4. Summary

The FIB system was extensively used in the work described in this thesis as a powerful und universal tool for the investigation of biological materials. It was used as an *in situ* laboratory for microscopy, structural investigations, sample preparation, fixation and mechanical testing.

The combination in one system of a microscope and a milling tool allows the FIB to be used for structural investigations, for the site specific and highly accurate preparation of cross-sections or TEM samples. The experience and results gained in the experiments show that a wide range of biological materials, such as insect's cuticle (chitin-fibre composite), wood (cellulose-fibre composite), horse hair (keratin) and spider silk (protein fibre) can be milled. The success with others, like tooth enamel and dentin, is described in the literature.

Best results in milling biological materials are achieved when a stepwise milling technique is used. For structural investigations, for example, first a large box or "stair" is milled close to the region of interest using a high beam current (> 1000 pA). This is followed by milling boxes in a stepwise approach to the region of interest using first medium beam currents (350 pA to 70 pA) for volume cutting and then the fine cleaning mode (< 70 pA) of the FIB system to achieve a smooth surface of the cut. Finally, the cutting edge is "polished" using low beam currents (<11 pA), again in the fine cleaning mode. A dwell time of 1.0 µs, and overlap of 50 % worked well in the experiments described here. The advantage of the fine cleaning mode is that the ion-beam cuts along a line and the line progresses into the material. Especially in the cutting of free-standing structures, like setae or fibres, the fine cleaning mode of the FIB was used and gave good results. No redeposition or flipping of the remaining material in the field of view was observed.

The influence of the ion beam on the sample material was investigated qualitatively on biological materials and quantitatively on the reference material polyimide (Kapton®). Kapton® was chosen as a reference material, because its material properties are known. All biological materials investigated were non-mineralised and therefore assumed to be comparable to Kapton® in structure and behaviour. The temperature rise in the sample material during ion bombardment could be calculated using the models developed by Melngailis (1986) and Ishitani and Kaga (1995). Even for medium beam currents (70 pA) this temperature rise was calculated to be significant (> 100 K). The ion implantation depth was

calculated using the Monte-Carlo-Simulation TRIM (Ziegler and Biersack, 2004). In Kapton® the mean depth was found to be 36.0 nm and the maximum depth 72.9 nm. A noticeable increase of 22 % in the Young's modulus was measured on Kapton®, after it had been exposed to the ion beam with a fluence of 1.01×10^{18} ions/cm² (measured by nanoindentation in a depth of 30 nm, Chapter 1.6.4). No change in the mechanical properties was found at an indentation depth larger than 100 nm. This shows how important it is to take such effects into account in material investigations using the FIB. Exposure to the ion beam should be minimised. In the mechanical investigations presented in the thesis such effects could, however, be neglected, since the volume affected by an ion implantation depth of ~100 nm was small in comparison to the overall sample dimensions (>1µm) and since the thermal conductivity of biological samples is small.

A novel *in situ* method for mechanical testing was developed which provides a force and strain resolution ideal for mechanical testing of biological samples in the µm-range. The force measuring device consists of a micromanipulator equipped with a piezoresistive AFM tip. The AFM tip can measure forces up to 1300 µN with a resolution of 20 µN. The strain is measured from micrographs taken during the tests. A FIB system was used for sample preparation. Different preparation techniques for different shaped samples are described. Beam bending experiments were performed inside an SEM by moving the AFM tip stepwise against the cantilever and monitoring the force and deflection. Tensile tests were performed inside a FIB system. The tungsten deposition of the FIB was used to glue the samples between the AFM tip and a supporting metal block. The load was applied by moving the AFM tip stepwise away from the metal block.

The mechanical properties of a hair from a horse tail and a single spruce wood cell wall were measured in bending and an individual seta of the hairy attachment system of a beetle, wind-receptor-hairs of the filiform sensors of crickets and natural and artificial spider silk fibres were tested in tension. The results of the mechanical tests are listed in Table 4.1, the experiments are described in detail in Chapter 3.

Table 4.1: Results of the mechanical tests.

Sample	Material	Testing mode	Young's modulus [GPa]	Tensile strength [MPa]	Strain to failure [%]
Kapton®	Polyimide	Bending	3.73±0.60 (Loading) 3.60±0.54 (Unloading)		
Horse hair	Keratin	Bending	6.28±0.66 (Loading) 6.37±0.64 (Unloading)		
Spruce wood cell wall	Cellulose-fibre composite	Bending	28.2±4.0 (Loading) 25.6±4.0 (Unloading)		
Beetle seta	Chitin-fibre composite	Tension	11.2±1.0	309±60	2.51±0.06
Filiform 1	Chitin-fibre composite	Tension	16.3±1.2	396±55	2.84±0.06
Filiform 2	Chitin-fibre composite	Tension	27.6±2.2	544±72	2.56±0.05
Natural spider silk	Protein fibre	Tension	11.5±1.0	474±20	8.84±0.06
Artificial silk 1	Protein fibre	Tension	5.8±0.3	124±14	2.94±0.06
Artificial silk 2	Protein fibre	Tension	6.9±1.4	232±58	14.53±0.05

All samples were dry-mounted in the vacuum of the SEM and the FIB. It is well known that the moisture content has a significant influence on the material properties of biological materials. In general, a decrease in the moisture content of biological samples leads to an increase in Young's modulus and tensile strength, whereas the strain to failure decreases. This is discussed in detail in Chapter 3.4. The values measured with the *in situ* method fall in the range of the literature values obtained by less advanced techniques. The advantage of the testing of dry specimens in vacuum is that it acts as a reference state, which is quickly and reproducible achieved. The "natural" or "fresh" state of the samples often stated in literature is much less well defined: since biological materials start to dehydrate once they are removed

from the living organism. It must, however, be noted that the values measured will not be exactly the expected *in vivo* values. Further experiments and new testing methods are required, which allow the testing of wet samples, or even *in vivo* testing.

The newly developed *in situ* force measuring device is remarkably versatile. It is not restricted to testing in bending or tension nor to the use inside an SEM or FIB. It can be also applied for compression tests and is equally well suited for use in an environmental scanning electron microscope or *ex situ* in a light microscope. However, so far the FIB system is required for sample preparation, no other method allows similar sample preparation accuracy. Especially the mounting of samples using tungsten tapes can currently not be overcome by any other technique, which operates under ambient conditions. Even if the samples are only prepared in the FIB (with a short exposure to the vacuum) and then tested after acclimatisation in air, the mechanical properties will differ to those of the "fresh" material which was never dried before (Vincent and Wegst, 2004). Future work will focus on an investigation and quantification of the effect of the moisture content on the structure and mechanical properties of small-scale biological samples. Therefore a novel system will be designed to mount or glue μm sized samples with similar precision as it is achieved with tungsten tapes.