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Imaging Macromolecular Structural Dynamics with Low-Dose, Time-Resolved Transmission Electron Microscopy (19-FS-038)

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Abstract

All well-established experimental methods for investigating the structure of biological macromolecules have spatial and/or temporal limitations inherent to the technique that limit their applicability to studying macromolecular dynamics. The ability to measure macromolecular dynamics is key to gaining predictive mastery of biological systems. This work demonstrates that sufficient data can be acquired from low-Z soft materials using dynamic transmission electron microscopy (DTEM) to potentially allow measurements of macromolecular motion. While a dynamic measurement of macromolecular motion or structural change requires further effort, it is evident from this work that this is feasible under specific experimental conditions that can be achieved using low-dose, time-resolved transmission electron microscopy.

Background and Research Objectives

Most biological processes involve concerted atomic and/or molecular motion. These concerted movements occur in large, flexible proteins or macromolecular complexes and, along with intermolecular interaction, confer function to these molecular machines. While it is clearly essential to capture the dynamic molecular states of these machines in order to understand their function, unfortunately all well-established experimental methods for investigating the structure of biological macromolecules have spatial and/or temporal limitations inherent to the technique that limit their applicability to studying macromolecular dynamics.

Direct imaging in a conventional transmission electron microscope (TEM) of the structure and dynamics of biological macromolecules is impaired by electron beam damage at the typical kinetic energies of electrons in a TEM (80–300 keV) [1], as well as by the limitations of beam-induced and Brownian motion of the specimen during image acquisition. While ultrafast X-ray techniques have demonstrated the capability to outrun radiation damage and collect high-resolution diffraction patterns (“diffract-and-destroy”) [2-5], imaging and diffraction with electrons provides some advantages over the use of X-rays for structural studies of biological macromolecules. For example, the scattering cross-section for electrons is ~10^3 times greater than that for X-rays [6,7] and electrons are capable of providing a significantly higher information/damage ratio [1]. Considering 8-keV X-rays, the damage per elastic scattering event is 10^3 times higher than that for 80–500-keV electrons [1,8], and elastic scattering (diffraction) of electrons or X-rays provides the high-resolution structural information.

Short-pulsed electron beams may be able to overcome the limitations of a conventional TEM, enabling the study of biological macromolecular dynamics with high spatial and temporal resolution to provide a complementary platform to existing techniques. This would provide significant improvement in the consistency and reliability of measurements to link macromolecular structure with function, enabling an experimental platform in the TEM to explore conformational changes in biological macromolecules initiated by, for example, localized laser heating, photoactivation, or laser flash photolysis.

The overall goals of this study were to assess radiation damage using a pulsed electron beam and observe intramolecular motion of biological macromolecules and thereby elucidate molecular function using nanosecond pulsed dynamic transmission electron microscopy (DTEM) [9,10]. The experimental plan was comprised of three proof-of-principle experiments, as illustrated in Figure 1. Within this scope, the proposed feasibility study planned to: 1) determine the damage accumulation rate for crystalline paraffin, a surrogate for bio-macromolecules; 2) demonstrate observable diffraction signal resulting from molecular motion in 2D crystalline bacteriorhodopsin (bR), a light-activated proton pump; and 3) demonstrate observable imaging signal resulting from molecular motion in non-crystalline myosin, a motor protein. As will be discussed in subsequent detail, the damage accumulation experiments provide insight to future opportunities for low-dose bioimaging experiments using pulsed electron beams; the diffraction studies with bR and liquid-cell imaging experiments show promise but require further effort.

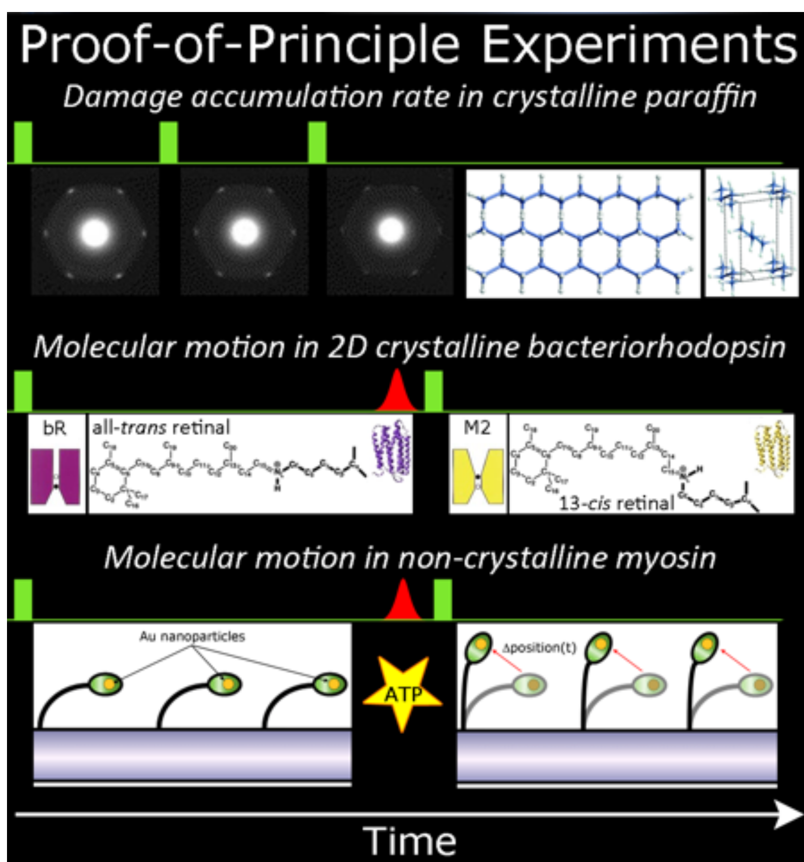

Figure 1: Schematic representation of the three experiments to demonstrate feasibility of low-dose, time-resolved imaging of bio-macromolecular structural function.

Scientific Approach and Accomplishments

The aim of this work was to determine the feasibility of acquiring dynamic measurements from biological macromolecules. First, it was demonstrated that short electron pulses provide a low-dose mode of operation to acquire data from specimens without radiation damage. It was then shown that signal can be acquired from biological macromolecules, though dynamic measurements require more significant efforts in specimen preparation and consideration of the timescales of dynamic processes relative to the temporal windows accessible using DTEM, as described below.

Materials

Saturated solutions of paraffin were prepared from dry granules in several solvents. Serial dilutions were prepared, and 2 µl of each solution were deposited onto formvar-coated lacy carbon grids and allowed to air dry.

Purple membranes (PM) were isolated from H. salinarum strain S9 using previously described procedures [11] and diluted to 3 mg/ml in protein. PM solutions were incubated for 6–8 days at 21°C with 6 mM Octyl glucoside detergent in phosphate buffer to break up the PM into soluble patches and to form larger crystalline domains. The bacteriorhodopsin (bR) 2D crystal solution was washed by pelleting via centrifugation at 16000 rpm for 1 hour until the supernatant was clear and then resuspended in water. The sample was deposited onto both few-layer graphene covered Cu mesh and hydrophobic 15-nm Si_3N_4 grids using several methods and concentrations of bR, principally by 1) dropping 2 μl of 1.5 mg/ml bR onto the grid, incubating for 1 min, blotting excess liquid with filter paper, and following the same process with a 2% glucose solution, or 2) dropping 2 μl of 0.15 mg/ml bR in 2% glucose in water onto the grid and allowing the solution to fully air dry without blotting.

Steptavidin conjugated 40 nm Au nanoparticles were purchased from Abcam at 10 OD and diluted at 1:25 in water. 2 μl of the diluted solution was deposited on 15 nm Si_3N_4 windows and either allowed to fully air dry or incubated for 1 min and excess liquid was blotted with a piece of filter paper.

Experimental

Radiation Damage Studies with C_{36} Paraffin

Figure 2 presents plots of intensity as a function of electron dose for C_{36} paraffin. The intensity is given as $(I/I_0)_{\{110\}}$, where I is the intensity in the {110} Bragg reflections after the indicated electron dose, as indicated in the figure, and I_0 is the intensity of the {110} Bragg reflections prior to any electron irradiation. These intensities were measured by radial integration of the intensity within the {110} reflections, and for both I and I_0, the intensities were normalized to the intensity in the {000} transmitted electron beam of the specific diffraction pattern, which accounts for any variability in both the specimen and electron pulses between measurements. The inset diffraction patterns, acquired using single 50-ns electron pulses, show states of the C_{36} paraffin samples prior to irradiation (labeled Initial) and after a final electron dose, as indicated. For all radiation damage studies with C_{36} paraffin, a pulse train comprised of four 50-ns electron pulses separated by 2.5 μs was used. The intensity (electrons/pulse) of the 50-ns electron pulses was measured using a Faraday cup that can be inserted into the column of the TEM. The intensity of the pulses can be controlled by varying the bias on the Wehnelt cap in the electron gun and changing the size of the second condenser aperture (C2) of the TEM.

The plot in Figure 2(a) presents the change in intensity using a dose per electron pulse of 8.8×10^{-5} $e^-/Å^2$. The inset diffraction patterns show the difference between the initial intensity of the {110} Bragg reflections and the intensity after a total dose of 3.18 $e^-/Å^2$. There is only a 5.5% change in intensity, and this total dose was achieved by exposing the sample to 36,000 50-ns electron pulses. For reference, the critical dose for C_{26} paraffin is on the order of 5 $e^-/Å^2$ using 100 keV electrons [1]. Here, 200 keV electrons were used, and it is evident that it is possible to acquire sufficient signal from a low-Z material such as paraffin without damaging the specimen. A typical DTEM measurement will use between 1 and 50 electron pulses, clearly well below the damage threshold in this case.

For comparison, the plot in Figure 2(b) presents in the change in intensity using a dose per electron pulse of 7.6×10^{-4} $e^-/Å^2$. In this case, a significant decrease in the intensity of the {110} Bragg reflections and a reduction in crystallinity, as indicated by the loss of sharp Bragg reflections in the diffraction pattern, was observed after only 12,000 pulses, corresponding to a total dose of 9.17 $e^-/Å^2$. Thus, it was demonstrated that it is possible to damage the C_{36} paraffin, and proper choice of experimental imaging parameters is essential

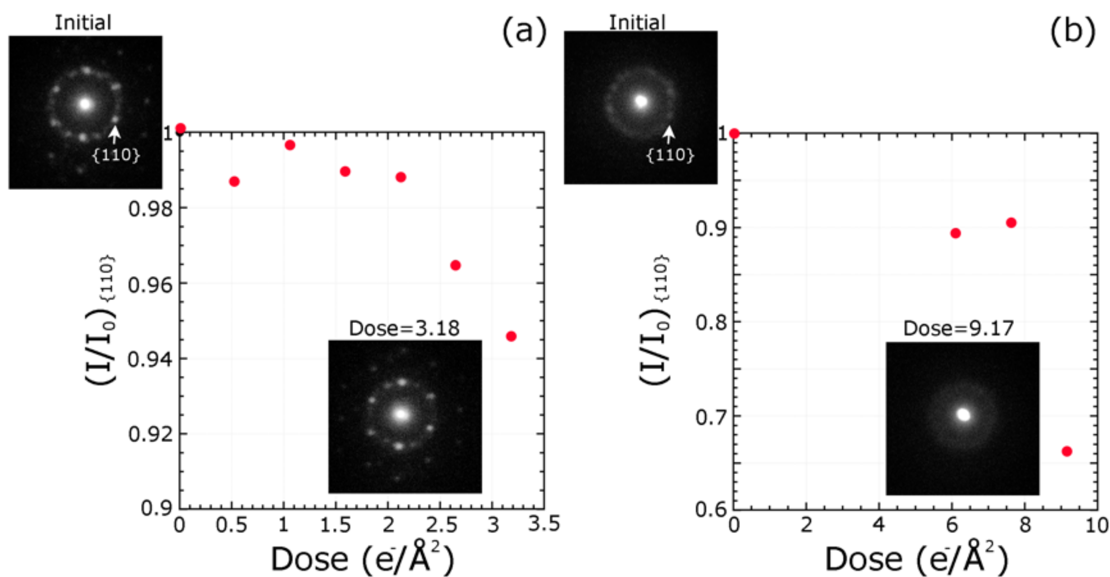

Figure 2: Intensity as a function of electron dose for C_{36} paraffin using a dose/per electron pulse of (a) 8.8×10^{-5} and (b) 7.6×10^{-4} $e^-/Å^2$.

The results of these radiation damage studies with C_{36} paraffin indicate significant opportunities for biological imaging experiments, as it is possible to acquire data from low-Z materials such as paraffin without damaging the specimen. The critical dose for damage is material dependent, and both the dose and dose rate can be varied in the DTEM, providing a platform with enormous flexibility to explore biological imaging. This warrants further study with numerous biological specimens to determine dose thresholds for data acquisition.

Pulsed Electron Diffraction with Bacteriorhodopsin

Electron diffraction experiments with two-dimensional (2D) bacteriorhodopson (bR) membrane protein crystals were conducted using 50-ns electron pulses, with an average dose per pulse of 7.6×10^{-4} $e^-/Å^2$. bR provides a good test specimen for in situ dynamic studies because: bR is arguably the most studied membrane protein; bR is naturally crystalline; and bR is light-activated. Before conducting in situ dynamic experiments, it must first be demonstrated that signal can be acquired from bR crystals in the DTEM.

Figure 3 shows electron diffraction patterns acquired from three different 2D bR crystalline specimens (Figure 3(a), (b), and (c)). The diffraction patterns were all acquired with fifty 50-ns electron pulses, for a total dose of 0.038 $e^-/Å^2$ per acquisition. Due to the low dose in a single 50-ns electron pulse, it was possible to integrate the signal from these fifty electron pulses to increase signal without damaging the specimen. Multiple diffraction patterns were acquired from each bR specimen, as shown in Figure 3(a), without damaging the sample after a total dose of 0.608 $e^-/Å^2$.

The diffraction patterns in Figure 3 demonstrate that measurable signal can be acquired from biological samples such as 2D bR crystals. These diffraction patterns are consistent with the bR crystal structure (P_3 symmetry with unit cell parameters $a = b = 63$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$), as shown in prior studies [12,13].

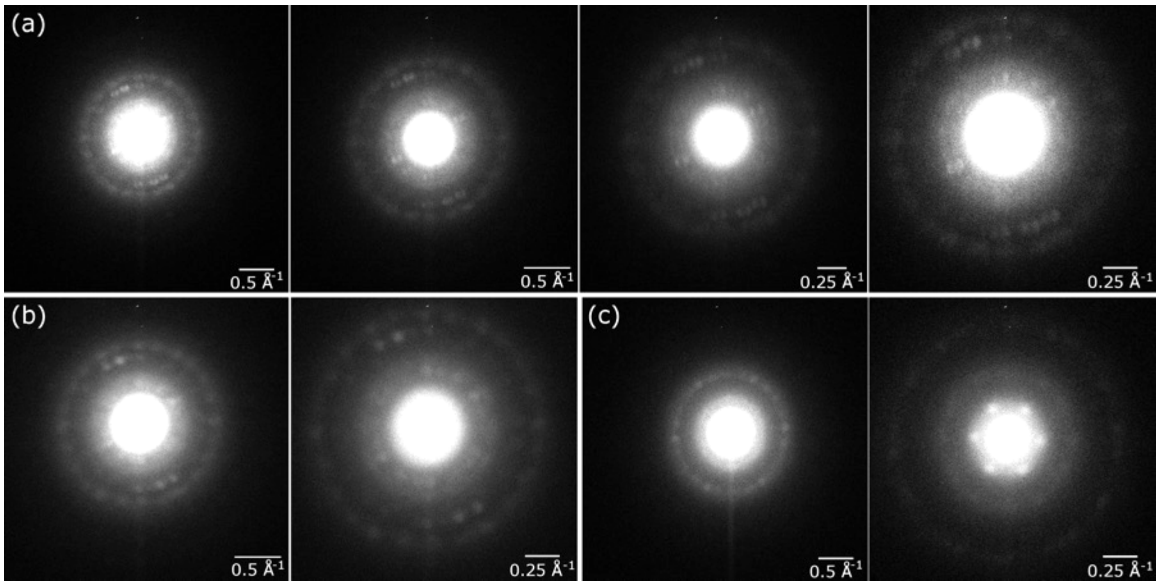

Figure 3: Electron diffraction patterns acquired from three (a, b, and c) different 2D bR crystalline specimens.

The next step with the 2D bR membrane protein crystals is to conduct time-resolved experiments to capture light-induced conformational changes. Intensity differences between diffraction patterns obtained from the ground state (unpumped) compared to photo-activated (pumped) can be transformed (using phases from known structures) to reveals shifts in charge densities that correspond to molecular motion. Experiments with three-dimensional (3D) bR protein crystals will also be investigated, as these will provide increased signal relative to the 2D crystals. However, experiments with 3D crystals require an environmental cell to keep the membrane protein crystals hydrated (the 2D experiments shown here were conducted in the vacuum of the TEM column), which creates new challenges, as will be discussed subsequently.

Liquid-Cell Imaging with Au Nanoparticles

Molecular motion in non-crystalline materials could be detectable in image mode with DTEM using contrast agents. Observable differences in the position of these contrast agents, such as Au nanoparticles, would provide evidence of motion. These types of experiments require extensive specimen preparation, and the measurements shown in Figure 4 were conducted to assess the resolution of DTEM imaging using 40-nm Au nanoparticles prior to beginning the necessary specimen preparation for time-resolved imaging experiments.

Figure 4 presents DTEM images acquired from streptavidin-conjugated 40-nm Au nanoparticles using 50-ns electron pulses. For comparison, images were acquired in the vacuum of the TEM column, shown in Figure 4(a), and in an environmental liquid cell, shown in Figure 4(b) and 4(c). As a critical electron dose is unknown for streptavidin, as well as for future biological macromolecules of interest such as myosin, images were acquired using 1, 5, 10, 20 and 50 electron pulses for comparison. The critical dose for hydrated muscle fibers is ~3.1 $e^-/Å^2$ [14,15], and all of these electron pulses are below this dose.

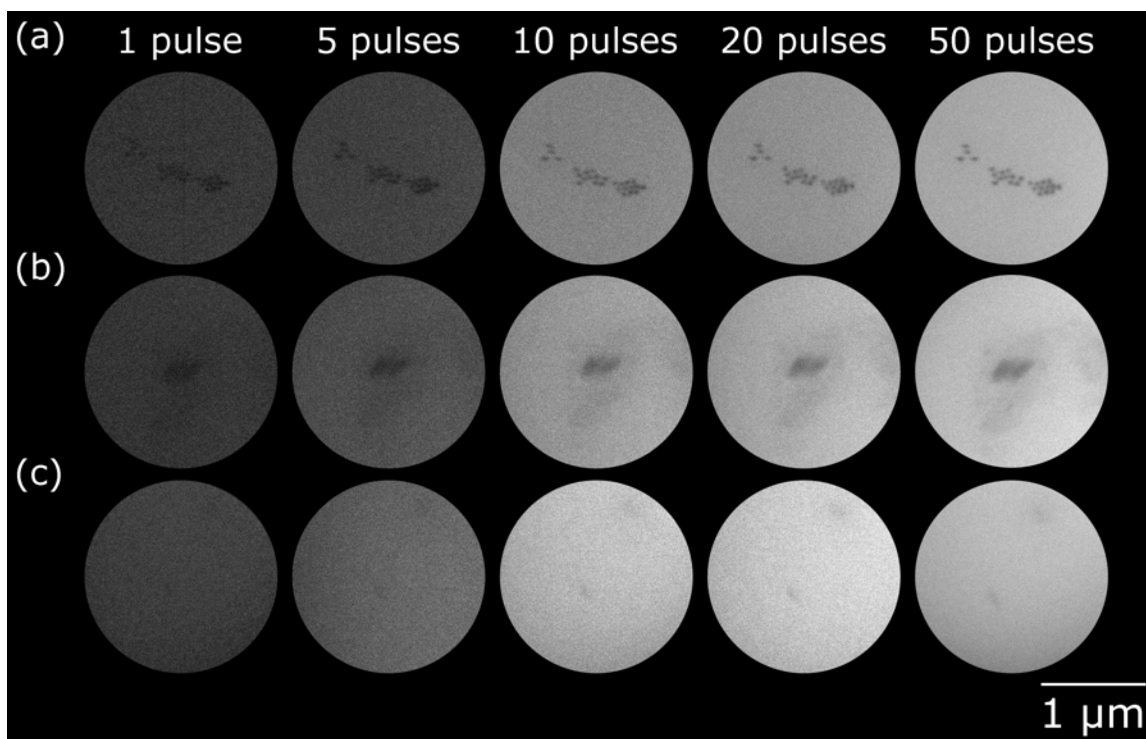

Figure 4: DTEM images acquired from streptavidin-conjugated 40-nm Au nanoparticles in (a) the vacuum of the TEM and (b,c) water in an environmental liquid cell. Images were acquired using 1, 5, 10, 20, and 50 electron pulses, each of 50-ns temporal duration.

As expected, as the number of electron pulses increases the resolution and signal to noise improve. The number of pulses that can be used depends not only on the critical electron dose of the specimen, but also on the timescale of the process of interest. As most macromolecular motion occurs on timescales of ms–s, using 20–50 pulses is likely feasible. The larger complication is spatial resolution in the environmental liquid cell. The 50-nm Au nanoparticles are clearly resolved in all of the images acquired in the vacuum of the TEM column (Figure 4(a)). Even in the 1-pulse image, there is sufficient signal to resolve the nanoparticles. However, in the environmental liquid cell (Figure 4(b,c)), the nanoparticles could not be resolved regardless of the number of electron pulses. Further efforts are required to improve the resolution. This can be accomplished by decreasing the fluid path length in the liquid cell by using a smaller volume of water and ensuring that the silicon nitride window membranes of the liquid cell remain hydrophilic to encourage wetting, typically accomplished by plasma cleaning prior to loading the cell.

Impact on Mission

Biological macromolecular dynamic studies are significant for Laboratory biosecurity missions, and this work will have an impact in the fields of electron microscopy, structural biology, and systems biology. Follow-on studies could show that time-resolved electron microscopy is a complementary method of choice for studying structural dynamics. In turn, structural dynamics could fundamentally change our understanding of how macromolecules work as molecular machines.

The potential to image biological macromolecules and their dynamics in functional states at relevant timescales would open new areas of research to benefit LLNL's Core Competency of "Bio-science and Bio-engineering" and Mission Focus Area of "Chemical and Biological Countermeasures." By leveraging LLNL's existing strengths in time-resolved and biological imaging

capabilities, this project will provide a competitive advantage to the Laboratory to attract funding from federal agencies with a strong interest in bioimaging, such as BER at the Office of Science and the National Institutes of Health (NIH).

Conclusion

We have demonstrated that it is possible to acquire signal from biological macromolecules without damaging the specimen using short, nanosecond electron pulses. These studies provide insight to future opportunities for low-dose, time-resolved bioimaging experiments using pulsed electron beams. A large spatio-temporal parameter space still remains unexplored using dynamic transmission electron microscopy with biological macromolecular specimens. Future work will aim to explore this parameter space, as well as to improve on the work shown here. Next steps for this research will continue through industrial collaboration and support from other funding agencies such as NIH.

Acknowledgments

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