Epilogue

When I started doing research I was sometimes puzzled by the fact that so many labs were competing to tackle similar questions using similar techniques. Were there only so few questions left or were there just too many scientists? Looking back at my thesis period, however, I realize that it is actually quite beneficial to have a community of researchers working on similar projects. In most groups that study individual motor proteins, projects proceed rather slowly and are often limited by experimental challenges that are not necessarily specific to that project. Because various groups tried to establish similar techniques, I could often profit from the ideas and solutions presented by others. In this final chapter, I will briefly summarize the major results of my thesis work and explain their relation to earlier and simultaneous work performed by other groups.

Five years ago, many single-molecule laboratories were specialized in a specific technique, either single-molecule fluorescence, optical tweezers, magnetic tweezers or scanning-force microscopy. Establishing such a technique requires a lot of time and every technique has its own specific demands and often requires dedicated custom-built setups. As optical filters and objectives continued to improve and solid state lasers and high-sensitivity cameras became cheaper, several labs considered combining multiple techniques in one instrument. Chapter 3 describes our version of a high-sensitivity fluorescence microscope combined with optical tweezers. After subsequent modifications (Chapters 4, 6 and 7), this microscope now allows for simultaneous detection of three different fluorophores down to the single-molecule level combined with the possibility of mechanical manipulation and force measurements with two independent optical traps. Chapter 3 shows that, instrumentally, such a combination is relatively straightforward to establish and depends to a large extent on the right selection of optical filters and laser wavelengths. However, it also demonstrates that fluorophores suffer significantly from the presence of the high intensity near-infrared laser and bleach faster, which greatly increases the challenge of experiments exploring individual proteins using these two techniques. While various labs have now established similar combinations of optical tweezers and fluorescence there is still a large barrier between proof-of-principle experiments and actual biophysical measurements. The pioneering work from Toshio
Yanagida’s lab ([70], performed in 1998!) continues to be the most exciting example of a truly successful combination. In their experimental approach, a filament is optically trapped with two optical traps and the binding of individual fluorescent ATP molecules to a motor protein located in between the two traps can be monitored in correlation with the filament displacement induced by the action of the motor protein.

At the time I started working on Eg5, not much biophysical experiments had been performed on this motor protein and I could start my experiments without the need to go down to the single-molecule level (Chapter 4), thereby avoiding the challenges mentioned above. In fact, many other labs also turned their attention towards non-conventional kinesins at that time and in a few years many different kinesins have been assayed (Chapter 2). Establishing new assays for these motors proteins was not always an easy task. It appears that the firstly discovered kinesin, Kinesin-1, is in many ways an ideal motor protein. It can easily be tissue-purified in high concentrations, is highly processive in many different buffer conditions and can be attached to glass surfaces using non-specific interactions, interactions that can be prevented by pre-coating with different proteins. For many other kinesins, more elaborate protein expression systems, surfaces preparation techniques and balanced buffer conditions are required to obtain consistent results. The work of me and many others have shown that the most successful strategies use polyethylene glycol-based surface blocking in combination with antibody or biotin-streptavidin mediated attachments (Chapter 4 and 7, [36, 147, 59, 155]). In addition, motor proteins are now commonly expressed in prokaryotic or eukaryotic expression systems to yield fusion constructs of a motor and a genetically encoded fluorescent protein (Chapter 5 and 7, [112]).

The experiments described in Chapter 4 directly demonstrated the ability of a bipolar motor to slide microtubules apart and revealed a sorting mechanism that can generated polarity specific microtubule arrays. In principle, these findings did not come as a surprise, since such a mechanism had already been proposed based on structural and functional evidence and the real advance of this work was more experimental than conceptual. The careful attachment and surface-blocking strategies combined with the use of optical tweezers to position filaments in well-defined orientations established a new level of reconstitution of a cell mechanical processes and opened the way towards building increasingly complex systems. A similar movement towards higher-order systems can be observed in recent studies of DNA-organizing factors, such as H-NS [33] and Rad51 [150].

In addition to the observation of relative sliding of microtubules, the work in Chapter 4 revealed the capability of Eg5 to tether microtubule ends, which suggests the presence of an additional microtubule binding mode. Furthermore, our results indicated that Eg5 was likely to be a processive motor, contrary to common belief. Before we could examine the relative sliding induced by Eg5 in more detail, i.e. measuring forces between microtubules and studying the effects of opposing motors, experiment that examine single-molecules of Eg5 were required. Exploring the single molecule be-
behavior of Eg5 proved to be a confusing enterprise that took most of the remainder of my thesis work, but a consistent picture of Eg5 motility is now emerging.

Chapter 5 describes the creation of our Eg5-GFP construct and shows that this construct can functionally replace endogenous Eg5 in spindle formation. We found that, like Kinesin-1, this motor can move processively, which means that it can take multiple steps along a microtubule before detaching. However, unlike Kinesin-1, the motility of Eg5 is very irregular and looks more like a biased random walk. In the presence of either ADP or the specific Eg5-inhibitor monastrol, we only observed one-dimensional diffusion along the microtubule. While we performed our experiments on a full-length tetrameric construct, another group successfully created a dimeric construct and found it capable of moving on average eight steps, without apparent diffusive features [147]. This indicates that the diffusive features we found are likely to correspond to a full-length specific region, presumably located in the tail region of Eg5. Surprisingly, when we studied Eg5 motility at elevated salt concentration, closer to physiological, we found that the diffusive motility mode dominated completely. Motors still attached to the microtubule, but instead of moving in a specific direction they diffused along the microtubule lattice regardless of whether ATP or ADP was present (Chapter 6). This suggested that the motors were in an inhibited state and that binding to an additional microtubule was required to activate their directional motility. Figure 6.4 is perhaps the most exciting picture of this thesis, because it directly demonstrates that Eg5 indeed gets activated upon binding to a second microtubule, which reveals a novel regulatory mechanism for molecular motors. It thus appears that Eg5 saves its powers, until it can do what it is supposed to: generating force between microtubules.

In the same period that we observed one-dimensional for Eg5, various others groups reported similar behavior for the microtubule-binding proteins that they were examining (Chapter 2) and a recent paper even showed that the actin-based motor Myosin V can diffuse along microtubules [5]. It thus appears that the charged surface of microtubules facilitates a very general type of interaction for many proteins. Indeed, when we examined the microtubule crosslinker Ase1 in single-molecule experiments, we again observed such diffusion (Chapter 7). This allowed us to explore the process with a less complex protein and we could distinguish two different mechanisms for this process. In addition, we found that this protein can multimerize along the microtubule lattice in such a way that it facilitates the specific targeting of the protein to zones of microtubule overlap.

Because of the interesting behaviors of individual Eg5 molecules, I have had little opportunity to follow up on Chapter 4 by measuring the forces that single and multiple Eg5 molecules generate between two microtubules, as well as the effect of opposing motors. These experiments will certainly require a combined optical tweezers/single-molecule fluorescence setup, because one needs to quantify the number of motors that interact with the microtubules. In fact, such experiments that probe motor action by detecting filament displacement might more generally prove to be the best way to examine
molecular motors in a combined tweezers/fluorescence setup. In such a geometry (as in Yanagida’s), the traps can be kept away from the region where motors function, preventing the enhanced bleaching described earlier. I am curious to see who will succeed to integrate all these elements and study the single-molecule and collective force generation of Eg5 and opposing motors between the two microtubules that they crosslink.