Computed tomography based on cryo X-ray microscopic images of unsectioned biological specimens

Dissertation
zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultäten
der Georg-August-Universität zu Göttingen

vorgelegt von
Daniel Weiß
aus Köln

Göttingen 2000
Synopsis

This thesis describes the application of computed tomography based on cryo X-ray microscopic images (CTXM) to unstained hydrated specimens of the unicellular green alga *Chlamydomonas reinhardtii* and of cell nuclei of *Drosophila melanogaster*, in order to reconstruct the three-dimensional specimen structure.

The cryo transmission X-ray microscope (CTXM) at the BESSY I electron storage ring was used to image the specimens at 2.4 nm wavelength and at a cryogenic temperature of 110 K, so that a large number of high-resolution images could be acquired of each cryogenic specimen.

The influence of the narrow-bandwidth illumination used in the X-ray microscope on the lateral resolution and on the depth of focus was investigated theoretically, showing that the X-ray microscopic image of a specimen can be used to obtain a good approximation of the projected linear absorption coefficient.

To permit the acquisition of tilt series for computed-tomography reconstruction, the CTXM was modified to accommodate a tilt stage. Thin-walled borosilicate glass capillaries were used as rotationally symmetric object holders, permitting tilt image acquisition over the full angular range of 180°. By reinforcing the capillaries with an electroplated nickel coating, the mechanical stability was sufficiently increased to be able to acquire high-resolution images of cryogenic specimens.

Live specimens of *C. reinhardtii* were frozen by plunging into liquid nitrogen, and high-resolution tilt images of a specimen were acquired for 42 tilt angles spanning 185°. Colloidal gold spheres were used as fiducial markers to align the images to a common axis of rotation, and a multiplicative algebraic technique was used to reconstruct the local linear absorption coefficient. The resulting reconstruction visualizes the three-dimensional specimen structure with a resolution of 60 - 70 nm as measured by edge sharpness. Fourier-space based analysis indicates that reconstructed structures finer than 250 nm and modulated perpendicular to the axis of object rotation have been partially interpolated from insufficient data. No such restriction applies to structures modulated in the direction of the rotation axis.

The volumetric absorption data of the *C. reinhardtii* specimen was segmented into organelles, and the volume and the average linear absorption coefficient of the organelles were measured. In addition, the accumulated dose for each reconstruction voxel was calculated. In spite of a high accumulated dose between 10⁸ and 10⁹ Gy, the specimen showed no radiation damage to the biological structures.

To obtain better preservation of the biological structures during the vitrification process, specimens of *C. reinhardtii* were vitrified by plunging into liquid ethane and imaged with the CTXM, resulting in a much improved specimen preservation. In collaboration with S. Vogt CTCXM was also applied to immunogold-labeled cell nuclei of male *Drosophila melanogaster* fruit fly cells.

The technique described in this thesis is the only microscopic method that is capable of imaging the three-dimensional structure of complete frozen-hydrated biological specimens in a near-native state, without the need for fixation or staining, for object thicknesses of up to 10 µm and showing specimen details down to a size of approx. 30 nm.
# Contents

## Introduction

1. **Image formation in the X-ray microscope** 3  
   1.1 Interaction of soft X-rays with matter 3  
   1.2 Imaging with Fresnel zone plates 6  
     - 1.2.1 Incoherent image formation 7  
     - 1.2.2 Influence of the finite monochromaticity 8  
     - 1.2.3 Lateral resolution and depth of focus 9  
   1.3 Image acquisition with the CCD camera chip 12  
     - 1.3.1 Image contrast and signal-to-noise ratio in amplitude contrast mode 13  
     - 1.3.2 Local CCD pixel sensitivity 15

2. **Experimental setup** 19  
   2.1 The cryo transmission X-ray microscope 19  
   2.2 Object holders for computed-tomography experiments 20  
     - 2.2.1 Strip holders 22  
     - 2.2.2 Capillary holders 23  
   2.3 Vitrification 30

3. **Tilt series alignment** 33  
   3.1 Alignment using fiducial markers 33  
     - 3.1.1 Creating a 3D marker model 37  
     - 3.1.2 In-plane projection translation $d_i$ 38  
     - 3.1.3 Projection scale $s_i$ 39  
     - 3.1.4 In-plane rotation angle $\alpha_i$ 39  
     - 3.1.5 Tilt angle $\theta_i$ 40  
     - 3.1.6 Iterative alignment algorithm 40  
     - 3.1.7 Finding the common in-plane rotation angle 42  
   3.2 Creation of the aligned tilt series 44  
     - 3.2.1 Interpolation methods 44  
     - 3.2.2 Comparison 48

4. **Computed tomography** 52  
   4.1 Principles 53  
     - 4.1.1 Radon transform and central section theorem 53  
     - 4.1.2 Reconstruction of a bounded object function 55  
   4.2 Reconstruction techniques 59  
     - 4.2.1 Filtered backprojection (FBP) 59
List of Figures

1.1 The soft X-ray ‘water window’ ........................................ 5
1.2 Fresnel zone plate (schematic drawing) .......................... 6
1.3 Spectral distribution of the monochromatized BESSY illumination ............................... 10
1.4 Point spread function for monochromatic and for narrow-bandwidth illumination .................. 11
1.5 Modulation transfer functions for monochromatic and for narrow-bandwidth illumination .................. 12
1.6 Typical X-ray microscopic flat-field image ........................ 16
1.7 Local pixel sensitivity map of the CCD chip .......................... 17

2.1 Experimental setup of the BESSY I cryo X-ray microscope .......... 20
2.2 Schematic drawing of the cryo X-ray microscope experimental setup used for CTCXM .................. 21
2.3 Reduced X-ray transmission at high tilt angles for strip holders ........ 23
2.4 Reinforced capillary holder for cryo experiments .................. 26
2.5 X-ray transmission of ice-filled borosilicate glass capillary at 2.4 nm .... 27
2.6 Detachable tilt stage .................................................. 30
2.7 Plunge-freezer used to vitrify microscopic specimens for CTCXM ........ 31

3.1 Set of alignment parameters describing the orientation of a projection .... 36
3.2 Interpolation on a quadratic grid ..................................... 45
3.3 Comparison of interpolation methods by modulation transfer function .................. 50
3.4 As above, with rotated coordinate system .......................... 51

4.1 Radon transform and central section theorem ....................... 54
4.2 Effect of bounded object function ................................... 57
4.3 FBP and MART reconstruction on a quadratic grid .................. 61
4.4 Cone weighting function used for weighted area sampling .......... 66
4.5 Single reconstruction plane A of Chlamydomonas reinhardtii ........ 70
4.6 Single reconstruction plane B of C. reinhardtii ....................... 70
4.7 DPR and FRC of reconstruction plane A ......................... 71
4.8 DPR and FRC of reconstruction plane B ............................ 72

5.1 X-ray microscopic image of ice-embedded C. reinhardtii specimen .... 74
5.2 Schematic diagram of C. reinhardtii .................................. 75
5.3 Tip of capillary holder filled with specimens of C. reinhardtii .......... 76
5.4 X-ray microscopic image of capillary holder with C. reinhardtii specimen ........................................ 77
5.5 Animated tilt series and reconstruction of LN2-frozen C. reinhardtii specimen ............................ 78
5.6 Slice representation of the LN2-frozen C. reinhardtii specimen .... 80
5.7  Comparison of electron and X-ray microscopic image of *C. reinhardtii* 82
5.8  Animated tilt series and reconstruction of liquid-ethane frozen *C. reinhardtii* specimen ........................................ 83
5.9  Slice representation of the liquid-ethane frozen *C. reinhardtii* specimen 84
5.10 X-ray microscopic image of immunogold-labeled cell nucleus of *D. melanogaster* ......................................................... 86
5.11 Animated tilt series and reconstruction of liquid-ethane frozen *D. melanogaster* cell nuclei ................................................... 87
5.12 Slice representation of liquid-ethane frozen *D. melanogaster* cell nuclei 88

6.1  Watershed segmentation of *C. reinhardtii* ............................ 91
6.2  *C. reinhardtii* organelles in a surface representation ............... 93
6.3  Accumulated local dose in the *C. reinhardtii* specimen ............. 95
6.4  Total object absorption and average accumulated dose in ice of the *C. reinhardtii* specimen .............................................. 97

A.1 XALIGN: work area window .................................................. 103
A.2 XALIGN: tool bar ............................................................... 104
A.3 XALIGN: fiducial alignment .................................................. 106
A.4 XALIGN: resampling parameters ............................................ 108
Introduction

Microscopic techniques have found applications in many areas of scientific investigation. In biology and medicine, confocal fluorescence microscopy is used for structural and functional imaging of living cells. The spatial resolution that can be obtained is limited by the wavelength of the visible light to about 200 nm in the lateral and 500 - 800 nm in the axial direction [Paw95]. 4Pi-confocal fluorescence microscopy with two-photon excitation improves the axial resolution by a factor of approx. 3 - 4 over that of a standard confocal microscope [SH96]. Lateral resolution on the order of 80 nm has been shown by near-field fluorescence microscopy of biological specimens [HPM+97]; however because it relies on the interaction between a tip and a surface, near-field optical microscopy can only be applied to specimen surfaces.

If fixed specimens are studied instead of living ones, far-field microscopic techniques with significantly improved resolution become available. Electron microscopy of serial sections can be used to image fixed biological specimens with a lateral resolution of 4 nm and an axial resolution of at best 30 nm [KGT+97]. If complete specimens are imaged, a maximum sample thickness of approx. 1 µm can be achieved by energy-filtering the electrons, i.e., minimizing the loss of resolution and contrast due to chromatic aberrations [SHM90]. However, because for electron microscopy the specimens have to be fixed and are usually also stained, there is a danger of introducing preparation artifacts.

Soft X-ray microscopy in the 2.34 - 4.38 nm wavelength range offers the possibility of imaging biological specimens of up to 10 µm thickness in their natural wet environment, showing specimen details down to 30 nm size [SRNC80, KJH95]. The intrinsic absorption contrast between protein and water in the so-called ‘water window’ [Wol52] permits the acquisition of high-contrast images of unstained specimens in the amplitude contrast mode of the X-ray microscope, using microscopic Fresnel zone plates as X-ray objectives [SR69, SRNC80]. By introducing a phase-shifting and absorbing structure in the Fourier plane of the zone plate objective, phase contrast can be used to image small object details with increased contrast [SR87, SRG+95].

Due to the small numerical aperture of the zone plate objectives, in the X-ray microscope the lateral resolution of approx. 50 nm is coupled with a depth of focus of several microns. X-ray microscopic images acquired in amplitude contrast mode can therefore be used to obtain good approximations of the projected object absorption [Leh97b]. Since the object structures are layered in the image, the usefulness of the image decreases as the objects become more complex. While stereo-pair recording creates a good visual impression of the three-dimensional structure of an object [Leh95], it is of little use in separating features of a continuously varying function. Also, there is only limited possibility of evaluating stereo-pairs quantita-
In general, there exist two approaches to resolve the ambiguities that are present in single two-dimensional images. The first is the ‘optical sectioning’ approach, also known as ‘through-focusing’. The microscopic specimen is imaged with a number of different focus settings; the images of the resulting focus series show only a particular depth level ‘sharp’, while the other parts of the object contribute only to a defocused background. This approach is usually pursued if the depth of focus is on the order of the lateral resolution, such as in confocal microscopy. If on the other hand the depth of focus is very much larger than the lateral resolution (such as in soft X-ray microscopy), it is advantageous to use a ‘tomographic’ approach, where projections of the object are acquired from different directions and merged computationally to obtain a ‘reconstruction’ of the three-dimensional object.

Computed tomography (CT) based on projections is well established in diagnostic radiology [Cor80, Hou80], but it has also been used in other fields such as radio astronomy [Bra56, BR67] and geology [Bev93]. Cryo electron microscopy has employed tomographic methods to visualize microscopic specimens ranging in size from complete cells of up to 750 nm thickness [GSR+98] down to individual macromolecules [Fra96, KGT+97], with three-dimensional resolutions between 20 - 40 nm and approx. 5 nm, respectively. Due to the large depth of focus of the X-ray objectives, computed tomography is also the method of choice for soft X-ray microscopy, and several groups have investigated the possibility of CT reconstruction based on X-ray microscopic images. Haddad et al. reconstructed a micro-fabricated gold pattern [HMT+94], Lehr the mineral sheaths of bacteria *Leptothrix ochracea* [Leh97b, Leh97a], Wang et al. frozen-hydrated mouse 3T3 fibroblasts. Lee et al. proposed a reconstruction technique suitable for a very small number of viewing angles [LDF+97]. Wolf examined the feasibility of using glass capillaries as object holders for CT experiments with the X-ray microscope [Wol97], concluding that water-filled capillaries have sufficient transmission to allow image acquisition with reasonable exposure times.

To acquire a high-resolution X-ray microscopic image of a hydrated biological specimen in amplitude contrast mode, a dose of approx. $10^7$ Gy must be applied to the specimen [Sch98]. If a tilt series of images is acquired for CT reconstruction, this dose is multiplied by the number of tilt images. At room temperature, the accumulated dose of about $10^9$ Gy causes severe radiation damage in the hydrated specimen [Sch92]. By imaging the specimen at cryogenic temperatures (approx. 110 K), the tolerable specimen dose can be increased by several orders of magnitude [Sch98], and the acquisition of a tilt series of high-resolution images becomes feasible. CT experiments with biological specimens must therefore be performed at cryogenic temperatures. The aim of this work was to apply computed tomography to high-resolution X-ray microscopic images of frozen-hydrated biological specimens.
Chapter 1

Image formation in the X-ray microscope

In the experiments described in this work, the X-ray microscope at the electron storage ring BESSY I was used to acquire magnified images of a three-dimensional microscopic object. The process of image formation consists of three steps. In the first step, the illuminating radiation interacts with the object, creating an intrinsic object contrast. The second step is the creation of a magnified image of the object contrast, which can then be acquired with a CCD camera chip. In the following sections, these steps are described in detail.

The aim of this work is to use the X-ray microscopic images for computed-tomography reconstruction of the microscopic object. Computed tomography reconstructs a function from its geometric projections (cf. chapter 4). It is therefore necessary to investigate to what degree the X-ray microscopic images can be used to obtain (approximate) projections of a local object property. This property will turn out to be the linear absorption coefficient.

1.1 Interaction of soft X-rays with matter

For the soft X-ray wavelength range between 0.1 and 10 nm wavelength, the interaction of X-radiation with matter is described in good approximation by the high frequency limit of classical anomalous dispersion theory [Hen81].

An electromagnetic planar wave of wavelength λ propagating in vacuum with a wave vector \( \mathbf{k} \) parallel to the z-axis can be described by the complex scalar electric field

\[
E(z, t) = E_0 e^{i(\omega t - kz)},
\]

with \( E_0 \in \mathbb{C} \) the amplitude of the electric field, \( \omega = 2\pi f \) the circular frequency of the planar wave, and \( k = |\mathbf{k}| = 2\pi/\lambda \) the magnitude of the wave vector. If the planar wave traverses a layer of homogeneous bulk material of thickness \( \Delta z \), orientated perpendicular to the z-axis, it is attenuated and phase shifted. This can be expressed by multiplying Eq. (1.1) with a complex factor:

\[
E'(z, t) = E_0 e^{i(\omega t - kz)} \times e^{-i\omega(n-1)\Delta z/c},
\]

where \( c \) is the vacuum light velocity and \( n \) is the complex index of refraction of the bulk material. It is customary to write the index of refraction as \( n = 1 - \delta - i\beta \). The
amplitude transmission $T$ of the bulk material is obtained by dividing Eq. (1.2) by Eq. (1.1):

$$T = e^{-i\omega(n-1)\Delta z/c} = e^{-2\pi\beta\Delta z/\lambda} \times e^{2\pi i\delta\Delta z/\lambda} \in \mathbb{R}, \text{ absorption} \quad |...| = 1, \text{ phase shift}$$

(1.3)

Thus $\beta$ measures the absorption, and $\delta$ the phase shift introduced by the material. The complex amplitude $E$ cannot be measured directly; experimentally accessible is the intensity $I = |E|^2$. The intensity transmission is given by

$$\frac{I'}{I} = |T|^2 = e^{-4\pi\beta\Delta z/\lambda}.$$  

(1.4)

The absorption measured by $\beta$ can also be expressed by one of the following:

$$\mu = \frac{4\pi\beta}{\lambda} \quad ([m^{-1}], \text{linear absorption coefficient})$$

(1.5)

$$\sigma_a = \frac{\mu}{N} \quad ([m^2], \text{atomic cross section})$$

(1.6)

$$\mu_m = \frac{\mu}{\rho} \quad ([m^2/kg], \text{mass attenuation coefficient}),$$

(1.7)

where $\rho$ is the mass density of the material, and $N$ the number of scattering atoms per unit volume$^1$.

The bulk material consists of atoms, each of which individually interacts with the radiation. This interaction is characterized by the complex-valued atomic scattering factor $f = f_1 + if_2$. The macroscopic index of refraction is related to the microscopic atomic scattering factor as

$$n = 1 - \frac{NX^2r_e^2}{2\pi} f,$$

(1.8)

with the classical electron radius $r_e = 2.8 \times 10^{-15} \text{ m}$. Henke et al. have measured $f$ for all elements up to $Z = 92$ and for photon energies between 50 and 30,000 eV, corresponding to a wavelength range of 0.04 to 24.8 nm [HGD93]. Between the K absorption edges of oxygen at 2.34 nm and of carbon at 4.38 nm wavelength lies the so-called ‘water window’ ([Wol52], cf. Fig. 1.1). At the low-wavelength end of the window, the 1/e transmission thickness of water is almost 10 $\mu$m, while that of a model protein $C_{94}H_{139}N_{24}O_{31}S$ with $\rho_{protein} = 1.35 \text{ g/cm}^3$ is ten times smaller. At 2.4 nm wavelength, hydrated biological specimens can therefore be imaged with high intrinsic absorption contrast.

In addition to the photoelectric absorption characterized by the atomic cross section $\sigma_a$ (cf. Eq. (1.6)), the X-rays are also attenuated by elastic scattering, with a cross section

$$\sigma_e = \frac{8}{3}\pi r_e^2(f_1^2 + f_2^2),$$

(1.9)

and by inelastic or Compton scattering (cross section $\sigma_i$). However, in the water window wavelength range $\sigma_a \gg \sigma_e \gg \sigma_i$ [HGD93, HGO80], so that the last two kinds of interaction can in good approximation be neglected.

$^1$While the linear absorption coefficient is often called $\mu_l$, for simplicity it will be referred to as $\mu$ in this work.
Figure 1.1: The $1/e$ transmission thickness ($\equiv 1/\mu$, approx. 37% transmission) as a function of wavelength for water and a model protein $C_{94}H_{139}N_{24}O_{31}S$. The soft X-ray ‘water window’ lies between the K absorption edges of oxygen (at 2.34 nm) and carbon (at 4.38 nm). By operating the X-ray microscope at $\lambda = 2.4$ nm, hydrated biological specimens (i.e., protein structures in water) can be imaged in water layer thicknesses of up to 10 $\mu$m with high intrinsic absorption contrast. Data from [HGD93].

Using the linear absorption coefficient $\mu$, Eq. (1.4) becomes:

$$\frac{I'}{I} = e^{-\mu \Delta z}, \quad (1.10)$$

or equivalently,

$$\ln I - \ln I' = \mu \Delta z, \quad (1.11)$$

where $\ln I$ denotes the natural logarithm of $I$. For inhomogeneous material, the complex index of refraction (and thus $\mu$) is no longer a constant. In this case (propagation along the $z$-axis), $\mu = \mu(z)$, and Eq. (1.11) becomes:

$$\ln I - \ln I' = \int_{z_1}^{z_2} \mu(z) \, dz, \quad (1.12)$$

with $[z_1, z_2]$ the extent of the layer of material. In this form, Eq. (1.12) describes a computed-tomography experiment: the initial intensity $I$ and the transmitted intensity $I'$ can be measured, yielding the integral absorption of the specimen. In order to obtain the local linear absorption coefficient $\mu(z)$, measurements of the integral specimen absorption must be made for different projection angles (cf. chapter 4).
1.2 Imaging with Fresnel zone plates

In the transmission X-ray microscope (TXM), magnified images of an object are created using a Fresnel zone plate as an X-ray objective. A Fresnel zone plate is a circular diffraction grating consisting of concentric zones with radially decreasing zone widths ([Sor75], cf. Fig. 1.2). The radius of the nth zone is given in good approximation by

\[
r_n^2 = n\lambda f + \frac{1}{4}(n\lambda)^2 \frac{M^3 + 1}{(M + 1)^3},
\]

(1.13)

where \( f \) is the focal length at the wavelength \( \lambda \) and \( M \) the imaging magnification. For small zone numbers \( n \) this can be further approximated as

\[
r_n^2 = n\lambda f,
\]

(1.14)

or by differentiating for \( r_n \),

\[
\text{dr}_n = \frac{\lambda f}{2r_n}.
\]

(1.15)

A zone plate diffracts the diverging spherical wave emerging from a source point into a spherical wave converging onto the image point. For more than 100 zones, the optical properties of a zone plate are those of a thin lens [Mic86]. Especially, the Rayleigh resolution \( \delta \) for monochromatic illumination and incoherent image formation is given by the numerical aperture \( \text{NA} = \frac{r_N}{f} \) of the zone plate objective, with \( r_N \) the radius of the outermost zone, as

\[
\delta = 0.61 \frac{\lambda}{\text{NA}} = 0.61 \frac{M}{r_N} = 1.22 \text{dr}_N.
\]

(1.16)

To improve the resolution, it is therefore necessary to decrease the outermost zone width \( \text{dr}_N \).
Equation (1.16) is a measure of the highest periodicity in the focused object plane (i.e., of the smallest object structures) that can be imaged with the microzone plate objective. However, when imaging three-dimensional objects, it is also necessary to consider the depth of focus $\delta_z$ of the objective, i.e., the extent of the focused region in the direction of the optical axis. It is given by:

$$
\delta_z = 0.61 \frac{\lambda}{(\text{NA})^2}
= 2.44 \frac{(\text{dr}_N)^2}{\lambda}
$$

(1.17)

Eqs. (1.16) and (1.17) apply to incoherent image formation with monochromatic radiation. The influence of the finite monochromaticity of the radiation on the lateral resolution and the depth of focus is investigated in the following sections.

1.2.1 Incoherent image formation

The computed-tomography experiments presented in this work are all based on tilt series of images acquired using the amplitude contrast mode of the TXM. In amplitude contrast mode, the microscope images the intrinsic photoelectric absorption contrast of the object (cf. section 1.1). By introducing a phase-shifting and absorbing ring structure in the Fourier plane of the zone plate objective, it is also possible to image objects in phase contrast mode [SRS’94, SRG’95].

In amplitude contrast mode, the image formation is influenced both by the illuminating condenser and by the imaging X-ray objective. The condenser zone plate used for these experiments (KZP7, [HR98]) has an outermost zone width of $\text{dr}_N = 54$ nm. At 2.4 nm wavelength, the numerical aperture $\text{NA}_{\text{cond}}$ is given by Eq. (1.15) as $\text{NA}_{\text{cond}} = \lambda/(2\text{dr}_N) = 0.0222$. Correspondingly, the numerical aperture of the X-ray objective micro zone plate ([WPS98]) with an outermost zone width of 40 nm is $\text{NA}_{\text{obj}} = 0.03$. Note that while the imaging micro zone plate has a solid or full-cone aperture, the condenser zone plate has a central stop to block the undiffracted radiation from the field of view. The illuminating aperture is therefore shaped like a hollow cone. The presence of a shadow aperture influences the modulation transfer to some degree [Sch98, NGH’00]; this effect is neglected in the following considerations. The ratio of the apertures is called the degree of partial coherence $m$; in this case, $m = \text{NA}_{\text{cond}}/\text{NA}_{\text{obj}} = 0.74$.

In the case of illumination with a plane wave ($\text{NA}_{\text{cond}} = m = 0$), or coherent illumination, the image formation process can be described as a linear system operating on the complex amplitude $E$ in the object plane. For incoherent illumination ($m \geq 1$), image formation can be described as a linear system operating on the intensity $I$ in the object plane [BW99]. In both cases, the transformation from object to image plane can be described by a convolution in the spatial domain, and by a corresponding multiplication in the Fourier domain. The linear system is therefore characterized by its response at different spatial frequencies, or modulation transfer function (MTF).

In order to be able to employ the useful properties of linear systems to calculate the lateral resolution and the depth of focus of the microscope, it is desirable to model the image formation as either coherent or incoherent, even if neither of these extreme cases is exactly given. For the experiments described in this work, this means approximating $m = 0.74$ either as $m = 0$ or as $m = 1$. 
Hopkins and Barham calculated the influence of the relative condenser aperture on the Rayleigh resolution of two pinholes [HB50], showing that by reducing the degree of partial coherence from \(m = 1\) to \(m = 0.74\), the Rayleigh resolution is reduced by only 8%. Therefore, the image formation in the TXM is assumed to be in good approximation incoherent, and can be described as a linear system acting on the intensity in the object plane. This means that the intensity in the image plane \(I_{\text{im}}\) is given by a convolution of the intensity in the object plane \(I_{\text{ob}}\) with the so-called point spread function (PSF) \(I_{\text{PSF}}\):

\[
I_{\text{im}}(x, y) = \iint I_{\text{ob}}(x', y')I_{\text{PSF}}(x - x', y - y')dx'dy' \tag{1.18}
\]

According to the convolution theorem, Eq. (1.18) corresponds to a multiplication in the Fourier domain:

\[
\tilde{I}_{\text{im}}(k_x, k_y) = \tilde{I}_{\text{ob}}(k_x, k_y)\tilde{I}_{\text{PSF}}(k_x, k_y), \tag{1.19}
\]

where \(\tilde{I}\) is the Fourier transform of \(I\). The modulus of \(\tilde{I}_{\text{PSF}}\) is called the modulation transfer function. It describes the contrast transfer from object to image intensity as a function of spatial frequency.

### 1.2.2 Influence of the finite monochromaticity

If the micro zone plate objective is illuminated with monochromatic radiation and used in one selected focusing diffraction order, the optical performance is that of a thin lens. In particular, the zone plate PSF for incoherent image formation is given by the intensity distribution in the vicinity of the geometric focal point of a thin lens. This intensity \(I_{\text{PSF}}\) can be calculated by approximating the Huygens-Fresnel integral for monochromatic images of a point source by a circular aperture [BW99]. In cylindrical coordinates,

\[
I_{\text{PSF}}(u, v) = \left(\frac{2}{\lambda}\right)^2 \left[|U_1^0(u, v)|^2 + |U_2^0(u, v)|^2\right]I_0, \tag{1.20}
\]

with

\[
u = \frac{2\pi}{\lambda} \left(\frac{a}{f}\right)^2 \text{ and } v = \frac{2\pi}{\lambda} \left(\frac{a}{f}\right) \sqrt{x^2 + y^2}, \tag{1.21}
\]

where \(I_0\) is the intensity at the geometric focal point, \(\lambda\) the wavelength of the monochromatic radiation, \(a\) the radius and \(f\) the focal length of the lens, and

\[
U_k(u, v) = \sum_{s=0}^{\infty} (-1)^s \left(\frac{u}{v}\right)^{k+2s} J_{k+2s}(v) \tag{1.22}
\]

are Lommel functions, with \(J_k(v)\) the Bessel function of \(k\)th order. The cartesian coordinates \((x, y, z)\) are relative to the geometric focal point, with the \(z\)-axis being the optical axis.

The intensity in the focal plane is obtained for \(u = z = 0\). In this case, Eq. (1.20) reduces to

\[
I_{\text{PSF}}(0, v) = \left[\frac{2J_1(v)}{v}\right]^2 I_0, \tag{1.23}
\]

the Airy formula for Fraunhofer diffraction at a circular aperture.
In the TXM, a combination of a condenser zone plate and a pinhole is used to monochromatize the polychromatic synchrotron radiation and to illuminate the object \([SRN+93]\). The spectral distribution of the illuminating radiation is approximately Gaussian in shape, characterized by a mean wavelength \(\lambda_0\) and a full width at half maximum of \(\Delta\lambda\). The ratio \(\lambda_0/\Delta\lambda\) is called the monochromaticity of the radiation. For a point X-ray source, a simple geometric consideration yields

\[
\frac{\lambda_0}{\Delta\lambda} = \frac{D}{2d'},
\]

where \(D\) is the diameter of the condenser zone plate, and \(d\) the diameter of the pinhole. For high resolution imaging, the monochromaticity of the illuminating radiation has to be equal to the number of zones of the micro zone plate X-ray objective \([Thi88]\).

For an extended X-ray source such as the bunches of electrons stored in the BESSY I storage ring, an improved approximation of the spectral distribution can be obtained by raytracing calculations. For each wavelength, a large number of random ray origins are created inside the BESSY source, and the rays are traced through the condenser zone plate. A tally is kept of how many rays go through the monochromator pinhole, yielding the relative intensity of this wavelength in the object illumination \([Gut82, Neu96]\). Based on software written by U. Neuhaeusler, this distribution was calculated for the experimental setup used for the CTCXM experiments (cf. Fig. 1.3). The resulting monochromaticity is \(\lambda_0/\Delta\lambda = 200\). This is somewhat smaller than the value \(\lambda_0/\Delta\lambda = 225\) given by Eq. \((1.24)\), which assumes a solid condenser aperture, while in fact the inner part of the KZP7 condenser is obstructed.

### 1.2.3 Lateral resolution and depth of focus

To calculate the effective point spread function for this kind of narrow-bandwidth illumination, the monochromatic PSF of Eq. \((1.20)\) was computed for a large number of (equidistantly spaced) wavelengths from the wavelength interval shown in Fig. 1.3, and the different monochromatic PSFs were added up, using the relative intensities as weights. Image formation is thus again modeled as being completely incoherent, with the contributions from different illuminating wavelengths adding up to form the total image intensity.

Fresnel zone plates are chromatic objectives, i.e., the focal length is a function of wavelength: \(f(\lambda) = 2a d_{\text{NN}}/\lambda\). The cartesian coordinates of Eq. \((1.21)\) are relative to the geometric focal point \(f(\lambda)\): for two wavelengths \(\lambda_1\) and \(\lambda_2\), the relative origin \((u, v) = (0, 0)\) lies at different points on the optical axis. Adding up the monochromatic PSFs therefore results in an elongation of the PSF (cf. Fig. 1.4). Assuming incoherent image formation, this three-dimensional narrow-bandwidth PSF determines two important properties of the X-ray microscope, namely the lateral resolution and the depth of focus.

As has been pointed out, image formation in the microscope can be described as a two-dimensional convolution (cf. Eq. \((1.18)\)). However, there is more than one object plane: in addition to the focused plane, there are parallel out-of-focus planes in the three-dimensional object space. Image formation for each of these planes is described by a different two-dimensional section of the three-dimensional PSF shown in Fig. 1.4. The lateral resolution is defined for the focused object plane;
Figure 1.3: Spectral distribution of the illumination produced by the linear monochromator, calculated by raytracing for each wavelength 100,000 rays with random origin inside the BESSY source, and determining the fraction that passes through the monochromator pinhole. Calculation parameters: mean wavelength $\lambda_0 = 2.4$ nm, inner diameter KZP7 2.25 mm, outer diameter 4.5 mm, distance BESSY source - KZP7 15 m, pinhole diameter 20 $\mu$m. With a full width at half maximum of $\Delta \lambda = 0.012$ nm, the monochromaticity is $\lambda_0/\Delta \lambda = 200$.

The corresponding two-dimensional PSF is that section of $I_{PSF}(u, v)$ that is defined by $u = z = 0$. For monochromatic radiation, this is the Airy diffraction pattern of Eq. (1.23).

Since the three-dimensional PSF of Eq. (1.20) is by definition circularly symmetric, so is the modulus of the Fourier transform of a two-dimensional section defined by a constant value of $u$, i.e., the modulation transfer function (MTF) of a selected object plane. Because the MTF does not depend on the orientation of an object structure, it can be plotted as a function of the modulus $k = |k|$ of the spatial frequency vector $k = (k_x, k_y)$. In addition, $I_{PSF}(u, v) = I_{PSF}(-u, v)$, so that defocused object planes have identical modulation transfer functions regardless of the sign of the defocus [BW99].

In order to characterize the imaging properties of the X-ray microscope, MTFs were calculated for different object planes both for monochromatic radiation and for the narrow-bandwidth radiation described above (cf. Fig. 1.5). The MTF for the focused plane ($z = 0$ $\mu$m) and monochromatic radiation has a form which is well known for incoherent image formation. Only object structures with the spatial frequency $k = 0$ $\mu$m$^{-1}$ show a modulation transfer of 1, meaning that the inte-
1.2 Imaging with Fresnel zone plates

Figure 1.4: The normalized point spread function $I_{\text{PSF}}/I_0$ (intensity near the geometric focal point, cf. Eq. (1.20)) of a zone plate objective as a function of radius $r$ and of defocus $z$ for (a) monochromatic radiation with $\lambda = 2.4$ nm and (b) narrow-bandwidth radiation with a mean wavelength $\lambda_0 = 2.4$ nm and a monochromaticity $\lambda_0/\Delta\lambda = 200$ (cf. Fig. 1.3), for a zone plate with an outermost zone width $d_{rN} = 40$ nm and a numerical aperture $a/f = 0.03$. The $z$-axis has been condensed by a factor of 10. Note that the narrow-bandwidth PSF is more elongated than the monochromatic PSF, indicating a greater depth of focus. In the focal plane, the secondary maxima are more pronounced in (b) than in (a), indicating decreased lateral resolution for narrow-bandwidth radiation. From [WSN+00].

Gral intensity is preserved under imaging. Object structures with higher spatial frequencies have reduced modulation transfer. The cut-off frequency of 25 $\mu$m$^{-1}$ corresponds to a periodicity of 40 nm, i.e., the outermost zone width of the imaging micro zone plate objective. The periodicity of the Rayleigh resolution, $1.22 d_{rN}$, corresponds to a spatial frequency of 20.5 $\mu$m$^{-1}$. As can be seen from the graph, at this frequency there is a modulation transfer of approx. 9%.

For narrow-bandwidth radiation with $\lambda_0/\Delta\lambda = 200$, the modulation transfer in the focal plane is considerably worse than for monochromatic radiation, both for high and low spatial frequencies. For example, the 9% modulation transfer corresponding to the Rayleigh resolution occurs at 16.7 $\mu$m$^{-1}$, with a resolution equivalent of 59.9 nm.

The diameter of the object mainly studied in this work is approx. 8 $\mu$m (cf. section 5.1). If the focused plane is adjusted to go through the center of the object, this means that the maximum defocus for any part of the object is $z = \pm 4$ $\mu$m. It is therefore necessary to compare the MTF for a defocus of $z = \pm 4$ $\mu$m to that of the focal plane at $z = 0$ $\mu$m (cf. Fig. 1.5). For the narrow-bandwidth MTF, there is little difference between the focused and the defocused plane, while the monochromatic MTF shows substantially different modulation transfer at $z = \pm 4$ $\mu$m. 9% modulation transfer is reached at approx. 6 $\mu$m$^{-1}$, corresponding to a periodicity of 167 nm. For higher spatial frequencies, the modulation transfer does not exceed 5%, and the MTF has zero modulation transfer at 7.5 $\mu$m$^{-1}$ and 17 $\mu$m$^{-1}$.

Summing up, the narrow-bandwidth radiation generated by the linear monochromator decreases the lateral resolution in the focused object plane compared to that of monochromatic radiation. At the same time it significantly increases the depth of focus, so that for an object with a diameter of 8 $\mu$m, the modulation transfer function is approximately the same for the whole object. In the case of a geometric projection, the image contrast is equal to the photoelectric absorption contrast of the object for all spatial frequencies. In the case of X-ray microscopic images, the image contrast is the result of a linear transformation of the photoelectric
![MTF graph]

Figure 1.5: The modulation transfer function (MTF) as a function of spatial frequency for different values of the defocus \( z \) (zone plate parameters as in Fig. 1.4). In the focused plane \( (z = 0 \, \mu m) \) the modulation transfer for narrow-bandwidth radiation with \( \lambda_0/\Delta\lambda = 200 \) is considerably worse than for monochromatic radiation. The object mainly used in the tomography experiments has a diameter of approx. 8 \( \mu m \), the maximum defocus for any object part is therefore \( z = \pm 4 \, \mu m \). For this defocus, the narrow-bandwidth MTF is much more favorable than the monochromatic MTF. From [WSN^00].

absorption contrast. The main difference between geometric projections and X-ray microscopic images acquired with narrow-bandwidth illumination is that high spatial frequencies are attenuated in the microscopic images, however nearly equally for all parts of the object. This effect is neglected in the tomographic reconstructions presented in chapter 5.

### 1.3 Image acquisition with the CCD camera chip

In order to make the magnified X-ray microscopic images accessible for evaluation, the intensity in the image plane must be recorded. In the current experimental setup, a CCD camera chip is used to integrate the local image plane intensity during the time of image acquisition. To describe this process properly, it is necessary to abandon the model of a continuous intensity in the image plane, and to consider the X-ray photons as discrete quanta to be registered in a statistical process characterized by a Poisson distribution. Consequently, in addition to the limitation imposed by the finite resolution of the X-ray microscope (cf. section 1.2), there is...
another limitation owing to the statistical nature of the photon detection process.

Because the X-ray microscopic images are to be used quantitatively (to reconstruct the local linear absorption coefficient of a three-dimensional specimen), the measurement of the local intensity should be as accurate as possible. It is therefore desirable to measure the local sensitivity of the CCD pixels, and to compensate for changes in the sensitivity.

### 1.3.1 Image contrast and signal-to-noise ratio in amplitude contrast mode

The photon detector used for the experiments described in this work is a charge-coupled device (CCD), a two-dimensional array of \(1024 \times 1024\) pixels, each of which acts as an integrating X-ray photon detector [Wil94]. Due to the relatively large energy of an X-ray photon (517 eV at 2.4 nm wavelength), many electron-hole pairs are generated in the band structure of the semiconductor for every X-ray photon incident to the CCD chip (approx. 90 at 2.4 nm wavelength [Wil94]). The electrons are locally stored in a potential well whose extent defines the size of the pixel. The number of photons that can be registered by one pixel during one exposure is limited by the full well capacity of the pixel, i.e., the number of electrons that can be stored in the potential well of the pixel. As is shown below, in amplitude contrast mode the full well capacity determines the image contrast that can be detected with a given signal-to-noise ratio (SNR).

The detection of X-ray photons in the CCD pixel is a statistical process, and the number of photons counted during one exposure of the CCD chip is a random variable. If a mean number of photons \(N\) is counted in a pixel during one exposure of the CCD chip, the probability \(p_n\) of counting exactly \(n\) photons is given by the Poisson distribution:

\[
p_n = e^{-N} \frac{N^n}{n!}
\]  

This distribution is not a property of the specific photon detector used, but a fundamental consequence of the temporal coherence of the synchrotron radiation\(^2\) [Pau85]. There are additional sources of noise that influence the signal-to-noise ratio of the CCD-acquired images, such as the noise generated by the on-chip amplifier [Wil94]; however, since the Poisson distribution inherent to photon detection is by far the predominant source of noise, other sources are not considered.

In the following, the contrast and the signal-to-noise ratio of the CCD-acquired image of an object with low photoelectric absorption contrast are calculated. For this purpose, two CCD pixels are considered: the 'background pixel' counts a mean number \(N_0\) of photons coming from the background, and the 'object pixel' counts a mean number \(N = xN_0\) of photons coming from the object (with \(x \lesssim 1\), hence \(N_0 > N\)). The image contrast \(k\) is given by

\[
k = \frac{N_0 - N}{N_0 + N} = \frac{1 - x}{1 + x}.
\]

As has been pointed out, \(N\) and \(N_0\) are the mean values of two random variables with Poisson distribution; namely, the number of X-ray photons coming from

\(^2\)Radiation emitted by thermal light sources, for example, exhibits a Bose-Einstein distribution of the photon numbers [Pau85].
the object, and the number of photons coming from the background. The object can be detected if the difference between the photon numbers of object and background pixel is statistically significant. The statistical properties of this difference \( D \) can be characterized by the signal-to-noise ratio, i.e., the ratio of the mean to the square root of the variance. Since the background pixel and the object pixel are statistically independent of each other, the variance of \( D \) is given by the sum of the variances of the photon numbers [BS91, p. 673]. For a Poisson-distributed process, the variance is equal to the mean [BS91, p. 666], so that the SNR of the difference \( D \) is

\[
s = \text{SNR}(D) = \frac{E(D)}{\sqrt{V(D)}} = \frac{N_0 - N}{\sqrt{N_0 + N}} = \frac{1 - x}{\sqrt{1 + x} \sqrt{N_0}}.
\]  

(1.27)

Dividing Eq. (1.26) by Eq. (1.27) yields

\[
\frac{k}{s} = \frac{1}{\sqrt{N_0(1 + x)}},
\]  

(1.28)

or equivalently,

\[
x = \frac{s^2}{N_0 k^2} - 1.
\]  

(1.29)

At the same time, rearranging Eq. (1.26) yields

\[
x = \frac{1 - k}{1 + k}.
\]  

(1.30)

Combining Eqs. (1.29) and (1.30) yields

\[
k^2 - \frac{s^2}{2N_0} k - \frac{s^2}{2N_0} = 0,
\]  

(1.31)

a quadratic equation in \( k \). The solutions are given by

\[
k = \frac{s^2}{4N_0} \pm \sqrt{\frac{s^4}{16N_0^2} + \frac{s^2}{2N_0}}.
\]  

(1.32)

The Rose criterion specifies that the SNR should be in the range of \( s = 3 - 5 \) [Ros48]. The 16-bit CCD chip used in these experiments stores up to \( 2^{16} - 1 = 65535 \) counts per pixel. An X-ray photon of 2.4 nm wavelength creates approx. 13 counts [Wil94]. The maximum number of photons that can be registered by a pixel is therefore \( N_0 = 65535/13 = 5041 \). With these values of \( s \) and \( N_0 \), in Eq. (1.32) the second term under the square root is larger than the first by more than a factor of 1000, so that

\[
k \approx \frac{s^2}{4N_0} \pm \frac{s}{\sqrt{2N_0}}.
\]  

(1.33)

Since the image contrast is positive (cf. Eq. (1.26)),

\[
\frac{k}{s} \approx \frac{1}{\sqrt{2N_0}} + \frac{s}{4N_0} \approx \frac{1}{\sqrt{2N_0}}.
\]  

(1.34)

With \( N_0 \approx 5000 \), the ratio \( k/s \) is approximately 0.01. If the contrast \( k \) is given in per cent, \( k \approx s \).
Using the 16-bit CCD chip, a given small image contrast will therefore be detected with a signal-to-noise ratio that is equal to the value of the image contrast if the camera chip is exposed to its full capacity. In amplitude contrast mode, the modulation transfer function is always \( \leq 1 \) (cf. Fig. 1.5). Therefore, if the Rose criterion of \( s = 3 - 5 \) is to be fulfilled, only object details of at least several per cent photoelectric amplitude contrast can be imaged, since the photoelectric absorption contrast is degraded by the modulation transfer function of the microscope.

There are two ways of dealing with this problem. The image contrast of small biological structures can be greatly enhanced by using phase contrast mode [SRG+95], without a similar increase in exposure time and dose. However, for the computed-tomography reconstruction of the object absorption, the X-ray microscopic images must be acquired in amplitude contrast mode.

On the other hand, the mean photon numbers \( N \) and \( N_0 \) can be increased by using several CCD pixels to accumulate X-ray photons for one resolution element, i.e., by increasing the imaging magnification from the resolution-adapted case to a super-sampling case. At the expense of a decreased field of view, small object structures can thus be imaged with increased statistical significance. Prior to reconstruction, the X-ray microscopic images acquired in this work were reduced in size by a factor of 2 (cf. section 3.2.2). The resulting pixel size of approx. 28 nm permits the representation of object structures down to this size. The size reduction increases the maximum number of photons per pixel \( N_0 \) by a factor of 4. However, since the biological specimens are imaged inside ice-filled glass capillaries with an average transmission of 14% (cf. Fig. 2.5), the effective \( N_0 \) is approximately 2800, so that \( k/s \approx 0.0134 \) (cf. Eq. (1.34)). Demanding a SNR of \( s = 3 \) is therefore equivalent to demanding a minimum image contrast of 4%.

### 1.3.2 Local CCD pixel sensitivity

As can be seen from Fig. 1.6, a number of small dirt particles adheres to the surface of the CCD chip. Most of these particles are only a few pixels in size. If the X-ray microscopic images are to be used in a qualitative manner (e.g., for morphological studies), the dirt particles do not notably deteriorate the image quality.

In this work, the X-ray microscopic images are used to approximate the integral object absorption, and computed tomography is used to reconstruct the local absorption coefficient of the specimen (cf. chapter 4). For this quantitative image evaluation it is desirable to take into account the local CCD pixel sensitivity. Ideally, all CCD pixels have the same sensitivity, i.e., when illuminated with the same number of photons, all pixels yield the same number of counts (disregarding the Poisson noise inherent to the detection of X-ray photons.)

In the experiment, the dirt particles on the chip surface reduce the sensitivity of the underlying pixels by absorbing some of the incident photons before they can be detected. However, if the pixel sensitivity is known for all pixels, this effect can be corrected by dividing each pixel of the original X-ray microscopic image by its sensitivity.

One way of creating a map of the local pixel sensitivity would be to illuminate the CCD chip homogeneously. The resulting image is a direct measurement of the pixel sensitivity. In order for this to be applicable to the tomography experiments, the chip would have to be illuminated with the correct wavelength, \( \lambda = 2.4 \) nm. Also the chip would have to be removed from the camera chamber. Quite possibly
1.3 Image acquisition with the CCD camera chip

Figure 1.6: An X-ray microscopic image showing an empty field of view, or flat-field image. Due to the critical illumination, the intensity in the image plane is characterized by an elliptical distribution. The most obvious detrimental effect associated with image acquisition is the presence of Poisson photon noise. In addition, small dirt particles lie on the chip surface and reduce the local pixel sensitivity. The process of removal and reinstallation of the camera chip would introduce new dirt particles, thus rendering the experiment pointless.

Therefore, in this work a different method was used to measure the local pixel sensitivity. In the course of a tomography experiment, a large number of flat fields (images showing only the background illumination) are acquired to correct the background illumination of the X-ray microscopic images. These flat fields show the elliptical illumination distribution that is created by the condenser zone plate; apart from noise, the local intensity varies slowly (cf. Fig. 1.6).

In order to measure the sensitivity of a pixel A, the average intensity measured by the neighbouring pixels is calculated; this is done by convolving the flat field with a two-dimensional Gaussian low-pass filter. Due to the nature of the illumination distribution, the illuminating intensity is approximately constant over a small area of the CCD chip, and the illuminating intensity for the pixel A can be taken to be the local average intensity as calculated by the low-pass filtering. The sensitivity of pixel A is then computed by dividing the intensity actually measured by the average intensity given by the low-pass filter.

For all pixels of the flat field, the intensity measured is subject to Poisson noise. The average intensity can be determined with sufficient statistical significance by choosing a sufficiently broad Gaussian low-pass filter. In this work, a standard deviation of 5 pixels was chosen. The measured intensity, however, is determined only by a single value, the value of pixel A in the flat field. In order to determine the pixel sensitivity with sufficient statistical significance, an average sensitivity
Figure 1.7: Local pixel sensitivity map for the CCD chip, calculated from 123 flat-fields in 15 iterations. The sensitivity ranges from 30% to 117%; in the image, black corresponds to 95% sensitivity and white to 105%. Small dirt particles and the remains of a dried liquid reduce the sensitivity and appear dark, while pincer marks appear white, indicating increased sensitivity due to the abrasion of absorbing material.

The accuracy of the sensitivity map can be further improved by iterating the process: once the local sensitivity has been calculated for each pixel, the flat fields can be corrected with this map before the low-pass filtering step. In this way, even large dirt particles can be compensated for.

Figure 1.7 shows the local pixel sensitivity of the CCD chip calculated from 123 flat fields. By using a relatively large number of flat fields, the noise in the sensitivity map is much reduced, and absorbing material can be identified even if it absorbs only one per cent of the radiation. In addition to the small dirt particles visible in individual flat fields, the dried remains of a liquid can be recognized. Surprisingly, the pixel sensitivity is greater than 100% for some pixels. These areas
have a distinctive shape and are probably pincer marks created while handling the chip. If the pincer tips abrade some of the chip material that lies above the photosensitive region, fewer photons are absorbed before entering the photosensitive region, thus increasing the pixel sensitivity.

Once the sensitivity map has been calculated, all X-ray microscopic images can be corrected by dividing them pixel-by-pixel by the sensitivity map.
Chapter 2

Experimental setup

2.1 The cryo transmission X-ray microscope

This section describes the experimental setup of the cryo transmission X-ray microscope at BESSY I, operational till November 1999, where all of the X-ray images used in this work were acquired. Although BESSY I has been decommissioned by the time of this writing, the microscope design is described in the present tense.

The transmission X-ray microscope (TXM) built and operated by the Institute for X-ray Physics (Universität Göttingen) is located at a dipole magnet beamline at the 800 MeV electron storage ring BESSY I. Relativistic electrons stored in the ring pass through the gap of the magnet and are accelerated perpendicular to their velocity vector \[\text{Att99}\]. They emit synchrotron radiation which is used as the X-ray source for the TXM.

The polychromatic synchrotron radiation passes through a 50 nm chromium layer that filters out high-energy photons, and is collected by a 9 mm diameter condenser zone plate \[\text{HR98}\]. The condenser focuses the X-rays onto the object, thereby increasing the illuminating intensity by orders of magnitude compared to the divergent beam coming from the dipole magnet. In addition, the condenser in combination with a 10 - 20 \(\mu\)m diameter pinhole acts as a linear monochromator. Since zone plates are chromatic objectives \(f \propto 1/\lambda\), the polychromatic synchrotron radiation has to be monochromatized to permit imaging.

The object under investigation is placed at the focus of the condenser zone plate, and is kept at atmospheric pressure. This enables easy object access and replacement. The microscopic objects are either placed inside a thin object chamber for hydrated biological specimens \[\text{VSS}^{+00}\], or mounted on support foils. The microscope stage permits moving the object in the x-, y-, and z-direction with an accuracy of approx. 0.1 \(\mu\)m. To inspect and pre-align the object, the condenser zone plate can be moved downwards, and a light microscope can be lowered in its place. For cryo experiments, a thermally insulating cryo object chamber can be fitted to the microscope stage (cf. Fig. 2.1).

The cryo chamber is designed to keep the object at a constant cryogenic temperature, which can be set between 90 K and room temperature \[\text{SNG}^{+95}\]. Liquid nitrogen flows into the chamber at a controlled rate. By evaporating, it creates an atmosphere of cold nitrogen gas inside the chamber which cools the object (cf. Fig. 2.2). Access to the object chamber is via a lid at the top of the chamber. In this way, the object can be changed without having to replace the atmosphere of cold nitrogen gas, and little or no humidity from the ambient air is introduced into the
2.2 Object holders for computed-tomography experiments

In order to obtain a three-dimensional image of an X-ray microscopic specimen using computed tomography, the specimen must be rotated around an axis perpendicular to the microscope optical axis, and imaged under a large range of viewing angles, ideally spanning 180° (cf. chapter 4). In this work, the expressions ‘tilting’ and ‘rotating’ are used interchangeably. In the imaging transmission X-ray microscope at BESSY I, the space available for the specimen is determined by the air gap between the vacuum windows (cf. Fig. 2.2). For room-temperature experi-
2.2 Object holders for computed-tomography experiments

Figure 2.2: Schematic drawing of the cryo X-ray microscope at BESSY I. The condenser zone plate illuminates the object inside the thermally insulated object chamber. The cold nitrogen atmosphere above the liquid nitrogen level at the bottom of the chamber keeps the object at a constant cryogenic temperature of down to 90 K. The axis of object rotation points out of the page. The micro zone plate creates a magnified image of the object which is acquired with the CCD camera. The relative sizes of the different parts are not drawn to scale. Modified from [SNG+95].

ments, this air gap is usually adjusted to 200 - 400 µm.

The X-ray absorption of room-temperature air at 2.4 nm is characterized by a linear absorption coefficient of $\mu_{\text{air}} = 1.75 \times 10^{-3} \, \mu\text{m}^{-1}$, or equivalently, $1/\mu_{\text{air}} = 570 \, \mu\text{m}$. The X-rays traversing the air gap are therefore significantly attenuated by the atmosphere, and it is important to minimize the air gap in order to keep exposure times at a minimum. At the same time, the air gap must be large enough to permit moving the object holder (such as a support foil) in order to image different parts of an extended sample. The cryo experiments described in this work were performed at a temperature of approx. 110 K, where the atmospheric density is increased by a factor of 2.7 compared to room-temperature air (assuming ideal gas conditions). The $1/e$ transmission thickness is reduced to about 210 µm, thus it is even more important to minimize the air gap than at room temperature. For the experiments described in this work, an air gap size of 300 µm was used.

The available object space is further defined by the extent of the stops with the X-ray windows perpendicular to the optical axis (cf. Fig. 2.2). The radius of these stops is 3 mm. The object and object holder must therefore fit into a cylindrical
volume with a diameter of 6 mm and a height of 200 - 300 μm (for cryo experiments). For conventional X-ray microscopy, this requirement is met by the thin object chamber for hydrated biological specimens, and by the support foils. However, due to their large extent perpendicular to the optical axis (several centimeters), these object holders can only be tilted by ±3° before coming into contact with the stops. The desired tilt range of ±90° cannot be achieved.

### 2.2.1 Strip holders

In order to increase the tilt range, the extent of the object holder perpendicular to the axis of object rotation must be reduced. If the perpendicular extent can be reduced to less than the air gap size (300 μm), the holder can be rotated through 360°. In the past, two object holder geometries have been investigated that permit a larger angular tilt range. Lehr used thin strips of copper foil with 30 - 150 μm diameter holes as object holders to acquire stereo-pair images of dry objects: diatoms, spores of the moss Dawsonia superba, and mineral sheaths of bacteria Leptothrix ochracea [Leh95]; Schneider used similar strips to image green algae Chlamydomonas reinhardtii under cryo conditions [Sch98]. In these experiments stereo-pair images were acquired for stereo viewing angles between 5° and 12.5°.

In order to have X-ray transparent areas on the strip holder, holes are drilled into the strips and covered with polyimide foil. The advantage of using these strips as object holders is that, apart from their small width, they closely resemble the support foils used for conventional X-ray microscopy. It should therefore be possible to adapt the established methods of growing cells on support foils [SSGO98, VSS00] to the strip holders.

However, there are several disadvantages if strip holders are used for computed-tomography experiments. The reconstruction methods described in chapter 4 are based on the assumption that the complete object (i.e., everything in the air gap that absorbs X-rays) is visible in each of the tilt images. Since the reconstruction is computed in planes perpendicular to the horizontal axis of object rotation, this requirement must only be fulfilled in the vertical direction, i.e., there should be a safe margin showing only the background illumination above and below the object in each of the tilt images. It is easy to see that by going to high tilt angles, new parts of the support foil and eventually of the strip holder will become visible, even if the specimen itself is completely inside the microscopic field of view for each tilt angle. In consequence, reconstruction artifacts arise from the inconsistency of the absorption data.

If the strip holders are used for cryo experiments, another problem arises. In this case, the specimen is embedded in ice, and the ice fills the hole drilled into the strip. Since the hole has a much larger diameter (up to 150 μm) than the thickness of the copper strip (approx. 10 μm), the geometry of the ice is that of a rectangular slab with a thickness of approx. d₀ = 10 μm. If this slab is illuminated at an oblique angle (i.e., for angles of incidence θ ≠ 0°), the effective slab thickness as seen by the X-rays varies as

\[ d = \frac{d₀}{\cos θ}, \quad (2.1) \]

cf. Fig. 2.3. Consequently, the X-ray transmission is reduced for high tilt angles. If reduction by a factor of 3 and a corresponding increase in exposure times is acceptable, tilt images can be acquired for an angular range of ±60°. By further
Figure 2.3: For a specimen embedded in a rectangular slab of ice (e.g., in a strip holder), the effective thickness of the ice layer is a function of the angle of incidence $\theta$ of the X-ray illumination: $d = d_0 / \cos \theta$. The illumination angle is equal to the tilt angle of the specimen (a). For a typical ice layer thickness $d_0 = 10 \, \mu m$, the transmission at $\theta = 0^\circ$ is 33%. It is reduced to 10% at $\theta = 60^\circ$ and vanishes at $\theta = 80^\circ$ (b).

increasing the exposure time by a factor of two, this range can be extended to $\pm 70^\circ$.

Under these conditions, there will be a missing angular ‘wedge’ of $40^\circ$, i.e., more than one fifth of the total angular range of $180^\circ$. This part of the object Fourier transform, associated with high tilt angles, is not measured (cf. chapter 4). However, due to the rectangular form of the object function, the high-tilt angle part of the Fourier transform requires a higher angular projection density (i.e., a smaller tilt angle increment) than the low-tilt angle part, in order to properly sample the underlying function (cf. section 4.1.2). Instead of acquiring no tilt images at high projection angles, more projections per angular interval should be acquired at high than at low tilt angles.

Consequently, the ‘missing wedge’ in Fourier space contributes to the object function with more weight than is given by simply considering the relative area of the wedge (given by the ratio $40^\circ / 180^\circ = 0.22$), resulting in noticeable reconstruction artifacts. It is therefore highly desirable to employ a rotationally symmetric holder permitting image acquisition over the full angular range of $180^\circ$. Such a geometry is provided by object holders made of glass capillaries.

2.2.2 Capillary holders

The use of glass capillaries as object holders for X-ray microscopy was first investigated by A. Wolf [Wol97]. A well-established method to produce very thin glass capillaries exists in connection with the patch clamp technique of electrophysiology. There, glass micropipettes are filled with a solution and pressed against the cell membrane of single cells to establish a seal with giga-Ohm resistance. In this
way ionic currents flowing across the cell membrane can be recorded. The type of
glass tubing used in the manufacture of the pipette, the initial thickness of the tub-
ing wall, the taper of the pipette tip, and the size of the tip opening are all critical
for the success of the experiment [Ken94]. Consequently, commercial apparatus
is available to manufacture micropipettes from glass tubing with fine control over
the different parameters of the pipette geometry [BF86].

The geometry of a micropipette can be characterized by the diameter and the
wall thickness of the hollow capillary. The optimal geometry of a capillary object
holder to be used for CTCXM experiments depends on the size of the object that
is to be investigated. It should be chosen so that it can easily accommodate the
objects, but not much larger. The upper limit of the (inner) capillary diameter is
given by the ice layer thickness that can still be imaged with ‘reasonable’ exposure
times. A typical value for this thickness is 10 µm, since at 2.4 nm wavelength, an
ice layer with a thickness of 10 µm has a transmission of 33%. The transmission
of the ice-filled capillary is further reduced by the capillary walls. These should be
as thin as possible (while still providing sufficient stability), and the specific type
of glass should be chosen to provide maximum X-ray transmission at 2.4 nm.

For smaller objects, it is desirable to manufacture a capillary holder with re-
duced diameter. Not only does reducing the holder diameter (and thus the ice
layer thickness) improve the holder transmission, but, for a given number of tilt
angles, the object can be reconstructed with increased Crowther resolution (cf. sec-
tion 4.1.2) by using an object holder with decreased diameter (cf. Eq. (4.15)).

Another parameter describing the capillary geometry is the taper of the capil-
lary tip, i.e., the angle of the capillary walls. Whereas in electrophysiology appli-
cations steeply tapering micropipettes are used for the patch clamp technique, the
object holders should have little or no taper to ensure a maximum usable length
of the X-ray transparent tip. A practical disadvantage of the capillary holder is
that the specimen arrangement is one-dimensional (the specimens occupy the hol-
low capillary in a row), whereas with support foils and strip holders, the specimen
arrangement is two-dimensional, leading to a much larger number of specimens
to choose from. Since there are relatively few specimens in a capillary tip to begin
with, the tip should be as long as possible (with constant diameter), or equivalently,
the taper of the tip should be minimal.

Summing up, the ideal capillary holder should have an inner diameter of ap-
prox. 5 - 10 µm (depending on the object size) with little tapering of the tip, and
thin capillary walls made of a glass with high X-ray transmission at 2.4 nm. In
electrophysiology, borosilicate or hard glass is most commonly used to produce the
micropipettes. A. Wolf investigated micropipettes pulled from borosilicate glass
tubing, concluding that borosilicate glass capillaries have relatively low X-ray ab-
sorption and are therefore suitable as object holders for computed-tomography
experiments [Wol97]. Consequently, the micropipettes used for the experiments
described in this work were pulled from the same borosilicate glass tubing (Fa.
Hilgenberg, cf. Table 2.1).

**Manufacturing the glass capillary**

The micropipettes used for the CTCXM experiments were manufactured using
a model P-87 Flaming/Brown type micropipette puller (Sutter Instrument Corp.,
[BF86]) made available by the Institut für Zoologie und Anthropologie, Abteilung
Zellbiologie. A circular filament is used as a heat source. On both sides of the filament, pulling clamps running on rollers can be used to exert a symmetric pulling force on the glass tubing. The tubing is inserted horizontally into the hollow filament, then the clamps on both sides are moved inwards and clamped on the tubing. While the clamps exert a symmetric pulling force, the filament heats the middle part of the tubing to above the melting point. The tubing softens and is drawn apart by the clamps, producing two tapering capillaries.

The pulling process is controlled by the four parameters HEAT, VEL., PULL, and TIME, which can be set via the input panel. HEAT specifies the filament current (in instrument units) and thus the filament temperature. Initially, a ramp test is executed to determine the melting point of the glass. Glass tubing is inserted into the filament, clamped, and the filament current is slowly increased. As soon as the glass begins to melt, the clamps begin moving apart and the ramp test terminates. The puller then displays the current setting of HEAT.

For micropipette pulling, the HEAT value from the ramp test should be increased by 10 - 15 units. The glass tubing is inserted and the pulling process started. As soon as the pulling velocity of the moving clamps exceeds a preset value VEL., a 'hard pull' is done, i.e., the pulling force is suddenly increased to a preset value PULL, for a preset length of time TIME. After that time, the filament and tubing are rapidly cooled by air from a pressurized air supply. This sequence constitutes one pulling step; it is possibly to perform several such pulling steps to create a specific tip geometry. The pulling process terminates when the middle part of the glass tubing breaks. Due to the symmetric instrument setup, two identical micropipettes are produced from each piece of glass tubing.

Available from Sutter Instrument Corp. is an expert system in the form of the software package GLASSWARE. For a given micropipette application, the pulling parameters can be optimized by specifying the current pulling parameters and the desired change in pipette geometry. The glass capillaries used in this work were pulled in a single pulling step using the pulling parameters shown in Table 2.2,
Table 2.2: The capillary holders used in this work were pulled on a P-87 Flaming/Brown micropipette puller using the parameters given below (in instrument units). The HEAT value was calculated by adding 15 to the result of the ramp test. Since the hard pull invariably breaks the micropipette and ends the pulling process, there is only a single pulling step.

<table>
<thead>
<tr>
<th>HEAT</th>
<th>PULL</th>
<th>VEL.</th>
<th>TIME</th>
</tr>
</thead>
<tbody>
<tr>
<td>700 - 740</td>
<td>30</td>
<td>45</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 2.4: Reinforced capillary holder for computed-tomography experiments at cryogenic temperatures. To ensure the mechanical holder stability under cryo conditions, the tapering tip of the borosilicate glass capillary is reinforced with an electroplated, 25 µm thick nickel coat (left image edge), leaving only the uppermost 500 - 800 µm of the tip X-ray transparent. The nickel coat prevents oscillations of the capillary. Prior to electroplating, the tip was sealed by dipping it into silicone rubber. This (empty) tip is still sealed with a drop of silicone rubber; the foremost 100 µm are coated with the rubber and must be removed to make the holder usable. Note the changing capillary wall thickness (decreasing from left to right) indicated by the interference colors.

determined by manual optimization according to the desired object holder geometry described above. The resulting capillaries have small taper of the capillary tip, the inner diameter varies from about 2 µm (at the very tip) to 15 - 20 µm approx. 500 µm from the tip. For borosilicate glass capillaries, the ratio of wall diameter to wall thickness is not changed by the pulling process. For the glass tubing used in this work, this ratio is given by 1000 µm / 40 µm = 25 (cf. Table 2.1). Correspondingly, the wall thickness increases with the capillary diameter, from about 100 nm at the very tip, to 400 nm for a diameter of 10 µm, and even greater wall thicknesses farther away from the tip. In the optical microscope, the changing wall thickness can be observed as changing interference colors (cf. Fig. 2.4).

Which part of the capillary is used in a CTCXM experiment depends on the size of the specimens inserted into the capillary. Assuming the objects to have a diameter of approx. 8 µm (such as the green alga Chlamydomonas reinhardtii, cf. section 5.1), a typical capillary diameter at the location of the specimen is 10 µm.
Based on the chemical composition given in Table 2.1 and the atomic scattering factors tabulated by Henke [HGD93], a linear absorption coefficient (cf. section 1.1) of 1.187 $\mu$m$^{-1}$ at 2.4 nm wavelength can be calculated for the borosilicate glass. The corresponding value for ice is 0.1094 $\mu$m$^{-1}$. If X-rays traversing the capillary holder are modeled as propagating along straight lines, the transmission of the ice-filled glass capillary can be computed as a function of the distance between the straight-line X-ray and the center of the capillary.

Figure 2.5 shows the X-ray transmission of an ice-filled glass capillary with an outer diameter of 10 $\mu$m and a wall thickness of 400 nm (produced with the pulling parameters given in Table 2.2). The high absorption of the capillary walls combines with the lower absorption of the ice to produce an almost constant transmission of 14% for the innermost 6 $\mu$m of the holder. In terms of transmission, the innermost 8 $\mu$m of the capillary holder are usable as background for an object; object parts that are closer to the capillary walls (i.e., farther away than 4 $\mu$m from the capillary center) are imaged with reduced signal-to-noise ratio because of the highly absorbing background.

The constant transmission of 14% can be compared to the variable transmission of a strip holder with maximum transmission of 33% at zero tilt angle (cf. Fig. 2.3). The strip holder transmission decreases to 14% at a tilt angle of 56°.
Table 2.3: Etching agent used to remove the NiCr electroplating base from the capillary tip.

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>8.25 g</td>
<td>ammonium cerium(IV)-nitrate (Merck)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 ml</td>
<td>aqua dest.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.5 ml</td>
<td>perchloric acid (60%, Merck)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Reinforcing the glass capillary

Experiments in the cryo object chamber of the CTXM have shown that the capillaries produced with the micropipette puller have insufficient stability. At cryogenic temperatures (approx. 110 K), the X-ray transparent glass tip oscillates at a frequency of several Hz with an amplitude of approx. 1 µm. Images acquired with a typical exposure time of several seconds are severely blurred and cannot be used for computed tomography. The oscillation was concluded to be the consequence of the high flexibility of the thin glass capillary. Evaporating nitrogen flows upwards from the bottom of the cryo chamber and past the capillary holder. The capillary acts as a stationary obstacle and causes the creation of a trail of vortices behind the obstacle, the Von Karman vortex street [SK89]. The shedding of these vortices from the capillary imparts an oscillating force to the capillary and causes the observed vibration.

In order to reduce or completely suppress this oscillation, it was decided to reduce the flexibility of the tapering capillary by covering it with a metallic layer. This was done by covering the capillary with a thin conductive layer as an electroplating base, and then electroplating a thicker support layer onto the base.

Experiments have shown that once the capillary tip has been filled with a fluid, it is not possible to completely remove that fluid. This means that the capillary holders can only be used once; it also means that before the capillary is filled with the microscopic specimens, any fluid must be prevented from entering the tip. Because the capillary has to be immersed in an electroplating bath, the tip must be sealed so that the bath is not drawn into the tip by capillary forces. To seal the tip, the capillary is dipped into an adhesive sealant (Silcoset 151, Ambersil Ltd.) that cures at room temperature to a resilient silicone rubber. The high viscosity of this sealant causes only the foremost tip of the capillary to be coated, leaving the usable part of the capillary (with a diameter of 8 - 15 µm) X-ray transparent (cf. Fig. 2.4).

After the tip has been sealed, the capillary is sputter-coated with a thin layer (approx. 150 nm) of NiCr. This material was chosen because NiCr has good conductivity and can be removed by wet etching, and because a sputter-coating device with a NiCr target was available [Düv99]. The resulting NiCr layer covers the whole capillary. However, only the lower part of the tapering capillary is to be electroplated, the upper part must remain X-ray transparent. The NiCr layer covering the first 500 - 800 µm of the capillary must therefore be removed before electroplating. This is done by dipping the NiCr-coated capillary tip into the etching agent described in Table 2.3.

The resulting capillary is electroplated in a commercially available nickel sulfamate plating bath (Blasberg). The capillary tip is inserted into the bath up to the point where the capillary begins to taper. The complete tip is thus submerged in the bath. Because the anode is located on one side of the capillary, the capillary is
slowly rotated during electrodeposition. Electroplating without rotation would result in an asymmetric nickel layer and, due to stress in the deposited layer, would bend the tip. The electroplating bath is kept at room temperature, and nickel is deposited on the capillary for 30 minutes, at a current of approx. 1.3 mA. The resulting nickel layer has a thickness of approx. 25 µm. This thickness provides sufficient stability under cryo conditions, and the reinforced capillary still fits into the 300 µm air gap. Figure 2.4 shows a reinforced capillary tip after electroplating.

The final manufacturing step consists in removing the rubber-coated tip of the capillary. This is done by placing the capillary tip on a razor blade (under light-microscopical control), and pushing down on it with a pincer. The capillary tip breaks off at the point of support (where it touches the razor blade). In this way the rubber-coated tip is removed and the reinforced capillary holder is ready for specimen insertion.

The detachable tilt stage

The capillary holders are suitable for microscopic specimens suspended in a liquid, e.g., specimens of the green alga *Chlamydomonas reinhardtii* in water (cf. chapter 5). To insert the specimens into the capillary, a sample of the specimen suspension is taken using a pipette (Eppendorf Reference) with an Eppendorf Microloader pipette tip (volume approx. 10 µl). The Microloader tip is then inserted into the large-bore end of the capillary, and the liquid sample is injected into the tapering tip of the capillary, taking care to avoid creating air bubbles which prevent the transport of specimens to the tip of the capillary. The liquid is drawn into the tapering capillary tip by capillary forces. In most cases, this process transports some specimens to the very tip of the holder, i.e., the part that can be imaged with the X-ray microscope.

The filled capillary holder is then inserted into a hollow steel axle (inner diameter 1 mm, outer diameter 2 mm) with an attached cog wheel, and adjusted so that the capillary tip protrudes 0.5 cm from the end of the hollow axle. This will put the capillary tip into the center of the yoke that forms the detachable tilt stage. The capillary is now glued into the hollow axle and the large-bore end is broken off. For room temperature experiments, the axle can now be inserted into the detachable tilt stage (cf. Fig. 2.6). Since the axle has to be inserted into the tilt stage with the fragile capillary tip first, a tool was custom-made to guide the axle during the insertion. The axle is secured by a spring that fits into a groove on the axle.

The assembled tilt stage is manipulated by means of a handle; it fits into a recess of the microscope cryo stage and attaches magnetically. In this way it is possible to rapidly attach the tilt stage to the microscope cryo stage. This is a necessary requirement in order to minimize the time during which the cryo chamber lid is opened, and thus avoid contamination of the cryo chamber interior with room-air humidity. However, since the tilt stage is only attached magnetically, it can still be rotated by a few degrees around the microscope optical axis when it is inserted into the recess. The axis of object rotation is therefore not parallel to the CCD camera chip. In the X-ray microscopic images, the axis of object rotation is itself rotated in the image plane by an unknown angle of a few degrees. This in-plane rotation angle is determined and compensated when the images are aligned to a common axis of rotation (cf. chapter 3).

Fixed to the microscope stage is a stepper motor (Faulhaber, Schönaich) that
Figure 2.6: The detachable tilt stage with the hollow axle into which the capillary holder is inserted. The capillary is adjusted so that the capillary tip is in the center of the yoke. The capillary is then glued into the axle and the end is broken off. The tilt stage is manipulated by means of the handle and attaches magnetically to the microscope cryo stage. The hollow axle and capillary holder are turned through the cog wheel.

turns a horizontal axle with a cog wheel. When the detachable tilt stage has been fitted into the recess of the microscope stage, the two cog wheels lock, and the capillary can be turned by means of the stepper motor. The stepper motor used for these experiments divides a complete rotation into 24 steps. In combination with a 1:485 reduction gear, 11640 motor steps rotate the capillary through 360°. The smallest possible tilt angle increment is therefore 0.031°. The accuracy of the tilt angle was measured to be better than 0.1°. Because the two cog wheels have some backlash, the capillary holder should be turned in only one angular direction during the acquisition of a tilt series. This ensures that the accuracy of the tilt angles is not reduced by the backlash.

2.3 Vitrification

G. Schneider designed a plunge-freezing device to vitrify biological specimens on support foils for cryo X-ray microscopy [SNG+95, Sch98]. This device was modified to accommodate capillary holders (cf. Fig. 2.7). Gaseous ethane from a pressurized supply is conducted into a small reservoir that is surrounded and cooled by means of a second reservoir filled with liquid nitrogen. The ethane condenses and fills the reservoir. The temperature of the liquid ethane can be measured, and kept at a constant value by heating it with a small resistor. The axle with capillary
Figure 2.7: Plunge-freezer used to vitrify microscopic specimens on support foils for cryo X-ray microscopy. The device has been modified to permit the plunging of capillary holders. In operational mode, the central reservoir is filled with liquid ethane to just below the rim, and the surrounding reservoir is filled with liquid nitrogen. The capillary is plunged by means of a compressed-air piston (top, not shown). The strings are used to raise the metal shield (with white insulating cover) in order to provide a cold gas reservoir when a capillary is removed from the liquid ethane.

is magnetically attached to the rod of a compressed-air piston. By releasing the piston, the rod is accelerated downwards and plunges the capillary into the liquid ethane at a speed of several m/s. The tip of the capillary (holding the specimens) is plunged to a depth of approx. 2.5 cm. After the piston stops, the capillary, hollow axle and cog wheel are completely immersed in liquid ethane. This ensures that the complete object is at the same cryogenic temperature.

In order to remove the capillary from the liquid ethane reservoir, a metal shield
is raised out of the liquid nitrogen reservoir. This creates a reservoir of cold nitrogen gas above the liquid ethane, and the piston can be drawn back to lift the capillary out of the liquid ethane. The capillary is still cooled by the cold nitrogen gas. It is then transferred to a styrofoam spoon filled with liquid nitrogen, and can be further handled preparatory to transporting to the microscope.
Chapter 3

Tilt series alignment

Due to gravitational sagging during the heating and pulling process, the tip of the capillary object holder is slightly curved. The rotating capillary tip describes a circle of approx. 100 µm diameter; rotating the object holder through a few degrees to the next tilt angle will therefore move the object out of the microscopic field of view. The microscope operator then has to move the microscope stage to bring the object back into the field of view. Since the object has also been moved in the direction of the optical axis, it must also be refocused. The manual alignment of the object with its position from the previous tilt angle cannot be done with a precision corresponding to the microscopic resolution. In consequence an unknown translation is applied to every tilt image; additionally there is an unknown in-plane rotation angle between the axis of rotation of the object and the coordinate system given by the CCD camera pixels. Aligning the images to a common axis of rotation must therefore be done after image acquisition.

For the experiments described in this work, colloidal gold spheres with 60 - 100 nm diameter were used as fiducial markers, and the X-ray microscopic images were aligned to a common axis of rotation using these markers. The exact method is described below; for each tilt angle, five alignment parameters are calculated with sub-pixel accuracy. However, if no fiducial markers are available, other methods may be used to align the images. Correlation-based methods exploit the fact that tilt images separated by a small tilt angle show very nearly the same object. The cross correlation between the images is computed, and the location of the cross-correlation maximum indicates the translation vector. Once the relative translation between successive tilt images is known, an aligned tilt series can be created. Rotation and scale-invariant registration methods have been proposed which permit the additional calculation of the in-plane rotation angle and the relative scale of the two images [CDD94, RC96].

3.1 Alignment using fiducial markers

One way of establishing a common system of reference for a set of X-ray microscopic images is to use fiducial markers. These fiducial markers can either be part of the investigated specimen, or suitable objects can be added to the specimen suspension. In finding the common system of reference, the fiducial markers are used as a set of three-dimensional points in the object space. In order to provide such a point set, fiducial markers should be spherical, so that their image does not depend on
3.1 Alignment using fiducial markers

the viewing angle, and near the microscopic resolution limit, so that the location of the associated point can be determined with resolution-limited accuracy. If the markers are perfectly spherical (or ellipsoidal), they need not be small as well, because the center of gravity of the sphere (or ellipsoid) can be used to define the associated 3D point with even better than resolution-limited accuracy.

The markers should be clearly discernible even if they come to lie before or behind object structures. For X-ray microscopy, the marker images should still be usable even if they are somewhat defocused. For amplitude contrast X-ray microscopy, this means choosing a marker material of high photoelectric absorption. At 2.4 nm wavelength, gold is such a material, with a characteristic 1/e attenuation length of only 44 nm. Gold spheres are routinely manufactured for electron microscopy purposes with diameters ranging from a few nanometers to several microns. The X-ray microscopic image of such a gold sphere is a function of the intrinsic absorption contrast of the sphere, determined by the sphere diameter; and of the optical setup used to image this intrinsic contrast (cf. section 1.2). Given the microscopic resolution, the diameter of the gold spheres can be chosen so that the image of the gold marker has sufficient contrast. In the experiments described in this work, colloidal gold spheres with 60 and 100 nm mean diameter were used (cf. chapter 5).

The fiducial-marker alignment algorithm, originally developed by Lawrence [Law83] and independently by Berriman et al. [BBFL84], is based upon treating the microscopic images as parallel projections of some object property; in the case of amplitude contrast X-ray microscopy, this property is the local linear absorption coefficient (cf. section 1.1). The alignment algorithm seeks to minimize the least-squares error between the measured marker coordinates on the microscopic images and the coordinates of the markers reprojected from a three-dimensional marker model. For this purpose, the measured marker coordinates are corrected using transformations for rotation of the projection plane in 3D space, for in-plane translation of the projection, and for magnification changes. In its most general form, the resulting equation is non-linear. However, since the equation is differentiable, the minimization can be attempted by one of the available numerical algorithms, for example conjugate gradients [PTVF92]. Even though in principle a minimum error can be found, the equation is not only non-linear, but the alignment parameters to be determined are also highly correlated.

Motivated by the need for a fast and robust alignment algorithm based on fiducial markers, Penczek et al. [PMBF95] developed such an algorithm for electron tomography. Using a number of simplifying assumptions, they found that each of the required alignment parameters, taken separately, can be estimated using closed-form solutions (i.e., not requiring iterations). Marker coordinates in 3D space are estimated, and, using explicit solutions for each alignment parameter, these coordinates are iteratively corrected. The algorithm is terminated when the projections of the 3D markers match the alignment-corrected coordinates of the fiducial markers in the images with sufficient accuracy.

The purpose of the fiducial-marker alignment algorithm is to determine a common coordinate system for a set of two-dimensional projections, given the locations of fiducial markers in these projections. A set of alignment parameters describing the orientation of a projection in three-dimensional space includes three Eulerian angles, a two-dimensional vector describing the in-plane translation and, to ac-
count for possible magnification changes, a projection scale\(^1\). In the following it is assumed that the microscope optical axis is parallel to the z-axis and the tilting axis coincides with the x-axis. In this case, the angular orientation can be described using only two Eulerian angles. Scale changes are assumed to be isotropic; thus there is only one scale parameter per projection. Finally, it is assumed that while the other alignment parameters may have random values, the tilt angles are already known to some accuracy, and only have to be corrected somewhat in respect to that initial estimate. Considering the experimental setup (cf. section 2.2), where the tilt angle is incremented by a known amount for each new tilt image, this assumption is justified. Under the above assumptions, each projection of a tilt series is characterized by four alignment parameters (Fig. 3.1).

The set of alignment parameters to be found has to correspond to the least-squares solution of the following fundamental set of equations, which relates the reprojected markers from the three-dimensional model (left-hand side) to the measured markers after correction with the alignment parameters (right-hand side):

\[
P(\theta_i) \mathbf{x}^j = \mathcal{R}(\alpha_i) s_i \mathbf{p}^i_j + \mathbf{d}_i, \tag{3.1}
\]

where \(i = 1, \ldots, \nu\), with \(\nu\) the number of projections (X-ray microscopic images), \(j = 1, \ldots, s\), with \(s\) the number of markers in each projection,

\[
\mathbf{x}^i = \begin{pmatrix} x^i \\ y^i \\ z^i \end{pmatrix}
\]

are the coordinates of the \(j\)th marker in three-dimensional space,

\[
P(\theta_i) = \begin{pmatrix} 1 & 0 & 0 \\ 0 & \cos \theta_i & -\sin \theta_i \end{pmatrix}
\]

is the projection matrix that projects \(\mathbf{x}^i\) perpendicular to the x-axis under the \(i\)th tilt angle \(\theta_i\) (cf. Fig. 3.1 (d)),

\[
\mathcal{R}(\alpha_i) = \begin{pmatrix} \cos \alpha_i & -\sin \alpha_i \\ \sin \alpha_i & \cos \alpha_i \end{pmatrix}
\]

is the rotation matrix that rotates the \(i\)th projection in-plane by the rotation angle \(\alpha_i\), \(s_i\) is the isotropic scale of the \(i\)th projection,

\[
\mathbf{p}^i = \begin{pmatrix} p^i_{x_i} \\ p^i_{y_i} \end{pmatrix}
\]

are the original measured coordinates of the \(j\)th marker on the \(i\)th projection, and

\[
\mathbf{d}_i = \begin{pmatrix} d_{x_i} \\ d_{y_i} \end{pmatrix}
\]

is the in-plane translation vector of the \(i\)th projection.

\(^1\)For a micro zone plate objective with focal length \(f \approx 1 \, \mu m\) and a magnification of approx. \(1740 \times\) (cf. section 3.2.2), moving the object in the 300 \(\mu m\) air gap results in a magnification change of at most 0.016%, or 1.6 nm for an object size of 10 \(\mu m\). There are therefore no significant scale changes during the acquisition of a tilt series.
3.1 Alignment using fiducial markers

Figure 3.1: Set of alignment parameters describing the orientation of a two-dimensional projection in three-dimensional space. The cartesian coordinate system was chosen so that the microscope optical axis is parallel to the z-axis and the tilting axis (here: of a non-microscopic sample) coincides with the x-axis. The orientation of the ith projection (X-ray microscopic image) of a tilt series can be described using four alignment parameters: (a) the projection translation vector $d_i$, (b) the projection scale $s_i$, (c) the in-plane rotation angle $\alpha_i$, and (d) the tilt angle $\theta_i$.

Thus, given the marker coordinates $p_i^j$, the alignment parameters $\theta_i$, $\alpha_i$, $s_i$, and $d_i$ have to be found. The total number of equations is $2sv$: the two equations (one for the $x$-, and one for the $y$-coordinate) of Eq. (3.1) for each of the $s$ markers in the $v$ projections. The number of parameters to be determined is given by the five unknown alignment parameters $\theta_i$, $\alpha_i$, $s_i$, $d_{x,i}$, and $d_{y,i}$, times the number of projections $v$. However, since the rotation of the system around the x-axis is not fixed, one of the angles $\theta_i$ can be arbitrarily set to zero. The same is true for the scale of the
whole 3D model, thus one of the scales $s_i$ can be set to one. Therefore, the number of parameters to be determined is $5\nu - 2$. Eq. (3.1) has a unique solution only if the number of equations is at least equal to the number of unknown parameters:

$$2s\nu \geq 5\nu - 2$$

$$\implies s \geq \frac{5}{2} - \frac{1}{\nu}$$

$$\implies s \geq 3.$$  (3.7)

In practice, the marker coordinates in the projections can only be measured with finite precision. To minimize the overall error, more than 3 markers should be used. Overdetermination of the problem reduces the final error and allows rejection of invalid markers. Markers in a projection are called ‘invalid’ if the marker images cannot be evaluated with sufficient precision, i.e., if the markers are severely blurred or if they lie in an image region with low transmission and reduced signal-to-noise ratio of the image intensity. The algorithm presented here permits excluding such invalid marker positions from the alignment procedure.

3.1.1 Creating a 3D marker model

Eq. (3.1) has the following expanded form:

$$\begin{pmatrix} 1 & 0 & 0 \\ 0 & \cos\theta_i & -\sin\theta_i \end{pmatrix} \begin{pmatrix} \chi^j_i \\ \gamma^j_i \\ \zeta^j_i \end{pmatrix} = \begin{pmatrix} \cos\alpha_i & -\sin\alpha_i \\ \sin\alpha_i & \cos\alpha_i \end{pmatrix} s_i \begin{pmatrix} p_{x_i}^j \\ p_{y_i}^j \end{pmatrix} + \begin{pmatrix} d_{x_i} \\ d_{y_i} \end{pmatrix}$$  (3.8)

On the left-hand side of Eq. (3.8), the $j$th 3D marker $\mathbf{x}^j$ is projected onto the plane of the $i$th projection. This reprojected 3D marker position should be equal (in a least-squares sense) to the position of the measured 2D marker $\mathbf{p}^j_i$ after alignment correction, which is given on the right-hand side. The order of alignment corrections is the following: first the 2D coordinates are scaled with the projection scale $s_i$, then rotated by the in-plane rotation angle $\alpha_i$, and finally translated by the projection translation vector $\mathbf{d}_i$.

Eq. (3.8) can be written in explicit form as a system of equations:

$$\begin{cases} 
\chi^j_i = p_{x_i}^j, \\
\gamma^j_i \cos\theta_i - \zeta^j_i \sin\theta_i = p_{y_i}^j,
\end{cases}$$  (3.9)

with $i = 1, \ldots, \nu$, $j = 1, \ldots, s$, where the right-hand side of Eq. (3.8) has been designated as $(p_{x_i}^j, p_{y_i}^j)$. The least-squares solution of the first set of equations of Eq. (3.9) is given by the average value of $p_{x_i}^j$:

$$\tilde{\chi}^j = \frac{1}{\nu} \sum_{i=1}^\nu p_{x_i}^j,$$  (3.10)

Assuming that $\cos\theta_i \neq 0 \ \forall \ i$, the second set of equations of Eq. (3.9) can be rewritten as

$$\zeta^j (\tan\theta_i) + \gamma^j = \frac{p_{y_i}^j}{\cos\theta_i},$$  (3.11)
which has the form of
\[ A^j X_i + B^j = Y^j_i, \] (3.12)
which shows that Eq. (3.11) forms \( s \) sets of linear equations, each consisting of \( \nu \) equations and each having 2 unknowns \( A^j = z^j_i \) and \( B^j = y^j_i \). In terms of the model, for each marker \( j \) there are \( \nu \) pairs of values \( (X_i, Y^j_i) \) which are functions of the markers’ positions and tilt angles, since \( X_i = -\tan \theta_i \) and \( Y^j_i = p'_{yi} / \cos \theta_i \).

In order to obtain values for the alignment-corrected marker coordinates \( p'_{xi} \) and \( p'_{yi} \), the alignment parameters are set to plausible initial values: \( d_i = 0, s_i = 1, \) and \( \alpha_i = 0 \) (unless there is some common in-plane rotation angle, cf. section 3.1.7, as indeed there is in the experimental setup, cf. section 2.2.2, in which case this common angle should be used as the initial estimate). As can be seen, at this point it is necessary to have non-trivial estimates of the tilt angle \( \theta_i \) in order to evaluate Eq. (3.11).

The least-squares solution of Eq. (3.12) can be easily found using linear regression. The abscissa of the fitted line is the y-coordinate \( \tilde{y}_j \), its slope is the z-coordinate \( \tilde{z}_j \) of the \( j \)th 3D marker. Together with the x-coordinate given by Eq. (3.10), these are the coordinates of the \( j \)th 3D marker \( \tilde{x}_j \) specified by the measured 2D marker positions and the current set of alignment parameters.

The availability of this ‘3D marker model’ makes it possible to determine all alignment parameters separately. For this purpose, the 3D markers are reprojected onto the projection planes using the left-hand side of Eq. (3.8). In the projection planes there are now available (a) the measured 2D markers corrected by the current alignment parameters \( \tilde{p}_i^{(j)} \), and (b) the 2D markers \( \tilde{p}_i^{(n)} \) reprojected from the current 3D marker model. These two sets of 2D markers are used to find new values for the alignment parameters \( d_i, s_i, \alpha_i, \) and \( \theta_i \).

### 3.1.2 In-plane projection translation \( d_i \)

Generally, an alignment parameter is calculated by optimizing (in a least-squares sense) the agreement between the two point sets \( \tilde{p}_i^{(j)} \) and \( \tilde{p}_i^{(n)} \) using that alignment parameter alone, i.e., by minimizing the residual error
\[ e([\tilde{p}_i^{(j)}], [\tilde{p}_i^{(n)}]) = \left[ \sum_{i,j} |\tilde{p}_i^{(n)} - \tilde{p}_i^{(j)}|^2 \right]^{1/2}, \] (3.13)
where the alignment-corrected markers \( \tilde{p}_i^{(j)} \) are functions of each of the alignment parameters. Correspondingly, the least-squares solution for the in-plane projection translation vector \( d_i \) (cf. Fig. 3.1 (a)) is determined by the minimum of the following functional:
\[ L_d(d_i) = \sum_{j=1}^{s} |\tilde{p}_i^{(n)} - \tilde{p}_i^{(j)} - d_i|^2, \] (3.14)
which has the solution
\[ \tilde{d}_i = \frac{1}{s} \left( \sum_{j=1}^{s} \tilde{p}_i^{(n)} - \sum_{j=1}^{s} \tilde{p}_i^{(j)} \right), \] (3.15)
i.e., the translation vector is given by the difference between the centers of gravity of the two marker sets.
3.1.3 Projection scale $s_i$

The projection scale is found using the so-called symmetric error, which has the advantage that it does not depend on the choice of reference nor on the rotation angle between the two sets of points [Hor87]. The corresponding functional has the following form:

$$L_s(s_i) = \sum_{j=1}^{s} \left| \frac{1}{\sqrt{s_i}} p_i^{(j)} - \sqrt{s_i} p'_i \right|^2,$$

(3.16)

which can be written as

$$L_s(s_i) = \frac{1}{s_i} \sum_{j=1}^{s} |p_i^{(j)}|^2 + s_i \sum_{j=1}^{s} |p'_i|^2 - 2 p''_i p'_i,$$

(3.17)

where $x^T$ denotes the transpose of vector $x$. Calculating the derivative over $s_i$ and setting it to zero yields

$$\frac{\partial}{\partial s_i} L_s(s_i) = -\frac{1}{s_i^2} \sum_{j=1}^{s} |p_i^{(j)}|^2 + \sum_{j=1}^{s} |p'_i|^2 = 0,$$

(3.18)

which has the solution

$$\tilde{s}_i = \left[ \frac{\sum_{j=1}^{s} |p_i^{(j)}|^2}{\sum_{j=1}^{s} |p'_i|^2} \right]^{1/2}.$$

(3.19)

3.1.4 In-plane rotation angle $\alpha_i$

The in-plane rotation angle $\alpha_i$ is found by calculating the minimum of the functional

$$L'_\alpha(\alpha_i) = \sum_{j=1}^{s} |p_i^{(j)} - \mathcal{R}(\alpha_i)p'_i|^2,$$

(3.20)

where $\mathcal{R}(\alpha_i)$ is a rotation matrix, or equivalently, by finding the maximum of

$$L_\alpha(\alpha_i) = \sum_{j=1}^{s} p''_i \mathcal{R}(\alpha_i)p'_i,$$

(3.21)

which can be expanded to

$$L_\alpha(\alpha_i) = \sum_{j=1}^{s} (p''_{x_i} p'_{x_i} + p''_{y_i} p'_{y_i}) \cos \alpha_i + \sum_{j=1}^{s} (p''_{y_i} p'_{x_i} - p''_{x_i} p'_{y_i}) \sin \alpha_i.$$

(3.22)

Calculating the derivative over $\alpha_i$ and setting it to zero yields

$$\tan \tilde{\alpha}_i = \frac{\sum_{j=1}^{s} (p''_{y_i} p'_{x_i} - p''_{x_i} p'_{y_i})}{\sum_{j=1}^{s} (p''_{x_i} p'_{x_i} + p''_{y_i} p'_{y_i})}.$$

(3.23)
3.1.5 Tilt angle $\theta_i$

Whereas for the alignment parameters $d_i$, $s_i$, and $\alpha_i$ there exist closed-form, least-squares solutions, such a solution cannot be found for the tilt angle $\theta_i$. The reason is that Eq. (3.11) is non-linear with respect to $\theta_i$. However, taking into account that initial estimates are known for the tilt angles, an approximate solution can be found using the following reasoning.

The projection of a two-dimensional vector can be described as a rotation of the coordinate system. For example, in the model described in the preceding sections a 3D marker is projected perpendicular to the x-axis, i.e., in the (z,y)-plane. If the marker $x^j$ is to be projected under the angle $\theta_i$ (measured from the z-axis, cf. Fig. 3.1 (d)), this can be achieved by rotating the (z,y)-coordinate system by $-\theta_i$:

$$\begin{pmatrix} p^i_{z_j} \\ p^i_{y_j} \end{pmatrix} = \begin{pmatrix} \cos \theta_i & \sin \theta_i \\ -\sin \theta_i & \cos \theta_i \end{pmatrix} \begin{pmatrix} z^j \\ y^j \end{pmatrix}$$

(3.24)

As can be seen, the term for $p^i_{z_j}$ in Eq. (3.24) is the same as in Eq. (3.8), which describes the projection of $x^j$ perpendicular to the x-axis. While the first projected coordinate $p^i_{z_j}$ cannot be measured in the experiment (it is projected at right angles to the current view), it can be computed from the coordinates $z^j$ and $y^j$ given by the 3D marker model and the tilt angle $\theta_i$. Using the constraint

$$(p^i_{z_j})^2 + (p^i_{y_j})^2 = (p^i_{z_j})^2 + (p^i_{y_j})^2$$

(3.25)

and the current value of the alignment-corrected y-coordinate $p^i_{y_j}$, Eq. (3.25) can be solved for $p^i_{z_j}$ as

$$p^i_{z_j} = \frac{p^i_{z_j}}{|p^i_{z_j}|} \left[ (p^i_{z_j})^2 + (p^i_{y_j})^2 - (p^i_{y_j})^2 \right]^{1/2},$$

(3.26)

where $p^i_{z_j}$ has been assumed to have the same sign as $p^i_{z_j}$, i.e., the reprojected 3D marker lies on the same side of the plane as the corresponding alignment-corrected marker in the projection.

There is now available a set of reprojected 3D markers $(p^i_{z_j}, p^i_{y_j})$, and, using Eq. (3.26), a corresponding set of measured markers $(p^i_{z_k}, p^i_{y_k})$. The two sets differ by a common rotation angle in the (z,y)-plane, which can be found similar to the in-plane rotation angle $\alpha_i$ using Eq. (3.23) with z-coordinates substituting x-coordinates. The increment to the tilt angle $\theta_i$ is therefore given by

$$\tan \delta \theta_i = \frac{\sum_{j=1}^{s} (p^i_{y_j} p^i_{z_k} - p^i_{y_k} p^i_{z_j})}{\sum_{j=1}^{s} (p^i_{y_j} p^i_{z_k} + p^i_{y_k} p^i_{z_j})}.$$

(3.27)

Note that differing from the preceding sections, it is the increment to the alignment parameter $\delta \theta_i$ that is computed.

3.1.6 Iterative alignment algorithm

Using the solutions for the alignment parameters found in sections 3.1.2 - 3.1.5, the alignment algorithm is as follows:
1. For each projection, measure the coordinates \((p_{xj}^i, p_{yj}^i)\) of the fiducial markers. For the experiments described in this work, this was done interactively using the XALIGN program (cf. appendix A). The marker positions of a given projection are initially measured relative to the origin of the CCD camera chip, and then translated so that the common center of gravity (i.e., the average marker position) lies at the origin.

2. Use plausible initial estimates for the alignment parameters: \(d_i = 0\), \(s_i = 1\), and \(\alpha_i = 0\) (unless there is a common in-plane rotation angle, cf. section 3.1.7), and the tilt angles \(\theta_i\) known from the experiment.

3. Correct the measured marker coordinates \((p_{xj}^i, p_{yj}^i)\) using the right-hand side of Eq. (3.8) with the current alignment parameters. This provides the set of alignment-corrected marker coordinates \((p_{xj}^{\prime i}, p_{yj}^{\prime i})\).

4. Using the alignment-corrected marker coordinates \((p_{xj}^{\prime i}, p_{yj}^{\prime i})\), calculate the corresponding 3D marker coordinates \((x_j^i, y_j^i, z_j^i)\) – the ‘3D marker model’ (Eqs. (3.10) and (3.11)). Translate the 3D marker positions so that the center of gravity lies at the origin.\(^2\)

5. Reproject the 3D marker coordinates \((x_j^i, y_j^i, z_j^i)\) onto the projections using the left-hand side of Eq. (3.8). This yields the set of reprojected marker coordinates \((p_{xj}^{\prime \prime i}, p_{yj}^{\prime \prime i})\).

6. Calculate the residual error of the 3D marker model using Eq. (3.13).

7. For an alignment parameter to be found, calculate a corrected value using the two marker sets \(\{p_{xj}^{\prime i}\}\) and \(\{p_{xj}^{\prime \prime i}\}\), and one of the Eqs. (3.15), (3.19), (3.23), or (3.27).

8. Repeat steps 3, 4, 5, 6, and 7 for all alignment parameters that have to be corrected.

9. Repeat steps 3, 4, 5, 6, 7, and 8 until the residual error of the 3D marker model becomes smaller than a given value or cannot be reduced anymore.

Instead of always accepting the corrected alignment parameter calculated in step 7, the suggested change can be evaluated to see whether the residual error is really reduced; and rejected if that is not the case. If a marker is obscured or badly defocused in a projection (so that the marker coordinates cannot be measured), the missing values of \((p_{xj}^i, p_{yj}^i)\) are omitted from step 3 of the algorithm. The generation of the 3D marker model in step 4 uses marker positions from all projections, so the missing marker can also be omitted from the average of Eq. (3.10) and the linear regression of Eq. (3.11). As an extreme case, a marker need be visible in only two projections in order to make linear regression possible and calculate the y- and z-coordinate of the 3D marker. In experiments conducted at BESSY I, an individual marker was typically visible and sufficiently well-focused to permit measuring in roughly 50% of the X-ray microscopic images constituting a tilt series.

\(^2\)This translation of the 3D markers is not described in [PMBF95]; it was found to be necessary in order for the iterative alignment algorithm to converge.
3.1.7 Finding the common in-plane rotation angle

As has been shown in the preceding sections, fiducial markers can be used to determine all relevant alignment parameters of the X-ray microscopic images, among them the in-plane rotation angle $\alpha$ (cf. Fig. 3.1 (c)). While the alignment algorithm based on Eq. (3.1) provides a robust estimate of the alignment parameters for each tilt image, experiments have shown that if only a few fiducial markers are available, the algorithm may calculate the alignment parameters with unacceptable error. In such cases it is desirable to use the marker information from all images to determine a common alignment parameter with increased accuracy.

From the experimental setup it is known that the in-plane rotation angle $\alpha_i$ is mainly due to a small angular mismatch between the magnetically attached object holder and the CCD camera chip (cf. section 2.2.2). The angle $\alpha_i$ is therefore approximately the same for all tilt images. In the following, an algorithm is described which was developed to determine the common in-plane rotation angle from all available fiducial markers in all images.

The starting point is again Eq. (3.1), which describes all relevant alignment parameters. Because the rotation matrix $\mathcal{R}(\alpha_i)$ can be inverted, Eq. (3.1) can be solved for $p^i_1$:

$$p^i_1 = \frac{1}{s_i} \mathcal{R}^{-1}(\alpha_i)(\mathcal{P}(\theta_i)x^i - d_i)$$

$$= \frac{1}{s_i} \mathcal{R}^{-1}(\alpha_i)\mathcal{P}(\theta_i)x^i - \frac{1}{s_i} \mathcal{R}^{-1}(\alpha_i)d_i,$$

where $\mathcal{R}^{-1}(\alpha_i)$ denotes the inverse of matrix $\mathcal{R}(\alpha_i)$.

Now consider two fiducial markers $x^i$ and $x^k$. Since all X-ray microscopic images are translated by an unknown translation vector, the absolute coordinates of the projected markers $p^i_1$ and $p^k_1$ are not available from the tilt images. However, the difference vector $p^i_1 - p^k_1$ does not depend on the translation and can therefore be measured. It is given by

$$\delta_{i,k} = p^i_1 - p^k_1$$

$$= \frac{1}{s_i} \mathcal{R}^{-1}(\alpha_i)\mathcal{P}(\theta_i)x^i - \frac{1}{s_i} \mathcal{R}^{-1}(\alpha_i)d_i - \frac{1}{s_i} \mathcal{R}^{-1}(\alpha_i)\mathcal{P}(\theta_i)x^k + \frac{1}{s_i} \mathcal{R}^{-1}(\alpha_i)d_i$$

$$= \frac{1}{s_i} \mathcal{R}^{-1}(\alpha_i)\mathcal{P}(\theta_i)(x^i - x^k).$$

Denoting $x^i - x^k$ as $(\Delta x_i, \Delta y_i, \Delta z_i)$, this can be written as

$$\delta_{i,k} = \frac{1}{s_i} \mathcal{R}^{-1}(\alpha_i) \begin{pmatrix} 1 & 0 & 0 \\ 0 & \cos \theta_i & -\sin \theta_i \\ 0 & \sin \theta_i & \cos \theta_i \end{pmatrix} \begin{pmatrix} \Delta x_i \\ \Delta y_i \\ \Delta z_i \end{pmatrix}$$

$$= \frac{1}{s_i} \mathcal{R}^{-1}(\alpha_i) \begin{pmatrix} \Delta x_i \\ \Delta y_i \cos \theta_i - \Delta z_i \sin \theta_i \\ \Delta z_i \cos \theta_i \end{pmatrix}.$$  

For any given pair of markers $x^i$ and $x^k$, $\Delta y_i$ and $\Delta z_i$ are constants. Defining

$$f(\theta_i) \equiv \Delta y_i \cos \theta_i - \Delta z_i \sin \theta_i$$

and using

$$\mathcal{R}^{-1}(\alpha_i) = \begin{pmatrix} \cos \alpha_i & \sin \alpha_i \\ -\sin \alpha_i & \cos \alpha_i \end{pmatrix},$$

(3.32)
Eq. (3.30) yields:

$$\left( \delta_{x_i}^{j,k}, \delta_{y_i}^{j,k} \right) = \frac{1}{s_i} \begin{pmatrix} \cos \alpha_i & \sin \alpha_i \\ -\sin \alpha_i & \cos \alpha_i \end{pmatrix} \begin{pmatrix} \Delta_x \\ f(\theta_i) \end{pmatrix}$$

$$= \frac{1}{s_i} \begin{pmatrix} \Delta_x \cos \alpha_i + f(\theta_i) \sin \alpha_i \\ -\Delta_x \sin \alpha_i + f(\theta_i) \cos \alpha_i \end{pmatrix}$$

(3.33)

Multiplying the first equation of Eq. (3.33) with $s_i \cos \alpha_i$, the second with $-s_i \sin \alpha_i$, and adding the two equations yields (since $\cos^2 \alpha_i + \sin^2 \alpha_i = 1$):

$$s_i \delta_{x_i}^{j,k} \cos \alpha_i - s_i \delta_{y_i}^{j,k} \sin \alpha_i = \Delta_x,$$  (3.34)

or, if $\sin \alpha_i \neq 0$,

$$\delta_{y_i}^{j,k} = \frac{\cos \alpha_i}{\sin \alpha_i} \delta_{x_i}^{j,k} - \frac{\Delta_x}{s_i \sin \alpha_i}.$$  (3.35)

Because of the experimental setup, it can be assumed in good approximation that the in-plane rotation angle is the same for all tilt images ($\alpha_i = \alpha \forall i$), and that all tilt images have the same magnification, which can therefore be set to unity ($s_i = 1 \forall i$), so that Eq. (3.35) becomes

$$\delta_{y_i}^{j,k} = \frac{\cos \alpha}{\sin \alpha} \delta_{x_i}^{j,k} - \frac{\Delta_x}{\sin \alpha}.$$  (3.36)

Plotting the pairs of values $(\delta_{x_i}^{j,k}, \delta_{y_i}^{j,k})$ for all tilt angles $\theta_i$, one therefore expects to see a linear graph of the form $y = ax + b$. The slope $a$ and abscissa $b$ of this graph can be determined using linear regression. The in-plane rotation angle $\alpha$ is given by

$$\alpha = \arctan \left( \frac{1}{a} \right).$$  (3.37)

Once $\alpha$ is known, the difference in the x-coordinate of the fiducial markers can be calculated from the abscissa $b$ as

$$\Delta_x = -b \sin \alpha.$$  (3.38)

Usually, more than two fiducial markers will be available. The common in-plane rotation angle can be computed for every pairing of $n$ available markers. There are

$$\binom{n}{2} = \frac{n!}{(n-2)!2!} = \frac{n(n-1)}{2}$$

such pairings. For a typical number of fiducial markers (10 - 15), this means that the common in-plane rotation angle $\alpha$ can be calculated as the average value of Eq. (3.37) for a large number (50 - 100) of marker pairings. To improve the accuracy, only those marker pairs $(j, k)$ should be used that are sufficiently apart in the tilt images for the relative errors of $\delta_{x_i}^{j,k}$ and $\delta_{y_i}^{j,k}$ to be small.

Experiments have shown that some tilt images have an in-plane rotation angle $\alpha_i$ that is significantly different from the average angle. They can be identified by the fact that the points $(\delta_{x_i}^{j,k}, \delta_{y_i}^{j,k})$ belonging to these images are significantly farther apart from the fitted line than the other points. However, by setting $s_i = 1$ in Eq. (3.34) and using the value for $\Delta_x$ that has been computed from the abscissa of the linear regression using Eq. (3.38), Eq. (3.34) becomes a non-linear equation for $\alpha_i$:

$$\delta_{x_i}^{j,k} \cos \alpha_i - \delta_{y_i}^{j,k} \sin \alpha_i - \Delta_x = 0,$$  (3.40)
which can be solved using one of the usual numerical procedures for root finding, such as the Newton-Raphson method \cite{PTVF92}.

In practice the common in-plane rotation angle $\alpha$ calculated using Eq. (3.37) was usually taken as a first estimate for the individual rotation angles $\alpha_i$, which were then individually corrected using the full fiducial marker algorithm described in section 3.1.

### 3.2 Creation of the aligned tilt series

Once the five alignment parameters for all tilt angles are known, the original X-ray microscopic images have to be shifted, rotated, and possibly scaled\(^3\) to create a tilt series aligned to a common axis of rotation. In order to make the best use of the sub-pixel accuracy of the alignment parameters, these operations should also be performed with sub-pixel accuracy.

At this time it may also be desirable to change the image size. Reduction of the image size can be combined with an improved signal-to-noise ratio of the intensity data. In addition, a reduction in size is often necessary because the original image size would result in a very large volume size, implying a time-consuming reconstruction and prohibiting interactive handling of the reconstructed volume on the available hardware.

It is helpful to formulate the task of creating the aligned tilt series in the terms of sampling theory: given the original intensity data measured on the grid points of a Cartesian coordinate system (the illumination-corrected CCD pixel values), and given a second coordinate system determined by the alignment parameters, sample the original intensity data at all grid points of the second (shifted, rotated, and possibly scaled) coordinate system. The set of samples constitutes the aligned tilt image.

The image-processing task is thus the task of interpolating a function value from a discrete set of available function values. Depending on the desired quality of the interpolation result, different interpolation methods are used. Increased interpolation quality usually also means increased computational cost, i.e., increased execution time.

#### 3.2.1 Interpolation methods

The X-ray microscopic image acquired by the CCD camera is modeled as follows. For a real number $x \in \mathbb{R}$, let $\lfloor x \rfloor$ be the integer number $n$ with $n \leq x < n + 1$. The intensity in the CCD chip plane is assumed to be known at all grid locations\(^4\), i.e., for all locations $(x, y)$ with $x = \lfloor x \rfloor$ and $y = \lfloor y \rfloor$. A given sample location $x$ is surrounded by the four neighbouring grid locations $(\lfloor x \rfloor, \lfloor y \rfloor)$, $(\lfloor x \rfloor + 1, \lfloor y \rfloor)$, $(\lfloor x \rfloor, \lfloor y \rfloor + 1)$, and $(\lfloor x \rfloor + 1, \lfloor y \rfloor + 1)$ (cf. Fig. 3.2).

Nearest-neighbour interpolation assigns the interpolated function value $f_{\text{NN}}(x)$

\[^3\text{No individual scaling of the X-ray microscopic images is necessary to compensate magnification changes occurring during acquisition of the tilt series (cf. footnote on p. 35); however, a global scale change (i.e., size reduction) is often desirable.}\]

\[^4\text{This models the CCD measurement as point-sampling the intensity in the image plane, and does not take account of the fact that the intensity is integrated over the area of the CCD pixel.}\]
Figure 3.2: Interpolation of the function value \( f(x, y) \) based on the function values at neighbouring grid points of a quadratic grid. Nearest-neighbour and bilinear interpolation use only the function values of the four neighbours shown above (cf. Eqs. (3.41) and (3.42)), bicubic interpolation also uses the partial derivatives and the cross derivative at the grid points.

the value at the nearest grid location. With \( \alpha = x - \lfloor x \rfloor \) and \( \beta = y - \lfloor y \rfloor \),

\[
f_{\text{NN}}(x) = \begin{cases} 
  f([x], [y]) & \text{for } \alpha, \beta < 0.5, \\
  f([x] + 1, [y]) & \text{for } \alpha \geq 0.5, \beta < 0.5, \\
  f([x], [y] + 1) & \text{for } \alpha < 0.5, \beta \geq 0.5, \\
  f([x] + 1, [y] + 1) & \text{for } \alpha, \beta \geq 0.5.
\end{cases}
\] (3.41)

\textit{Bilinear interpolation} uses the four neighbouring grid locations to linearly interpolate the function value in \( x \) and \( y \). The interpolated function value is then given as

\[
f_{\text{bilinear}}(x) = (1 - \alpha) (1 - \beta) f([x], [y]) \\
+ \alpha (1 - \beta) f([x] + 1, [y]) \\
+ (1 - \alpha) \beta f([x], [y] + 1) \\
+ \alpha \beta f([x] + 1, [y] + 1).
\] (3.42)

While \( f_{\text{NN}} \) is discontinuous, \( f_{\text{bilinear}} \) is a continuous function. However, its partial derivatives are discontinuous for \( x = [x] \) or \( y = [y] \). \textit{Bicubic interpolation} also takes into account the partial derivatives and the cross derivative at the four neighbouring grid locations (usually by computing them from the 16 nearest neighbours of \((x, y), [PTVF92])\), and has continuous partial derivatives across grid lines.
The three interpolation methods described so far only use a very small neighbourhood of the sample location to interpolate the sample value. Other interpolation methods use all of the available data to interpolate a function value. B-spline interpolation does this by computing polynomials of degree \( D \) that smoothly approximate the known function values for one dimension, e.g., along the rows of the image. To interpolate the function at \((x, y)\), these polynomials are evaluated for \(x\), and a new fitting polynomial is computed for the newly created image column and evaluated at \(y\). Optimized implementations exist that permit fast interpolation of large data arrays \(\text{[Ebe]}\).

### Two-dimensional sampling theory

Finally, it is necessary to consider interpolation methods that have well-defined behaviour in Fourier space. In the point-sampling model used to describe the CCD image acquisition, the X-ray microscopic images are characterized by the sampling interval in the object plane. This sampling interval is given by the CCD pixel size divided by the imaging magnification. The CCD camera used for these experiments has a pixel size of 24 \(\mu\)m. The imaging magnification used in the different CTCXM experiments was between 1740\(\times\) and 1850\(\times\); an imaging magnification of 1740\(\times\) will be used as a numerical example\(^5\). In this case, the sampling interval in the object plane is 13.79 nm.

The process of CCD image acquisition is equivalent to point-sampling the continuous function \(f\) (the intensity in the image plane) on a quadratic grid. Following [Goo88], the sampled function \(f_s\) is given by

\[
f_s(x, y) = \text{comb}\left(\frac{x}{\Delta}\right) \text{comb}\left(\frac{y}{\Delta}\right) f(x, y),
\]

with the comb function

\[
\text{comb}(x) = \sum_{n=-\infty}^{\infty} \delta(x - n) \tag{3.44}
\]

and \(\Delta\) the spacing of the quadratic grid. Since \(f_s\) is obtained by multiplying \(f\) with a two-dimensional comb function, the Fourier transform (or spectrum) \(F_s\) of \(f_s\) is the result of convolving the spectrum \(F\) of \(f\) with the spectrum of the comb function (which is itself a comb function):

\[
F_s(k_x, k_y) = \mathcal{F}\left\{ \text{comb}\left(\frac{x}{\Delta}\right) \text{comb}\left(\frac{y}{\Delta}\right) \right\} \otimes F(k_x, k_y) = \sum_{n=-\infty}^{\infty} \sum_{m=-\infty}^{\infty} F\left(k_x - \frac{n}{\Delta}, k_y - \frac{m}{\Delta}\right). \tag{3.45}
\]

The spectrum of the sampled function is therefore given by duplicating the original spectrum \(F\) at all locations \((n/\Delta, m/\Delta)\) in the Fourier plane.

For any sampling interval \(\Delta\), there is also a special spatial frequency \(f_c\), called the Nyquist critical frequency, given by

\[
f_c \equiv \frac{1}{2\Delta}. \tag{3.46}
\]

\(^5\)The images of the LN\(_2\)-frozen \textit{C. reinhardtii} specimen were acquired with a magnification of 1740\(\times\), cf. section 5.1.
Note that the Nyquist frequency is half of the spatial frequency that is associated with the sampling interval. The Whittaker-Shannon theorem of sampling theory says that if a continuous band-limited function is sampled with a spatial frequency that is at least twice as high as the highest spatial frequency occurring in the function, the continuous function is completely determined by the discrete samples. Equivalently, the spatial frequencies in the continuous function must not exceed the Nyquist frequency of the sampling. In this case, the original spectrum $F$ has nonzero components only within a rectangular region $[-f_c, f_c] \times [-f_c, f_c]$ of the Fourier plane, and the ‘extra copies’ of the spectrum that are created by the sampling process (cf. Eq. (3.45)) do not overlap the original spectrum. The original function can be reconstructed by inverting the sampling process, i.e., by removing those ‘extra copies’ of $F$ by multiplying the spectrum of the sampled data $F_s$ with a suitable transfer function.

A suitable transfer function is a two-dimensional rectangle function that is equal to one in the region $[-f_c, f_c] \times [-f_c, f_c]$, and equal to zero everywhere else:

$$H(k_x, k_y) = \text{rect}(k_x \Delta) \text{rect}(k_y \Delta)$$  \hspace{1cm} (3.47)

Multiplication of the spectrum $F_s$ with this transfer function yields the spectrum of the original function:

$$F_s(k_x, k_y) \text{rect}(k_x \Delta) \text{rect}(k_y \Delta) = F(k_x, k_y)$$  \hspace{1cm} (3.48)

In the spatial domain, the original function is obtained by convolving the sampled function with the inverse Fourier transform of $H$, the impulse response $h$:

$$f(x, y) = f_s(x, y) \otimes h(x, y),$$  \hspace{1cm} (3.49)

with

$$h(x, y) = \int \int \text{rect}(k_x \Delta) \text{rect}(k_y \Delta)e^{2\pi i (k_x x + k_y y)} dk_x dk_y$$

$$= \frac{1}{\Delta^2} \text{sinc} \left( \frac{x}{\Delta} \right) \text{sinc} \left( \frac{y}{\Delta} \right),$$  \hspace{1cm} (3.50)

where $\text{sinc}(x) = \sin(\pi x)/(\pi x)$. Because the sampled function is nonzero only at the grid points $(n \Delta, m \Delta)$, Eq. (3.49) can be written as a sum:

$$f(x, y) = \sum_{n=-\infty}^{\infty} \sum_{m=-\infty}^{\infty} f_s(n \Delta, m \Delta) \text{sinc} \left( \frac{x}{\Delta} - n \right) \text{sinc} \left( \frac{y}{\Delta} - m \right)$$  \hspace{1cm} (3.51)

Interpolation of the sampled function is thus achieved by a weighted sum over the sampled function values. The sum originally extends over all samples, but in order to make the interpolation computationally feasible, a finite filter support must be used, i.e., only samples within a given neighbourhood of $(x, y)$ are included in the sum. In the case of a finite rectangular filter support, simply using the filter weights given by Eq. (3.51) is equivalent to multiplying the impulse response with a rectangle function. In the Fourier domain, the transfer function Eq. (3.47) is therefore convolved with a product of sinc functions, resulting in a ‘ringing’ transfer function that attenuates some spatial frequencies below the cut-off frequency $f_c$, and passes some frequencies above.
This effect can be ameliorated by multiplying the filter weights in Eq. (3.51) with a 'window' function \( w \) that is equal to one at the center of the filter support, smoothly falls to zero at the edges of the support, and is equal to zero outside the support. Some of the window functions that have been used are [PTVF92]:

\[
\begin{align*}
    w(d) &= 1 - d, & \text{(Bartlett window)} \\
    w(d) &= 1 - d^2, & \text{(Welch window)} \\
    w(d) &= (1 + \cos(\pi d))/2, & \text{(Hann window)}
\end{align*}
\]  

for \( d \leq 1 \) and \( w(d) = 0 \) for \( d > 1 \), where \( d \) is the normalized distance from the filter center (\( d = 1 \) at the edge of the filter support).

This kind of interpolation is also able to correctly handle a reduction in size. Since a size reduction is equivalent to an increased sampling interval, the Nyquist frequency of the sampling process is reduced (cf. Eq. (3.46)). The data must therefore be low-pass filtered before resampling to ensure that the Nyquist frequency is not exceeded. This can be done simply by changing the transfer function of Eq. (3.47) to account for the new Nyquist frequency:

\[
H(k_x, k_y) = \text{rect}\left(\frac{k_x \Delta}{s}\right) \text{rect}\left(\frac{k_y \Delta}{s}\right),
\]  

where \( s \) is the scaling factor (\( s < 1 \) for a reduction in size). The corresponding impulse response is given by

\[
h(x, y) = \left(\frac{s}{\Delta}\right)^2 \text{sinc}\left(\frac{sX}{\Delta}\right) \text{sinc}\left(\frac{sY}{\Delta}\right).
\] 

### 3.2.2 Comparison

The process that leads from the X-ray microscopic specimen to the aligned tilt series used to reconstruct the specimen absorption can be broken down into several steps. The first of these steps, the creation of a magnified image of the specimen in the image plane, can be described in good approximation by a space-invariant linear system with a finite frequency response (cf. section 1.2).

The next step in the process is the acquisition of the magnified image by the CCD chip. Assuming that the image acquisition can be described in good approximation as point-sampling the continuous intensity in the image plane, and neglecting the influence of noise, the transition from continuous intensity to discrete intensity samples can be exactly described in the terms of sampling theory, by duplicating the band-limited spectrum of the continuous intensity on a grid in the Fourier plane, with a grid spacing given by the sampling interval (cf. Eq. (3.45)).

For an imaging magnification of 1740×, the sampling interval in the object plane was shown to be 13.79 nm, corresponding to an object plane sampling frequency of 72.5 \( \mu \text{m}^{-1} \) and a Nyquist frequency of 36.3 \( \mu \text{m}^{-1} \). For a micro zone plate objective with outermost zone width \( d_{\text{r}_{\text{n}}} = 40 \) nm, the modulation transfer function for monochromatic illumination and incoherent image formation has a cut-off frequency of 25 \( \mu \text{m}^{-1} \) (for all object planes, cf. section 1.2.3). The X-ray microscopic images are therefore band-limited, and oversampled by a factor of 36.3/25 = 1.45.

In section 1.2.3, the modulation transfer function for narrow-bandwidth illumination was calculated, and a modulation transfer of 9% (corresponding to the Rayleigh resolution criterion) in the focused object plane was found to occur for the
spatial frequency of 16.7 µm\(^{-1}\) (cf. Fig. 1.5). The Nyquist frequency of the sampling performed by the CCD camera exceeds this Rayleigh resolution-equivalent frequency by a factor of \(36.3/16.7 = 2.17\). Thus, the original microscopic images can be reduced in size by a factor of 2 (in each direction, the number of pixels is reduced by a factor of 4) while preserving all object information up to and exceeding the Rayleigh resolution as calculated for narrow-bandwidth illumination.

The third step of the process is the interpolation (translation, rotation, and possibly scaling) of the CCD images to create the aligned tilt series. In the model of sampling theory, the mathematically correct way to do this is to invert the point-sampling that has been performed by the CCD chip, by applying the Whittaker-Shannon theorem. This is done by means of a weighted sum over the CCD pixel values in the neighbourhood of the sampling location, and can be combined with a low-pass filter if the images are to be scaled. Since this weighted-sum interpolation is also a space-invariant linear system, it can be characterized by a modulation transfer function (MTF).

It is clear from the above description that weighted-sum interpolation is best suited to the task of interpolating the X-ray microscopic images. To evaluate the different interpolation methods described above, intensity data from X-ray microscopic images was interpolated, and experimental MTFs were determined for the different interpolation methods.

In order to evaluate the interpolation methods, a quadratic image with 256 × 256 pixels (part of an original X-ray microscopic image) was resampled with an interpolation offset of 0.5 pixels in each direction, i.e., the sample locations were chosen to lie in the middle of the grid cells. The weighted-sum interpolation was performed with a radially symmetric transfer function (compare with the quadratic transfer function of Eq. (3.55))

\[
H(k_x, k_y) = \text{rect} \left( 2\Delta \sqrt{k_x^2 + k_y^2} \right),
\]  

with a cut-off frequency of half the original Nyquist frequency, to enable a reduction in size by a factor of 2. The corresponding impulse response is given by

\[
h(x, y) = \frac{J_1 \left( \left(\pi/2\right) \sqrt{x^2 + y^2} \right)}{4\sqrt{x^2 + y^2}},
\]  

with \(J_1(x)\) the Bessel function of the first kind, order one. The filter support is also circular, with a radius of 25 pixels. A Hann window was used to avoid ringing artifacts.

The original image as well as the resampled images were Fourier transformed using a fast Fourier transform algorithm [FJ98]. The power spectrum of each image was calculated by averaging the squared modulus of the Fourier transform over circular rings in the two-dimensional Fourier plane. To obtain modulation transfer functions for the interpolation methods, the power spectra of the interpolated images were divided by the power spectrum of the original image, and the square root was taken. The resulting graph shows the modulation transfer as a function of spatial frequency (cf. Fig. 3.3).

If the coordinate system used for interpolation is rotated, the MTFs of nearest-neighbour, bilinear, and bicubic interpolation vary wildly, while the MTFs of b-spline interpolation and weighted-sum interpolation stay the same (cf. Fig. 3.4).
3.2 Creation of the aligned tilt series

Figure 3.3: Comparison of interpolation methods by modulation transfer function. A 256 \times 256 pixel sample X-ray microscopic image was interpolated for a pixel off-set of (0.5, 0.5), and experimental MTFs were determined by comparing the power spectra of original and interpolated data (see text). Bilinear and bicubic interpolation do not have a well-defined MTF. Apart from nearest-neighbour interpolation, which simply replicates the original image, all interpolation methods act as low-pass filters, but only the weighted-sum interpolation has a clearly defined passband and suppresses spatial frequencies above the cut-off frequency efficiently.

However, only the weighted-sum interpolation provides a clearly defined passband with an adjustable cut-off frequency, and permits to resample the intensity data with an increased sampling interval without violating the Nyquist condition.

By using weighted-sum interpolation, the low-pass filter used for size reduction and the sub-pixel accuracy interpolation necessary to correct image shift and rotation can be incorporated into a single spatial domain filter operation. Weighted-sum interpolation is therefore adopted as the method of choice for resampling the X-ray microscopic images to create the aligned tilt series. As has been pointed out, low-pass filtering the data increases the signal-to-noise ratio of the intensity data. Reduction of the image width and height by a factor of 2 increases the number of counts per pixel by a factor of 4, and thus improves the SNR by a factor of \( \sqrt{4} = 2 \). The comparatively large filter support radius of 25 pixels is necessary to achieve a sufficiently steep transition from passband to stopband in the low-pass filter (cf. Fig. 3.3). It should be noted that the high interpolation quality comes at the expense of increased computational cost: with a filter support radius of 25 pixels, approx. 1960 CCD pixels contribute to each weighted sum.

The software program XALIGN was written to permit the interactive selection
3.2 Creation of the aligned tilt series

of fiducial markers in a tilt series, to compute alignment parameters for each tilt image using the algorithm described in section 3.1, and to resample the images using one of the interpolation methods described above. The XALIGN manual can be found in appendix A.

Figure 3.4: Comparison of interpolation methods for a rotated coordinate system. Same parameters as in Fig. 3.3, but in addition to the interpolation offset, the interpolated coordinate system was rotated by 5° (typical in-plane rotation angle for a CTCXM experiment). Because the Fourier transform is not rotationally invariant, the resampled images have to be rotated by −5° afterwards in order to compare the power spectra. Thus, two consecutive interpolations are performed on the data. In between interpolations, the data is stored as single-precision floats. This causes the noise that is observable for the weighted-sum interpolation at spatial frequencies greater than 0.55 of the Nyquist frequency. In practice, only one interpolation is performed on the intensity data, so the filter performance (suppression of high-frequency content) is better than shown here.
Chapter 4

Computed tomography

The term *computed tomography* (CT, also known as computerized tomography or CAT, computer assisted tomography) means, in a mathematical sense, the reconstruction of a function from its line integrals. The most prominent example of an application of computed tomography is the use of transmission CT in diagnostic radiology. A cross-section of the human body is scanned by a thin X-ray beam whose intensity loss is recorded by a detector and processed by a computer to produce a two-dimensional image which in turn is displayed on a screen [Cor63, Hou73, BDC76]. In addition, CT has found applications in many other areas, among them geology [Bev93], radio astronomy [Bra56] and electron microscopy [HH80, KGT+97, GSR+98].

The application of CT in diagnostic radiology closely resembles the way in which CT was used in this work, namely to reconstruct the local linear absorption coefficient of a microscopic specimen based on cryo X-ray microscopic images (CTCXM). In many respects the difference between the two applications is simply a change of scale. The characteristic size of a ‘specimen’ in diagnostic radiology is on the order of decimeters, while an X-ray microscopic specimen is smaller by five orders of magnitudes, with a characteristic size of several micrometers [SRN+93]. At the moment, the corresponding change in spatial resolution only spans 3 - 4 orders of magnitude: from approx. 200 µm for medical CT [BB96], to 100 - 250 nm for frozen-hydrated mouse 3T3 fibroblasts [WJMO00], and 60 - 70 nm for frozen-hydrated algae *Chlamydomonas reinhardtii* in this work (cf. section 5.1). Other authors have reported results for non-biological objects: a visible structure size of 50 nm for the mineralized sheaths of bacteria *Leptothrix ochracea* [Leh97a], and the resolving of 100 nm structures for a micro-fabricated gold pattern [HMT+94, LDF+97].

There are a number of experimental challenges associated with the change of scale that explain why a corresponding increase of resolution has not yet been achieved. To obtain specimen information at the target resolution of 50 nm, the specimens have to be exposed to a very high radiation dose, precluding the study of live specimens. Cryo microscopy can be used to stabilize the specimens against radiation damage [SNG+95]; however, for high-resolution images it is necessary to avoid specimen drift during image acquisition, which is more difficult for experiments at cryogenic temperatures than at room temperature.

While there have been efforts to acquire high-resolution image information without the use of X-ray optics [MSC98, MCKS99], this has not yet been demonstrated on biological specimens, and all available high-resolution data of such specimens has been acquired using some sort of imaging system, i.e., an X-ray micro-
4.1 Principles

4.1.1 Radon transform and central section theorem

The possibility of reconstructing a three-dimensional object from its projections follows from Radon’s general theory on the determination of functions through their integrals over certain manifolds [Rad17]. According to Radon, an object can be reconstructed uniquely from its line projections if all line projections are known. In this general form, the Radon theorem is of limited use because it does not address the questions of how to reconstruct the object function from a limited number of experimental (i.e., noisy) projections to a finite resolution, and whether for a limited number of projections there does indeed exist a unique reconstruction.

The parallel projection geometry used to describe image formation in the X-ray microscope (cf. section 1.2) is a special case of Radon’s theory where the integrals are performed along parallel lines. This geometry implies a reconstruction problem with cylindrical symmetry. The unknown three-dimensional object function (in this case, the local linear absorption coefficient \( \mu(x) \)) can be reconstructed in independent reconstruction planes perpendicular to the axis of object rotation, reducing the 3D reconstruction problem to a set of 2D problems that share a common geometry. The transformation that maps the 2D object function \( f(x, y) \) of such a reconstruction plane onto the set of its 1D projections \( p_\theta(x') \) is called the Radon transform:

\[
R_\theta : f(x, y) \rightarrow p_\theta(x'),
\]

where \( \theta \) is the projection angle and \( x' \) is the projection coordinate (cf. Fig. 4.1 (a)).

Eq. (4.1) describes the (idealized) CTCXM experiment for a single reconstruction plane: for a specimen with unknown object function \( f(x, y) \), an X-ray microscopic image is acquired under the tilt angle \( \theta \); this image is used to obtain (an approximation of) the projection \( p_\theta(x') \) of \( f(x, y) \). In order to ‘reconstruct’ \( f(x, y) \) from the projections, the Radon transform must be inverted.

This inversion can be accomplished by exploiting the relationship between the projections and the Fourier transform of the object function that is described by the central section theorem, also known as the projection theorem. The central section theorem is implied in the mathematical definition of the Fourier transform. Following [Goo88], the Fourier transform of a two-dimensional function is defined as

\[
F(k_x, k_y) = \hat{f}(f(x, y))(k_x, k_y)
= \int f(x, y)e^{-2\pi i(k_xx+k_yy)}dx\,dy
\]

Similarly, the function \( f(x, y) \) can be represented in terms of its Fourier transform \( F(k_x, k_y) \) by applying the inverse Fourier transform:

\[
f(x, y) = \hat{F}^{-1}(F(k_x, k_y))(x, y)
= \int F(k_x, k_y)e^{2\pi i(k_xx+k_yy)}dk_x\,dk_y
\]
4.1 Principles

(a) parallel projection in real space  
(b) corresponding central section in Fourier space (central sections for other tilt angles are indicated)

Figure 4.1: The Radon transform maps the 2D object function $f(x, y)$ by parallel projection onto the set of 1D functions $p_\theta(x')$, where $\theta$ is the projection angle and $x' = x \cos \theta + y \sin \theta$ (a); according to the central section theorem (Eq. (4.6)), the Fourier transform $P_\theta(k')$ of such a projection forms a central section of the 2D Fourier transform $F(k_x, k_y)$ of the object function (b).

‘Projecting’ the function $f(x, y)$ is defined as calculating the line integrals along a given direction. For example, the projection $p_\theta(x')$ under the projection angle $\theta = 0$ (where $x' = x$) is calculated by integrating $f(x, y)$ over $y$. With Eq. (4.3), the integration yields:

$$p_0(x) = \int f(x, y) dy$$
$$= \int\int F(k_x, k_y) e^{2\pi i (k_x x + k_y y)} dk_x dk_y dy$$
$$= \int F(k_x, k_y) e^{2\pi i k_x x} \left( \int e^{2\pi i k_y y} dy \right) dk_x dk_y$$
$$= \int F(k_x, 0) e^{2\pi i k_x x} dk_x$$
$$= \mathcal{F}^{-1}_{k_x}[F(k_x, 0)](x),$$

where $\mathcal{F}^{-1}_{k_x}[F(k_x, 0)](x)$ denotes the inverse one-dimensional Fourier transform of $F(k_x, 0)$ in $k_x$, with $k_y = 0$. This result also holds for projection directions other
than along the y-axis [DEK80]:

\[ p_\theta(x') = \mathfrak{F}_k^{-1}(F(k', \theta))(x'), \quad (4.5) \]

or equivalently,

\[ F(k', \theta) = \mathfrak{F}_x(p_\theta(x'))(k') = P_\theta(k'). \quad (4.6) \]

This means that for a two-dimensional function \( f(x, y) \), the one-dimensional Fourier transform \( P_\theta(k') \) of a projection \( p_\theta(x') \) is equal to a central section of the 2D Fourier transform \( F(k_x, k_y) \) (cf. Fig. 4.1 (b)). An analogous relationship exists between the two-dimensional projection of a three-dimensional object and the corresponding 2D central section plane of its 3D Fourier transform. The central section theorem suggests that an unknown three-dimensional function can be 'reconstructed' by filling the Fourier space with data on two-dimensional central planes that are derived by 2D Fourier transform from the projections of the unknown function.

If during the course of a CT experiment \( N \) projections of the object function are to be acquired, it is intuitively clear that without further knowledge of the Fourier transform \( F(k_x, k_y) \), the \( N \) projections should be distributed equidistantly over the angular range of 180° (or \( \pi \)), with a constant angular increment \( \Delta \theta = \pi/N \). In this way, the corresponding central sections will form a star pattern about the origin of the Fourier plane, and all parts of the underlying function \( F(k_x, k_y) \) are sampled equally, though with decreasing density as the spatial frequency increases (cf. Fig. 4.1 (b)). It is also clear why no projections for tilt angles \( \theta > \pi \) need be acquired: such a projection simply mirrors the projection acquired under the tilt angle \( \theta - \pi \).

In practice, projections obtained through a CT experiment have finite resolution, and the unknown object function is reconstructed to finite resolution. The effect of restricting the reconstruction problem to finite resolution can be understood by considering the projection theorem and taking into account the boundedness of the object [Hop69].

### 4.1.2 Reconstruction of a bounded object function

A bounded object function is localized within a finite area and has the function value zero outside that area. In mathematical terms, a bounded object \( o(x) \) can be described as the product of an arbitrary unbounded function \( \hat{o}(x) \) that has the same function values as \( o(x) \) within the object boundary, and a binary shape function \( s(x) \) that has the value 1 within the object boundary, and the value 0 without:

\[ o(x) = \hat{o}(x)s(x) \quad (4.7) \]

The Fourier transform of the bounded object is then given by

\[
\begin{align*}
O(k) & = \mathfrak{F}(\hat{o}(x)s(x))(k) \\
& = \mathfrak{F}(\hat{o}(x))(k) \otimes \mathfrak{F}(s(x))(k) \\
& = \hat{O}(k) \otimes S(k),
\end{align*}
\]

\quad (4.8)
where $\otimes$ denotes convolution. This means that every Fourier component $\hat{O}(k)$ of the unbounded function is surrounded by the shape transform $S(k) = \hat{s}(x)(k)$.

For the experiments described in this work, borosilicate glass capillaries were used as object holders for the CTCXM specimens (cf. section 2.2.2). The 2D shape function of these cylindrical capillaries is a circular disc:

$$s(x) = \begin{cases} 
1 & \text{for } |x| \leq D/2, \\
0 & \text{otherwise},
\end{cases} \quad (4.9)$$

where $D$ is the diameter of the circle (capillary). In the experiment the glass capillary is kept at ambient pressure, surrounded and cooled by a cryogenic atmosphere. The mean linear absorption coefficient of this atmosphere is approx. $4.7 \times 10^{-3} \mu m^{-1}$ (cf. section 2.2), i.e., about 23 times smaller than that of ice ($0.11 \mu m^{-1}$). In good approximation, the shape function $s(x)$ can therefore be said to be equal to zero for $|x| > D/2$.

The Fourier transform of a circularly symmetrical function can be evaluated using the Fourier-Bessel transform. For the circular disc (cf. appendix B),

$$S(k) = \mathcal{B}\{s(x)\}(k) = \frac{D}{2k} J_1(\pi k D), \quad (4.10)$$

with $k = |k|$ and $J_1$ the Bessel function of the first kind, order one. Thus the (arbitrary) Fourier transform $\hat{O}(k)$ is convoluted with a circularly symmetrical function with the profile given by Eq. (4.10) (cf. Fig. 4.2 (a)). The effect of this convolution is to smooth the Fourier transform $O(k)$ of the bounded object function. A characteristic distance in Fourier space over which $O(k)$ may be considered to be smooth is given by the first $k$-axis crossing $k_c$ of the smoothing function $S(k)$. Because the first zero of the Bessel function $J_1(x)$ occurs at $x \approx 1.22 \pi$, the characteristic distance $k_c$ is given by:

$$\pi k_c D = 1.22 \pi \iff k_c = \frac{1.22}{D}. \quad (4.11)$$

The Crowther resolution criterion combines the characteristic distance $k_c$ with the tilt angle increment $\Delta \theta$ to estimate the resolution of the reconstruction in the following way. As has been shown in section 4.1.1, for a tilt series consisting of $N$ projections, the tilt angle should be chosen according to

$$\theta_{n+1} = \theta_n + \Delta \theta, \quad (4.12)$$

with $n = 1, \ldots, N - 1$, where $\Delta \theta = \pi/N$ is the constant angular increment. Consider two neighbouring projections separated by an angle of $\Delta \theta$. In the Fourier plane, there are two corresponding central sections separated by $\Delta \theta$, sampling an underlying function $O(k)$ with a smoothness that is characterized by the characteristic distance $k_c$. A critical spatial frequency $k_0$ can be defined for which the two central sections are separated by $k_c$ (cf. Fig. 4.2 (b)):

$$\frac{k_c}{k_0} = \tan(\Delta \theta) \quad (4.13)$$

Using Eq. (4.11) yields

$$k_0 = \frac{1.22}{\tan(\Delta \theta) D}. \quad (4.14)$$
4.1 Principles

Figure 4.2: The boundedness of the object function (capillary with diameter \( D = 9 \ \mu m \)) means that the (arbitrary) object Fourier transform is convoluted with a circularly symmetric shape transform (a), with the first k-axis crossing at \( k_c = 1.22/D = 0.136 \ \mu m^{-1} \); the **Crowther resolution criterion** (b) specifies the critical spatial frequency \( k_0 \) for which two neighbouring central sections are separated by \( k_c \), as \( k_0 = k_c / \tan(\Delta\theta) \).

For spatial frequencies greater than \( k_0 \), the Fourier plane is insufficiently sampled by the available central sections to be able to reconstruct the underlying function \( O(k) \), and thus its inverse Fourier transform, the unknown object function \( o(x) \). In order to form a rough estimate of Eq. (4.14), assuming \( \tan(\Delta\theta) \approx \Delta\theta \), replacing 1.22 with 1 and using the periodicity \( d_0 = 1/k_0 \) associated with the spatial frequency \( k_0 \) yields

\[
d_0 = \Delta\theta D, \quad (4.15)
\]

where \( d_0 \) is called the **Crowther resolution** of the reconstruction [CDK70, Hop69]. Eq. (4.15) can also be written as

\[
N = \frac{\pi}{\Delta\theta} = \frac{\pi D}{d_0}. \quad (4.16)
\]

In this form it specifies the number of projections \( N \) that must be acquired to reach a Crowther resolution \( d_0 \) for a specimen of diameter \( D \).

In the case of projections obtained experimentally, an upper limit can be set for the achievable Crowther resolution. Such projections are always available in a discrete form. The highest spatial frequency that can be represented with a pixel size \( s_{pix} \) is the **Nyquist frequency** \( k_{Ny} = 1/(2 s_{pix}) \), corresponding to a periodicity of two pixels. The ratio of the critical spatial frequency \( k_0 \) to the Nyquist frequency is

\[
k_0 \approx \frac{k_{Ny}}{1} = \frac{1}{2 s_{pix}}.
\]
given by Eq. (4.16) as

\[
\frac{k_0}{k_{Ny}} = \frac{2 s_{pix} N}{\pi D} \approx 0.64 \frac{N}{D_{pix}},
\]

where \(D_{pix}\) is the capillary diameter in projection pixels. Finally, if the object function is to be reconstructed to a Crowther resolution that is equivalent to the Nyquist frequency,

\[
N = \frac{\pi}{2} D_{pix}
\]

projections must be acquired.

The Crowther resolution is not a measure of the size of the structures that are visible in the reconstruction. In the case of the CTCXM experiments described in this work, the central sections corresponding to the experimental projections have Fourier information up to the resolution of the X-ray microscope; therefore, structures with corresponding periodicity are present in the reconstruction, even though the Crowther resolution might be considerably worse than the X-ray microscopic resolution owing to the relatively small number of projections that could be acquired in the experiment.

Rather, the Crowther resolution specifies the highest spatial frequency \(k_0\) of the object function that can be interpolated for all directions of \(k\). For spatial frequencies greater than \(k_0\), the validity of the reconstructed object function depends on the orientation: a spatial modulation with a wave vector \(k\) that lies on one of the central sections provided by the projections has been (indirectly) measured in the experiment and can therefore be considered ‘valid’ in the reconstruction, while for modulations whose wave vector lies between central sections, \(O(k)\) must be interpolated from the values of \(O(k)\) on the central sections.

The shape function describing current experiments is circularly symmetric (cf. Eq. (4.9)). As has been described in section 2.2.1, thin strips of X-ray translucent material may also be used as object holders. The corresponding (idealized) 2D shape function is that of a rectangle; the shape transform is no longer circularly symmetric, but a product of sinc functions:

\[
S(k) = \text{sinc}(s_{k_x} k_x) \text{sinc}(s_{k_y} k_y)
\]

Objects imaged using such strip holders will have a thickness of at most 10 \(\mu m\) to permit image acquisition within reasonable exposure times, but their extent in the plane of the holder will probably be on the order of 20 - 30 \(\mu m\), e.g., if complete cells are imaged. For such a non-quadratic shape function, the scales \(s_{k_x}\) and \(s_{k_y}\) are different. Consequently, the Fourier transform \(\hat{O}(k)\) is convoluted with an elongated shape transform, and the resulting Fourier transform \(O(k)\) of the object function has a characteristic distance \(k_c\) that depends on the direction in \(k\)-space. The angular sampling scheme of a constant angular increment (cf. Eq. (4.12)) is therefore no longer optimal. Several schemes have been proposed for variable angular increment, all of them decreasing the increment for high tilt angles. Most widely adapted has been the scheme proposed by Saxton et al. [SBH84] and modified by Grimm et al. [GSR+98], where the tilt angle varies as

\[
\theta_{n+1} = \theta_n + \arcsin(\sin(\Delta \theta) \cos \theta_n).
\]
4.2 Reconstruction techniques

In the preceding section, principles underlying the reconstruction of an unknown function from its line integrals have been presented. In the case of CTCXM and the cylindrical symmetry of the current experimental setup, the three-dimensional reconstruction problem is reduced to the reconstruction of an unknown two-dimensional function $f(x, y)$ in a reconstruction plane perpendicular to the axis of object rotation, and assembling the 3D function by stacking the reconstruction planes on top of each other. The reconstruction algorithms presented below are therefore described for a two-dimensional geometry.

Direct implementation of the central slice theorem is equivalent to populating the Fourier plane of the unknown function by interpolating between the central sections given by Fourier transforms of the projections. While reconstruction algorithms working in Fourier space have been implemented [Rad92], they are computationally expensive due to the necessary interpolation in Fourier space. Restricting the area contributing to an interpolated Fourier component by means of a moving window reduces the computational cost [LBS93, LB94], however the results are found to be identical to those produced by filtered backprojection (see below).

In addition to the above-mentioned Fourier methods, there exists a class of algorithms that reconstruct the object function in real space. Filtered backprojection is one such algorithm, and it is also the most widely used reconstruction algorithm. A further class of algorithms is known as iterative algebraic reconstruction methods. The CTCXM specimens investigated in this work were reconstructed with such an iterative algorithm, MART. Filtered backprojection and the MART algorithm are presented in the following sections.

4.2.1 Filtered backprojection (FBP)

To derive the filtered backprojection algorithm (FBP, [RL71, RK82]), also known as weighted backprojection, consider the unknown function $f(x, y)$ expressed in terms of its Fourier transform (cf. Eq. (4.3)):

$$f(x, y) = \int_{-\infty}^{\infty} \int_{-\infty}^{\infty} F(k_x, k_y) e^{2\pi i (k_x x + k_y y)} dk_x dk_y$$

(4.21)

The cartesian coordinates $(k_x, k_y)$ can be expressed in terms of polar coordinates $(k, \theta)$ as

$$k_x = k \cos \theta$$

and $$k_y = k \sin \theta,$$

(4.22)

so that with $dk_x dk_y = k dk d\theta$, Eq. (4.21) becomes

$$f(x, y) = \int_0^{2\pi} \int_0^{\infty} dk F(k, \theta) e^{2\pi i k (x \cos \theta + y \sin \theta)}.$$  

(4.23)

This integral can also be evaluated by choosing the integration ranges as $k \in [-\infty, \infty]$ and $\theta \in [0, \pi]$. In this case, $dk_x dk_y = |k| dk d\theta$, and by substituting
4.2 Reconstruction techniques

\[ x' = x \cos \theta + y \sin \theta \]

Eq. (4.23) becomes:

\[
f(x, y) = \int_{0}^{\pi} \int_{-\infty}^{\infty} dk \, |k| \, F(k, \theta) e^{2\pi i kx'}
= \int_{0}^{\pi} \int_{0}^{\infty} \tilde{F}_k^{-1}(|k| F(k, \theta))(x')
\]

(4.24)

According to the central slice theorem (cf. Eq. (4.6)),

\[
F(k, \theta) = \tilde{F}_{x'}(p_{\theta}(x'))(k),
\]

(4.25)

where \( p_{\theta}(x') \) is the projection of \( f(x, y) \) for the projection angle \( \theta \). Resubstituting \( x' \) in Eq. (4.24) yields the filtered backprojection algorithm:

\[
f(x, y) = \int_{0}^{\pi} \int_{0}^{\infty} \tilde{F}_k^{-1}(|k| \tilde{F}_{x'}(p_{\theta}(x')))(x \cos \theta + y \sin \theta)
\]

(4.26)

Thus, the high-pass filtering step of FBP consists of Fourier transformation of the projections \( p_{\theta}(x') \), multiplication with \(|k|\), and inverse Fourier transformation. In the second step, the filtered projections \( p_{\theta}^*(x') \) are backprojected, i.e., they are ‘smeared out’ into a 2D body by translation into the direction normal to the projection. Mathematically, a point \((x, y)\) is projected for all angles \( \theta \in [0, \pi] \) using \( x' = x \cos \theta + y \sin \theta \), and the corresponding values of the filtered projections are integrated over the angular range.

Reconstruction on a quadratic grid

The mathematical model of the filtered backprojection algorithm (cf. Eq. (4.26)) describes the reconstruction of a continuous function \( f(x, y) \) from continuous, high-pass filtered projections \( p_{\theta}^*(x') \) of the function. However, projections obtained in a CT experiment are usually only available as a discrete set of values. In the case of CTCXM, the projections are obtained from X-ray microscopic images which have been acquired with a CCD camera and consist of a discrete set of pixel values (cf. section 2.1).

In order to apply a reconstruction method to the measurements acquired during a CT experiment, the reconstruction algorithm is implemented in software and executed on a computer to perform the necessary calculations, either running on general-purpose hardware such as a modern PC or workstation, or on dedicated hardware that is adapted to the specific calculation task. In either case the function is reconstructed in discrete form. While for filtered backprojection discretization is necessary to enable computational reconstruction of the underlying continuous function, iterative algebraic reconstruction methods such as MART inherently operate on discrete functions (cf. section 4.2.2).

Regardless of the actual reconstruction algorithm used, the cartesian coordinate system is chosen so that the axis of object rotation coincides with the z-axis, and the local linear absorption coefficient \( \mu(x) \) is reconstructed in two-dimensional reconstruction planes perpendicular to the z-axis. A reconstruction plane is therefore characterized by its z-coordinate \( z_{\text{plane}} \). The continuous function \( \mu(x, y, z_{\text{plane}}) \) is
reconstructed on a quadratic $M \times M$ grid, i.e., for values $\mu_j$ at the locations $(x_j, y_j)$ with $j = 1, \ldots, M^2$ (cf. Fig. 4.3). The dimensionless values $\mu_j$ correspond to the product of $\mu(x_j, y_j, z_{\text{plane}})$, with a dimension of $1 / \text{length}$, and the size of the unit cell, with a dimension of length. The unit cell of the grid together with the spacing of the two-dimensional reconstruction planes along the $z$-axis define the volume element (‘voxel’) that is associated with the value $\mu_j$.

When the locations $(x_j, y_j)$ are projected under the angle $\theta$ for FBP reconstruction, the corresponding projection coordinate $x' = x_j \cos \theta + y_j \sin \theta$ is usually not
an integer; the high-pass filtered projection \( p^*_{\theta} (x') \) must then be interpolated. In practical tests, nearest neighbour interpolation has been found insufficient, while linear interpolation produces acceptable results [Row79].

Assuming that there are \( N \) projections acquired under projection angles \( \theta_n, n = 1, \ldots, N \), the local linear absorption coefficient \( \mu_j \) of voxel \( j \) is calculated by modifying Eq. (4.26) to account for discretization of the reconstruction plane and of the angular range:

\[
\mu_j(x_j, y_j) = \sum_{n=1}^{N} p^*_{\theta_n}(x_j \cos \theta_n + y_j \sin \theta_n),
\]

(4.27)

where \( p^*_{\theta_n}(x') \) has to be interpolated from the \( N \times M \) discrete integral absorption values \( p_i, i = 1, \ldots, N \times M \), that make up the projections associated with one reconstruction plane. In practice, the projections are first high-pass filtered using a one-dimensional fast Fourier transform algorithm [FJ98], and then linearly interpolated.

While Eq. (4.26) is still a mathematically correct expression for \( f(x, y) \) and contains no approximation, the summation in Eq. (4.27) is necessarily restricted to the tilt angles \( \theta_n \) available from the experiment. This means that the (discrete) two-dimensional Fourier transform of \( \mu_j \) resembles the star pattern shown in Fig. 4.1 (b). Disregarding noise created by the limited accuracy of the interpolation of \( p^*_{\theta_n}(x') \), the 2D Fourier transform contains only the central sections available from the experimental projections via Eq. (4.25), and all other Fourier components are set to zero. Especially, the finite extent of the object function and the corresponding smoothness of its Fourier transform (cf. section 4.1.2) are not used to interpolate the missing Fourier components.

### 4.2.2 Multiplicative algebraic reconstruction (MART)

At the moment, CTCXM experiments are characterized by a significant mismatch between the number of projections that can be acquired of a specimen, and the spatial resolution at which the specimens can be imaged using the X-ray microscope. This mismatch results in a low Crowther resolution of the reconstructions compared to the Rayleigh resolution of the images. As a consequence, the reconstruction quality depends critically on the way that the missing Fourier components are interpolated. By using other reconstruction schemes than filtered backprojection (such as MART), the reconstruction quality can be improved significantly both qualitatively and quantitatively.

In the experiments conducted so far at the electron storage ring BESSY I, the number of tilt images that could be acquired of a specimen was limited by the manual operation of the microscope (specifically, by having to move the stage and refocus the specimen for each new tilt angle, cf. chapter 2). At the moment, a typical tilt series of X-ray microscopic images comprises 40 - 50 viewing angles, spanning an angular range of 180° (cf. chapter 5).

For the CTCXM experiments described in this work, a typical imaging magnification of 1740× - 1850× was used. With a CCD pixel size of 24 μm, the pixel size in the object plane is approx. 13 - 13.8 nm. After the microscopic images have been reduced in size by a factor of 2 (cf. section 3.2.2), the pixel size is approx. 25.9 - 27.6 nm. The outer diameter of a capillary holder is approx. 9 μm, so the projection...
4.2 Reconstruction techniques

width is between $9000 \text{ nm} / 27.6 \text{ nm} = 326$ and $9000 \text{ nm} / 25.9 \text{ nm} = 347$ pixels. Using Eq. (4.17), the inverse Crowther resolution is between 0.07 - 0.1 of the Nyquist frequency of the images. Of the Fourier plane region that contains object information (the resolution domain), less than one percent is therefore adequately sampled by the star pattern of central sections (cf. Fig. 4.1 (b)) to permit interpolation of the underlying function for all directions of $k$.

Since the experimentally measured Fourier components are distributed sparsely in the Fourier plane, the reconstruction is strongly influenced by the way that the reconstruction algorithm interpolates missing Fourier components. For this kind of problem, the multiplicative algebraic reconstruction technique (MART) has been shown to perform better than filtered backprojection [Ver93], and has been used for computed tomography of X-ray microscopic specimens (the mineral sheaths of bacteria *Leptothrix ochracea*) by Lehr [Leh97b], implementing the algorithm suggested in [Ver93].

MART is one of a class of iterative algebraic reconstruction methods. The unknown function is reconstructed in a series of iterative steps. Different from analytical algorithms such as FBP, MART inherently operates on a discrete function. The local linear absorption coefficient is reconstructed as values $\mu_j$, $j = 1, \ldots, M^2$, on a quadratic $M \times M$ grid. Available from the experiment are $N$ one-dimensional projections for each reconstruction plane, with $M$ pixels per projection\(^1\). For each projection pixel $p_i$, $i = 1, \ldots, N \times M$, a ‘projection ray’ issuing from the projection pixel is traced through the reconstruction plane (cf. Fig. 4.3). A weight $w_{i,j}$ is assigned to each voxel $j$, specifying the amount with which that voxel contributes to projection ray $i$ (see below).

For each projection ray, the product of voxel weight and voxel value is summed along the ray, yielding a linear equation in $\mu_j$:

$$
\begin{align*}
p_1 &= w_{1,1} \mu_1 + w_{1,2} \mu_2 + \cdots + w_{1,M^2} \mu_{M^2} \\
p_2 &= w_{2,1} \mu_1 + w_{2,2} \mu_2 + \cdots + w_{2,M^2} \mu_{M^2} \\
\vdots &
\end{align*}
$$

\(4.28\)

This system of linear equations can be written in matrix notation as

$$
p = W\mu,
$$

\(4.29\)

with the vector of projections $p = (p_i)$, the vector of absorption coefficients $\mu = (\mu_i)$, and the matrix of vector weights $W = (w_{i,j})$.

To obtain the unknown absorption coefficients, Eq. (4.29) must be inverted. An inverse matrix $W^{-1}$ exists only if the determinant $\text{det}(W) \neq 0$ [Fis86]. In this case, the reconstruction problem could be formally solved by matrix inversion [CDK70]:

$$
\mu = W^{-1}p
$$

\(4.30\)

Classical approaches to matrix inversion are prohibited by the sheer size of $W$: since the projections of a glass capillary have a width of $M = 330 - 350$ pixels (see above), there are $108900 - 122500$ voxels to a reconstruction plane, and with $N = 40$

\(^1\)Note that FBP uses the high-pass filtered projections $p_0^*(x')$, and MART the unfiltered projections $p_0(x')$. 

- 50 projections, the \((N \times M) \times M^2\) matrix \(W\) has \(1.44 \times 10^9\) - \(2.14 \times 10^9\) elements. Only a small fraction (approx. \(1/M\)) of all voxels contribute to a given projection ray, so \(W\) is a sparse matrix. Still, even storing only the non-zero elements \(w_{i,j} \neq 0\) would require a very large amount of storage space.

Instead, the solution to Eq. (4.30) is calculated in a series of iterations, numbered with the superscript \(q \in \mathbb{N}\). To initialize the iterative process, an initial estimate \(\mu_1^q\) is formed by assigning all voxels a constant, nonnegative value:

\[
\mu_1^q = \mu_0 \quad \text{for } j = 1, \ldots, M^2, \tag{4.31}
\]

where \(\mu_0\) is the average voxel value, obtained by taking the average value of a projection pixel and dividing by the average length of a projection ray:

\[
\mu_0 = \frac{1}{NM^2} \sum_{i=1}^{N \times M} p_i \tag{4.32}
\]

For one iteration of the algorithm, the current estimate \(\mu_j^q\) of the absorption coefficients is projected according to the projection geometry, i.e., the integral absorption along each projection ray \(i\) is calculated as

\[
g_i^q = \sum_{j=1}^{M^2} w_{i,j} \mu_j^q. \tag{4.33}
\]

This calculated line integral of \(\mu_j^q\) along ray \(i\) is compared to the measured line integral along the ray, which is given by the projection pixel \(p_i\), and the voxel values \(\mu_j\) contributing to the ray are accordingly changed.

In the algebraic reconstruction technique (ART, [Kar37, GBH70]), the discrepancy between \(g_i^q\) and \(p_i\) is subtracted from the object estimate along the projection ray, so that perfect agreement is achieved for the particular projection direction considered. In the simultaneous iterative reconstruction technique (SIRT, [Gil72]), the discrepancies of all projections are simultaneously corrected.

MART calculates a multiplicative correction factor for each voxel:

\[
c_{i,j}^q = \begin{cases} 
1 - \varepsilon (w_{i,j}/w_{i,j}^{\max})(1 - p_i/g_i^q) & \text{for } g_i^q \neq 0, \\
1 & \text{otherwise,}
\end{cases} \tag{4.34}
\]

where \(w_{i,j}^{\max}\) is the maximum of all weights \(w_{i,j}\) contributing to ray \(i\) and \(\varepsilon\) is a relaxation parameter. After the correction factors have been calculated for all voxels contributing to a ray, these voxels are instantly corrected using

\[
\mu_j^{q+1} = \mu_j^q \cdot c_{i,j}^q. \tag{4.35}
\]

For one iteration of the algorithm, the line integral along each of the \(N \times M\) projection rays is calculated for the current estimate \(\mu_j^q\) using Eq. (4.33), and the voxels contributing to the projection ray are instantly multiplicatively corrected using Eq. (4.34).

**Assigning the voxel weights contributing to a projection ray**

There are several possibilities for assigning the weight \(w_{i,j}\) with which voxel \(j\) contributes to projection ray \(i\). Lehr defined a set of equidistant points on the projection ray, with a spacing equal to the grid spacing. For each such point, weights \(w_{i,j}\) were assigned to the 4 neighbouring voxels using bilinear interpolation [Leh97b].
The task of assigning voxel weights to form a projection ray is equivalent to rendering a line of finite width on a regular grid. This is a common task in computer graphics, known as drawing an anti-aliased line. Of the several methods that can be used for this purpose, in this work the method of weighted area sampling was implemented [FvDFH96]. It is based on the following consideration: the projection ray can be characterized by a line equation and a non-zero line width (cf. Fig. 4.3). The ray profile perpendicular to the ray direction is assumed to be rectangular and to have unit width. These specifications define a two-dimensional ray function in the reconstruction plane, with function value 1 ‘on’ the ray, and 0 elsewhere.

This ray function is to be sampled at the voxel centers, i.e., with a finite sampling interval equal to the grid spacing. In order to fulfill the sampling theorem (cf. section 3.2), the ray function has to be low-pass filtered prior to sampling, with a cut-off frequency equal to the Nyquist frequency of the sampling. This can be accomplished in real space by convolving the ray function with a suitable smoothing function (either a product of sinc functions or a first-order Bessel function divided by its argument, cf. Eq. (3.58)), and sampling the resulting function at the voxel centers. However, because the resulting function has to be evaluated only at the voxel centers, in order to calculate the voxel weight for one voxel, it is sufficient to integrate the product of the ray function and the smoothing function centered on the voxel center. The smoothing function is therefore used as a weighing function for integration of the ray function, and the voxel weight is calculated in the same manner as the interpolated function value in section 3.2.

In section 3.2, an interpolated function value was computed as a weighted sum over the set of discrete pixel locations in the vicinity of the interpolation location. Here, the ray function is continuous, and integration must be used instead of summation. However, the ray function assumes only two values, 0 and 1. The integration must therefore be extended only over the support of the ray function. Because the function value in this region is constant (equal to 1), the product of ray function and weighting function is equal to the weighting function, and the resulting integration is performed over that part of the weighting function that lies on the projection ray (cf. Fig. 4.4).

The original weighting function is the Fourier transform of the two-dimensional low-pass filter function. It has infinite support, i.e., every voxel contributes to every projection ray. To make the calculation of Eq. (4.33) computationally feasible, the original weighting function is approximated by a weighting function with finite support. For the experiments described in this work, a cone function (cf. Fig. 4.4) with a finite support radius equal to the grid spacing was used. The cone weighting function constitutes a reasonable compromise between computational cost and quality [FvDFH96].

The voxel weight $w_{i,j}$ is calculated as the ratio $W_S / W$ of the subvolume $W_S$ of the cone weighting function that lies on the projection ray, to the complete volume $W$ of the cone weighting function. Because the cone weighting function is circularly symmetric, the voxel weight depends only on the distance from the voxel center to the projection ray. For a given projection ray width (usually one pixel) and weighting function (in addition to the cone function, square as well as Gaussian-shaped weighing functions have been used [FvDFH96]), the voxel weights can therefore be precomputed as a function of distance, and fast voxel weight assignment can be performed using a look-up table.
4.3 Resolution measures

Once the CTCXM specimens have been reconstructed, it is important to assess the reconstruction quality. This can be done qualitatively by visualizing single voxel planes $\mu_j(x_j, y_j, z_{\text{plane}})$ with constant $x_j$, $y_j$ or $z_{\text{plane}}$, i.e., by ‘slicing’ the reconstructed volume parallel to one of the coordinate axes. The local absorption coefficients can be displayed as lighter and darker shades of gray, and the resulting ‘image’ can be compared to the X-ray microscopic images underlying the reconstruction. Since the reconstruction takes place in planes perpendicular to the axis of object rotation (with constant $z_{\text{plane}}$), it is appropriate to evaluate these reconstruction planes to assess the reconstruction quality (see below).

In evaluating the reconstruction planes, it is important to distinguish between two different aspects of the visible structures, namely their ‘sharpness’ and their ‘validity’. Sharp structures are associated with high spatial frequencies, and in an X-ray image acquired using absorption contrast, the sharpness is determined by the X-ray microscopic resolution (cf. section 1.2). While the modulation transfer function of the X-ray microscope attenuates high spatial frequencies, all spatial frequencies up to the resolution limit are transmitted to some degree, and hence, all image structures in the X-ray microscopic image are based on corresponding structures in the object. As has been shown in section 4.1.2, the sharpness of the object structures in the reconstruction is essentially equal to the sharpness of the structures in the images, and is only degraded by misalignment of the projections.

The ‘validity’ of the object structures, however, is determined by the Crowther
resolution of the reconstruction. While the sharpness of object structures can be easily assessed qualitatively, the validity of object structures is not immediately apparent from the reconstruction plane. In this context, it is customary to speak of ‘artifacts’ in the reconstruction. This appellation is somewhat unfortunate, because the name artifact (Latin \textit{arte factum}, “made by art”) implies the introduction of additional ‘artifical’ structures into the reconstruction that have no counterpart in the real object. However, as has been shown in section 4.2.1, especially for filtered backprojection most artifacts arise from the \textit{absence} of object information in the reconstruction. The FBP artifacts are due to the missing Fourier components that lie between central sections.

Since the reconstruction process is naturally described in Fourier space, it is clear that a quantitative resolution measure taking into account the validity of object structures might also be based upon some evaluation of the Fourier transform of a reconstruction plane. In the following, two such resolution measures are presented and applied to CTCXM experimental data.

### 4.3.1 Differential phase residual (DPR)

The differential phase residual (DPR, [Cro71, FVB81, Fra96]) as well as the Fourier shell correlation described in the following section both make use of the discrete Fourier transform of the reconstructed object function (here: the local linear absorption coefficient) to define a resolution measure. The DPR resolution is defined as the highest spatial frequency of the reconstructed object function for which missing Fourier components are still interpolated \textit{consistently} by the reconstruction algorithm. In this way it is similar to the Crowther resolution which specifies to what spatial frequency Fourier components can be interpolated, taking into account the boundedness of the object function. However, instead of estimating this spatial frequency from the number of projection angles and the diameter of the object (cf. Eq. (4.17)), DPR provides a direct measurement of the \textit{phase consistency} of the reconstructed function.

By applying a reconstruction algorithm to the aligned tilt series of projections, the reconstructed object function is obtained for a given reconstruction plane. This reconstructed object function can be Fourier transformed using FFT techniques [FJ98]. If only one Fourier transform is available, there is no way of measuring the phase consistency of the Fourier components. To overcome this problem, two ‘independent’ reconstructions are created by partitioning the aligned tilt series of projections into two subsets of equal size, and reconstructing the object function from each subset. It is clear that the Crowther resolution of each of these reconstructions, based on half of the available projections, is only half that of the original reconstruction; this has to be taken into account when the resulting DPR plot (see below) is interpreted.

Since the DPR is a measure of correlation between the reconstructions from the two projection subsets, it is of crucial importance that no correlations are unwittingly introduced when the tilt series is partitioned. Partitioning the tilt series into a set of even- and a set of odd-numbered images avoids the introduction of such correlations. Each projection subset is used to reconstruct the local linear absorption coefficient, leading to the reconstruction planes $\mu_1(x)$ and $\mu_2(x)$ (with $x$ on the grid given by $(x_i, y_j, z_{plane})$). Let $F_1(k)$ and $F_2(k)$ be the discrete Fourier transforms of the two reconstruction planes, with the spatial frequency $k$ assuming all values on the
regular Fourier grid \((k_x, k_y)\) within the Nyquist range. The complex Fourier components can be written as \(F_1(k) = A_1(k)e^{i\phi_1(k)}\) and \(F_2(k) = A_2(k)e^{i\phi_2(k)}\). The phase difference is then given by
\[
\Delta \phi(k) = \phi_2(k) - \phi_1(k),
\]
and the differential phase residual is defined as
\[
\bar{\Delta} \phi(k, \Delta k) = \frac{\sum_{|k|,|\Delta k|} (\Delta \phi(k))^2 (|F_1(k)|^2 + |F_2(k)|^2)}{\sum_{|k|,|\Delta k|} (|F_1(k)|^2 + |F_2(k)|^2)}^{1/2}.
\]

The sums are computed over Fourier components falling within circular rings in 2D Fourier space. These rings are defined by an inner spatial frequency radius \(k_1 = |k|\) and an outer radius \(k_2 = k_1 + \Delta k\). Thus, for any spatial frequency, \(\bar{\Delta} \phi\) gives a measure of the (amplitude-weighted) phase consistency. In principle, as in the case of the Fourier shell correlation below, the entire curve of \(\bar{\Delta} \phi(k, \Delta k)\) for all values of \(k\) is needed to characterize the degree of consistency between the two reconstructions. However, it is convenient to use a single figure, \(k_{45}\), for which \(\bar{\Delta} \phi(k_{45}, \Delta k) = 45^\circ\). As a conceptual justification for the choice of this value, consider the effect of superimposing two sine waves with a phase difference \(\Delta \phi\). If \(\Delta \phi\) is significantly smaller than \(45^\circ\), the waves interfere constructively, whereas for \(\Delta \phi > 45^\circ\), destructive interference begins to occur.

Since good agreement between the two Fourier transforms \(F_1(k)\) and \(F_2(k)\) means a small differential phase residual (for identical reconstruction planes it is equal to zero), the expected graph of \(\bar{\Delta} \phi(k, \Delta k)\) has small values for low spatial frequencies, and increasing values for higher spatial frequencies.

### 4.3.2 Fourier ring correlation (FRC)

*Fourier ring correlation* (FRC, [SB82], also known as Fourier shell correlation (FSC) in 3D), is similar in concept to the differential phase residual as it is based on a comparison of two Fourier transforms. As for DPR, the tilt series is partitioned into two subsets and the object function is reconstructed from each subset. The Fourier ring correlation is also evaluated by summing over circular rings in 2D Fourier space. Instead of the phase consistency, however, the correlation of \(F_1(k)\) and \(F_2(k)\) is averaged:
\[
\text{FRC}(k, \Delta k) = \frac{\sum_{|k|,|\Delta k|} F_1(k)F_2^*(k)}{\left(\sum_{|k|,|\Delta k|} |F_1(k)|^2 \sum_{|k|,|\Delta k|} |F_2(k)|^2\right)^{1/2}}.
\]

where \(F_2^*(k)\) denotes the complex conjugate of \(F_2(k)\).

To establish a resolution criterion, the FRC of the reconstruction (as determined by Eq. (4.38)) is compared to the correlation expected for pure noise, \(c/(N_k,\Delta k)^{1/2}\), where \(N_k,\Delta k\) denotes the number of Fourier coefficients in the circular ring with inner radius \(k\) and thickness \(\Delta k\), and \(c\) is a safe multiple, originally chosen to be \(c = 2\) [SB82], although a value of \(c = 3\) appears to give better agreement between FRC and DPR [Fra96]. Using \(c = 2\) is equivalent to a signal-to-noise ratio of \(\text{SNR} = 0.2\), while the DPR resolution criterion of \(\bar{\Delta} \phi(k_{45}, \Delta k) = 45^\circ\) is equivalent to \(\text{SNR} = 1\) [Rad88].

The FRC graph shows inverse behaviour to that of DPR. High correlation occurs at low spatial frequencies, resulting in FRC values close to 1, while smaller FRC values for higher spatial frequencies indicate decreasing correlation.
4.3.3 DPR/FRC evaluation of CTCXM data

In the following, the two resolution measures are applied to a CTCXM-reconstructed specimen of the green alga *Chlamydomonas reinhardtii* (cf. chapter 5). This specimen was chosen because the associated tilt series has the highest image quality of any acquired in this work. Tilt images were acquired for 42 tilt angles spanning 185°. To improve the signal-to-noise ratio, three images were acquired for each tilt angle and added. The specimen had a large number of gold markers in the vicinity (cf. Fig. 5.4); 18 of these markers were used to align the tilt series (cf. section 3.1). The results obtained for this specimen can therefore be considered representative of what CTCXM is capable of at the moment.

The DPR and FRC resolution can be compared to the Crowther resolution of the specimen. After reduction in size by a factor of 2, the outer diameter of the glass capillary holder used for the C. reinhardtii specimen was approx. 315 pixels, with a pixel size of 27.6 nm. With a tilt angle increment of 4.52° ≡ 0.079 radians, the inverse Crowther resolution given by Eq. (4.17) is therefore 0.08 of the Nyquist frequency, or 1.46 μm⁻¹, corresponding to a structure width of 342 nm.

The complete specimen was reconstructed using filtered backprojection (FBP), with bilinear interpolation of the high-pass filtered projections, and the multiplicative algebraic reconstruction technique (MART), with 5 iterations and a relaxation parameter ε = 0.3. The number of iterations and the value of the relaxation parameter are not the result of an optimization process (e.g., an exhaustive search in parameter space for maximum resolution as measured by the DPR/FRC criteria). Instead, they are based on previous computed-tomography experiments [Leh97b, Wol97]. Using numerical simulations, Wolf showed that with the MART algorithm only a small number of iterations is needed for convergence, and a relatively low relaxation parameter (only 30% of the computed changes is actually applied to the voxel values) tends to reduce noise in the reconstruction [Wol97].

After computed-tomography reconstruction of the linear absorption coefficient of the specimen, two reconstruction planes were selected; plane A lies at the upper end of the specimen and has little object detail (cf. Fig. 4.5), while plane B cuts midway through the specimen and shows parts of the chloroplast and pyrenoid (cf. Fig. 4.6). In this way, any influence of the object structure on the resolution as measured by DPR and FRC will be detected.

The DPR and FRC graphs of reconstruction plane A are plotted in Fig. 4.7. The graphs plotted for the FBP reconstruction allow easy interpretation: starting at approx. 5°, the DPR graph rises steeply and reaches 45° at 0.9 μm⁻¹, while the FRC graph begins with complete correlation and fulfills the FRC criterion at 1.1 μm⁻¹, resulting in a resolution of approx. 1 μm⁻¹. The resolution of the FBP reconstruction thus lies below the inverse Crowther resolution of 1.46 μm⁻¹.

The graphs plotted for the MART reconstruction show substantial phase consistency and correlation up to 4 μm⁻¹. The FRC graph shows correlation of approx. 30% up to the Nyquist frequency, making the application of the resolution criterion more difficult. However, strictly applying the two resolution criteria defined above yields resolution values of 2 μm⁻¹ for DPR and 4.6 μm⁻¹ for FRC. The consistency of Fourier component interpolation in the MART algorithm therefore exceeds the inverse Crowther resolution.

Corresponding graphs for reconstruction plane B are plotted in Fig. 4.8. The FRC resolution criterion yields the same values as for reconstruction plane A. How-
Figure 4.5: Single reconstruction plane A (398x398 pixels) of a specimen of *Chlamydomonas reinhardtii* inside an ice-filled glass capillary, reconstructed using (a) filtered backprojection (FBP) with bilinear interpolation, and (b) the multiplicative algebraic reconstruction technique (MART), with 5 iterations and a relaxation parameter $\varepsilon = 0.3$. Of the specimen, only parts of the cell wall are visible. Attached to the upper half of the glass wall is a gold marker with high absorption. Note that the visible reconstruction noise as well as the streaking artifacts generated by the gold marker are more prevalent in the FBP reconstruction.

Figure 4.6: Single reconstruction plane B of *C. reinhardtii*, reconstructed as in Fig. 4.5. The reconstruction plane contains specimen details such as the fibrous chloroplast and, embedded in it, the approximately spherical pyrenoid. As in Fig. 4.5, the visual quality of the MART reconstruction exceeds that of the FBP reconstruction as regards noise and streaking artifacts.
Figure 4.7: Differential phase residual $\Delta \phi(k, \Delta k)$ (DPR, cf. Eq. (4.37)) and Fourier ring correlation $FRC(k, \Delta k)$ (FRC, cf. Eq. (4.38)) of reconstruction plane A (cf. Fig. 4.5). The Fourier ring thickness $\Delta k$ was chosen so that the 2D Fourier plane is divided into 100 rings. The Nyquist frequency associated with the projection pixel size of 27.6 nm is 18.1 $\mu$m$^{-1}$; the $k$-axis range goes up to 10 $\mu$m$^{-1}$. The inverse Crowther resolution $k_0 = 1.46$ $\mu$m$^{-1}$ is indicated. The DPR gives a resolution of 0.9 $\mu$m$^{-1}$ for the FBP reconstruction and of 2 $\mu$m$^{-1}$ for MART, while FRC gives 1.1 $\mu$m$^{-1}$ for FBP and 4.6 $\mu$m$^{-1}$ for MART (with the noise correlation multiple chosen to be $c = 3$). The quantitative criteria bear out the qualitative assessment of the two reconstruction techniques, with MART providing better resolution by a factor of 2 (DPR) and 4 (FRC).

However, for the MART reconstruction the degree of correlation at higher spatial frequencies is decreased, and consequently, so are the values of the DPR and FRC resolution. Since the main difference between planes A and B is the amount of object matter present, the reduced correlation appears to be a consequence of the increased object complexity.

To obtain an average specimen resolution for all CTCXM experiments performed so far, the three specimens described in chapter 5 were evaluated using the DPR criterion (which corresponds to a higher signal-to-noise ratio than the FRC criterion, cf. section 4.3.2). For each specimen, DPR graphs were calculated for all reconstruction planes. The DPR graphs were averaged and the spatial frequency $k_{45}$ corresponding to the DPR resolution criterion $\Delta \phi(k_{45}, \Delta k) = 45^\circ$ was determined from the average DPR graph. Table 4.1 shows that the DPR resolution somewhat...
Figure 4.8: Differential phase residual (DPR) and Fourier ring correlation (FRC) of reconstruction plane B (cf. Fig. 4.6), with parameters as in Fig. 4.7. The FBP reconstruction yields the same results as in plane A (0.9 µm\(^{-1}\) for DPR and 1.1 µm\(^{-1}\) for FRC). For the MART reconstruction, the degree of correlation at higher spatial frequencies has been reduced in comparison to plane A, possibly due to the increased presence of object detail, and the resulting measures are 1.9 µm\(^{-1}\) for DPR and 2.5 µm\(^{-1}\) for FRC.

Table 4.1: Specimen diameter D, inverse Crowther resolution \(k_0 = 1/d_0\), and average DPR resolution \(k_{45}\) of the three reconstructed CTCXM specimens. The Crowther resolution \(d_0\) was calculated using Eq. (4.15) with a common tilt angle increment \(\Delta \theta = 4.52^\circ\).

<table>
<thead>
<tr>
<th>specimen</th>
<th>D [µm]</th>
<th>(k_0) [µm(^{-1})]</th>
<th>(k_{45}) [µm(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN(_2) frozen alga</td>
<td>8.7</td>
<td>1.5</td>
<td>2.0</td>
</tr>
<tr>
<td>liquid-ethane frozen alga</td>
<td>7.1</td>
<td>1.8</td>
<td>2.1</td>
</tr>
<tr>
<td>D. cell nuclei</td>
<td>8.15</td>
<td>1.6</td>
<td>1.8</td>
</tr>
</tbody>
</table>

exceeds the inverse Crowther resolution for all three specimens. The average DPR resolution of the specimens is approx. 2 µm\(^{-1}\), corresponding to a periodicity of 500 nm or a structure width of 250 nm.
Chapter 5

Experiments with biological specimens

5.1 *Chlamydomonas reinhardtii*

In the preceding chapters, the method of computed tomography based on cryo X-ray microscopic images (CTCXM) was presented. As the first biological specimen for CTCXM, the unicellular green alga *Chlamydomonas reinhardtii* was chosen. *C. reinhardtii* is a well-studied organism of considerable biological interest. It lends itself well to CTCXM for several reasons:

- With a typical adult cell diameter of 8 - 10 µm [Har88], a complete, ice-embedded alga can be imaged at 2.4 nm wavelength with a typical exposure time of a few seconds. Because the alga is approximately spherical with a diameter of 10 µm, it fits into a capillary holder and a tilt series spanning 180° can be acquired, with each tilt image showing the complete specimen. This is a necessary requirement if reconstruction artifacts due to a limited angular range or inconsistent absorption data are to be avoided (cf. chapter 4).

- *C. reinhardtii* has already been investigated with the CCTXM at BESSY I [Sch98], and high quality X-ray microscopic images of specimens in conventional, non-tilt holders are available (Fig. 5.1). These images show that while the organelles of *C. reinhardtii* are sufficiently X-ray absorbing to provide high image contrast, no parts of the alga are X-ray opaque. As a whole, the alga is sufficiently X-ray translucent for the image intensity (and the intensity-derived integral object absorption) to have a high signal-to-noise ratio even in the darkest regions.

- As a single-cell organism, *C. reinhardtii* consists of several distinctive organelles which can be identified in the X-ray microscopic images and in the CTCXM reconstruction of the local linear absorption coefficient (Fig. 5.2). The characteristic size of the organelles spans a broad range: from several micrometers (the pyrenoid) to a few nanometers for the fibrous strands of the chloroplast (cf. Fig. 5.7) or the internal structure of the flagella. The CTCXM resolution can therefore be estimated by examining reconstructed specimen details of different sizes.

- Like other green algae, *C. reinhardtii* has a thick cell wall and thrives in many aquatic environments [Har88]. For example, live specimens can be added to
Figure 5.1: An ice-embedded specimen of the green alga *Chlamydomonas reinhardtii*. The alga was rapidly cooled by plunging into liquid ethane, and the image was acquired using the amplitude contrast mode of the CTXM, with an exposure time of 5 s and an accumulated dose in ice of about $4 \times 10^6$ Gy. Of the specimen organelles, the flagella (F), the flagellar roots (FR), several spherical vesicles (Ph), the chloroplast (Chl), the pyrenoid (Py), and the cell wall (W) can be identified (cf. Fig. 5.2). The curved outline of the cell wall is unbroken by ice crystals and indicates good vitrification. From [Sch98].

- A suspension of colloidal gold and will continue to live and swim about for hours. This makes it possible to add fiducial markers (the colloidal gold) to a suspension of specimens, transfer the resulting specimen-marker suspension to the capillary holder, and cryo-immobilize the specimens by plunge-freezing.

- *C. reinhardtii* has been the subject of several electron microscopic studies, so that results obtained using CTCXM can be easily verified at a resolution exceeding that of CTCXM. The comparison with electron microscopic images also offers the possibility of discovering preparation-induced artifacts, either in the electron micrographs or in the X-ray microscopic images.

- Several strains of *C. reinhardtii* are part of the Sammlung von Algenkulturen (collection of alga cultures, SAG) at Göttingen, so specimens from an expertly maintained strain could be obtained for X-ray microscopy at short notice.
5.1 Specimen preparation

Specimens of the 11-32b strain (SAG classification, corresponding to classifications CCAP 11/32A and UTEX 90) of *Chlamydomonas reinhardtii* Dangeard were obtained on agar from the Sammlung von Algenkulturen at Göttingen. The specimens were transferred to a petri dish and a suspension of gold colloids (British BioCell International) was added. The spherical gold particles are suspended in water, with a 0.01% concentration of HAuCl$_4$. The mean diameters of the gold particles used were 60 nm (EM.GC60, with $2.6 \times 10^{10}$ particles/ml) and 100 nm (EM.GC100, with $5.6 \times 10^9$ particles/ml).

*C. reinhardtii* is phototropic. After the specimens become motile, they aggregate in that part of the petri dish that is turned towards the main light source. Using a pipette (Eppendorf Reference) with an Eppendorf Microloader pipette tip, a liquid sample of approx. 10 µl was taken from a site of high specimen concentration. The Microloader tip was then inserted into the large-bore end of a reinforced capillary, and the liquid sample was injected into the tapering tip of the capillary. If no specimens are transported to the X-ray transparent part of the capillary, the motility of the *C. reinhardtii* specimens has another advantage: by swimming about ran-
Figure 5.3: Tip of borosilicate glass capillary holder filled with specimens of *C. reinhardtii*, imaged with a light microscope. The blur is due to the movement of the specimens. The large specimen in the center of the image is momentarily at rest; the form of the chloroplast can be discerned, as well as the presence of the flagella.

domly inside the capillary, the specimens will eventually get trapped in the very tip (Fig. 5.3). This process can be accelerated by illuminating the tip with a light source, so that the phototropic algae will swim preferentially towards the tip.

The volume available to the algae inside the capillary is very small, especially when the algae fill the inside in a continuous line (cf. Fig. 5.3). In such a case it might be expected that the water surrounding the algae is quickly depleted of nutrients, eventually killing the specimens. However, experiments have shown that the algae will continue to move inside the capillary for up to one hour. It is not known whether they undergo any morphological changes at the X-ray microscopic resolution during that time, so care was taken to cryo-immobilize the specimens shortly after injection into the capillary.

5.1.2 Liquid-nitrogen frozen specimen

A first CTCXM experiment with *C. reinhardtii* was undertaken to determine the reconstruction quality that could be obtained for this kind of specimen; the exact preservation of the biological structures was considered to be of secondary importance. Therefore, liquid nitrogen (LN$_2$) was used as a convenient primary cryogen.

Specimens of *C. reinhardtii* were added to a 60 nm diameter colloidal gold solution. After the specimens had become motile, a sample was taken and injected into a reinforced borosilicate glass capillary. The capillary was glued into the hollow axle and inserted into the detachable tilt stage (cf. Fig. 2.6). The tilt stage was then immediately plunged by hand into liquid nitrogen and transferred to the cryo object chamber while still immersed in LN$_2$. A suitable specimen was selected from those visible inside the holder, and a tilt series of images was acquired at a magnification of 1740×. The X-ray objective used for imaging was a nickel micro zone plate (Ni 3.4 W, [WPS98]) with an outermost zone width $d_{r_N} = 40$ nm and an initial measured diffraction efficiency of 10%. However, because the micro zone plate had been in use for several months, the diffraction efficiency at the time of
Figure 5.4: LN$_2$-frozen specimen of *C. reinhardtii*, embedded in ice, in a borosilicate glass capillary holder, imaged with the CTXM. The exposure time was 20 s. The numerous small dark spots are the 60 nm diameter colloidal gold markers. The flagella visible on the left does not belong to this specimen.

The CTCXM experiment was probably degraded to 5 - 8%\textsuperscript{1}. The angular increment of the tilt stage was set to 146 steps of the stepper motor, or approx. 4.52°. For each tilt angle, the specimen was placed in the center of the microscopic field of view, and the optimal focus setting was determined by acquiring several low-dose exposures at different focus settings. In order to minimize the specimen dose deposited during adjustment and focusing, these tasks were performed using the binning mode of the CCD chip.

After finding the optimal focus setting, three images were acquired of the specimen for each tilt angle. The exposure time was chosen to avoid oversaturation of

\textsuperscript{1}This efficiency degradation is believed to be the consequence of carbon deposition from the vacuum windows onto the micro zone plate, reducing the overall transmission.
Figure 5.5: Aligned tilt series (a) and reconstructed local linear absorption coefficient (b) of a liquid-nitrogen frozen specimen of *C. reinhardtii*. Click on the images to start the animation.

The CCD chip, i.e., the brightest pixels in the image were filled to capacity. For capillary holders, the largest pixel counts occur just above and below the walls of the capillary (cf. Fig. 5.4). Oversaturation of the camera chip was avoided because it might damage the chip, and because oversaturated areas (which are clamped to the maximum pixel value) cannot be normalized with a flat-field image. Also, while no stage drift was observed during these experiments, very long exposure times accumulate the effect of stage drift and can produce directional blurring.

Tilt images were acquired for 42 tilt views, corresponding to an angular range of 185.1°. A tilt image acquired under 180° can be mirrored in the vertical direction and compared to the tilt image acquired under 0°. In this way, any changes that have occurred during the acquisition process are made visible. Also, for every tilt angle (and thus, for every focus setting), a flat-field image was acquired showing the inhomogeneous background illumination.

The three images belonging to a tilt angle were added and normalized using the corresponding flat-field image. In the resulting images, the positions of 18 different gold markers (on average visible in approx. 50% of the images) were measured with the XALIGN program, and alignment parameters were computed using the algorithm described in section 3.1. The images were interpolated and reduced in size by a factor of 2 to create an aligned tilt series, using the weighted-sum interpolation described in section 3.2 (cf. Fig. 5.5, panel (a)).

The interpolated intensity data was transformed into integral absorption data by taking the negative natural logarithm. The local absorption coefficient was
reconstructed using the MART algorithm (cf. section 4.2.2) with 5 iterations and a relaxation parameter $\varepsilon = 0.3$ (cf. section 4.3.3). Figure 5.6 shows slices through the reconstructed linear absorption coefficient, taken parallel to the axis of object rotation. The absorption coefficient was visualized by mapping low absorption ($\mu \leq 0.07 \, \mu m^{-1}$) to white, and high absorption ($\mu \geq 0.7 \, \mu m^{-1}$) to black, so that the slices resemble conventional two-dimensional X-ray microscopic images.

The slice representation conveys a clear impression of the inner structure of the specimen, much more so than the single X-ray microscopic image. The prominent features of the cell (cell walls, fibrous chloroplast, pyrenoid with starch hull imbedded in the chloroplast, cf. Fig. 5.2) are all known from electron microscopic sections of dried thin sections [Har88]. Another striking feature of the reconstructed volume are several X-ray dense spherical vesicles. The presence of these vesicles in X-ray microscopic images of C. reinhardtii has already been reported [Sch98, SFP+97], but the availability of volumetric data now makes it possible to assess their relative volume (approx. 1.5% of the total alga volume, cf. chapter 6.1). It can also be seen from the slices that these vesicles have the highest linear absorption coefficient of all specimen organelles. Finally, there is evidence of the flagellar roots (Fig. 5.6, bottom of panel (e)), but not of the flagella themselves. They probably became disattatched when the specimen was moving inside the capillary.

While several specimen organelles can be clearly identified in the reconstruction, in place of the nucleus and nucleolus there appears a honeycomb-like structure in the lumen of the chloroplast. This structure was concluded to be an artifact of imperfect vitrification, produced by using liquid nitrogen as a primary cryogen. Because of the inferior cooling properties of this cryogen, local cooling rates at the center of the specimen were probably low enough for ice crystal formation to take place. Considering that the solubility of the protein contents of the cytoplasm is lower in ice than in water, an explanation can be found for the honeycomb-like structure. Spontaneous nucleation of ice crystals in the region of the nucleus forms several expanding ice crystals. The expanding ice crystals meet to form a honeycomb-like structure, and the protein transported by the phase fronts delineates the cell walls of this structure.

Other specimen organelles that cannot be recognized in the reconstruction are the eyespot, the mitochondria, and the Golgi apparatus, all of which have probably also been destroyed by ice crystal segregation.

As has been described in section 4.3, there are several possible definitions for the ‘resolution’ that has been achieved in the reconstructed linear absorption coefficient of the specimen. The differential phase residual and the Fourier ring correlation are measures of the highest spatial frequency of the reconstructed object function for which missing Fourier components are still interpolated consistently by the reconstruction algorithm. Using these resolution measures, the liquid-nitrogen frozen specimen has been evaluated in section 4.3.3, yielding a maximum value of $2.58 \, \mu m^{-1}$ for the Fourier ring correlation, corresponding to a structure width of 194 nm.

A resolution measure that can be more easily compared with the known Rayleigh resolution of the images is given by the ‘sharpness’ of the reconstruction. To estimate the resolution in these terms, the edge sharpness of the glass walls and the full width at half maximum (FWHM) of selected colloidal gold spheres were measured. This was done by sampling the linear absorption coefficient along given lines (across the glass wall or across gold spheres), and determining the FWHM.
Figure 5.6: Local linear absorption coefficient of the LN$_2$-frozen *C. reinhardtii* specimen, reconstructed using the MART algorithm with 5 iterations and $\varepsilon = 0.3$. Panel (a) shows the first of the 42 X-ray microscopic images constituting the tilt series, panels (b) - (h) show slices of the reconstructed linear absorption coefficient. The slices are parallel to the axis of object rotation, viewed at the same viewing angle as the X-ray microscopic image, 27.6 nm thick (equal to the voxel size) and spaced at 0.6 $\mu$m. Of the reconstructed volume, only the parts inside the capillary walls are shown. Low absorption maps to light gray, and high absorption to dark gray. From [WSN$^+$$^00$].
of the gold sphere profile, or in the case of the glass walls, the slope of the profile. The FWHM of the gold spheres ranges from 70 to 100 nm, for a nominal diameter of 60 nm; however, many of the ‘gold spheres’ in the reconstruction were probably really clusters of two or more gold spheres, resulting in an elongated profile.

The glass wall edge sharpness corresponds to a FWHM of 60 to 70 nm. For comparison, the Rayleigh resolution for narrow-bandwidth illumination computed in section 1.2 was 16.7 µm⁻¹, with a resolution equivalent of 59.9 nm. Therefore, the reconstruction resolution measured by edge sharpness is only marginally worse than that of the X-ray microscopic images. This also implies that the alignment of the tilt series was of high quality; otherwise, the reconstruction resolution would have been further reduced by misalignment.

A slice through the reconstructed linear absorption coefficient has an effective ‘thickness’ that is equal to the reconstruction voxel size, 27.6 nm. It is therefore interesting to compare this virtual slice with a real median section of a C. reinhardtii specimen imaged with a transmission electron microscope (cf. Fig. 5.7). While the resolution is much higher in the electron microscopic image, it is only a single section. For three-dimensional imaging of the specimen, the complete cryo-fixated specimen has to be physically sliced into many such sections using a cryo microtome, the sections have to be imaged separately, and the section images have to be aligned to form a stack corresponding to the original specimen structure. This is an experimentally challenging and time-consuming task.

Also, the contrast in the electron microscopic image is based on heavy-metal staining; in the CTCXM slice, the intrinsic photoelectric absorption contrast of the protein structures has been visualized without any staining methods or sectioning artifacts.

### 5.1.3 Liquid-ethane frozen specimen

Although it was possible to acquire high-resolution images of the liquid-nitrogen frozen C. reinhardtii specimen, and to reconstruct the local absorption coefficient with high resolution, the biological significance of the result is reduced by the presence of vitrification artifacts. In more recent experiments, liquid ethane was therefore used as the primary cryo-ogen.

The inner reservoir of the plunge-freezing device (cf. section 2.3) was filled with liquid ethane to just below the rim, and the ethane temperature was adjusted to 113 K. The C. reinhardtii specimens were prepared as described in the previous section. However, after filling the reinforced capillary and glueing it into the hollow axle, the axle was magnetically attached to the piston of the plunge-freezing device (cf. Fig. 2.7), and immediately plunged into the ethane reservoir, to a depth of approx. 2.5 cm. After the piston stopped, the hollow axle and cog wheel were completely immersed in liquid ethane. The metal shield was drawn up to provide a reservoir of cold gaseous nitrogen, and the hollow axle was transferred to a magnetic holder inside a styrofoam spoon filled with liquid nitrogen.

To transport the capillaries to the microscope, cylindrical containers made of aluminum have been constructed. The styrofoam spoon was transferred to a styrofoam basin filled with LN₂, and the hollow axle was magnetically attached to a aluminum rod fitted with a screw thread. The rod was then screwed into a hole in the lid of the LN₂-filled transport container, so that the fragile capillary tip was immersed in LN₂ and protected from contact with the container walls. Care was
Figure 5.7: Comparison of imaging techniques. Panel (a) shows a median section through a wild-type cell of Chlamydomonas reinhardtii, with the nucleus (N) and nucleolus (NU), chloroplast (C), pyrenoid (P), and other cellular features which are depicted schematically in Fig. 5.2, imaged with a transmission electron microscope at a magnification of ×11,700 (from [Har88]). Panel (b) shows the central slice of the reconstructed local absorption coefficient of a specimen of C. reinhardtii obtained with CTCXM, with the absorption coefficient visualized as in Fig. 5.6.

taken to keep the capillary tip immersed in LN$_2$ at all times. The cylindrical containers were stored inside a large dewar filled with LN$_2$, and transported to the microscope.

At the microscope, the transport containers were transferred to another styrofoam basin filled with LN$_2$. All parts of the following transfer procedure were executed inside the LN$_2$-filled basin. The rods were removed from the transport containers. Using a custom-made guiding device, the hollow axle was inserted into the detachable tilt stage (cf. Fig. 2.6) and secured by the spring. The tilt stage was then transferred to a LN$_2$-filled styrofoam spoon and inserted into the cryo object chamber, where it was magnetically attached to the microscopic stage.

X-ray microscopic images of a liquid-ethane frozen C. reinhardtii specimen were acquired for 42 tilt angles spanning 185.1° (same settings as for the LN$_2$-frozen
5.1 *Chlamydomonas reinhardtii*

(a) Aligned tilt series of 42 X-ray microscopic images spanning 185.1°  
(b) Reconstructed local linear absorption coefficient (visualized as in Fig. 5.6)

Figure 5.8: Aligned tilt series (a) and reconstructed local linear absorption coefficient (b) of a liquid-ethane frozen specimen of *C. reinhardtii*. Click on the images to start the animation.

specimen) using a nickel micro zone plate objective (Ni 4098/4) with an imaging magnification of 1850 x. The initial measured diffraction efficiency of the micro zone plate was 12.5% [Peu00]; as for the liquid-nitrogen frozen specimen, this efficiency had probably been degraded to 5 - 10% at the time of the CTCXM experiment. Since for the LN₂-frozen specimen the capillary holder had sustained some radiation damage during acquisition of the tilt series, for the liquid-ethane frozen specimen only one image was acquired for each tilt angle (instead of three images). The resulting tilt series showed that no visible radiation damage occurred in either the specimen or the capillary holder. The X-ray microscopic images were aligned using 9 different gold markers and reduced in size by a factor of 2, and the linear absorption coefficient was reconstructed using the MART algorithm with 5 iterations and $\varepsilon = 0.3$ (cf. Fig. 5.8, panel (a)).

At the time when the tilt series of the liquid-ethane frozen specimen was acquired, the BESSY X-ray source was significantly larger than when the LN₂-frozen specimen had been imaged. The increased source size causes a decreased monochromaticity of the object illumination and correspondingly, a decreased X-ray microscopic resolution. Consequently, the image quality is not as high as for the LN₂-frozen specimen, and the resolution of the reconstruction is also lower than that of the LN₂-frozen specimen (cf. Fig. 5.9). However, the preservation of the internal structures is much better. The most obvious difference is the complete preservation of the nucleus and nucleolus (panels (e) - (h)). Whereas for the LN₂-frozen specimen much of the specimen interior has the same linear absorption coefficient as the surrounding water (approx. 0.1 $\mu$m$^{-1}$, cf. Fig. 5.6), for the liquid-ethane frozen specimen only the vacuole has the same linear absorption coefficient as water (panels
Figure 5.9: Slice representation of a liquid-ethane frozen specimen of *C. reinhardtii*, reconstruction and visualization as in Fig. 5.6. Panel (a) shows the original X-ray microscopic image from the tilt series, while panels (b) - (i) show slices of the linear absorption coefficient parallel to the axis of rotation, spaced at 0.36 µm. The dark edges in panel (b) are part of the glass walls.
(g) - (i)), and the cytosol surrounding the organelles is characterized by a homogeneous absorption coefficient of approx. 0.25 \( \mu m^{-1} \), indicating that on the resolution scale of the X-ray microscope, the biological material has not been segregated by ice crystal formation.

The flagella and flagellar roots have been well preserved: panel (d) shows the flagella entering the cell wall, panel (e) shows the interconnected flagellar roots. The flagella themselves wrap around the alga at about the height of the flagellar roots; in the slices, the flagella appear as dark blots or rings on the left and right of the alga. The vacuole shown in Fig. 5.2 is visible in the basal part of the specimen; different from the schematic view, it appears well rounded. Due to the lower resolution, the fibrous structure of the chloroplast is not as apparent as in the LN\(_2\)-frozen specimen. The cell wall is only visible for the basal part of the alga. It is not as well rounded as in Fig. 5.1, indicating that the cryo preservation is still not perfect.

5.2 *Drosophila melanogaster*

As has been shown in the preceding section, the strong intrinsic absorption contrast between protein and water in the water window wavelength range permits to reconstruct the three-dimensional protein structure of a microscopic specimen based on unstained X-ray microscopic images of the specimen. However, many biological questions concern not the morphology of a specimen, but the distribution of a specific protein in the specimen. In order to distinguish this protein from other proteins, it must be labeled in some way, i.e., some sort of marker must be selectively attached to it. In fluorescence microscopy, different fluorescent colors are used as labels. For absorption contrast X-ray microscopy, the contrast of a label is based on its absorption. By bringing highly absorbing elements such as gold and silver into close proximity of the investigated protein, the protein distribution in the specimen can be visualized.

S. Vogt investigated the distribution of the dosage compensation protein MSL-1 in the cell nuclei of male *Drosophila melanogaster* fruit fly cells, by using silver-enhanced immunogold labeling of the MSL-1 protein \[VSS^{+00}\]. Cells of the SL2 cell line (ATCC number: CRL\#1963) were grown in Schneider’s Drosophila medium (Life Technologies, Karlsruhe), fixed with 4% formaldehyde, and extracted with 0.5% Triton X-100 (Sigma-Aldrich Chemie, Deisenhofen). They were incubated with the primary antibody (rabbit, against c-MSL-1) and afterwards with a secondary antibody, 1 nm colloidal gold conjugated goat F(ab’)\(_2\) anti-rabbit IgG (British Biocell). The colloidal gold was then enhanced with a silver enhancement kit (LI Silver, Nanoprobes Inc., Stony Brook).

In this way, Vogt was able to show that immunogold-labeled nuclear structures can be investigated with the transmission X-ray microscope (cf. Fig. 5.10), at a resolution exceeding that of confocal laserscan microscopy by about a factor of five \[VSS^{+00}\]. It was therefore attempted to apply CTCXM to the immunogold-labeled cell nuclei, in the hope of visualizing the three-dimensional distribution of the label. For this purpose, cell nuclei were fixed, extracted, and labeled according to the procedure described above; however, in order to obtain a specimen suspension that could be injected in a capillary holder, the cell nuclei were not prepared on support foils, but in suspension.
Figure 5.10: X-ray microscopic image of a cell nucleus of a male *Drosophila melanogaster* cell. The dosage compensation protein MSL-1 was labeled using gold conjugated antibodies, and the absorption of the label was increased by silver enhancement. From [VSS+00].

100 nm colloidal gold markers were added to the suspension of cell nuclei, and the specimen concentration was increased by centrifugation. Suspension samples were taken using a Microloader pipette tip and injected into the capillary. Because the cell nuclei are not motile, they must be transported to the X-ray transparent part of the capillary as the suspension is drawn into the tip by capillary forces. It was observed that if no cell nuclei were visible inside the capillary tip, they could sometimes be moved there by repeatedly striking the large-bore end of the capillary holder against a hard surface. The specimens were frozen in liquid ethane using the procedure described in section 5.1.3.

At the microscope, several capillary holders were examined to find suitable labeled cell nuclei. However, the distinct localization of the label known from two-dimensional support foils (cf. Fig. 5.10) was not observed. The absence of conclusive labeling is probably a consequence of the changed preparation protocol; the cell nuclei were prepared in suspension, whereas before they were attached to support foils.

In spite of the absence of visible label in the cell nuclei, a group of three nuclei
was selected for CTCXM, and 46 tilt images spanning 203.2° were acquired (cf. Fig. 5.11, panel (a)). The micro zone plate objective used for image acquisition was the same as for the liquid-ethane frozen *C. reinhardtii* specimen (cf. section 5.1.3). One image was acquired for each tilt angle. The linear absorption coefficient was reconstructed with 5 iterations of the MART algorithm and \( \epsilon = 0.3 \). Figure 5.12 shows a slice representation of the reconstructed cell nuclei. Note that the original X-ray microscopic image in panel (a) shows no conclusive labeling. The *D. melanogaster* cell nuclei are different from the *C. reinhardtii* specimens in that they cannot be subdivided into easily distinguishable sub units such as the alga organelles. Instead, the approximately spherical cell nuclei present a uniform appearance. The fine texturing of the nuclei may be an artifact of the fixation and extraction process. Of the three cell nuclei, only one (visible in the upper left part of the slices) appears to have a sub structure of several inclusions with reduced absorption coefficient.

At 2.4 nm wavelength, the linear absorption coefficient of gold is \( \mu = 23.4 \, \mu m^{-1} \). The corresponding values for the model protein \( C_{94}H_{139}N_{24}O_{31}S \) and ice are \( \mu = 1.35 \, \mu m^{-1} \) and \( \mu = 0.109 \, \mu m^{-1} \), resp. For bulk material, the reconstructed absorption coefficient of gold is therefore greater than that of protein and ice by a factor of 17 and 215, resp. This would make it very easy to identify the gold label. However, a single gold marker has a diameter of only 1 nm, and hence a volume of 0.5 nm³. The volume of a reconstruction voxel, by comparison, is \((30 \, nm)^3 = 27.000 \, nm^3\). In order to double the linear absorption coefficient of a protein-filled reconstruction voxel, \(54.000/17 = 3176\) 1 nm gold markers must therefore be present inside the voxel. If the number of markers is smaller by one or two degrees of magnitude, the absorption of those markers must be increased by this factor, e.g., by silver
Figure 5.12: Slice representation of the liquid-ethane frozen *D. melanogaster* cell nuclei, reconstruction and visualization as in Fig. 5.6. The X-ray microscopic image from about halfway through the tilt series shows a fissure in the ice (a), this is an artifact of radiation damage. The slices in panels (b) - (i) are spaced at 0.42 µm.
Summing up, it cannot be determined whether in this first attempt there were gold markers attached to the MSL-1 protein, and the silver enhancement step failed, or whether the MSL-1 labeling failed altogether. To reconstruct the distribution of labeled proteins in the cell nuclei, it is necessary to either establish a labeling protocol for cell nuclei in suspension that works as well as the protocol for cells on support foils, or else to label the cell nuclei on the support foils, select interesting nuclei with the light microscope, and transfer only those nuclei to the capillary holder. This could possibly be done using a micro manipulator and micro injection device attached to the light microscope. More experiments are necessary to determine the feasibility of either approach.
Chapter 6

Quantitative analysis

Apart from the possibility to visualize the local linear absorption coefficient by displaying slices through the reconstructed volume, the availability of volumetric data enables meaningful quantitative measurements of specimen characteristics. In the following, two such quantitative measurements are performed on the microscopic specimen with the highest reconstruction quality, namely the LN$_2$-frozed specimen of *C. reinhardtii*. The fact that this specimen was somewhat damaged in the freezing process reduces the biological significance of the quantitative results, but not the viability of the measurements themselves.

6.1 Volume and mean linear absorption coefficient of *C. reinhardtii* organelles

The purpose of the specimen analysis presented below is to identify the different organelles of the *C. reinhardtii* specimen, and to measure two exemplary quantities for each of the organelles, namely the volume and the mean linear absorption coefficient.

Methodically speaking, identification of the specimen organelles is equivalent to a segmentation of the reconstructed volume. Each voxel of the volume has an associated gray value$^1$. Based on this value, each voxel is to be assigned a label designating the specimen organelle that it belongs to. Additional labels must be assigned to that part of the alga interior that does not belong to any organelle, and to the alga exterior.

A simple way of segmenting a volume is to apply a threshold to the voxel gray values. For a given gray value $t$, the set of voxels is segmented into two subsets: voxels with gray values greater than or equal to $t$, and voxels with gray values less than $t$. In many medical CT applications, a specific range of gray values corresponds to a specific type of tissue or bone, so that by choosing an appropriate threshold, only those voxels that belong to the tissue can be selected. The resulting subset of voxels is usually compact (because it corresponds to a specific part of the body) and can be visualized as a surface. Several methods exist for creating a surface composed of polygons for a compact set of voxel locations, most notably the

---

$^1$Because medical tomographic volumes are usually displayed in shades of gray, it is customary to speak of the ‘gray value’ of a voxel regardless of the physical property that it represents. In this case, the gray value is the local linear absorption coefficient, but segmentation can be applied to any local specimen property.
Figure 6.1: Watershed segmentation of the LN$_2$-frozen C. reinhardtii specimen. The original gray values (a) are smoothed with a median filter and the morphological gradient is computed (b). Starting from user-defined label locations, the gradient map is ‘flooded’ with organelle labels, resulting in the segmented volume (c). Note that all of these steps operate on 3D neighbourhoods, but only slices through the 3D data are displayed.

‘Marching Cubes’ algorithm and variants thereof.

However, threshold segmentation is not a suitable method for the C. reinhardtii specimen, because the specimen organelles do not have unique, characteristic gray values. Instead, organelles contain a whole range of gray values, e.g., the fibrous structure of the chloroplast contains both light and dark voxels (cf. Fig. 5.6).

Instead, a technique known as ‘watershed segmentation’ is used [BM92]. The volume is segmented in three steps. First, the original gray values are smoothed with a median filter and the morphological gradient is computed. For a given three-dimensional voxel neighbourhood, this gradient is defined as the difference between the maximum and the minimum gray value occurring in the neighbourhood. In the resulting gradient map homogeneous regions have low values, while high gradient values denote inhomogeneity, e.g., at the interfaces of adjacent regions (cf. Fig. 6.1).

In the second step, some voxels have to be assigned a label by hand. In practice, this means displaying the gray values with some kind of image editing software, and ‘painting’ the several organelles with different colors. These colors are then converted into labels and assigned to the voxels that have been painted. This should be done for several of the slices constituting the volume. It is not necessary or indeed desirable to attempt to paint all the voxels belonging to an organelle. The organelle interfaces should be determined by the segmentation algorithm, so it is advisable to paint only some of the voxels at the center of the organelles. In addition to the organelles, the remaining alga interior and the alga exterior must be labeled with specific labels.
Table 6.1: Quantitative analysis of *C. reinhardtii* organelles, based on the segmented linear absorption coefficient (LAC, cf. Fig. 6.2), showing the absolute and relative volume as well as the average LAC. To exclude the borders of the segmented regions from the LAC average, the regions were eroded with a 3 pixel radius before averaging. The standard deviation of the average LAC is given in brackets. The relatively high values indicate the structural inhomogeneity of the organelles (cf. Fig. 5.6). From [WSN+00].

<table>
<thead>
<tr>
<th>organelle</th>
<th>vol. [µm³]</th>
<th>vol. [%]</th>
<th>LAC [µm⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>chloroplast</td>
<td>129.13</td>
<td>47.54</td>
<td>0.385(91)</td>
</tr>
<tr>
<td>pyrenoid</td>
<td>3.42</td>
<td>1.26</td>
<td>0.441(66)</td>
</tr>
<tr>
<td>spherical vesicles</td>
<td>4.00</td>
<td>1.47</td>
<td>0.711(86)</td>
</tr>
<tr>
<td>flagellar roots</td>
<td>0.14</td>
<td>0.05</td>
<td>0.206(39)</td>
</tr>
<tr>
<td>other</td>
<td>134.91</td>
<td>49.67</td>
<td>0.187(80)</td>
</tr>
<tr>
<td>total</td>
<td>271.60</td>
<td>100.00</td>
<td>0.306(137)</td>
</tr>
</tbody>
</table>

In the final step, the hand-labeled voxels are used as seeds for the segmentation algorithm. The name ‘watershed segmentation’ stems from the way in which the remaining voxels (those that have not been labeled by hand) are assigned a label. Each hand-labeled voxel is regarded as a ‘label source’ from which the label is propagated to the immediate 3D neighbourhood. Whether a label is propagated to an adjacent unlabeled voxel depends on the gradient map: labels automatically ‘flow down’ to low gradients, but to reach higher gradient values, the overall ‘water level’ has to be gradually increased until all voxels are labeled. Due to the high gradient values at the interfaces of adjacent regions, those interfaces act as watersheds for the flooding process and separate the regions efficiently (cf. Fig. 6.1 (c)). The exact implementation of the flooding process is based on ordered queues [Vin90, BM92] and can be found in appendix C.

The described process was applied to the *C. reinhardtii* specimen. Chloroplast, pyrenoid, spherical vesicles and flagellar roots were identified as distinct organelles in the slice representation of the specimen (cf. Fig. 5.6). Because the watershed segmentation starts with hand-labeled voxels at the center of the organelles and works outward, the resulting labeled regions are compact and disjunct and can be visualized as surfaces (Fig. 6.2).

Based on this segmentation, quantitative analysis can be performed on the organelles. Two exemplary measurements of the four organelles are their volume and their average linear absorption coefficient (LAC, cf. Table 6.1). The relatively large standard deviation of the average LAC is due to the inhomogeneity of the organelles. This is especially true of the chloroplast, which has a very fibrous structure. As can be seen, the chloroplast takes up almost 50% of the alga interior, while the spherical vesicles, which feature very prominently in two-dimensional X-ray microscopic images, occupy only 1.5% of the total alga volume. This result is in stark contrast with other measurements based on two-dimensional images, which estimated the relative volume of the spherical vesicles to be as high as 20% [SFP+97].

Evaluating the volume outside the alga gives a LAC value of 0.098(14) µm⁻¹ for ice. Based on the atomic scattering factors tabulated by Henke [HGD93], theoretical LAC values of 0.109(2) µm⁻¹ for ice and 1.35(14) µm⁻¹ for a model protein
Figure 6.2: Several organelles of the *C. reinhardtii* specimen identified by watershed segmentation of the reconstructed linear absorption coefficient: chloroplast (green), pyrenoid (blue), spherical vesicles (red), and flagellar roots (brown). One quadrant of the chloroplast has been cut away to reveal the pyrenoid. The gray values of the chloroplast have been mapped to the chloroplast surface as light and dark green. Also displayed is one slice of the reconstructed linear absorption coefficient. Visualization using the Amira system developed at ZIB (http://amira.zib.de). From [WSN′00].
Table 6.2: Selected cellular components and their linear absorption coefficient (LAC). Data from [HGD93].

<table>
<thead>
<tr>
<th>cellular component</th>
<th>density [g/cm$^3$]</th>
<th>LAC [$\mu$m$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>protein C$<em>{94}$H$</em>{139}$N$<em>{24}$O$</em>{31}$S</td>
<td>1.35</td>
<td>1.351</td>
</tr>
<tr>
<td>nucleic acid C$<em>{38}$H$</em>{63}$N$<em>{16}$O$</em>{40}$P$_{4}$</td>
<td>1.7</td>
<td>1.347</td>
</tr>
<tr>
<td>lipid C$<em>{18}$H$</em>{34}$O$_2$</td>
<td>1.0</td>
<td>0.98</td>
</tr>
<tr>
<td>H$_2$O (ice)</td>
<td>0.98</td>
<td>0.109</td>
</tr>
</tbody>
</table>

with the chemical composition C$_{94}$H$_{139}$N$_{24}$O$_{31}$S and $\rho_{\text{protein}} = 1.35(14)$ g/cm$^3$ were calculated. With the complete-cell average LAC of 0.306(137) $\mu$m$^{-1}$, this yields a dry protein content of the alga of 17(11)%. Again, the large standard deviation does not denote experimental error, but rather the inhomogeneity of the specimen.

The above calculation assumes that the specimen is composed only of ice and the model protein. Table 6.2 shows the linear absorption coefficients for several other cellular components. The values are about an order of magnitude greater than for ice, so that the above calculation of the percentage of dry biological material also applies (at least roughly) if different kinds of biological material are considered.

While the X-ray dense spherical vesicles show the highest LAC value, the expected value of $\geq 1$ $\mu$m$^{-1}$ of biological material (cf. Table 6.2) is not reached. This is due to the fact that even inside the vesicles, the biological material is present in hydrated form, so that the mean LAC is lower than that of the dry material.

### 6.2 Accumulated local dose and radiation damage in the C. reinhardtii specimen

In addition to the quantitative measurements described in the preceding section, the volumetric absorption data can be used to reenact the CT experiment in a numerical simulation. The purpose of this simulation is to determine the accumulated local dose (ALD), i.e., the dose that each spatial resolution element (voxel) of the specimen has accumulated during the acquisition of the tilt series. The ALD is of interest for several reasons. It can be compared to the upper dose limit under cryo conditions predicted by model calculations [Sch94]; and localized radiation damage can be correlated to the local dose. The calculation of the accumulated local dose is again demonstrated for the LN$_2$-frozen C. reinhardtii specimen.

During the CT experiment, flat-field images were acquired for every tilt angle in order to normalize the intensity of the images showing the specimen. These flat-field images show the intensity distribution incident to the specimen holder (in CCD camera counts), and they can be used to simulate the CT experiment.

As a first step, each flat-field image is scaled according to the total exposure time that was used to acquire images under the corresponding tilt angle. For example, for the C. reinhardtii specimen three specimen images but only one flat-field were acquired for each tilt angle, so that the flat-field intensity must be multiplied by a factor of approx. three (approximately because the BESSY ring current is different for each image). The intensity must be further scaled to account for the diffraction efficiency of the micro zone plate objective and for all absorbing elements between the object and the CCD camera. The cumulative effect of the degraded MZP diffrac-
6.2 Accumulated local dose and radiation damage in the \textit{C. reinhardtii} specimen

Figure 6.3: Accumulated local dose in the \textit{C. reinhardtii} specimen (one slice of the 3D data). High dose maps to light gray, and low dose to dark gray. During acquisition of the tilt series, the specimen was illuminated from the front, the right, and the back (185° angular range), hence the dose values on the right are higher than those on the left side. The dose in ice ranges from $1 \times 10^8$ to $3 \times 10^8$ Gy, for the protein structures from $3 \times 10^8$ to $1.5 \times 10^9$ Gy, and for the glass capillary from $5 \times 10^8$ to $5 \times 10^9$ Gy. From [WSN+00].

The result of the above step is an accumulator volume with the number of photons.
tons that have been absorbed by each voxel. The size of the voxel is known, and the material density is assumed to be a uniform $\rho = 1 \text{ g/cm}^3$. The absorbed photons can thus be converted to the accumulated local dose.

Because the tilt series covers $185^\circ$, the specimen has not been illuminated from all sides, and the accumulated local dose is not radially symmetric (Fig. 6.3). The dose in ice ranges from $1 \times 10^8$ to $3 \times 10^8$ Gy, in the protein structures from $3 \times 10^8$ to $1.5 \times 10^9$ Gy. The glass capillary has sustained doses between $5 \times 10^8$ Gy and $5 \times 10^9$ Gy. The average dose is therefore in the range of the critical dose computed by Schneider for which a significant number of H$_2$O molecules are dissociated by the radiation [Sch94].

To further investigate possible radiation damage in the specimen it is interesting to see whether mass loss has occurred. Mass loss refers to the loss of absorbing material in the specimen during acquisition of the tilt series. It occurs when the gaseous products of radiation-induced reactions leave the object and thereby reduce its absorption. The tilt series of aligned projections of the object absorption provides an accurate measurement of the total object absorption, since each of the projections shows the same volume of object space and since the object is completely visible in each of the projections.

Figure 6.4 shows the total object absorption of the tilt series, computed as the sum over all pixels in the absorption projections. Apart from changes due to the imperfect illumination correction of the X-ray microscopic images, the total object absorption is constant, indicating that no mass loss has occurred.
Figure 6.4: The total object absorption (computed as the sum over all pixels in the absorption projections) and the average accumulated dose in ice (which ranges from $1 \times 10^8$ to $3 \times 10^8$ Gy, cf. Fig. 6.3) of the 42 aligned projections of the *C. reinhardtii* specimen. The dose increases approximately linearly, but apart from changes due to the imperfect illumination correction of the X-ray microscopic images, the total object absorption is constant, indicating that no mass loss has occurred. From [WSN+00].
Summary and Outlook

The cryo transmission X-ray microscope (CTXM) at the BESSY I electron storage ring images hydrated biological objects at a cryogenic temperature of down to 90 K, showing specimen details down to 30 nm size. In this way, a radiation dose of up to $10^9$ Gy can be used to acquire a large number of high-resolution images of a single cryogenic specimen without incurring radiation damage at the X-ray microscopic resolution level. The intrinsic absorption contrast between protein and water at 2.4 nm wavelength permits to visualize the structure of unstained biological specimens in their natural wet environment. The aim of this work was to use the methods of computed tomography to reconstruct the three-dimensional structure of cryogenic biological specimens from X-ray microscopic images.

The influence of the narrow-bandwidth illumination used in the X-ray microscope on the lateral resolution and on the depth of focus was investigated theoretically. It was shown that for a mean wavelength of 2.4 nm and a micro zone plate objective with an outermost zone width $d_{\text{rr}} = 40$ nm, the X-ray microscopic image of a specimen with a typical diameter of 8 $\mu$m can be used to obtain a good approximation of the projected linear absorption coefficient of the specimen.

For computed-tomography reconstruction of a three-dimensional object it is necessary to acquire projections of the object absorption for a series of viewing angles. To permit the acquisition of such tilt series, the CTXM was modified to accommodate a detachable tilt stage. Using this tilt stage, cryogenic biological specimens in suitable object holders can be rotated around an axis perpendicular to the microscope optical axis. If the object holders are not rotationally symmetric, tilt images can only be acquired for a restricted angular range, giving raise to severe reconstruction artifacts due to the ‘missing wedge’ effect. Therefore, thin-walled borosilicate glass capillaries with a diameter of approx. 10 $\mu$m were used as rotationally symmetric object holders, permitting tilt image acquisition over the full angular range of 180°. The specimens were inserted into the capillary holders by suspending them in water and injecting the suspension into the capillaries.

With an X-ray transmission of the water-filled capillaries of about 14%, it was possible to image the specimens inside the capillaries with exposure times of a few seconds. Initial experiments showed an insufficient mechanical stability of the thin glass capillaries at cryogenic temperatures. By reinforcing the capillaries with an electroplated nickel coating, the mechanical stability was increased sufficiently to be able to acquire high-resolution images of the cryogenic specimens.

With the current experimental setup of the X-ray microscope, the high-precision alignment of the specimen necessary for computed-tomography reconstruction cannot be done by means of the microscope stage alone. Therefore, colloidal gold spheres with 60 - 100 nm diameter were added to the specimen suspensions as fiducial markers. In the X-ray microscopic images, the positions of these mark-
ers were used to determine a set of alignment parameters necessary to align the images to a common axis of rotation.

In a first experiment, living specimens of the unicellular green alga *Chlamydomonas reinhardtii* were inserted into a reinforced capillary holder and frozen by plunging into liquid nitrogen, without any other fixation or preparation steps. High resolution tilt images of a specimen were acquired for 42 tilt angles spanning 185°. Using colloidal gold spheres as fiducial markers, the X-ray microscopic images were resampled to create an aligned tilt series. At the same time they were also reduced in size by a factor of 2, so that the three-dimensional specimen volume could be reconstructed and handled reasonably fast on the available hardware. This size reduction preserves all image information up to the Rayleigh resolution calculated for narrow-bandwidth illumination, while increasing the signal-to-noise ratio of the image intensity by a factor of 2. The integral object absorption was calculated from the normalized intensity of the aligned tilt series, and a multiplicative algebraic technique was used to reconstruct the local linear absorption coefficient in reconstruction planes perpendicular to the axis of object rotation.

By displaying parallel slices through the reconstructed linear absorption coefficient, the three-dimensional specimen structure was visualized much more clearly than in the original X-ray microscopic images. The appearance of most specimen organelles agrees well with light and electron microscopic studies, although some damage due to imperfect vitrification was observed. The resolution of the reconstruction as measured by edge sharpness was 60 - 70 nm and thus somewhat worse than the Rayleigh resolution of the original X-ray microscopic images, the degradation being due to a small misalignment of the tilt series. In addition to the sharpness-based resolution measure, the Fourier space based resolution measures *differential phase residual* (DPR) and *Fourier ring correlation* (FRC) were applied to the reconstructed specimen. These resolution measures calculate the spatial frequency up to which Fourier coefficients are consistently interpolated by the reconstruction algorithm. Due to the relatively low number of tilt images, the average DPR resolution of the specimens investigated in this work was only approx. 500 nm, meaning that reconstructed structures finer than 250 nm and modulated perpendicular to the axis of object rotation should be interpreted with caution. Note that since the reconstruction takes place in independent reconstruction planes perpendicular to the rotation axis, no such restriction applies to structures modulated in the direction of the rotation axis.

The availability of volumetric absorption data allows quantitative analysis of specimen properties. The volumetric data of the *C. reinhardtii* specimen was segmented into organelles, and the volume and the average linear absorption coefficient of the organelles were measured. Based on the reconstructed three-dimensional absorption coefficient and the flat-field images acquired with the tilt series, the accumulated dose for each reconstruction voxel was calculated. In spite of a high accumulated dose between $10^8$ and $10^9$ Gy, the specimen showed no radiation damage to the biological structures.

To obtain better preservation of the biological structures during the vitrification process, specimens of *C. reinhardtii* were vitrified by plunging into liquid ethane, and imaged with the CTXM. Although the image quality (and thus the reconstruction quality) was inferior to that of the liquid-nitrogen frozen specimen, the specimen preservation was much better, with complete preservation of the nucleus, nucleolus and the neuromotor apparatus composed of flagella and flagellar roots.
In order to extend the possibilities of computed-tomography reconstruction to the study of selected proteins, in collaboration with S. Vogt this method was also applied to the cell nuclei of male *Drosophila melanogaster* fruit fly cells. The dosage compensation protein MSL-1 was labeled in the cell nuclei using immunogold labeling in conjunction with silver enhancement, and a tilt series of images was acquired. However, no clear evidence of label could be found in the reconstruction. Further efforts should be made to acquire tilt series of images of specimens containing labeled protein. In future experiments, suitable labeled cell nuclei could be selected using a light microscope, and transferred to the capillary holder with a micro manipulator and micro injection apparatus.

For those extended biological specimens that do not fit into the capillary holders, such as cells grown on a support foil, the possibilities of using strip holders for computed-tomography experiments should be further investigated. Whereas the limited angular range and the associated ‘missing wedge’ artifacts are of a fundamental nature, the fact that the specimen extends beyond the microscopic field of view can be addressed by choosing an extended-volume reconstruction algorithm.

In order to increase the Crowther resolution of the reconstruction, efforts should be made to substantially increase the number of tilt views acquired for a tilt series. Since the acquisition of a tilt series is already a very time-consuming task even for the limited number of views acquired in experiments so far, it is necessary to further automate the process, especially the alignment and refocusing of the specimen necessary for each tilt angle. In case of radiation damage it is also possible to use dose fractionation, i.e., to distribute a constant total specimen dose among an increased number of images. In this case, the alignment would have to be done for images with reduced signal-to-noise ratio, and correlation-based alignment techniques might have to be used. Alternatively, the need for alignment and refocusing of the specimen might be substantially reduced through the use of a high-precision goniometer-based tilt stage.

The new transmission X-ray microscope at the BESSY II electron storage ring promises to deliver high resolution images with reduced exposure times. For the purposes of computed tomography, it will be especially interesting to study the effect of the hollow-cone illumination. This illumination is expected to extend the depth of focus, thus making the images more projection-like and thus more suitable for computed tomography. On the other hand, model calculations predict that the modulation transfer at relatively low spatial frequencies will be somewhat reduced; this would probably have to be compensated, e.g., by using a weighted-sum interpolation that amplifies those spatial frequencies.

The technique described above has some unique advantages over other microscopic techniques. It is the only microscopic method that is capable of imaging the three-dimensional structure of complete frozen-hydrated biological specimens in a near-native state, without the need for fixation or staining, for object thicknesses of up to 10 µm and showing specimen details down to a size of approx. 30 nm. Further research efforts should concentrate on increasing the number of tilt angles in a tilt series, since the single most important shortcoming of current reconstructions is the low DPR resolution perpendicular to the axis of object rotation. This is a fundamental consequence of the relatively low number of tilt images that have been acquired so far, and as has been shown in this work, can only be partially compensated by using an interpolating reconstruction algorithm.

Future computed-tomography experiments will be able to benefit from new mi-
cro zone plate objectives with decreased outermost zone widths of 20 - 25 nm, and hence, increased microscopic resolution. If the effective depth of focus is sufficiently large to encompass complete microscopic specimens, the use of such micro zone plates will directly benefit the reconstruction resolution in the direction of the axis of object rotation, and will also permit to reconstruct finer specimen details perpendicular to that axis. The increased depth of focus that is expected for the hollow-cone illumination is therefore a necessary requirement in order to effectively use X-ray objectives with increased microscopic resolution for computed-tomography reconstruction.
Appendix A

Manual for the XALIGN software

The 16-bit intensity data measured by the CCD camera chip is stored by the image acquisition software PMIS in the proprietary image format PMI, data type unsigned short. As the first processing step, the X-ray microscopic images acquired during the CTCXM experiment are read from the image files, the dark current is subtracted, and the individual pixel counts are cast to the 4 byte floating-point data type float. Each image is then normalized by dividing it by its flat-field image, yielding an intensity range \([0, \ldots, 1]\). If the image intensity values exceed those of the flat field owing to Poisson noise, the intensity is clamped to 1. At this time, the images can also be corrected by a local pixel sensitivity map (cf. section 1.3.2). The normalized intensity data is then stored in floating-point format as a continuous image stack, dimensions \(x \times y \times z\), where \(x\) and \(y\) are the image dimensions (usually \(x = y = 1024\)), and \(z\) is the number of tilt images acquired.

The remaining processing task prior to reconstruction is to align the images of this stack to a common tilt axis, possibly including a reduction in size (to improve the signal-to-noise ratio). This task can be accomplished in two steps. In the first step, a common system of reference has to be established for all tilt images using fiducial markers (cf. section 3.1); the second step interpolates the original image stack using the common system of reference to create the aligned tilt series. The software XALIGN was written to perform both of these steps.

XALIGN was written in ANSI-C on a Silicon Graphics (SGI) OCTANE workstation. While XALIGN uses the OpenGL graphics library supported by SGI workstations to display the X-ray microscopic images and overlaid markers, it should be possible to build an XALIGN executable on other platforms for which free OpenGL implementations are available (e.g. Mesa for the Linux operating system [Paul]). The graphical user interface (GUI) was created using Xforms, a free GUI toolkit based on Xlib for X Window Systems. Xforms is available in binary form for several Unix platforms [ZO].

The program XALIGN is invoked as xalign [filename]. The file name of the image stack can be specified as the optional parameter. Since the image stack contains only the image data and no header information, XALIGN attempts to read the stack dimensions from the filename. If the file name is of the form name.XxYxZ, the dimensions will be set to \(x\), \(y\), and \(z\), and the image stack will be read to memory. Otherwise the user has to specify the dimensions explicitly. If no file name is given, XALIGN starts up with an empty work area and the image stack can be loaded using the File/Load projections menu entry.

The main form of the program is shown in Fig. A.1. It contains the work area
Figure A.1: XALIGN work area window with main menu and overlaid marker positions

window showing the current image from the image stack at the current magnification, as well as the main menu. The content of the work area window can be moved using the sliders at the right and bottom of the window, or by pressing the middle mouse button and moving the mouse.

Data display

Most program functions are accessed via the tool bar (cf. Fig. A.2). The uppermost group of objects (Map) controls the display mode, i.e., the way that the normalized intensity values are visualized. The intensity values are mapped to screen colors using one of several color maps. The linear gray map reproduces the images as displayed by the PMIS software, while linear RGB increases the visual contrast by
mapping the intensity values to a color spectrum. Since the dynamic range of the intensity data exceeds the dynamic range that can be displayed on a computer monitor, the displayed data range can be reduced by setting the two sliders in the Map group. The upper slider controls the lower range cutoff, the lower slider the upper range cutoff. The new, reduced data range is then visualized either by clipping the data range at the specified values (button Clip), or by increasing the on-screen contrast to fit the specified data range (button Enhance).

Maximum screen contrast is achieved by using the Enhance mode and setting the sliders to the minimum and maximum intensity values of the currently visible area. This can be done automatically by checking the Options/AutoEnhance menu entry. In AutoEnhance mode, the minimum and maximum of the currently visible intensity values are calculated every time the window content changes, and the slider settings are updated to maintain maximum screen contrast. At high magnification, this mode maximizes the screen contrast for small image areas and is helpful in finding markers that are ‘invisible’ at low magnification.

Tool modes

The group below (Tools) comprises the six currently available tool modes: zoom mode, point marker mode, ellipse marker mode, ROI selection mode, line selection mode, and FBP reconstruction mode. The depressed button indicates the currently active tool. Only one tool may be active at any time.
Zoom mode

By clicking on the magnifying glass the program enters zoom mode. A rectangular area can be drawn by pressing the left mouse button and moving the mouse to indicate the extent of the area, then releasing the button. The program then displays the selected area, increasing the magnification if necessary. Clicking the left mouse button zooms in on the image in magnification steps of +1, while pressing the right mouse button zooms out in magnification steps of -5. The magnification factor may also be set using the counter zoom in the tool bar.

Pressing the middle mouse buttons moves the current image under the window (this functionality is available in all tool modes). Alternatively, the window content may be moved using the sliders at the right and bottom of the work area. Movement in the z-direction of the stack (between tilt images) is effected via the projection counter of the tool bar. Shortcuts for decreasing and increasing the current projection number are the ‘z’ and ‘x’ keys, respectively.

Point marker mode

The hand icon turns on the point marker mode. This is the tool used to measure the positions of the fiducial markers (which, for the purposes of alignment, are assumed to be points). The currently active marker number is shown on the third counter (marker) of the tool bar; it can be modified using the arrows. Pressing the left mouse button in the work area places the currently active marker at the position of the mouse cursor. At magnification settings greater than one, this position is evaluated with sub-pixel accuracy. It is advisable to measure the marker positions at high magnification where the marker center can be identified with sub-pixel accuracy. Right-clicking deletes the marker under the mouse.

Initially, the measured marker positions are indicated as red dots, with the marker number next to the dot. As soon as a common system of reference is available (after executing the fiducial alignment, see below), the marker position reprojected from the current 3D marker model is also shown (cf. section 3.1): in green, if a measured marker position is available in the currently visible projection, in yellow if the marker has not been measured in the current projection. For an example, consider Fig. A.1. Point markers 1, 2, 5, and 6 have been measured in this projection (red); the reprojected positions of the markers (green) lie very close to the measured markers, indicating good agreement between the measured marker positions and the 3D marker model. Point markers 4, 7, 8, and 9 have been measured in other projections, so a corresponding 3D marker position is available and has been reprojected (yellow), but they have not yet been measured in this projection. The display of the reprojected 3D marker model (colors green and yellow) can be turned on and off by toggling the Options/Show 3d model menu entry. The yellow markers are very useful in locating the approximate position of a marker that has not yet been measured.

To help with the measurement of the marker center, there exists an experimental automatic-measuring mode which can be turned on by checking the Options/AutoMarking menu entry. In AutoMarking mode, left-clicking the mouse is interpreted as a first estimate of the marker center position. The intensity data is then used to identify all pixels belonging to the marker, by smoothing and thresholding the image pixels. The marker center is calculated as the center of gravity of
Figure A.3: XALIGN fiducial alignment form

all pixels belonging to the marker. This tool should be used with caution.

The dashed-ellipse icon enters ellipse marker mode. This is identical to point marker mode, but instead of setting point markers, ellipsoidal markers can be delineated by drawing an ellipse around them. The center of the ellipse is used as the marker center. The currently active ellipse marker number is selected via the marker ellipse counter of the tool bar. In ellipse marker mode, automarking is not available.

ROI selection mode

The dashed-rectangle icon enters the region of interest (ROI) selection mode. By clicking the left mouse button, moving the mouse and releasing the mouse button, one ROI can be drawn for each projection. Right-clicking the mouse deletes the current ROI. The ROI indicates the size of the area that is to be resampled for the aligned image stack.

Fiducial alignment

As soon as some marker positions have been measured in the images, a corresponding 3D marker model can be calculated. The availability of the reprojected markers (in yellow) is often a great help in finding the markers, especially if they are obscured by object structures.

The fiducial-alignment form is brought up by clicking the Actions/Fiducial alignment menu entry. It is shown in Fig. A.3. The object group in the upper left corner of the form selects the alignment parameters. There are four alignment parameters per projection (cf. Fig. 3.1). By checking the corresponding box, the parameter is included in the alignment process, i.e., it is iteratively adjusted to minimize the residual error between the measured and the reprojected marker positions. Otherwise, the parameter is kept at the initial value.

Next to the parameter selection, the total number of different markers is displayed, as well as the percentage of missing (i.e., yellow) markers. Below that,
the initial (before alignment) and the final residual error are given in pixels, cf. Eq. (3.13); also the number of iterations performed and the total time needed for the alignment.

Next to this, several parameter input fields are grouped in the Parameter group: the initial estimate of the in-plane rotation angle, the maximum number of iterations per parameter, and the tolerance. This last value is the relative amount by which the residual error must be decreased in order to continue the alignment process. If the residual error increases, or decreases by less than this amount, the alignment procedure is terminated. The total number of iterations (and thus the time necessary for alignment) can be influenced by changing the tolerance value and the maximum number of iterations per parameter.

Below the Parameter group is a list of all marker numbers currently in use. Initially, all markers are valid (indicated by highlighting in yellow). By clicking marker numbers in this list, the marker status is changed from ‘valid’ to ‘invalid’ and vice versa. Only valid markers are included in the alignment procedure. This way of designating invalid markers is useful when marker positions have been measured and one or several markers show large disagreement between the red and the green marker positions. In this case, the marker may have been misidentified in some projections, resulting in an invalid 3D marker. Instead of deleting the marker positions of this marker by hand in all projections, the marker can be declared invalid, and is then excluded from the alignment procedure. It is also possible to quickly observe the effect of using only a subset of all markers for the alignment.

The alignment procedure is started by clicking the Align button. A log is kept of all parameter changes; this log is displayed in the Process log window. After the alignment procedure terminates, the current values of three of the four alignment parameters are displayed in the graph window on the right of the form. In-plane rotation angle, uniform scale, and change (!) in tilt angle are plotted over the projection number. Clicking the graph window cycles between the three parameters.

Resampling

Once the fiducial alignment is satisfactory – i.e., the red and green markers are close together, and the final residual error is below 1 pixel –, the aligned tilt series can be interpolated from the original image stack. Clicking the Actions/Resample projections menu entry brings up the resampling parameters input form (cf. Fig. A.4). At the top of the form, the interpolation method is selected: nearest neighbour, bilinear, bicubic, or custom filter, listed in order of increasing quality and execution time. Usually an aligned tilt series is created using a fast interpolation method, such as bilinear interpolation, and inspected by eye by displaying it as a movie (e.g., using the Cantata software [Kho]). If the alignment quality is satisfactory, it is advisable to recreate the tilt series with a custom filter. Only by using a custom filter with a well-defined modulation transfer function can the original image information at different spatial frequencies be preserved, and the signal-to-noise gain concurrent with a reduction in size be achieved (cf. section 3.2.2).

The scaling factor input field specifies the isotropic scaling factor. A reduction in size can thus be obtained by using values smaller than 1. If a custom filter is used, the scaling factor should not be smaller than the cutoff frequency of the filter, to avoid aliasing. The padding value is the value that XALIGN substitutes if the
interpolation exceeds the bounds of the image. This is usually the case for non-zero in-plane rotation angles. If a normalized-intensity image is interpolated, the padding value should be 1. Using the *projections* input field, the resampling can be restricted to a subset of the projections. In this way it is possible to resample only the currently visible image.

If a custom filter is to be used, the file containing the filter weights must be selected by clicking the button inside the custom filter group. This brings up a file selector. XALIGN expects a file containing only the raw filter weights (in 4 byte *float* format), so it attempts to read the filter parameters from the file name. The first three numbers contained in the file name are read into the three input fields below. If the file name does not contain the filter parameters, they must be specified by the user.

When interpolating the X-ray microscopic images using a custom filter, XALIGN examines circular neighbourhoods for each sample location, assigns each pixel within the neighbourhood a weight depending on its distance from the sample location, and calculates the weighted sum of intensities over the neighbourhood (cf. section 3.2.2). The contents of the custom filter file are interpreted by XALIGN as the filter weights, with the first value belonging to distance zero, the last value belonging to the largest distance used (the filter radius $r_f$), and the other weights distributed equidistantly between 0 and $r_f$.

The number of filter weights is specified using the *filesize* input field, the filter radius $r_f$ using the *radius* field. The *samples per pixel* input field contains the number of weights per unit (i.e., pixel) distance. The filter specification is redundant, the three filter parameters are related via

$$filesize - 1 = radius \times samples \text{ per pixel}. \quad (A.1)$$

Because the filter file also contains the weight for distance zero, the *filesize* entry must be reduced by 1. For an example, consider Fig. A.4. The number of samples (weights) per pixel is 17, the total number of weights 423. The filter radius is therefore $(423 - 1)/17 = 24.8235$. 

![Figure A.4: XALIGN resampling parameters input form](image)

**Table:**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>radius</strong></td>
<td>24.8235</td>
</tr>
<tr>
<td><strong>samples per pixel</strong></td>
<td>17</td>
</tr>
<tr>
<td><strong>filesize</strong></td>
<td>423</td>
</tr>
<tr>
<td><strong>scaling factor</strong></td>
<td>0.5</td>
</tr>
<tr>
<td><strong>padding value</strong></td>
<td>1</td>
</tr>
<tr>
<td><strong>projections</strong></td>
<td>0–41</td>
</tr>
</tbody>
</table>


Interpolation using a custom filter is a computationally expensive task. For a filter radius of 24.8235, an average of 1936 neighbourhood pixels have to be evaluated for each sample. However, only by using a large filter radius can a steep cutoff of the filter be achieved.
Appendix B

Fourier-Bessel transform of a circular disc

The two-dimensional Fourier transform of a circularly symmetric function \( f(x, y) = f(r) \) is itself circularly symmetric and can be written as \( F(\rho) \). It is given by the Fourier-Bessel transform, also known as the Hankel transform of zero order. In polar coordinates [Goo88],

\[
F(\rho) = \mathcal{B}\{f(r)\}(\rho) = 2\pi \int_0^\infty r f(r) J_0(2\pi \rho r) \, dr, \tag{B.1}
\]

where \( J_0 \) is the Bessel function of the first kind, zero order. A circular disc is given by

\[
\text{circ}(r) = \begin{cases} 
1 & \text{for } r \leq D/2, \\
0 & \text{otherwise},
\end{cases} \tag{B.2}
\]

where \( D \) is the diameter of the disc. Using Eq. (B.1), the Fourier-Bessel transform of the circular disc is

\[
\mathcal{B}\{\text{circ}(r)\}(\rho) = 2\pi \int_0^{D/2} r J_0(2\pi \rho r) \, dr. \tag{B.3}
\]

Substituting \( r' = 2\pi \rho r \) yields

\[
\mathcal{B}\{\text{circ}(r)\}(\rho) = \frac{1}{2\pi \rho^2} \int_0^{\pi \rho D} r' J_0(r') \, dr'. \tag{B.4}
\]

Using the identity [BS91, p. 442]

\[
\int_0^x \xi J_0(\xi) \, d\xi = x J_1(x), \tag{B.5}
\]

with \( J_1 \) the Bessel function of the first kind, order one, the integral can be evaluated to yield

\[
\mathcal{B}\{\text{circ}(r)\}(\rho) = \frac{D}{2\rho} J_1(\pi \rho D). \tag{B.6}
\]
Appendix C

Ordered-queue based watershed algorithm

In the course of the watershed segmentation, new queue entries (or clients) must be created, and the entries for segmented voxels can be deleted. Using the general-purpose C functions malloc() and free() for these tasks means incurring a considerable administration overhead, and possibly increased memory consumption. Instead, a large continuous memory area is allocated as a client pool, from which queue entries are allocated and to which they are returned after processing.

The client data are the x-, y-, and z-coordinates of the corresponding voxel. Because each client is an entry in a queue, the client data also contains a pointer to the next entry in the queue:

```c
typedef struct _oqc { /* ordered queue client */
    unsigned short x, y, z; /* coordinates of corresp. voxel */
    unsigned int next; /* index of next client in queue */
} oqc;
```

For large volumes, the total number of voxels is on the order of \(10^7 \sim 10^8\). During processing, old queue entries are deleted and new entries are added; therefore, only a fraction of the total number of voxels will be queue entries at any one time. The memory requirement for the ordered queues is still quite large; the memory requirement for a single queue entry should therefore be minimal.

A suitable data type for the (positive) voxel coordinates \(x, y, z\) is unsigned short, with a range of \([0...65535]\) and a size of 2 bytes. Clients in the client pool are addressed using an unsigned int pointer next, with a range of \([0...4.3 \times 10^9]\) and a size of 4 bytes. The address range is well in excess of the number of voxels of a typical volume. The \(x\)-coordinate also doubles as a flag for the client state. Initially, all \(x\)-coordinates are set to 65535 (the maximum unsigned short value), indicating that the client is available for allocation. On deallocation, this value is restored. The client allocation algorithm is then a simple search for a client with \(x\) set to 65535.

The total size of a client is \(3 \times 2 + 4 = 10\) bytes, so the memory requirement for \(10^7\) clients will be 95 Megabyte. In addition, there are the memory requirements for the gradient map of the volume, and for the segmented volume itself.
The segmentation is initialized by scanning the label map for the initial markers and populating the queues accordingly:

```c
/* create oq clients from map block */
for (z = 0, ni = 0; z < zsize; z++)
    for (y = 0; y < ysize; y++)
        for (x = 0; x < xsize; x++, ni++) {
            /* has this voxel a label? */
            if ((label = map[ni]) != iooi) {
                /* compute corresponding queue */
                level = CAST(farray[ni], min, diff, levels);

                /* insert client into this queue */
                oqc_insert(pool, &pool_p, first, last, level,
                           x, y, z, 0, oqcp_size, &pc);
            }
        }
```

The volume is then segmented by processing the ordered queues, starting with the highest priority:

```c
/* process clients with highest priority first */
activeq = 0;

/* loop until all clients are processed */
for (;;) {
    /* extract client from active queue; stop if empty */
    if ((activeq = oqc_extract(pool, first, activeq,
                               &x, &y, &z, levels, &pc)) == levels) break;

    /* get voxel label from label map */
    label = map[z * ysize * xsize + y * xsize + x];

    /* loop over pixel neighbourhood: */
    for (c = 0; c < nbh.n; c++) {
        /* compute coordinates of neighbourhood voxel */
        nz = z + nbh.dz[c];
        ny = y + nbh.dy[c];
        nx = x + nbh.dx[c];

        /* is this voxel inside the volume? */
        if ((nx >= 0) && (nx < xsize) && (ny >= 0) &&
            (ny < ysize) && (nz >= 0) && (nz < zsize)) {
            /* compute index for the voxel */
            ni = nz * ysize * xsize + ny * xsize + nx;

            /* has this voxel a label? */
            if (map[ni] == iooi) {
                /* propagate label to the new voxel */
                map[ni] = label;
            }
        }
    }
}
```
/* compute queue for new voxel */
level = CAST(farray[ni], min, diff, levels);

/* insert the new voxel into this queue */
oqc_insert(pool, &pool_p, first, last, level,
    nx, ny, nz, activeq, oqcp_size, &pc);

This code uses the routine oqc_insert() to insert a client into an ordered queue:

#define NULL_UINT (unsigned int) -1
#define UNUSED_USHORT (unsigned short) -1

void oqc_insert(oqc *pool, unsigned int *pool_p,
    unsigned int *first, unsigned int *last,
    int level, unsigned short x,
    unsigned short y, unsigned short z, int activeq,
    unsigned int oqcp_size, unsigned int *pc) {
    /* inserts a new client in the ordered queue at level level,
     * if level >= activeq, else at activeq */
    unsigned int p, c;

    /* allocate new client */
    for (c = 0, p = *pool_p; c < oqcp_size; c++) {
        if (pool[p].x == UNUSED_USHORT) break;
        if ((++p) == oqcp_size) {
            p = 0;
            printf("restarting pool\n");
        }
    }
    if (c == oqcp_size) {
        fprintf(stderr,
            "cannot allocate new oq client, abort.\n");
        exit(-1);
    }

    /* write data to client */
    (*pc)++;
    pool[p].x = x; /* store voxel coordinates */
    pool[p].y = y;
    pool[p].z = z;
    pool[p].next = NULL_UINT; /* last client in queue */
    if (level < activeq)
        level = activeq; /* sorry, queue’s shut */
    if (first[level] == NULL_UINT) /* no clients */
Clients are extracted from the queues with the routine `oqc_extract()`:

```c
int oqc_extract(oqc *pool, unsigned int *first, int activeq,
    unsigned short *x, unsigned short *y,
    unsigned short *z, int levels,
    unsigned int *pc) { /* extracts a client from the ordered queue at activeq
    (if there are clients) and returns its coordinates.
    Returns the current active queue (=levels if all queues
    exhausted) */

    unsigned int p;

    /* no clients in active queue? -> search
     for next active queue or end of queues */
    if (first[activeq] == NULL_UINT)
        do activeq++;
    while ((first[activeq] == NULL_UINT) &&
        (activeq < levels));
    if (activeq == levels) return levels;

    /* get pointer to first element in active queue */
    p = first[activeq];
    (*pc)--;
    /* store pixel coordinates of that client */
    *x = pool[p].x;
    *y = pool[p].y;
    *z = pool[p].z;
    /* push head-of-queue pointer to next client */
    first[activeq] = pool[p].next;
    /* mark client as unused */
    pool[p].x = UNUSED_USHORT;
    return activeq;
}
```
Bibliography


[BF86] K.T. Brown and D.G. Flaming. *Advanced Micropipette Techniques for Cell Physiology*. Wiley, Great Britain, 1986. 2.2.2, 2.2.2


<table>
<thead>
<tr>
<th>Reference</th>
<th>Title</th>
<th>Publication Information</th>
</tr>
</thead>
</table>
Bibliography


[Fa. Hilgenberg. Technische Daten unseres Glases Nr. 500. personal communication. 2.1]


dreidimensionalen Bildgebungsverfahren für die Röntgenmikroskopie.*
PhD thesis, Georg-August-Universität Göttingen, Institut für Röntgenphysik, 1997. (document), 4.2.2, 4.2.2, 4.3.3

[MCKS99] Jianwei Miao, Pambos Charalambous, Janos Kirz, and David Sayre.
Extending the methodology of x-ray crystallography to allow imaging


[MSC98] J. Miao, D. Sayre, and H.N. Chapman. Phase retrieval from the magni-
Am. A*, 15:1662–1669, 1998. 4

[Neu96] Ulrich Neuhaeusler. Untersuchungen zum elementspezifischen Kon-
trast an Absorptionskanten mit dem Röntgenmikroskop. Master's the-
thesis, Georg-August-Universität Göttingen, Institut für Röntgenphysik, 1996. 1.2.2

[NGH+00] B. Niemann, P. Guttmann, D. Hambach, G. Schneider, D. Weiß, and
G. Schmahl. The Condenser-Monochromator With Dynamical Aper-
ture Synthesis For The TXM At An Undulator Beamline At BESSY II.
In W. Meyer-Ilse, T. Warwick, and D. Attwood, editors, *X-Ray Mi-
Physics. 1.2.1

[Pau] Brian Paul. The mesa 3-d graphics library. Available from
http://www.mesa3d.org. A

schweig, 1985. 1.3.1, 2


[Peu00] Markus Peuker. *Elektronenstrahl lithographie und Nanostruk-
turübertragung zur Herstellung von hochauflösenden diffraktiven
Röntgenoptiken aus Nickel.* PhD thesis, Georg-August-Universität
Göttingen, Institut für Röntgenphysik, 2000. 5.1.3

[PMBF95] P. Penczek, M. Marko, K. Buttle, and J. Frank. Double-tilt electron
tomography. *Ultramicroscopy*, 60:393–410, 1995. 3.1, 2

merical Recipes in C.* Cambridge University Press, Cambridge, 2nd
edition, 1992. 3.1, 3.1.7, 3.2.1, 3.2.1

[Rad17] J. Radon. Über die Bestimmung von Funktionen durch ihre Integralw-
erte längs gewisser Mannigfaltigkeiten. In *Berichte über die Verhand-
lungen der Königlich Sächsischen Gesellschaft der Wissenschaften zu


Acknowledgements

I would like to thank Prof. Dr. G. Schmahl for affording me the opportunity to work on this fascinating subject, investigating ‘classical’ X-ray microscopical techniques in conjunction with techniques from very different scientific areas, such as computed tomography. He demonstrated a continuous and vivid interest in my work, while at the same time giving me the opportunity to explore those avenues of research that I thought to be promising.

My next big thank you goes to Priv. Doz. Dr. Gerd Schneider. His interest in all aspects of CTCXM was very stimulating and his many suggestions often helpful – if sometimes arduous in their implementation. Long discussions about Fourier optics helped to improve my understanding of image formation in the X-ray microscope. Last but certainly not least, I was lucky to be able to use the cryo stage for the X-ray microscope that he developed together with Dr. Bastian Niemann (whom I would also like to thank), and without which none of the experiments on biological specimens would have been possible. I was also able to profit from his plunge-freezing device.

Dr. Peter Guttmann did a very good job babysitting the microscope (and its users) at BESSY. His constant good humour, even in the face of microscope malfunctions, was a great help during the hours of tilt series acquisition. It was also nice of him to let me wash down the capillaries with LN₂ (inside the cryo chamber), a somewhat dangerous action which might well have cost him a precious micro zone plate. Thanks to the BESSY staff for providing suitable illumination, and for making it possible to use the X-ray microscope until the very last days of BESSY I.

To my predecessors Dr. Johannes Lehr and Alexander Wolf I am grateful for investigating and assessing many of the experimental and numerical methods I was able to use for my experiments.

I thank Stefan Vogt for his cooperation on reconstructing Drosophila melanogaster cell nuclei. While the cell nuclei have yet to prove such grateful CTCXM subjects as Chlamydomonas reinhardtii, I am now reluctantly convinced that their interest to biologists might actually be greater.

Thanks to the friendly ladies at the Sammlung von Algenkulturen in Göttingen, who provided me with a never-ending supply of ‘green yeast’, trusty Chlamydomonas; and to Dr. Rolf Heblich for showing me how to operate the micropipette puller.

To Peter Nieschalk of the mechanical workshop I am grateful for bringing his skill and craftsmanship to bear on my amateurish drafts. Thanks also to Hans Dübener of the electronic workshop. Jochen Herbst I would like to thank for his help in the lab.

I enjoyed talking about tomography and microscopy with my successor, Christian Knöchel, and solemnly invest him with that venerable garment, the mantle of tomography.

My thanks to Stefan Vogt, Christian Knöchel, Gerd Schneider, and my father for reading this thesis, and suggesting improvements — most of which were imple-
I am deeply honoured to be the recipient of an award commemorating a very special man, Werner Meyer-Ilse, and would like to express my gratitude to the award committee.

During my years in Göttingen, my parents supported me generously in every possible way. I would like to thank them for all they have done for me.

I am very grateful to my wife for supporting me throughout this work. I could not have finished this thesis in time without her patience, love, and gentle urging. She will be relieved to know that all questions about formatting issues are now finally laid to rest.
Lebenslauf


1978-1982 Besuch der Grundschule in Detmold

1982-1991 Besuch des Gymnasiums Leopoldinum in Detmold

1988-1989 Besuch der Central High School in Omaha, NE, USA

Juni 1991 Abitur


1992-1997 Studium der Physik an der Universität Göttingen

1996 Studentische Hilfskraft an der Forschungseinrichtung Röntgenphysik der Universität Göttingen

1996-1997 Diplomarbeit an der Forschungseinrichtung Röntgenphysik der Universität Göttingen

Thema: “Optimierung der Kunststoffform bei der galvanischen Herstellung von Mikrozonenplatten aus Nickel”
Leiter: Prof. Dr. G. Schmahl

Mai 1997 Diplomprüfung in Physik

seit Juni 1997 Wissenschaftlicher Mitarbeiter am Institut für Röntgenphysik der Universität Göttingen

Promotion am Institut für Röntgenphysik der Universität Göttingen

Thema: ‘Computed tomography based on cryo X-ray microscopic images of unsectioned biological specimens’
Leiter: Prof. Dr. G. Schmahl

1999 Werner Meyer-Ilse Memorial Award for Excellence in X-ray Microscopy

09.06.2000 Heirat mit Michaela Amelung