Review

Effects of radiation damage in studies of protein-DNA complexes by cryo-EM

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\textbf{A B S T R A C T}

Nucleic acids are responsible for the storage, transfer and realization of genetic information in the cell, which provides correct development and functioning of organisms. DNA interaction with ligands ensures the safety of this information. Over the past 10 years, advances in electron microscopy and image processing allowed to obtain the structures of key DNA-protein complexes with resolution below 4 Å. However, radiation damage is a limiting factor to the potentially attainable resolution in cryo-EM. The prospect and limitations of studying protein-DNA complex interactions using cryo-electron microscopy are discussed here. We reviewed the ways to minimize radiation damage in biological specimens and the possibilities of using radiation damage (so-called ‘bubblegrams’) to obtain additional structural information.

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\textbf{Contents}

1. Introduction ...................................................................................................................... 57
2. Structural basis of protein-DNA interactions studied by cryo-EM ................................ 58
   2.1. Large protein-DNA complexes .................................................................................. 58
   2.2. Nucleoid in \textit{E. coli} ................................................................................................... 59
   2.3. Viral protein-DNA complexes .................................................................................. 60
3. Radiation damage in cryo-EM ....................................................................................... 61
4. ‘Bubblegrams’ reveal the disguised protein structure ..................................................... 62
5. Conclusions ..................................................................................................................... 62
Acknowledgements ............................................................................................................. 62
References ............................................................................................................................ 62

1. \textbf{Introduction}

DNA organization into chromatin protects its primary structure from damage, provides vital functions of the cell, and mediates the transfer of genetic material between cells. Ligands (ions, antibiotics, high-molecular weight compounds) actively participate in the formation of the chromatin structure (Alberts et al., 2002; Boyle et al., 2008 Travers and Muskhelishvili, 2015).

Low molecular weight substances, such as metal ions, the only traces of which can be found in cells, strongly affect the structural characteristics of DNA by introducing single- and double-strand breaks which lead to inhibition of the protein synthesis (Sissi et al., 2005). Copper, manganese and nickel ions (Desoize, 2002) bear strong carcinogenic and mutagenic effects on DNA. Simultaneously, metals such as platinum are used in various drugs, for instance cisplatin (Bury et al., 1987; Desoize and Madoulet, 2002; Mohamed and Soliman, 2010).

The second type of DNA-binding ligands includes small organic molecules, such as dyes, antibiotics and anti-cancer drugs. These ligands can be divided into two classes, depending on their interactions with the DNA: (i) ligands with a chain structure (Fig. 1A)

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Since the conformational state of the macromolecular complex generally reflects its functional activity (Alberts et al., 2002), knowledge of the spatial structure enables the researcher to interpret and predict the changes in the functioning molecule. Interactions of DNA with ions, low molecular weight substances and small proteins are investigated by X-ray crystallography, computational docking and molecular dynamic simulations (Lengauer and Rarey, 1996; Rossmann, 2000; Volokh et al., 2015). Complexes of DNA with larger proteins are investigated using cryo-electron microscopy (cryo-EM).

The cryo-EM approach is set in motion by spreading soluble homogenous protein-DNA complexes across a hole in a carbon film, creating a thin layer from a few hundred to a few thousand Ångstroms (Milne et al., 2013; Thompson et al., 2016). Then, the film is promptly frozen by plunging it into cryogen (McDonald and Auer, 2006; Thompson et al., 2016; Tivol et al., 2008). Imaging of vitrified specimens, choice of electron source, electron detector and hardware have been previously discussed in detail (Cheng, 2015; Milne et al., 2013; Thompson et al., 2016). Image processing of 2D projections to a 3D reconstruction and its refined structure is achieved using specialized computer programs, such as the: EMAN (Ludtke et al., 1999), RELION (Scheres, 2012), SPRING (Desfosses et al., 2014), Bsoft (Heymann et al., 2008), Frealign (Lyumkis et al., 2013), etc.

One of the main limitations related to DNA-ligand structural evaluation is lack of pure and stable complexes with no or little flexibility. To improve and facilitate applications of electron microscopy to those objects both sample preparations, as well as different microscopy techniques, are being developed. During the last decade, the resolution, achieved for 3D reconstructions of DNA-protein complexes went down from about 30 Å to 3.9 Å (Table 1).

The latest developments include stain-free sample preparation using a super-hydrophobic surface with further imaging using high-resolution transmission electron microscopy (HRTEM) (Gentile et al., 2012). Using this approach we can detect the DNAs double helix and measure its helical characteristics without stain-agent bias, which can lead to new opportunities for studying DNA in different complexes with ligands, including small organic and inorganic molecules with a potential resolution of 1.5 Å (Marini et al., 2016). Along with the development of classical TEM methods, a DNA damage response imaging technique with use of positron emission tomography (PET) and single-photon emission computed tomography (SPECT) was recently developed (Knight et al., 2017). These promising approaches use DNA ligands radiotracers to visualize DNA damage response, which can lead to new opportunities in the oncology field.

2. Structural basis of protein-DNA interactions studied by cryo-EM

2.1. Large protein-DNA complexes

3D structures of large protein complexes with nucleic acid (RNA), obtained from single particle reconstruction, have been studied since the 1960s. In fact, due to their large size, relative stability and the availability of established purification protocols, the 70S ribosome and plant RNA-containing viruses were the first to be analyzed by single particle electron microscopy (Frank, 2002). In contrast, with the exception of the DNA itself, DNA-containing protein complexes have avoided cryo-EM studies for a long time. In particular, using the cryo-approach, the hexagonal packing of DNA in the head of the giant bacteriophage (Fokine et al., 2005, 2004) was resolved.

Structural studies of DNA-protein complexes began with the interpretation of chromatin on raw cryo-images (Athey et al., 1990). This approach did not provide much resolution, but demonstrated (Davey et al., 2002) that fit into grooves on the DNA surface and interact with phosphate groups and bases; (ii) intercalating agents (Fig. 1B) which lodge between DNA base pairs, push them apart and deform the structure of the helix (examples: transcription blockers distamycin-A and actinomycin-D). Interactions with small ligands change the physical, chemical and mechanical properties of the DNA helix, its flexibility and the distance between bases. Small ligands can disrupt the interactions of DNA with large enzymes, which, in turn, lead to transcription blocking, and are also often used for therapeutic purposes (Sheng et al., 2013).

Large proteins form the third type of DNA-ligand interactions and are generally found in chromatin (Fig. 1C) and its multiple remodeling factors (Fig. 1D). The structural-functional role of nuclear chromatin in eukaryotes is substantial: it exposes the DNA to cellular enzyme systems, executing the natural mechanism of gene activity regulation. DNA fragments that are associated with the nucleosomes become inaccessible to nucleases (Noll, 1974; Wigler and Axel, 1976) and small intercalating ligands (Hayes et al., 1990).
Table 1
The protein-DNA complexes, solved by cryo-EM and image processing.

<table>
<thead>
<tr>
<th>Protein-DNA complex</th>
<th>Resolution, Å (FSC)</th>
<th>Number of particles used</th>
<th>Microscope/ Image Detector</th>
<th>Software package used for image processing</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>N4 virus</td>
<td>30 (0.5)</td>
<td>2096–2506</td>
<td>CM200 (Philips)/Zeiss scanner Tecnai F20 (FEI)/CCD camera (Gatan)</td>
<td>EMAN</td>
<td>(Choi et al., 2008)</td>
</tr>
<tr>
<td>SWR1</td>
<td>28 (0.5)</td>
<td>32,000</td>
<td></td>
<td>EMAN</td>
<td>(Nguyen et al., 2013)</td>
</tr>
<tr>
<td>RSC–nucleosome</td>
<td>25 (0.5)</td>
<td>~37,000</td>
<td>CM200 (Philips)/Zeiss scanner Tecnai F20 (FEI)/Zeiss scanner Tecnai 12 (FEI)/CCD camera (Gatan)</td>
<td>EMAN</td>
<td>(Chaban et al., 2008)</td>
</tr>
<tr>
<td>SWI/SNF–Nucleosome</td>
<td>23 (0.5)</td>
<td>no information</td>
<td></td>
<td>EMAN</td>
<td>(Dechassa et al., 2008)</td>
</tr>
<tr>
<td>Elongation complex</td>
<td>22 (0.5)</td>
<td>8500</td>
<td>JEM-2100 (JEOL)/UltraScan 4000 CCD (Gatan)</td>
<td>EMAN</td>
<td>(Gaykalova et al., 2015)</td>
</tr>
<tr>
<td>OCCM</td>
<td>14</td>
<td>~90,000</td>
<td>JEM-2101F (JEOL)/UltraScan 4000 CCD (Gatan)</td>
<td>EMAN</td>
<td>(Sun et al., 2013)</td>
</tr>
<tr>
<td>PolII-clamp-exonuclease–tc</td>
<td>8 (0.143)</td>
<td>63,215</td>
<td>Titan Krios (FEI)/K2 summit (Gatan)</td>
<td>MOTIONCORR</td>
<td>CTFFIND3 RELION 1.3</td>
</tr>
<tr>
<td>Nucleosome-NuA4</td>
<td>7.9 (0.143)</td>
<td>168,802–390,201</td>
<td>Titan Krios/Falcon II (FEI)</td>
<td>CTFFIND3</td>
<td>EMAN2 RELION 1.3</td>
</tr>
<tr>
<td>MCM2–7 helicase</td>
<td>3.8</td>
<td>85,366</td>
<td>Titan Krios (FEI)/K2 summit (Gatan)</td>
<td>RELION 1.3</td>
<td>EMAN2 RELION 1.3</td>
</tr>
<tr>
<td>CMG helicase</td>
<td>3.7–4.8 (0.143)</td>
<td>687,794</td>
<td>Titan Krios (FEI)/K2 Summit (Gatan)</td>
<td>RELION 1.3</td>
<td>EMAN2 RELION 1.3</td>
</tr>
<tr>
<td>Nucleosome core</td>
<td>3.9 (0.143)</td>
<td>26,060</td>
<td>Titan Arctica/Falcon2 (FEI)</td>
<td>RELION 1.3</td>
<td>(Chua et al., 2016)</td>
</tr>
</tbody>
</table>

the variable thickness of 30 nm chromatin fibers in vitrified ice and allowed the authors to suggest a solid-solenoid model of the chromatin structure. This model was later supported by Robinson and co-authors (Robinson et al., 2006). Another raw-imaging cryo-EM study held ten years later (Bednar et al., 1999) suggested that, during transcription through the nucleosome, the histone octamer remains structured and does not lose single histones. Thus, as a part of chromatin, nucleosomes are stable upon changes in the DNA structure (Odeli et al., 2013).

Large DNA-containing complexes are well suited to be studied with the single particle cryo-EM approach (Fig. 1B,C).

Using cryo-EM, several reconstructions of large chromatin-remodeling factors were obtained: SWR1 (~1 MDa) (Nguyen et al., 2013), SWI/SNF (~1.2 MDa) (Dechassa et al., 2008), RSC (~1.2 MDa) (Chaban et al., 2008), ISW1a–ΔATPase in complex with nucleosome (Fig. 1D) (~500 kDa) (Yamada et al., 2011). All complexes are composed of protein and at least a small fragment of DNA. The resolution of all reconstructions was below 2 nm, mostly because of their flexibility. There is a common practice of using crystal structures of known domains and/or subcomplex parts for the construction and interpretation of a molecular model of the whole complex (Wriggers and He, 2015). In the absence of the crystal structures in databases, homology modeling is a good alternative that may be used. Homology modeling is based on the observation that the spatial structure of proteins is much more conservative than their amino acid sequence (Pils et al., 2005). 30% sequence identity is considered to be sufficient for the construction of models. To interpret the cryo-EM structure of the ISW1a–ΔATPase complex with nucleosome, crystal structures of HSS-loc3 and nucleosome were docked into the EM density (Davey et al., 2002). It has been demonstrated that ISW1a may interact with two portions of the DNA linker simultaneously. First, the ISW1a complex binds to the linker, which will later be translocated. Once the linker section reaches the desired length, the ISW1a blocks further translocation. For the interpretation of the SWR1 structure, the crystal structure of the RuvBL1 hexamer (Matias et al., 2006) was docked into the electron density (Nguyen et al., 2013).

Recently, a breakthrough in cryo-EM methodology (Glaser, 2016a), due to the invention of direct electron detectors and subsequent correction of beam-induced specimen movement (Henderson, 2015), allowed to receive high-resolution structures of DNA–protein complexes: the nucleosome (Chua et al., 2016) and the transcription initiation complex, which included the RNA polymerase and DNA in open and closed conformations (Plaschka et al., 2016). At high resolution, the side chains of the amino acids are detectable. But, in this case, inevitable electron damage should be taken into account. Experience shows that most sensitive to radiation are the cysteine, aspartate and glutamate residues (Fig. 2D) (Allegritti et al., 2014). Some disulfide bonds in a protein molecule become distorted at an electron dose as little as 5 e/Å², and an accelerating voltage of 1 MeV (Baker and Rubinstein, 2010).

2.2. Nucleoid in E. coli

In prokaryotes, the circular DNA does not form a pronounced ‘chromatin’, but has been shown spatially ordered (Ryan and Shapiro, 2003; Shapiro and Losick, 2000). A number of DNA-binding proteins in bacteria that control the dynamic reorganization of bacterial nucleoids form a dense net in the cytoplasm and are capable to produce an even more complex structure, which resembles the mitotic apparatus in eukaryotes (Niki et al., 2000; Niki and Hiraga, 1998). Interestingly, DNA-binding proteins in bacteria are capable of crystallization under stress conditions (Frenkel-Krispin et al., 2004). A range of scientific papers published recently (Gallat et al., 2014; Koopmann et al., 2012) suggest in vivo crystallization as a new line of research in the field of structural biology. The advantages of in vivo crystallization are: the production of post-translationally modified proteins, easiness of isolation by spinning down the crystals after cell lysis, and the possibility of analyzing crystals by TEM.

The structure of in vivo crystals can be investigated using electron tomography. This approach deals with large cellular organelles (Bharat and Sheres, 2016) and whole prokaryotic cells (Kishimoto-Okada et al., 2010; Murata et al., 2016). Moreover, recently it
has demonstrated good results with single molecules (Wan and Briggs, 2016). The electron tomography approach consists of taking a series of images of the object at different angular orientations to obtain a better angular resolution; the sample may be turned by 90° to collect a new series of images. Currently, images of macromolecular complexes may be obtained at a resolution greater than 0.85 nm (Beck and Baumeister, 2016) by combining and processing many subtomograms. The main obstacle of working with the tomographic reconstruction — is radiation damage of samples obtained by prolonged exposure to the electron beam. Therefore, the total radiation dose in electron tomography should not exceed 50e/Å².

### 2.3. Viral protein-DNA complexes

Likewise, pro- and eukaryotic DNA, like viral DNA, forms complexes with proteins more often than previously thought. A number of internal phage proteins were discovered by proteomics analyses (Thomas et al., 2016, 2012). They can serve as scaffolds, proteases, RNA polymerases, or perform transport functions. Often, they can be found attached to the bacteriophage portal, such as the vertex ‘core’ of the T7 phage, via which DNA enters the capsid during assembly and exits during infection. It has a cylindrical form (Steven et al., 1983) and consists of three proteins that possess different symmetries (Agirrezabala et al., 2005; Cerritelli et al., 2003; Guo et al., 2013).

In the nineteen eighties, the much larger internal protein structure which spanned the capsids of giant phiKZ-like bacteriophages, encased within genomic DNA, was discovered. The authors called it the ‘inner body’ (Krylov et al., 1984, 1978; Krylov and Zhaxykov, 1978). It forms an elongated cylindrical structure (Sokolova et al., 2014; Wu et al., 2012) and is currently thought to serve as a structural device for arranging genomic DNA inside a giant phage head.

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**Fig. 2.** The source and results of electron damage. (A) The schematic for progress of radiation damage in the TEM; (B) Influence of radiation damage onto resolution of the 3D reconstruction of protein molecule. Reconstruction of amino acids 124–144 of F420 hydrogenase calculated from images obtained (left) with low electron dose ~10 e/Å² (resolution 3.36 Å), (middle) an increased dose of ~24 e/Å² (resolution 3.84 Å), and (right) high-dose ~49 e/Å² (resolution 4.16 Å). The side chains of Asp125 and G1u132 underwent damage already after the first irradiation. Reprinted with permission from (Allegretti et al., 2014). (C) Electron damage in cryo-EM leads to a bubble formation. Bacteriophage EL, frozen in amorphous ice, no damage (left). Similar bacteriophage after irradiation with the high dose of electrons (right). Noticeable are bubbles of hydrogen gas (arrows). Reprinted with permission from RAS publishing agency.
3. Radiation damage in cryo-EM

In an electron microscope, electrons are the source of image formation. Energy transmitted from the electrons to the sample can ionize atoms and thereby cause X-ray emission and other forms of radiation damage and a consequent rearrangement of chemical bonds. As a result, the fine details are lost and the raw data may not adequately reflect the original structure (Glaeser, 2008; Karuppasamy et al., 2011). The precise effect of radiation damage depends on the chemical composition of the molecule (Fromm et al., 2015). Thus, radiation damage is a limiting factor to the achievable resolution in biological specimens (Glaeser, 2008; Karuppasamy et al., 2011).

The energy of electrons is significantly higher (more than hundreds of electron volts) than the energy of covalent bonds (few electron volts). The greatest damage to the sample occurs at highest exposure, when the incident electrons lose from ~5 to ~100 eV of their energy (on average ~20 eV) (Glaeser et al., 1971; Grant and Grigorieff, 2015; Langmore and Smith, 1992). The study of the effect of radiation damage on the tobacco mosaic virus structure revealed its first visible effects as the dose increased beyond 14.3 e/Å². Initially, radiation affects carboxylate residues (mainly, negatively charged), followed by other intermediate and large size side chains, which leads to resolution deterioration of the main chain (Fromm et al., 2015). Radiation damage effects are detectable at high spatial frequencies, but can be observed at low resolution too (Conway et al., 1993).

Electron losses are responsible for the valence electron excitation (which forms covalent bonds), breaking ties, the emission of secondary electrons, and the appearance of free radicals in biological objects. This process is called the primary damage. Free radicals, in turn, trigger a cascade of chemical reactions referred to as the secondary damage (Fig. 2A). Breaking of chemical bonds occurs at all temperatures; therefore, cooling does not affect the primary damage of samples. However, liquid nitrogen temperature slows down the motion of the molecules, i.e. inhibits the secondary damage (Grant and Grigorieff, 2015; Henderson et al., 1990).

The Cryo-EM technique developed several approaches to minimize damage caused by electron radiation: (i) the reduction of the exposure time. Typical electron dosages for biological objects range from 1 to 20 e/Å². Although some biological samples can withstand 100–500 e/Å² (depending on the chemical composition and temperature), the structural details required for high-resolution reconstruction undergo irreversible changes at exposures ~10 e/Å² or less. Thus, radiation damage determines experimental conditions and limits the resolution of 3D structures (Grant and Grigorieff, 2015; Orlova and Saibil, 2010). (ii) The reduction of the electron dose. Low-dose systems are widely used to reduce electron damage in search, alignment and focus modes, until, finally, to block the beam before the final step, capturing an image. However, short exposures lead to noisy images and, as a consequence, to the lack of high-resolution data. This, in turn, complicates subsequent image processing. The ability of the detector to count each electron becomes critical for such short exposures (Baker and Rubinstein, 2010; Karuppasamy et al., 2011; McMullan et al., 2014) (iii) The introduction of direct electronic detectors to cryo-EM not only allowed to omit the intermediate step of signal-to-light-conversion of electrons (Faruqui and Henderson, 2007), but also to have fast readout rates of up to 400 frames per second in ‘movie mode’ (Shrum et al., 2012). Through a special procedure called ‘motion correction’ (Henderson, 2015) all images of particles obtained from the selected frames are aligned and summed (Fig. 2B). Thus, the beam-induced movement of particles in ice during the exposure is compensated, and a higher resolution of the 3D reconstruction may be obtained. Moreover, this multiple frame technique allows adjusting the effects of radiation damage. For this aim, only a fraction of images with minimal damage is chosen for further processing. The combination of these approaches significantly improved the resolution of the 3D reconstructions in the past two or three years – up to 3–4 Å (Allegritti et al., 2014; Campbell et al., 2015; Liu et al., 2016).

Tertiary protein damage, due to the formation of gas bubbles, occurs when samples are subjected to high energy electron irradiation (more than 40 e/Å²) (Fig. 2C). Hydrogen gas bubbles appear in the irradiated sample during a process described below (Dubochet et al., 1988; Leapman and Sun, 1995). First, radiolysis
of water occurs under the influence of an electron beam: \( \text{H}_2\text{O} \rightarrow \text{H}^+ + \text{OH}^- \). In ice, free radicals are combined back into \( \text{H}_2\text{O} \), but in close proximity to the protein molecule they react with the hydrogen atoms of organic compounds: \( \text{OH}^+ + \text{R}^- \rightarrow \text{RO}^- + \text{H}_2 \) (Leapman and Sun, 1995). Bubbles are generally formed near that surface of the molecule that is in contact with the ice, and is not inside the protein (Hankamer et al., 2007). The gas is released in such amounts that the pressure inside the bubbles may reach thousands of atmospheres (Leapman and Sun, 1995).

4. ‘Bubbles’ reveal the disguised protein structure

Interestingly, in some rare cases, electron damaging effects may provide useful structural information. This especially concerns complexes formed by protein and DNA. Upon irradiation of such heterogeneous systems by an increased electron dose (=40–50 e/Å²), gas bubbles formed faster and at a lower dose than upon irradiation of the protein–protein complexes. It is thought that tightly packed DNA prevents the diffusion of hydrogen gas away from the protein, which leads to a rapid local accumulation of radiolysis products and to earlier bubbling of proteins (Black and Thomas, 2012; Sokolova et al., 2014; Wu et al., 2012). As a result, gas bubbles may form a pattern that precisely outlines the protein–DNA complex. Interestingly, the same electron dose does not induce bubbles in free DNA embedded in ice (Chen et al., 2008). The pattern of radiation damage is clearly visible and may help in situations where the protein is indistinguishable against the background of the surrounding nucleic acid, providing information about the existence and size of the protein structures. Some authors have suggested the term ‘bubbles’ for this phenomenon (Cheng et al., 2014; Wu et al., 2012).

Bubbles were initially observed in structural studies of bacteriophage capsids containing DNA (Sokolova et al., 2014; Thomas et al., 2008). When taking a pair of images of the same phage particle at a lower (=10–20 e/Å²) and higher electron dose (=30–40 e/Å²), it was noticed that, while particles in the first image (Fig. 3A) were not damaged and the ‘inner body’ was invisible in the background of the surrounding DNA, in the second high dose image, bubbles of hydrogen were formed, outlining a specific elongated shape of the internal protein structure (Fig. 3B). The linear dimensions of bubbles allowed to predict the approximate size and position of this ‘inner body’. Both high-dose and low-dose images have identical orientations. Thus, they made possible to determine the Euler angles of the intact inner body within the non-radiated capsid. These angles were used to calculate the three-dimensional reconstruction of the inner body (Wu et al., 2012).

Another example reveals the location of the portal vertex in the Lusuz4-like bacteriophage (Fig. 3C). This phage has an icosahedral capsid and a short conical tail. Its inner protein core is located near the phage vertex. When the phage particles were subjected to high electron dose radiation, the hydrogen gas produced the bubble at the protein core end, which is normally concealed by the surrounding DNA. After aligning the particles and producing the class-sum images, the orientation of the vertex can be revealed (Fig. 3D).

Recently, the birth of the gas bubble process was studied in detail using the T7 bacteriophage (Cheng et al., 2014). It turned out that the emission of gas begins only after the accumulation of a critical concentration of radiolysis products. Power electron dose causes the occurrence of bubbles and must be evaluated beforehand to minimize the damage. Another option to be considered – temperature. Diffusion of radiolysis products from their place of origin is that much slower, the lower the temperature. Therefore, the accumulation of radiolysis products at helium temperatures can lead to earlier electron-beam damage than at the temperature of liquid nitrogen. This type of radiation damage has been observed in electron tomographic experiments (Bammes et al., 2010).

5. Conclusions

Latest achievements in cryo-EM that we have started using in past two to three years, and particularly the direct detectors, enabled to collect movies and use only several frames that accumulated the low electron dose for the final reconstruction. Additionally, the phase plates provide improved contrast and signal-to-noise ratio. This approach enabled the three-dimensional reconstructions of macromolecules with resolutions close to 2 Å (Bartesaghi et al., 2015; Banerjee et al., 2016; Kimanius et al., 2016) or even below (Merck et al., 2016). Still, obtaining a reconstruction with a resolution below 3 Å remains challenging. A large number of DNA-protein complexes remain in low resolution or have even avoided structural investigations altogether.

Yet, electron beam damage of macromolecules doesn’t seem to be a resolution limiting factor anymore. Now other factors came to the fore: beam-induced motion, the detective quantum efficiency (DQE) of detectors, the flexibility of macromolecules (Bartesaghi et al., 2015; Glaser, 2016b). Moreover, even the electron-beam damage could provide additional structural information. New approaches in image collection have recently made it possible to use radiation damage to localize objects concealed in vitrified ice, determine their orientation and, subsequently, determine their three-dimensional structure.

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