

# Structure, Dynamics and Function of Membrane Proteins using 4D Electron Microscopy

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My research aims to understand the synergistic relationship between **protein structure, dynamics and function**. Using a powerful combination of **single particle cryo-EM** and **4D cryo-EM**, my team and I will image protein structural dynamics with unprecedented spatial (sub-nanometer) and temporal (from picosecond to seconds) resolution, observing hydrated biological dynamics in real-time.

I have two specific aims:

***Specific Aim 1: Determination of structural dynamics leading to changes in membrane potential.*** 4D EM will be used to resolve the structural dynamics of membrane proteins that alter the membrane potential either directly or *via* a signaling cascade. The principle is similar to that of time-resolved X-ray crystallography of proteins, with the major advantage that 4D EM can diffract crystals either too thin, or too small, to scatter X-rays.

***Specific Aim 2: Development of laser-based methods to enhance traditional cryo-EM imaging of single-molecule membrane proteins and whole cells.*** The interfacing of an ultrafast, pulsed laser to a conventional transmission electron microscope may lead to improvements in conventional cryo-EM imaging or electron cryotomography. I would like to explore these possibilities and directly apply laser-based methods to cryo-electron imaging of membrane proteins both as “single particles” and within the cell membrane.

## Introduction

Structural biology is entering a new and exciting age. It is clear that the structure-function paradigm is insufficient to fully establish the molecular mechanisms of many biological processes and that the integration of the fourth dimension, time, into structural biology methods is fundamental to our understanding of the relationship between structure, dynamics and function (1).

Electron microscopy (EM) is an immensely powerful tool for obtaining atomic-resolution images of proteins but in conventional EM the resulting images are static *i.e.* they have been time-averaged over seconds. The ideal structural biology technique would combine the spatial resolution of electron microscopy with the temporal resolution of laser spectroscopy thus enabling the direct visualization of changes in protein structure during their functional operation.

Such a novel and exciting form of microscopy has recently been developed and is known as “Four-Dimensional Electron Microscopy” or 4D EM (2) (Fig. 1). The concept is as follows: a pulsed laser delivers a train of picosecond ( $10^{-12}$  s) or nanosecond ( $10^{-9}$  s) optical pulses to the electron source generating timed single electron packets that are used to image the sample (the probe pulse). The other optical beam (clocking or pump pulse) is used to heat or excite the sample and delivers initiating pulses at the specimen thus defining the zero of time.

By adjusting the time delay between the initiation of structural change by the pump pulse and the recording of an image or “frame” by the probe pulse, different timescales (ranging from picosecond to seconds) can be explored. Through the combination of successive frames, this revolutionary technique yields movies of atomic processes with (up to) femtometer spatial sensitivity (3) and femtosecond temporal resolution (4).

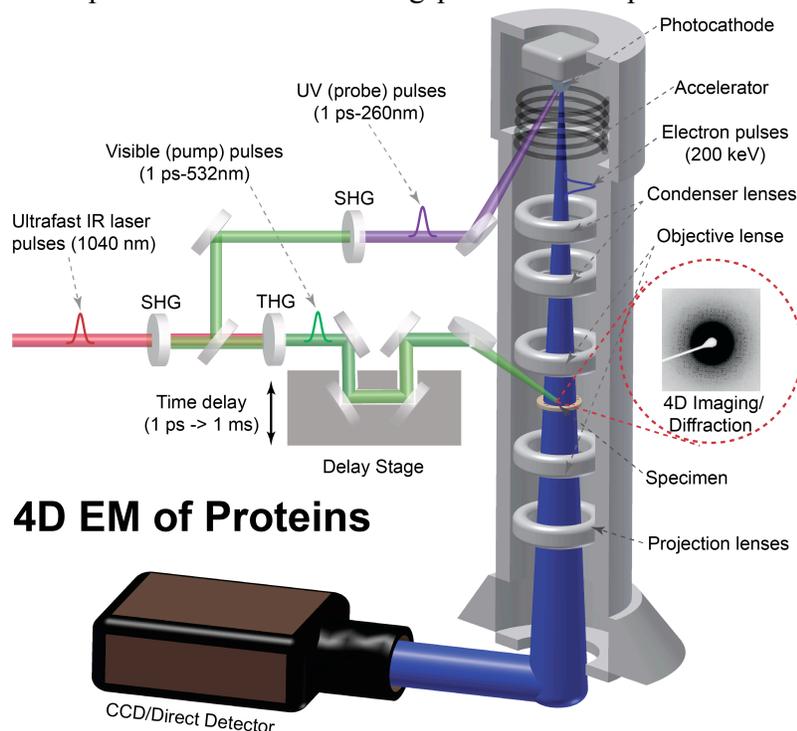


Fig. 1. The 4D Electron Microscope. A Transmission Electron Microscope (TEM) is interfaced with a picosecond/nanosecond laser to create a probe pulse for imaging, diffraction and spectroscopy at well-defined times after a clocking or pump pulse is used to heat or excite the sample.

## 4D Electron Crystallography

I have recently demonstrated that 4D cryo-electron microscopy, in which a protein macromolecule is embedded in glassy ice, permits the visualization of picosecond or nanosecond dynamics of proteins in a fully hydrated, native-like state (5). In this proof-of-principle experiment, from a series of time-resolved diffraction patterns of protein macromolecules in a thin layer of vitrified water at cryogenic temperatures, I was able to detect picometer movements of protein molecules on a nanosecond time scale (Fig. 2).

Together with traditional cryo-EM techniques, the application of 4D cryo-EM to membrane proteins is the central aim of my research proposal. While traditional cryo-EM will allow my team to determine atomic-resolution 3D structures of protein complexes (6), 4D cryo-EM will permit the examination of protein dynamics in the hydrated state with unprecedented spatiotemporal resolution (5). Indeed, the conformational changes in biologically active 2D membrane protein crystals (7) and 3D micro- or nanocrystals are often much larger than the picometer-scale movements I have detected in amyloid-like microcrystals (Fig. 2), and many of these movements occur on the picosecond, or faster, time scales (8). Below, I outline in detail each of my specific aims using a novel combination of 4D cryo-EM (Figs. 2, 3) and laser-assisted single-particle cryo-EM (Fig. 4) to determine the structure and dynamics of proteins in the hydrated state.

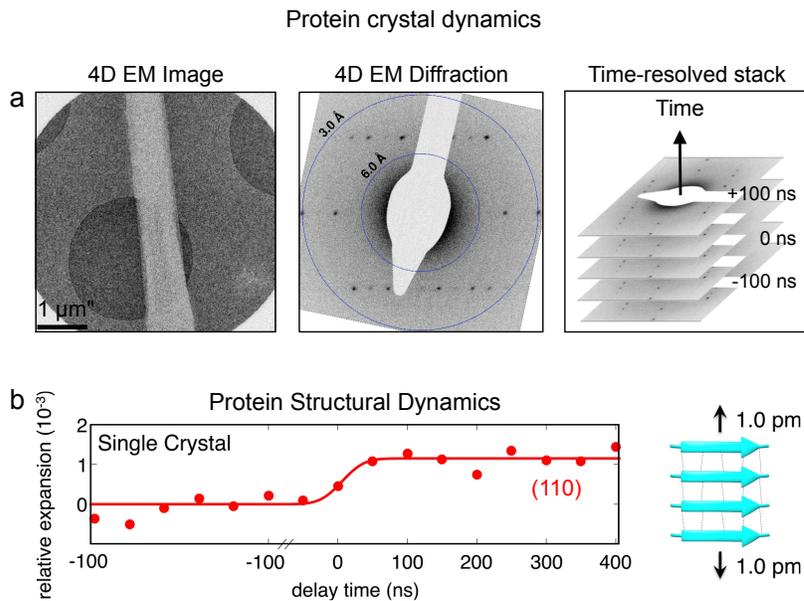


Fig. 2. 4D EM of protein microcrystals. **(a, left)** An image of a sub-micron protein crystal taken with a 4D electron microscope and **(a, middle)** its diffraction pattern. Note that using the pulsed electron beam, spots at 2.8 Å are observed indicating near-atomic resolution. **(a, right)** Diffraction patterns are taken at discrete time delays forming a time-resolved “movie” stack. **(b)** Algorithms are used to detect the expansion of the crystals’ constituent

$\beta$ -sheets resulting in a decreased separation between peaks (in reciprocal space) upon excitation by the pump pulse. Importantly, the protein crystals do not shatter under significant laser-induced pressure (~5 MPa) and are suitable for investigating protein dynamics using 4D EM.

*Specific Aim 1: Determination of structural dynamics leading to changes in membrane potential.*

### **Structure and Dynamics of Membrane Proteins using 4D Electron Crystallography**

Electron crystallography is an immensely powerful technique for obtaining atomic-resolution structures of membrane proteins. It has the distinct advantage of using a lipid bilayer as the medium for crystallization, unlike X-ray crystallography (or solution NMR), which generally studies membrane proteins solubilized in a detergent micelle (9). Crucially, the more natural membrane environment is likely to favor a native conformation and potentially to allow conformational changes in response to external stimuli such as light (10) or heat (11).

I propose to use 4D EM to resolve the structural dynamics of membrane proteins that alter membrane potential either directly (ion channels/pumps) or *via* a signaling cascade (G-Protein Coupled Receptors). To directly visualize the opening and closing of an ion channel, the light-gated ion channels **channelrhodopsin** (12) and **KR2** (13) will be used. The conformational changes involved in signal transduction will be determined using **rhodopsin** (14). Finally, I plan to investigate the structural changes involved in the opening and closing of a voltage-gated ion channel, **KvAP**, by using pulsed infrared light to reversibly alter the electrical capacitance of the lipid bilayer leading to depolarization of the membrane and pore opening (15).

### **Pipeline for 4D Electron Crystallography**

The pipeline for 4D electron crystallography is shown schematically in Figure 1.5. Briefly, after expressing and purifying the protein, screening for large 2D crystals or sub-micron-sized 3D crystals is necessary. Once we have identified likely candidates, crystals are embedded in vitreous ice (cryo-EM) and screened using 4D EM to check that they (i) do not shatter under the (pump) laser beam and (ii) diffract to high-resolution using the electron beam. At this stage, a well-defined time-delay,  $t_n$  (e.g. +5 ps), between laser (pump) and electron (probe) beams is set and tilt-series data on a single crystal is collected. Images (for phase information) and diffraction patterns (for amplitudes) at the time-delay,  $t_n$ , are recorded. Using phase extension methods (16), a high-resolution 3D membrane protein structure at time delay,  $t_n$ , can be solved. This procedure is then repeated for the next time delay,  $t_{n+1}$  (e.g. +10 ps), by changing the optical path length of the pump beam and recording a tilt series and solving the 3D structure at this later time. Owing to radiation damage, it may be necessary to obtain tilt-series at different time delays from different crystals, but the inherent assumption of no crystal-to-crystal variation is similar to the sample homogeneity requirement for “single-particle” approach commonly used to solve protein structure using traditional cryo-EM (6). The 3D structures solved at multiple time delays can be put together sequentially to form frames of a movie showing the conformational changes of the protein of interest.

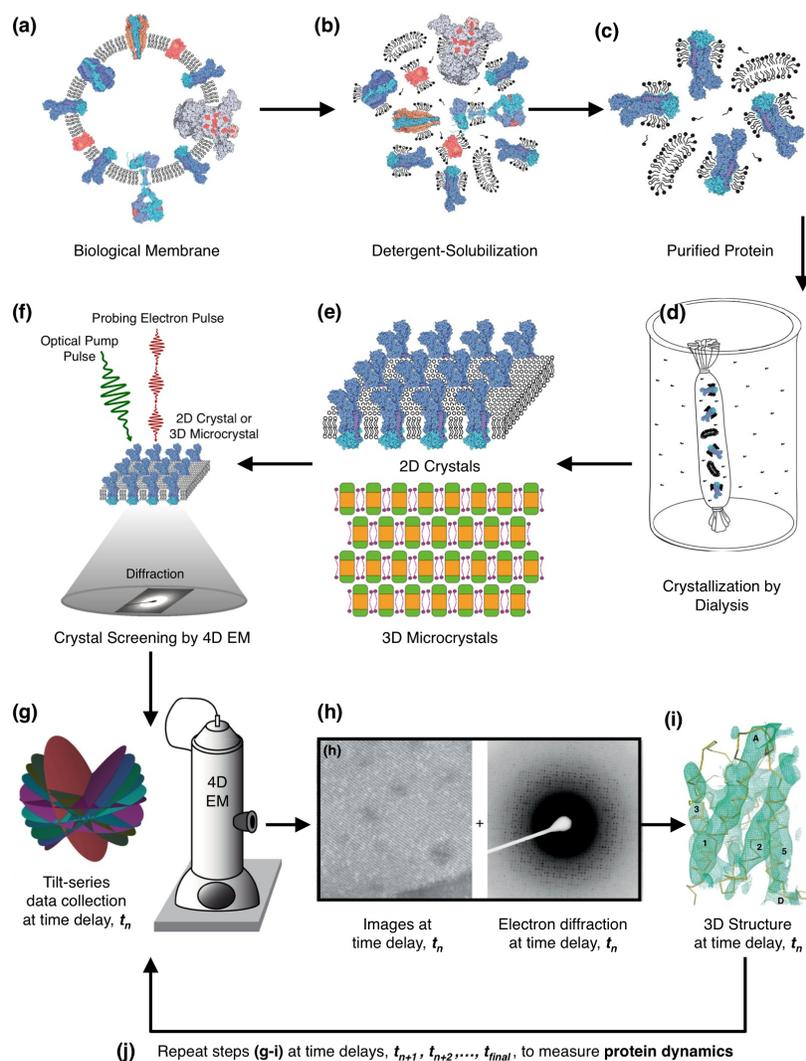


Fig. 3. Structure determination by 4D Electron Crystallography begins with vesicles derived from a biological membrane, which could be either from natural sources or from a heterologous expression system. This biological membrane has a heterogeneous population of membrane proteins embedded in a lipid bilayer (a). Detergent is used to solubilize this membrane (b), thus placing each of the proteins in a mixed micelle of lipid and detergent. (c) Extra lipid is added to the preparation and dialysis is then used to remove the detergent, thus reconstituting the purified membrane protein back into a lipid bilayer (d). Two-dimensional, or 3D micro-crystals are formed, that are thin enough to diffract electrons. (f) These crystals are then screened using the 4D EM to check that they do not shatter under the (pump) laser beam and diffract to high-resolution using the electron beam. (g, h) 4D Images and diffraction patterns of a single crystal are recorded at various tilt-angles using a time-delay  $t_n$ . Amplitudes from the diffraction patterns are combined with phases from the images and after merging data together from a wide variety of tilt angles, a three-dimensional structure at time-delay  $t_n$  is generated (i). This procedure is then repeated for the next time delay,  $t_{n+1}$ , by changing the optical path length of the pump beam and recording a tilt series and solving the 3D structure at this later time. The 3D structures solved at multiple time delays can be put together sequentially to form frames of a movie showing the conformational changes of the protein of interest. Image adapted from (9).

*Specific Aim 2: Development of laser-based methods to enhance traditional cryo-EM imaging of single-molecule membrane proteins and whole cells.*

**Pulsed imaging for single particle reconstruction**

In light of the recent exciting developments in the attainable resolution of single particle cryo-EM reconstructions using direct electron detectors (17), which allow a movie of a sample to be recorded over the course of its exposure to the electron beam thus permitting beam-induced motion to be computationally corrected by aligning the frames, I would like to investigate whether a pulsed electron beam in a 4D electron microscope, delivered by photoemission from the cathode, gives any improvement over a continuous electron beam, as used in conventional electron microscopy.

Radiation damage of a protein sample occurs on two timescales (18). Chemical bonds are broken on the order of femtoseconds and mass loss occurs between 1 picosecond and 1 microsecond. It requires approximately  $1 \times 10^8$  electrons to form an image of a protein molecule. Therefore, if a pulsed laser can generate enough photoelectrons to image a protein molecule before mass loss occurs, the overall resolution of the single particle cryo-EM reconstruction would be improved (in particular, for smaller protein complexes where mass loss is a more significant problem than for larger, robust complexes (19)).

**Development of a Ponderomotive Phase Plate**

Cryo-EM imaging of proteins relies on phase contrast, producing two-dimensional phase-resolved images based on the delays experienced by electron wave fronts as they pass through the protein macromolecules embedded in vitreous ice. Since a perfectly focused lens produces minimum phase contrast, cryo-EM images of proteins complexes, or cells, are taken at high defocus (Fig. 4b, left (20)). While this increases the phase contrast of the specimen, there is a resulting loss of information at both low and high spatial frequencies, limiting resolution of the resulting cryo-EM/tomographic reconstruction.

In principle, the post-specimen electron beam can be phase-corrected and the most common type of phase plate that can be inserted into the microscope is a carbon film with a small hole (Zernike phase plate). This plate alters the phase of scattered electrons by  $90^\circ$  with respect to the unscattered beam, resulting in dramatically enhanced contrast (Fig. 4b, right (20)). However, thin-film phase plates are prone to charging, degrading in performance in only a few hours, can cause incoherent scattering, resulting in a loss of information, and are prone to contamination.

I would like to develop a “phase plate” that does not suffer from these deleterious effects and allows **in-focus phase contrast of cryo-EM specimens**. By focusing a pulsed laser to intersect the post-specimen electron beam (Fig. 4a), the ponderomotive potential of the focused laser crossover produces a scattering-angle-dependent phase shift in the electrons, thus resulting in a highly tunable contrast transfer function, with significantly enhanced contrast of the protein macromolecules or cells under inspection.

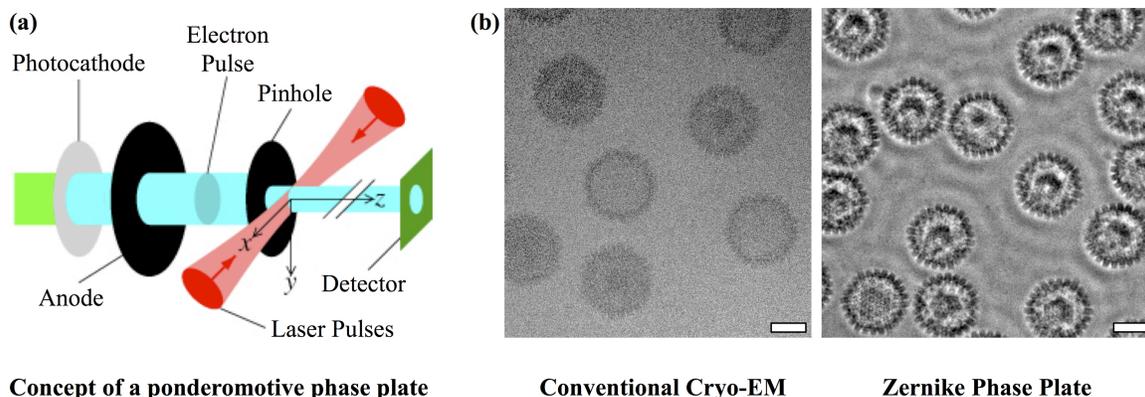


Fig. 4. Phase-shifted cryo-EM of protein macromolecules. **(a)** Schematic of a ponderomotive phase plate. **(b, left)** Image of ice-embedded HSV-1 B capsids collected using conventional cryo-EM. **(b, right)** Equivalent image collected using Zernike phase plate. Scale bar, 50 nm. Images in panel **(b)** taken from (20).

## Conclusions

In this short proposal, I have briefly set forth my vision of how 4D electron microscopy could yield new and exciting insights into the structure, dynamics and function of membrane proteins. Determining the membrane protein structural dynamics involved in controlling the flow of ions into and out of the cell is fundamentally important for understanding how cells communicate. Development of novel laser-based methods may lead to significant improvements in conventional cryo-EM or tomography of biological specimens. **There is a wealth of unexplored phenomena that cannot be captured by conventional transmission electron microscopy but *can* be visualized with 4D electron microscopy** and I would very much like to be given the opportunity to develop this new technology in the Chemistry department at the University of Cambridge.

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