monitoring is essential to ensure that the structure and functions of macromolecular complexes labeled through this approach remain intact [41]. It is also worth noting that in this multicomponent system, the linker between the encapsulin particle and the target is approximately 20 nm long, which undoubtedly affects the accuracy of target localization. Finally, the authors found that the kinetics of tag binding to the target depends on the target concentration [41].

METABOLIC LABELING

Metabolic tags are diverse molecules that are consumed by cells during various cellular processes. What distinguishes this approach from the above-described methods is its focus on targeting subcellular compartments rather than specific proteins. The use of metabolic tags, along with immunoEM techniques, can be considered as one of the first approaches to imaging subcellular components using EM. For example, the Golgi apparatus was specifically labeled at the ultrastructural level by incubating cells with ceramide conjugated to a fluorescent molecule. Subsequent photooxidation of diaminobenzidine upon the tag irradiation with visible light formed the electron density in the *trans*-Golgi zone [90].

The target of this labeling procedure is the endosomal-lysosomal compartment, which occupies a significant portion of the cell's volume and interacts with both endoplasmic reticulum and the Golgi apparatus. The loading of tags into the endosomal compartment is performed by the natural cellular mechanism of endocytosis, which automatically solves the issue of tag delivery. Either nanoparticles are absorbed directly or bovine serum albumin (BSA) is used as the label carrier, which can be conjugated with both fluorescent dye and metal particles. A combination of this approach with light microscopy has been applied to study the restructuring the cell vacuolar system in infections caused by *Salmonella enterica* and *Salmonella typhimurium* [91]. Recently, bimodal BSA was obtained, which carried both a fluorescent group and a gold particle as an electron-dense. It was tested for applicability in several microscopic modalities, including fluorescence microscopy, correlation light fluorescence, EM in ultrathin sections with chemical fixation, and correlative cryoET, in which such bimodal labels are in high demand [92]. In addition to the main function (marking of the endosomal-lysosomal compartment lumen), contrasting gold particles conjugated with BSA can be used as fiducial markers for the alignment of a series of angular projections during tomogram reconstruction [92] (Fig. 1e). Moreover, the proposed design and the method of its synthesis protect the fluorophore from quenching by closely

spaced metal atoms, which, as the authors pointed out, is a serious problem in the design and synthesis of bimodal labels [92]. Therefore, metabolic labeling is an efficient and physiological approach to labeling subcellular compartments in cryoET, even though it is applicable to a limited range of tasks.

CONCLUSIONS

Kaufman et al. [20] articulated the motivations behind the development of labeling techniques compatible with cryofixation and cryoEM analysis of cellular and tissue samples, including a need to detect a cell of interest or a rare event in a heterogeneous cell population, identify a studied molecular ensemble or a specific molecule in an EM image [25], or reveal a yet unknown process or structure based on the involvement of the studied molecule in it. And while the first two tasks can be solved using genetically encoded fluorescent labels and the correlation approach (CLEM), the remaining tasks require the use of labeling technologies at a completely different level.

Two questions arise. How does the preservation of the native state of biomolecules achieved by cryofixation combined with the use of labels affect the functioning and behavior of the labeled molecule in a living cell? What new information can be obtained using compromise solutions to the first of these issues?

The first question is rather difficult to answer in general because the methods of ultrastructural labeling are not yet widely used in the cryo-electronic format, and their pitfalls will appear gradually with a wider introduction of such methods into laboratory practice. Considering the experience accumulated for the application of GFP and its analogs, there is hope that possible solutions to emerging problems will be found quickly, as the studies on the optimization of such techniques are already underway [66, 86].

What problems requiring the use of cryoET are difficult to solve without using the labels? The answer is: (i) localization of specific genomic sequences in the intact nucleus, (ii) obtaining three-dimensional structures of globular proteins in their native environment, and (iii) determination of the orientation of protein subunits in native multimeric intracellular complexes.

While having the ability to use an electron-dense label to mark proteins like TALEs (transcription activator-like effectors) [93, 94] and dCas [95], researchers can identify specific sequences in the genome and, by attaching labels to the regulatory components of multimeric enzymatic complexes, identify areas in which the studied process (transcription, replication, DNA repair, processing of certain species of RNA) take place. Such targets are indistinguishable on the chromatin background, and the use of labels seems necessary to solve the problem of their localization. The ability to implement such labeling in combination with cryofixation will significantly expand our understanding of the molecular mechanisms of these processes because of the preservation of the native or near-native structures in which the process occurs and involved proteins.

Finally, the ability to label a protein in such a way that its orientation in space can be determined using the label makes it possible to solve the problem of determining the position and, possibly, to identify the role of a given molecule in the functioning of the entire structure. Such attempts have been made, although not in intact cells, but in the isolated axoneme [96]. Experiments were conducted to establish the relative position and orientation of protein molecules using labeling with gold NPs.

Similar attempts have been made with the preparations of the basal and apical plasma membranes, in which the N-terminal FerriTag label was used to localize the Hip1r protein that links the clathrin coating of endocytic vesicles with actin filaments [97]. The position of the label indicated the parallel orientation of Hip1r dimers with the C-terminal parts directed toward the membrane of the forming vesicle and the labeled N-terminus directed into the cytoplasm, either perpendicular or at an angle to the clathrin mesh. Notably, in this study, FerriTag was detected without loading it with iron ions, but based only on the shape of the ferritin cage.

None of the described technologies is universal (Fig. 1, Table 1), especially when compared with fluorescent immunocytochemistry and chimeric fluorescent protein technology in visible light microscopy. Each method has a number of features that may seem as disadvantages, but the variety of labels and methods of their delivery allows researchers to choose the most suitable system for solving a specific research problem, taking into account such factors as known or supposed location of biological object, its tolerance to labeling conditions, duration of the experiment, etc.

Many works cited in the review are proof-of-concept studies. Successful application of the described methods for visualizing a specific target in a specific cell type does not guarantee the detection of the same or another target in another type of cells. To transfer the protocols to other objects, physiological controls are necessary to evaluate the effect of an excessively massive or potentially toxic label on cell physiology.

All described methods will undoubtedly benefit from the integration of a fluorescent component, allowing for the monitoring of the labeling efficiency and physiological state of cells during the experiment, selection of cells for the study, and identification of areas for subsequent lamella fabrication [20].

Table 1. Tested labeling methods compatible with cryofixation

Method	Label	Label carrier/ label-target linkage	Cell permeable?	Advantages	Disadvantages
Live immunogold labeling	gold NPs	antibodies/ covalent, *ex vivo*	no	established methodology	only for labeling extracellular membrane targets
Metallothioneins	metal NPs	chimeric protein/ covalent during synthesis in the cell	synthesized in the cell	established methodology, small label size, no unbound label	requires incubation of cells with heavy metal salts
FerriTag	proteinaceous nanocage + iron ions	chimeric protein/ rapamycin-induced binding in the cell	synthesized in the cell	well-developed methodology	requires incubation of cells with heavy metal salts
SPOT	wedge-shape DNA origami	chimeric protein with GFP/via RNA aptamer	no	target localization accuracy, bimodal system	complex design, only for labeling extracellular membrane targets
Encapsulins GEM2	protein nanocage	chimeric protein/ rapamycin-induced binding in the cell	synthesized in the cell	do not contain metals, no effect on the target molecule prior to the rapamycin signal	large size, long linker, complex stoichiometry
Metabolic labeling	gold NPs + fluorophore	BSA/adsorption + covalent binding *ex vivo*	endocytosis	bimodal, biologically inert, works as a fiducial marker for aligning a series of sections	only for labeling the endosome-lysosomal compartment

CryoET, as perhaps the most promising and powerful implementation of cryoEM used for solving the problems of cell biology, has already taken its place in the arsenal of researchers, but the development of labeling systems for the cryo-format is slow. This is largely due to the objective complexity of the problem itself. The second reason for the slow development of selective labeling tools may be that the current demand for transmission cryoelectron microscopes significantly exceeds the availability of high-tech equipment for these studies. This puts researchers developing such labeling systems in a difficult situation when they have to risk expensive instrument time. However, the appearance of works, such as recent preprints by Wang et al. [70] and Sun et al. [97], who used FerriTag to localize protein molecules in macromolecular complexes to establish their structure and orientation, perfectly illustrates both this thesis and the general demand for the development in this study field.

At present, it is difficult to predict the direction in which the next breakthrough will occur, and whether it will occur at all. Perhaps, a universal labeling system for cryoET will never be invented, and researchers will need to choose a method from a set of specialized techniques. It is also possible that a completely different solution to the problem of the object localization will be found, for example, the application of the elemental composition analysis for localization of “elemental labels” [98, 99] (although such method requires even more specialized equipment) or by implementing effective algorithms for solving the inverse problem, namely, identification of a protein in a cryo-tomogram based on its known or predicted structure (which no longer can be considered labeling). These methods are already entering research practice, and a still small number of examples of their application is gradually increasing [100, 101]. Or, perhaps, the progress will follow the path of improving the correlation microscopy and will result in the creation of a visible light fluorescence microscope with a subdiffraction resolution of about 30 nm or even less that would operate under cryogenic conditions [19] and will be supported by the introduction of a unified format for the data transfer between different instruments.

Contributions. E.P.K. and S.A.G. developed the concept and wrote the text of the article; I.I.K. edited the manuscript.

Funding. The work was supported by the Russian Science Foundation (project no. 23-74-00021).

Ethics approval and consent to participate. This work does not contain studies involving human or animal subjects.

Conflict of interest. The authors of this work declare that they have no conflicts of interest.

REFERENCES

1. Osumi, M. (2012) Visualization of yeast cells by electron microscopy, *J. Electron. Microsc.*, **61**, 343-365, https://doi.org/10.1093/jmicro/dfs082.
2. Knott, G., and Genoud, C. (2013) Is EM dead? *J. Cell Sci.*, **126**, 4545-4552, https://doi.org/10.1242/jcs.124123.
3. Sengupta, R., Poderycki, M. J., and Mattoo, S. (2019) CryoAPEX – an electron tomography tool for subcellular localization of membrane proteins, *J. Cell Sci.*, **132**, jcs222315, https://doi.org/10.1242/jcs.222315.
4. Walther, P., Schmid, E., and Höhn, K. (2013) High-pressure freezing for scanning transmission electron tomography analysis of cellular organelles, *Methods Mol. Biol.*, **931**, 525-535, https://doi.org/10.1007/978-1-62703-056-4_28.
5. Moor, H. (1987) Theory and practice of high pressure freezing, in *Cryotechniques in Biological Electron Microscopy*, Berlin, Heidelberg, Springer Berlin Heidelberg, pp. 175-191, https://doi.org/10.1007/978-3-642-72815-0_8.
6. Studer, D., Humbel, B. M., and Chiquet, M. (2008) Electron microscopy of high pressure frozen samples: bridging the gap between cellular ultrastructure and atomic resolution, *Histochem. Cell Biol.*, **130**, 877-889, https://doi.org/10.1007/s00418-008-0500-1.
7. Dubochet, J., and McDowall, A. W. (1981) Vitrification of pure water for electron microscopy, *J. Microsc.*, **124**, 3-4, https://doi.org/10.1111/j.1365-2818.1981.tb02483.x.
8. D'Imprima, E., Fung, H. K., Zagoriy, I., and Mahamid, J. (2024) Cryogenic Preparations of Biological Specimens for Cryo-Electron Tomography, in *Cryo-Electron Tomography: Structural Biology in situ*, Cham, Springer International Publishing, 85-114, https://doi.org/10.1007/978-3-031-51171-4_6.
9. Williams, D.B. and Carter, C.B. (2009) "Transmission Electron Microscopy: A Textbook for Materials Science," 2nd Edition, Springer, New York, pp. 1-757.
10. Tokuyasu, K. T. (1973) A technique for ultracryotomy of cell suspensions and tissues, *J. Cell Biol.*, **57**, 551-565, https://doi.org/10.1083/jcb.57.2.551.
11. McDowall, A. W., Chang, J. J., Freeman, R., Lepault, J., Walter, C. A., and Dubochet, J. (1983) Electron microscopy of frozen hydrated sections of vitreous ice and vitrified biological samples, *J. Microsc.*, **131**, 1-9, https://doi.org/10.1111/j.1365-2818.1983.tb04225.x.
12. Al-Amoudi, A., Norlen, L. P., and Dubochet, J. (2004) Cryo-electron microscopy of vitreous sections of native biological cells and tissues, *J. Struct. Biol.*, **148**, 131-135, https://doi.org/10.1016/j.jsb.2004.03.010.
13. Marko, M., Hsieh, C., Moberlychan, W., Mannella, C. A., and Frank, J. (2006) Focused ion beam milling of vitreous water: prospects for an alternative to cryo-ultramicrotomy of frozen-hydrated biological samples, *J. Microsc.*, **222**, 42-47, https://doi.org/10.1111/j.1365-2818.2006.01567.x.
14. Marko, M., Hsieh, C., Schalek, R., Frank, J., and Mannella, C. (2007) Focused-ion-beam thinning of frozen-hydrated biological specimens for cryo-electron microscopy, *Nat. Methods*, **4**, 215-217, https://doi.org/10.1038/nmeth1014.
15. Rigort, A., Bäuerlein, F. J., Villa, E., Eibauer, M., Laugks, T., Baumeister, W., and Plitzko, J. M. (2012) Focused ion beam micromachining of eukaryotic cells for cryoelectron tomography, *Proc. Natl. Acad. Sci. USA*, **109**, 4449-4454, https://doi.org/10.1073/pnas.1201333109.
16. Frangakis A. S. (2024) Principles of tomographic reconstruction, in *Cryo-Electron Tomography. Focus on Structural Biology* (Förster, F., and Briegel, A., eds) Springer, Cham, Vol. 11, doi.org/10.1007/978-3-031-51171-4_26.
17. Xue, L., Lenz, S., Zimmermann-Kogadeeva, M., Tegunov, D., Cramer, P., Bork, P., Rappsilber, J., and Mahamid, J. (2022) Visualizing translation dynamics at atomic detail inside a bacterial cell, *Nature*, **610**, 205-211, https://doi.org/10.1038/s41586-022-05255-2.
18. Jin, W., Zhou, Y., and Bartesaghi, A. (2024) Accurate size-based protein localization from cryo-ET tomograms, *J. Struct. Biol.*, **10**, 100104, https://doi.org/10.1016/j.yjsbx.2024.100104.
19. DeRosier, D. J. (2021) Where in the cell is my protein? *Q. Rev. Biophys.*, **54**, e9, https://doi.org/10.1017/S003358352100007X.
20. Kaufmann, R., Grünewald, K., and Baker, L.A. (2024) Targeting, Localisation and Identification, in *Cryo-Electron Tomography. Focus on Structural Biology* (Förster, F., and Briegel, A., eds) Springer, Cham, vol. 11, https://doi.org/10.1007/978-3-031-51171-4_6.
21. Beales, C. T., and Medalia, O. (2022) Gold nanomaterials and their potential use as cryo-electron tomography labels, *J. Struct. Biol.*, **214**, 107880, https://doi.org/10.1016/j.jsb.2022.107880.
22. Groysbeck, N., Hanss, V., Donzeau, M., Strub, J. M., Cianférani, S., Spehner, D., Bahri, M., Ersen, O., Eltsov, M., Schultz, P., and Zuber, G. (2023) Bioactivated and PEG-protected circa 2 nm gold nanoparticles for in cell labelling and Cryo-electron microscopy, *Small Methods*, **7**, e2300098, https://doi.org/10.1002/smtd.202300098.

23. Eltsov, M., Maclellan, K. M., Maeshima, K., Frangakis, A. S., and Dubochet, J. (2008) Analysis of cryo-electron microscopy images does not support the existence of 30-nm chromatin fibers in mitotic chromosomes in situ, *Proc. Natl. Acad. Sci. USA*, **105**, 19732-19737, https://doi.org/10.1073/pnas.0810057105.
24. Nishino, Y., Eltsov, M., Joti, Y., Ito, K., Takata, H., Takahashi, Y., Hihara, S., Frangakis, A. S., Imamoto, N., Ishikawa, T., and Maeshima, K. (2012) Human mitotic chromosomes consist predominantly of irregularly folded nucleosome fibres without a 30-nm chromatin structure, *EMBO J.*, **31**, 1644-1653, https://doi.org/10.1038/emboj.2012.35.
25. Plitzko, J. M., and Bollschweiler, D., (2024) Fundamentals of Instrumentation and Electron Optics for Cryo-Electron Tomography, in *Cryo-Electron Tomography. Focus on Structural Biology* (Förster, F., and Briegel, A., eds) Springer, Cham, vol. 11, https://doi.org/10.1007/978-3-031-51171-4_1.
26. Wang, Q., Mercogliano, C. P., and Löwe, J. (2011) A ferritin-based label for cellular electron cryotomography, *Structure*, **19**, 147-154, https://doi.org/10.1016/j.str.2010.12.002.
27. De Boer, P., Hoogenboom, J. P., and Giepmans, B. N. (2015) Correlated light and electron microscopy: ultrastructure lights up!, *Nat. Methods*, **12**, 503-513, https://doi.org/10.1038/nmeth.3400.
28. Horisberger, M., and Rosset, J. (1977) Colloidal gold, a useful marker for transmission and scanning electron microscopy, *J. Histochem. Cytochem.*, **25**, 295-305, https://doi.org/10.1177/25.4.323352.
29. Hainfeld, J. F., and Furuya, F. R. (1992) A 1.4-nm gold cluster covalently attached to antibodies improves immunolabeling, *J. Histochem. Cytochem.*, **40**, 177-184, https://doi.org/10.1177/40.2.1552162.
30. Bendayan, M. (1995) Colloidal gold post-embedding immunocytochemistry, *Progr. Histochem. Cytochem.*, **29**, 1-159, https://doi.org/10.1016/s0079-6336(11)80027-6.
31. Connor, E. E., Mwamuka, J., Gole, A., Murphy, C. J., and Wyatt, M. D. (2005) Gold nanoparticles are taken up by human cells but do not cause acute cytotoxicity, *Small*, **1**, 325-327, https://doi.org/10.1002/smll.200400093.
32. Yi, H., Strauss, J. D., Ke, Z., Alonas, E., Dillard, R. S., Hampton, C. M., Lamb, K. M., Hammonds, J. E., Santangelo, P. J., Spearman, P. W., and Wright, E. R. (2015) Native immunogold labeling of cell surface proteins and viral glycoproteins for cryo-electron microscopy and cryo-electron tomography applications, *J. Histochem. Cytochem.*, **63**, 780-792, https://doi.org/10.1369/0022155415593323.
33. Dahan, I., Sorrentino, S., Boujemaa-Paterski, R., and Medalia, O. (2018) Tiopronin-protected gold nanoparticles as a potential marker for cryo-EM and tomography, *Structure*, **26**, 1408-1413.e3, https://doi.org/10.1016/j.str.2018.06.009.
34. Jiang, J., Cheong, K. Y., Falkowski, P. G., and Dai, W. (2021) Integrating on-grid immunogold labeling and cryo-electron tomography to reveal photosystem II structure and spatial distribution in thylakoid membranes, *J. Struct. Biol.*, **213**, 107746, https://doi.org/10.1016/j.jsb.2021.107746.
35. Petersen, J. D., Mekhedov, E., Kaur, S., Roberts, D. D., and Zimmerberg, J. (2022) Endothelial cells release hybrid extracellular vesicles: microvesicles that secrete exosomes, *Biophys. J.*, **121**, 293a, https://doi.org/10.1016/j.bpj.2021.11.1285.
36. Koifman, N., Nir-Shapira, M., and Talmon, Y. (2023) Selective labeling of phosphatidylserine for cryo-TEM by a two-step immunogold method, *J. Struct. Biol.*, **215**, 108025, https://doi.org/10.1016/j.jsb.2023.108025.
37. Kireev, I., Lakonishok, M., Liu, W., Joshi, V. N., Powell, R., and Belmont, A. S. (2008) *In vivo* immunogold labeling confirms large-scale chromatin folding motifs, *Nat. Methods*, **5**, 311-313, https://doi.org/10.1038/nmeth.1196.
38. Orlov, I., Schertel, A., Zuber, G., Klaholz, B., Drillien, R., Weiss, E., Schultz, P., and Spehner, D. (2015) Live cell immunogold labelling of RNA polymerase II, *Sci. Rep.*, **5**, 8324, https://doi.org/10.1038/srep08324.
39. Fontana, J., Tzeng, W. P., Calderita, G., Fraile-Ramos, A., Frey, T. K., and Risco, C. (2007) Novel replication complex architecture in rubella replicon-transfected cells, *Cell. Microbiol.*, **9**, 875-890, https://doi.org/10.1111/j.1462-5822.2006.00837.x.
40. Teng, K. W., Ishitsuka, Y., Ren, P., Youn, Y., Deng, X., Ge, P., Lee, S. H., Belmont, A. S., and Selvin, P. R. (2016) Labeling proteins inside living cells using external fluorophores for microscopy, *eLife*, **5**, e20378, https://doi.org/10.7554/eLife.20378.
41. Fung, H. K. H., Hayashi, Y., Salo, V. T., Babenko, A., Zagoriy, I., Brunner, A., Ellenberg, J., Müller, C. W., Cuylen-Haering, S., and Mahamid, J. (2023) Genetically encoded multimeric tags for subcellular protein localization in cryo-EM, *Nat. Methods*, **20**, 1900-1908, https://doi.org/10.1038/s41592-023-02053-0.
42. Yadav, A., Verma, N. C., Rao, C., Mishra, P. M., Jaiswal, A., and Nandi, C. K. (2020) Bovine serum albumin-conjugated red emissive gold nanocluster as a fluorescent nanoprobe for super-resolution microscopy, *J. Phys. Chem. Lett.*, **11**, 5741-5748, https://doi.org/10.1021/acs.jpclett.0c01354.
43. Tan, Y., He, K., Tang, B., Chen, H., Zhao, Z., Zhang, C., Lin, L., and Liu, J. (2020) Precisely regulated luminescent gold nanoparticles for identification of cancer metastases, *ACS Nano*, **14**, 13975-13985, https://doi.org/10.1021/acsnano.0c06388.
44. Phan, M. D., Kim, H., Lee, S., Yu, C. J., Moon, B., and Shin, K. (2017) HIV peptide-mediated binding behaviors of nanoparticles on a lipid membrane, *Langmuir*, **33**, 2590-2595, https://doi.org/10.1021/acs.langmuir.6b04234.

45. Quan, X., Sun, D., and Zhou, J. (2019) Molecular mechanism of HIV-1 TAT peptide and its conjugated gold nanoparticles translocating across lipid membranes, *Phys. Chem. Chem. Phys.*, **21**, 10300-10310, https://doi.org/10.1039/c9cp01543d.
46. Morgan, E., Doh, J., Beatty, K., and Reich, N. (2019) VIPERnano: improved live cell intracellular protein tracking, *ACS Appl. Mater. Interf.*, **11**, 36383-36390, https://doi.org/10.1021/acsami.9b12679.
47. Ahwazi, R. P., Kiani, M., Dinarvand, M., Assali, A., Tekie, F. S. M., Dinarvand, R., and Atyabi, F. (2020) Immobilization of HIV-1 TAT peptide on gold nanoparticles: a feasible approach for siRNA delivery, *J. Cell. Physiol.*, **235**, 2049-2059, https://doi.org/10.1002/jcp.29105.
48. Jiménez-Mancilla, N., Ferro-Flores, G., Santos-Cuevas, C., Ocampo-García, B., Luna-Gutiérrez, M., Azorín-Vega, E., Isaac-Olivé, K., Camacho-López, M., and Torres-García, E. (2013) Multifunctional targeted therapy system based on (99m) Tc/(177) Lu-labeled gold nanoparticles-Tat(49-57)-Lys(3) -bombesin internalized in nuclei of prostate cancer cells, *J. Labell. Compounds Radiopharmaceut.*, **56**, 663-671, https://doi.org/10.1002/jlcr.3087.
49. Keppler, A., Gendreizig, S., Gronemeyer, T., Pick, H., Vogel, H., and Johnsson, K. (2003) A general method for the covalent labeling of fusion proteins with small molecules *in vivo*, *Nat. Biotechnol.*, **21**, 86-89, https://doi.org/10.1038/nbt765.
50. Los, G. V., Encell, L. P., McDougall, M. G., Hartzell, D. D., Karassina, N., Zimprich, C., Wood, M. G., Learish, R., Ohana, R. F., Urh, M., Simpson, D., Mendez, J., Zimmerman, K., Otto, P., Vidugiris, G., Zhu, J., Darzins, A., Klaubert, D. H., Bulleit, R. F., and Wood, K. V. (2008) HaloTag: a novel protein labeling technology for cell imaging and protein analysis, *ACS Chem. Biol.*, **3**, 373-382, https://doi.org/10.1021/cb800025k.
51. Vu, T. Q., Lam, W. Y., Hatch, E. W., and Lidke, D. S. (2015) Quantum dots for quantitative imaging: from single molecules to tissue, *Cell Tissue Res.*, **360**, 71-86, https://doi.org/10.1007/s00441-014-2087-2.
52. Giepmans, B. N., Deerinck, T. J., Smarr, B. L., Jones, Y. Z., and Ellisman, M. H. (2005) Correlated light and electron microscopic imaging of multiple endogenous proteins using Quantum dots, *Nat. Methods*, **2**, 743-749, https://doi.org/10.1038/nmeth791.
53. Huang, X., and Tang, M. (2021) Research advance on cell imaging and cytotoxicity of different types of quantum Dots, *J. Appl. Toxicol.*, **41**, 342-361, https://doi.org/10.1002/jat.4083.
54. Nielson, K. B., Atkin, C. L., and Winge, D. R. (1985) Distinct metal-binding configurations in metallothionein, *J. Biol. Chem.*, **260**, 5342-5350, https://doi.org/10.1016/S0021-9258(18)89027-5.
55. Mercogliano, C. P., and DeRosier, D. J. (2006) Gold nanocluster formation using metallothionein: mass spectrometry and electron microscopy, *J. Mol. Biol.*, **355**, 211-223, https://doi.org/10.1016/j.jmb.2005.10.026.
56. Ziller, A., and Fraissinet-Tachet, L. (2018) Metallothionein diversity and distribution in the tree of life: a multifunctional protein, *Metallomics*, **10**, 1549-1559, https://doi.org/10.1039/c8mt00165k.
57. Nordberg, M., and Nordberg, G. F. (2000) Toxicological aspects of metallothionein, *Cell. Mol. Biol.*, **46**, 451-463.
58. Ruttkay-Nedecky, B., Nejdl, L., Gumulec, J., Zitka, O., Masarik, M., Eckschlager, T., Stiborova, M., Adam, V., and Kizek, R. (2013) The role of metallothionein in oxidative stress, *Int. J. Mol. Sci.*, **14**, 6044-6066, https://doi.org/10.3390/ijms14036044.
59. Mercogliano, C. P., and DeRosier, D. J. (2007) Concatenated metallothionein as a clonable gold label for electron microscopy, *J. Struct. Biol.*, **160**, 70-82, https://doi.org/10.1016/j.jsb.2007.06.010.
60. Bouchet-Marquis, C., Pagratis, M., Kirmse, R., and Hoenger, A. (2012) Metallothionein as a clonable high-density marker for cryo-electron microscopy, *J. Struct. Biol.*, **177**, 119-127, https://doi.org/10.1016/j.jsb.2011.10.007.
61. Risco, C., Sanmartín-Conesa, E., Tzeng, W. P., Frey, T. K., Seybold, V., and de Groot, R. J. (2012) Specific, sensitive, high-resolution detection of protein molecules in eukaryotic cells using metal-tagging transmission electron microscopy, *Structure*, **20**, 759-766, https://doi.org/10.1016/j.str.2012.04.001.
62. Barajas, D., Martín, I. F., Pogany, J., Risco, C., and Nagy, P. D. (2014) Noncanonical role for the host Vps4 AAA^+ ATPase ESCRT protein in the formation of Tomato bushy stunt virus replicase, *PLoS Pathog.*, **10**, e1004087, https://doi.org/10.1371/journal.ppat.1004087.
63. Nishino, Y., Yasunaga, T., and Miyazawa, A. (2007) A genetically encoded metallothionein tag enabling efficient protein detection by electron microscopy, *J. Electron Microsc.*, **56**, 93-101, https://doi.org/10.1093/jmicro/dfm008.
64. Diestra, E., Fontana, J., Guichard, P., Marco, S., and Risco, C. (2009) Visualization of proteins in intact cells with a clonable tag for electron microscopy, *J. Struct. Biol.*, **165**, 157-168, https://doi.org/10.1016/j.jsb.2008.11.009.
65. Diestra, E., Cayrol, B., Arluison, V., and Risco, C. (2009) Cellular electron microscopy imaging reveals the localization of the Hfq protein close to the bacterial membrane, *PLoS One*, **4**, e8301, https://doi.org/10.1371/journal.pone.0008301.
66. Hirabayashi, A., Fukunaga, Y., and Miyazawa, A. (2014) Structural analysis of the PSD-95 cluster by electron tomography and CEMOVIS: a proposal for the application of the genetically encoded metallothionein tag, *Microscopy*, **63**, 227-234, https://doi.org/10.1093/jmicro/dfu006.

67. Grandinetti, G., Goetz, D., Santas, A. J., Chinthalapudi, K., and Trout, A. (2024) Tracking intracellular proteins of interest with cryo-electron microscopy, *Microsc. Microanal.*, **30**, ozae044.387, https://doi.org/10.1093/mam/ozae044.387.
68. Jutz, G., van Rijn, P., Santos Miranda, B., and Böker, A. (2015) Ferritin: a versatile building block for bionanotechnology, *Chem. Rev.*, **115**, 1653-1701, https://doi.org/10.1021/cr400011b.
69. Clarke, N. I., and Royle, S. J. (2018) FerriTag is a new genetically-encoded inducible tag for correlative light-electron microscopy, *Nat. Commun.*, **9**, 2604, https://doi.org/10.1038/s41467-018-04993-0.
70. Wang, C., Iacovache, I., and Zuber, B. (2024) Genetically encoded FerriTag as a specific label for cryo-electron tomography, *bioRxiv*, https://doi.org/10.1101/2024.09.10.612178.
71. Ni, T. W., Staicu, L. C., Nemeth, R. S., Schwartz, C. L., Crawford, D., Seligman, J. D., Hunter, W. J., Pilon-Smits, E. A., and Ackerson, C. J. (2015) Progress toward clonable inorganic nanoparticles, *Nanoscale*, **7**, 17320-17327, https://doi.org/10.1039/c5nr04097c.
72. Jiang, Z., Jin, X., Li, Y., Liu, S., Liu, X. M., Wang, Y. Y., Zhao, P., Cai, X., Liu, Y., Tang, Y., Sun, X., Liu, Y., Hu, Y., Li, M., Cai, G., Qi, X., Chen, S., Du, L. L., and He, W. (2020) Genetically encoded tags for direct synthesis of EM-visible gold nanoparticles in cells, *Nat. Methods*, **17**, 937-946, https://doi.org/10.1038/s41592-020-0911-z.
73. Silvester, E., Vollmer, B., Pražák, V., Vasishtan, D., Machala, E. A., Whittle, C., Black, S., Bath, J., Turberfield, A. J., Grünewald, K., and Baker, L. A. (2021) DNA origami signposts for identifying proteins on cell membranes by electron cryotomography, *Cell*, **184**, 1110-1121.e16, https://doi.org/10.1016/j.cell.2021.01.033.
74. Rothemund, P. W. (2006) Folding DNA to create nanoscale shapes and patterns, *Nature*, **440**, 297-302, https://doi.org/10.1038/nature04586.
75. Jiang, Q., Shang, Y., Xie, Y., and Ding, B. (2024) DNA origami: from molecular folding art to drug delivery technology, *Adv. Mater.*, **36**, e2301035, https://doi.org/10.1002/adma.202301035.
76. Mallik, L., Dhakal, S., Nichols, J., Mahoney, J., Dosey, A. M., Jiang, S., Sunahara, R. K., Skiniotis, G., and Walter, N. G. (2015) Electron microscopic visualization of protein assemblies on flattened DNA origami, *ACS Nano*, **9**, 7133-7141, https://doi.org/10.1021/acsnano.5b01841.
77. Aissaoui, N., Mills, A., Lai-Kee-Him, J., Triomphe, N., Cece, Q., Doucet, C., Bonhoure, A., Vidal, M., Ke, Y., and Bellot, G. (2024) Free-standing DNA origami superlattice to facilitate Cryo-EM visualization of membrane vesicles, *J. Am. Chem. Soc.*, **146**, 12925-12932, https://doi.org/10.1021/jacs.3c07328.
78. Raab, M., Jusuk, I., Molle, J., Buhr, E., Bodermann, B., Bergmann, D., Bosse, H., and Tinnefeld, P. (2018) Using DNA origami nanorulers as traceable distance measurement standards and nanoscopic benchmark structures, *Sci. Rep.*, **8**, 1780, https://doi.org/10.1038/s41598-018-19905-x.
79. Bouvier-Müller, A., and Ducongé, F. (2018) Application of aptamers for in vivo molecular imaging and theranostics, *Adv. Drug Deliv. Rev.*, **134**, 94-106, https://doi.org/10.1016/j.addr.2018.08.004.
80. Hong, H., Goel, S., Zhang, Y., and Cai, W. (2011) Molecular imaging with nucleic acid aptamers, *Curr. Med. Chem.*, **18**, 4195-4205, https://doi.org/10.2174/092986711797189691.
81. López-Colón, D., Jiménez, E., You, M., Gulbakan, B., and Tan, W. (2011) Aptamers: turning the spotlight on cells, *Nanomed. Nanobiotechnol.*, **3**, 328-340, https://doi.org/10.1002/wnan.133.
82. Valdés-Stauber, N., and Scherer, S. (1994) Isolation and characterization of Linocin M18, a bacteriocin produced by *Brevibacterium linens*, *Appl. Environ. Microbiol.*, **60**, 3809-3814, https://doi.org/10.1128/aem.60.10.3809-3814.1994.
83. Moon, H., Lee, J., Min, J., and Kang, S. (2014) Developing genetically engineered encapsulin protein cage nanoparticles as a targeted delivery nanoplatform, *Biomacromolecules*, **15**, 3794-3801, https://doi.org/10.1021/bm501066m.
84. Sutter, M., Boehringer, D., Gutmann, S., Günther, S., Prangishvili, D., Loessner, M. J., Stetter, K. O., Weber-Ban, E., and Ban, N. (2008) Structural basis of enzyme encapsulation into a bacterial nanocompartment, *Nat. Struct. Mol. Biol.*, **15**, 939-947, https://doi.org/10.1038/nsmb.1473.
85. Contreras, H., Joens, M. S., McMath, L. M., Le, V. P., Tullius, M. V., Kimmey, J. M., Bionghi, N., Horwitz, M. A., Fitzpatrick, J. A., and Goulding, C. W. (2014) Characterization of a Mycobacterium tuberculosis nanocompartment and its potential cargo proteins, *J. Biol. Chem.*, **289**, 18279-18289, https://doi.org/10.1074/jbc.M114.570119.
86. McHugh, C. A., Fontana, J., Nemecek, D., Cheng, N., Aksyuk, A. A., Heymann, J. B., Winkler, D. C., Lam, A. S., Wall, J. S., Steven, A. C., and Hoiczyk, E. (2014) A virus capsid-like nanocompartment that stores iron and protects bacteria from oxidative stress, *EMBO J.*, **33**, 1896-1911, https://doi.org/10.15252/embj.201488566.
87. Giessen, T. W., Orlando, B. J., Verdegaal, A. A., Chambers, M. G., Gardener, J., Bell, D. C., Birrane, G., Liao, M., and Silver, P. A. (2019) Large protein organelles form a new iron sequestration system with high storage capacity, *eLife*, **8**, e46070, https://doi.org/10.7554/eLife.46070.
88. Sigmund, F., Massner, C., Erdmann, P., Stelzl, A., Rolbieski, H., Desai, M., Bricault, S., Wörner, T. P., Snijder, J., Geerlof, A., Fuchs, H., Hrabě de Angelis, M., Heck, A. J. R., Jasanoff, A., Ntziachristos, V., Plitzko, J.,

and Westmeyer, G. G. (2018) Bacterial encapsulins as orthogonal compartments for mammalian cell engineering, *Nat. Commun.*, **9**, 1990, https://doi.org/10.1038/s41467-018-04227-3.

89. Sigmund, F., Berezin, O., Beliakova, S., Magerl, B., Drawitsch, M., Piovesan, A., Gonçalves, F., Bodea, S. V., Winkler, S., Bousraou, Z., Grosshauser, M., Samara, E., Pujol-Martí, J., Schädler, S., So, C., Irsen, S., Walch, A., Kofler, F., Piraud, M., Kornfeld, J., Briggman, K., and Westmeyer, G. G. (2023) Genetically encoded barcodes for correlative volume electron microscopy, *Nat. Biotechnol.*, **41**, 1734-1745, https://doi.org/10.1038/s41587-023-01713-y.
90. Pagano, R. E., Sepanski, M. A., and Martin, O. C. (1989) Molecular trapping of a fluorescent ceramide analogue at the Golgi apparatus of fixed cells: interaction with endogenous lipids provides a trans-Golgi marker for both light and electron microscopy, *J. Cell Biol.*, **109**, 2067-2079, https://doi.org/10.1083/jcb.109.5.2067.
91. Zhang, Y., and Hensel, M. (2013) Evaluation of nanoparticles as endocytic tracers in cellular microbiology, *Nanoscale*, **5**, 9296-9309, https://doi.org/10.1039/c3nr01550e.
92. Fermie, J., de Jager, L., Foster, H. E., Veenendaal, T., de Heus, C., van Dijk, S., Ten Brink, C., Oorschot, V., Yang, L., Li, W., Müller, W. H., Howes, S., Carter, A. P., Förster, F., Posthuma, G., Gerritsen, H. C., Klumperman, J., and Liv, N. (2022) Bimodal endocytic probe for three-dimensional correlative light and electron microscopy, *Cell Rep. Methods*, **2**, 100220, https://doi.org/10.1016/j.crmeth.2022.100220.
93. Miyanari, Y., Ziegler-Birling, C., and Torres-Padilla, M. E. (2013) Live visualization of chromatin dynamics with fluorescent TALEs, *Nat. Struct. Mol. Biol.*, **20**, 1321-1324, https://doi.org/10.1038/nsmb.2680.
94. Ma, Y., Wang, M., Li, W., Zhang, Z., Zhang, X., Tan, T., Zhang, X. E., and Cui, Z. (2017) Live cell imaging of single genomic loci with quantum dot-labeled TALEs, *Nat. Commun.*, **8**, 15318, https://doi.org/10.1038/ncomms15318.
95. Chen, B., Gilbert, L. A., Cimini, B. A., Schnitzbauer, J., Zhang, W., Li, G. W., Park, J., Blackburn, E. H., Weissman, J. S., Qi, L. S., and Huang, B. (2013) Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system, *Cell*, **155**, 1479-1491, https://doi.org/10.1016/j.cell.2013.12.001.
96. Song, K., Awata, J., Tritschler, D., Bower, R., Witman, G. B., Porter, M. E., and Nicastro, D. (2015) *In situ* localization of N and C termini of subunits of the flagellar nexin-dynein regulatory complex (N-DRC) using SNAP tag and cryo-electron tomography, *J. Biol. Chem.*, **290**, 5341-5353, https://doi.org/10.1074/jbc.M114.626556.
97. Sun, W. W., Michalak, D. J., Sochacki, K. A., Kunamaneni, P., Alfonzo-Méndez, M. A., Arnold, A. M., Strub, M. P., Hinshaw, J. E., and Taraska, J. W. (2024) Cryo-electron tomography pipeline for plasma membranes, *bioRxiv*, https://doi.org/10.1101/2024.06.27.600657.
98. Stahlberg, H. (2024) Cryo-electron microscopy in color, *Nat. Methods*, **21**, 2233-2234, https://doi.org/10.1038/s41592-024-02427-y.
99. Pfeil-Gardiner, O., Rosa, H. V. D., Riedel, D., Chen, Y. S., Lörks, D., Kükelhan, P., Linck, M., Müller, H., Van Petegem, F., and Murphy, B. J. (2024) Elemental mapping in single-particle reconstructions by reconstructed electron energy-loss analysis, *Nat. Methods*, **21**, 2299-2306, https://doi.org/10.1038/s41592-024-02482-5.
100. Gao, J., Tong, M., Lee, C., Gaertig, J., Legal, T., and Bui, K. H. (2024) DomainFit: Identification of protein domains in cryo-EM maps at intermediate resolution using AlphaFold2-predicted models, *Structure*, **32**, 1248-1259.e5, https://doi.org/10.1016/j.str.2024.04.017.
101. Maurer, V. J., Siggel, M., and Kosinski, J. (2024) What shapes template-matching performance in cryogenic electron tomography *in situ*? *Acta Crystallogr. D. Struct. Biol.*, **80**, 410-420, https://doi.org/10.1107/S2059798324004303.