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 holography with low energy electrons as well as coherent diffraction and is assisted by microstructuring techniques using a focussed gallium ion beam device. Our current activities are divided in the following interconnected individual projects:

- **Electron Holography**
  The overall idea is to apply holography with low energy electrons to investigate the structure of individual biological molecules. Major experimental challenges are to improve the interference resolution in electron holograms, 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. This is achieved by back-propagating the object wave information, recorded in the hologram plane, by employing a numerical algorithm to solve the integrals governing this coherent optics problem.

- **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 XFEL (X-Ray Free Electron Laser) project.

- **Fluorescent Microscopy**
  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, we also address the energetics of a single DNA molecule. Appropriate DNA modifications for attaching fluorescent proteins to are designed by Clondiag Chip Technologies in Jena and shall serve us in our efforts to obtain structural information about proteins by electron holography.

- **Coherent Low-Energy Electron Diffraction Microscopy**
  This is our most recent approach of using coherent electron wave fronts for structural biology at the single molecule level. It is based on an iterative phase retrieval scheme pioneered by John Miao from the University of California at Los Angeles with whom we collaborate in this project. Below we shall discuss the recent achievements in this project in some more detail.

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. Nevertheless, a strong desire to gain structural data from just a single molecule is emerging. We are working towards the objective of deriving atomic structure in-

formation 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 its oversampled coherent low-energy electron diffraction pattern.

15.1 Diffraction microscopy and the Oversampling Method

X-ray and electron diffraction microscopy have traditionally been limited to crystalline samples. The repeating crystalline structure amplifies the signal in certain directions, so that the far-field diffraction pattern shows discrete Bragg peaks. Such an X-ray diffraction pattern lacks information on the phase of the object wave upon scattering which is needed to provide unambiguous information on the real space location of the atoms. The recovery of a single molecule structure from its diffraction pattern constitutes an inverse problem, which requires a solution to the phase problem. Historically, Gabor's invention of holography [1], dating back to 1948, was the first approach towards solving the phase problem by recording the interference of the object wave with a reference wave. In 1952, Sayre first proposed the possibility of recovery of phase information of non-crystalline samples by oversampling its diffraction pattern [2]. In 1972, Gerchberg and Saxton proposed the first iterative algorithm to recover the object structure from the amplitude of the scattered wave [3]. This algorithm was successfully applied to recover an object from its oversampled diffraction pattern by Miao et al. [4]. They demonstrated that the phase retrieval algorithm converges (after several 1000 iterations) if the initial conditions are such that the surrounding of the molecule is known. Given that the diffraction pattern is sampled above Nyquist frequency (oversampled), the uniquely-defined phase distribution can be recovered from the scattering record [5]. Thus, the experimental recording of a far-field diffraction pattern followed by numerical recovery of the object structure constitutes the novel class of high resolution coherent diffraction imaging techniques. In what follows, we summarize the state of the art as well as recent achievements in this fairly active field, which are to some extent also catalyzed by the emerging XFEL facilities around the world. CDI has successfully been applied to image nanocrystals [6], single cells [7] and individual bacteria [8] with X-rays. A carbon nanotube was imaged at atomic resolution with high energy electrons [9]; three-dimensional objects could be reconstructed from a single diffraction pattern [10]. Most applied phase retrieval methods are based on hybrid input-output and error reduction algorithms [11]. However, a number of problems associated with the reconstruction procedure remain: non-uniqueness of the solution due to unavoidable noise in experimental diffraction patterns, missing data in the overexposed central area of the diffraction pattern, complex-valued transmission functions likely to be exhibited by imaged objects, and finally, stagnation of the iterative process often at partial solutions. These shortcomings explain why there is still an ongoing search for better reconstruction methods and alternative experimental techniques. Modifications of the reconstruction methods with the goal to optimize the convergence of the iterative process, such as charge-flipping [12] and the so called shrink-wrap [13] algorithms have recently been suggested. Furthermore, novel experimental techniques to simplify the reconstruction process without sacrificing high resolution, such as Fresnel Diffraction Imaging [14] and Fourier Transform Holography [15] have recently been proposed. In the latter a small hole next to the sample provides the reference wavefront which in turn reduces the reconstruction process to a single Fourier transform while, at the same time, the achievable resolution remains as high as in conventional CDI.

15.2 Schematic of the Coherent Electron Diffraction Microscope and experimental realization

We have embarked onto using 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. 15.1. 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 (see Fig. 15.3) 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.

Our diffraction microscope has been designed and built featuring a dedicated detector system, available since March 1st 2011, for obtaining oversampled diffraction patterns. It comprises a microchannel plate, a small grain phosphor screen di-

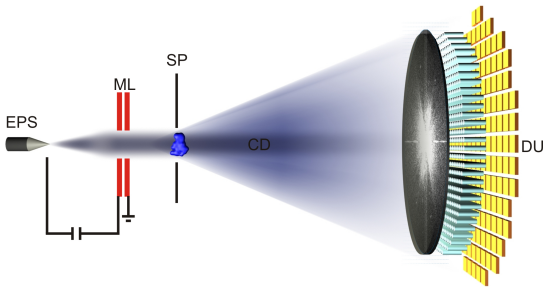

Fig. 15.1 – The coherent electron diffraction microscope for imaging individual biomolecules. (EPS): coherent electron point source, (ML): low aberration micro-lens to form a parallel electron wavefront, (SP): sample imbedded inside the coherent electron beam, (CD): coherent diffraction pattern, (DU): detector unit, featuring a high spatial resolution low-energy electron detector that fulfils the oversampling condition.

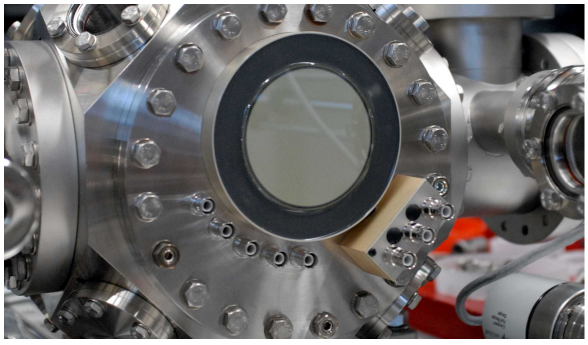

Fig. 15.2 – Ambient pressure side of the 75 mm diameter detector showing the fiber optic plate and connectors for the micro-channel-plate detector.

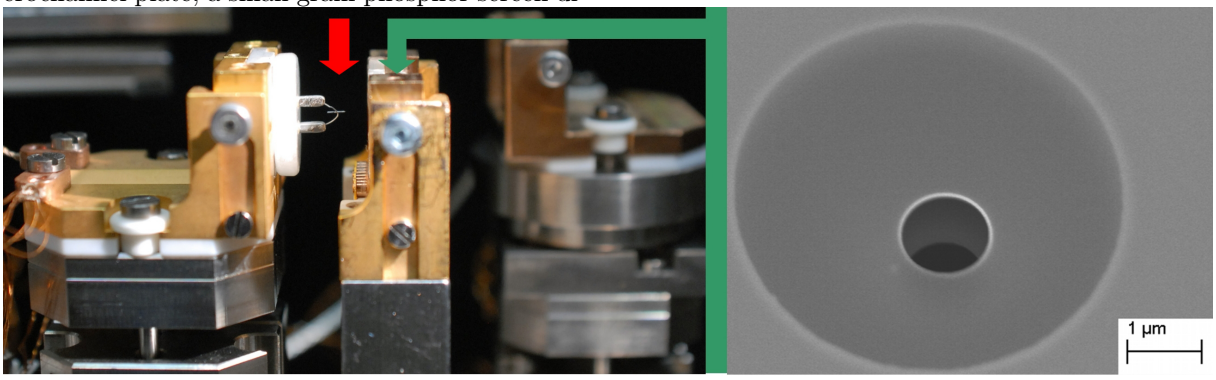

Fig. 15.3 – Formation of a low-energy coherent parallel electron wavefront. The electron point source (marked by the red arrow) is mounted onto a nanometer precision Piezo stage and positioned close to a micro machined lens (green arrow) exhibiting a 1micron bore. In this way a parallel beam of sub-micron width is formed beyond the lens. A SEM image of the lens is shown at the right.

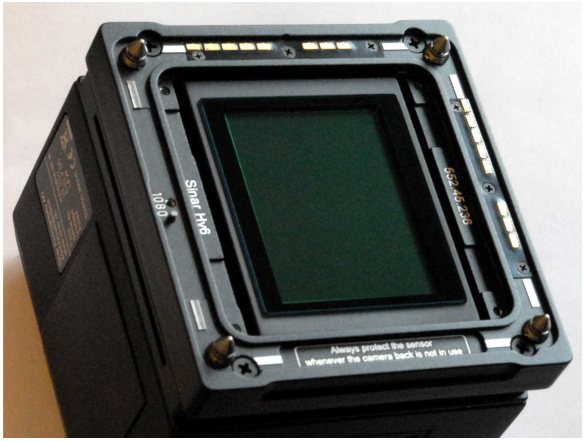

Fig. 15.4 – A high resolution CCD chip (Sinar AG, Zurich) featuring 8000x6000 pixels is optically coupled to the fiber optic plate for recording the diffraction pattern with high spatial resolution and a 16 bit dynamics.

rectly deposited onto a fibre optic plate (see Fig. 15.2) and a high resolution (8000x6000 pixels) CCD chip (Fig. 15.4). An electrochemically etched single W[111] crystal serves as the source of coherent low-energy electrons. It is mounted on a three-axis manipulator so that the tungsten tip can be positioned with sub-nanometer precision. The sample is mounted on a separate four-axis manipulator to enable centering it in the electron beam. An additional degree of freedom for rotating the sample has been included so that sets of diffraction patterns at various tilt angles can be acquired.

15.3 Conditions for non-destructive imaging of a single biomolecule

Radiation damage is the major problem that obstructs imaging an individual biological molecule for structural analysis. Due to the strong inelastic scattering of X-rays and high-energy electrons there is little hope for obtaining structural information from just one molecule. Even the expected results of the XFEL projects currently under development involve averaging. In fact, more than 10^5 diffraction patterns of identical molecules must be recorded in order to obtain structural detail at a resolution of 3 , even with a 10 fs X-ray pulse containing 10^{12} photons at 1.5 wavelength. This implies that currently there is still no tool or concept at hand based on X-rays or high-energy electrons that has a realistic chance of imaging just one single biological molecule at atomic resolution.

In contrast to the current state-of-the-art imaging methods as briefly sketched above, we could recently show that a molecule as fragile as DNA withstands irradiation by coherent low-energy electrons and remains unperturbed even after a total dose of at least 5 orders of magnitude larger than the permissible dose in X-ray or high-energy electron imaging [16]. These recent findings demonstrate that coherent low-energy electrons are the only non-damaging Angstrom wavelengths radiation known today. This is the pre-requisite for the current proposal: with coherent low-energy electrons it shall thus be possible to look at truly just one entity if it comes to high resolution diffraction microscopy of individual biomolecules.

15.4 Recent achievements in coherent diffraction microscopy

15.4.1 Published results

Apart from radiation damage, electron lens aberrations are a major barrier limiting the resolution of electron microscopy. By scaling down an electron lens by several orders of magnitude, we circumvented the problem of intrinsic lens aberrations and ended up with a conceptually simple one micron sized lens exhibiting aberrations comparable to those of magnetic objective lenses used in modern high end electron microscopes [18].

By using our coherent electron source in combination with such a micro-lens it has been possible to form a parallel beam and direct it towards a carbon nanotube bundle suspended over a micromachined slit in a silicon nitride membrane. In this way the first coherent electron diffraction pattern, albeit not yet fulfilling the oversampling condition needed for reconstruction, of a single nanometer-sized object using low-energy electrons has been obtained [19].

15.4.2 Imaging biological samples

While a dedicated UHV instrument is in operation and micro-lens fabrication is meanwhile also routinely possible, a major focus must now be directed towards appropriate preparation of single biomolecules, detecting their diffraction pattern with high spatial resolution at the detector level and retrieving the phase of the object-wave by a fast converging iterative numerical algorithm. It has turned out that it is feasible to obtain a hologram of a biological object prior to recording a diffraction pattern of the very same biological sample. This provides a great benefit, not available in any other diffraction setup. Usually, the central region of the diffraction pattern providing the low resolution information about the object is missing due to the overexposure of the zero and low order diffraction. In our setup, this usually missing information is provided by first gathering a hologram of the very same object just before the micro-lens is activated to form a parallel beam to obtain a diffraction pattern of the very same object. This is illustrated in Figs. 15.5 and 15.6 showing electron holograms and the corresponding diffraction patterns of a tobacco mosaic virus (TMV) (sample by courtesy of the University of Strasbourg) and an individual double wall carbon nanotube, stretched over a 200 nm wide opening in a silicon nitride membrane.

While both diffraction patterns have not yet been sampled at high rates, since the 8000x6000 pixel CCD chip has just been delivered (March 1st), the nanotube diffraction pattern displayed in Fig. 15.6 has been obtained already with the enlarged MCP detector of 75 mm in diameter. The orange circle in Fig. 15.6 indicates a k-vector that corresponds to a resolution of currently 3.9 . In future experiments this value shall be pushed into the 2 regime by detecting even higher orders of the diffraction pattern.

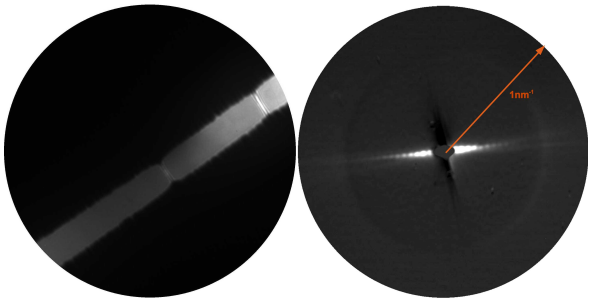

Fig. 15.5 – Left: Hologram of a TMV taken with 78 eV electrons. Right: Coherent diffraction pattern taken of the very same TMV with electrons of 137 eV kinetic energy. The modulations apparent in the diffraction pattern correspond to a length of 18 nm in real space - the width of an individual TMV.

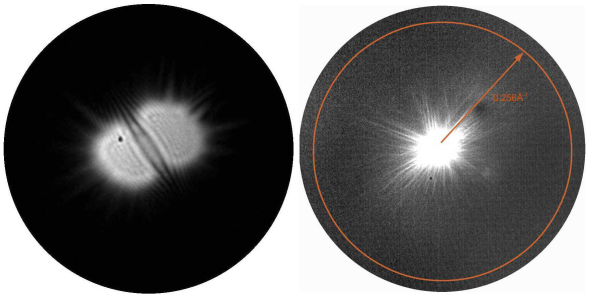

Fig. 15.6 – Left: Hologram of a double wall carbon nanotube (DWCNT) taken with 62 eV electrons. Right: Coherent diffraction pattern taken of the very same DWCNT with electrons of 127 eV kinetic energy.

15.5 Novel Fourier-domain constraint for Fast Phase Retrieval [17]

The distribution of the scattered object wave in the far-field $O_0(X,Y)$ is given by the Fourier transform of the object transmission function $o(x,y)$: $O_0(X,Y) = FT(o(x,y))$. Although the object shape cannot easily be recognized in its far-field image, it can be uncovered by an iterative reconstruction processes. While there is an intensive search for optimal constraints in the object plane to improve the reconstruction routines, possibilities to apply constraints in the Fourier domain have not yet been explored. Recently, we proposed

a novel, simple and powerful Fourier-domain constraint providing fast and reliable reconstructions. In common phase retrieval methods, the phase of the iterated field O_i is adapted for the next iterative loop, while the amplitude is replaced by the square root of the measured intensity:

$$O_{i+1} = \frac{O'_i}{|O'_i|}\sqrt{I_0}, \qquad (15.1)$$

where $I_0 = O_0 O_0^*$. We propose using the following constraint instead:

$$O_{i+1} = \frac{O'_i}{|O'_i|^2 + \epsilon} O_0 O_0^* = l O_0 O_0^*. \qquad (15.2)$$

This formula is derived via a direct analogy to holography, where the transmission of a recorded hologram H is given by $H \sim R^* O + R O^*$. Here, R denotes the reference wave and O the object wave. In the reconstruction process, the hologram is multiplied with the reference wave resulting in the straightforward reconstructed object wave $RH/|R|^2 = O$. This approach can be applied for reconstructing a diffraction pattern if we formally treat the measured intensity of the diffraction pattern $O_0 O_0^*$ as a hologram H and O'_i as the reference wave R see Eq. 15.2. This novel constraint, unlike the one given by Eq. 15.1, does not only account for the phase of O'_i, but also for its amplitude. The latter must gradually approach the amplitude of O_0. In order to avoid division by zero, a constant ϵ is added in the denominator. Any additional constraint in the object plane can still be selected independently of the Fourier-domain constraint.

The performance of our novel constraint is demonstrated by the reconstruction of a simulated and experimental light optical diffraction pattern, the result of the latter is shown in Fig.15.7. We also compared the performance of our novel reconstruction algorithm to those of established reconstruction methods. Among these, the so called "shrink-wrap" algorithm [13] is considered to be the most effective. By using the "shrink-wrap" algorithm the simulated diffraction pattern was already reconstructed after about 70 iterations. However, the "shrink-wrap" algorithm failed when applied to our experimental diffraction pattern. The reason could be that the "shrink-wrap" algorithm relies on a continuously updated support mask, which could not be well defined for our experimental sample as there were some fine scratches around the object as verified by optical microscopy.

This novel algorithm has a number of advantages in comparison to other algorithms: any initial guess about the shape of the object is irrelevant for the convergence of our routine. This independence of the object topology shall be highly beneficial for the reconstruction of objects that cannot be confined within a certain area suitable for "masking", or for objects that are surrounded by some features which cause unwanted contribution to the diffraction pattern. A low-resolution reconstruction of the object is already achieved after a few iterations. This low-resolution reconstruction can then be either further refined by additional iterations or it can be used as an input for any other conventional phase retrieval method.

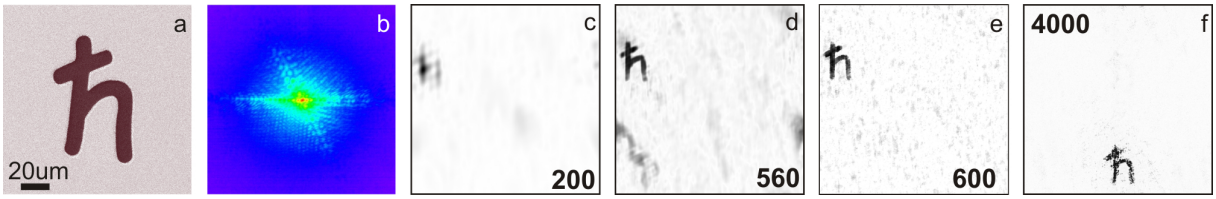

Fig. 15.7 – Recovery of the letter "h" from its experimental diffraction pattern. (a) SEM image of the object. (b) Recorded light optical diffraction pattern displayed in a logarithmic intensity scale. (c) Low-resolution image of the object recovered after 200 iterations. (d) Recovered amplitude of the object after 560 iterations, (e) after 600 iterations and (f) after 4000 iterations using a routine with a tight mask.

15.6 Summary of recent achievements in Coherent Diffraction Microscopy

The following achievements towards structural biology of single molecules have been established:

- Micro-lens design and routine fabrication
- Minimal spherical aberrations by a downscaling concept
- First coherent diffraction pattern with low-energy electrons of an inorganic sample
- First coherent diffraction pattern of a biological sample, a TM-virus
- Implementation of a new low-energy electron detector exhibiting 15 micron spatial resolution
- Implementation of a 8000x6000 pixel detector with 16bit dynamics
- A novel fast and reliable numerical method for the reconstruction of diffraction patterns
- Method for retrieving the 3-dimensional object distribution by 3-dimensional deconvolution
- The relation between a hologram and a coherent diffraction pattern of the same object
- Freeze drying biological sample preparation

15.6.1 Biological sample preparation

While we initially envisioned using tips, well defined by field ion microscopy prior to their use as a sample holder, we dropped this strategy in favour of planar samples for supporting a single biological molecule. The reason for this is the advantage of being able to post-accelerate the parallel beam emerging from the micro-lens towards the sample. By this means, the deBroglie wavelength can be adjusted in a simple manner, while a non-planar sample, such as a tip, would introduce unwanted electron optical aberrations. Figure 15.8 displays preliminary efforts in using graphene and double wall carbon nanotubes as sample holders. While it turns out that graphene is in fact sufficiently transparent even for our low-energy electrons, as evident from Fig. 15.8a and 15.8b, the cleanliness of graphene remains to be a serious problem. At present, the level of dirt on a free-standing graphene flake is just too high to allow its use as a reliable sample holder for proteins. However, efforts to obtain really clean graphene are underway worldwide and there is thus a fair chance that one of the pioneers of using graphene in TEM studies, namely Prof. Jannik Meyer from the Boltzmann Institute in Vienna, shall provide us with clean graphene samples in the near future.

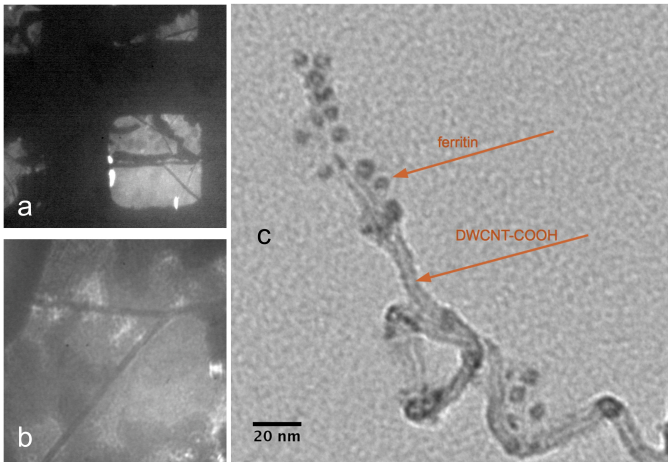

Fig. 15.8 – a) Graphene flakes deposited onto an 8 micron square copper grid show transmission for 450eV electrons. b) Even at just 65 eV kinetic energy, a significant transmission through graphene is observed; however it is apparent that the graphene sheet is highly contaminated. c) TEM image of ferritin molecules attached to a carbon nanotube.

In the meantime, we focussed on using functionalized carbon nanotubes or DNA molecules as templates for presenting the desired protein to the coherent planar electron wavefront. In both cases these elongated structures shall be placed over holes 200 nm in diameter milled in thin silicon nitride membranes by our focussed ion beam machine. An example of ferritin attached to the outer wall of a double wall carbon nanotube, taken with a standard TEM at our University, is shown in Fig. 15.8c. For modifying DNA molecules and attaching proteins to the double helix, we have enrolled the support of Clondiag Chip Technologies, a company based in Jena, Germany.

15.7 Electron column

Beyond the micro-lens, a second electron optical element, for instance an Einzel-lens exhibiting three apertures of one millimeter in diameter each shall be incorporated to bring the following important improvements to the coherent diffraction microscope: First, the divergence angle of the beam impinging onto the sample could be further reduced and consequently the spatial coherence of the parallel electron beam would increase. This shall ameliorate the quality of the reconstructions. Second, the diameter of the parallel electron beam at the sample shall effectively be reduced to 50nm without losing intensity. The oversampling criterion requires that the reconstructed region is at least twice as big as the illuminated region of the sample. Because the size of the reconstructed region is inversely proportional to the pixel size of the detector, a reduction of the beam diameter shall relax the strong requirement on the spatial resolution of the detector and facilitate the fulfillment of the oversampling criterion. Third, the addition of a millimeter-sized Einzel-lens would permit to build a complete electron column with an overall size of just a few millimeters. Simulations performed in the frame of the Master thesis of Flavio Wicki have shown that the size of the focus which could be obtained with such an electron column shall be as small as 5nm. The size of the electron column in combination with our low-energy electron point source shall permit us to build a SEM with a significantly reduced size compared to conventional SEMs. Furthermore, kinetic electron energies below 100 eV shall provide high surface sensitivity while ultra high vacuum compatibility is ensured. Such a machine shall be a worldwide novelty in the field of scanning electron microscopy.

We have dedicated a system, formerly used for electron holography, to the task of implementing such a mini electron-column. It is displayed in Fig. 15.9 together with the first design of such mini-lens which is readily being built in our machine shop and shall be subject to a first test in the frame of an ongoing Master thesis. We envision that the implementation of such an electron column shall be combined with a secondary-electron detector and a scanning system. Once such a mini SEM is working, it shall also be employed for coherent diffraction imaging experiments on single biomolecules. It will just be a matter of adjusting the voltage on the lenses to go from a focused beam to parallel one.

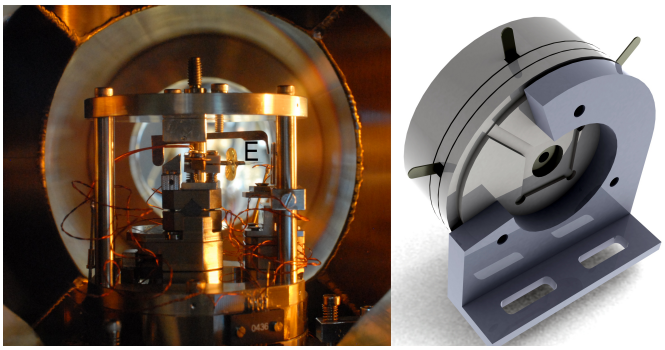

Fig. 15.9 – At left, inside view of a modified electron holography system which shall incorporate an electron column of some millimeters in size. The electron emitter is marked with the letter E. At right, a design for a miniature electron column is shown which combines a micro-lens with an Einzel-lens in one column.

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