15 Physics of Biological Systems

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The structural investigation of individual biological objects by employing coherent low-energy electrons is the primary goal of our research. It involves inline holography with low energy electrons, Fourier transform holography as well as coherent diffraction imaging and is assisted by micro-structuring techniques using a focused gallium ion beam device as well as a focused helium ion beam available to us at the at the Swiss Federal Laboratories for materials science and technology (EMPA) in Dübendorf. Our current activities are divided in the following interconnected individual projects:

- **Electron Holography and Coherent Diffraction**
  Major experimental challenges are to improve the interference resolution, establish methods for creating free standing thin films of graphene transparent for low-energy electrons as well as appropriate techniques to present a single protein to the coherent electron wave front. Next to these experimental issues, a second, equally important aspect for achieving high resolution structural information is the reconstruction of the electron holograms, respectively iterative phase retrieval, in coherent diffraction. This is achieved by employing newly developed numerical algorithms to solve the integrals governing these coherent optics problems.

- **Lens-less Imaging by Fourier Transform Holography (FTH)**
  FTH with low-energy electrons is a high resolution lens-less imaging method based on the use of an extended reference where a specimen of biological or non-biological nature is non-destructively imaged. The recording is performed by illuminating the specimen and reference object or pinhole with a parallel beam of low-energy electrons. The result of the interference between the wave scattered by the specimen and the reference wave results in an intensity distribution, or holographic diffraction pattern, whose Fourier transform represents the autocorrelation of the transmission function of the specimen under study.

- **Electron and Ion Point Sources**
  Field Ion Microscopy and related techniques are employed for fabricating and applying novel electron and ion point sources. In collaboration with the PSI, field emitter arrays are characterized and specified for their use as bright electron sources for the X-Ray Free Electron Laser (XFEL) project.

- **DNA and Proteins in the Liquid Phase**
  The aim of this project is to directly observe the dynamics of single DNA molecules in liquids by video fluorescent microscopy. In combination with molecular anchoring techniques, adopted from Clondiag Chip Technologies in Jena, we also address the energetics of a single DNA molecule. Appropriate DNA modifications for attaching fluorescent proteins are also designed by and shall serve us in our efforts to obtain structural information about proteins by electron holography and coherent diffraction. Thermal desorption spectroscopy of water from

fluorescent proteins shall help us to judge under what thermal conditions proteins are still in their native state in a vacuum environment.

Most of the protein structural information available today has been obtained from crystallography experiments by means of averaging over many molecules assembled into a crystal. Since biological molecules exhibit different conformations, averaging smears out structural details. That is why a strong desire to gain structural data from just a single molecule is emerging. We are working towards the objective of deriving atomic structure information from experiments carried out on just one individual molecule subject to the interaction with a coherent low-energy electron wave. Meanwhile, it has been thoroughly established that electrons with kinetic energies below 200 eV are the only radiation known today where elastic scattering dominates. Radiation damage-free imaging of a single biological molecule is thus possible by recording holograms and coherent low-energy electron diffraction patterns [1].

Retrieval of the Phase of the Scattered Wave - When Holography Meets Coherent Diffraction

The phase problem is inherent to crystallographic, astronomical and optical imaging where only the intensity of the scattered signal is detected and the phase information is lost and must somehow be recovered to reconstruct the object's structure. Modern imaging techniques at the molecular scale rely on utilizing novel coherent light sources like X-ray free electron lasers for the ultimate goal of visualizing such objects as individual biomolecules rather than crystals [2]. Here, unlike in the case of crystals where structures can be solved by model building and phase refinement, the phase distribution of the wave scattered by an individual molecule must directly be recovered. There are two well-known solutions to the phase problem: holography and coherent diffraction imaging (CDI). Both techniques have their pros and cons. In holography, the reconstruction of the scattered complex-valued object wave is directly provided by a well-defined reference wave that must cover the entire detector area which often is an experimental challenge. CDI

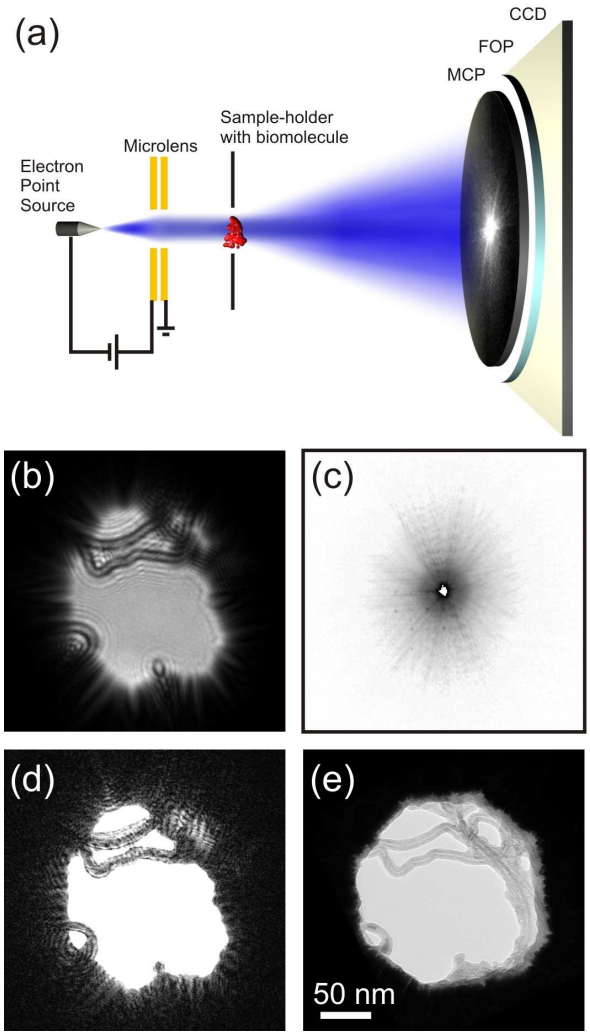

FIG. 15.1 – *HCDI reconstructions of a coherent low-energy electron diffraction pattern of individual carbon nanotubes. (a) Schematics of the low-energy electron microscope, the distance between electron source and detector amounts to 68 mm. The detector components are: microchannel plate (MCP), fiber optical plate (FOP) and CCD chip. (b) Hologram recorded with electrons of 51 eV kinetic energy. (c) Diffraction pattern recorded with electrons of 145 eV kinetic energy. (d) Reconstructed amplitude distribution using HCDI. (e) TEM image recorded with 80 keV electrons. In (b) and (d) the central parts of the images, with 600x600 pixels are shown.*

provides highest possible, only wavelength limited, resolution, but the phase recovery is an iterative process which requires some pre-defined information about the object and whose outcome is not always uniquely-defined. Moreover, the diffraction patterns must be recorded under oversampling conditions, a pre-requisite to be able to solve the phase problem. We have shown how holography and CDI can be merged into one superior technique: holographic coherent diffraction imaging (HCDI). An inline hologram can be recorded by employing a modified CDI experimental scheme. We demonstrated that the amplitude of the Fourier transform of an inline hologram is related to the complex-valued visibility, thus providing information on both, the amplitude and the phase of the scattered wave in the plane of the diffraction pattern. With the phase information available, the condition of oversampling the diffraction patterns becomes obsolete, and the phase problem can be solved in a fast and unambiguous manner. We demonstrated the reconstructions of various diffraction patterns of objects recorded with visible light as well as with low-energy electrons as shown in Fig. 15.1 [3].

Non-destructive Imaging of an Individual Protein

The mode of action of proteins is to a large extent given by their ability to adopt different conformations. This is why imaging single biomolecules at atomic resolution is one of the ultimate goals of biophysics and structural biology. The existing protein database has emerged from X-ray crystallography, NMR or cryo-TEM investigations. However, these tools all require averaging over a large number of proteins and thus over different conformations. This of course results in the loss of structural information. Likewise it has been shown that even the emergent X-FEL technique will not get away without averaging over a large quantity of molecules.

We have recently obtained the first recordings of a protein at sub-nanometer resolution obtained from one individual ferritin by means of low-energy electron holography. One single protein could be imaged for an extended period of time without any sign of radiation damage. Since ferritin exhibits an iron core, the holographic reconstructions could also be cross-validated against TEM images of the very same molecule by imaging the iron cluster inside the molecule while the protein shell is decomposed.

The protein of interest needs to be free-standing in space when exposed to the low-energy electron beam. Therefore the protein is attached to a carbon nanotube suspended over a hole. Strictly speaking, arrays of small holes of 240 nm in diameter are milled in a carbon coated silicon nitride membrane by means of a focused gallium ion beam. The nanotube-ferritin complex is kept in aqueous solution of which a droplet is applied onto the holey membrane. Once the droplet has dried out some of the nanotubes remain across holes providing free-

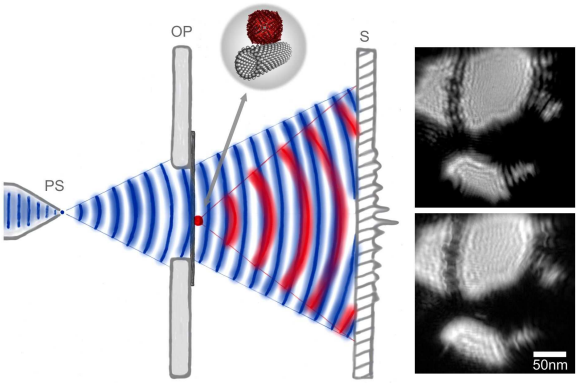

FIG. 15.2 – *Left: Scheme for recording the low-energy electron hologram of a protein. Conduction electrons confined in a pointed W(111) single crystal wire are field emitted into vacuum at an atomic-sized emission area providing a coherent low-energy electron point source (PS). At the less than 1 micron distant object-plane (OP), part of the coherent electron wave is scattered by a ferritin attached to a carbon nanotube constituting the object wave indicated in red. At a distant detector screen (S), the far-field interference pattern between object- (red) and reference-wave (blue) - the hologram - is recorded and its digital record is subject to the numerical reconstruction of the protein. Right: A hologram (top) and its reconstruction (bottom) show individual ferritins attached to a carbon nanotube.*

standing ferritin molecules. These molecules can then be examined in our low-energy electron microscope.

Prior to the described preparation procedure the carbon nanotubes undergo acid treatment in order to form carboxyl groups on the outer wall and hence disperse efficiently in ultra highly purified water. Adding a buffered solution of proteins, the latter eventually bind to the nanotubes by dipole forces, as schematically illustrated in the inset of Fig. 15.2. This preparation does not rely on specific features of ferritin and hence is applicable to a large class of biomolecules [4].

Recent Achievements in Coherent Diffraction Microscopy

We have decided to use the damage-free radiation provided by coherent low-energy electrons to realize coherent diffraction imaging of single molecules. The overall setup of our coherent electron diffraction imaging microscope is sketched in Fig. 15.3 (left) together with a recently achieved coherent diffraction pattern of free-standing graphene recorded in the transmission mode with a 8000x6000 pixels CCD chip. A sharp W-tip acts as an electron point source emitting a coherent spherical electron wave with kinetic energies between 50 and 300 eV. A micro-lens placed a few microns away from the electron emitter forms a coherent parallel wave that impinges onto a molecule attached to a micro-structure some distance behind the lens in a field-free region. At a distant detector, the intensity of the diffraction pattern corresponding to the amplitude-square of the Fourier transform of the object is recorded with high spatial resolution. In order to sample this pattern with sufficiently high frequency to match the oversampling requirement, the object must be surrounded by a no-density region.

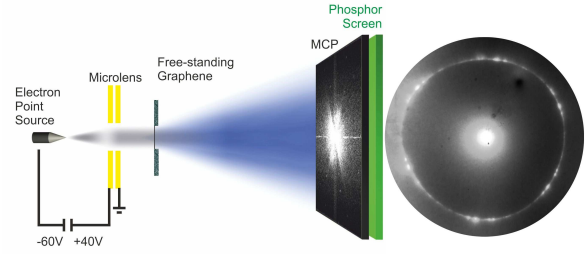

FIG. 15.3 – *Left: Schematic representation of the coherent electron diffraction microscope for imaging individual biomolecules. Right: Coherent diffraction pattern of a free-standing graphene flake mounted on a micro machined holey silicon nitride membrane. With a kinetic energy of 400 eV, the highest order diffraction signal corresponds to a resolution below 2 Å in real space.*

Lens-less Imaging by Fourier Transform Holography: Nano-Patterning Using the Helium Ion Microscope

The Orion helium ion microscope, see Fig. 15.4, at the EMPA in Dübendorf allows structuring of thin films in the nanometer regime. FTH is a lens-less imaging method based on the use of a reference scatterer [5–8]. If coherent low-energy electrons are used, FTH turns into a high-resolution imaging technique where a specimen of biological or non-biological nature is non-destructively imaged.

The recording is performed by illuminating the specimen and reference scatterer with a parallel beam of electrons whose energy range is 50-200 eV. The interference between the wave scattered by the specimen and the reference wave results in an intensity distribution, or holographic diffraction pattern, whose Fourier transform represents the auto-

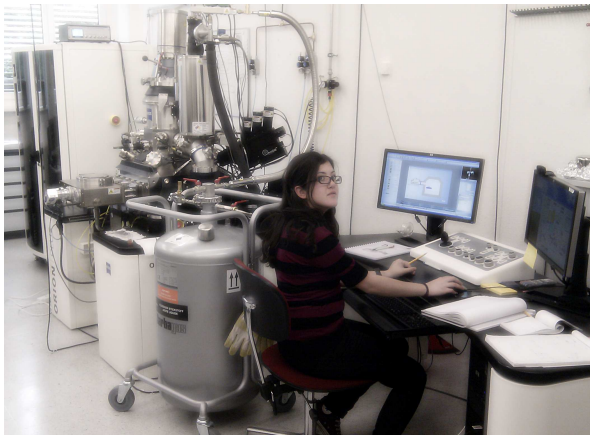

FIG. 15.4 – *Mirna Saliba working on the helium ion microscope at the EMPA in Dübendorf.*

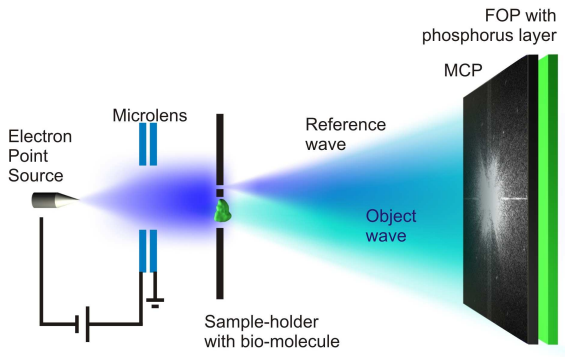

FIG. 15.5 – *Schematic of FTH: From left to right: a divergent beam of low-energy electrons impinges on an electrostatic micro-lens which collimates the beam. It is incident on the sample plane that includes a specimen and a pinhole.*

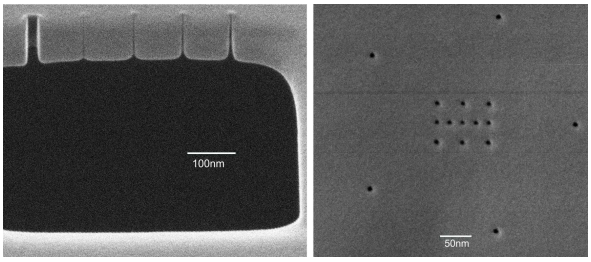

FIG. 15.6 – *Left: 1 pixel width lines milled with varying doses across a structure. The line on the far right clearly shows a width below 5 nm. Right: Patterning of a mask suitable for Fourier transform holography: the object to be reconstructed consists of an array of 7 nm holes surrounded by 5 pinholes to serve as reference scatterers.*

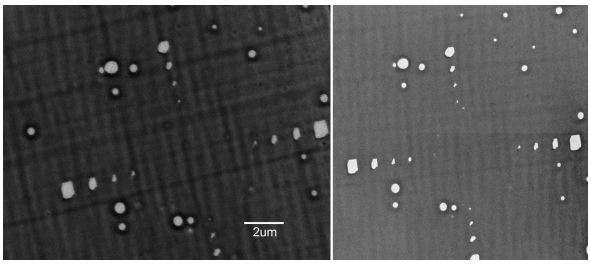

FIG. 15.7 – *Left: Helium ion micrograph in transmission mode of several milled holes in a 15 nm thin carbon membrane. Right: Transmission electron micrograph of the same region at 120 kV.*

correlation of the transmission function of the specimen under study. FTH (see Fig. 15.5) highlights the most advantageous aspects of both holography and coherent diffraction imaging, i.e. it is a hybrid technique that naturally encrypts the phase information by the use of a reference scatterer and yields high resolution complex-valued images of the specimen of interest in a single computational step.

FTH with low-energy electrons requires the fabrication of pinholes with just 5-20 nm diameter serving as reference scatterers. The latter is achieved by a Helium Ion Microscope (HIM) [9]. Obtaining the minimal pinhole size (5 nm) with the HIM is crucial to push the resolution limit, i.e the smaller the milled pinhole, the higher the resolution of the reconstructed image. So far, nano-patterning has been routinely achievable using the HIM [11]. The milling has been performed in metal coated silicon nitride substrates as well as in free-standing carbon membranes, as shown in Figs. 15.6 and 15.7

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[11] We kindly acknowledge the support of Ivan Shorubalko and Fabio Lamattina with the Orion helium ion microscope at the EMPA in Dübendorf