

# A cylindrical specimen holder for electron cryo-tomography

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### Abstract

The 'missing wedge' is a long-standing problem in electron tomography, caused by the use of slab-like flat specimens, which increase in thickness when tilted to high angles. Attempts have been made to reduce the undesirable effects caused by the missing wedge, but the problem remains, particularly for the radiation-sensitive frozenhydrated specimens used for high resolution imaging.

Specimens with cylindrical symmetry offer a way to overcome this problem, since the thickness remains constant at all viewing angles. However, while this has been suggested before, it has never been demonstrated for frozen-hydrated specimens. In this work, I present a way to make cylindrical specimens for electron cryo-tomography, using thin-walled carbon tubes as specimen holders.

The tubes are made in a multi-step process, involving carbon deposition on glass micropipette templates and subsequent removal of the glass. Tube diameters are typically a few hundred nanometres, with a wall thickness of 10–20 nm. To make frozen-hydrated specimens, the tubes are filled with an aqueous sample and then plunge-frozen in liquid ethane.

Electron images acquired from the tubes have equal quality at all viewing angles, with a tilt range restricted only by the physical limits of the microscope. Tomograms from specimens such as gold particles and liposomes show that the effects of the missing wedge are substantially reduced, with much improved resolution along the electron beam axis. Structural features oriented in all directions are visible in the reconstructions, in marked contrast to tomograms acquired over a more restricted angular range. These results are promising, however some technical challenges remain before this method can be used routinely.

### Declaration

This thesis:

- is my own work and contains nothing which is the outcome of work done in collaboration with others, except where specified in the text;
- is not substantially the same as any that I have submitted for a degree or diploma or other qualification at any other university; and
- does not exceed the prescribed limit of 60,000 words.

Colin Palmer September 2012

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# Contents

List of Figures 1				
List of abbreviations				15
1	Introduction			17
	1.1	Electro	on cryo-tomography	17
		1.1.1	A brief history	17
		1.1.2	Limitations	19
	1.2	Image	reconstruction from projections	21
		1.2.1	The projection theorem	22
		1.2.2	Fourier reconstruction	23
	1.3	The m	issing wedge problem	25
		1.3.1	Effects of the missing wedge	26
		1.3.2	Reducing the missing wedge	31
	1.4	Cylind	lrical specimens for tomography	34
		1.4.1	Inherently cylindrical specimens	34
		1.4.2	Machining of cylinders from larger specimens	36
	1.5 Carbon nanopipettes		n nanopipettes	37
		1.5.1	A note on nomenclature	39
	1.6	Outlin	e of this work	39
2	Mat	erials a	and methods	41
2.1 Manufacture of carbon nanopipettes		facture of carbon nanopipettes	41	
		2.1.1	Pulling of glass micropipettes	41
		2.1.2	Carbon coating	41
		2.1.3	Oxygen plasma etching	43
		2.1.4	Hydrofluoric acid etching	44

		2.1.5 Imaging and characterisation		
	2.2	Specimen preparation		
		2.2.1 Attaching CNPs to grids		
		2.2.2 Preparation of liquid specimens		
		2.2.3 Specimen loading		
		2.2.4 Freezing and cryo-transfer		
	2.3	Electron microscopy		
	2.4	Image processing 50		
3	Man	ufacture and characterisation of carbon nanopipettes 51		
	3.1	Overview		
	3.2	Pulling of micropipettes 53		
	3.3	Carbon deposition		
	3.4	Oxygen plasma etching		
	3.5	Hydrofluoric acid etching		
	3.6	Characterisation of carbon nanopipettes 59		
	3.7	Discussion of the manufacturing process		
4 Cryo-tomography		o-tomography 67		
	4.1	Specimen preparation I		
		4.1.1 Nanopipette loading		
		4.1.2 Freezing and support		
	4.2	Tomography of gold particles 71		
	4.3	Tomography of ribosomes 73		
	4.4	Specimen preparation II		
	4.5	Tomography of liposomes 82		
	4.6	Tomography of whole bacteria 88		
5	Disc	pussion 93		
	5.1	Summary and significance of results		
	5.2	Limitations		
		5.2.1 Tube damage		
		5.2.2 Specimen positioning		
		5.2.3 Contamination and devitrification		
	5.3	Prospects for the future		
		5.3.1 Improving carbon nanopipette fabrication		

	5.3.2	Specimen support in the TEM	<b>)</b> 9
	5.3.3	Improving specimen loading	)0
	5.3.4	Combining nanopipettes with FIB milling	)2
5.4	Conclu	ısion	)4

### References

# LIST OF FIGURES

1.1	The principle of tomography	18
1.2	The principle of 3D reconstruction by Fourier synthesis	23
1.3	Discrete sampling in Fourier space	24
1.4	Synthetic reconstructions to demonstrate the missing wedge (part I) .	27
1.5	Synthetic reconstructions to demonstrate the missing wedge (part II)	29
1.6	Tomography acquisition schemes	32
2.1	Photograph of the furnace used for chemical vapour deposition $\ldots$	42
2.2	Apparatus used to support micropipettes during HF etching	44
2.3	Apparatus used for gluing grids to nanopipettes	45
3.1	Overview of the carbon nanopipette manufacturing process	52
3.2	Glass and carbon micropipettes	53
3.3	Micropipettes after carbon deposition	55
3.4	Micropipette measurements before and after oxygen plasma etching .	57
3.5	Carbon nanopipette measurements after HF etching	59
3.6	SEM image of a carbon nanopipette tip	60
3.7	Elemental maps and longitudinal EDX linescan of a carbon nanopipette	61
3.8	Transverse EDX linescan of a carbon nanopipette	62
4.1	Transmission electron micrograph of a blocked carbon nanopipette tip	68
4.2	A carbon nanopipette attached to a grid	69
4.3	Image from tilt series of gold particles in a carbon nanopipette	71
4.4	A slice from the reconstruction of the tube shown in Figure 4.3	72
4.5	Images from tilt series of ribosomes in a nanopipette	75
4.6	Slices from a reconstruction of the ribosome specimen	76
4.7	Fluorescence microscopy of a carbon nanopipette filled with liposomes	81

4.8	Projection images of liposomes inside a carbon nanopipette	82
4.9	Comparison of reconstructions from unprocessed and cutout images	83
4.10	Slices and line profiles from the liposome reconstruction	85
4.11	Volume rendering of tomograms from a large and small angular range	87
4.12	Photographs of a normal and modified Polara cartridge	89
4.13	Images from a tilt series of <i>C. crescentus</i> cells inside a carbon nanopipette	90
4.14	Slices from a reconstruction of C. crescentus cells in a carbon nanopipette	91
5.1	Bacterial growth inside a nanopipette	101
5.2	FIB milling of nanopipettes	103

## LIST OF ABBREVIATIONS

2D	Two dimensional
3D	Three dimensional
BHF	Buffered hydrofluoric acid
BSA	Bovine serum albumin
C. crescentus	Caulobacter crescentus
CNP	Carbon nanopipette
Cryo-ET	Electron cryo-tomography
СТ	Computed tomography
CVD	Chemical vapour deposition
E. coli	Escherichia coli
EDX	Energy-dispersive X-ray spectroscopy
EM	Electron microscopy
FIB	Focussed ion beam
HF	Hydrofluoric acid
MFC	Mass flow controller
sccm	Standard cubic centimetres per minute
SEM	Scanning electron microscope
SIRT	Simultaneous iterative reconstruction technique

- SNR Signal-to-noise ratio
- STEM Scanning transmission electron microscope
- TEM Transmission electron microscope
- WBP Weighted back-projection

# CHAPTER 1

#### INTRODUCTION

## 1.1 Electron cryo-tomography

#### 1.1.1 A brief history

Electron tomography is a three-dimensional (3D) imaging technique which can provide structural information about biological specimens with a resolution of a few nanometres. It has recently gained widespread popularity due to its ability to produce 3D images of cellular material at molecular resolution. It can therefore be used to bridge the 'resolution gap' between structural studies at the molecular and cellular levels. The highest resolutions are achieved using cryogenically-fixed specimens in the technique called electron cryo-tomography (cryo-ET), and the quality of the results of this method can be illustrated with reference to a small selection of recent examples (Beck et al., 2004; Brandt et al., 2010; Briggs et al., 2009; Scheffel et al., 2006). The technique has by now been reviewed many times; good reviews have recently been written by Plitzko and Baumeister (2007), Tocheva et al. (2010), Vanhecke et al. (2011), and Yahav et al. (2011). The review by Koning and Koster (2009) includes a comprehensive list of structural studies performed using cryo-ET before that date, while a detailed examination of many aspects of electron tomography can be found in the various contributions to the book by Frank (2006a).

The principle of tomography is to reconstruct a three-dimensional object by recombining images collected from it by projection at a range of angles (Figure 1.1). In the field of electron microscopy, this idea was developed independently by three research groups at around the same time (De Rosier and Klug, 1968; Hart, 1968; Hoppe, 1974; Hoppe et al., 1968). The method of De Rosier and Klug (1968) was initially the most



**Figure 1.1:** The principle of tomography. (a) Projection images of an object are collected at a range of angles. (b) Backprojection is performed computationally to produce a reconstruction of the object. Figure reproduced from Baumeister (2004) with kind permission from De Gruyter. The original article is available online at http://www.degruyter.com.

successful, since the bacteriophage tail specimen that was examined in their work possessed helical symmetry, which effectively allowed many different projection images to be extracted from a single micrograph. De Rosier and Klug (1968) were therefore able to produce the first fully three-dimensional reconstruction of a biological object obtained from transmission electron microscope (TEM) images. Subsequent results extended this method to the study of icosahedral viruses (Crowther et al., 1970a,b), and eventually led to the development of the structural technique of single-particle electron microscopy. This progression has been reviewed by Crowther (2010).

The more general method of tilting a unique, asymmetric specimen in the microscope to obtain multiple projection images was very cumbersome with the equipment available at that time. It would take another twenty years, and the advent of electronic cameras and computer control systems, before automation would allow tomography to become a routine technique for the investigation of biological specimens (Dierksen et al., 1992; Koster et al., 1992).

During the 1980s, the development of cryo-fixation for electron microscopy (reviewed by Dubochet et al., 1988) allowed biological material to be examined in a frozenhydrated, near-native state, free from the artifacts which plagued more traditional specimen preparation techniques (Al-Amoudi et al., 2004; Dubochet and Sartori Blanc, 2001; Murk et al., 2003). The combination of cryo-fixation with automated tomography techniques allowed the subsequent development of electron cryo-tomography (Bullitt et al., 1997; Dierksen et al., 1995; Grimm et al., 1997).

#### 1.1.2 Limitations

Despite its benefits, cryo-ET has some significant limitations, which were reviewed in detail by Plitzko and Baumeister (2007). Two of these are shared with electron tomography of plastic-embedded specimens: the specimen thickness must be small (well under 1  $\mu$ m), making observation of larger objects difficult; and typical specimens are wide and flat, preventing observation from high tilt angles. This leads to an effect called the 'missing wedge' problem, which will be discussed in much more detail in section 1.3.

Other limitations apply much more significantly to frozen-hydrated specimens: image contrast in unstained biological material is very weak, leading to images with a very low signal-to-noise ratio (SNR); such specimens are also rapidly damaged by the passage of the electrons used for imaging.

A variety of techniques have been used and developed to reduce the severity of these limitations. Contrast in unstained material can be increased by the use of large defocus settings in the TEM. This increases the phase contrast at low spatial frequencies, allowing objects to be seen in the images, but causes oscillations in the contrast transfer function at higher frequencies which complicates interpretation of the images if a high resolution is required. A high defocus also requires the use of a highly coherent electron source, in the form of a field-emission gun (Koster et al., 1997).

Specimen damage caused by the incident electron radiation is inherently reduced by the use of frozen specimens, since cooling to 90 K has a significant protective effect. Additional gains were expected from further reduction of the temperature to 4 K (Koster et al., 1997), but recent results have failed to show a benefit at this temperature (Comolli and Downing, 2005; Iancu et al., 2006). The only other way in which electron damage has been substantially reduced has been the development of efficient low-dose techniques, in which over 95% of the electrons passing through the specimen are used for the recording of projections (Koster and Bárcena, 2006).

With these techniques, the allowable electron dose becomes a relatively simple compromise between increased image SNR as a result of higher doses, versus progressive damage to the specimen which restricts the achievable resolution. For single-particle electron microscopy studies aiming for sub-nanometre resolution, the allowable dose is approximately 10–20 e<sup>-</sup>/Å<sup>2</sup>, while for tomographic observation at a resolution of 2–6 nm, a much higher total dose of 50–200 e<sup>-</sup>/Å<sup>2</sup> can be used (Baker and Rubinstein, 2010).

The problem of specimen thickness has been approached in many ways. A primary method of increasing the usable thickness has been to increase the accelerating voltage of the microscope. This reduces the strength of the interaction between the electrons and the specimen, allowing the electrons to penetrate thicker specimens and reducing electron damage. However, the reduced interaction with the specimen also reduces the available contrast, limiting the gains which are available with this method. Consensus has settled on a compromise of 200–300 kV, which allows the use of specimens up to a thickness of approximately 500 nm (Plitzko and Baumeister, 2007). At these thicknesses, though, inelastic scattering of electrons causes significant degradation of the image, through both multiple scattering effects and chromatic aberration.

A major improvement in cryo-ET of thick specimens was achieved with the use of energy filtering (Grimm et al., 1997), which allows the inelastically-scattered electrons to be removed from the image. However, with very thick specimens, most of the incident electrons will undergo inelastic scattering and be removed, meaning that few electrons are available to form an image. This situation can be improved a little by most-probable loss energy filtering (Bouwer et al., 2004; Grimm et al., 1996), and there is the potential for further improvement in future with the use of aberration-corrected TEMs. However even this will not be able to prevent the degradation of the image by multiple scattering events in thick specimens.

The other major approach to the problem of specimen thickness has been to simply reduce the specimen size. Perhaps the easiest way in which this can be achieved for whole cells is to use the smallest cell type for which the biological question of interest is still relevant (Gan et al., 2011; Henderson et al., 2007). Beyond this, physical thinning of the specimen is required. Sectioning frozen material with a microtome has developed into a useful technique for 2D electron microscopy (Al-Amoudi et al., 2004; Dubochet, 2012; Zuber et al., 2005), and has also been extended for use in cryo-tomography (Al-Amoudi et al., 2007; Bouchet-Marquis and Hoenger, 2011; Hurbain and Sachse, 2011; Pierson et al., 2010). The benefit is that thin sections can be cut from material of almost any size, and examined by electron tomography at molecular resolution. However, cryo-sectioning suffers from a multitude of artifacts and is technically very challenging, which has so far restricted its widespread use (Dubochet, 2012).

A very interesting recent development has been the use of focussed ion beam (FIB) milling to prepare thin sections from frozen biological material (Hayles et al., 2010; Marko et al., 2007; Rigort et al., 2010, 2012a,b; Wang et al., 2012). Results from

this technique are promising, and specimens prepared in this manner suffer from none of the artifacts which affect cryo-sections. However, technical challenges remain, in particular the problem of locating a desired specimen sufficiently accurately (at a sub-cellular level) as to be able to isolate it in a thin lamella with the FIB. Recent advances in correlative microscopy techniques will undoubtedly help with this (Rigort et al., 2012b), and it will be exciting to follow developments in this field in the next few years.

### **1.2 Image reconstruction from projections**

The theory of the reconstruction of an object from its projections, which is fundamental to all forms of tomography, was first derived by Radon (1917, translated into English in 1986). However this work did not become widely known until much later, and so the same theory was derived independently in several fields where such reconstruction techniques are useful, including medical radiology (Cormack, 1963, 1964), radio astronomy (Bracewell and Riddle, 1967), and electron microscopy (De Rosier and Klug, 1968).

The Radon transform of an object of *n* dimensions consists of the complete set of projections of the object onto (n-1)-dimensional hyperplanes. For a two-dimensional object, the Radon transform is the set of line integrals in all directions through the object, and is often referred to as the sinogram because the projection of a single point follows a sinusoidal path in the transform.

The task of tomography is to invert this projection transform in order to recover the original object from a set of its projections. There are several approaches to the problem, including direct inversion of the Radon transform (Cormack, 1963, 1964; Zeitler, 2006), back-projection in real space (Gilbert, 1972a; Radermacher, 2006), and synthesis of the object's Fourier transform followed by back-transformation (Crowther et al., 1970b; De Rosier and Klug, 1968). In the simple case of 2D projections taken around a single tilt axis, the problem is reduced to the reconstruction of individual two-dimensional slices from one-dimensional projections at each position along the tilt axis, and in this case the three reconstruction methods just mentioned can be shown to be mathematically equivalent (Radermacher, 2006). Because of this equivalence, I will discuss reconstruction in terms of the Fourier synthesis method, since this offers the most intuitive understanding of the problems caused by missing information.

#### **1.2.1** The projection theorem

Fourier reconstruction methods are based on the projection theorem (also called the central section theorem or the Fourier slice theorem). The derivation of this theorem is simple, and it is worth explaining so that the 'Fourier synthesis' used in the reconstruction methods can be understood.

The projection theorem states that the Fourier transform of a projection of an object is identical to a central section (i.e. a section passing through the origin) of the Fourier transform of the complete object. This result follows directly from the definition of the multi-dimensional Fourier transform. Following the notation used by Hawkes (2006), the three-dimensional Fourier transform is defined as:

$$F(X, Y, Z) = \iiint \rho(x, y, z) \exp\left\{-2\pi i (Xx + Yy + Zz)\right\} dx \, dy \, dz \tag{1.1}$$

where *x*, *y*, *z* and *X*, *Y*, *Z* represent the coordinates in real and reciprocal space, respectively; *F* represents the Fourier transform; and  $\rho(x, y, z)$  is the density function which defines the object.

Any central section of this Fourier transform can be found (with appropriate choice of coordinates) by setting Z = 0:

$$F(X, Y, 0) = \iiint \rho(x, y, z) \exp\{-2\pi i (Xx + Yy)\} dx dy dz$$
$$= \iint \left\{ \int \rho(x, y, z) dz \right\} \exp\{-2\pi i (Xx + Yy)\} dx dy$$
(1.2)

The expression  $\int \rho(x, y, z) dz$  is the line integral of the density function in the *z*-direction at position (x, y), which is equal to the projected mass of the object along this line and can be represented as  $\mu(x, y)$ . Equation (1.2) can then be rewritten as:

$$F(X, Y, 0) = F(X, Y) = \iint \mu(x, y) \exp\{-2\pi i (Xx + Yy)\} dx dy$$
(1.3)

which is simply the two-dimensional Fourier transform of a projection of the object.

When the projection theorem is used in electron microscopy, the implicit assumption is that transmission electron images represent true projections of a physical aspect of the object's structure (such as the mass density or the electrical potential density). The question of how well-justified this assumption is has been examined in detail by Hawkes (2006). It is sufficient here to say that small deviations are likely, particularly



**Figure 1.2:** The principle of 3D reconstruction by Fourier synthesis. Figure adapted from De Rosier and Klug (1968) with kind permission from Macmillan Publishers Ltd.

for thick specimens, but the deviations are not expected to be large enough to cause concern at the resolutions typically obtained in cryo-tomography.

#### **1.2.2** Fourier reconstruction

Using the projection theorem, it can be seen that the complete three-dimensional Fourier representation of an object could be built up by combining the sections obtained from projection images (Figure 1.2). Ideally, with an infinite number of projections, each sampled at an infinitesimally small spacing, the object could be reconstructed perfectly, since Fourier space would be entirely filled by the synthesis of all of the projection transforms. Of course, in reality this is unattainable for several reasons: the number of projection images is limited by both practicality and the electron dose limit for biological specimens, the spacing of sampling points is limited by the pixel size of the camera (or the grain size in the case of film), and most significantly, the images are corrupted by various sources of noise. All of these factors limit the resolution to



**Figure 1.3:** Discrete sampling in Fourier space. The lines represent the Fourier transforms of projection images, with coefficients available at the points marked with circles. The hollow squares represent the coefficients required for the inverse transform used to reconstruct the object. Interpolation is required to obtain these coefficients from the known ones, and where the distance between known and required points is too large this interpolation will produce errors in the reconstruction. Figure reproduced from Frank (2006b) with kind permission from Springer Science and Business Media.

which full information about the object is available.

This can be seen graphically in Figure 1.3, where (in a single plane representing a 2D section of the full 3D Fourier space) the available Fourier coefficients from the projection images are shown as circles regularly spaced along the black lines. The spacing of these points and the length of the lines are determined by the image magnification, in combination with the pixel size and the width of the camera. The grid of squares in the figure represents the Fourier coefficients which are required for back-transformation of the object into Cartesian coordinates in real space. It can be seen that in many cases, the required coefficients do not precisely overlap with those available from the projections. However, interpolation can be used to find the desired coefficients to a tolerable degree of accuracy when the known values are not too distant. This makes it clear that the available resolution depends on the angular spacing (marked in Figure 1.3 as  $\Delta \theta$ ) as well as the size and sampling of the individual images, since beyond a certain distance from the centre of Fourier space, the available values will be spaced too widely for accurate interpolation.

The precise form of this relationship was derived in an off-cited paper by Crowther et al. (1970b).<sup>1</sup> They established that for equally-spaced projections around a single axis, the necessary number of views, n (referred to as m by Crowther et al. (1970a); n

<sup>&</sup>lt;sup>1</sup>Also often mis-cited as Crowther et al. (1970a).

is more commonly used in subsequent literature), is related to the size of the object, D, and the desired resolution, d, by a simple formula:

$$n = \frac{\pi D}{d} \tag{1.4}$$

The same relationship was also found independently by Bracewell and Riddle (1967), but in the field of electron tomography this formula is commonly referred to as the 'Crowther criterion'. A vital assumption of this calculation is that the projection images are equally spaced over a full range of 180°. Crowther et al. (1970b) showed that when this condition is not met, the resolution drops substantially, and equation (1.4) is no longer valid. The implications of this are discussed in the next section.

### 1.3 The missing wedge problem

Typical specimens used for electron tomography take the form of flat, extended slabs, because they are prepared as a thin layer on a supporting metal grid. This is convenient for several reasons, and methods for preparing specimens in this form are well developed, though optimising the process for new specimens is still laborious. However, there is a key disadvantage of this type of specimen when used for tomography. As discussed in the previous section, an ideal data collection scheme for tomography involves taking projection images from an evenly-spaced set of tilt angles covering a full angular range. However, when a slab-like specimen is tilted, the effective thickness increases. At low tilt angles this is not a significant problem, but at high angles this increase becomes severe: the thickness is multiplied by factor of two at a tilt angle of 60°, three at 70°, and almost six at an angle of 80°.

Because thickness is so important in determining the quality of TEM images, this effect means that there is a substantial range of tilt angles where projection images cannot be obtained. Reconstruction of an incomplete tilt series leads to a wedge-shaped section of Fourier space where no information is available, and therefore this is commonly referred to as the 'missing wedge' problem.

For convenience in discussing the reconstruction process and the missing wedge, I will use the conventional coordinate system for 3D reconstructions from a single-axis tilting scheme. In this system, the coordinates x, y and z refer to positions within the specimen. The y-axis is the tilt axis, the z-axis is the 'vertical' direction (which is aligned with the electron beam axis when the specimen is not tilted), and the x-axis

is perpendicular to the other two.<sup>2</sup> The equivalent directions in Fourier space are referred to as X, Y and Z.

#### 1.3.1 Effects of the missing wedge

The primary effect of the missing wedge is to remove information from the reconstruction about spatial frequencies which are oriented in directions within the wedge. In practice, this means that extended structures (such as cell membranes) running parallel to the plane of the specimen are entirely lost from the reconstruction, while compact objects become smeared and stretched along the *Z*-axis.

These effects can be seen most clearly in reconstructions generated from synthetic data, where the effects of noise are absent and any image degradation can be attributed entirely to the reconstruction algorithm.

Figure 1.4a shows a phantom image composed of a large circle, linear features oriented in several directions, and a small filled circle which breaks the symmetry of the image. Next to this is the sinogram (or Radon transform) of the image, obtained by projecting the image onto a line at 1° intervals. The sinusoidal trace from the small circle stands out, while the overlapping fainter sinusoids from the straight lines in the image are also visible. Below the phantom and its sinogram, six different reconstructions of the image are shown, using restricted sets of the projections to simulate the effects of the missing wedge. The 2D image shown is equivalent to one slice from a single-axis 3D reconstruction, with the *x*-axis horizontal and the *z*-axis vertical. These reconstructions were all calculated using the weighted back-projection algorithm (WBP; Radermacher, 2006) as implemented in the IMOD software package (Kremer et al., 1996).

The reconstruction from a full angular range  $(\pm 90^{\circ})$  is a good likeness of the original phantom image, though some contrast has been lost. The next reconstruction  $(\pm 89^{\circ})$  looks similar, though some horizontal white bands are visible extending from horizontally-oriented objects: most obviously the horizontal straight lines, but also the horizontal parts of the outer circle. (On close inspection of the  $\pm 90^{\circ}$  figure, some radial linear features are visible in what should be empty space. These are caused by the same phenomenon, and are artifacts of the tilt increment used in the reconstruction.)

As the angular range is further reduced to  $\pm 80^{\circ}$ , these white bands widen into

<sup>&</sup>lt;sup>2</sup>The handedness of tomographic reconstructions is often ill-defined unless great care is taken to correctly identify and preserve it. None of my work is concerned with the handedness of the specimens, and so I have made no attempt to ensure its preservation or correctness. For convenience when discussing the reconstruction process I will generally refer to a right-handed system of coordinates.



**Figure 1.4:** Synthetic reconstructions to demonstrate the missing wedge (part I). Full explanations are given in the text. (a) A phantom figure, its sinogram, and several reconstructions with variable angular range. (b) A comparison of the WBP and SIRT algorithms for the  $\pm 60^{\circ}$  reconstruction from part (a).

wedges, and the horizontal straight lines in the object become quite poorly resolved. The same effect starts to degrade the completeness of the outer circle, though at  $\pm 80^{\circ}$  the entire circle can still be clearly identified. Below this angular range, however, the circle becomes broken, and the wedge artifacts broaden to form strong linear features in the reconstruction, which strikingly extend at a tangent from the points where the image features cease to be resolved. When the tilt range decreases to  $\pm 45^{\circ}$ , these artifacts also appear from the diagonal lines in the figure, indicating that these too would be lost if the range were further reduced.

The effect of the reconstruction algorithm is shown in Figure 1.4b. Here, the  $\pm 60^{\circ}$  WBP reconstruction is shown again, and compared with reconstructions using different numbers of iterations (20 and 60) of the Simultaneous Iterative Reconstruction Technique (SIRT; Gilbert, 1972b), also as implemented in the IMOD package. At twenty iterations, this algorithm has a slight low-pass filtering effect which makes features stand out more prominently but with blurred edges. At sixty iterations, the high-frequency information is restored, and (ignoring the circular artifacts around the image edges) the image quality is similar to the WBP reconstruction. This demonstrates that the SIRT algorithm cannot compensate for the missing wedge. In these circumstances, SIRT appears to offer no benefits, though with more realistic noisy images, the improvement over WBP can be more significant.

At this point it is interesting to note that most electron tomography experiments are carried out using an angular range between  $\pm 45^{\circ}$  and  $\pm 60^{\circ}$ . Looking at the reconstructions in Figure 1.4a, it is clear that a substantial amount of information is lost, and horizontal objects are simply not resolved. The ends of the horizontal lines can be seen as localised features, but it is far from clear what they represent in the absence of knowledge of the full structure. In a noisy reconstruction they would almost certainly be lost completely. The star-like object in the centre of the figure shows that the horizontally-oriented parts of more complicated shapes can be lost just as much as isolated horizontal objects.

However, a legitimate objection is that most biological objects do not contain such straight lines and tend to have more irregular shapes. (Although, of course, many important biological questions are concerned with the shapes of membranes and protein filaments. At the typical few-nanometre resolution of electron tomography, such objects can be entirely straight and smooth, and the reconstructions in Figure 1.4a show just how much information about such objects might be lost due to the missing wedge.) To examine the effect of the missing wedge on more irregular objects, a



**Figure 1.5:** Synthetic reconstructions to demonstrate the missing wedge (part II). Full explanations are given in the text. **(a)** A phantom figure (adjusted from that presented in Figure 1.4a) with straight lines replaced by wavy ones, and several reconstructions. **(b)** A phantom figure with full-width horizontal lines (which extended for eight times the width shown here), and a reconstruction showing that the lines disappear entirely even with an almost-complete tilt range.

second phantom image was created with the straight lines replaced by wavy ones. This phantom and reconstructions of it are shown in Figure 1.5a. Here, the reconstruction artifacts are similar, but the horizontal features are still resolved even with an angular range of  $\pm 45^{\circ}$ . However it is striking that they have become severely elongated in the vertical direction, and their contrast is much lower than that of objects in other orientations.

A final instructive demonstration is shown in Figure 1.5b. Another phantom image was created from the original shown in Figure 1.4a, by the addition of two horizontal lines. The phantom was widened (not shown) so that these lines extended for eight times the original image width. A reconstruction of this, even at an almost-complete angular range of  $\pm 89^{\circ}$ , entirely fails to resolve the horizontal lines. This illustrates that, when a horizontal feature is so wide as to be effectively infinite, it can only be resolved by including a projection which is precisely aligned with it. This is directly analogous to the extended slab of the specimen in normal tomography experiments, and explains why the layer of plastic or ice is usually not directly visible in the reconstruction. The presence and thickness of the layer are instead normally inferred from features assumed to be on the layer's surface.

This discussion and the examples in Figures 1.4 and 1.5 make it clear that the resolution in tomograms with a missing wedge is anisotropic. As discussed before (section 1.2.2, equation (1.4)), the resolution formula derived by Crowther et al. (1970b) applies for tomograms with an even angular sampling. Crowther et al. (1970b) also derived a formula for the resolution when the angular range is limited, but their calculation assumes the resolution remains isotropic, and so it severely underestimates the resolution in the well-sampled region. A refinement of this calculation was given by Radermacher (1988) who states that, for regularly-spaced projections in a restricted range around a single axis, the Crowther relation (equation (1.4)) remains valid for the resolution in the *x* direction. The resolution in the *y* direction is determined directly by the resolution in the individual projection images and their alignment accuracy, while the z resolution depends on the missing wedge. Radermacher (1988) expresses the z resolution as the product of the x resolution and an elongation factor which depends on the maximum tilt angle. This is based on an approximation of the three-dimensional point-spread function as an ellipsoid. For compact objects, this seems reasonable, but a simple elongation factor fails to capture the way in which linear or planar objects can entirely disappear if oriented appropriately with respect to the missing wedge.

#### 1.3.2 Reducing the missing wedge

Because of the problems caused by the missing wedge, various attempts have been made to reduce the severity of its effects. The ideal solution is to remove the missing wedge completely. However, this can only be achieved by moving away from the flat specimens normally used for tomography, and instead using compact specimens, ideally with cylindrical symmetry. This is the approach used in this work, and therefore it is discussed in detail in section 1.4.

Without complete removal of the missing wedge, there are two ways in which its effects can be reduced. The amount of missing information can be reduced by changing the geometry of data collection, or prior information can be used in the reconstruction to remove artifacts. The latter technique has been developed for materials science applications, where prior constraints on the reconstruction can be very strong, for example the knowledge that the specimen is composed entirely of only one or a few types of atoms. In this case, accurate reconstructions can be obtained from relatively few projections by using an algorithm which forces the reconstruction to take on discrete values, and in favourable cases the effects of the missing wedge can be entirely removed (Batenburg et al., 2009; Myers et al., 2010; Roelandts et al., 2012). A similar effect can be obtained using the technique of compressed sensing. For this method, the object (technically, any transformation of the object into a suitable representational basis) is assumed to be 'sparse', meaning that only a small proportion of the coefficients used to describe the object have non-zero values. Applying a sparseness condition during reconstruction can allow accurate tomograms to be generated from very few projection images (Saghi et al., 2011). The discrete tomography technique can be viewed as a particular form of compressed sensing. Both methods are discussed in reviews by Leary et al. (2012) and Saghi and Midgley (2012).

However, these kinds of prior information are generally not available for biological specimens.<sup>3</sup> Here, attempts to reduce the effects of the missing wedge have focussed on alternative data collection geometries which can increase the coverage of Fourier space in the reconstruction. The simplest of these is dual-axis tomography (also known as double-tilt tomography; Figure 1.6b), in which a tilt series is collected around a single axis, after which the specimen is rotated by 90° and a second tilt series is collected.

<sup>&</sup>lt;sup>3</sup>I am not aware of any ways in which biological objects at tomographic resolution could be represented in the sparse manner required for compressed sensing, but I am also unaware of any proof that this is not possible. If such a representation could be found, then biological electron tomography might be able to exploit the benefits of this approach.



**Figure 1.6:** Tomography acquisition schemes. Each image is represented by a plane in Fourier space, representing the central section derived from it. Empty regions show areas of Fourier space which are not sampled. (a) Single-axis tilt, where the missing volume is in the shape of a wedge. (b) Dual-axis tilt, where the missing volume forms a pyramid. (c) Conical tilt, where the missing volume forms a cone. Figure reproduced from Lanzavecchia et al. (2005) with kind permission from Elsevier.

ted around an axis orthogonal to the first (Mastronarde, 1997; Penczek et al., 1995). This has become routine for plastic-embedded specimens, but it was only more recently that suitable equipment was developed to allow the use of dual-axis tomography with frozen-hydrated specimens (Iancu et al., 2005; Nickell et al., 2003). In dual-axis tomography, the missing wedge is reduced to a 'missing pyramid', with a corresponding reduction in the proportion of Fourier-space information which is absent. The angle often used to estimate the size of the missing wedge (for example by Nudelman et al., 2011) is  $\pm$ 70°. When this range is used, tilting around a single axis gives coverage of 78% of Fourier space, while dual-axis tilting to the same angle covers 93%. However, the maximum tilt angle which provides useful information is often much less than 70°, and the coverage of Fourier space will be correspondingly reduced.

In any electron tomography experiment, there is a compromise between the allowable electron dose and the number of images which can be taken. In dual-axis tomography, the total dose must be divided between two separate tilt series. Though the signal-to-noise ratio (SNR) in the complete tomogram is determined by the total dose (McEwen et al., 1995), the individual images must retain a sufficiently high SNR to allow their mutual alignment. In a dual-axis scheme, this may require the angular increment to be increased so the total dose is not split between too many images. However, restriction of the angular increment reduces the available resolution. For frozen-hydrated specimens the dose limitation is particularly severe, and the gains in isotropy of resolution from a double-tilt scheme may be offset by the increased electron dose or reduced number of images per tilt series which is required to obtain images from both axes. Certainly, dual-axis tomography is not routinely used for cryogenic specimens, though since its development (Iancu et al., 2005; Nickell et al., 2003) several groups have made at least some use of the technique (Dudkina et al., 2010; Komeili et al., 2006; Rouiller et al., 2008; Zheng et al., 2009).

The double-tilt technique has also been extended to multiple-axis tilting (Messaoudi et al., 2006). This increases the number of faces of the missing pyramid and so reduces its volume, however this moves to an extreme position in the compromise between electron dose and information completeness. This approach is therefore only feasible for plastic-embedded specimens which are not too sensitive to electron damage. Even with radiation-tolerant specimens, this method may not provide an optimal compromise between the electron dose and the level of resolution isotropy thus obtained.

The final data collection scheme which has been used for tomography is conical tilt (Figure 1.6c), in which the specimen is tilted to a fixed angle and then rotated in-plane around a full 360° (Lanzavecchia et al., 2005; Zampighi et al., 2005, 2011). This reduces the missing wedge to a 'missing cone', and further increases the Fourier space coverage when compared to dual-axis tomography at the same maximum tilt angle. The technique also has the additional benefit of providing a relatively even sampling of Fourier space. However, it has the notable disadvantage that all images are taken at a high tilt angle where the quality of the images is lowest, reducing resolution and making image alignment more difficult. The technique has so far only been used for thin (< 50 nm) plastic-embedded or metal replica specimens, which are relatively insensitive to radiation and for which the image deterioration at high tilt is not too severe. To my knowledge, a suitable holder for conical tomography of frozen specimens has not been developed, and, as for the multiple-tilt technique, it is doubtful whether conical tomography would provide any improvement for thick frozen specimens.

In all of these cases, the proportion of missing information in Fourier space is reduced by the addition of more images at different angles. Much of the work on these image acquisition schemes demonstrates that the resolution does become more isotropic, as intended (but at the cost of higher radiation doses or reduced angular increments). However, there is always still some missing information: in particular, the spatial frequency information along and near the *z*-axis is still absent, meaning

that extended structures running within the plane of the specimen (including the slab of the specimen itself) are not resolved at all in the reconstructions, and there is still always at least a small amount of elongation and smearing of compact structures. The only way to prevent these problems is to obtain information about the entirety of Fourier space, which can most economically be done by using a full 180° rotation around a single axis. (As well as being a simple extension of the single-tilt scheme, a 180° single-axis rotation is also the limit of the conical tilting method when the tilt angle is increased to 90°.) To avoid a prohibitive thickness in any viewing direction, full tilt around one axis requires the use of a specimen which is compact in at least the other two axes. The most natural shape which fits this requirement is a cylinder; the use of cylindrical specimens for tomography is discussed in the next section.

### 1.4 Cylindrical specimens for tomography

In principle, there are two ways to obtain a cylindrically-shaped specimen. Either a specimen can be prepared which is naturally cylindrical in shape, or a cylinder can be cut from a larger piece of bulk material. Both of these methods have been used for tomography, and they will be discussed in turn.

### 1.4.1 Inherently cylindrical specimens

The idea of using cylindrical specimens for tomography is not a new one. In the field of medicine, X-ray computed tomography (CT) has always used specimens which are roughly cylindrical in shape, and from its initial development onwards has made use of projection images from an angular range of at least  $180^{\circ}$  (Hounsfield, 1973). The resolution of medical CT imaging is on the order of millimetres, and the technique is therefore not useful for research in cell and molecular biology. The recent development of zone plate lenses has allowed the related technique of soft X-ray micro-tomography to reach resolutions of approximately 50 nm (Larabell and Le Gros, 2004; McDermott et al., 2012; Schneider et al., 2002, 2010), using cylindrical specimens of frozen-hydrated cells mounted in glass micropipette capillaries. For cell biological studies, this technique has certain technical advantages over electron tomography: it does not require the specimen to be in a vacuum, and the natural absorption profile of soft X-rays allows the imaging of biological material with good contrast, even when embedded in a thick (10 µm) layer of ice and surrounded by glass (Le Gros et al., 2005). Observation

of frozen specimens of this size by TEM is simply not possible. However, X-ray tomography simply cannot match the resolution available in electron tomography when more detailed imaging is required.

In a different area of research (using warm, dry, non-biological material), a modified X-ray micro-tomography technique using multiple-wavelength imaging has allowed the absorption signatures of individual chemicals to be identified, permitting threedimensional chemical mapping (Hitchcock et al., 2008). Interestingly, for this study the X-ray absorption of glass at certain wavelengths caused problems, and so carbon nanopipettes – to be discussed in section 1.5 – were used as specimen holders.

In contrast to this successful use of cylindrical specimens in several forms of X-ray tomography, progress towards effective isotropic imaging in electron tomography has proved to be more challenging. Over twenty years ago, Turner, Barnard and colleagues published the results of the first use of cylindrical specimens for biological electron tomography. In a conference paper (Turner et al., 1991) and then a full report (Barnard et al., 1992), they presented their development of a full-rotation stage for cylindrical specimens in the transmission electron microscope. They demonstrated the use of this stage with a specimen of dry puffball spores attached to the outside of a standard glass micropipette, and successfully reconstructed a tomogram from a tilt series covering a full angular range of 180°. However, imaging through the glass walls of the micropipette required the use of a high-voltage (1 MeV) electron microscope and extremely high radiation doses (approximately 3,200 e<sup>-</sup>/Å<sup>2</sup>), and the resolution of their images was poor. This group has not reported any subsequent results from this system, and their paper (Barnard et al., 1992) remains the only published example of full-tilt electron tomography of biological specimens.

More recently, Lee and Subramaniam (2004) reported, at a conference, a replication of the glass capillary method. Few details are available, but their abstract suggests that they succeeded in obtaining tomograms of pre-stained bacteriophage particles inside glass micropipettes. The acceleration voltage that they used is not mentioned, nor is there any indication of the quality of the results, except for a note that some beam-induced charging effects seen with the glass capillaries were eliminated by the deposition of a layer of carbon on their outer surface. The authors have published nothing further on the subject in the years since.

#### 1.4.2 Machining of cylinders from larger specimens

After its original development in biological sciences, the technique of electron tomography has recently been gaining popularity for nano- and atomic-scale studies in materials science (Bar Sadan et al., 2008; Saghi and Midgley, 2012; Weyland et al., 2006). (For technical reasons, tomography of hard materials is usually performed in a scanning transmission electron microscope, or STEM.) Concurrently with these developments, the use of focussed ion beam (FIB) technology for nanofabrication and (S)TEM specimen preparation has rapidly become widespread (Leer et al., 2007). The FIB is a versatile tool, and has been used to create pillar-shaped specimens for atom probe microscopy as well as TEM examination (Lozano-Perez, 2008; Sugiyama and Sigesato, 2004). Many groups have presented different ways of modifying this FIB milling technique for the production of thin planar or cylindrical specimens suitable for TEM or STEM tomography (Fukuda et al., 2004; Hernández-Saz et al., 2012; Jarausch et al., 2009; Kawase et al., 2007; Koguchi et al., 2001; Yaguchi et al., 2008). Others have developed various specimen stages to allow full rotation of specimens inside the STEM (Guan et al., 2011; Jornsanoh et al., 2011; Kamino et al., 2004a; Tanigaki et al., 2012; Yaguchi et al., 2008), and an interesting analysis has been published on the properties of tomographic reconstructions from thick cylindrical specimens (Kato et al., 2008).

Some of the FIB-based preparation techniques developed for materials science might be applicable to plastic-embedded biological material. Indeed, two groups have published reports demonstrating this idea. Heymann et al. (2006) and Kamino et al. (2004b) both used FIB milling to create pillar-shaped specimens from a block of resinembedded yeast cells, and both groups used a lift-out strategy in which a lamella is cut with the FIB and then extracted for further milling. Kamino et al. (2004b) made use of repeated STEM observations to guide the milling process, and finished by producing a rectangular pillar a few micrometres across. This contained some embedded cells which could be seen relatively clearly in bright field STEM images, though the authors do not report any use of tomography. (This is perhaps not surprising, since in a specimen of this thickness, beam broadening effects would be expected to limit the resolution of STEM images in a depth-dependent manner (Hohmann-Marriott et al., 2009). This would make tomographic reconstruction difficult as the images would no longer represent true projections of the specimen.) Heymann et al. (2006) used a dual beam system, allowing the milling process to be guided by SEM imaging within
the same instrument. This group produced a cylindrical needle with a diameter of approximately 1  $\mu$ m, which was observed by TEM and shown to contain parts of several yeast cells. Again, no tomography was reported.

Despite successful results, these FIB milling techniques developed for materials science appear to be technically challenging even with robust specimens at room temperature, and it is difficult to envisage the successful use of any of them with frozen-hydrated material without very substantial modifications. Similarly, the full-rotation stages that have been made for materials science experiments do not appear suitable for work with cryogenic specimens. However, FIB milling techniques have recently been adapted with some success to the production of thin lamellae for biological cryo-tomography (Hayles et al., 2010; Rigort et al., 2010; Wang et al., 2012). This is a promising and rapidly-developing field, and the idea of using a FIB to produce a cylindrical specimen from frozen-hydrated biological material has been suggested (Leis et al., 2009), but the technical challenges to actually achieving this are substantial.

For my work, starting with a cylindrically-shaped specimen seemed to be more feasible. This approach avoids the need to find and extract the specimen from a large mass of material, which is one of the more serious limitations of the subtractive preparation approach. In principle, the use of an inherently cylindrical specimen is just the same method as used by Barnard et al. (1992). However, for use at higher resolution and in an intermediate-voltage electron microscope, it is necessary to move away from glass as a material for the tube walls.

Carbon is extensively used as a support film for electron microscopy due to its strength, conductivity and transparency. Therefore, thin-walled carbon tubes of the correct size for electron tomography (approx.  $0.5 \ \mu$ m) would be expected to make useful specimen holders. Tubes like this have already been made, under the name of carbon nanopipettes. They were mainly intended for use as analytical probes for electrophysiology, and their production and use will be discussed in the next section.

# 1.5 Carbon nanopipettes

Carbon nanopipettes (CNPs) were first produced by the group of Haim Bau (Kim et al., 2005a). Their development arose from a combination of two previous lines of research. Micropipettes and nanopipettes, usually made of glass, had been investigated for a wide variety of uses including microinjection of cells, electrochemical sensing, patch clamping for electrophysiology, and scanning microscopy techniques such as scanning

ion-conductance microscopy. These and more of the many uses of nanopipettes are described in more detail in reviews by Morris et al. (2010), Actis et al. (2010), and Ying (2009).

Meanwhile, research on carbon nanotubes had led to a method for production of small-diameter carbon pipes by templated chemical vapour deposition in a porous alumina membrane (Che et al., 1998). Initially these pipes were – confusingly – referred to as nanotubes, despite being an order of magnitude larger than true molecular nanotubes. However, more recently they have been consistently referred to as carbon nanopipes (Whitby and Quirke, 2007). Research on the hydrodynamic properties of carbon nanopipes established that they were hydrophilic and could be filled with liquids and solid particles (Kim et al., 2005b; Rossi et al., 2004).

The desire to integrate carbon nanopipes into larger devices resulted in the development of carbon nanopipettes (Kim et al., 2005a). These were made by chemical vapour deposition of carbon on all surfaces of glass micropipette templates, followed by removal of the carbon outer layer and etching of the glass at the tip to reveal a short protrusion of the inner carbon surface. (A schematic of this process is shown later in Figure 3.1.) These nanopipettes were established to be optically transparent, and electrical ion transport through them was investigated, demonstrating that they could be used as electrodes.

Subsequent work by the same group refined the manufacturing method and found that, due to their conductive carbon layer, CNPs could be used for both microinjection and electrochemical measurement (Schrlau et al., 2008a,b, 2009). They were also found to be more robust than glass micropipettes for repetitive penetration of cell membranes, and further research established that the surface chemistry of the carbon layer could be adjusted by variations in the manufacturing process (Vitol et al., 2009).

Another research group extended the method to produce nanopipettes with smaller tip diameters, and found that insertion of these into cells caused significantly less perturbation to cellular physiology than insertion of similarly-sized glass micropipettes (Singhal et al., 2010). This report also described the use of high-temperature annealing to increase the strength of the carbon tubes, and further refined the manufacturing process. The development of carbon nanopipettes is reviewed by Schrlau and Bau (2009, 2011).

Importantly, both groups found that carbon nanopipes and nanopipettes were largely transparent in the TEM (Kim et al., 2005b; Schrlau et al., 2008b; Singhal et al., 2010). This suggested that using nanopipettes as specimen holders for electron microscopy was feasible. Indeed, in their review, Schrlau and Bau (2009) suggested this possibility, though it should be noted that members of our research group had already discussed this idea with Prof. Bau in 2007.

## 1.5.1 A note on nomenclature

There is some inconsistency in the literature about whether and when the prefixes *micro-* and *nano-* should be used to describe fine-tipped pipettes. This is discussed by Actis et al. (2010), who suggest a definition of nanopipettes as those with tip diameters below 200 nm, and micropipettes as those in the range  $0.2-20 \mu m$ . This suggestion is sensible but by no means universal. For consistency with the majority of the literature (such as Schrlau et al., 2008b), in this work I generally refer to any carbon-tipped pipette with a size in the micrometre or sub-micrometre range as a carbon nanopipette (or simply a nanopipette), while glass-tipped pipettes, because they are produced using a machine called a micropipette puller. This terminology is not self-consistent with respect to the actual sizes of the pipette tips, but does help to maintain the distinction between glass pipettes undergoing processing, and the carbon-tipped pipettes used for tomography.

## 1.6 Outline of this work

In this thesis, I show that it is possible to produce cylindrical, frozen-hydrated specimens for electron tomography, by using carbon nanopipettes as specimen holders.

In chapter 3, I discuss my fabrication of carbon nanopipettes, while in chapter 4, I show the results obtained by using these nanopipettes for electron cryo-tomography. Finally, in chapter 5, I discuss the advantages and limitations of the method, and present suggestions for future improvements.

# CHAPTER 2

## MATERIALS AND METHODS

# 2.1 Manufacture of carbon nanopipettes

### 2.1.1 Pulling of glass micropipettes

Quartz glass capillary tubes (1.0 mm outer diameter, 0.7 mm inner diameter, 75 mm length; Sutter Instrument) were pulled into fine-tipped micropipettes using a P-2000 laser-based micropipette puller (Sutter Instrument).

Various pulling parameters were tried to begin with, but the successful programme used for most of this work was as follows: one step, heat 900, filament 2, velocity 30, delay 130, pull 200. (Each parameter is a digital value on an arbitrary scale.) The laser heating time is determined automatically based on the separation velocity of the glass, and this programme usually produced a heating time of about 3.6 s, and micropipette tip sizes of around 25–35 nm.

#### 2.1.2 Carbon coating

The processing steps of carbon coating, oxygen plasma etching and HF etching were all performed in a clean room at the University of Cambridge Nanoscience Centre.

Carbon coating was carried out in a tube furnace (model HST-600; Carbolite) with a programmable electronic control unit (model 3216; Eurotherm) and an over temperature protection control unit (model 2132; Eurotherm). The furnace was equipped with a work tube made of mullite (25 mm inner diameter, 750 mm length; Carbolite). Micropipettes were stacked in batches of up to twenty in a porcelain combustion boat (Coorstek 60035; 7.5 mL capacity; Sigma-Aldrich) which was then placed into the centre of the work tube for carbon coating. The tube ends were sealed with stainless



**Figure 2.1:** A photograph of the furnace used for chemical vapour deposition. The furnace itself is in the upper right corner, with its control unit in the lower right corner. Between the two are mounted the control unit for the mass flow controllers (the MFCs themselves are behind, out of sight) and the pressure gauge monitoring unit. Just below and left of the furnace are the valves used for controlling the vacuum pump exhaust and a nitrogen source for venting the work tube. On the left is the computer, used to control the gas flows and monitor the process pressure.

steel fittings (made by the Nanoscience Centre workshop) equipped with fluoroelastomer O-rings (material V76F; Precision Polymer Engineering) and gas connection ports. A photograph of the furnace is shown in Figure 2.1.

Stainless steel tubing was used to carry gases from cylinders to the furnace. The work tube was filled with argon (Pureshield argon; BOC) while the furnace heated up and cooled down, and with methane (4.5 grade; Air Products) for the chemical vapour deposition process. Gas flows were controlled individually by mass flow controllers (Type 1479A; MKS Instruments) operated by an electronic control system (Type 146; MKS Instruments).

Work tube pressure was monitored by a pair of pressure transducers with maximum readings of 2 and 500 Torr (Type 722A; MKS Instruments) and an electronic gauge unit (model PDR2000; MKS Instruments). The tube exhaust was passed to a rotary vacuum pump via an adjustable valve (Swagelok).

The electronic control units for the mass flow controllers and pressure gauges were connected to a computer with RS-232 cables. Work tube pressure and gas flows were monitored and controlled by the computer using a custom-written control programme, allowing semi-automatic operation of the system. To achieve the desired working pressure, the tube was first pumped to a rough vacuum, then the gas flow was started and the exhaust valve position manually adjusted until the pressure settled at the intended value. The system would then run without further adjustment until the end of the process.

For programmed operation, the furnace controller was set to ramp slowly (approximately 30 °C per minute) up to the process temperature, remain there for ten minutes longer than the process duration, and then switch off to allow the work tube to cool for several hours. The computer control programme was started at the same time as the furnace and set to ensure a flow of argon for five minutes longer than the furnace's ramping time. Gas flow was then switched to methane for the desired process duration, and back to argon for 35 minutes when the furnace had been switched off and had started to cool. After this the gas flow was switched off, allowing the work tube to return to a rough vacuum while it cooled back to ambient temperature.

Occasionally (usually after three or four cycles of carbon deposition) excess carbon was removed from the inside of the work tube by oxidation. The tube was heated to 800 °C while a mixture of 20% oxygen (standard grade; BOC) in argon was passed through for one or two hours at a pressure of 200 Torr. The porcelain boats used to hold the tubes during carbon deposition were cleaned after each deposition cycle by oxygen plasma etching (as described below) for two minutes.

## 2.1.3 Oxygen plasma etching

An aluminium plate was used to support carbon-coated micropipettes during oxygen plasma etching. The micropipettes were placed upright in holes (1.1 mm diameter) drilled part-way through the plate, which was then placed in the chamber of a microwave-based plasma barrel etcher (model MRC100; Cambridge Fluid Systems). This positioned the tips of the micropipettes close to the centre of the etching chamber. The system was pumped to a rough vacuum and this was maintained while a flow of oxygen gas (4.75 litres per minute; N5.0 purity; BOC) was passed through the chamber. Microwave emission was then activated to generate a plasma for ten seconds, at a power of 100 W.



**Figure 2.2:** A photograph of the apparatus used to support micropipettes during HF etching. When in use, the cluster of vertical white rods in the centre of the picture would each hold a single micropipette, tip downwards. In each of the round holes in the piece of black plastic beneath would sit a plastic beaker containing HF or water for rinsing.

## 2.1.4 Hydrofluoric acid etching

Hydrofluoric acid etching was performed in a dedicated fume hood in the Nanoscience Centre clean room. Micropipettes were held in batches of 15 in a custom-built dipping apparatus made almost entirely of HF-resistant plastic. This supported three plastic beakers (50 mL capacity), containing buffered hydrofluoric acid (BHF; 20 mL) for the etching, and deionised water for the subsequent rinsing steps. A photograph of the apparatus is shown in Figure 2.2.

The acid was prepared by taking a standard stock solution of 7:1 BHF (consisting of 35% NH<sub>4</sub>F and 6.3% HF in water; J. T. Baker) and diluting with an equal volume of deionised water to yield a solution with an HF concentration of approximately 3%. Micropipettes were dipped into this solution to a depth of a few millimetres for one to three minutes. They were rinsed by dipping into the two beakers of deionised water, and left to dry for at least 24 hours before removal from the dipping apparatus.

### 2.1.5 Imaging and characterisation

Micropipettes were imaged using a scanning electron microscope (SEM) at various stages of the production process. The SEM (LEO 1530 VP; Zeiss) was operated at



**Figure 2.3:** A photograph of the apparatus used for gluing grids to nanopipettes. A nanopipette is shown with a grid attached after the epoxy has been cured. The nanopipette shaft is clamped down into its channel by a clamp off the lower edge of the picture, while the grid is held by the clamp visible in the top of the photograph.

an accelerating voltage of 3.0 kV with a  $30 \text{ }\mu\text{m}$  aperture. The working distance was typically 2.7 mm and secondary electron images were collected using the in-lens detector.

Energy-dispersive X-ray analysis (EDX) was carried out using a detector attached to the SEM (Inca model 7426; Oxford Instruments). The SEM specimen support was made of aluminium, so to avoid overlap of the X-ray peaks from the background (Al K $\alpha$  at 1.5 keV) and the silicon in the glass (Si K $\alpha$  at 1.7 keV), a copper plate was placed below the specimens. The working distance when using the EDX was typically 7 mm, and the detector was placed approximately 30 mm away from the specimen.

# 2.2 Specimen preparation

## 2.2.1 Attaching CNPs to grids

Carbon nanopipettes were glued to standard slot grids (3.05 mm diameter with a 2 x 1 mm slot; Agar Scientific) using either silver-loaded conductive epoxy or rapidcuring epoxy. A metal support apparatus (manufactured in the LMB workshop) was used to hold a grid and a CNP in the correct arrangement for gluing. A photograph of the apparatus with a glued CNP and grid is shown in Figure 2.3.

To carry out the procedure, a grid was first rested in place and clamped into

position with the sprung metal clip seen in the top of the photograph. (Not visible in the photograph are the downward-projecting prongs which actually make contact with the grid and press it to the base plate.) After this, a CNP was placed into a channel cut into the base plate and clamped down loosely. The nanopipette was rotated so that the tip was at its highest point, and then moved along the channel until the tip was over the grid. The nanopipette was then rotated to lower the tip until it was just above the grid. The grid was carefully aligned to ensure that the nanopipette was perpendicular to the slot with the nanopipette tip in the centre of the grid. The nanopipette was rotated further so the tip made contact with the grid, and then clamped firmly to prevent further movement.

The two parts of the epoxy were then measured out and thoroughly mixed, and a very small drop was placed on the junction of the grid and the nanopipette, working under a binocular microscope for precision. A pointed wooden skewer was used both to place the drop of glue and then to manipulate it and ensure that it made good contact with both nanopipette and grid.

When using conductive epoxy, the gluing apparatus was placed on a hotplate at 100 °C for 20 minutes to activate the curing process. Using rapid-curing epoxy the apparatus was simply left on the bench for five minutes. After this, the epoxy was solid enough that the CNP could be removed from the gluing apparatus, with the grid firmly attached. The removal had to be performed carefully to prevent damage. The procedure, in detail, was as follows: the nanopipette was first held down by hand while the clamp on the shaft was released and swung away. While continuing to hold the nanopipette down, the clamp on the grid was released by sliding the point of a pair of tweezers underneath it and levering the clamp upwards off the grid and rotating it away to the side. A larger pair of tweezers was then used to grab the shaft of the nanopipette (in a slot cut into the base plate for this purpose) and the nanopipette was lifted out of its channel while continuing to apply downward pressure by hand. Once the nanopipette was clear of the apparatus, this pressure was released and the nanopipette was always positively held and was never free to bounce or rattle against the apparatus.

## 2.2.2 Preparation of liquid specimens

Colloidal gold for fiducial markers was prepared by mixing 990  $\mu$ L of gold colloid (BBInternational) with 10  $\mu$ L of a 10 mg/mL solution of bovine serum albumin (BSA;

Sigma), to give a BSA concentration of 100  $\mu$ g/mL. This allowed the gold to be pelleted and resuspended without aggregation.

Ribosomes were provided by C. Neubauer, as a 10  $\mu$ M solution in buffer G: 50 mM KCl, 10 mM NH<sub>4</sub>Cl, 10 mM Mg(CH<sub>3</sub>COO)<sub>2</sub>, 5 mM HEPES pH 7.5 (Selmer et al., 2006). This was diluted tenfold in buffer G to give a 1  $\mu$ M solution. 2  $\mu$ L of this was added to 18  $\mu$ L of gold colloid (15 nm particle size, nominal concentration 1.4×10<sup>12</sup> particles/mL) to make the solution used for tomography.

Liposomes were prepared from *Escherichia coli* total lipid extract (Avanti Polar Lipids). 40  $\mu$ L lipid solution (25 mg/mL in chloroform) were mixed with 30  $\mu$ L methanol and 100  $\mu$ L chloroform, and dried in the base of a glass vial with a stream of dry nitrogen gas to form a lipid film. This was further dried for one hour in a vacuum desiccator. 0.5–1 mL of a buffer solution (25 mM Tris–HCl, pH 7.4, 150 mM NaCl) was added to the vial and sonicated for 3 min. Liposomes were produced by extruding this repeatedly through a filter with a nominal pore size of 0.1  $\mu$ m.

The liposomes provided by D. Ghosal were prepared in this manner before solutions of *E. coli* MinC and MinD proteins were added to give a final concentration of 20  $\mu$ M of each protein. ATP and MgCl were also added at concentrations of 1 mM. The liposomes were then kept at 25 °C for 15 min to allow the proteins to bind to the liposomes and then stored at 4 °C until use. For the tomography specimen, 50  $\mu$ L gold colloid (15 nm particle size, nominal concentration  $1.4 \times 10^{12}$  particles/mL) was spun in a microcentrifuge (14,680 rpm, 1 min) to form a loose pellet. 3  $\mu$ L of the pellet was transferred to 24  $\mu$ L of the liposome solution, and 3  $\mu$ L of a 1 mg/mL stock of the membrane dye FM4-64 (Molecular Probes) was added. (This is ten times the manufacturer's recommended concentration; high concentrations were required for observation of the stain with short exposure times when using low magnification objective lenses in the fluorescence microscope.)

*Caulobacter crescentus* cells (strain CB15N) were grown at 30 °C in PYE medium (0.2% Bacto peptone (Difco), 0.1% yeast extract (Difco), 1 mM MgSO<sub>4</sub> and 0.5 mM CaCl<sub>2</sub>) supplemented with 10 µg/mL ampicillin. 500 µL of a stationary-phase culture was pelleted and then resuspended in 50 µL of the growth medium. FM4-64 and BSA were added to final concentrations of 10 µg/mL and 1 mg/mL respectively. Gold colloid with a high BSA concentration was prepared by pelleting 1 mL of gold colloid (20 nm particle size, nominal concentration 7.0×10<sup>11</sup> particles/mL), resuspending in 90 µL distilled water and adding 10 µL of 10 mg/mL BSA to give a final concentration of 1 mg/mL. To prepare the tomography specimen, 20 µL of the *C. crescentus* mixture

was pelleted and resuspended in 20  $\mu$ L of the gold and BSA mixture.

### 2.2.3 Specimen loading

Many of the details of the specimen loading procedures are given in sections 4.1 and 4.4. Additional details are as follows. Small amounts of liquids were placed into the shaft of a nanopipette using a standard 20  $\mu$ L adjustable laboratory pipette fitted with a Microloader pipette tip (Eppendorf). The pipette was set to draw up 3  $\mu$ L of liquid, and used to transfer a small amount of the mixture of the specimen to the neck of a carbon nanopipette. The actual volume transferred was less than the nominal 3  $\mu$ L for two reasons: drawing fluid up into the Microloader tips was very slow and would take minutes to reach the full volume, and some liquid was left in the Microloader tip to avoid the formation of bubbles inside the nanopipette. After loading, pressure was applied to the shaft of the nanopipette when necessary using an oil-filled microinjector pump (CellTram Oil; Eppendorf).

For specimens where further liquid movement was required, the nanopipette tip was dipped into a drop of liquid held in the end of a standard 10  $\mu$ L plastic laboratory pipette tip, fitted to a standard adjustable pipette which was mounted in a custom-made clamp attached to a micromanipulator (MC-35A; Narishige, Japan). This process was performed at the focal point of a Nikon Eclipse E800 microscope, fitted with a selection of objective lenses. Those used for this work were a Nikon Plan Fluor 10×, numerical aperture 0.3, working distance 16 mm; and a Nikon Plan 40×, numerical aperture 0.65, working distance 0.56 mm.

## 2.2.4 Freezing and cryo-transfer

When ready for freezing, liquid ethane was prepared by condensation in a brass cup cooled by liquid nitrogen. The ethane was cooled until some solid ethane was visible on the sides of the cup, indicating that the liquid was at its freezing point and therefore as cold as possible. The nanopipette to be frozen was held with the grid at the tip pressed down onto a rubber mat. A diamond scribe was gently rubbed across the glass of the nanopipette where it crossed the edge of the grid until the tube separated. The grid was then picked up with fine-tipped tweezers and plunged into the liquid ethane, before transfer to a cryo-storage box under liquid nitrogen.

Grids were loaded into cartridges for imaging in the TEM, making sure that the grid slots were aligned perpendicular to the microscope's tilt axis. The grids were

loaded with the glued side facing downwards, which ensured the largest angular viewing range for the tip of the carbon tube. The loading procedure was carried out under or just above the surface of liquid nitrogen in an insulated cryo-station, and was performed as quickly as possible, to reduce condensation of ice contamination onto the tubes.

# 2.3 Electron microscopy

Transmission electron microscopy was performed at liquid nitrogen temperature using a Tecnai G<sup>2</sup> Polara (FEI Company) operating at an accelerating voltage of 300 kV and equipped with a post-column energy filter (Tridiem 864; Gatan) and a  $4k \times 4k$  pixel camera (UltraScan 4000; Gatan). Tilt series were collected using SerialEM image acquisition software (Mastronarde, 2005). Single images were collected using either SerialEM or DigitalMicrograph (Gatan).

Unless otherwise stated, images in tilt series were acquired with the following conditions: magnification was 41,000×, and defocus was set at around  $-10 \ \mu\text{m}$ . A 70  $\ \mu\text{m}$  C2 aperture was used along with a 100  $\ \mu\text{m}$  objective aperture. The camera was binned by a factor of two to record 2k × 2k images. In some cases the images were energy filtered to select zero-loss electrons using a 20 eV bandwidth. Beam intensity and exposure time were constant for all images in a tilt series, with a typical electron dose of 1 e<sup>-</sup>/Å<sup>2</sup> per image, meaning the cumulative dose for the series was around 150 e<sup>-</sup>/Å<sup>2</sup>.

Tilt series were acquired using SerialEM's low dose mode, meaning that specimen tracking and focussing were performed at a position offset from the target area. With cylindrical specimens, this required careful adjustment, since the cross-correlation calculation used for these procedures was prone to failure. In particular, if the target area was at the very tip of a nanopipette, and the tracking area was further back along the nanopipette's shaft, it was essential to make sure that the tracking area was magnified and positioned to include only one side of the tube. If both sides were visible, any change in the tube diameter caused by tilting would lead to huge tracking errors as the software restored the diameter by moving longitudinally along the tapering cylinder. Additionally, it was essential that the tracking area contained a strong feature on the side of the tube to allow it to correctly align successive images. This sometimes required the tracking area to be repeatedly redefined during a tilt series as various surface features on the tube (such as crystals of ice contamination) moved in and out of view.

# 2.4 Image processing

Tilt series were processed using the IMOD package (Kremer et al., 1996). The images were aligned using gold fiducial markers, and usually binned by a factor of two to give aligned images which were 1k × 1k pixels in size. The full dynamic range of the images (16 bits per pixel) was retained at all stages of processing.

Tomograms were reconstructed using either the *R*-weighted backprojection algorithm in IMOD, or the simultaneous iterative reconstruction technique (SIRT) using Tomo3D (Agulleiro and Fernandez, 2011). SIRT was typically continued for 40–60 iterations. Isotropic low-pass filtering of tomograms was performed with IMOD, and TomoAND (Fernández and Li, 2003; Fernández et al., 2007) was used to carry out anisotropic nonlinear diffusion, using automatic tuning of parameters where possible.

Simple image adjustments (such as rotation, resizing, contrast adjustment and scale bar placement) were carried out using Adobe Photoshop, and line profiles were generated in ImageJ (Schneider et al., 2012). Volume rendering of tomograms was performed using PyMOL (Schrödinger, LLC, 2010), after using IMOD to bin the processed tomograms by a factor of two to reduce the computer memory requirements.

# CHAPTER 3

## MANUFACTURE AND CHARACTERISATION OF

CARBON NANOPIPETTES

## 3.1 Overview

Carbon nanopipettes (CNPs) were produced in a multi-step manufacturing process adapted from the method used by Schrlau and colleagues (2008b). The principle of the method was to use a glass micropipette as a template for deposition of a layer of carbon, with subsequent removal of the glass from the tip, leaving a carbon tube extending from the remaining glass. This method has the advantage that the very small carbon tube is smoothly integrated into the larger glass capillary tube, allowing simple handling and the ability to add fluids to the tube relatively easily.

A schematic overview of the manufacturing method is shown in Figure 3.1, and photographs of a typical glass micropipette and a CNP after manufacture are shown in Figure 3.2. The process begins with a quartz glass capillary tube which is heated and pulled apart to form a pair of micropipettes. Both surfaces of the micropipettes are coated with carbon by chemical vapour deposition (CVD), after which the carbon layer is removed from the outer surface by oxygen plasma etching, and the glass is then removed from the end of the pipette by etching with hydrofluoric acid (HF). The nanopipettes are observed in the scanning electron microscope (SEM) at various stages, allowing the process to be monitored and the pipette sizes to be measured. For more detailed characterisation, energy-dispersive X-ray spectroscopy (EDX) can be used in combination with the SEM to obtain elemental maps of the tips of the nanopipettes.



**Figure 3.1:** Overview of the carbon nanopipette manufacturing process. (a) Glass micropipettes are drawn from capillary tubing. (b) Carbon is deposited over all surfaces of the pipette by chemical vapour deposition. (c) Oxygen plasma is used to remove the carbon layer from the pipette's outer surface. (d) Hydrofluoric acid etching removes glass from the pipette tip, exposing the original inner layer of carbon.



**Figure 3.2:** Glass and carbon micropipettes. Top: a glass micropipette after it has been pulled. Bottom: a carbon nanopipette after the complete manufacturing process. For scale, the capillary tube diameter (before it narrows towards the tip) is 1 mm.

# 3.2 Pulling of micropipettes

Quartz glass capillary tubes were used for the micropipettes due to their very high softening temperature of approximately 1700 °C. This was required because the carbon deposition process was carried out at over 900 °C, and the other glass types commonly used for micropipettes – borosilicate and aluminosilicate – deform substantially at these temperatures (Sutter Instrument Company, 2003). This necessitated the use of a laser-based micropipette puller, since standard wire filament-based pullers cannot reach a temperature high enough to pull quartz pipettes.

Extensive trials were performed to find parameter settings which would reliably produce micropipettes with a suitable shape and size. It was impractical to search exhaustively, however, as the micropipette puller has five independent parameters for each laser heating cycle, and can chain multiple cycles together in sequence, giving a very large range of possible programmes. Despite many attempts, I could not find parameters which would consistently produce micropipettes with tip diameters in the desired size range of a few hundred nanometres (i.e. about the size of bacterial cells). Most programmes produced tip diameters which were either very small (below 50 nm) or too large (over 2  $\mu$ m), and a small number of programmes occasionally produced pipettes with sizes in the desired range, but inconsistently and with variable tip shapes.

The final parameters chosen (detailed in section 2.1.1) worked reliably to produce micropipettes with a smooth tapered shape finishing with a tip diameter of approx-

imately 30–40 nm. After all of the subsequent processing steps, these were actually found to produce CNPs with the desired tip diameter; this will be discussed further in section 3.7.

The tips of the micropipettes were slightly offset from the central axis of the capillary tube (easily visible in Figure 3.2), indicating that the focal point of the laser was set towards one side. This turned out to be an advantage when attaching the pipettes to EM grids, since the eccentric location of the tip enabled very fine adjustments of position to be made by rotating the shaft of the pipette. (See section 2.2.1 for details.) The laser was therefore not adjusted to correct this. However, it did mean that the cross-section of the micropipettes was often slightly elliptical rather than perfectly circular.

# 3.3 Carbon deposition

In their original method, Schrlau and colleagues (2008b) deposited a thin layer of a metal catalyst (ferric nitrate) on the inner walls of glass capillary tubes before pulling them into micropipettes. Carbon was deposited preferentially on the catalyst-coated surface by CVD from a methane and argon mixture (in 2:3 ratio), flowing at 500 standard cubic centimetres per minute (sccm) and heated to 900 °C for two hours at atmospheric pressure (Schrlau et al., 2008b, and H. H. Bau, personal communication).

My attempts to replicate this procedure were unsuccessful. In particular, the catalyst deposition (achieved by filling capillary tubes with a solution of ferric nitrate in isopropyl alcohol and leaving to dry in air) always led to a visible orange residue of ferric nitrate at one end of the tube and occasionally a small number of visible grains of ferric nitrate elsewhere. Subsequent CVD produced a carbon coating only on areas where the catalyst had been visible. Despite consultation on more precise details of the method used (M. G. Schrlau, personal communication), I was never able to produce the continuous layer of catalyst that was apparently required for effective carbon deposition.

The CVD furnace used in my work was of a slightly different design to that used for the published method (H. H. Bau, personal communication), only having one heated zone rather than three, and with an exhaust which had to be pumped to a partial vacuum rather than venting to the atmosphere at ambient pressure. This did not appear to be significant for my lack of success in reproducing the published method, though, since successful carbon deposition did occur where the catalyst was visibly



**Figure 3.3:** Micropipettes after carbon deposition. A few micropipettes are shown in the porcelain boat used to support them during the CVD process. For scale, the boat is approximately 10 mm wide.

present.

When I attempted CVD at higher temperatures (above 900 °C) for several hours, I found a very thin layer of carbon deposited over all surfaces (in addition to the much thicker carbon produced on the grains of catalyst). This catalyst-independent method of carbon deposition was therefore optimised to produce a reliable CVD procedure.

Experiments showed that increasing pressure, temperature, time and the proportion of methane in the gas mixture all caused an increase in the thickness of deposited carbon, as judged by the visible darkening of the glass tubes. Decreasing the methane flow rate (while keeping the same pressure) also increased the carbon deposition thickness. A suitable CVD procedure for carbon nanopipette manufacture was determined by combining all of these factors: untreated quartz micropipettes (i.e. without ferric nitrate deposition) were heated to 925 °C for 1.5–2 hours in undiluted methane flowing at 100 sccm at a pressure of 400 Torr. Further details can be found in section 2.1.2. A picture of several micropipettes after the CVD process is shown in Figure 3.3.

Some batches of micropipettes were kept at the deposition temperature of 925  $^{\circ}$ C under a flow of argon for three hours after the deposition had finished. This was intended to anneal the carbon to increase its strength, though ideally a higher temperature of 1200  $^{\circ}$ C would be used for this (Singhal et al., 2010). This step produced a small decrease in the measured tip diameters of the finished nanopipettes, and so was used for most of my later experiments.

After this procedure had been established, another group published a similar, catalyst-free method for manufacturing CNPs (Singhal et al., 2010), also based on the method of Schrlau et al. (2008b). These results demonstrated a CVD rate that was strongly affected by the length and geometry of the glass micropipettes, and the authors elegantly showed that even without a layer of catalyst, it is possible to tune the CVD conditions to produce significantly more carbon deposition inside the pipettes than on the outer surface. However, their reported conditions (the same as Schrlau

et al. (2008b) but at a lower of temperature of 875 ℃ and for a shorter time of 0.5–1 h) would be expected to produce even less uncatalysed carbon deposition than the original catalysed method, and it is unclear why this was not observed in my results or those of Schrlau et al. (2008b). Since Singhal and colleagues (2010) demonstrated such strong dependence on local geometry, it seems likely the difference is due to unreported details of the equipment and methods used in each case. Their method offered no compelling advantages over the method I had already established, so this was not investigated further.

# 3.4 Oxygen plasma etching

In their studies, both Schrlau et al. (2008b) and Singhal et al. (2010) were able to proceed directly from carbon deposition to glass removal, since their CVD conditions avoided carbon deposition on the outside of the glass. However, in the first published description of CNPs (Kim et al., 2005a), a different CVD procedure was used which coated both surfaces of the glass with carbon. An oxygen plasma etching step had therefore been used to remove the carbon from the outer surface. A similar technique was also used for one of the variations in the method reported by Vitol and colleagues (2009).

Using a similar method, I found that oxygen plasma worked well to remove carbon from the outside of coated micropipettes. A ten-second etch was sufficient to remove all carbon from the outer surface, with no visible effect on the inner carbon layer (judged visually by the darkness of the tube). When tested with full-size capillary tubes, carbon removal was observed on the inner surface as well, starting at the end and etching to leave clear glass progressively further away from the tip as the plasma etching time was increased. This effect was also observed when etching two large micropipettes with tip diameters of 30 and 70  $\mu$ m, though the rate of carbon removal from the inner surface was decreased when compared with the full-size pipette (which had an inner diameter of 700  $\mu$ m). These results suggest the possibility that plasma was generated only outside the tubes and entered any openings by diffusion, which was exactly the desired behaviour for etching only the outer surface of the pipettes. However, the details of the mechanism were not investigated further, since the method appeared to produce good results.

Schrlau and Bau (2009) mentioned that one of their reasons for developing the catalysed method for CVD was that their previous method (Kim et al., 2005a) suffered



**Figure 3.4:** Micropipette tip diameter measurements before carbon deposition and after oxygen plasma etching. For each set of measurements, the box shows the inter-quartile range, the horizontal line across the box marks the median, and the whiskers show the full range. The maximum micropipette diameter after etching was 500 nm; the axis has been shortened for clarity.

from low yields due to damage of the CNP tips during the oxygen plasma etching step. I have found this to be a slight problem, but not a serious one. The tip diameters of a representative sample of my micropipettes were measured by SEM (see section 3.6) before carbon deposition and again after plasma etching. From the sample of 116 micropipettes, eleven (9%) were damaged during handling between processing steps and the measurements of these were ignored. The sizes of the remaining 105 micropipettes are plotted in Figure 3.4. The majority of these showed very small increases in size (median diameter before CVD, 30 nm; after  $O_2$  etch, 40 nm), though a minority showed larger increases (upper quartile before CVD, 40 nm; after  $O_2$  etch, 90 nm). This increase was generally not large enough to be a cause for concern, since the desired final tip size of the finished CNPs was intended to be substantially larger still (300–800 nm).

# 3.5 Hydrofluoric acid etching

Once the outer carbon layer had been removed, HF was used to remove glass from the pipette tips. This was an incomplete etch, intended to remove only the surface layer of the part of the glass which was submerged in the HF solution. Because the glass walls become thinner towards the tip, this surface etching was sufficient to remove all of the glass from the last few micrometres of the pipette, while leaving a tapering glass layer beyond this to provide mechanical support to the carbon. (This can be seen schematically in Figure 3.1 c-d.)

Publication	BHF ratio	Etch time	Length exposed
Schrlau et al. (2009)	6:1	30 s	< 1 µm
Schrlau et al. (2008a)	6:1	4 min	5–15 µm
Vitol et al. (2009)	6:1	4 min	approx. 45 µm
Singhal et al. (2010)	6:1	15 min	approx. 200 µm
Personal communication,	5:1	5 min	60 µm
M. G. Schrlau to J. Salje (2007)			

Table 3.1: Reported HF etching conditions for CNP manufacture

Using this method, etching for longer periods of time would be expected to lead to a greater length of exposed carbon. However, the literature is inconsistent on the exact conditions which might be suitable. All authors who have reported the manufacture of CNPs (see Table 3.1) made use of buffered hydrofluoric acid (BHF, also known as buffered oxide etch), which is a mixture of 40% NH<sub>4</sub>F and 49% HF solutions, typically in a volumetric ratio of between 5:1 and 7:1. This has an expected etching rate of approximately 100 nm per minute for 5:1 BHF at room temperature (Williams and Muller, 1996), and the rate is slightly lower at the 6:1 or 7:1 ratio as the concentration of HF is correspondingly reduced.

Table 3.1 summarises the reported lengths of carbon exposure for various etching conditions. The carbon exposure lengths mentioned for Vitol et al. (2009) and Singhal et al. (2010) were estimated from micrographs shown in their papers. Confusingly, given their relatively short reported etching time, Vitol et al. (2009) show an image of a CNP with a well-defined step at the end of the glass section, which would be expected for complete etching with very shallow immersion in the acid solution, rather than the deeper immersion and incomplete etching which is generally reported.

The slight inconsistency in these reported results is presumably at least partly due to differences in the geometry of the glass micropipettes used in each case, since the thickness profile of the glass should determine how much carbon is exposed when a certain thickness of glass is removed. These reports therefore provided a useful starting point, but it was not expected that these results would be directly applicable to my manufacturing process.

Statistics for the CNPs that I have made are shown in Figure 3.5, where the tip diameters and exposed lengths of carbon are plotted against the etching time. The etching solution used in these experiments was a two-fold dilution of 7:1 BHF in water. (A number of CNPs were completely broken during the manufacturing process, and



**Figure 3.5:** Carbon nanopipette measurements after HF etching. (a) Tip diameter plotted against HF etching time. (b) Length of exposed carbon plotted against HF etching time. For each set of measurements, the box shows the inter-quartile range, the horizontal line across the box marks the median, and the whiskers show the full range. Only two CNPs were tested with an etch time of 0.5 min, and these are plotted individually.

these are excluded by showing only measurements for the CNPs with a tip diameter below 2  $\mu$ m in the graphs.) The tip diameters are quite variable (Figure 3.5a), with no obvious correlation with the HF etching time. In Figure 3.5b, it can be seen that the lengths of exposed carbon show a clear relationship (as expected) between increased HF etching time and the length of tube exposed.

Most of the CNPs intended for electron tomography were produced with an etching time of 1–3 minutes, giving an average of 1–4  $\mu$ m of carbon tube exposure. This was chosen because more exposure was unnecessary and potentially counter-productive. Further back along the tubes, the tube diameter becomes too large for effective TEM imaging when filled with ice. Since the presence of glass is only a problem where TEM imaging is intended, keeping glass on the tube until very close to the tip should help to provide mechanical support to the fragile carbon.

## **3.6 Characterisation of carbon nanopipettes**

After manufacture, CNPs were examined in the SEM, allowing measurement of the tip diameter and the length of the glass-free section of the tube. A typical SEM image of a good CNP is shown in Figure 3.6, and clearly shows the shape of the tip and the distinction between the remaining glass and the exposed carbon.



**Figure 3.6:** SEM image of a carbon nanopipette tip. The remaining glass after HF etching stands out in bright contrast against the darker exposed carbon. The measured diameter of the tip was 390 nm, and the length of the fully-exposed carbon was 2.9 µm. Scale bar: 1 µm

To confirm this interpretation of the SEM images, elemental maps and linescans were obtained using energy-dispersive X-ray spectroscopy (EDX). Figure 3.7 shows a reference SEM image, along with EDX maps of the silicon and carbon distributions in the image, and an EDX linescan along the length of the tube. Figure 3.8 shows another reference image marking the position of a transverse linescan across the width of the tube, with the results of the linescan shown below.

There were problems with image drift in the SEM, presumably due to electrostatic charging effects, since most of the outer surface of the tubes was made of insulating quartz glass. In general it was possible to obtain reasonable images at moderate magnification, but high magnification images (above approximately 160,000×) were often blurred. Even at low magnification, the drift made it difficult to acquire good EDX spectra, since X-ray count rates were low and long acquisition times (minutes) were needed to achieve a reasonable signal-to-noise ratio.

The effects of this drift, and the correspondingly limited exposure times, can be seen in the two-dimensional EDX maps shown in Figure 3.7. The X-ray emission signal from silicon is reasonably strong, so there is relatively little background noise and the position of the glass tube stands out clearly (Figure 3.7b), but the signal does fade away as the glass thickness decreases towards the tip of the tube. The signal from oxygen is much weaker, so the oxygen map is much more noisy (Figure 3.7c). The position of the exposed carbon can just about be seen, but the edges of this part are poorly defined. Acquiring these maps was a compromise, as the signal could be seen continuously building above the noise background as the exposure lengthened, but with gradual specimen drift causing blurring if the exposure time was too long.

Despite the relatively low strength of the signal, it can be seen that the mapped locations of silicon and carbon broadly match the contrast difference seen in the



**Figure 3.7:** Elemental maps and longitudinal EDX linescan of a carbon nanopipette. (a) SEM reference image. (b) EDX map of the distribution of silicon in the image area. (c) EDX map of the carbon distribution. (d) Graph of the results of an EDX linescan along the full length of the tube, showing recorded X-ray emissions for four elemental emission lines: copper La1 (red), silicon Ka1 (green), oxygen Ka1 (cyan) and carbon Ka1 (purple). Scale bar: 10  $\mu$ m



**Figure 3.8:** Transverse EDX linescan of a carbon nanopipette. (a) SEM reference image showing the location of the linescan (yellow line). (b) Graph of the results of an EDX linescan across the tube at the marked position, showing recorded X-ray emissions for four elemental emission lines: copper L $\alpha$ 1 (red), silicon K $\alpha$ 1 (green), oxygen K $\alpha$ 1 (cyan) and carbon K $\alpha$ 1 (purple). Scale bar: 10  $\mu$ m

SEM reference image, suggesting that the visible contrast does correctly indicate the positions of the glass and exposed carbon.

The specimen drift situation is improved when taking one-dimensional linescans, because the scanning time of the electron beam is much reduced and so the signal from each point of the scan is significantly stronger, allowing shorter exposure times. The results of a longitudinal linescan of the same tube are shown in Figure 3.7d, with simultaneous recording of emissions from silicon and oxygen (the major constituents of the quartz glass, which is almost pure SiO<sub>2</sub>), carbon, and copper. The copper signal comes from a backing plate positioned several millimetres behind the tube. Any

electrons which pass through the tube will hit the copper plate, allowing the electron transparency of the tube to be measured.

This graph shows a substantial amount of information. The silicon and oxygen signals decrease smoothly to a background level, suggesting a gradual thinning of the glass layer before it disappears completely in the last few micrometres of the pipette. The copper signal increases strongly in concert with the decrease in silicon and oxygen, showing that the tube becomes increasingly transparent as the glass layer thins. At the same time, the weak carbon signal increases in strength, indicating exposure of the carbon layer from underneath the glass, and drops back to background levels in the last micrometre of the scan beyond the end of the tube. The copper signal also shows a slight jump at this point, but it is hard to separate any real effect here from the large fluctuations due to noise. The effect is seen more clearly in Figure 3.8b, which shows the results of a transverse scan across the exposed carbon part of the tube. In this case, it is clear that the carbon and copper signals move in opposition to each other, indicating the expected inverse relationship between carbon thickness and electron transparency. The carbon profile shows peaks at the edges, and a smooth curve decreasing to a minimum between the peaks, which is exactly what would be expected for a recording of the projected thickness of a hollow cylinder. Importantly, this graph also shows that the copper background signal in the middle of the tube is between 85% and 90% of its background level, suggesting the tube is largely transparent to electrons (even at the 3 keV energy used in recording these images). This is obviously good news for a tube intended for use as a TEM specimen holder!

# 3.7 Discussion of the manufacturing process

The manufacturing process presented here has generally worked well, producing carbon tubes of the target size (a few hundred nanometres) with a reasonable success rate of approximately 70%. (The remaining 30% consisted of roughly equal numbers of intact nanopipettes which were over 800 nm in diameter, and nanopipettes which were broken during manufacture.) However, it proved quite difficult to optimise the various steps, and there is still significant potential for improving the process. The main reason for the difficulty of optimisation has been the lack of a way to observe and characterise the inside of the tubes. Effectively, the only way to check the state of the carbon on the inner surface was to etch away the glass from the end and observe the carbon that remained. This made it hard to separate the effects of the different

processing steps.

Another factor which made optimisation difficult was variation in pipette sizes both within and between batches. This meant that to identify trends in the results as a consequence of changing the manufacturing conditions, the production of several batches was often needed. It has therefore not been possible to fully test the effects of many of the possible variations.

A particular curiosity was that the tube size at the end of the process was substantially greater than at the start (median tip diameter of micropipettes pulled initially, 35 nm; after HF etching, 600 nm). Because of the difficulty of characterising the inner carbon layer in the intermediate steps, it is impossible to give a definite reason for this, though there are a few possibilities.

Given the small size of the micropipettes initially, and the carbon deposition thickness of 10–20 nm (measured in tomographic reconstructions), it is quite possible that the carbon built up to the point that the tube was completely blocked. This would then mean that further carbon deposition would have occurred only from gas which had diffused along the tube from the open end, and so the deposition rate near the blocked tip would be expected to drop significantly. This in turn would mean that the carbon near the tip was thinner and therefore weaker than the carbon further back along the pipette, and so when the glass was removed it would be more likely for the carbon to break at the tip.

However, this effect cannot fully explain the size increase, because in a small sample of micropipettes which were pulled with a larger initial tip diameter, a substantial size increase was still observed (sample size, 14; median tip diameter before processing, 370 nm; after HF etching, 1.85  $\mu$ m). This could perhaps be explained if, in the carbon removal step, the oxygen plasma entered the tip and partially or completely removed the inner carbon layer near the end. This would lead to thin or entirely absent carbon at the tip, again increasing the final tip diameter. These or other reasons may well explain the observed increase in size, but further work would be necessary to investigate the cause more thoroughly.

A final point to note is the inherent compromise between CNP strength and electron transparency which arises when using CNPs as specimen holders for TEM. In all previous work on CNPs (Kim et al., 2005a; Schrlau et al., 2008a,b, 2009; Singhal et al., 2010), the tip strength could be increased by using a very thick layer of carbon. However, a thick layer of carbon also has the effect of severely reducing the electron transparency of the tube walls, meaning that fewer electrons are available for imaging the specimen inside the tube. In this work I have therefore tried to keep the carbon walls as thin as possible, but this makes the tubes very fragile. Finding a good compromise between strength and transparency was difficult, because often damage to the tubes was only visible once they had been fully processed, loaded with a specimen and observed in the TEM. Even with very strong tubes, some damage would be inevitable, and so finding a suitable carbon thickness required an iterative process of producing batches of tubes, observing the failure rate, and then making adjustments for subsequent batches.

# CHAPTER 4

### Cryo-tomography

# 4.1 Specimen preparation I

In order to use carbon nanopipettes (CNPs) as specimen holders for electron cryotomography (cryo-ET), there are three requirements. First, an aqueous specimen must be placed in the tip of a CNP, where the tube walls are made of thin, electrontransparent carbon and the tube diameter is small enough for tomography. Second, the specimen must be frozen into amorphous (or 'vitreous', that is, non-crystalline) ice, and third, the CNP must somehow be supported inside the electron microscope for image collection.

### 4.1.1 Nanopipette loading

The first requirement (specimen positioning within the nanopipette) was fulfilled for homogeneous aqueous specimens by placing a small amount of the liquid inside the shaft of the nanopipette, and then allowing capillary action to move the liquid to the nanopipette's tip. This was assisted by applying pressure to the liquid via a microinjection pump attached to the shaft of the pipette. Usually, it was observed that once the liquid meniscus was pushed to the point where the nanopipette's diameter became sufficiently small (typically 10–15% of the diameter of the shaft), the capillary force took over and pulled the liquid to the tip of the nanopipette, even if the external pressure was released. (Before this point, if the pressure was released the liquid would move back to a position close to its starting point.)

This observation provides several pieces of information about the tubes. The fact that capillary action pulls the liquid towards the tip shows that the carbon surface



**Figure 4.1:** Transmission electron micrograph of a blocked carbon nanopipette tip. Some surface ice contamination is visible. Scale bar: 500 nm.

on the inside of the tube is hydrophilic. (This was also suggested by the observation of small droplets of condensation on the inside of the pipette ahead of the liquid meniscus.)

The amount of pressure required to move the liquid gives an indication of the size of the hole at the tip, since (given the hydrophilic nature of the tube walls) the only force preventing the liquid's movement along the tube is the compression of the air ahead of the liquid, and the magnitude of this compression will depend on the size of the hole at the tip through which the air can escape. The pressure was never directly measured, but subjectively this provided a useful guide as to the state of the nanopipette. When the liquid moved very easily to the tip (or in extreme cases, moved to the tip spontaneously before the microinjector pump could be attached), the tip was invariably later found to have a large diameter of several micrometres. Conversely, the nanopipettes which required the most pressure while filling usually had the smallest tip diameters on later observation.

In some cases, the maximum amount of force that could be supplied by the microinjector was insufficient to cause the tube to fill, suggesting a blockage in the tube. In this situation, if the nanopipette tip was gently touched to any solid surface, the tube would then fill easily and later be observed to be broken. One such nanopipette which was suspected to be blocked was imaged by TEM, and this confirmed that the tip was sealed (Figure 4.1).

A final and somewhat confusing observation was that in some cases, the liquid meniscus moved rapidly by capillary action to within a few micrometres of the end of the tube, and then stopped. Gentle pressure applied with the microinjector was



Figure 4.2: A carbon nanopipette attached to a grid with silver-loaded conductive epoxy.

then sufficient to push the liquid all the way to the end. I have no explanation for this behaviour, since the capillary force should only increase as the tube diameter shrinks towards the tip and so would be expected to fill the tube completely.

## 4.1.2 Freezing and support

The other two requirements for CNPs as specimen holders (rapid freezing for vitrification, and support in the TEM) were fulfilled by attaching the nanopipettes to standard electron microscopy slot grids, as shown in Figure 4.2. This allowed the CNPs to be handled in the same way as normal TEM specimens, using established procedures for rapid freezing and cryo-transfer to the microscope.

Initially, the attachment was done using a silver-loaded epoxy resin with high electrical and thermal conductivity, because of fears that CNPs might show heating and charging effects in the TEM. Even though the epoxy only made contact with the insulating quartz glass exterior of the nanopipette (and not the conductive carbon layer), I thought that using a conductive glue for the attachment might help to reduce the severity of any heating and charging problems. However, the conductive epoxy required a prolonged curing process at elevated temperature, making the attachment procedure quite laborious and inconvenient. Once it was established that the CNPs did not show heating or charging effects, a rapid-curing (but non-conductive) epoxy was tested. This made no apparent difference to the specimens' behaviour in the TEM and so was used for most subsequent experiments. More details of the attachment procedure are given in section 2.2.1.

After nanopipettes had been attached to grids and filled with a liquid specimen, the grid and nanopipette tip were detached from the shaft of the nanopipette. This was done by breaking the glass at the point where it crossed the edge of the grid, using a diamond scribe tool to scratch the glass gently and repeatedly until it separated. The grids were then immediately frozen by the standard method of plunging into liquid ethane cooled to its freezing point by liquid nitrogen.

Once frozen, the grids with attached nanopipette tips could be handled and loaded into the microscope using normal procedures, with only slight modifications to the loading process. The Polara microscope used for this work uses a cartridge system to hold the grids, with each cartridge supporting one grid which is held in place by a thin C-clip ring. Normally the grid is simply lowered into the cartridge and a C-clip is placed on top. For loading nanopipette specimens, after placing the grid into the cartridge, an extra step was added in which the grid was rotated so that the grid slot was perpendicular to the long axis of the cartridge, ensuring that the nanopipette was aligned with the microscope's tilt axis. More care was also needed with the C-clips, since slot grids are thicker than normal grids. This meant that the C-clips were less likely to stay in place correctly, and so it was necessary to position them in the cartridges more precisely than usual, and to use only the highest-quality C-clips. (Many of the clips, even when new, are slightly bent and will not rest completely flat, and these have a much higher failure rate when used with slot grids.)

An obvious objection to using slot grids to support the nanopipettes is that the edges of the grid will obscure the specimen at high tilt angles. This is correct, but not actually a problem in practice due to other limitations in the equipment used for this work: the microscope's specimen stage is physically limited to a tilting range of  $\pm 80^{\circ}$ .<sup>1</sup> Backlash in the tilting mechanism means that even this is a slight overestimate of the true range.

A more significant problem is that the Polara microscope's cartridges obscure the specimen at much lower tilt angles than would be accessible if the slot grid or the microscope stage were the only limitations. For most specimens, the tilting limit of the cartridges is  $\pm 70^{\circ}$  or even lower, but with careful positioning of the specimen within a few micrometres of the centre of the grid horizontally, and a little below the plane of the grid vertically (achieved with my specimens by loading the grid with the CNP on the underside), the achievable tilt range increases to  $\pm 72-73^{\circ}$ . It has also been possible to achieve larger tilt ranges by physically altering the cartridge (see section 4.6).

<sup>&</sup>lt;sup>1</sup>It is possible to increase the physical tilting range of the stage to  $\pm 90^{\circ}$ , and this has been done for a similar microscope in the Netherlands. However, due to the design of the stage and control system there is no guarantee that the movement of the stage would remain eucentric at such high tilt angles, making it difficult or impossible to acquire high-quality tilt series over this range without a more thorough redesign of the stage for this purpose (A. J. Koster, personal communication).



**Figure 4.3:** An image from a tilt series of gold particles in a carbon nanopipette. The large gold particles have a nominal diameter of 50 nm, and the mixture also contained 10 nm particles which are not obviously visible in this image. The tube is approximately 1.8  $\mu$ m in diameter. The slight bulge visible at the upper right corner is the edge of a larger drop of ice which protruded from the end of the tube. Scale bar: 500 nm.

# 4.2 Tomography of gold particles

Initial testing of carbon nanopipettes as specimen holders was performed using colloidal gold particles, which have high density and so can be seen with high contrast in the TEM. A mixture of 50 nm and 10 nm diameter gold particles was prepared, put into a CNP, frozen and transferred into the microscope as described in the previous section. A tilt series was collected over an angular range of  $\pm 70^{\circ}$  in 1° steps at a nominal magnification of 13,500×.

One of the images from the tilt series is shown in Figure 4.3. The tube diameter is approximately 1.8  $\mu$ m, which is much too large for good observation of a frozenhydrated specimen. Illustrating this, dark patches and streaks were visible in the tube at some viewing angles, indicating that the ice inside the tube was crystalline. This is not unexpected after plunge freezing of a specimen of this thickness. Despite this, the large 50 nm diameter gold particles can clearly be seen, though the 10 nm particles are not really visible. (From the tomogram, it is known that the 10 nm particles are



**Figure 4.4:** A slice from the reconstruction of the tube shown in Figure 4.3. The large 50 nm gold particles are clearly visible, and 10 nm particles can be seen along the tube walls, most prominently in the upper left corner. Also visible are strong white streaks from the large gold particles, which are artifacts of the reconstruction process. The slice thickness is 3.4 nm. Scale bar: 500 nm.

almost exclusively arranged on the inner surface of the nanopipette, and on very close inspection of this area it is just possible to identify some of them in this image.)

The tilt series was aligned by using some of the gold particles as fiducial markers, and a tomographic reconstruction was calculated by weighted backprojection. The reconstruction was then isotropically filtered to remove high-frequency noise. A slice through the reconstruction is shown in Figure 4.4. Gold particles of both sizes are clearly visible. The gold seems to adhere to the carbon surface, but a small number of particles are visible embedded in the ice inside the tube.

Despite the large size of the tube, this reconstruction demonstrates that it is possible to use carbon nanopipettes as specimen holders for cryo-tomography. Importantly, the images look good considering the ice thickness, suggesting that electrical charging of the specimen is not a serious problem.

However, the tube's size does indicate a problem. This particular nanopipette had a measured tip diameter of only 400 nm at the end of the manufacturing process, however the diameter in the TEM is almost five times as large. This indicates that the tip of the nanopipette was broken at some point during the specimen preparation process: because the nanopipette tapers towards the tip, removal of a section at the end will leave a new tip which is wider than the previous one. This will also have the
effect of reducing the length of tube which is completely devoid of glass. In cases such as this one where the diameter has increased substantially, it is possible that all of the exposed carbon section has been lost, in which case the images may actually include a thin layer of glass as well as the carbon and ice.

A hint of this can be seen along the bottom wall of the tube in Figure 4.4. A thin dark line can be seen, above which is a wider, less dark region, with an uneven top surface on which the gold particles are seen. A reasonable interpretation of this is that the thin dark line is glass, and the uneven layer is the carbon layer, with the ice and gold inside. It is noticeable that a similar feature is absent from the top wall of the tube, suggesting that perhaps the carbon exposure at this part of the tube is asymmetric. This would not be entirely unexpected, since SEM images of the completed CNPs show that the carbon exposure is uneven around the tube's circumference (see Figures 3.6 and 3.7a).

# 4.3 Tomography of ribosomes

Having shown the feasibility of using carbon nanopipettes for cryo-tomography, the next step was to test them with biological specimens. This proved to be more difficult than expected: various relatively large (100 nm range) objects were tested, but without much success. The most extensively tested specimen was bacteriophage T4: many tomograms were collected, of tubes with a variety of tip sizes, but phage particles were never observed in the reconstructions. Perhaps the most likely explanation for this is the difficulty of ensuring that a particle of this size is statistically likely to be found in the volume of liquid contained in the useful part of a CNP tip. For a tip with a diameter of 500 nm and a useful length of 5  $\mu$ m, this volume is approximately 1×10<sup>-18</sup> m<sup>3</sup>, or one femtolitre. To achieve an average of one particle in this volume requires a numerical concentration of 1×10<sup>15</sup> particles per litre. Though this is equivalent to a relatively low molar concentration of 1.7 nM, for large particles such as bacteriophage this concentration proved difficult to reach.

To solve this problem of ensuring a specimen's presence in the nanopipette tip, two possibilities were apparent: either to use smaller objects such as protein complexes, which are routinely prepared at micromolar or nanomolar concentrations; or to use larger, discrete objects with some kind of label allowing their position to be visualised, and then to control the fluid movement while loading to ensure that the target object is positioned correctly. Accordingly, three biological specimens were tested with at least some success: ribosomes, liposomes, and whole bacterial cells. In this section, I discuss the tomography of ribosomes, while liposomes and bacterial cells are the subjects of sections 4.5 and 4.6 respectively.

Ribosomes were chosen because they are known to be visible in tomographic reconstructions of frozen prokaryotic cells. They tend to be more visible than other cellular protein complexes because of their large size (approximately 25 nm diameter) and their relatively high density, due to the high proportion of nucleic acids they contain. Ribosomes were also easily available in the laboratory, since they are prepared in large quantities for X-ray crystallography studies.

For my tomography experiments, a solution of ribosomes from *Thermus thermo-philus* (Selmer et al., 2006) was kindly provided by C. Neubauer. This was mixed with colloidal gold (15 nm particles, for use as fiducial markers) and diluted to a final concentration of 100 nM, then added to a carbon nanopipette and frozen as previously described (section 4.1). From the manufacturer's specification, the expected concentration of gold particles in the final mixture was 2.1 nM, and therefore ribosomes would be expected to outnumber the gold particles by a ratio of approximately 50:1.

The tip diameter of the chosen nanopipette was approximately 400 nm after fabrication. After loading into the TEM, the diameter a short way back from the end of the tube was measured to be 450 nm, indicating that (unlike the tube containing gold particles discussed previously) the tip of this tube had not been broken to any significant extent. However, this was not a perfect specimen: the tube surface was contaminated with large ice crystals, and it became apparent while tilting the specimen that the ice inside the tube was crystalline, as images at certain angles showed various stripes and mottled patterns indicative of crystal diffraction. Despite these concerns, a tilt series was collected over an angular range of  $\pm 73^{\circ}$ , in 1° steps. The magnification was nominally 50,000×, and the defocus was set to  $-6 \,\mu$ m. Images were energy filtered to select zero-loss electrons with a 20 eV slit width, and the total electron dose for the series was 180 e<sup>-</sup>/Å<sup>2</sup>. An example of a good image from the series is shown in Figure 4.5 along with an image showing the effects of diffraction from crystalline ice in the tube.

Surface ice contamination, as seen on this tube, occurred noticeably more often with cylindrical specimens than with normal grids. It is possible this is caused by the slightly slower cryo-transfer procedure required with the nanopipette specimens (see section 4.1.2), but there seemed to be no correlation between the degree of contamination and the time taken during loading. More likely is that these specimens are



**Figure 4.5:** Images from a tilt series of ribosomes and 15 nm gold particles inside a carbon nanopipette. (a) A normal image. (b) An image showing the effects of diffraction, indicating crystalline ice inside the tube. The tube is approximately 450 nm in diameter. Scale bars: 100 nm.

inherently more vulnerable to such contamination: with a normal grid, a boundary layer of cold air will be present across the whole grid area during handling, helping to reduce mixing with the humid air and therefore reducing the formation of ice crystals. However, with the slot grids, the entire space surrounding the nanopipette tip is open, and air will be free to flow close to the tube on all sides. Ice crystals would therefore be expected to form and attach to the tube more often than to a typical central part of a normal grid.

Crystalline ice was also observed inside the tubes more often than expected. Certainly, some tubes, even of quite large diameters up to almost 1  $\mu$ m, were successfully vitrified and remained in this state throughout the microscopy process. (For an example, see section 4.6.) It therefore seems likely that the freezing process itself is usually successful at cooling the specimens rapidly enough to form amorphous ice. The cryo-transfer step is inherently the most variable part of the preparation procedure, and the part where the tubes are most exposed to the environment. Because of the greater air flow (compared to normal grids) likely to be experienced in the vicinity of the nanopipette tips during this handling, I suspect it is at this stage that some of the specimens were warmed sufficiently to cause de-vitrification of the ice. (Such de-vitrification is known to occur at approximately 135 K (Dubochet et al., 1988).



**Figure 4.6:** Slices from a reconstruction of the ribosome specimen. (a) Slice perpendicular to the microscope's optical axis. (b) Slice perpendicular to the tilt axis. The position of the slice is marked by the vertical dashed line in part A. (c) Magnified view of the boxed area in part (a). (d) Magnified view of the boxed area in part (b). The slice thickness in all cases is 9.3 nm. Some 15 nm gold particles are clearly visible, along with a number of particles embedded in the ice. Scale bars: (a) & (b) 100 nm; (c) & (d) 20 nm.

According to the temperature monitor on the microscope's cryo-transfer arm, temperatures of up to 100 K are routinely experienced during transfer, so it is not unlikely that a narrow cylinder with a high ratio of surface area to volume could easily warm up substantially more than this if exposed to a stream of warmer air.) However, this explanation cannot be easily confirmed.

The tilt series was aligned using the fiducial gold particles, and reconstructed using the Simultaneous Iterative Reconstruction Technique (SIRT) algorithm. Slices from the tomogram, in two different orientations, are shown in Figure 4.6. The gold particles stand out clearly in the reconstruction (though some look less clear because they are only partially contained in the slice shown). Other particles, less dense than the gold, are also visible, embedded throughout the ice. The size of these particles is slightly smaller than expected for full 70S ribosomes, and though not terribly clear, they look somewhat heterogeneous. It therefore seems likely that most of these are dissociated 50S and 30S ribosomal subunits, while some of the larger ones might be complete 70S ribosomes. The number of these particles is substantially greater than the number of gold particles. This would be expected from the composition of the initial mixture, though the precise ratio of the two particle types in the tomogram has not been quantified. (Subtomogram averaging was attempted to see if the shape of the particles could be identified, but no improvement was seen; presumably this is because the reconstructed particles do not contain sufficient information for correct orientations and classes to be found. However, this approach was not tried extensively and so perhaps more could be achieved with a persistent attempt.)

Importantly, the particles are still visible reasonably clearly in the direction of the missing wedge (vertical in Figure 4.6b,d). This is a substantial improvement over typical cryo-tomograms, and demonstrates the potential of this approach to achieve isotropic resolution if the remaining (mechanical) limitations on the tilt range could be removed.

Also visible at the end of the tube is some kind of irregular structure arranged in thick, wavy sheets, with a reasonably clear boundary separating it from the rest of the material inside the tube. Similar features were often observed at the open end of my nanopipette specimens. It was also observed in some nanopipettes which were warmed up, thoroughly dried and then again observed by TEM, ruling out the possibility that it is some kind of ice-related deformity. It is likely to be some kind of deposit of salt, protein or both, caused by evaporation at the end of the tube. (This would explain the variability in its occurrence, since the tubes were frozen in batches and hence exposed to the air for a variable amount of time between filling and freezing.) However, this phenomenon has not been investigated in any detail and so the true cause is unknown.

# 4.4 Specimen preparation II

The other specimens successfully tested in carbon nanopipette holders were liposomes and whole bacterial cells. Neither of these can feasibly be concentrated to the nanomolar concentrations required to ensure presence in the volume of liquid contained in a nanopipette tip. However, liposomes can be concentrated to a reasonable degree, and both have the advantage that they can very easily be visualised in the light microscope using fluorescent membrane dyes.

To actually position large, stained structures such as liposomes in the nanopipette tip, though, requires a different method for specimen preparation. The fluid flow in the tip must be controlled, so that by moving the liquid, the discrete structures suspended in it can be moved as desired. An obvious way to do with would be to simply apply pressure to push liquid out of the tip, and so move objects in the liquid towards the end of the tube. However, this is not possible using the loading method as previously described (section 4.1), because of the effect of surface tension. In particular, for nanopipette tips which are small enough to be useful for tomography, the pressure required to overcome surface tension and push liquid out of the end of the tip is too large to be applied with sufficient control.

The reason for this can be shown quite simply, using the Young–Laplace equation for surface tension of a spherically-curved surface,

$$\Delta p = \frac{2\gamma}{R}$$

where  $\Delta p$  is the pressure difference across an interface,  $\gamma$  is the surface tension, and R is the radius of curvature. Because of its reciprocal relationship with curvature, the pressure increases dramatically as the radius of curvature decreases. Under the assumption that to push liquid out of a nanopipette tip, a hemispherical surface will be formed before drops can escape, the required pressure can be calculated. For water at 20 °C, the value of  $\gamma$  is 72.8 mN·m<sup>-1</sup> (Vargaftik et al., 1983), and so to form a hemispherical water drop with a radius of 500 nm, the pressure required is 290 kPa, or almost three atmospheres. Decreasing the radius to 250 nm (for a typical carbon nanopipette tip with a diameter of 500 nm), this rises to 580 kPa, or almost six atmospheres.

This effect was apparent during nanopipette loading experiments. Along with the difference in the ease of filling noted earlier (section 4.1.1), it was possible to identify nanopipettes with large tip diameters because drops of liquid would very easily be formed on the end of the tube when pressure was applied via the microinjector pump. This was not possible with small-tipped nanopipettes: applying high pressure would either cause the tip to break, after which liquid would flow as with any large nanopipette, or the nanopipette would start to slide free of the O-ring seal which held it to the pump, without any sign of liquid flow from the tip. Perhaps with a stronger seal, it would be possible to push liquid through small-tipped nanopipettes, but the chance of tip damage would only increase, and if flow could be induced it is not clear how easily it could then be controlled.

The solution to this problem was to dip the end of the nanopipette into another drop of an aqueous fluid. This removes the air-water interface responsible for the surface tension, allowing flow in and out of the end of the nanopipette. This had to be done in a light microscope to allow the process to be observed. Various configurations were tried, but one of the most successful was the simplest method. For this, a liquid-filled nanopipette was attached parallel to the surface of a glass microscope slide, allowing the movement of the nanopipette to be controlled using the microscope's normal specimen movement controls. A standard laboratory adjustable pipette, with a small drop of liquid in its tip, was then fitted to a micromanipulator, and positioned with its tip close to the microscope's optical axis. By watching through the microscope and adjusting the positions of both the large pipette and the nanopipette, it was then possible to dip the nanopipette's tip into the liquid inside the tip of the large pipette. Pressure could then be applied in either direction using the microinjection pump, causing liquid to flow in or out of the nanopipette.

This method was used for a variety of specimens, including the liposomes which will be discussed in the next section. It had the advantage of being relatively simple and quick to set up, meaning specimens were not exposed to the environment for long periods of time. It was also possible to perform this method with nanopipettes which were already attached to slot grids, by cutting the tip of the large pipette at a small angle and approaching the nanopipette at an angle of about 45°. This allowed the method to be used with nanopipettes which had been glued to grids using conductive epoxy (see section 4.1.2), though this had two serious disadvantages. Firstly, the handling was quite difficult, and many nanopipettes were destroyed through accidental collisions with the large pipette caused by mishandling or vibration. Secondly, the nanopipettes had to be oriented with the grids aligned vertically to allow the large pipette to fit into the slot. This in turn meant that the microscope objective lens could be no closer than about 2 mm to the nanopipette tip, which decreased the accuracy of observation since

only lenses with a long working distance, and hence a low numerical aperture, could be used.

Once I discovered that rapid-curing epoxy could be used to attach grids to the nanopipettes (section 4.1.2), it became possible to perform the specimen loading and manipulation with a bare nanopipette, and attach the grid afterwards (though this did lead to an inevitable delay of a few minutes between specimen preparation and freezing, while the grid was attached and the epoxy hardened). Using this variation, the large pipette and nanopipette could approach each other coaxially, which substantially reduced the chance of accidental collisions. This also allowed the use of a lens with a shorter working distance, improving the observation of the nanopipette. The optical quality was still fairly poor, however, as can be seen in Figure 4.7.

One disadvantage which was found with this method was that while the fluid flow was occurring, the walls of the large pipette prevented observation of the nanopipette tip. The liquid movement therefore had to be performed iteratively, by a process of repeated dipping and flow followed by withdrawal to allow the nanopipette tip to be observed. Several variants on the technique were tested to avoid this limitation, with some success at the cost of increased technical difficulty. These experiments established that observing the fluid flow process itself was actually not particularly useful: because the nanopipette tip was the smallest constriction in the flow, the liquid here moved at its fastest, meaning that even with reasonably fine control of the pressure controlling the flow, it was impossible to stop the flow precisely enough to keep any desired object in the crucial final section of the nanopipette. This will be explained further in section 5.2.2.

In the case of liposomes, this inability to directly monitor the flow as it occurred was not a serious concern, since the iterative method worked reasonably well. The loading procedure was found to work most successfully when the nanopipette was initially filled with water containing only gold particles, and then dipped into a mixture containing liposomes, gold particles and a fluorescent membrane dye. (Bovine serum albumin was also included in both mixtures because it helped to weaken the adhesion of the liposomes to the carbon walls of the nanopipette.) Some of the liposome mixture was then drawn up into the nanopipette. At this point it was generally found that liposomes were visible (by fluorescence) inside the nanopipette, but not close to the tip.

After dipping again and pushing some liquid back out of the nanopipette, the liposomes were seen to have moved towards the tip and in some cases could be seen



**Figure 4.7:** Fluorescence microscopy of a carbon nanopipette filled with liposomes. (a) Brightfield transmitted light image. (b) Epifluorescence image of the FM4-64 membrane dye. (c) Overlay of the two images. Liposomes can be seen distributed throughout the nanopipette, including the section at the tip. Scale bar: 10 μm

at the extreme end of the tube (Figure 4.7). Presumably this behaviour is caused by the liposomes adhering to the carbon of the nanopipette so they are drawn in rapidly but then expelled only slowly, though the exact cause is uncertain.

This method generally gave better results than the alternative of putting the liposomes inside the nanopipette initially, and simply trying to move liquid out of the tube to move them to the tip. In this case the liposomes (and bacterial cells, which were also tested with this method) seemed to adhere firmly to the tube walls where the tube narrows to a few micrometres in diameter. This apparently caused a blockage, because the liposomes would then never move any closer to the tip despite many attempts at subsequent liquid flow.

Though I had previously tried experiments with pure liposomes, those shown in Figure 4.7 were mixed with the membrane-binding MinCD protein complex from *Escherichia coli* (Raskin and de Boer, 1999), and were kindly provided by D. Ghosal. These were used because there was some evidence that the MinCD complex forms filaments on the surface of liposomes which can be seen by cryo-ET. After the liposomes had been moved to the nanopipette tip, the nanopipette was attached to a slot grid with rapid-curing epoxy and plunge-frozen as already described (section 4.1.2).



**Figure 4.8:** Projection images of liposomes and 15 nm gold particles inside a carbon nanopipette. (a) Unprocessed image taken from the aligned tilt series. (b) Processed image with background removed and set to a neutral grey value. Scale bars: 200 nm.

# 4.5 Tomography of liposomes

The nanopipette containing frozen liposomes was transferred to the electron microscope, and a tilt series was collected. The angular range was  $\pm 72.5^{\circ}$ , with images taken at tilt increments of 1°. The defocus was  $-10 \mu m$  and images were energy filtered to select zero-loss electrons with a slit width of 20 eV. An image from the series is shown in Figure 4.8a. Like the ribosome specimen previously discussed (section 4.3), images in the series showed dark and striped patterns at certain tilt angles, suggesting that the ice inside the tube was at least partly crystalline. However, unlike the ribosome specimen, this nanopipette had very little ice contamination on its surface.

A reconstruction was generated using the SIRT algorithm, and a slice from this reconstruction is shown in Figure 4.9a. Clusters of liposomes were clearly visible



**Figure 4.9:** Comparison of reconstructions from unprocessed and cutout images. (a) Slice from the reconstruction of the original unprocessed tilt series. (b) Equivalent slice from the reconstruction of the processed tilt series. See the text for more details. Slice thickness in both cases is 1.2 nm. Scale bars: 200 nm.

inside the tube, with at least part of each cluster positioned very close to the tube wall, suggesting that the liposomes were helped to remain in the nanopipette tip by adhering to the carbon surface during the filling process. In the reconstruction, the tip was seen to be elliptical, with major and minor axis diameters of 610 and 530 nm, giving an average thickness of 570 nm. This nanopipette had a measured diameter of 420 nm after manufacture, suggesting that a small part of the tip was probably broken off during specimen preparation.

Though the liposomes could be seen clearly in the reconstruction, I was concerned that the reconstruction algorithm might be affected by the extreme contrast in the images between the tube and the vacuum surrounding it. (The image intensity outside the tube was typically over ten times that measured inside the tube.) I therefore processed the tilt series images to replace the tube surroundings with a flat neutral grey, of the same intensity as the mean value inside the tube. Figure 4.8b shows the processed image corresponding to the original image shown above (Figure 4.8a), and the tube contents can be seen more clearly in the processed image. There is of course no new information here, and it is perfectly possible to see the same level of detail inside the tube in the unprocessed image by adjusting the display contrast settings. However, it was hoped that the removal of the high intensity background might allow the reconstruction algorithm to enhance the contrast of the tube contents more effectively.

In this case, this approach gave good results: a slice from the reconstruction of the processed series is shown in Figure 4.9b, and the liposomes are substantially more visible than in the equivalent slice from the original reconstruction. Importantly, the difference is now more than simply an adjustment of the image contrast; no amount of contrast adjustment of the original reconstruction can reproduce the clarity seen in the processed one. Because of this improvement, further results presented in this section are all based on the processed series reconstruction. (This approach does not always produce such improvements: it was also tried with the ribosome specimen presented in section 4.3, and made no apparent difference to the visibility of features in the reconstruction.)

Magnified, orthogonal slices of the tomogram are presented in Figure 4.10 parts a, b and c. Using a coordinate system where the *z*-axis is the optical axis of the TEM and the *y*-axis is the tilt axis (with the *x*-axis orthogonal to both), these figures show slices in the *xy*, *yz* and *xz* planes respectively. Though the missing wedge is smaller here than in typical cryo-tomograms, its effects are still apparent: the resolution is anisotropic, clearly best within the *xy* plane, and worst along the *z*-axis. Importantly, though, complete circular cross-sections of liposomes are still visible in the *xz* and *yz* planes.

In some places, the membranes of two liposomes come together to form a thicker line. This provides a useful way to estimate resolution in the tomogram. Line profiles across these boundaries (averaged across a line with a thickness of 10.5 nm) consistently show that, in directions within or near the *xy* plane, the two membrane layers are resolved as a pair of minima, with a separation of approximately 7.5 nm. Two such profiles are plotted in Figure 4.10 parts d and e. Similar profiles along directions close to the *z*-axis fail to resolve the individual membrane layers, instead showing a single broad minimum (Figure 4.10f). The positions of these line profiles are marked by



**Figure 4.10:** Slices and line profiles from the liposome reconstruction. (a), (b) and (c): Slices in the *xy*, *yz* and *xz* planes, respectively. (d), (e) and (f): Line profiles (averaged across a width of 10.5 nm), coloured to match their marked positions in parts (a) and (b). The line plotted in (d) is entirely within the *xy* plane. The line in (e) is angled slightly away from the *y* direction, and the line in (f) is angled slightly away from the *z* direction. (d) and (e) show two closely-spaced membranes as a pair of minima with a separation of 7.5 nm, while (f) shows that a similar feature in the *z* direction is not resolved. Slice thickness is 12 nm for parts (a) and (b), and 6 nm for part (c). Scale bars: 100 nm.

correspondingly-coloured boxes in Figure 4.10 parts a and b.

This membrane separation of 7.5 nm therefore provides an upper estimate of the tomogram's resolution in the xy plane. Qualitatively, the reconstruction's appearance suggests that the resolution is not much better than this, so 7.5 nm seems like a reasonable estimate for the resolution. Although the resolution is worse in the z direction, the equal quality of images at all tilt angles from cylindrical specimens means that, with technical improvements, tilt series could be acquired over a full angular range and so achieve an isotropic resolution at least as good as this.

A point to note is that no protein filaments were visible in this specimen. The MinCD complex would be expected to form filaments on the surface of the liposomes, and to induce deformities in their shapes. Since the protein filaments are not seen, it is possible either that the quality of the tomogram is insufficient to identify them, or that they have been detached from the liposomes during preparation. Given the high visibility of the membranes in this tomogram, and the fact that most of the visible liposomes are spherical in shape, it seems more likely that the protein filaments are simply absent, rather than present but unseen.

Finally, the high contrast in this reconstruction made it an ideal candidate for visualisation using three-dimensional volume rendering. This allows structures to be observed in three dimensions without segmentation, and when used interactively on a computer this can quickly give the user a powerful insight into the overall shape and structure of a reconstruction, in a way which is more intuitive to see and manipulate than the normal slice-by-slice visualisation commonly used for tomograms. However, in volume rendering the effects of the missing wedge become very apparent. To illustrate this effect, a second reconstruction was generated from the same tilt series as the first, but omitting some images so the total tilt range was only  $\pm 50^{\circ}$ . To enhance the membrane structures in both tomograms, anisotropic nonlinear diffusion (Frangakis and Hegerl, 2001; Fernández and Li, 2003) was used to remove noise and sharpen linear and planar structures. Equivalent slices from the full range and restricted range reconstructions are shown in Figure 4.11a and Figure 4.11e respectively, demonstrating that the quality of the tomogram in the *xy* plane is barely affected by the reduction in angular range.

The remaining parts of Figure 4.11 show equivalent views of the volume renderings of the two tomograms. (Precise equivalence is difficult to achieve in volume renderings of two separate reconstructions, but the images have been prepared to look as similar as possible.) In Figure 4.11b and f, a view along the *z*-axis is shown, roughly equivalent



**Figure 4.11:** Volume rendering of tomograms from a large and small angular range. (a)–(d): Images from the tomogram already presented, reconstructed from an angular viewing range of  $\pm$ 72.5°. (e)–(h): Equivalent images from a tomogram reconstructed from an angular range of  $\pm$ 50°. (a) and (e): Slices through the tomograms, after anisotropic nonlinear diffusion to reduce noise and enhance planar structures. Slice thickness is 2 nm. (b) and (f): Volume rendering of the tomograms looking along the *z*-axis. The large cluster of liposomes in the centre of the images is the same as that visible in parts (a) and (e). (c) and (g): Volume renderings looking along the *x*-axis at a slightly magnified view of the same large cluster of liposomes. (d) and (h): The same volume renderings, now looking along an oblique view roughly intermediate between the *x*- and *z*-axes. Scale bars: 200 nm.

to the slices presented above. At least three separate clusters of liposomes can be seen, showing the value of this visualisation technique for obtaining an overview of an entire volume. In this view, both tomograms look similar. Also visible in these pictures is a diffuse cone of background density. (With the rendering thresholds required to visualise the liposomes clearly, this background density is unavoidable. This graphically illustrates the effect of the tube thickness on the tomogram: near the tip, the background is almost absent, but where the tube thickness increases the background rapidly comes to dominate the reconstruction. However, this is probably an artifact, since in the reconstruction from the original unprocessed tilt series the interior of the tube has a more even intensity. An analysis by Kato et al. (2008) suggests that a reduced signal-to-noise ratio is to be expected in the centre of a thick cylindrical specimen, and this presumably explains the phenomenon.)

Figure 4.11c and g show views in the *yz* planes of the reconstructions. This is the direction where the effects of the missing wedge are strongest, and both tomograms show poor resolution along the *z*-axis. The full-range reconstruction looks a little better than the restricted-range one, but any improvement in the rendered shape of the liposomes is difficult to see above the general background blur along the direction of the missing wedge.

The most dramatic contrast between the two reconstructions is seen in the final pair of images (Figure 4.11d and h). This view is taken from an oblique angle, roughly intermediate between the two previous viewing directions. Here, in the full-range reconstruction the shape of the liposomes is still very clear, while in the restricted-range tomogram the information about the shape of the liposomes in the vertical direction has been lost. This effect is even more striking when viewing these volume renderings interactively: in the *xy* plane, both tomograms appear equally detailed. However as soon as the viewing angle is changed, it becomes clear that the liposomes in the restricted-range reconstruction the liposome shape is visible from a much wider range of angles and the impression of observing a three-dimensional object is sustained.

# 4.6 Tomography of whole bacteria

The primary purpose of this work was to create a cylindrical holder which could be used for structural studies of whole bacterial cells. As such, many attempts were made to collect tomograms from bacteria inside carbon nanopipettes, using the loading



**Figure 4.12:** Photographs of **(a)** a normal cartridge for the Polara microscope, and **(b)** a modified cartridge where the sides have been cut away to increase the angular viewing range of the specimen.

methods already described (sections 4.1 and 4.4) and others. In general, this has been unsuccessful, due to difficulties in keeping suitably-sized nanopipettes intact while simultaneously positioning bacteria in the useful part of the tip.

However, one nanopipette, which had been prepared with cells of *Caulobacter crescentus*, was successfully imaged by electron tomography, and does contain structures which have the appearance of cells, though the level of detail is poor (and, in fact, the presence of cells inside the tube was not initially recognised). This nanopipette was prepared using the 'simple' loading method described in section 4.1, using a specimen of *C. crescentus* cells mixed with fiducial gold particles, bovine serum albumin and a fluorescent membrane dye. After loading, it was observed that fluorescence from the dye was present a few micrometres away from the pipette tip, but not in the tip itself. However, the diameter of this nanopipette after fabrication was approximately 450 nm, while in the TEM the diameter was measured to be 830 nm, indicating that the tip had been broken during the specimen preparation process. This means it is entirely possible that the cells that were seen a short distance away from the tip before freezing were then present at the very end of the nanopipette after it had broken.

One significant alteration to the preparation procedure for this nanopipette was the use of a modified cartridge for supporting the grid in the Polara microscope. In an effort to increase the available tilt range for my specimens in the Polara, the laboratory workshop cut away some material from the sides of a normal cartridge, allowing a specimen at the centre to be observed from a wider range of angles. A photograph of this modified cartridge is shown in Figure 4.12, alongside a normal cartridge for comparison.

The sides of the modified cartridge were cut away to the point that the C-clip used to retain the grid became the piece which limited the available tilt range. If loaded carefully, with a nanopipette positioned correctly in the centre of the grid, and with



**Figure 4.13:** Images from a tilt series of *C. crescentus* cells inside a carbon nanopipette. (a) Tilt angle  $-79^{\circ}$ . (b) Tilt angle  $0^{\circ}$ . (c) Tilt angle  $+79^{\circ}$ . It is very clear that the general appearance of the tube does not change significantly at high tilt angles, unlike the slab-like specimens normally used for tomography. Scale bars: 200 nm.

the gap in the C-clip aligned with one side of the cartridge, it was possible to obtain images of the specimen over the Polara's entire available tilt range of  $\pm 80^{\circ}$ . Achieving all of these conditions simultaneously was difficult. This was compounded by the fact that with the modified cartridge, the C-clip ring – which was already slightly tricky to load correctly using slot grids in normal cartridges – was held even less firmly than normal due to the removal of material which would normally contribute to securing it. Loading this cartridge therefore often took many attempts before the C-clip would hold the grid in place securely.

In order to avoid having to carry out these repeated loading attempts in cryogenic conditions when loading the microscope, I tried loading the cartridge at room temperature immediately after specimen preparation, and then plunge freezing the entire cartridge and grid at once. This was never tested with a normal grid (and is perhaps only possible for specimens where blotting is not required), but, perhaps remarkably, this technique did produce successful vitrification of ice inside carbon nanopipettes.

This is seen in the tilt series collected from this particular nanopipette, which shows no signs of crystalline diffraction in any of the images. The tilt series was



**Figure 4.14:** Slices from a reconstruction of *C. crescentus* cells in a carbon nanopipette. (a) Slice perpendicular to the microscope's optical axis. (b) Cross-sectional slice at the position of the yellow dashed line in part (a). (c) Magnified view of a slice at the position of the yellow box in part (a). (e) Magnified view of the position marked by the red box in part (b). (d) and (f) Reproduction of parts (c) and (e) with red lines tracing the path of features assumed to be cell membranes. Slice thicknesses: (a) 21 nm, (c) and (d) 32 nm, (b), (e) and (f) 53 nm. Scale bars: (a) and (b) 200 nm, (c)–(f) 50 nm.

collected over a range of  $\pm 79^{\circ}$ , in 1° increments. The magnification was 22,500×, the defocus was set to  $-6 \ \mu\text{m}$ , and the total electron dose for the series was 110 e<sup>-</sup>/Å<sup>2</sup>. The

energy filter was not used. Images from the series are shown in Figure 4.13, showing that images obtained at all angles of tilt are similar in quality.

As mentioned above, the presence of cells inside this tube was missed initially, because they cannot be seen either in the projection images or in thin slices of a simple weighted backprojection reconstruction. However, on close inspection of thicker slices from the reconstruction, it became apparent that the tube does contain some structures which bear a close resemblance to the double membrane of a Gram-negative bacterial cell envelope. A higher quality reconstruction was therefore calculated using the SIRT algorithm, and slices from this reconstruction are shown in Figure 4.14.

These features, though not easily traced through the whole reconstruction, are clearly extended, continuous sheets, and importantly they are always paired, with a consistent distance separating each pair. This separation is similar to that observed between the cell membranes in other tomograms of *C. crescentus* (Briegel et al., 2006). These two factors strongly suggest that these are indeed the envelopes of bacterial cells.

It is notable that these structures are seen most clearly at the tube edges. This would be expected if the tube thickness is the problem preventing the cells from being seen more clearly: the centre of the tube is observed through the tube's full diameter at all tilt angles, whereas the edges are seen through a thinner chord of the cylinder from at least some angles, and so the signal-to-noise ratio of these areas will be improved. (Significantly for the structures seen here, the viewing angles with the lowest projected thickness are those which contribute information about spatial frequencies in the radial direction, which is ideal for visualising planar structures running around the tube's circumference.)

This tomogram can be seen as a proof of principle, demonstrating that with a sufficiently small missing wedge, the reconstruction is almost free of the artifacts and anisotropic resolution which a large missing wedge typically causes. (This is nicely demonstrated by the essentially complete circumference of the tube visible in Figure 4.14b, though it is noticeable that the missing wedge still prevents resolution of the cell membranes in the vertical direction.) However, this tomogram also demonstrates the challenge of getting good results from this method, since this is the only reconstruction where bacterial cells have been seen out of many attempts, and the quality is well below that required for a useful imaging technique. If the technical challenges can be solved, though, then the potential of this approach is clear.

# CHAPTER 5

## Discussion

## 5.1 Summary and significance of results

The results presented in the previous chapter demonstrate, using three different biological subjects (ribosomes, liposomes and whole bacterial cells), that electron cryotomography can be successfully performed using cylindrical specimens. To the best of my knowledge, this is the first time this has been achieved.

For all of the specimens discussed, images were collected with essentially equal quality at all tilt angles, and it would be possible to continue this over a complete tilt range if the mechanical limitations preventing this were removed. These cylindrical specimen holders would therefore be capable of producing tomograms with isotropic resolution, if a suitable support could be constructed to allow their full rotation in the TEM. With suitably thin cylinders, the achievable resolution of such tomograms should be similar to or better than that seen in typical cryo-tomograms of small whole cells, and this method could therefore provide a very useful tool for ultrastructural studies where isotropic resolution is required.

Importantly, several anticipated difficulties were found not to cause problems. Carbon nanopipette specimens displayed no sign of any effects (such as image distortions or thermally-induced drift) from heating or electrical charging in the beam of the electron microscope. Despite being only supported and cooled from one side, the tips of the nanopipettes did not show noticeably greater image drift than specimens prepared on standard cryo-EM grids. Freezing of the specimens was successful, showing that a layer of carbon surrounding the specimen does not slow heat transfer enough to prevent vitrification of the ice. Finally, when attached to EM grids, handling of the frozen nanopipettes was relatively simple, requiring minimal adjustments to normal cryo-transfer procedures.

An additional advantage of this method which has not been discussed in any detail is the avoidance of a blotting step during specimen preparation. One of the major challenges in preparing standard cryo-EM specimens is to find an effective and reproducible way of blotting the excess liquid from the grids, to leave an ice layer of the desired thickness without allowing excessive evaporation of the medium which can cause severe osmotic changes (Dubochet et al., 1988). With liquid-filled nanopipettes, the specimen thickness is defined entirely by the dimensions of the carbon tube, and so blotting is not required. A small amount of evaporation can occur from the exposed liquid at the extreme tip of the tube, but in contrast to the situation encountered with normal cryo-EM grids, here the surface area exposed for evaporation is very small in comparison to the liquid volume. Any osmotic changes induced by evaporation will be correspondingly reduced, or at least spatially limited to the end of the tube. (It is likely that evaporation caused the effects seen at the end of the ribosome specimen in Figure 4.6a, but it is notable that these effects are limited to the final 200–300 nm of the tube.)

Despite these successes and the clear advantage of using cylindrical specimens for tomography (as discussed in the Introduction), challenges remain before this technique can be used routinely. Most significant of these is the problem of accurately preparing specimens with a structure of interest correctly positioned within a cylinder that is thin enough for high-resolution tomography. The challenges and limitations of the method are discussed in more detail in the next section, followed by a discussion of possible ways in which the technique could be improved in section 5.3.

## 5.2 Limitations

There are several limitations and challenges which have so far prevented the full success of this method. Solutions to some of these could prove technically difficult, but none of them are fundamental problems, in that no basic physical principles prevent a solution from being possible.

As with much of electron microscopy, good specimen preparation remains the major challenge. In my work on this technique, the greatest difficulty has been in trying to position a desired specimen correctly in the thin, usable carbon-walled part of a nanopipette, while simultaneously ensuring that the nanopipette tip remains unbroken and the ice remains vitreous throughout the process of loading, freezing and

electron microscopy. This difficulty is caused by the interaction of several individual problems which merit further discussion.

#### 5.2.1 Tube damage

Firstly, there is the problem of tube fragility: nanopipettes are broken and lost at all stages of the process. During manufacturing, losses occurred, but these were not too much of a concern since the rate was relatively low. Overall, approximately 15% of nanopipettes were discarded during or after fabrication. At this stage, many nanopipettes were processed in parallel, and all were measured after manufacture and so those which were damaged could be simply removed without substantial wasted effort.

Of greater concern were breakages during the specimen preparation process, since nanopipettes were handled individually at this point and each one broken represented a significant investment of time and effort. Some broken nanopipettes could be identified relatively easily during the liquid filling process (discussed at length in sections 4.1 and 4.4), but for those broken less badly or at a later stage, the damage was only discovered once the specimens had been loaded into the TEM for tomography. Around 60–70% of nanopipette tips were found to be larger in diameter – and therefore almost certainly broken – when measured in the TEM than they had been after manufacture (though some of these were still thin enough to be usable).

When a thick layer of carbon is used, carbon nanopipettes are known to be very strong: in fact, Schrlau et al. (2008b) discuss how their CNPs, made with a wall thickness of over 80 nm, are stronger than glass micropipettes, and can buckle and then recover their shape when pushed into solid objects. For transmission electron microscopy, though, it is important to try to reduce the carbon thickness as much as possible to reduce attenuation of the electron beam by the tube walls. This inherently decreases the strength of the nanopipettes, making them vulnerable to damage during normal handling.

## 5.2.2 Specimen positioning

The second problem to be discussed is the difficulty of positioning a specimen in the correct part of a nanopipette tip. As discussed in the previous chapter, for homogeneous liquid specimens this was not a problem since it was relatively simple to move the liquid to the end of the nanopipette, which would ensure the specimen's presence

in the carbon tip section. However this was much more difficult for larger, discrete specimens. For large particles which could not be easily stained or visualised in the light microscope, such as bacteriophage particles, it was essentially impossible to position them correctly.

Membrane-bound specimens such as liposomes and Gram-negative bacteria could be made to fluoresce with a membrane dye, and it was therefore possible to monitor their movement in the light microscope, as discussed in section 4.4. Theoretically, this approach should allow very fine control over specimen positioning and movement, and ideally would allow an individually-chosen target (displaying a desired selection of fluorescent markers, for example) to be selected from the solution and drawn up into a nanopipette tip. However, due to technical difficulties in both microscopy and fluid control, I was not able to achieve this.

For the microscopy, optical quality was generally not ideal for high-precision imaging of the end of the nanopipette and any structures within it. With the method described in section 4.4, the nanopipette was observed in air using an objective lens with moderate magnification and working distance, and therefore a relatively low numerical aperture ( $40\times$ , N.A. 0.65). Furthermore, the lens was designed for imaging liquid specimens through a glass coverslip, and optical aberrations were apparent when observing nanopipettes in air (see, for example, the fringing effects at the nanopipette tip in Figure 4.7a). A variant of the technique was tested which allowed imaging of the specimen in more ideal conditions – in liquid and under a flat coverslip – and the aberrations were improved. However the optical quality was still insufficient to monitor specimen movements in detail. Ideally an objective lens with high magnification and high numerical aperture working very close to the surface of the coverslip. Again, this was tried, but almost all nanopipettes tested in this way were destroyed by collisions with the glass.

The more significant problem, though, was the difficulty of controlling specimen movement and stopping liquid flow when the desired object was in the correct position. Due to the constricting effect of the nanopipette tip, fluid flow velocity was greatest in the very tip of the tube where it was intended to position the specimen. This caused two undesirable effects. Firstly, objects would accelerate dramatically while passing through this part of the tube, to the point that even at very low flow rates it was impossible to follow the objects by eye: they would slowly approach the tip, before suddenly accelerating and appearing again at slow speed on the other side of the tip before I could react. Secondly, the high flow rate had the effect of keeping the nanopipette tip clear of any obstructions. Liposomes or cells would often adhere to the tube walls further back inside the tube, but if any did adhere in the narrowest part of the tip they would swiftly be washed clear by the rapid flow.

Because these problems were caused by the constriction of the tip, they were at their most severe when the tip diameter was smallest. Smaller tip diameters are essential for improving the quality of the eventual tomograms, and so this makes finding a solution essential if these nanopipette specimen holders are to become a viable alternative to established specimen preparation techniques.

#### 5.2.3 Contamination and devitrification

As discussed in section 4.3, cylindrical specimens were observed to contain crystalline ice, or have surface ice contamination, more often than standard cryo-tomography specimens. I suspect that both of these effects are caused by the exposed nature of a cylinder projecting into the centre of the empty space in a slot grid, allowing much more air flow around the specimen than would typically occur at any given point of a specimen in the form of a flat sheet. This is a cause for concern, though since some nanopipettes, even at larger sizes (such as the one filled with bacterial cells discussed in section 4.6) have been successfully observed with no sign of crystalline ice and very little surface contamination, it is clearly possible to prepare and load specimens without these problems.

The fact that specimens have been successfully vitrified even with large diameters approaching 1  $\mu$ m suggests that the freezing process is successful. It is possible that variations in the freezing process are responsible for some tubes containing crystalline ice, but because of the relative lack of scope for variability during freezing (particularly compared to normal grids where blotting has such a substantial effect) it seems more likely that devitrification during the cryo-transfer process is the cause. Devitrification of a specimen which has been successfully vitrified on freezing is thought to be much less damaging to biological structures than the formation of large crystals which occurs when freezing is too slow (Cyrklaff and Kühlbrandt, 1994; Dubochet, 2007), and so if this is the cause of the crystallinity seen in some of my specimens, it is not likely to be a severe problem.

# 5.3 **Prospects for the future**

There are several ways in which it should be possible to improve the techniques presented in this work. Improvements in the manufacturing process would allow carbon nanopipettes to be produced more reliably and with higher quality, while better methods of specimen preparation could increase the chances of producing good, thin cylindrical specimens containing subjects of biological interest. Perhaps most interesting is the possibility of combining nanopipette-based specimens with the focussed ion beam (FIB) milling technique which has recently been shown to have great potential in other areas of cryogenic specimen preparation (Rigort et al., 2010; Hayles et al., 2010).

### 5.3.1 Improving carbon nanopipette fabrication

The process of improving nanopipette manufacture has been an ongoing one, with each new batch used to test small variations in the procedure. This has produced a gradual improvement in nanopipette quality and yield, and such incremental improvement could certainly be continued. The quality of the carbon layer is a particular area which would benefit from further research. Ideally, nanopipettes would be produced with stronger carbon walls, which could then be made thinner without sacrificing robustness. One way to achieve this would be to find a way to make the carbon surface smoother: in the current process the carbon surface has a very rough texture, as can be seen in the tomograms. Assuming the overall strength is determined by the thinnest areas, a smoother layer would have a higher strength with the same quantity of carbon. It is not entirely clear how a smoother carbon layer could be produced, but there are many variations in the chemical vapour deposition process which could be explored, including the use of alternative carbon feedstock gases (such as ethylene or acetylene) and different rates of deposition (by varying temperature and gas flow rates).

A second way to improve the carbon quality would be to anneal it at high temperature after deposition to increase the graphite content. This was already tested at the deposition temperature of 925 °C (see section 3.3) where the effect was small, but higher temperatures of 1200 °C would be expected to be much more effective (Singhal et al., 2010). Achieving this temperature would require modification of the furnace system used in this work, but might lead to significant improvements in the carbon strength. Annealing would also be likely to change the surface properties of the carbon, but it is hard to say if this would be an advantage or a disadvantage without testing. If problems did arise then other possibilities exist for subsequent modification of the carbon surface chemistry (Vitol et al., 2009).

### 5.3.2 Specimen support in the TEM

A significant limitation on the technique presented here is the way in which carbon nanopipettes are supported inside the TEM. Attaching the nanopipettes to standard EM grids using epoxy has certain advantages, such as physical protection of the nanopipette tip and simple integration of the nanopipettes into normal TEM specimen handling procedures. However there are important disadvantages as well. If a grid is attached before a specimen is put into the nanopipette, the specimen can be frozen immediately after preparation, but handling of the nanopipette during specimen loading is seriously restricted by the presence of the grid. Alternatively if the specimen is prepared first, there is then a delay while the grid is attached before the specimen can be frozen. In both cases, it is then necessary to detach the grid and nanopipette tip from the nanopipette shaft, which is potentially a cause of tip damage. This step is also completely irreversible, with the significant disadvantage that it is not possible to examine a nanopipette in the TEM before specimen preparation.

A much better option would be to find a reversible way to support nanopipettes in the TEM. If this could be done while keeping at least a short length of the nanopipette shaft attached to the tip, it would then be possible to examine nanopipettes in the TEM both before and during the specimen preparation process, allowing the process to be characterised more fully. The difficulty with this approach is that the nanopipette with a useful length of shaft would be a minimum of several millimetres long, meaning the TEM specimen stage would require significant alteration.

It is possible that this might be achieved using the Polara microscope's cartridge system, since the cartridge is substantially larger than a normal grid and so some space is potentially available for a mechanism to hold a nanopipette. Perhaps more feasible would be to adapt the tip of a standard side-entry holder. This approach was taken by Barnard et al. (1992) for their room-temperature cylindrical specimens, though obviously this would be more difficult for a holder to be used under cryogenic conditions. An advantage of this approach is that it might be possible to add some form of rotational control of the nanopipette accessible from outside the microscope. With the currently-available microscope tilt range of  $\pm 80^{\circ}$ , it would only be necessary

to rotate the nanopipette by a further 20°, independently of the stage tilt, to allow access to a complete angular range for imaging.

A more complicated way to approach this problem could be to adapt one of the various full-tilt specimen stage designs which have recently been developed for tomography in materials science (Guan et al., 2011; Tanigaki et al., 2012; Yaguchi et al., 2008). The difficulty here is that these stages are relatively complicated and the use of cryogenic specimens was not considered in their design. The addition of cryogenic cooling would be very challenging if not impossible, though these stages could more feasibly be used as inspiration for an entirely new design, intended from the beginning to be used with cryogenic specimens. An alternative but probably equally complex way to approach this problem would be to redesign the microscope stage itself, to add support for full angular tilting and nanopipette specimens. This seems somewhat unlikely to happen, though if the benefits of full-tilt tomography are judged important enough by the scientific community in future then such a redesign might become a possibility.

## 5.3.3 Improving specimen loading

As mentioned before, specimen positioning in the nanopipettes was one of the greatest challenges in this work. The biggest single improvement which could be made to this method would be to find a way to place a desired specimen into a carbon nanopipette tip reliably and without damage. It is possible that adjustments to the methods already tested might provide substantial improvement, though for the reasons discussed in section 5.2 this may be unlikely.

If the existing method is to be improved, better microscopy is essential. The only way to achieve more accurate control of liquid flow and object positioning is to observe the process more closely. This would require the use of a high-magnification, high-quality objective lens and would therefore require working under a coverslip and at a very small working distance. Though this has already been attempted without success, it is possible that some kind of apparatus could be constructed which would allow working in these conditions without the nanopipette damage problems which have obstructed progress so far.

Another requirement for full control over the process would be to immobilise the desired specimen within the liquid medium, or at least to significantly restrict its movement. This would allow a target to be identified before bringing the nanopipette



**Figure 5.1:** Bacterial growth inside a nanopipette. A single bacterium placed near to the tip of a nanopipette (a) could be incubated in growth medium to allow the cell to divide (b) and form a colony. If the colony expanded along the bore of the nanopipette, some cells might end up in the tip (c), where they could be observed by tomography.

to its position and drawing it into the tip. In the current method, Brownian motion prevents this from being done with any kind of accuracy. Such motion could perhaps be restricted by embedding the specimen in a gel matrix or attaching it to a glass surface. In either case, the more accurate microscopy just discussed would be essential for controlling the movements of specimen and nanopipette sufficiently carefully to ensure specimen transfer into the nanopipette tip.

A more radical way to move a specimen into the nanopipette tip would be to allow it to move or grow there by itself. For example, a bacterial cell could be positioned within a short distance of the tip and then allowed to grow and divide, expanding until the colony filled the tip (Figure 5.1). Alternatively if the inner surface of the nanopipette could be appropriately prepared, crawling eukaryotic cells could move to the tip by chemotaxis, or cells with long processes such as neurons could be encouraged to grow into and through the carbon section of the tube. It is possible that the restricted diffusion of oxygen and nutrients to a cell growing in such confined conditions would prohibit growth, but there is evidence that bacteria can grow in channels which are restricted to only 250 nm in one dimension (Männik et al., 2012), and it would certainly be interesting to test such growth in a nanopipette where two dimensions are similarly restricted.

#### 5.3.4 Combining nanopipettes with FIB milling

The problems experienced with this method so far are mostly concerned with specimen thickness. If nanopipettes break during preparation, they may still contain an object of interest but are usually too large for good imaging in the TEM. If the nanopipettes do not break, those with the smallest diameters are most difficult to load with a specimen, while at larger diameters the specimen preparation becomes much easier. In particular, for studies of whole bacterial cells, the ideal specimen would consist of a single cell surrounded by as thin a layer of ice as possible. This would require placing a bacterium into a nanopipette where the tip diameter matches that of the cell, but it is doubtful if this could ever be reliably achieved.

The recent application of focussed ion beam milling to cryogenic specimens offers a possible solution to this problem. In a number of recent publications, several groups have demonstrated that FIB milling of frozen-hydrated material can give good results, with few of the artifacts associated with alternative preparation methods such as ultramicrotomy (Hayles et al., 2010; Rigort et al., 2010, 2012a,b; Wang et al., 2012). This work has generally aimed at the preparation of thin wedges or lamellae, using the FIB to remove material from a target area to reduce the thickness, and allowing tomography to be performed on parts of specimens (such as whole eukaryotic cells) which are normally much too thick for TEM observation. In a review, Leis et al. (2009) suggested that a similar method could be applied to high-pressure frozen material to create cylindrical specimens, though no results making use of this idea have yet been published.

Due to its ability to precisely remove small amounts of material from frozen specimens, the FIB seems to be an ideal tool for thinning carbon nanopipettes for tomography (Figure 5.2). Specimens could be prepared with a larger diameter, making it much easier to place a desired object in the correct position. (The required accuracy of positioning would also be much lower, since the target would not be required to be in the extreme tip of the tube.) FIB milling could then be used to remove the walls of the nanopipette and to thin the remaining ice, leaving the specimen exposed as a narrow frozen cylinder. This would be expected to dramatically increase the quality



**Figure 5.2:** FIB milling of nanopipettes. A nanopipette to be prepared by FIB milling could be made substantially wider than normal (a), making it much easier to position a specimen (such as a bacterial cell) near to the nanopipette tip. The FIB could then be used to remove material from the marked areas. Rotation around the nanopipette's axis and repetition of the milling would produce a thin, cylindrical protrusion containing the specimen surrounded by a minimal layer of ice (b).

of the tomograms, since ice thickness is a significant limiting factor.<sup>1</sup>

With the ability to thin the tubes after freezing, the nanopipette thickness during initial preparation would be limited only by the vitrification depth achievable using the plunge freezing technique. This would be expected to be a few micrometres. (It could also be possible to use simple glass micropipettes for the initial specimen preparation, though the low thermal conductivity of glass might prevent successful vitrification in which case carbon-walled tubes would still be necessary.) As well as making preparation easier, the greater size of the initial specimen could also allow larger objects to be

<sup>&</sup>lt;sup>1</sup>The precise way in which the FIB should best be used to thin a cylinder would have to be established by experiment. It is possible that milling from the side, as shown schematically in Figure 5.2, would be effective, and rotation of the cylinder to two or more positions would allow the production of a prism-shaped protrusion. However, if the FIB milling accuracy is insufficient for this approach without causing damage to the remaining material, it might instead be necessary to adopt a variant of the annular milling method (Miller et al., 2005). This would require milling with the FIB in an axial direction from the end of the tube to remove material using an annular pattern. This approach would allow a protective layer of metal to be deposited in front of the target area, which would not be possible with the tangential milling approach.

used, which would widen the scope of possible specimens to include eukaryotic cells.

This technique would have notable advantages over that previously proposed (Leis et al., 2009), due to the fact that the target specimen would be embedded in a much smaller quantity of material. With a specimen prepared by high-pressure freezing, a large amount of material would have to be removed using a microtome before a target could be identified, and FIB milling would then be required to remove a substantial amount more. Locating a desired specimen could also be very difficult.

With a specimen prepared in a nanopipette, no microtomy would be necessary, and only small amounts of excess material would need to be removed with the FIB. However, locating the target specimen accurately within the tube – to allow it to be exposed precisely by the milling process – could still be challenging. Optical microscopy would probably be the best way to locate a fluorescently-labelled specimen, and it might be that simply measuring the position of the fluorescence peak relative to the tube walls (perhaps with a correction for the effects of refraction) would be accurate enough for guidance of the milling process. Two viewing directions would be required to locate the target precisely in three dimensions, and some form of fiducial mark (perhaps a small droplet of epoxy resin on the tube) would be necessary to allow the orientation of the tube to be uniquely identified in both the optical microscope and the FIB.

## 5.4 Conclusion

The results presented here demonstrate that it is possible to perform electron cryotomography at nanometre-scale resolution with a minimal missing wedge. The method is technically challenging, but future developments could allow full-tilt tomography to become widespread and routine. The benefits of this approach would be far-reaching: it would enable isotropic examination of cellular ultrastructure, and this could provide insights into a range of important biological questions where missing information currently makes accurate and complete observation impossible.

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