Summary

Cells represent the basic building blocks of living organisms. They are constantly dividing and copying their DNA genetic material to produce offspring. DNA replication, the duplication of the genomic material just before cell division, is a process ubiquitous to all living cells. Such DNA copying is performed by a number of specialized proteins, so-called DNA polymerases, in a highly coordinated process. For a unicellular organism, such as the bacterium *Escherichia coli* (*E. coli*) - this means generating a complete new – and identical – individual, including the faithful duplication of millions of base pairs of genetic information.

*E. coli* contains five different types of DNA polymerases (DNA Pols): Pols I and III replicate DNA under normal conditions with high accuracy and are unable to copy damaged DNA; Pols II, IV and V are expressed in response to DNA damage and perform error-prone translesion (TLS) DNA synthesis across damage sites. Cells are constantly exposed to harsh conditions: UV light from the sun, starvation, extreme pH and temperature are all factors that may severely impact cellular metabolism and lead to DNA damage.

Decades of research have led to a detailed understanding of how these different polymerases work, but a large number of important questions are still unanswered: how can the cell faithfully replicate its DNA in the presence of damage on its DNA template? How does the cell coordinate which type of DNA polymerase is used to copy DNA? This thesis focuses on the development of fluorescence microscopy tools to visualize individual DNA polymerases inside living bacterial cells and attempts to address these and related questions.

In Chapter 1, I provide background on the mechanism of bacterial DNA replication and introduce the proteins involved in this complicated and highly coordinated process. I first introduce the replisome as the DNA replication factory, discuss the accessory proteins involved, and provide a brief description of how the activities of the different DNA polymerases are coordinated in the replisome. Further, I describe what is known about the consequences to the cell when DNA is damaged. In this Chapter, I will discuss in detail the molecular mechanisms underlying the bacterial SOS response, a concept central to my thesis. Bacterial cells respond to environmental stress by activating global patterns of gene expression. During the SOS response, a number of different error-prone DNA polymerases are expressed in response to damage; this mechanism represents a necessary step for the cell to restart replication. To limit the introduction of too many mutations, extra layers of regulation are required to control when the different types of error-prone polymerases become activated.

While the biochemical activities of DNA polymerases have been extensively characterized, many questions about their regulation and competition *in vivo* remain unanswered. It is not known, for example, how and when a stalled DNA poly-
merase III – the main replisomal polymerase – is displaced from damage sites in vivo, or whether TLS polymerases are able to gain access to undamaged templates. Such questions are highly relevant to our understanding of mutagenesis and its role in evolution. The cellular environment is exceedingly complex and crowded. With the development of high-sensitivity and high-resolution microscopy and modern genetic engineering techniques, we are now in a position to modify proteins inside living cells and track them in real time! Following any molecular process in live organisms is perhaps best comparable to trying to find a friend wearing a black t-shirt in a rock festival with hundreds of thousands of people – at night. Recent advances in imaging and genetic engineering would allow your friend to wear a hat with a stroboscopic light. He may be not as hard to find as before, but it would still be challenging. In the final part of Chapter 1, I briefly compare ensemble and single-molecule strategies, present the limitations of each, discuss the difficulties of in vivo experimentation and describe recent single-molecule studies of DNA replication. Applying state-of-the-art techniques involves developing the analysis tools and establishing new experimental protocols. In Chapter 2, I describe open-source software that I developed to streamline many of the analyses performed in my thesis research. The software is developed as a plugin for the well-known image-analysis software ImageJ.

In Chapter 3, I describe an experimental artefact that we encountered when developing fluorescent labeling approaches to be used in E. coli. We observed that the popular and frequently used red fluorescent protein mKate2 associates with the inner membrane of the bacterial cell while it should be freely diffusing through the cytosol. Remarkably, this mislocalization is only seen at very low protein concentrations. This finding directly impacts the interpretation of work done by us and other researchers in the field and results in a requirement for additional experimental controls in single-molecule cellular localization studies.

Chapter 4 describes a study exploring how Escherichia coli responds to high levels of DNA damage. Damage in chromosomal DNA blocks replication, which in turn induces the SOS damage response. Initially, these mechanisms support the restarting of replication by using non-mutagenic DNA-repair strategies such as nucleotide or base-excision repair. If these strategies fail, the accumulation of damage sites triggers the expression of error-prone DNA polymerases in a last-resort attempt to synthesize DNA past the lesions. This work focusses on DNA Polymerase V (Pol V), an enzyme that is expressed late in the SOS response and that has been reported as highly mutagenic. Because of its high intrinsic mutagenicity, Pol V levels are kept low in the cell. We use single-molecule fluorescence microscopy to visualize UmuC (a subunit of Pol V) inside living cells and to characterize its behavior in space and time. We show that Pol V is controlled by a hitherto undiscovered layer of regulation with the enzyme temporarily being placed at the membrane to prevent it accessing DNA before other, less mutagenic TLS polymerases have had a chance to act first.

What are the molecular mechanisms involved in selecting the right polymerase to be active on the DNA? The long-held molecular view is based on polymerase switching, a model with the different polymerases finding their position within the replisome by mass action, driven by the concentration of each DNA polymerase and
its affinity to the DNA and replisome.

Chapter 5 explores this multi-polymerase scenario in live *E. coli* cells. Although the biochemical activities of the TLS polymerases are well known, there are many open questions surrounding the regulation and competition between these enzymes *in vivo*. Using genetic engineering and single-molecule imaging, we were able to track the cellular replisome and Pol V in *E. coli* strains lacking the other TLS polymerases Pol II and/or Pol IV. Removing these proteins allows us to test to what extent mass-action driven mechanisms regulate polymerase exchange and access to the DNA template. We found that the presence of Pols II and IV have little effect on the number of Pol V molecules bound to replisomes or other sites on the chromosome.

Finally, in Chapter 6 I will discuss the implications of this work for the current models on TLS polymerase exchange. Specialized DNA polymerases are key players in evolution. They promote genetic diversity and boost adaptation by increasing genetic variety. In particular, understanding the molecular pathways that drive evolution in harsh cellular conditions could explain why bacteria develop resistance to antibiotics or why cancer cells fail to respond to certain treatments.