Chapter 1

Introduction

1.1 The importance of quantitative *in vivo* approach in biophysical studies

Life is all but easy. If this statement is generally accurate for most human lives, it is definitely true when attempting to understand the functioning and behavior of living systems. Life is indeed very complicated. Living systems can accomplish incredibly complex tasks at an incredibly wide range of length and time scales. Consider a mammal for example. Its size lies usually on the meter length scale while its vital organs are one order of magnitude smaller. In turn organs like the intestine or the brain are often composed of subunits lying again one lower order of magnitude in size and each organ subunit and tissue is ultimately composed of cells, the building bricks of life, which are six orders of magnitude smaller than the individual itself.

In order to carry out complex vital tasks, all the cells forming multicellular organisms are capable of differentiation, tissues formation, coordination, interaction and communication amongst different cell types, such as to provide the individual with astonishing abilities like moving around in space, growing and reproducing (just to cite some of the most important ones). Nowadays, we are still far from understanding in deep how such interactions emerge and are organized. In fact a comprehensive mechanistic and predictive description of the underlying phenomena at the basis of such complex behaviors is still elusive and sometimes presents mysterious features (e.g. think about self awareness and conscious thought originating from the interplay among billions of neurons in a human brain).

Physicists are used to simplify a problem when it seems too complicated to describe mathematically, by reasoning on what are the essential features and "degrees of freedom" that can explain most of the behavior of a process, still giving major insights on the actual nature
of the system. When modeling a purely physical phenomenon the "higher order corrections" are often identified and neglected. Nevertheless when trying to apply this approach to experimental biology, biophysicists face many difficulties. Following a reductionist approach, e.g. similar to that adopted by solid state or statistical physics where the properties of the macroscopic system are linked by the theory to the features and interactions of single atoms or molecules, it makes sense to start studying life from its fundamental constituents: cells.

It turns out cells are not really mere 'bricks’ of life but are on the contrary incredibly complicated out-of-equilibrium entities composed essentially of organic macromolecules, water, metabolites and ions that are also capable of coordinating, interacting and communicating between themselves again on a wide range of length scales (from microns to nanometers, three orders of magnitude). Think of the "simplest" single-celled organisms: bacteria. They are composed of a lipid bilayer membrane (some of them like E. coli have even two and a cellular rigid sugar wall between them) which encloses millions of proteins [1] and the whole genetic information necessary to grow and reproduce. All in just $\sim 1\mu m^3$ volume. Proteins and genes inside cells can interact in wonderful ways via a dense network of transactions such as to provide bacteria with the ability to move, sense food (chemotaxis), produce energy, grow, measure sub-cellular position, divide via binary fission, replicate and segregate their chromosomes, adapt to different environments, respond to specific stress with specific expression patterns, exchange DNA with other bacteria, acquire resistance to antibiotics, change their phenotype according to the environment, and the list could continue for a while..

Just have a look at the modeled metabolic network in E. Coli shown in Fig.1.1.1, covering only a very small fraction of all the types of interactions mentioned above.

![Fig. 1.1.1 Recent model of the metabolic network of E. coli (K12, MG1655) obtained from the KEGG database](image-url)
Looking at Fig. 1.1.1 it looks like every single cell is already quite a universe on its own! A very different system from solid state physics where mean field theories and nearest neighbor interactions are already enough to beautifully capture and predict the properties of a crystal for instance. In fact, most cellular components have multiple (known) interaction partners and often times multiple functions and ‘tasks’ as well. The number of different cellular constituents is just too high to attempt a purely reductionist approach, given such a dense interaction network.

In a way this issue is reminiscent of how Newtonian dynamics failed to describe the macroscopic behavior of thermodynamic systems by solving the equation of motion of \(10^{23}\) number of particles. Statistical mechanics solved this problem in the most elegant way. Nevertheless, if on one hand statistical mechanics was able to link the microscopic and macroscopic features of thermodynamical systems composed of order of Avogadro’s number of particles in thermodynamic equilibrium, life is on the contrary and by definition an out of equilibrium system. Life needs to produce and consume energy to sustain itself. Thus not all the branches of statistical mechanics, which can still give many insights into biological systems, can be employed to describe what is happening inside cells and for sure cannot provide (so far) a precise predictive power not even of single cells behavior.

Of course there is some degree of reductionism that can be employed when studying living cells, for instance by identifying subcellular structure and components and studying them separately. In bacteria one can define the membrane, nucleoid, cytoskeleton, and more in detail identify specific sets of proteins or genes to study separately. Though, differently from solid state physicists, biophysicists working with living systems should always keep in mind that the cell is not the a bare collection of passive components but rather an incredibly complex network of active interactions (look at Fig. 1.1.1 again) and processes that are intrinsically intertwined. Thus the biophysicist should always maintain a holistic approach to the study of life at all scales. Given the complexity of biology, there is a lot of value in studying cells and organisms in vivo, where all the interactions mentioned before are taking place naturally but under controlled conditions.

The modern approach of biophysicists consists in applying models, data collection and analysis methods borrowed from physics to biological data (often from living cells). Many incredibly successful applications of the theories of diffusion, optics, statistical mechanics and electronics (amongst many others) to biological data and experimentation on biological system are found in the literature. Feeding quantitative data into theories coming from classical physics is providing very deep insights on the mechanistic nature of biological processes, insights that are unaccessible when attempting qualitative descriptions. From in vivo biophysical studies we know the localization patterns and dynamics of many important
proteins in many different organisms, we know a lot about how DNA is organized, stored and replicated, how cell grow and divide, how they move and so on.

Nevertheless, when studying biological systems in vivo, it becomes key to try interpreting results and experimental data acquired on specific cellular components (as is possible only to measure few of the many observables in a cell) at the system level, thinking in holistic terms and being aware that the measured data is always the output of many intricate intracellular transactions with different weights.

1.2 Localization and mobility: two fundamental features of proteins in bacteria

Though very complicated, bacteria are amongst the simplest living systems one can study. A lot of scientific work has been carried out since the discovery of microorganisms (which happened between 1665 and ca. 1678 [2]) to an extent that the gram-negative bacterium E. coli became with time a model organism for microbiology. Throughout few centuries of research we gained an astonishing control and reproducibility on bacterial growth, physiology and genome editing such that a huge variety of genome engineering tools, molecular biology assays combined with high-tech imaging techniques are nowadays available to researchers approaching the study of bacteria in vivo [3]. With these cutting-edge tools and techniques is possible (just to cite the most relevant for this work) to knock in or out specific genes of interest on the chromosome, introduce expression-controlled plasmids producing protein of interest inside the cell and fuse fluorescent proteins to target proteins to visualize them in living cells using fluorescent microscopy [4].

Thanks to such technical advances it became possible, since a couple of decades ago, to design complex and well controlled experiments where specific proteins or set of proteins species are monitored over time with high spatio-temporal resolution and throughput in living bacteria. In recent years researchers employing such methods reported a huge variety of evidence about the importance of precise sub-cellular localization of specific protein species (see[5–9] and [10] for a general review on the topic of protein localizations in bacteria). Alongside with that, the ability of some other protein species to move by means of passive diffusion in the cytoplasm or on the membrane became clearly essential for many vital cellular processes like protein complexes assembly, cellular division and motility [11–13].

One of the most striking process requiring both precise cellular localization and mobility of proteins, which are the topics at the core of this thesis work, is binary fission (one of the mechanism for bacterial reproduction) where an elongated growing cell bearing at least two
copy of the bacterium’s chromosome splits into two daughter-cells of approximately the same length, volume and DNA content [14]. This process is extremely steady, reproducible and under certain growth conditions it can take place as fast as every 20 minutes [15].

In order for binary fission to take place, bacteria like *E. coli* need to gather some kind of positional information about where the middle cell is and then pinch the membrane at that location shrinking it until the mother cell is divided into two. Membrane pinching is accomplished by a protein named FtsZ which polymerizes in ring-shaped bundles precisely at middle cell and contracts to squeeze the membrane and achieve division [16], providing a fundamental example of how protein localization is key for bacteria.

On the other hand the position at which FtsZ polymerizes is dictated by a very peculiar process involving protein mobility. Among other proteins with related functions, the so called "min system" includes a protein (MinD) that is capable of producing spatial oscillations of its concentration on the inner side of the cytoplasmic membrane. These oscillations form on average an inhomogeneous spatial profile as the oscillating proteins spend more time at the poles than at the middle cell. Researchers have found convincing anti-correlation between the minD density along the cell long axis and the position of the division site and FtsZ polymerization [17] which happens where the average concentration of minD is minimum (see Fig 1.2.1).

![Fig. 1.2.1](image)

**Fig. 1.2.1** An example of the importance of localization of mobility *in vivo*: minD oscillations determine the position of FtsZ ring formation (A) MinD::EGFP oscillations. Each frame (from left to right) is acquired every 7s, image adapted from [18] (B) Overlay of MinD and FtsZ fluorescence images. Oscillatory movement of MinD drives FtsZ polymerization at mid-cell.
Another relevant cellular process which requires precise protein localization, assembly and mobility is chemotaxis [19]. The chemotaxis network is a relatively simple protein network providing bacteria with the ability to sense food or repellent gradients and swim in the direction of a favorable environment. For this network to function, a set of mobile signaling cytoplasmic proteins serving as signal carriers and a set of clustered membrane proteins, serving as attractant/repellent concentration sensors, are required. Details about the chemotaxis network can be found later in this chapter. Importantly, many of these proteins have to be placed at specific cellular positions while others need to be able to displace from a cellular region to another for the network to function.

The spatio-temporal organization of the nucleoid also provides plenty of examples showing how specific proteins have to be mobile and/or precisely localized. Obvious examples are repressors and transcription factors which have to be both mobile to find their binding DNA target sequence by diffusion [20] and also to have a high affinity for their specific target and, in the case of repressors, be able to precisely co-localize with a specific gene promoter region. Also a set of so-called Nucleoid Associated Proteins (discussed more in detail later in the book) are known to co-localize with the nucleoid in order to spatially organize it by different binding modes [21], but still exhibiting mobility [22], indicating a binding-unbinding dynamics on the nucleoid.

Finally we mention the protein MreB as an example of spatial organization and mobility of proteins. This protein can polymerize on the inner membrane in different patterns and its activity is related to building new cell wall material as the cell is growing [23]. Experimental evidences in the literature show that its mobility on the membrane, which is associated with energy consumption, could in turn influence the mobility of other proteins that are not associated with ATP burning [24]. We could go on with many other examples where both localization and mobility are key to bacterial homeostasis but in this work we will focus on chemotaxis proteins and proteins involved in nucleoid spatial organization.

1.3 Spatial organization of membrane receptor proteins is key to bacterial chemotaxis

Motile bacteria are usually equipped with molecular machineries that provide them with the ability to move towards more favorable environments, where attractants concentrations are higher. This process goes under the name of chemotaxis. Micro-organisms can sense food gradients and escape from higher repellents concentrations. Many bacteria like *E. coli*, *Salmonella*, *R. sphaeroides*, *Pseudomonas* and *Vibrio* have a dedicated set of proteins
form a biochemical network of molecular interactions and signal feedbacks which goes under the name of chemotaxis network [25].

In E. coli the input of such network is an extracellular change in concentration of either attractants or repellents while the response integrates to a bias in the rotation of flagella which influences the frequency at which the bacterium changes direction of swimming. This bias, in the framework of the so called ‘run-and-tumble’ motility [26], is sufficient to make bacteria climb up food gradients and run down repellent gradients [27].

Thus chemotaxis defines the ability of an organism to move in response to a chemical stimulus by biasing its swimming in the direction parallel to the concentration gradient of food or antiparallel to the concentration gradient of repellents. More in detail, the chemotaxis signaling network modulates cell swimming by regulating the switch between the counter-clock wise (CCW) and clock-wise (CW) rotation biases of the flagella. CW rotation makes flagella bundle together thus producing a directed propulsion that results in a straight swimming ‘run’ state. On the other hand when flagella spin CCW the flagellar bundle falls apart and the cell randomly reorient its body during the so-called ‘tumble’ state. After tumbling, the bacterium initiate a new ‘run’ following a ’run-and-tumble’ dynamics which at large times can be considered as a random walk. This random walk becomes biased in the presence of a gradient of attractant or repellent.

From a biochemical point of view, transmembrane chemoreceptors sense changes in the concentration of chemical attractants in the periplasm and control the activity of the cytoplasmic receptor-associated kinase CheA, which in turn regulates motor rotation. When phosphorylated, CheA transfers the phosphoryl group to the response regulator CheY, which then induces cell tumbling by binding to the switch complex and changing the direction of motor rotation from CCW to CW. Other proteins are involved in the chemotaxis signaling network. The receptors-associated CheA and CheW, the signal terminator CheZ and the adaptors proteins CheR and CheB. CheR and CheB constitute the adaptation system that is used to restore sensitivity of the system in the presence of sustained attractant stimulation thus enabling measurement of changes in attractant concentrations, rather than absolute levels. This adaptation is mediated through the methylation of receptors on five of their glutamate residues which influences CheA activity in different ways for different methylation states. A sketch of the chemotaxis biochemical network is given in Fig. 1.3.1.
Fig. 1.3.1  **(A) Chemotaxis network and clustering of chemoreceptors.** Ligand molecules are bound by transmembrane receptors (gray) and the chemotactic signal is transmitted to the CheA kinase that gets subsequently phosphorylated and becomes active. CheR and CheB (orange and purple) constantly methylate/demethylate the receptors providing sensory adaptation. The activated CheA reach the motors (black) at the base of the flagella and transmit the chemotactic signal to the flagella. CheZ (cyan) is responsible for dephosphorylation of active CheA.  **(B)** Fluorescence image of labeled Tar receptors in live E. coli.  **(C)** Average of EM sub-tomograms of clustered receptors showing the hexagonal arrangement of single trimers of dimers. Asterisks indicate position of single receptor dimers. Scale bar 12nm. Image adapted from [28].

Chemoreceptors are known to assemble into clusters of various size forming a crowded environment [5, 29] on the cytoplasmic membrane (see Fig. 1.3.1B and C). Clusters are composed of trimers of dimers forming an hexagonal array as shown in Fig. 1.3.1C. Many independent researchers have shown that receptor clustering is functional for signal sensing, namely providing amplification of the signal by receptor cooperativity [30] allowing bacteria to sense extremely small concentration change. If much is known about the steady-state
function of receptor clusters, not many investigations have been carried out on their dynamics and stability under different ligand stimulation condition. Some results from literature actually draw contrasting conclusion about chemoreceptor cluster stability upon ligand binding ([31–36]).

First, chemical cross linking experiments suggest that receptors tend to form less dense clusters upon attractant binding while repellent binding tends to stabilize the clusters of *E. coli* cells [32], [33]. Yet another study (also using electron cryo-tomography) reported no change in the hexagonal packing, either upon stimulation or under different growth conditions [34]. Studies which employed optical diffraction-limited imaging have also reported conflicting results. On one hand, using immuno-fluorescence labeling, a number of studies have reported a nearly complete disruption of polar clusters in fixed *E. coli* and *B. subtilis* cells. In these works, strong increases in the fraction of cells exhibiting lateral clusters or diffuse localization patterns was observed upon stimulation with attractant, and the localization was restored after adaptation [31, 35]. In one of these studies, stimulus-dependent redistribution of receptors between clusters was observed not only with immuno-fluorescence, but also with receptors directly fused to the green fluorescent protein in live (unfixed) *B. subtilis* cells [35]. On the other hand, in a study that monitored receptor clusters via fluorescent fusions of CheZ, which tends to localize at the receptor complexes, no change in localization upon attractant stimulation was apparent in live *E. coli* cells [36].

In this thesis work we present the results of our attempt to clarify this controversy, using a combined approach of super resolution imaging in fixed cells (similar to [5]) and live-cell single-particle tracking to estimate cluster size and mobility before and after introducing a saturating ligand stimulation.

### 1.4 Macromolecular crowding in biological systems

Macromolecular crowding is a physico-chemical phenomenon causing attraction of macromolecules in solution at high concentration of total solute, mainly due to excluded volume interactions. The non-specific repulsion originating from the impenetrability of macromolecules in crowded environments, can generate purely entropic depletion forces [37] as the volume available for each single macromolecule to occupy is limited by the presence of the others. For example, if two large molecules in a crowded medium are found in close spatial proximity (e.g. due to thermal motion), they will exclude smaller crowders from the space in correspondence of their minimal distance point and feel a depletion attractive force as illustrated in Fig. 1.4.1. This phenomenon will create a depletion layer around each macromolecule and they will feel an attractive force every time their depletion layers (whose
size will depend on the size of the other crowders in solution) will overlap. Nevertheless we should keep in mind that this particular case does not cover all the possible scenarios and all the effects that can arise in macromolecular crowded environments: the magnitude, the nature and the implications of excluded volume effects can indeed strongly depend on the size distribution of the macromolecules in solution [38].

Inside living cells, about 20-30% of the cytoplasm is occupied by biological macromolecules like proteins or RNA [39] and thus excluded volume effects can be ubiquitous. The cytoplasm is indeed a very crowded environment as illustrated in Fig. 1.4.1A showing a data-driven artist impression of a cellular inside.

Fig. 1.4.1 Intracellular crowding generates depletion forces due to excluded volume. (A) Data-driven artist impression of bacterial cytoplasm giving an illustrating the high degree of crowding inside the cytoplasm of living cells (image courtesy of Goodsell lab.) (B) Illustration of depletion attraction due to excluded volume. Red smaller circles represent crowders while bigger cyan circles represent larger particles in solution. when crowders are excluded from the volume in between the larger particles, i.e. their depletion layers overlap, the larger particles feel an effective attractive force.

The constituents of the cytoplasm come in a very wide range of different sizes and thus effects of crowding can be very diverse on different protein species for example. As a consequence of macromolecular crowding, cytoplasmic diffusivity decreases with protein size and crowding levels [40, 41]. In addition, the cytoplasm (in which water is the most abundant component) contains many charged or polar molecular species and excluded volume effects can influence the rate of reactions depending on electrostatic, Van Der Waals or hydrophobic interactions.
Importantly, crowding and excluded volume effects has also been shown to influence the size of bacterial chromosomes inside artificial crowders solutions [43] also in the absence of the native Nucleoid Associated Proteins [44]: a higher crowding level leads to a more compact configuration of isolated chromosomes and this effect is thought to be important for nucleoid spatial organization inside living cells too [45].

Crowding effects seems to be relevant also in cellular membranes, it is indeed estimated that membrane proteins occupy 30-50% of the total membrane area [46]. Simulations on membrane diffusivity also show a decrease in diffusion coefficients of both lipids and protein with increased membrane crowding [47] and results from theory showed that crowding can affect the gating energies of mechanosensitive channels [48]. Experiments employing Fluorescence Correlation Spectroscopy (FCS), Fluorescence Recovery After Photobleaching (FRAP) and Single-Particle Tracking (SPT) also point in the same direction (see [49] for a review on the topic).

Since excluded volume influences diffusion of proteins both on the membrane and in the cytoplasm, crowding is likely to be involved in protein localization, mobility and clustering, thus affecting the processes described in the previous section of this chapter. Importantly for the cell, crowding has been shown to (differently) affect a variety of reaction rates in the cytoplasm both in silico [50] and in vitro [51]. Thus, vital functions like transport, enzymatic reactions, protein association, transcription, DNA replication protein polymerization and protein folding must cope with or exploit the high crowding levels in both the cytoplasm and the membrane. In this context, a recent review on the topic introduced the concept of crowding homeostasis highlighting the importance of crowding inside living systems and proposing the idea of crowding being actively maintained at certain levels throughout the life of a cell [52]. Despite crowding is raising increasing research interest, whether cells use it in their favor, what is its interplay with active processes and in general how important it is for the life of a cell is still subject of intense discussion and research. This is also due to the fact that testing the effect of crowding perturbations in vivo is experimentally challenging [53]. In fact, until recently [54] it was not possible to precisely quantify crowding levels in living cells. Also, introducing controlled perturbations to crowding without perturbing physiology or triggering unwanted cellular responses is not trivial due to the sensitivity of bacteria to external perturbations and to their variety of adaptation mechanisms.
1.5 Bacterial nucleoids are not random polymers and live in the crowded cytoplasm

Looking at bacterial chromosomes under electron or optical microscopes, gives the impression that they consist of a completely disorganized random polymer thrown into the cell’s cytoplasm [55]. By looking at electron micrographs, the contrast with eukaryotic DNA is pretty striking (see Fig 1.5.1A): as bacteria do not have histones, they don’t undergo mitosis and they lack a nuclear envelope enclosing their genetic material, the nucleoid at first glance looks like a completely random polymer blob. For this reasons the bacterial chromosome was historically named ‘nucleoid’ as to clearly state the difference with the eukaryotic nuclear organization.

By contrast, in recent years the nucleoid has been proven to possess a very fascinating and high degree of spatio-temporal organization, which is plausible given the wide variety of processes it has to carry out throughout the cell cycle. Such organization seems to be mediated by a set of DNA-binding proteins like Nucleoid Associated Proteins (NAPs, proteins like H-NS, Fis and HU have been shown to be capable of drastically influencing DNA folding both in vitro and in vivo [56–61]), Structural Maintenance of Chromosome proteins (SMCs) [62–64] but also by the passive action of intracellular crowding [43, 65, 66] acting via depletion forces resulting from purely entropic effects. Recent estimates report indeed a concentration of $\sim 3 \times 10^6$ proteins/$\mu m^3$ for the cytoplasm [67].

The volume of a typical bacterial cell is very small ($\sim 1\mu m^3$) and the nucleoid, which is one thousand times longer than the cell long axis when unraveled (1 mm as opposed to 1 um), has to fold approximately one thousand times to fit in the cellular volume reaching a very high degree of compaction already in the absence of crowding. Nevertheless, we know from polymer physics that a flexible polymer in a neutral solution and confined in a space much smaller than its radius of gyration would tend to occupy all the space available since that would be the most entropically favorable state [68]. Though, bacterial nucleoids do not fill the entire cellular space but only a fraction of it and this fraction is dependent on growth conditions (Fig. 1.5.1 C). Given the variety of (in vitro) experimental ad theoretical results on crowding cited earlier in this chapter, it is thought that intracellular crowding plays an important role in compacting the nucleoid to a size smaller than the cellular volume. Some recent experiments on isolated nucleoids from lysed living cells showed indeed that the chromosomes are somewhat similar to loaded spring: if the membrane of living E. coli is digested, the nucleoid undergoes a sudden, rapid and reproducible expansion [43], the main compressing force probably being crowding.
In addition, results in literature suggest that also active processes like RNAP activity contribute in shaping nucleoid morphology in vivo [69]. Consistently, when the metabolism of the cell is shut down, for instance by exposing cells to dinitrophenol (DNP), the nucleoid undergoes an expansion phase until it fills almost the whole cellular volume [70]. Thus, nucleoid compaction is likely to originate from the interplay of passive and active mechanisms but testing and studying the effect of crowding and active processes on nucleoid morphology inside living cells has remained experimentally challenging.

In this thesis work we present the results of our experimentations on the nucleoid organization under controlled crowding perturbations induced by osmotic shock in vivo (Chapter 5) in the physiologically relevant experimental framework provided by the mother machine PDMS device (described later in this chapter). This part of the work was aimed at clarifying the role of crowding in organizing the nucleoid in living cells and its relation to active cellular processes.

**Fig. 1.5.1** Bacterial nucleoid arrangement in the cell. A. comparison between electron micrographs of a bacterial nucleoid ([71], upper panel) and a mitotic human chromosome (DuPraw1968, lower panel). B. Live E. coli nucleoids labeled with HU::mCherry under different growth rate conditions showing distinct nucleoid morphologies.
1.6 Single molecule fluorescence imaging approach allow for studying protein organization, mobility and interactions

Recent technical advances in fluorescence microscopy and fluorescent protein engineering have opened the way for a wide range of new experimental possibilities both in live and fixed cells [72, 73]. In particular super resolution techniques have overcome the resolution limitations of wide-field fluorescence imaging due to diffraction limit which restricts the maximum resolution that can be achieved with an optical imaging system to half the wavelength of the emitted light. This hard physical limit states that any emitter object smaller than half the wavelength of the emitted light, would appear when imaged under an optical microscope as a bell shaped function called Point Spread Function (PSF, Fig 1.6.1A). The width of the PSF is about half of the wavelength of the emitted light. With the advent of Super Resolution (SR) techniques like PALM, STORM and STED ([74–76]) it became possible to image sub-cellular structures with ten times higher resolution and resolve spatial patterns that would otherwise result in a blurred image when employing diffraction limited imaging techniques. Super resolution though, come usually at the cost of fixing cells and thus give up on measuring dynamics of the cellular process under study in real time.

Both PALM and STORM rely on the ability to image single fluorescent molecules and estimate their position by fitting their PSF with a bell-shaped two dimensional function, usually a Gaussian. With the high sensitivity of modern detectors it is now pretty standard to image single fluorescent proteins in living and fixed cells. Another requirement for both PALM and STORM is to image single molecules in a well-separated fashion, avoiding PSF overlap in order to unambiguously estimate the position of individual emitters.

Producing a well-separated spatial profile of individual emitters became much easier with the introduction of photo-activatable (or photo-switchable) fluorophores which are special fluorescent protein that can be pushed from a dark state to an emitting one by UV light irradiation. Before the advent of such fluorophores, producing a sparse emitter spatial profile was challenging in living cells for most of the times the protein of interest comes in large copy number in the cell and the PSFs of individual labeled proteins tend to overlap rendering the single molecule position estimation hard if not impossible. The use of photo-activatable fluorophores [77] in biophysical experiments allows for stochastically activating the emission state of a subset of the total fluorophore population in the cell, by shining UV light on the sample. By tuning the intensity of UV irradiation is possible to acquire time lapse images having only few well-separated PSFs produced by stochastically photo-activated fluorophores.
1.6 Single molecule fluorescence imaging approach allow for studying protein organization, mobility and interactions in the sample. Such images (see Fig 1.6.1B) are suitable for estimation of emitters’ position at nanometer precision via 2D Gaussian fitting.

PALM and STORM imaging protocols consist of an image acquisition part, a data processing and an image reconstruction part. In the image acquisition phase, many (on the order of thousands) subsequent image frames with few well-separated emitting fluorophores each are recorded. In the data processing and image reconstruction part each such single frame is analysed and the PSFs of all the fluorophores in the frame are fitted to estimate the emitters’ positions with higher spatial precision. The localization precision will depend on the number of collected photons for each single PSF and neglecting higher order corrections can be estimated as $\Delta \sim \frac{\sigma}{\sqrt{N}}$ where N is the number of collected photons [78]. After each data frame has been processed this way, localizations are filtered for duplicates, multiple localization of the same emitter and sample drift [79] and eventually a single super-resolved image is produced by showing all the localizations coming from each data frame at the same time (see Fig. 1.6.1B).

Fig. 1.6.1 **PALM analysis protocol** (A) Representative image of a Point Spread Function and successive fitting procedure. After fitting the PSF with a 2D Gaussian distribution the emitter is localized with an average precision of 10nm. (B) After collecting multiple frames with well-separated emitters each PSF in each frame is fitted to localize the emitter with high precision. Subsequently all the super-resolved frames are superimposed to reconstruct the full SR image (image adapted from [74]).

PALM can also be employed for experiments *in vivo* [80, 81]. The main limitation of using PALM in living systems is that most of the labeled proteins inside cells are highly mobile and they can diffuse over large distances in the time required to complete PALM imaging. Thus, the final super-resolved image will be blurred by protein motion. On the other
hand such limitation can be turned into an advantage: by activating the emission of single labeled-proteins one can then perform single-molecule tracking on even very dense spatial profiles of labeled proteins [82, 83] to study their mobility (PALM-tracking). Nevertheless, since PALM tracks come from single fluorophores, they are limited by bleaching and if a decent localization precision is desired the excitation power has to be relatively high thus producing on average short (<10 frames) tracks.

To overcome the limitations of PALM-tracking we developed a hybrid single-particle tracking (SPT) technique that allows for reliable tracking of clustered proteins in crowded environments while achieving a much longer (up to a factor of 10) tracklength when compared to usual PALM tracking. Instead of using a wide-field 405nm activation profile, we focus the activation light into a diffraction limited spot and deliver a short (<10ms) focused 405nm pulse onto immobilized bacteria. When clustered proteins, like membrane chemoreceptors, are labeled with photo-switchable fluorophores multiple fluorophores belonging to the same clusters will become active after a localized activation pulse. Their bleaching behavior will not follow a single exponential curve and tracklength distributions will exhibit much longer tails as compared to those produced by PALM tracking. See Chapter 2 for a more detailed description of the technique.

1.7 Mother machine allows for nucleoid imaging in E. coli under physiological growth

When studying nucleoid organization in live bacteria, great care has to be taken in order to minimize perturbations to physiology. This is mainly because nucleoid morphology can vary a lot depending on growth conditions, stress and changes in the environment [71, 84, 70] or also in response to perturbations to other cellular processes or proteins [85, 86]. In general, cellular physiology can be strongly perturbed by standard experimental protocols for microscopy, that often expose bacteria to abrupt changes in buffer solutions, temperature and osmotic pressure (among others) during cell harvesting, sample preparation and imaging. Thus an ideal tool for experimental studies on nucleoid spatio-temporal organization would be a device allowing imaging experiments under well defined and steady physiological conditions, to minimize external perturbations that could trigger uncontrolled cellular and nucleoid responses. In many cases, it is indeed desirable to perform imaging on bacteria undergoing a precise growth phase. In fact nucleoid morphology, together with cell size and metabolic activity, can also drastically change throughout the different phases of bacterial growth [87, 88, 86].
Bacterial growth of an initial population in a finite volume of growth medium follows 4 different phases: the lag phase (where no growth is observed), the exponential phase, the stationary phase (when nutrients start to be scarce and growth is arrested) and finally the death phase. The standard growth phase at which cells are harvested from (liquid) cultures in most bacterial studies, is exponential growth. During exponential phase cells are healthy and growing at the maximum rate for that given growth condition. In practice it is quite difficult to achieve steady growth and keep cells in a precise growth phase while at the same time having them immobilized on a glass surface for carrying out microscopy on the nucleoid. Some techniques like agar or poly-acrylamide pads [89, 90] are capable of providing steady exponential growth and immobilization of cells but at a cost: medium exchange (for instance if a step-like perturbation mediated by a certain chemical needs to be introduced in the system) is pretty slow, challenging and inefficient.

Fig. 1.7.1 (A) Phase-contrast image of a representative field of view in a typical mother machine experiment. The upper ends of the growth channels are facing the main channel where fresh medium is continuously flowing. (B) Schematic of a mother machine device. Growth medium is constantly exchanged in the growth channels such that cells in the growth channels can grow exponentially at all times. The 'mother' cell (indicated with 'old pole' in the figure) will never get swiped away by the flow while all the other daughter cells eventually will. Image adapted from [14]
On the other hand standard microfluidic flow cells [91] allow for fast medium exchange but often utilize some chemical treatment of the glass surface (like Poly-L-Lysine, PLL) or antibody attachment that can perturb growth, cell shape and physiology [92]. A microfluidic tool that overcomes all these difficulties is the so called "mother machine" which allow for both rapid medium exchange and steady exponential growth of bacterial cells during imaging experiments [93]. See Fig 1.7.1A for an example of a typical field of view in a mother machine experiment.

The mother machine is a PDSM microfluidic device ([94]) consisting of few thousands of parallel growth channels of about 1 µm in width and 20µm in length arranged in a comb fashion. Each growth channel is closed at one end while the other open end is facing the main channel where fresh growth medium is usually flowing. Inlet and outlet are placed at both ends of the main channel. Thus, the main channel can provide continuous fresh growth medium flow, bypassing the nutrient limitations leading to stationary and death phase and ensuring that cells in the growth channels are always in exponential phase. Each growth channel can fit a line of (about 6) single cells. As they grow and elongate, the closest cell to the open end of the growth channel is periodically swept away by the medium flow. At the other closed end of each channel is where the 'mother cell' lie (see Fig. 1.7.1B). The name is appropriate since that is the only cell (or better the only 'pole') that will never be washed away by medium flow and is also the reason why the device itself is called 'mother machine'. In addition to these features, the medium flowing in the main channel can be exchanged rapidly by connecting the inlet to a different medium source.

The mother machine opened the way for a new class of experiments with unprecedented physiological relevance which in recent years unveiled fundamental features of bacterial growth, size regulation and gene expression [14, 95–98]. We decided to employ this device for our studies on nucleoid spatial organization under controlled crowding perturbations via osmotic shock. In this work, we took advantage of both the efficiency of medium switching and the non perturbative nature of the mother machine device to conduct pioneering experiments investigating the relationship between nucleoid morphology, macromolecular crowding and active processes inside the cell (see Chapter 5).

1.8 Application of Super Resolution imaging to non-biological systems

The features of super resolution techniques like PALM can also be exploited for investigations on non-biological systems [99–101]. The techniques developed in our laboratory for the
1.9 Thesis outline and scope

This thesis lies in the framework of a quantitative description of diffusion, binding and positioning of a series of different proteins in relation to fundamental biological processes inside living bacteria. The quantitative \textit{in vivo} approach at the basis of this work aims at understanding better the role of protein and DNA spatial organization, focusing on bacterial chemotaxis and nucleoid morphology in the context of macromolecular crowding.

\textbf{In Chapter 2} I describe the techniques we utilized for the experimental part of this work on chemoreceptor spatial organization (Chapter 4). First I introduce the PALM imaging approach and protocol for measuring chemoreceptor cluster size in fixed bacteria. I describe the detailed protocol for cluster size estimation under different attractant stimulation conditions. Subsequently I describe the technique I developed for reliable "single-particle" tracking of a population of fluorescently labeled proteins forming a crowded environment both in the cytoplasm and on the membrane. We named this technique Localized Photo-Activation Single-Particle Tracking (LPA-SPT). By genetically fusing the protein of interest to photoswitchable fluorophores (of the Eos family) we are able to locally switch the fluorescent emission from state1 to state2 of a subset of fluorophores falling in the vicinity of a diffraction-limited spot generated by focusing a 405nm laser onto the sample plane. This way only a small (tunable) fraction of the whole protein population will be in emitting state2, thus making it easy to track them even in crowded environments. This chapter has been published.

\textbf{In Chapter 3} I describe a set of experimental tools we developed and implemented in our laboratory for imaging experiments on bacterial nucleoids at a low level of physiological perturbation. I introduce a novel DNA-label (H-NS-dbd) produced by truncation of an endogenous DNA-binding protein (H-NS). Our construct can be expressed \textit{in vivo} and a set of results from different tests and validations show that H-NS-dbd interfere less with the nucleoid compared to labels based on the full H-NS. We find indeed a lower DNA binding affinity for H-NS-dbd. I show that H-NS-dbd is suitable for labeling chromosomes in cell
species that do not express the native H-NS. In addition, our toolkit includes techniques like PALM, 3D PALM and PALM-tracking for high resolution studies on nucleoid associated proteins. We present preliminary experiments that paved the way for future studies on global nucleoid spatial organization. Our efforts in developing a low perturbative assay for linking DNA compaction and gene expression resulted in a set of Fluorescent Repressor Operator System (FROS) strain with couples of specific labeled loci at controlled genomic distances. Preliminary tests showed how our FROS system is suitable for measuring end to end distances and tracking, even though the labels rely on the binding of only 6 TetR::YFP or LacI::mCherry. Finally, I describe the mother machine based experimental protocol that we employed to measure the crowding perturbations on nucleoid size and morphology under physiologically relevant conditions (see Chapter 5).

In Chapter 4 I present a study on the spatial distribution and mobility of clustered membrane chemoreceptors upon ligand binding. By means of PALM imaging and our novel single-particle tracking technique (LPA-SPT) we show that ligand binding can perturb cluster organization, mobility and size distribution of transmembrane chemoreceptor clusters. In particular, by combining these two approaches we provide orthogonal evidences indicating that the average size of clusters is modulated by sustained ligand binding in a time-dependent manner. Employing adaptation-deficient mutants we show that cells capable of chemotactic adaptation also restore their cluster size distribution to pre-stimulus levels while cheRB mutants do not. Our findings suggest that cluster size might be an important dynamic variable in chemotaxis and that cells seems to be able to modulate cluster size and mobility distributions under different stimulation conditions. Taker together, our spatial resolution experiments clarified the long standing controversy about stability of chemoreceptors clusters upon cognate ligand stimulation.

In Chapter 5 we employ a FRET crowding sensor to measure the level of molecular crowding inside live E. coli together with morphology, size and dynamics of fluorescently labeled chromosomes in growing cells subjected to osmotic shocks. We show that variations in intracellular crowding, induces by hyper osmotic shock, produce variations in nucleoid (and cell) size in vivo and for different growth conditions. On the contrary, nucleoids in cells subjected to hypo osmotic shock expands, suggesting that crowding is important for nucleoid compaction. By shutting down metabolism, we investigate the interplay between active and passive (e.g. crowding) mechanisms in steady state nucleoid morphology and during dynamic responses to osmotic shock. Taken together, our results provide insight into the different roles of intracellular crowding in nucleoid spatial organization in living cells, connecting nucleoid morphology to the properties of the cytoplasm and to metabolism.

In Appendix A I present a work done in collaboration with Eric Garnett’s group (AMOLF)
where I designed and executed the experimental part which involved a novel protocol for PALM imaging. The experiments were aimed at measuring carbon nanowires absorption profile at high spatial resolution, by measuring the intensity of single emitters stochastically activated at different distances from the wire. This work has been published.