SUPER-RESOLVED MICROSCOPY AS A TOOL TO UNVEIL INTRACELLULAR STRUCTURE IN BIOLOGICAL SYSTEMS

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Institute of Biomaterials and Biomedical Engineering
University of Toronto

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Abstract

We have employed a combination of super-resolution microscopy techniques to explore the spatial redistribution of the heat-stable nucleoid structuring (H-NS) protein within E. coli in response to high osmolarity. H-NS is an abundant nucleoid association protein in gram-negative bacteria and plays a dual role as a global gene regulator and nucleoid organizer. By imaging the spatial organization of H-NS, we hoped to gain insight into its complicated role in osmoadaptation.

While we saw no effect on the spatial organization of H-NS in osmotically stressed, exponentially growing cells, we observed a profound rearrangement of H-NS proteins in osmotically stressed, stationary phase cells. In this case, H-NS was dynamically translocated towards the cell periphery, over the course of 5-10 minutes, and appears to be excluded from a tightly condensed chromosome. While the mechanism remains elusive, we were able to connect this response to the overall superhelicity of the bacterial chromosome. By inhibiting DNA gyrase in exponential phase, we were able to observe a very similar reorganization of H-NS, and chromosomal collapse, that we previously observed only in stationary phase. This behavior implies that the superhelicity of the chromosome plays a role in regulating osmoadaptation.
To perform these studies, owing to the small size of bacteria and the diffraction limit of light, we established an optical setup on which we can conduct one- or two-color PALM or dSTORM in 3-dimensions. These single-molecule localization microscopy (SMLM) methods can achieve a resolution of roughly 20 nm. In addition, we developed image-processing tools to reconstruct accurate SMLM images and studied the photo-physics of various photo-switchable fluorophores to confirm their suitability for high-resolution SMLM. Further applications of these methods and developments are discussed herein.
“Let the beauty of what you love be what you do.”

~Rumi
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# Table of Contents

Chapter 1: Introduction ........................................................................................................... 1

Chapter 2: Super-Resolved Microcopy to Resolve Biological Systems beyond the Diffraction Limit * .......................................................................................................................... 5

1. ........................................................................................................................................... 5
2. ........................................................................................................................................... 5
2.1 Summary .......................................................................................................................... 5
2.2 Single-Molecule Localization Microscopy (SMLM) .......................................................... 5
   2.2.1 Direct Stochastic Optical Reconstruction Microscopy (dSTORM) ............................. 9
   2.2.2 Photo-Activated Localization Microscopy (PALM) .................................................. 12
   2.2.3 Super-Resolution Radial Fluctuations (SRRF) Algorithm ....................................... 12
2.3 Microscope Setup and Modes of Microscopy .................................................................. 16
   2.3.1 Modes of Microscopy ............................................................................................... 16
   2.3.2 Microscope Setup ..................................................................................................... 19
   2.3.3 Three-Dimensional (3D) Imaging ............................................................................. 22
   2.3.4 Measurements and Image-Processing Software in SMLM .................................... 23
      2.3.4.1 Table of Fluorophores Coordinates (Localization Table) ................................... 23
      2.3.4.2 Slicing (Ellipse) Filter ....................................................................................... 24
      2.3.4.3 Drift-Correction ................................................................................................. 25
      2.3.4.4 Merging Localizations into Blinks .................................................................. 26
      2.3.4.5 Image Rendering ............................................................................................... 27
2.4 Concluding Remarks ........................................................................................................ 28
Chapter 3: Chromatic Aberration Correction and Studying Alexa-647 and Atto-532 Photo-Physics in vitro by dSTORM* ................................................. 29

3. .................................................................................................................................................. 29
3.1 Summary .................................................................................................................................... 29
3.2 Two-Color Imaging and Chromatic Aberration Correction ......................................................... 29
3.3 Study Alexa-647 and Atto-532 Photo-Physics in vitro with dSTORM ......................................... 33
3.4 Concluding Remarks .................................................................................................................. 37

Chapter 4: Single-Molecule Localization Microscopy (SMLM) In-Silico for Molecular Counting* ................................................................................................................. 38

4. .................................................................................................................................................. 38
4.1 Summary .................................................................................................................................... 38
4.2 Theory of Counting Molecules in SMLM .................................................................................. 38
4.3 SMLM Simulation Procedure .................................................................................................... 40
4.3.1 Single Fluorophore .............................................................................................................. 40
4.3.2 Extract λ from an Array of Single Fluorophores ................................................................... 44
4.3.3 Array of Molecules Labeled with Fluorophores .................................................................. 46
4.4 In-silico Molecular Counting Results ...................................................................................... 47
4.5 Concluding Remarks .................................................................................................................. 48

Chapter 5: Revealing Intracellular Spatial Distribution of RNA Polymerase II in Mammalian Cells by dSTORM* ................................................................. 49

5. .................................................................................................................................................. 49
5.1 Summary .................................................................................................................................... 49
5.2 Transcription Factories in Stem Cells and Differentiated Cells .................................................. 49
5.3 Methods and Materials ........................................................................................................ 51
  5.3.1 Protein Labeling Using Immunofluorescence Technique ........................................ 51
  5.3.2 Cell Fixation and Sample Preparation ......................................................................... 52
  5.3.3 Two-Dimensional (2D) and Three-Dimensional (3D) dSTORM setup ....................... 54
5.4 dSTORM Images Results ................................................................................................. 55
  5.4.1 Two-Dimensional (2D) Imaging Results ...................................................................... 55
  5.4.2 Three-Dimensional (3D) Imaging Results .................................................................... 56
5.5 Concluding Remarks ........................................................................................................ 58

Chapter 6: Spatial Redistribution of the Global Transcriptional Regulator H-NS in E. coli under Osmotic Stress* .................................................................................... 59

6. ........................................................................................................................................ 59
  6.1 Summary .......................................................................................................................... 59
  6.2 Introduction ...................................................................................................................... 59

6.3 Materials and Methods ..................................................................................................... 63
  6.3.1 Labeling H-NS proteins with fluorescent protein mEos3.2 in E. coli ......................... 63
  6.3.2 Stressing the Bacterial Cells and Fixation .................................................................... 63
  6.3.3 Labeling Chromosome with DAPI ............................................................................. 64
  6.3.4 Mounting the Sample .................................................................................................. 65

6.4 Fluorescence Microcopy Methods .................................................................................... 65
  6.4.1 Super-Resolution Microscopy Methods ...................................................................... 65
    6.4.1.1 Photo-Activated Localization Microscopy (PALM) .............................................. 66
    6.4.1.2 Super-Resolution Radial Fluctuations (SRRF) ....................................................... 66
  6.4.2 DAPI-stained Nucleoid Imaging ................................................................................. 67
  6.4.3 Image-Processing and Analysis .................................................................................... 67
6.4.3.1 Spatial Analysis of Protein Distribution ........................................................... 68
6.4.3.2 Quantification of Chromosome Response ......................................................... 69
6.5 Results .................................................................................................................. 70
  6.5.1 Intracellular Reorganization of H-NS under Osmotic Stress at Different Phases of Bacterial Growth ........................................................................................................... 70
  6.5.2 Intracellular Reorganization of DNA-binding HU protein in E. coli in Response to Osmotic Stress .................................................................................................................. 74
  6.5.3 Spatial Organization Dynamics of Chromosome and H-NS in Response to Osmotic Stress 76
    6.5.3.1 H-NS and Chromosome Dynamics in Response to Osmotic Stress in Exponential Phase ......................................................................................................................... 76
    6.5.3.2 H-NS and Chromosome Dynamics in Response to Osmotic Stress in Stationary Phase 77
  6.5.4 How DNA Gyrase Inhibition Alters Chromosome/H-NS Response to Osmotic Stress 79
6.6 Concluding Remarks .............................................................................................. 82
6.7 Future Directions .................................................................................................... 83

Chapter 7: Conclusions and Future Directions ....................................................... 85

References .................................................................................................................... 90
List of Figures

Figure 1) Localization Precision .......................................................... 6
Figure 2) Principle of Single-Molecule Localization Microscopy (SMLM) .......... 7
Figure 3) Photo-switches Categories ....................................................... 8
Figure 4) Energy States of Fluorophores Switching Between On- and Off-States .... 11
Figure 5) Super-Resolution Radial Fluctuations (SRRF) Principle ..................... 14
Figure 6) SQUIRREL Analysis Workflow .............................................. 15
Figure 7) Resolution Scaled Error (RSE) and Resolution Scaled Pearson (RSP) vs Ring Radius . 16
Figure 8) Schematic of Modes of Microscopy ........................................... 17
Figure 9) Schematic of Single-Molecule Imaging Setup .................................. 21
Figure 10) Three-dimensional (3D) Super-Resolution Microscopy ...................... 22
Figure 11) Image-Processing Slicing Filter ............................................. 25
Figure 12) Drift-Correction Based on Cross-Correlation .................................. 26
Figure 13) Post-Processing Merging Analysis .......................................... 27
Figure 14) Image Representation in SMLM ........................................... 28
Figure 15) Micro-Fabricated Nano-Hole Array Application in Chromatic Aberration Correction .......................................................... 30
Figure 16) Nearest Neighbor Analysis to Calculate Experimental Localization Precision. .... 32
Figure 17) Localization Precision and Registration Error .................................. 33
Figure 18) SMLM Imaging Chamber ...................................................... 34
Figure 19) Photo-physics Analysis of Alexa-647 (red), Atto-532 (green) Imaged Through dSTORM at a Frame Rate of 20 Hz ............................................. 35
Figure 20) Duty Cycle Measurement ...................................................... 36
Figure 21) ON-, OFF- and Photobleaching (PB) States .................................. 41
Figure 22) Workflow of How to Simulate Single Photo-Switching Fluorophore SMLM Data ... 43
Figure 23) Blinking Number Characteristic (λ) versus Threshold ...................... 45
Figure 24) In-Silico Molecular Counting Platform .................................... 46
Figure 25) In-silico Molecular Counting Experiment ................................... 47
Figure 26) Immunofluorescence Labeling .............................................. 52
Figure 27) Imaging Chamber used To Study Mammalian Cells Adhered to the Coverslip ........54
Figure 28) RNAPII Spatial Distribution within Mouse Embryonic Stem Cell (A-B) and Mouse Cortex Cells (C-D) ..................................................................................................................56
Figure 29) Three-Dimensional (3D) dSTORM Images of RNAPII (tagged with Alexa-647) Spatial Distribution within a Mouse Cortex Cell. ..............................................................................................57
Figure 30) Bacteria cells Segmentation Using Nucleus Contours as the Seeds in ImageJ ........68
Figure 31) Spatial Analysis of Protein ..........................................................................................................................69
Figure 32) Chromosome Area Quantification ..............................................................................................................70
Figure 33) PALM Imaging: Intracellular Reorganization of H-NS in E. coli under Osmotic Stress in Stationary Phase of Growth ........................................................................................................71
Figure 34) Intracellular Reorganization of H-NS in E. coli under Osmotic Stress in Stationary Phase and Artefacts from Transfecting Bacteria with Plasmid that Encodes hns Gene (fused to meEos3.2) ........................................................................................................................................72
Figure 35) Axial Intensity of H-NS molecules in E. coli under Osmotic Stress at Stationary Phase ..................................................................................................................................................73
Figure 36) H-NS and Chromosome Redistribution in E. coli in Response to Osmotic Stress .....74
Figure 37) HU and Chromosome Redistribution in E. coli in Response to Osmotic Stress ....75
Figure 38) Dynamics of Intracellular Reorganization of H-NS and Chromosome in E. coli under Osmotic Stress in “Exponential Phase” ..................................................................................................77
Figure 39) Dynamics of Intracellular Reorganization of H-NS and Chromosome in E. coli under Osmotic Stress in “Stationary Phase” ........................................................................................................78
Figure 40) Quantification of H-NS and Chromosome Dynamics in Response to Osmotic Stress for Exponential and Stationary Phases ..................................................................................................79
Figure 41) Intracellular Reorganization of H-NS Molecules under Osmotic Stress in Coumermycin Pretreated E. coli ........................................................................................................................................80
Figure 42) Coumermycin Effect on Chromosome Area Compaction in E. coli .........................81
Figure 43) Quantification of Coumermycin Effect on Compaction of Chromosome in E. coli at Both Phases of Growth ........................................................................................................81
Figure 44) Schematic of E. coli Response (H-NS Spatial Redistribution) to 30-minute Osmotic Stress at Exponential Phase and Stationary Phase.
Chapter 1: Introduction

Fluorescence microscopy has opened a window into the world of microscopic biological systems and revolutionized our understanding of these systems. However, the level of detail we can observe is ultimately restricted by the wavelength of the light being applied to the system of interest\(^1\). This “diffraction limit” or “resolution limit” is the result of diffraction of light as it passes through an aperture (i.e., a microscope objective). The Abbe resolution limit (Equation 1) can quantify the minimum distance between two point-like light sources at which they can still be resolved in spite of the diffraction of light\(^1,2\).

\[
\text{Equation 1) } \quad \text{Abbe Resolution} = \frac{\lambda}{2\text{N.A.}}
\]

In the Abbe resolution limit, \(\lambda\) is the wavelength of the observed light and N.A. is the numerical aperture of the objective lens\(^1,2\). In practice, using visible light (wavelength in the spectrum range of 400 to 750 nm) and a high N.A. objective such as an oil immersion objective with a typical N.A. of 1.4 results in a resolution limit of approximately 200 nm\(^2\). This resolution limit was long thought to be a central limitation of classic light microscopy\(^1,2\). Although the resolution offered by classical light microscopy could be sufficient to study biological systems such as tissue morphology or entire-cell dynamics, it did not allow sub-cellular structures in small biological organism such as bacteria to be resolved. Thus, there was a need to break this diffraction limit barrier\(^2\). In the past two decades, scientists keenly worked to develop microscopy techniques to go around this barrier. They successfully established a number of super-resolved microscopy techniques such as stochastic optical reconstruction microscopy (STORM), direct STORM (dSTORM), photo-activated localization microscopy (PALM), fluorescent PALM (fPALM), structured illumination microscopy (SIM) or stimulated emission depletion (STED), to name a few, that can attain a resolution well beyond what is possible by classical light microscopy\(^3,4\).

Depending on the system of interest, the type of super-resolved microscopy technique employed can vary. For instance, in this Ph.D. project, we studied the spatial organization of two kinds of proteins: 1) RNA polymerase (RNAP) II in eukaryotic cells and 2) the heat-stable nucleoid
structuring (H-NS) protein in E. coli. To study RNAP II clusters, referred to as transcription factories, we applied dSTORM in conjunction with immunofluorescence labeling. To probe the spatial distribution of H-NS, we employed both PALM and super-resolution radial fluctuations (SRRF). dSTORM and PALM are two single-molecule localization microscopy (SMLM) techniques that can achieve a resolution at the scale of tens of nanometers. With SRRF, a super-resolved post-processing technique, we obtain poorer resolution than in SMLM, but we can still achieve a resolution of roughly 150 nm, which is sufficient to characterize our biological system of interest (i.e., reorganization of H-NS proteins). In chapter 2, we explain the principle of both dSTORM and PALM in detail, and present the workflow for SRRF.

As the first main step of this Ph.D. project, we built an optical setup (demonstrated in chapter 2) on which we could conduct dSTORM and PALM experiments to study the biological systems mentioned above. By inserting a range of laser lines and corresponding optics, we developed our setup to be compatible for a range of photo-switching fluorophores such as Alexa-637 and Atto-532 and subsequently suitable for two-color SMLM. We built our optical setup such that we can perform three modes of microscopy (i.e., wide-field, total internal reflection fluorescence (TIRF), and highly inclined and laminated optical sheet (HILO) microscopy). Furthermore, we designed our setup to enable three-dimensional (3D) SMLM, and we performed the calibration for the axial image-plane. In addition to building the setup, we developed a series of image-processing tools, presented in chapter 2, for reconstructing accurate SMLM images.

As mentioned above, we built our setup such that we can perform two-color (green and red) SMLM. However, to perform accurate two-color microscopy, it is necessary to correct for chromatic aberrations. Therefore, by employing an array of Nano-holes as fiducial markers, in chapter 3 we calibrated for chromatic aberrations. In addition to this, it is also necessary to utilize an appropriate pair of photo-switching fluorophores that display compatible photo-physics when they are residing in the same environment. Hence, in chapter 3 we studied the in vitro photo-physics of Alexa-647 and Atto-532 through dSTORM. We measured the number of photons collected by an EMCCD camera in each switching (‘blinking’) event between the fluorescent state and the dark state, and we calculated the duty cycle, i.e., the amount of time fluorophores spend in the dark state compared to the amount of time they stay fluorescent. Regarding these
characteristics, we confirmed that these fluorophores could be confidently utilized for one- or two-color dSTORM experiments and provide high-resolution SMLM images.

Moreover, in chapter 4, we employed the photo-physics characteristics of Alexa-647, and we established an in-silico SMLM experiment for examining the accuracy of a mathematical theory developed in our lab. This mathematical model can extract the number of target molecules (tagged with photo-switching fluorophores) from the number of blinks\cite{13}. To test this model, we first developed an SMLM in-silico experiment for calibration for the characteristic number of blinks per Alexa-647 molecule. Then, we established our main in-silico experiment that simulates an array of molecules tagged with Alexa-647 fluorophores undergoing blinking for a sufficiently long acquisition.

In addition, in chapter 5 we labeled RNAPII proteins with Alexa-647 through indirect immunofluorescence labeling. We could successfully conduct dSTORM and map RNAPII proteins within the nuclei of mouse cortex cells and embryonic cortex cells. Furthermore, we were able to image the organization of this protein within a 1 µm-thick section of the nucleus of the mouse cortex cell through 3D dSTORM. Despite these accomplishments, we had to stop that study due to the very slow throughput of dSTORM. At the time, we could only image 1 µm of the nucleus but mammalian cell nuclei are typically very large (≈10-20 µm). Therefore, to image each nucleus, 10-20 dSTORM images would have to be taken. Furthermore, to obtain reasonable statistics, we would have had to acquire the images of more than one cell nucleus. Subsequently, we should have processed all of these images before further quantitative analysis.

Due to the complications mentioned above, we shifted our study from the intracellular distribution of RNAPII proteins in eukaryotes to another biological system. In chapter 6, we studied the spatial reorganization of H-NS proteins in response to high osmolarity in E. coli. We explored this osmoadaptation behavior in both exponential and stationary phases of growth. We started our study by using PALM and we observed a pronounced osmotic response only in stationary phase. This osmotic response was so noticeable that we did not necessarily need ultra-fine resolution that we could attain by PALM. We hesitated to continue using PALM especially because of the slowness of this technique together with all the corresponding subsequent image-
processing analysis required. Therefore, for the rest of our study, we altered our microscopy technique from PALM to the much faster technique SRRF providing 150 nm of resolution, which was sufficient to observe the intracellular osmotic rearrangement of H-NS proteins.

In addition, in chapter 6, we studied the dynamics of the redistribution of H-NS proteins and the chromosome in response to osmotic stress at both phases. After exploring these dynamics, to uncover why we observed the osmotic response only in stationary phase, we altered the level of chromosome supercoiling in both phases. We investigated how the overall level of supercoiling could affect the response by inhibiting DNA gyrase, an enzyme which introduces negative supercoiling in bacterial chromosomal DNA\textsuperscript{14}. 
Chapter 2: Super-Resolved Microscopy to Resolve Biological Systems beyond the Diffraction Limit

*The introduction to the principle of SMLM presented in this chapter is used in the review article “Resolving Biology Beyond the Diffraction Limit with Single-Molecule Localization Microscopy*1. Contributions: Prof. Milstein and Dr. Mazouchi established the microscopy setup and Nafiseh Rafiei completed this setup by implementing 532 nm and 561 nm laser lines and corresponding optics into the excitation path, and inserting a high-speed filter wheel into the detection path. She integrated a cylindrical lens into the setup and performed the calibration for 3D imaging. Regarding the image-processing analysis, Dr. Mazouchi developed the drift-correction tool and Nafiseh Rafiei built all the other image-processing platforms presented in this chapter.

2.1 Summary

In this chapter, we present the overall principle of single-Molecule Localization Microscopy (SMLM) and two of the main techniques of this method photo-activated localization microscopy (PALM)*7 and direct stochastic optical reconstruction microscopy (dSTORM)*6. We describe the differences between these two methods and their applications in this Ph.D. project. In addition, we present a super-resolved post-processing microscopy called “super-resolution radial fluctuations (SRRF)*8 and explain how we apply this method in this work. Moreover, we explain three modes of fluorescence microscopy and display the experimental optic setup we utilize to conduct super-resolved microscopy. Furthermore, we report how we calibrated for the axial image-plane and performed three-dimensional (3D) SMLM with the same experimental setup by inserting a cylindrical lens within the detection path. Lastly, we introduce four MATLAB scripts we developed to process SMLM data and reconstruct an accurate SMLM image.

2.2 Single-Molecule Localization Microscopy (SMLM)

One technique to circumvent the diffraction limit of light is single-molecule localization microscopy (SMLM) that can generate images with resolutions of an order-of-magnitude better than diffraction limited (“classical”) fluorescence microscopy*7,9. SMLM relies upon determining the precise location of individual fluorescent labels within a diffraction-limited area at any one time. The intensity distribution, or “point-spread function” (PSF), of a single fluorophore is an Airy pattern. However, the central intensity peak of the PSF can be well approximated by and fitted with a Gaussian function to determine the spatial coordinates of the fluorophore*15 (Figure
The precision of this measurement (commonly referred to as the "localization precision", \( \sigma \)) is essentially limited by the number of photons that the fluorophore emits. More precisely, localization precision scales like \( \sigma = \frac{s}{\sqrt{N}} \), where \( s \) is the standard deviation of the fitted Gaussian and \( N \) is the number of detected photons \(^{16} \) (Figure 1). However, Equation 2 provides a more realistic quantification of the localization precision in each lateral dimension \(^{17} \). According to this equation, the localization precision (\( \sigma \) (nm)) depends on the pixel size (\( a \) (nm)), background noise (\( b \) (photons/pixel)), standard deviation of the PSF along x or y (\( s \) (nm)) and finally, but the most importantly, the number of collected photons (\( N \)) \(^{17} \). We depicted the concept of the localization precision in Figure 1.

![Figure 1](image)

**Figure 1** Localization Precision

(Top) Simulated camera images of a single fluorophore that emits 100 (A) and 1000 (B) photons (\( N \)). The star (*) displays the fitted center of the PSF (i.e., a localization). (Bottom) Blue circles that are Gaussian distributed represent multiple localizations of the fluorophore. Localization precision, i.e., the width of the Gaussian function, scales like about \( \frac{1}{\sqrt{N}} \). The localization precision is notably higher for the brighter fluorophore (B). This image is adapted from reference [1], (Rafiei et al., 2016).
Equation 2)

\[
\sigma = \sqrt{\frac{16}{9} \left( \frac{s^2 + a^2}{N} \right) + \frac{8\pi \left( s^2 + a^2 \right)b^2}{a^2 N^2}}
\]

As described above, it is straightforward to determine the coordinates of a single fluorophore that emits a sufficient number of photons. However, it is no longer feasible to localize fluorophores if there are multiple ones within very close proximity (i.e., a diffraction-limited area). In this case, the corresponding PSFs overlap and it is not possible to identify individual PSFs. The trick employed by SMLM is to separate overlapping PSFs in time and to then localize each by fitting a Gaussian function to the PSFs as before (Figure 2).

Figure 2) Principle of Single-Molecule Localization Microscopy (SMLM)

The solid and dotted lines represent two distinct fluorescent states either fluorescent (ON) and non-fluorescent (OFF) states or two spectrally separate states. A) Two fluorophores are not resolvable if the distance between them is less than the Abbe limit. B) When only one of the fluorophores fluoresces at the time, PSF can be fitted by a 2D Gaussian function to determine corresponding fluorophore localization (red circle). C) The process is repeated until all the fluorophores are localized and their localizations are summed up to generate a super-resolved image. This image is adapted from reference [1], (Rafiei et al., 2016).

In this context, the key factor that enables photo-activated localization microscopy (PALM) and direct stochastic optical reconstruction microscopy (dSTORM), both examples of SMLM, to overcome the diffraction limit is the employment of photo-switching fluorophores. These probes, or “photo-switches”, are able to transition irreversibly or reversibly between two distinct fluorescent states in a controllable manner. The transitions can be between fluorescent (On) and non-fluorescent (Off) states or between two spectrally separated fluorescent states (e.g.,...
green and red). Accordingly, photo-switches can be categorized into three groups: probes that 1) can be reversibly transitioned between On- and Off- states, 2) are initially Off, but upon activation by light, are irreversibly switched On, 3) are initially On but upon exposure to light, irreversibly shifted to another color. These categories of photo-switches (illustrated in Figure 3) are called, respectively, “photo-switchable”, “photo-activatable” and “photo-convertible” fluorophores, however, for convenience the term photo-activatable will be used for both of the last two classes. Photo-activatable fluorophores can typically be switched by exposure to a very low intensity “activation laser” with a wavelength of about 400 nm.

Photo-switches are either synthetic (organic dyes and quantum dots) or naturally occurring (fluorescent proteins). Cyanine (e.g., Cy3, Cy5 and Alexa-647), rhodamine (e.g., Alexa-488 and Atto-532) and oxazine dyes (e.g., Atto-655 and Atto-680) are all photo-switchable dyes and
Dronpa is a photo-switchable fluorescent protein\textsuperscript{12}. Fluorescent proteins (FPs) are genetically expressed whereas synthetic organic dyes should be introduced to the molecule of interest exogenously\textsuperscript{23}. Each of these classes has their own advantages and disadvantages: FPs can be fused to the molecule of interest using transgenic approaches, therefore they are highly specific and expressed in a 1:1 ratio with the molecule of interest, which is ideal for quantitative imaging\textsuperscript{24}. In contrast, to label the target molecule with synthetic dyes exogenous agents and coupling methods like immunofluorescence labeling are required. Using these exogenous methods causes the ratio of labeling not to be 1:1 necessarily, and can often lead to non-specific binding from the target molecule\textsuperscript{25}. However, synthetic dyes have some advantages over FPs. The most significant benefit is that synthetic dyes are much brighter than FPs. Another advantage is that synthetic dyes are available in numerous colors, while FPs are currently only available in a limited number of colors\textsuperscript{23}.

While similar in concept, the difference between PALM and dSTORM arises from the class of photo-switching fluorophores used in these techniques and the methods employed to “make” these probes switch (blink). In the following sections, we explain the differences between these two techniques and their implications for our studies.

2.2.1 Direct Stochastic Optical Reconstruction Microscopy (dSTORM)

dSTORM employs synthetic photo-switchable fluorophores that can switch between an On-state and an Off-state. In this context, we often talk about a fluorophore characteristic termed the “duty cycle” (D.C.), Equation 3. The duty cycle indicates the amount of time the fluorophore spends in the On-state ($T_{on}$) compared to the Off-state ($T_{off}$)\textsuperscript{12}.

\textbf{Equation 3) \[ D. C. = \frac{T_{on}}{T_{off}} \]}

To localize on average only one PSF at a time, the duty cycle should scale as $1/M$, where $M$ is the number of fluorophores located within a diffraction-limited area\textsuperscript{12}. Many synthetic fluorophores used in dSTORM should be tuned to display duty cycles of $10^{-4}$ to $10^{-5}$ to obtain super-solved images\textsuperscript{12}. The duty cycle of the fluorophores can be adjusted with the presence of millimolar (10-
200 mM) concentrations of reducing agents, such as thiol compounds like β-mercaptoethylamine (MEA) and β-mercaptoethanol (BME), included in the imaging-buffer⁹,²⁶.

In the On-state, fluorophores can transition from the ground state (S₀) to the excited singlet state (S₁), and after a few nanoseconds emit a red-shifted photon as they return to the ground state. However, a very small population of excited fluorophores (typically about 0.1%) can transit from the excited state to a non-fluorescent triplet state (T₁) through intersystem crossing²⁷. Upon interaction with oxygen molecules in the environment, fluorophores in the triplet state eventually (after a few micro- to milli- seconds) return to the ground state and the fluorescence cycle. However, fluorophores in the excited states (singlet or triplet) exposed to oxygen and other molecules can be altered to a permanently non-fluorescent photobleached state¹⁹,²⁷.

In dSTORM, initially, almost all the fluorophores are in the On-state under exposure to a very intense excitation laser (1-10 kW/cm²)²⁷. Then, upon interaction with the thiol reducing elements in the imaging-buffer, almost all of the excited fluorophores are reduced to a stable and reversible dark Off-state¹⁹,²⁶,²⁷, which can have a lifetime in a range of hundreds of milliseconds to several seconds⁹. The key to enable us to conduct a successful dSTORM measurement is to keep the majority of the fluorophores in the Off-state and stochastically return only a subset of them to the On-state¹⁹. This will be achieved by adjusting the concentration of reductants in the imaging-buffer and/or removing oxygen as the major quencher of the triplet/dark states from the buffer using an oxygen scavenger system¹²,¹⁹. In addition, either spontaneously or by irradiation of the activation laser (typically we employ a 405 nm laser) with very low intensity (0.1-30 W/cm²), a sparse subset of fluorophores in the Off-state is reactivated and returned to the singlet state while the majority of fluorophores remain in the Off-state¹². This switching between On- and Off- states is repeated until all the fluorophores are imaged individually⁹. We display a schematic of the photo-physics in Figure 4.
To perform dSTORM imaging, we use the conventional synthetic fluorophores Alexa-647 and Atto-532 with the excitation peaks at 650 nm and 532 nm and emission peaks at 665 nm and 553 nm, respectively. We acquired images at a frame rate of 20 Hz and excited Alexa-647 and Atto-532 fluorophores by employing 637 nm and 532 nm lasers with an intensity of about 1 kW/cm² and 4 kW/cm² respectively. To enable these fluorophores to become photo-switchable we use an imaging-buffer as described below. Using this imaging-buffer causes these fluorophores to reactivate spontaneously without the need to apply an activation laser (i.e., 405 nm).

**dSTORM imaging-buffer:** In this study, the imaging-buffer is Phosphate Buffered Saline (PBS) with a pH of 7.6, supplemented with thiol reducing compounds, (50 mM) MEA, and contains the oxygen scavenging system PCA-PCD, 50 mg/mL Protocatechuate Acid (PCA), 5 μM Protocatechuate 3,4-Dioxygenase (PCD). We preferred to use PCA-PCD rather than other commonly used oxygen scavenger system like GLOXY (glucose-oxidase) as PCA-PCD can maintain

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*Figure 4) Energy States of Fluorophores Switching Between On- and Off- States*

Fluorophore in the ground state S0 is excited to the excited S1 state upon exposure to the excitation light (Ex). After emitting red-shifted light, it returns to the ground state in few nanoseconds. Due to intersystem crossings, excited fluorophore can transit to the non-fluorescent triplet state (T1) or Dark state (D) that can last, in respect, few microseconds and few milliseconds (even minutes). This image is adapted from ref [29], (Nahidiazar et al., 2016).
the pH of the buffer. In the case of using GLOXY, the pH can drop with a rate of 2 pH/hour\(^2\), however, PCA-PCD can retain a stable pH over a long time (90 minutes)\(^2\). Maintaining pH is crucial in SMLM, because dropping pH can decrease the brightness of the fluorophores, which leads to a decrease the localization precision\(^1\).

### 2.2.2 Photo-Activated Localization Microscopy (PALM)

PALM utilizes photo-activatable fluorescent proteins (PA-FPs) instead of synthetic dyes. In addition, there is no need for a specific imaging-buffer to make the PA-FPs switch. In PALM, first by shining a near-ultraviolet laser, a sparse subset of PA-FPs within the sample are photo-activated. This subset of PA-FPs is excited and individually imaged until all the PA-FPs are photobleached. This cycle is repeated until all the PA-FPs are activated, excited, imaged and photobleached\(^7\) (see Figure 3,B,C).

To enable us to conduct PALM, our molecule of interest, H-NS, was fused to the green-to-red PA-FP mEos3.2, which is in the class of Eos fluorescent proteins\(^3\). Unlike mEos2, mEos3.2 is highly monomeric and does not aggregate at high concentration\(^3\),\(^4\). This will be particularly important for studying a highly expressed regulatory protein like H-NS. In addition, mEos3.2 provides a high number of photons (about 1000) per blink, which is important to get a better localization precision\(^3\). The emission and excitation peaks of mEoS3.2 in the green channel are respectively 506 nm and 519 nm\(^3\). Upon exposure to the activation laser (i.e., UV/405 nm), mEos3.2 converts to the new spectral fluorescent state (red channel) and the emission and excitation peaks are shifted to 573 nm and 584 nm\(^3\), respectively. For PALM, we utilized no imaging-buffer and activated mEos3.2 using a 405 nm laser line with very low intensity (about 1 W/cm\(^2\)) and image mEos3.2 in the red channel. For the excitation laser in the red channel, we employed a 561 nm laser line with very high intensity, about 1 kW/cm\(^2\) and recorded images at the frame rate of 20 Hz.

### 2.2.3 Super-Resolution Radial Fluctuations (SRRF) Algorithm

SRRF is a “post-processing” super-resolved analysis tool available as an open-source ImageJ plugin\(^8\). This threshold-free computational method analyzes a stack of images and directly
generates a super-resolved image. In contrast to SMLM techniques such as PALM and dSTORM, to generate a super-resolved image this algorithm can be applied to conventional wide-field images that have low signal-to-noise ratios to provide a resolution below 150 nm. Although, the resolution of SRRF is lower than PALM and dSTORM, the main advantage of this technique over these aforementioned techniques is the fast images acquisition time. To obtain a super-resolved PALM/dSTORM image reconstruction, tens of thousands of images should be collected. However, to obtain a super-resolved SRRF image, only hundreds of images need to be acquired (which takes under a minute).

In SRRF, there is no requirement to photo-switch the fluorophores. Instead, this approach first calculates the convergence of the local intensity gradient (“radiality”) across the entire frame on a sub-pixel basis and produces a radiality map for each single frame of the stack. Then, to enhance the contrast and de-noise the final super-resolved image, it analyzes the temporal correlations of the radiality maps, retains the fluorophore-induced radiality peaks and discards the noise-associated ones. Radiality peaks that are induced by noise are uncorrelated in time i.e., their temporal correlation will become zero at these locations. Unlike noise-induced radiality peaks, fluorophore-associated radiality peaks are highly correlated in time and are preserved. We depict the workflow of SRRF in Figure 5. To obtain SRRF images of H-NS fused to mEos3.2, we conducted classical fluorescence microscopy and imaged mEos3.2 in the green channel. We excited this fluorophore with a very low intensity 488 nm laser (about 8 W/cm², Coherent, OBIS) and the emission fluorescence was filtered through a 520/35 nm (Chroma) band-pass filter. We acquired 100 images at a frame rate of 20 Hz and post-processed the sequence of images with SRRF. The only parameter we adjusted in this plugin was the “ring radius” that is the radius from each point within the intensity map. This parameter calculates the convergence of intensity gradients surrounding each point and determines the radiality of the full-width at half maximum (FWHM) of PSF. The ring radius was set to vary between 0.1σ and 3σ, where σ is the PSF standard deviation. We changed this parameter by changing the coefficient of σ within the working range of 0.1 to 3.
To ensure we have chosen the correct value for the ring radius and to minimize the introduced artifacts into our super-resolved images, we used a computational tool termed “super-resolution quantitative image rating and reporting of error locations” (SQUIRREL) also implemented as an open-source ImageJ plugin\(^{33}\). This method requires two images as input: the super-resolved image and the diffraction-limited image as a reference image. SQUIRREL convolves the super-resolved image with an estimated/pre-known PSF, generates a scaled, convolved super-resolved image and compares this convolved image with the reference image pixel by pixel. It then generates an error map that indicates the local degrees of dissimilarity between the super-resolved image and the diffraction-limited one\(^{33}\). We illustrate the schematic of this computational method in Figure 6.
In SQUIRREL, error maps can be calculated based on two types of error: either a 1) “resolution scaled error” (RSE) representing a root-mean squared error or 2) a “resolution scaled Pearson coefficient” (RSP) that represents the Pearson-correlation coefficient between the diffraction-limited and super-resolved image. 

Figure 6) SQUIRREL Analysis Workflow
Super-resolved image (A), here dSTORM image of RNAPII molecules tagged with Alexa-647 in the cortex mouse cell, is convolved with the estimated or pre-known point-spread function (B) and generates a convolved image (C) that is compared to the reference (diffraction-limited) image (D) to produce spatial (resolution scaled) error map (E).
To set the optimized ring radius value for our analysis, we obtained the SRRF image of our system of interest for a range of ring radius values (i.e., about 15 SRRF images of the same sample). Having a diffraction-limited image of the system as a reference image, we obtained RSE and RSP values for each of these SRRF images and picked the ring radius such that RSE is minimum and/or RSP is maximum (i.e., in the range of 1σ to 2.5σ in the case of Figure 7).

2.3 Microscope Setup and Modes of Microscopy

2.3.1 Modes of Microscopy

To conduct super-resolved imaging, we used the microscopy setup that is built upon an inverted microscope with a high numerical aperture TIRF objective (N.A. >1.4) that enables us to operate all three modes of microscopy: epifluorescence (wide-field fluorescence), total internal reflection fluorescence (TIRF), and highly inclined and laminated optical sheet (HILO) microscopy.
The most conventional mode of microscopy is epifluorescence. However, the main problem of using this mode of microscopy is that it suffers from a high level of background from out of focus fluorophores. To overcome this limitation, we can apply TIRF or HILO microscopy to reduce background and achieve a much higher signal-to-noise ratio\textsuperscript{10,11}. We illustrate all of these modes of microscopy in Figure 8.

\textbf{Figure 8) Schematic of Modes of Microscopy.}

To illuminate the sample, light is focused at back focal plane (black dashed line). In Epi fluorescence microscopy, the focused beam is on the optical axis. In objective-based TIRF, the focused beam is translated away from the optical axis (red dashed line) at the back focal plane and the illumination and fluoresce light both are collected from the objective. In HILO, light is focused at the back focal plane and away from the optical axis but not as much as TIRF and the illumination depth is more than TIRF but less than Epi. Orange circles represent excited fluorophores and the blue ones stand for the unexcited probes.

TIRF microscopy is an optical method that only excites fluorophores within an extremely thin depth above the coverslip. This technique is based on the total internal reflection of light reflecting off the interface of two media with different refractive indexes (such as glass and water). Although the light is reflected, a portion of the electromagnetic field can penetrate the interface, although the field rapidly decays in intensity. The penetrating light is called an evanescent wave\textsuperscript{10}. The evanescent wave has the same frequency as the excitation light and it decays exponentially with distance from the interface, therefore, only fluorophores within 100-200 nm of the interface are efficiently excited and the background can be reduced by orders-of-magnitude compared to standard epifluorescence imaging\textsuperscript{10}.  

\textsuperscript{10}
To achieve TIRF, there are two main optical configurations: prism-based TIRF and objective-based TIRF. In prism-based TIRF microscopy, the illumination light is delivered to the interface through a prism and the fluorescence light is collected by the objective, while in objective-based TIRF light is both delivered and collected by the objective. The latter configuration requires an inverted fluorescence microscope and an objective with very high numerical aperture (N.A. ≥ 1.45)\textsuperscript{34,35}. In objective-based TIRF microscopy, which our optical setup is designed for, light should be focused and translated away from the optical axis at the back focal plane of the objective (Figure 8, middle) to ensure the incident light is collimated and hits the interface at the critical angle\textsuperscript{35}.

TIRF microscopy is widely used to study cell membranes because of the thin illumination penetration depth \textsuperscript{34}. However, it can only be applied if the region of interest is very close to the surface and cannot be used to investigate structures inside a cell, which may appear at depths of several microns above the coverslip\textsuperscript{10,34,35}. To obtain an improved signal-to-noise ratio compared to epifluorescence microscopy and to image within large cells, we instead apply HILO microscopy (or “dirty-TIRF”), which is built upon the TIRF approach\textsuperscript{11,36}. In HILO microscopy, the illumination beam is highly inclined and strikes the sample/coverslip interface with an angle slightly below the critical angle\textsuperscript{11,37}. A narrow sheet of light can pass through the coverslip at a highly-inclined angle illuminating a slice of the specimen with a thickness of about 7 \(\mu\)m, which is roughly two orders-of-magnitude larger than in TIRF mode but still much smaller than the fraction illuminated by epifluorescence\textsuperscript{11}. Hence, this sectional illumination in HILO microscopy can significantly reduce fluorescence background and therefore increase the signal-to-noise ratio by an order-of-magnitude compared with conventional epifluorescence microscopy\textsuperscript{11}.

In this Ph.D. project, depending on the biological system we examine, such as mammalian or bacterial cells, and the location of the intracellular structures (e.g., inside or at the membrane), we will use one of these modes of microscopy. For instance, to study transcription factories inside mammalian cells (such as mouse embryo stem cells) we employed HILO microscopy. However, to explore the spatial distribution of H-NS molecules in E. coli, we applied epifluorescence.
2.3.2 Microscope Setup

Our single-molecule (super-resolved imaging) instrumentation (Figure 9), and all the required optics, are bolted upon a vibration isolating air table inside a sufficiently dust-free room. We conducted our single-molecule experiments with an inverted Olympus IX71 microscope equipped with a 60X oil immersion objective (Olympus, APON 60XOTIRF) with an N.A. of 1.49 suitable for TIRF microscopy. To operate dSTORM and PALM imaging, by considering the maximum absorption wavelength of the photo-switching fluorophores we use to tag our molecules of interest, we implemented different laser lines into our setup. We employed 637 nm (World Star Tech, NRC B-2) and 532 nm (Laser Quantum, Gem) laser lines to excite Alexa-647 and Atto-532, respectively, for one-color/two-color dSTORM imaging, and a 561 nm laser line to excite mEos3.2 for PALM imaging. In addition, we placed a 405 nm laser line (Coherent, CUBE) in our setup as an activation laser for both the case of dSTORM and PALM imaging. Moreover, we have a 488nm laser line (Coherent, OBIS) to be able to image mEos3.2 in the green channel by conducting classical epifluorescence microscopy followed by SRRF.

There is an acousto-optical tunable filter (AOTF) in the excitation pathway to rapidly select the appropriate laser lines at a corresponding power. In addition, integrating a movable mirror inside the illumination pathway enables us to translate the focused light along back focal plane of the objective and switch between epifluorescence, TIRF and HILO microscopy. This mirror reflects the excitation beam and sends it to the filter cube of the microscope. Then the excitation laser is reflected from the dichroic mirror inside the filter cube, focused on the back focal plane of the objective, and sent to the sample. The same objective collects the fluorescence emission and transmits it through the same dichroic mirror followed by filtering by an emission filter inside the filter cube. Apart from the laser lines in our setup, we also employed an arc lamp (X-Cite, series 120Q), which we mainly utilized to excite DAPI (4’, 6-diamidino-2-phenylindole), a blue-fluorescent DNA stain, excited at 359nm and emitting at 461 nm.

To compensate for focal changes caused by thermal fluctuations and mechanical shift of the microscope, we implemented an auto-focus system called “continuous reflection interface sampling and positioning” (CRISP, ASI c-203) in the detection pathway. This auto-focus system
illuminates a mask with an IR (850 nm) LED, projects its image into the sample, collects the IR light reflected from the specimen and guides it to a position-sensitive-detector (PSD) which can detect lateral changes\textsuperscript{41}. The MS2000 controller amplifies the PSD signal sent as a feedback to the piezo stage (Nano-Drive, μsMCL-1720) to adjust the focal position. Therefore, this auto-focus system can maintain the axial stability and keep the specimen in focus for hours\textsuperscript{41}.

We also introduced a high-speed filter wheel (THORLABS fw103) in the detection pathway, which enables us to perform two-color imaging by rapidly rotating between the appropriate filter for each channel. After this high-speed filter wheel and before the camera, we implemented a telescope that could increase the total magnification of our setup up to 137 times to provide a pixel size of 117 nm and consequently, a PSF FWHM of 235 nm according to the Nyquist-Shannon sampling criteria. The fluorescence light is transmitted to a very sensitive electron multiplying charge-coupled device (EMCCD, Andor iXon3) camera. We record typically a stack of 12,000 to 25,000 images for dSTORM and PALM imaging and 100-1,000 images for classical light microscopy followed by SRRF with a frame rate of 20-40 Hz.
Figure 9) Schematic of Single-Molecule Imaging Setup

Consists of excitation laser lines: 561 nm, 637 nm, 532 nm and 488 nm as well as 405 nm laser line as an activation laser. An acousto-optic tunable filter (AOTF) is employed to select a wavelength and its amplitude. The laser light is focused onto the back focal plane of an oil-immersion objective with a numerical aperture of 1.49. Dichroic mirror in the detection path separates the excitation and emission light. Long-pass and/or band-pass filters inside the filter cube and filter wheel filter the fluorescence light that would be led through the telescope and focused on the EMCCD camera.
2.3.3 Three-Dimensional (3D) Imaging

We are able to obtain three-dimensional (3D) super-resolved imaged by the simple introduction of a cylindrical lens with a focal length of 0.5 m within the final imaging telescope (Figure 10, A). The lens produces an astigmatism that makes the shape of the PSF elliptical (Figure 10, B). We can then determine the axial position of the fluorophore in regard to this depth dependent ellipticity. Therefore, we should perform a calibration of the PSF at varying heights before data acquisition.

To obtain an axial calibration curve, we utilized a 20 nm fluorescent bead (excited by 532 nm) as a fiducial marker. We found the sharpest focus (defined here as zero μm) of the fiducial markers and moved the microscope stage along z with a step size of 20 nm from -1 μm to 1 μm by employing a piezo-stage. We then recorded the PSF of the marker at each step. We took twenty images at each z position and later averaged over these data points to obtain the smooth curves shown in Figure 10, C.

![Figure 10](image1.png)

**Figure 10** Three-dimensional (3D) Super-Resolution Microscopy

A) A cylindrical lens is introduced in the detection path to generate astigmatism. The image is adapted from reference [43] (Science, Huang et. al., 2008). B) As the z position is changed, the ellipticity of the PSF image varies accordingly. Scale bar is 500 nm. C) Calibration curve: the z stage moved from -1 μm to 1 μm with the step size of 20 nm. The FWHM of the PSF along x (blue curve) and along y (green curve) vary in accordance to the z position. Each data point is the average of twenty images taken at each z position. Focal plane is where these two curves intersect.
2.3.4 Measurements and Image-Processing Software in SMLM

To control our microscope, we use the open-source software package μManager which is developed as an ImageJ plugin\textsuperscript{43,44}. As mentioned above, we should acquire a stack of thousands of images for SMLM (dSTORM and PALM) experiments and a stack of hundreds of images for SRRF microscopy. After recording a stack of images for each of these techniques, we should apply a sequence of image-processing techniques that we discuss next.

2.3.4.1 Table of Fluorophores Coordinates (Localization Table)

As noted earlier, in dSTORM and PALM experiments, we activate a sparse subset of fluorophores in each frame and generate a large stack of images. Afterwards, the PSF of individual fluorophores in each frame of the stack can be approximated with a Gaussian function, \textbf{Equation 4}, where \( \sigma_x \) and \( \sigma_y \) are the standard deviations, \((x_c, y_c)\) are the coordinates of the center point, and \( A \) and \( B \) are the scaled amplitude and a bias from the background signal, respectively\textsuperscript{6}.

\begin{equation}
I(x_c, y_c, A, B) = \frac{A}{2\pi\sigma_x\sigma_y} \exp\left(-\frac{(x-x_c)^2}{2\sigma_x^2} - \frac{(y-y_c)^2}{2\sigma_y^2}\right) + B
\end{equation}

By fitting this function to the PSF of each spot in a frame, we can identify the coordinates of all active fluorophores in the stack. We refer to the list of single-molecule coordinates as a “localization table”. To obtain the localization table, we employ an open-source SMLM software package called rapidSTORM\textsuperscript{45}, which is commonly used due to its speed in generating a localization table. This software provides the localization table by performing two main tasks on each frame of the image stack: 1) identifying each fluorophore from noise and 2) fitting the optimal 2D Gaussian function to the identified PSF and evaluating the fit parameters. Fluorophores with a PSF below an intensity threshold are discarded\textsuperscript{6}. We display an artificial example of a portion of a localization table generated by rapidSTORM in Table 1. Note, rapidSTORM outputs the FWHM along x and y, abbreviated as PSFx and PSFy (\(\text{PSF}_{x,y} = 2\sqrt{2\ln 2}\sigma_{x,y}\)).
To generate the localization table with rapidSTORM, as stated above, a 2D Gaussian is approximated to fit the PSF of a fluorophore. In addition, the PSFs of the fluorophores vary in size based on their depth above the coverslip. RapidSTORM allows the user to select either a fixed ($\sigma_x = \sigma_y$) or variable fitting algorithm for the Gaussian fits. In both cases, rapidSTORM exclusively keeps localizations in which the integral of the PSFs are above the intensity threshold inserted as another central input parameter. The fixed width fit works well for 2D samples, but for most samples we need to employ the variable fitting routine. Consequently, by applying a variable fit to PSF$_x$ and PSF$_y$, many localizations with very large PSFs, usually corresponding to out of focus fluorophores and noise, are also identified. To discard false localizations and only keep the localizations of fluorophores that are in focus, we developed a MATLAB script to filter the localization table, limiting the acceptable range of values for PSF$_x$ and PSF$_y$. This program plots a 2D histogram of the PSF of the fluorophores (Figure 11, A) and finds PSF$_{x}^*$ and PSF$_{y}^*$, which are the mean values for PSF$_x$ and PSF$_y$ detected by the fitting algorithm. Subsequently, it then fits a 2D Gaussian to the histogram and identifies $S_x$ and $S_y$, the standard deviations of the fit. Afterward, a plot of PSF$_x$ vs PSF$_y$ of all the localizations is plotted (Figure 11, B). Along the major principle component of the data, an ellipse with the center at (PSF$_{x}^*$, PSF$_{y}^*$) and diameters of $w_1=\alpha S_x$ and $w_2=\alpha S_y$ is generated, where we typically choose $\alpha=3$. We retain only the localizations that have PSFs within this ellipse. In the next chapter, we present how choosing a fixed or variable fit to the PSF influences the quantification of our SMLM images.
In order to reconstruct a super-resolved image in dSTORM and PALM, it is essential to acquire a sufficient number of images. Therefore, these experiments usually last for several minutes (10-25 minutes). Because of the long image acquisition, besides vertical drift, a measurable lateral or x-y drift can be introduced in the final reconstructed image, which greatly affects the image quality and interpretation (even if it is in the nanometer range). Consequently, x-y drift in the image should be corrected. This correction is conducted either using a fiducial marker or by applying a post-processing image-correlation analysis.

For most of our images, we employed an image-correlation analysis, written in MATLAB. However, since in SMLM the activated fluorophores are very sparse in each frame, to perform a reliable cross-correlation analysis, it is necessary to combine the consecutive frames into segments with the length of \( f \), which defines a drift-correction time step. A typical value for \( f \) is on the order of \( f = 500 \) frames, however, the last segmentation may not contain exactly 500
frames. Our drift-correction program takes the stack of images, splits it into segments, merges all the localizations within each segment and generates corresponding SMLM image ($S_i$) for each time point, $i$. This analysis calculates the cross-correlation function ($C_{ij}$) between two successive segments ($S_i, S_j$) and by fitting a 2D Gaussian to $C_{ij}$ finds where the Gaussian is maximized. This is simply to identify the sample drift at time point $j$. After determining the lateral drift for each segment, we obtain the shift in each frame within the segment by interpolation and realign the individual frames of the image stack accordingly. We present the principle of drift-correction based on image-correlation in Figure 12.

![Drift-Correction Based on Cross-Correlation](image)

**Figure 12** Drift-Correction Based on Cross-Correlation

A) Stack of PALM images: each color refers to one temporal segmentation, which consists of $f$ images. The dashed arrow represents time. B) Super-resolved images corresponded to each segmentation. Drift between each two consecutive super-resolved images is found by measuring the cross-correlation between them. C) This graph displays the drifts along $x$ (blue) and $y$ (orange) for the PALM data (30,000 frames at a frame rate of 20 Hz). Each segmentation consists of 500 images.

### 2.3.4.4 Merging Localizations into Blinks

As explained earlier, in SMLM fluorophores are separated temporally and spatially. However, some activated fluorophores stay in the fluorescent state for more than one consecutive frame. The total number of photons emitted at each blinking event by these probes is therefore split...
into more than one frame and consequently, they are localized in all the successive frames they are present. The resulting localization coordinates of the same fluorophore in sequential frames are not exactly equal and can vary by tens of nanometers.

We developed a script (written in MATLAB) that takes the localization table, already drift-corrected, and first identifies all localizations occurring within a single, given frame. After determining all the coordinates belonging to one frame, it considers a circular area with a radius of R (usually, we select R= 100 nm) around each localization and examines if another localization appears within this area in the following frame. If that is the case, the two localizations are merged together and their corresponding intensities are added. We present a schematic of this post-processing method in Figure 13.

![Figure 13: Post-Processing Merging Analysis](image)

**Figure 13: Post-Processing Merging Analysis.** Green circles with red outlines represent the location of fluorophores that stay in the fluorescent state for a few consecutive frames located within the dashed circle with the radius of 100 nm. In merging process, localizations correspond to these fluorophore are identified, their intensities are added up and the mean value of the coordinates is replaced.

### 2.3.4.5 Image Rendering

Unlike conventional microscopy images, which display a spatial map of image intensities, SMLM image reconstructions reflect localization densities. One approach to representing the images
from the localization table is mapping them on a 2D plot and binning them into a mesh with a bin size of the localization precision. After binning the localizations, there are two possibilities: 1) summing up the number of events in each bin, 2) blurring each localization with a 2D Gaussian function that has a standard deviation which reflects localization precision, and adding up the blurred spot in each bin. The first method produces a pixelated super-resolved image in which every pixel displays the number of localizations detected in this area. The second method generates a pixelated super-resolved image in which the intensity of each pixel is corresponding to the local density of single-molecule localizations. We employed the latter approach (illustrated in Figure 14) to illustrate SMLM.

![Schematic SMLM image (left) and the zoom in (right). Each localization (cross) is blurred with a 2D Gaussian function (blue circles) with the width reflecting localization precision. The black pixel is the camera pixel and the red one is the super-resolved pixel.](image)

**Figure 14) Image Representation in SMLM**

2.4 Concluding Remarks

To conclude, we built the experimental setup to perform an accurate one- or two-color PALM and dSTORM for a range of fluorophores like Atto-532 and mEos3.2. We designed our setup such that we can conduct three-dimensional (3D) SMLM, and we calibrated the axial image-plane. In addition, we developed image-processing software to reconstruct the super-resolved images accurately.

After developing the experimental setup for conducting an accurate SMLM and generating analytical tools to process the SMLM raw data, in the next chapter we present two necessary steps towards a correct two-color SMLM: correction for the chromatic aberration, and investigating the photo-physics of the pair of fluorophores that are selected to be employed.
Chapter 3: Chromatic Aberration Correction and Studying Alexa-647 and Atto-532 Photo-Physics in vitro by dSTORM*

* Contributions: Nafiseh Rafiei designed the array of Nano-holes used for two-color calibration. Toronto Nanofabrication center manufactured this array. Nafiseh Rafiei performed the calibration experiment and developed an analytical tool for image registration. She conducted the photo-physics experiments and established a series of analytical methods to compute average number of collected photons, average number of consecutive On-frames, and duty cycle.

3.1 Summary

To achieve accurate two-color super-resolved images, first, we carefully corrected for chromatic aberration between red and green channels. Then, we studied the photo-physics of Alexa-647 and Atto-532 relevant for conducting dSTORM experiments. We confirmed that this pair of fluorophores is an appropriate candidate for two-color SMLM because they emit a high number of photons and display a low duty cycle under the same imaging buffer conditions.

3.2 Two-Color Imaging and Chromatic Aberration Correction

To study the interaction between any two types of biological molecules, one approach is to conduct two-color imaging. Therefore, the two molecules of interest should be labeled with fluorophores such as Alexa-647 and Atto-532 that are spectrally distinct from each other. One of the two-color imaging techniques we can pursue is two-color dSTORM. In order to conduct two-color imaging, we can acquire images of both fluorophores sequentially by rapidly alternating between two channels, while using a single EMCCD camera. In order to reconstruct an accurate two-color dSTORM image and correctly register the red (Alexa-647) channel image with the green (Atto-532) channel image, we corrected for the chromatic aberration between these two channels. For this calibration, we designed an array of Nano-holes as fiducial markers and employed it to estimate the function that maps one color channel to another. This array was manufactured at the Toronto Nanofabrication Centre (TNFC), at the University of Toronto via electron beam lithography by coating a coverslip (VWR, 22x22 mm, No. 1.5) with a chromium/silver layer with a thickness of 100nm and inserting 1000x1000 holes with diameters 100 nm located 1.5 μm apart (Figure 15).
We attached this array on a glass microscopy slide (Fisher scientific, 1 mm) using liquid glue on the edges. We inserted a green band-pass filter (520/66) in front of a white light source to mimic the green laser and illuminated the array by this optically filtered light. We imaged 1000 frames (512 X 512 pixels) at a frame rate of 20 Hz with EMG value of 5. We repeated this procedure for the red channel using a red band-pass filter (640/50 nm) in front of the white light.

After acquiring images of the array in green and red channels, we obtained the localization tables using rapidSTORM employing a threshold of 2000 counts (equivalently about 100 photons), PSF FWHM of 300nm and minimum spot distance of 10 pixels followed by drift correction in MATLAB using Nano-holes as the fiducial markers. After drift correction, we used the MATLAB image-processing toolbox (cp2tform) as Churchman suggested\textsuperscript{50} to compute the transformation function between these two channels and mapped the green channel to the red channel. We calculated the mean offset between the positions of Nano-holes in both channels after registration to quantify the “co-localization precision” which defines how precisely the images are overlaid. If all the points are considered as fiducials and subsequently, images are registered, then this offset is called the Fiducial Registration Error (FRE)\textsuperscript{51,50}. If each point as a target is excluded from the fiducials and the transformation function is calculated based on the remaining fiducials then this offset is called the Target Registration Error (TRE)\textsuperscript{51,50}. To analyze the co-

\textbf{Figure 15) Micro-Fabricated Nano-Hole Array Application in Chromatic Aberration Correction}
\textit{This array consists of 1,000,000 holes with 100nm diameters and their centers are 1.5 \textmu m apart.}
localization precision ($\delta_T$), TRE is commonly used which is typically reported below 10 nm\textsuperscript{51,50}. However, we measured both TRE and FRE values in this experiment. Total co-localization precision ($\delta_T$) can be calculated from Equation 5, where $\delta_G$ and $\delta_R$ are respectively localization precisions in the green and red channels\textsuperscript{52}.

\textbf{Equation 5)} \quad \delta^2_T = \delta^2_G + \delta^2_R + \text{TRE}^2

We measured the localization precision ($\delta$) in each channel (i.e., $\delta_G$ and $\delta_R$) by three methods: theory (Equation 2), standard deviation (STD) and nearest neighbor analysis (NNA).

To calculate $\delta$ from the standard deviation for this experiment, we measured the mean values of all localizations corresponding to each hole and how much these localizations are spread from the mean value (i.e., $\delta_i$ where $i$ is the index of the hole)\textsuperscript{16}. Finally, we averaged over all the holes within the field of view to obtain the total standard deviation (Equation 6).

\textbf{Equation 6)} \quad \delta = <\delta_i>

To obtain $\delta$ from NNA, we measured the distance between localizations originating from the same emitter (here Nano-array hole) in two adjacent frames and acquired the histogram of this parameter ($d$). By fitting Equation 7 to this distribution we can get $\delta$\textsuperscript{53}.

\textbf{Equation 7)} \quad P(d) = \frac{d}{2\delta^2} exp \left( \frac{-d^2}{4\delta^2} \right)

We present a NNA analysis for Nano-holes array images in the green and red channels in Figure 16. For all of the three aforementioned methods, we obtained a localization precision in both channels that is less than 0.5 nm. In addition, we computed the registration error TRE less than 3.5 nm (see Figure 17). Employing Equation 5 we could achieve a total co-localization precision of up to 3.6 nm.

\textbf{Equation 8)} \quad \delta_T = \sqrt{0.5^2 + 0.5^2 + 3.5^2} \approx 3.6 \text{ (nm)}
The distance ($d$) of localizations originated from the same fluorescent emitter in adjacent frames is measured. From the fitting curve $P(d) = \frac{d}{\gamma \delta^2} \exp\left(-\frac{d^2}{4\delta^2}\right)$, experimental localization precision ($\delta$) is obtained.

Figure 16] Nearest Neighbor Analysis to Calculate Experimental Localization Precision.
3.3 Study Alexa-647 and Atto-532 Photo-Physics in vitro with dSTORM

As previously described, photo-switchable fluorophores that emit a high number of photons in each blinking event and have a low duty cycle are required to obtain a high-resolution image reconstructed by SMLM\(^{12}\). To assure that Alexa-647 and Atto-532 satisfy these criteria, we measured their duty cycles and the number of detected photons per blinking event under the same experimental conditions (imaging-buffer, frame rate (20 Hz) and Electron-Multiplying (EM) gain (300)). To perform this experiment, we sparsely attached Alexa-647 and Atto-532 oligos, separately, to two clean glass Poly-L-lysine (PLL) coated coverslips (Fisher scientific, 0.17 to 0.25 mm).

*Figure 17* Localization Precision and Registration Error.

Images of Nano-holes array are acquired in green and red channels. Localization precision is calculated from different methods: nearest neighbor analysis (NNA), theory and standard deviation (STD). The image of green channel was transformed to the red channel. The error of this transformation, i.e., mean offset between the positions of Nano-holes in both channels after registration is calculated. This offset is represented by either fiducial registration error (FRE) or target registration error (TRE).
For all SMLM experiments, we should clean the coverslips thoroughly in advance to reduce background arising from artifacts on the slides (fluorescence impurities) as much as possible. For all of our experiments we cleaned the coverslips by sonication in 3 M potassium hydroxide (KOH) followed by another round of sonication in Ethanol (Sigma-Aldrich, 99%) each lasting for 30 minutes. After each sonication, we rinsed the coverslips five times with distilled water (after the final rinse, we let the coverslips air-dry). After cleaning the coverslips, we incubated them overnight with Poly-L-lysine (Sigma-Aldrich, 0.1% in H₂O) followed by an air-dry.

We then made an imaging chamber by adhering the coverslips to a glass microscope slide (Fisher scientific, 1 mm) using narrow (about 5 mm) strips of double-sided Scotch tape as a spacer (see Figure 18). To enable us to inject the imaging buffer, we drilled two holes about 1.5 cm apart in advance into the glass slide.

![Figure 18] SMLM Imaging Chamber
Consists of a glass microscope slide with two drilled holes, roughly 1.5 cm apart, handmade spacer made of double-sided tape and a coverslip attached on top.

To attach the Alexa-647-oligos and Atto-532-oligos to the pre-cleaned PLL coated coverslips, we loaded them into the chambers and let them incubate for about one hour followed by washing with distilled water to remove non-attached oligos. Afterward, we added the dSTORM imaging-buffer to each of these chambers and conducted dSTORM. To image Alexa-647 we applied a 637 nm laser with an intensity of 1.5 KW/cm², and to image Atto-532 we employed a 532 nm laser with an intensity of 4 KW/cm². For both cases, we acquired 20,000 images with a frame rate of 20 Hz. After conducting dSTORM, to obtain localization tables, we employed rapidSTORM using a threshold of 30,000 counts (or equivalently about 1500 photons), a PSF FWHM of 300 nm and a minimum distance threshold of 10 pixels (i.e., about 1.2 μm between two neighboring localizations). We analyzed both fluorophore’s localization tables using MATLAB to measure the
average number of collected photons per blinking event, the average number of frames these fluorophores remain fluorescent (On-frames) and their duty cycles.

To measure the average number of photons per blink and count the number of consecutive On-frames, we again merged multiple localizations from single blinks considering a radius of 300 nm around each localization. We found in our experiment that both Alexa-647 and Atto-532 remain fluorescent in about two successive frames (i.e., 100 ms). In addition, we collected on average 4500 photons from Atto-532 and 3600 photons from Alexa-647 per blinking event (see Figure 19). These photon numbers combined with the PSF standard deviation value \( \sigma = \frac{FWHM}{2\sqrt{2\ln(2)}} = \frac{300}{2\sqrt{2\ln(2)}} \approx 127 \text{ nm} \) in theory \( \delta = \frac{\sigma}{\sqrt{N}} \) provides us a very high localization precision, about 2 nm. However, this value substantially increases due to pixellation effects and background noise as shown previously in theory (Equation 2).

![Figure 19](image)

**Figure 19** Photo-physics Analysis of Alexa-647 (red), Atto-532 (green) Imaged Through dSTORM at a Frame Rate of 20 Hz

A) Histogram of photon number: Average number of detected photons per blinking event is 4500 for Atto-532 and 3600 for Alexa-647. B) histogram of successive On-frames: Atto-532 and Alexa-647 respectively stay for on average 2.2 and 1.9 consecutive frames.

To evaluate the duty cycle empirically, we considered a “sliding window” (T) with a size of 700 seconds. Within the window, we computed the “total” amount of time the fluorophores fluoresce \( T_{on} = \sum_i t_{on}^i \) where \( t_{on}^i \) represents the amount of time the \( i^{th} \) fluorophore fluoresces.)
By merging the localizations into blinks, we counted the number of florescent fluorophores within the window. Hence, we calculated the empirical duty cycle using Equation 9.

**Equation 9**

\[ D.C = \langle \frac{T_{on}}{T} \rangle, \]

where \( T \) is the size of the sliding window and the bracket denotes averaging over fluorescent molecules. As a result, we obtained the corresponding duty cycles of both Alexa-647 and Atto-532, which are quite similar and satisfactorily low, on the order of \( 10^{-4}-10^{-5} \) (Figure 20).

In summary, in the experimental conditions we ran dSTORM, Alexa-647 and Atto-532 respectively emit an average of 3600 and 4500 photons per blinking event and both have a duty cycles on the order of \( 10^{-4}-10^{-5} \). In addition, they remain florescent for on average 100 ms before transferring to the dark state. We employed these photo-physics parameters explicitly in the simulation we explain in the following section.

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**Figure 20** Duty Cycle Measurement

A) Schematic of Intensity traces of three fluorophores. Gray rectangular represents the sliding window in which the total On-time of all the detected fluorophores is recorded and divided by the width of the window to calculate duty cycle. B) Duty cycle of Alexa-647 (orange) and Atto-532 (green)
3.4 Concluding Remarks

As a conclusion, we corrected for chromatic aberration. We analyzed the error inherent in co-localization measurements and found the total co-localization precision in our experimental conditions is 3.6 nm, which is satisfactory enough to perform a successful image registration. Then, we studied the photo-physics of photo-switching fluorophores Alexa-647 and Atto-532 in vitro with dSTORM. Under our working imaging conditions, we observed that Alexa-647 emit about 3600 photons and Atto-532 emits about 4500 photons in each blinking event. Moreover, they have a duty cycle of about $10^{-5}$ and stay fluorescent for about 100 ms before transition to the dark state. Collectively, these results verify that these fluorophores are suitable candidates for single-color (used individually) or two-color (used as a pair) dSTORM.

In the next chapter, we employed the characteristics of these two photo-switching fluorophores explicitly into a simulation we developed in order to generate in-silico SMLM data.
Chapter 4: Single-Molecule Localization Microscopy (SMLM) In-Silico for Molecular Counting*

*This chapter is based upon our article “Molecular Counting with Localization Microscopy: A Bayesian Estimate Based on Fluorophore Statistics”. Contributions: Prof. Milstein and Daniel Nino established the mathematical theory for SMLM molecular counting and Nafiseh Rafiei developed the simulation utilized as the test platform for this theory.

4.1 Summary

In this chapter, we simulated dSTORM data of an array of molecules each labeled with a binomially distributed number (zero to four) of photo-switching organic fluorophores such as Alexa-647. We utilized this simulation to test the theory developed in our lab for counting the number of molecules (tagged with fluorophores) from the number of blinking events detected in SMLM experiments. Here, first we present how we simulated dSTORM data for photo-switching fluorophores like Alexa-647 to generate an image stack. Then, we explain the extension of this simulation to an “array” of single, resolvable fluorophores from which we could extract parameters like the characteristic number of blinks λ. Finally, we discuss the full, in-silico molecular counting experiment and we compare numerical to the Bayesian molecular counting theory we developed.

4.2 Theory of Counting Molecules in SMLM

Beyond imaging structure, SMLM has a high potential to be employed as an accurate technique to count the abundance of molecules (i.e., proteins and nucleic acids) in biological organisms. SMLM-based molecular counting can provide additional information about cellular structure and shed a light on the stoichiometry of interacting proteins. In addition, this technique can be employed to study the stochastic variation of molecules across a population of cells, which is unavailable to bulk measurements. Single cell variability in mRNA and protein copy number levels is thought to be a key element for many biological processes such as cell division or evolutionary adaptation.

Most classical microscopy-based molecular counting techniques are based on two methods: 1) observing stepwise photobleaching of fluorophores, and 2) measurement of the overall
amplitude of signals (fluorescence intensity calibration to a standard)\textsuperscript{56–58}. Despite the valuable insight these two techniques have provided in a wide range of cellular processes, both of these methods have their own limitations\textsuperscript{13}. Stepwise photobleaching can only detect small numbers of molecules (less than 10). In contrast, although intensity-based technique can identify more numbers of molecules, this technique is hindered by the stochastic variation of photons emitted by fluorophores\textsuperscript{13}. However, both techniques are similarly limited in the case of diffraction-limited fine structures because of an overlapping PSF from adjacent features\textsuperscript{13}.

As explained in chapter 2, SMLM relies on localization of spatial coordinates of a single fluorophore attached to a target molecule with a precision which scales like \( \frac{1}{\sqrt{N}} \), where \( N \) is the number of detected photons collected from the fluorophore\textsuperscript{1}. Therefore, SMLM typically requires the employment of photo-switching fluorophores that can be made to blink in such a way that only a random, sparse subset of fluorophores is visible in each frame\textsuperscript{59,60}. In SMLM, typically tens of thousands of images are recorded, spatial coordinates of the fluorophores are determined, and the resulting localizations are rendered into a final super-resolved image\textsuperscript{13}.

In our lab, we developed a Bayesian based, mathematical theory that can provide a maximum-likelihood estimate of the number of molecules of interest (labeled with photo-switching fluorophores) from the total number of blinking events detected in an SMLM experiment\textsuperscript{13}. In this theory, there are two key assumptions: 1) The distribution of the number of blinks from a single fluorophore is given by a geometric distribution\textsuperscript{13,61} (characterized by the parameter \( \lambda \)), 2) The probability of binding a fluorophore to a binding site on a target molecule is binomially distributed\textsuperscript{13}.

Applying these assumptions, the estimate of the most likely number of target molecules (\( \bar{M}_M \)) and the corresponding variance of this estimation (\( \sigma^2_M \)) are presented in Equation 10 and Equation 11, respectively. In this estimate, \( B \) is the total number of detected blinks and \( \lambda \) is the characteristic (not the same as average) number of blinking events of a specific fluorophore within the measurement time (\( T \)). Moreover, \( h \) is the possible number of binding (hybridization) sites on a target molecule where fluorophores can bind and \( \theta \) is the fractional occupancy which is \( 0<\theta<1 \)\textsuperscript{13}. Note, \( h \) and \( \theta \) are the pre-known parameters.
\textbf{Equation 10)} \quad \tilde{\mu}_M = \frac{B(e^\Lambda - 1)}{\theta h},

\textbf{Equation 11)} \quad \tilde{\sigma}^2_M = \frac{\tilde{\mu}_M^2 (e^\Lambda + 1 - \theta)}{B \frac{1}{e^\Lambda - 1}}.

Since I was not involved in the theoretical work that went into the above equations, I am not going to discuss their derivation here. Rather, I will focus on the simulations that were used to test the theory.

There are a number of practical considerations that might make it difficult to validate this theory experimentally. As an example, one of the complications in SMLM counting is that if the region of interest (ROI) is not illuminated homogenously, or if the local chemical environment changes temporally or across the sample, then the assumption about a geometric distribution of blinks characterized by a single parameter \( \lambda \) might not hold any more\textsuperscript{13}. To avoid addressing all the complications of experimental SMLM counting, we developed a simulated SMLM stack of images (with MATLAB) in which we could impose spatial and temporal uniformity to the statistics of the blinking events. In addition, another advantage of performing SMLM in-silico was that we knew exactly the number of target molecules we attempted to count, therefore, we could quantify the accuracy of our theoretical predictions\textsuperscript{13}.

Our simulated images, just like actual SMLM data, were processed to produce a localization table that is then analyzed to extract the number of molecules\textsuperscript{13}. To make the in-silico experiments more realistic, we employed the relevant photo-physics of Alexa-645 that we measured in the previous chapter. These parameters include the average number of detected photons per blinking event, the consecutive number of On-frames for the fluorophore and the duty cycle.

\section{4.3 SMLM Simulation Procedure}

\subsection{4.3.1 Single Fluorophore}

First, we simulated one photo-switching fluorophore located at the middle of 20 by 20 pixels field of view (FOV) with a pixel size of 117 nm and at a frame rate of 20 Hz both consistent with our experimental setup. This fluorophore switches from the On-state to the Off-state with a
probability of $P_{\text{Off}}=K_{\text{Off}}dt$, and from the Off-state to the On-state with a probability of $P_{\text{On}}=K_{\text{On}}dt$, in the time interval $dt$ that we assigned as 1ms for our simulations. $K_{\text{Off}}=1/T_{\text{On}}$ and $K_{\text{On}}=1/T_{\text{Off}}$ are the rates of the photo-switching events where $T_{\text{On}}$, $T_{\text{Off}}$ are the times the fluorophores are respectively in the On- and Off-states. In addition, we added the irreversible photobleaching state in an ad hoc manner without putting a rate for this process. We just sampled from a geometric distribution for each fluorophore to determine the max number of times each fluorophore could blink and used that as a cutoff for the number of blinks the fluorophore could undergo. If it reached the max number of blinks, then fluorophore would "photobleach" (see **Figure 21**). However, $K_{\text{Off}}$ can be easily obtained by implementing $T_{\text{On}}, 100$ ms, which we previously got in studying photo-physics of Alexa-647 (or, Atto-532). In addition, by plugging $T_{\text{On}}$ and duty cycle (D.C.), $10^{-3}$, into equation $T_{\text{off}}=T_{\text{on}}/\text{D.C.}$ we attained $T_{\text{off}}$ and correspondingly $K_{\text{On}}^{13}$.

![Figure 21](image)

**Figure 21)** ON-, OFF- and Photobleaching (PB) States

*Upon excitation by light, fluorophore transits from the OFF-state to ON-state with the rate of $K_{\text{on}}$ and transits from the ON-state to OFF-state with the rate of $K_{\text{off}}$. However, after reaching maximum number of blinking events, from the ON-state it goes to the irreversible photobleaching (PB) state.*

To generate a stack of (pixelated) images of a blinking fluorophore, first we divided the whole acquisition imaging time (15,000 frames x 50 ms) into time intervals $dt$ with the size of 1 ms. Then, we determined if the fluorophore should have been On or Off at each $dt$. Afterwards, we binned the time intervals into single frames each consisting of 50 bins.
At each time interval, depending on the corresponding (On-/Off-) state, we produced a uniformly random number like R1 (or R2). If R1<P\text{Off} then the fluorophore switches (stays) Off and likewise, if R2<P\text{On} then the fluorophore switches (stays) On. If the fluorophore is On at dt, then we assigned a Gaussian function with a full-width at half maximum (FWHM) of 300nm to the PSF of the fluorophore (or, equivalently $\sigma\text{PSF}=127$ nm\textsuperscript{13}). However, to allocate the amplitude (A) to this function we considered the fraction of time the fluorophore stays in the On-state to $T\text{On}$, multiplied by the average number of photons the fluorophore emits per binding event ($P$). That is $A=Pdt/T\text{On}$ photons where we applied 3,500 photons for $P$ according to our previous observation about Alexa-647 (or, Atto-532) photo-physics. Therefore, $A=30$ photons for the Gaussian function assigned to an On fluorophore in the interval dt.

However, in reality after few Poissonian transitions between On- and Off- states, fluorophores undergo photobleaching process that is a light-induced irreversible structural damage of fluorophore due to interaction with another molecule in the excited states\textsuperscript{62}. Therefore, to be more realistic and truncate the blinking process after sufficient time by photobleaching, we implemented photobleaching dynamics in our simulations by making the fluorophore blink according to a geometric distribution. We utilized “geornd” statistics toolbox to determine the maximum number of blinks the fluorophore can undergo (geornd(1-exp(-1/$\lambda$))) and set $\lambda=2$ as previously measured by other lab members\textsuperscript{63}.

After assigning a Gaussian function to an On fluorophore (not photobleached yet) at dt, we grouped the temporal bins into single frames and summed up the pixelated Gaussian functions within each bin. Then, to simulate shot noise from the fluorescence, we added the Poisson distributed shot noise Poisson to all the pixels by employing “the poissrnd” statistics toolbox. Likewise, to simulate background, we added a Gaussian white noise with a mean value of 48 and a standard deviation of 10 photons per pixel to each frame using “imnoise” image-processing toolbox\textsuperscript{13}. As note, we obtained these parameters for the white noise previously from our experimental SMLM data. Finally, we appended all the single frames to generate the stack of images. We provide a schematic of our simulated single fluorophore photo-switching in Figure 22.
Figure 22) Workflow of How to Simulate Single Photo-Switching Fluorophore SMLM Data

A) at each time interval, an image matrix is generated if the fluorophore is at the On-state and not photobleached yet. B) These matrices are binned together into a single image frame. C) A Gaussian white noise is added to each single frame. D) All the single frames are appended to each other in order to generate the SMLM stack of images.
4.3.2 Extract λ from an Array of Single Fluorophores

As mentioned earlier, the characteristic number of blinks (λ) is one of the key input parameters we need to extract the number of target molecules labeled with a fluorophore. Therefore, it is essential to measure λ in an SMLM experiment conducted under similar experimental conditions to the true SMLM counting experiment. These two experiments should be similar in the acquisition time, frame rate, imaging buffer, excitation and activation laser intensities. Consequently, as one should measure λ in an actual SMLM experiment, we also performed a SMLM simulation from that we could obtain λ. This simulation is an extension of our simulation for a single fluorophore, explained earlier, to an array of individual fluorophores located sparsely enough from each other such that their PSFs do not overlap even if they both fluoresce simultaneously.

To simulate a grid of fluorophores, we placed 1156 photo-switching fluorophores 7 pixels apart (≈ 820 nm, each pixel=117 nm) within the camera field of view and made them blink with the same photo-physics parameters we used to simulate single fluorophore in the previous section. We generated a stack of SMLM images consisting of 15,000 frames at a frame rate of 20 Hz\textsuperscript{13}. Afterwards, we analyzed the stack of images with the open-source software rapidSTORM using fixed 300 nm FWHM Gaussian function to fit to the PSF and obtained the localization table\textsuperscript{64}. Once we got the table, we merged the temporal consecutive localizations that are located within an area with a radius of 100 nm into one blink by employing the software we developed and explained in chapter 2. Subsequently, we built a histogram of blinking events by one fluorophore, displayed in the inset to Figure 23, and fitted an exponential function to the histogram to measure λ\textsuperscript{13}.

As an important note, as we described before, total number of localizations and correspondingly the total number of blinks is very sensitive to the photon number (counts) threshold value which in rapidSTORM indicates the integrated number of photons (counts) within the Gaussian fit\textsuperscript{13}. Hence, at too low values of threshold, it is very difficult to differentiate the actual localization of a fluorophore from the background noise (which in our simulation is 48 photons/pixel). Indeed, setting the threshold value too low can generate many false, random localizations in the
Therefore, to avoid counting these false localizations into our measurement, we evaluated $\lambda$ at higher thresholds followed by an extrapolation to the zero value threshold, Figure 23\textsuperscript{13}.

In practice, we first generate a plot of $\lambda$ versus threshold. We create a series of histograms of blinking number for a single fluorophore from the localization tables constructed upon different threshold values ranging from, approximately, 1000 to 35,000 counts ($\approx$ 50 to 1525 photons) with an increment of 2000 counts ($\approx$ 100 photons). Then, we fit an exponential distribution to each of these histograms to obtain $\lambda$. As displayed in Figure 23, $\lambda$ linearly decreases in the range of 15,000 to 30,000 counts (equivalently about 750 to 1500 photons). Fitting the data within this range and extrapolating to zero value provides an excellent agreement with what we explicitly implemented as an input in our simulation (i.e., 1.98 in comparison to 2)\textsuperscript{13}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure23.png}
\caption{Blinking Number Characteristic ($\lambda$) versus Threshold.}
\end{figure}

(Dashed line) Linear fit, from 15,000 to 30,000 counts, to the data points. (Solid blue line) Simulation input value, $\lambda$=2, which completely agrees with the extrapolation of the measurements at zero threshold. (Inset) Typical histogram of blinking events of a single fluorophore (for this example, threshold=21,000 counts). We extract $\lambda$ from the geometric fit (Solid red line). This figure is taken from ref [14] (D. Nino et al., Biophys. J, 2017).
4.3.3 Array of Molecules Labeled with Fluorophores

After performing a calibration for $\lambda$, we established our full simulation to conduct in-silico molecular counting. We built a 2D lattice of 1225 molecules (35 by 35 molecules) eight pixels (936 nm) apart from each other (Figure 24, A), sparse enough such that they are differentiable from each other. Each molecule has a maximum of four binding sites ($h=4$) to which a fluorophore can attach. However, the number of bound fluorophores per molecule varies according to a binomial distribution with a fractional occupancy of 75% ($\theta=0.75$) (Figure 24, B) and the fluorophores hybridized to each individual molecule scatter within two pixels that is an area with the size of about the diffraction limit.$^{13}$

After building the grid of molecules tagged with fluorophores, we made the fluorophores blink with the same parameters utilized in the calibration experiment in-silico i.e., $\lambda=2$, D.C.$=10^{-3}$, $T_{on}=100$ ms and average number of 3500 photons per blink. As before, fluorophores blinked according to a geometric distribution and the simulation lasted for 15,000 frames, a Gaussian white noise (mean value= 48, standard deviation= 10 photons) is added to each frame, at the frame rate of 20 Hz.$^{13}$

![Figure 24) In-Silico Molecular Counting Platform](image)

**Figure 24) In-Silico Molecular Counting Platform**

_A) Schematic of 1225 molecules distribution within the field of view. Molecules are eight pixels apart from each other. Each molecule has four binding sites to where a fluorophore (blue circle) can bind according to a binomial distribution with an occupancy fraction number of 0.75. B) Probability distribution of number of bound fluorophores to each molecule._
4.4 In-silico Molecular Counting Results

Similar to the calibration experiment, to yield the localization table, we analyzed the simulated dSTORM image stack with rapidSTORM using fixed-width PSF FWHM of 300 nm for a range of threshold values (i.e., 1000 to 35,000 counts with a step size of 2000 counts). To extract the total number of blinking events (B) and subsequently the most-likely number of molecules ($\bar{\mu}_M$) from each localization table (which is upon a different threshold), we merged the consecutive localizations located in a circular area of 100 nm centered at the first localization. We plotted blinks versus threshold and linearly extrapolated the data points within the range of 15,000 to 29,000 counts to zero threshold (Figure 25, A). We used the extrapolated B (5197) and $\lambda$ (1.98) in Equation 10 and Equation 11 to obtain, respectively, the expected number of molecules ($\bar{\mu}_M$) and the variance of our estimation ($\bar{\sigma}_M^2$). Correspondingly, we obtained $\bar{\mu}_M = 1138$ and $\bar{\sigma}_M = 27$ for our estimation, which is somewhat below the target of 1225 molecules.

As an alternative approach, we directly plotted $\bar{\mu}_M$ obtained from Equation 10 versus threshold using the corresponding B and $\lambda$ for that threshold and again extrapolated to the zero value to obtain an expected number of molecules. This extrapolation yields $\bar{\mu}_M = 1210$, which is very close to our target of 1225 molecules (Figure 25, B).

![Figure 25](image)

**Figure 25** In-silico Molecular Counting Experiment

Left) Total number of blinks versus threshold. Green solid line represents the actual total number of blinking (5345) of all the fluorophores extracted from simulation. Green circles illustrate total number of blinks at each threshold obtained by merging temporal successive localizations within a circle of 100 nm radius. Right) Average number of molecules versus threshold. Blue solid line displays the actual number of molecules (1225) implemented explicitly into the simulation. Blue circles are the average number of molecules at each threshold and are yielded directly by plugging B and $\lambda$ (corresponding to that threshold) into the theory. In both figures, dotted line represents the fit of measurements within the range of 15-29k to extrapolate at zero value threshold.
4.5 Concluding Remarks

To conclude, we successfully established an SMLM in-silico stack of images and obtained the total number of blinks from these data. We employed the molecular counting theory, and found that the estimated number of molecules was in an excellent agreement with the actual number of target molecules in the simulation.

Up to this chapter, we developed the experimental setup for 2D or 3D SMLM (dSTORM and PALM) for several fluorophores: Alexa-647, Atto-532 and mEos3.2. Moreover, we developed image-processing software to process all the SMLM data to reconstruct an accurate super-resolved image. In addition, we studied the photo-physics of photo-switching fluorophores Alexa-647 and Atto-532 to assure they emit enough photons per blinking event as well as to assess their duty cycle. Furthermore, we utilized the corresponding photo-physics parameters in the simulation we developed above. Now, having developed and validated the experimental and analytical setup, in the following chapters we apply these SMLM techniques to biological systems.
Chapter 5: Revealing Intracellular Spatial Distribution of RNA Polymerase II in Mammalian Cells by dSTORM*

* Contributions: Dr. Kaur at Prof. Mitchell’s Lab (University of Toronto) maintained mouse stem cells and cortex cells. Nafiseh Rafiei labeled RNAPII with Alexa-634 in cortex cells and Dr. Kaur conducted this labelling for stem cells. Dr. Mazouchi and Nafiseh Rafiei together performed 2D imaging and processed data. Nafiseh Rafiei conducted 3D dSTORM and analyzed the corresponding data.

5.1 Summary

One of the biological systems we were interested in was the spatial distribution of active RNA polymerase II (RNAPII) proteins within the nucleus of mammalian cells. Through an indirect immunofluorescence labeling, we tagged RNAPII molecules with Alexa-647, the photo-switchable fluorophore that we had studied the relevant photo-physics of in detail. Using dSTORM, we imaged the organization of RNAPII molecules within the nucleus of two types of mammalian mouse cells: cortex cells and embryonic stem cells. When multiple RNAPII proteins cluster within discrete foci, these clusters are referred to as “transcription factories”. Up to date, due to the transcription factories small size, which is well beyond the diffraction limit, the abundances and the size of these sites within the differentiated cells has been a matter of debate. Moreover, to our knowledge, transcription factories have not been studied at all in stem cells. Therefore, one can employ our dSTORM data to correlate the number and size of transcription factories and correlate between differentiated cells and stem cells.

We were able to image RNAPII molecules located within 1 μm thick sections of the nucleus of a mouse cortex cell by employing three-dimensional (3D) dSTORM. One can utilize this 3D dSTORM data to evaluate 3D clustering algorithms such as 3D FOCAL that is under development in our lab.

5.2 Transcription Factories in Stem Cells and Differentiated Cells

Gene transcription plays a significant role in the process of cell differentiation. In the nucleus of eukaryotic cells, it is thought that transcription occurs within discrete sites within the cell termed “transcription factories”. More precisely, transcription factories are locations where active
(phosphorylated) RNA polymerase II (RNAPII) cluster to transcribe genes\textsuperscript{5,66}. Up to date, almost all previous studies about transcription factories have been conducted in differentiated cells but not in stem cells\textsuperscript{66,68}. Stem cells are cells with the ability to renew themselves and differentiate into all types of cells that have specialized function\textsuperscript{69}. This characteristic of “pluripotency” is the fundamental feature of stem cells\textsuperscript{69,70}, which hold great promise for regenerative medicine\textsuperscript{71}.

One of the great challenges of modern biomedical engineering is to understand how RNAPII transcription determines the fate of stem cells, so that this fate can be altered and/or engineered in a controlled manner\textsuperscript{69}. Therefore, there is a need to characterize transcription factories both within stem cells and within differentiated cells. One of the difficulties to studying transcription factories is the small size of these sites. By electron microscopy they been estimated to be 45-100nm in diameter, beyond the resolution of classical light microscopy\textsuperscript{68}. Moreover, there is a large uncertainty in the reported number of transcription factories, from a few hundred to many thousands\textsuperscript{68}.

To better understand how the spatial organization of RNAPII molecules can influence the destiny of a stem cell, we employed dSTORM\textsuperscript{9}. With the enhanced resolution of this technique, we would be able to resolve a length scale that makes it appropriate for mapping the RNAPII molecules’ distribution in both mouse embryonic and differentiated cortex cells. In summary, for the scope of this thesis, we maintained differentiated cells (stem cells were prepared in Prof. Jennifer Mitchell’s lab at the University of Toronto), labeled RNAPII molecules through immunofluorescence labeling with Alexa-647 fluorophores, conducted dSTORM, employed image-processing tools we developed to process data, and reconstructed a super-resolved image. Using the calibration experiment that we performed previously and integrating a cylindrical lens into our setup, we performed 3D dSTORM to map RNAPII proteins in the mouse cortex cell and reconstructed the image with ThunderSTORM\textsuperscript{72}. To gain insight into how RNA transcription varies between stem cell and differentiated cells, one should quantify transcription factories abundances as well as characterize their corresponding sizes within both cellular types.

In addition, this study can be extended to investigate the interaction of RNAPII molecules and either of four crucial transcription factors such as Oct3/4, Sox2, Klf4 or Nanog using two-color
dSTORM. These core transcription factors mainly govern self-renewal and pluripotency in stem cells\textsuperscript{73}. In other words, small changes in the level of these factors will trigger stem cells to differentiate\textsuperscript{74}. Although the gene regulatory network of these central transcription factors is well studied, the interaction of these factors with active RNAPII proteins is not properly addressed. By colocalization of these factors with RNAPII molecules, characterizing the spatial organization of these transcription factors within the transcription factories as well as quantifying the abundances of these molecules in transcription factories in both stem cells and differentiated cells, one can shed a light on the underlying transcriptional mechanisms that control pluripotency.

5.3 Methods and Materials

5.3.1 Protein Labeling Using Immunofluorescence Technique

Immunofluorescence labeling is a common biochemical technique to label antibodies with fluorophores\textsuperscript{75}. An antibody is a Y shaped protein produced by a B cell against an antigen as an immune response. This Y shaped protein consists of two domains: the arms, which are variable and called the antigen binding site and the tail which is conserved and can be detected by other antibodies\textsuperscript{76}. One can conduct this technique by two main methods: direct immunofluorescence labeling and indirect immunofluorescence labeling\textsuperscript{75}. In the first approach, the antibody, which is chemically conjugated to a fluorophore, binds to the molecule of interest whereas in the latter, the unlabeled primary antibody binds to the molecule of interest and the secondary antibody, which is tagged by a fluorophore, will bind to the conserved domain of the primary antibody\textsuperscript{75}. We illustrate both methods in Figure 26.
Both direct and indirect immunofluorescence labeling techniques have their own advantages and disadvantages. For instance, direct immunofluorescence labeling is very expensive because of the cost associated with labeling the antibody but this approach can provide a very specific binding\textsuperscript{75}. On the other hand, indirect labeling is economic but can cause non-specific binding to sites other than molecule of interest\textsuperscript{75}. One of the main advantages of indirect labeling over direct labeling is the high level of signal we can obtain since more than one secondary antibody, which is tagged with fluorophores, can bind to the primary antibody\textsuperscript{75}.

For this study, we employed indirect immunofluorescence labeling to tag RNAP\textsubscript{II} with Alexa-647. As a note, RNAP\textsubscript{II} consists of twelve subunits\textsuperscript{77}. However, RPB\textsubscript{1} is the largest catalyst subunit of this protein and contains a carboxyl terminal domain (CTD) which consists of fifty-two tandem repeats of the serine-rich heptad motif YSPTSPS\textsuperscript{68}. During transcription, serine 2 and 5 become phosphorylated\textsuperscript{78}. Taking advantage of antibodies that can bind to phosphorylated serine 5 in CTD\textsuperscript{68}, as the primary antibody, we used the anti-RNA polymerase II CTD repeat YSPTSP (phospho S5) antibody (abcam, ab5131) and for the secondary antibody, we applied goat anti-rabbit (abcam, ab150079) labeled with Alexa-647.

5.3.2 Cell Fixation and Sample Preparation

We performed this study in fixed cells that we received from Prof. Jennifer Mitchell at the University of Toronto. Differentiated mammalian cells grew in high glucose phenol-red-free
DMEM (ThermoFisher) supplemented with L-glutamine (Gibco, 1%), sodium pyruvate (Gibco, 1%) and 10% Fetal Bovine Serum (FBS, ThermoFisher). However, for embryonic stem cells, two leukemia inhibitory factors (lif), PD0325901 (1 μM) and CHIR99021 (3 μM) should be added to the above media to prevent the cells from differentiating. Cells grew in the T25 flasks (Falcon), at 37°C and 5% CO₂. To passage the cells, 2 ml 0.25% trypsin EDTA (Gibco) was incubated with the cells for 2-3 minutes at 37°C followed by the addition of 2 ml of media, spinning at 1200 rpm for 4 minutes and resuspension in 6ml media. One ml of the resuspension should be added to 9 ml of media and incubated on the flask.

To be able to prepare the sample for microscopy, at the last stage, cells should grow and adhere upon a coverslip. Therefore, a sterile coverslip (Fisher scientific, 0.17 to 0.25 mm) was placed within each well of a 6-well tissue culture plate (Falcon) coated with 0.1% gelatin followed by aspiration of the extra gelatin, seeding the cells onto the coverslips and incubation at 37°C and 5% CO₂ overnight. To fix the cells, first each well is aspirated and washed with 1X PBS, then cells were incubated with Neutral Buffered 10% formalin for 20 minutes at room temperature followed by another wash with 1X PBS. To make the cells permeable and at the same time block the cells to prevent non-specific binding, cells were incubated for 30-45 minutes at room temperature with 1 ml blocking buffer which contained 10% FBS and 0.1% TritonX-100 (Sigma-Aldrich) in 1X PBS. After making the cells permeable, primary antibody (1000X diluted) in antibody buffer (0.2% FBS and 0.1% TritonX-100 in 1X PBS) is added to each well and incubated at 4°C overnight. The following day, before adding the secondary antibody, cells should be washed three times each for 5 minutes with 2 ml washing buffer that contained 0.1% Tween (Sigma-Aldrich) in 1X PBS to remove non-bound primary antibodies. After this washing step, the secondary antibody (2000X diluted) in the antibody buffer can be added to each well and should be incubated for 20 minutes at room temperature. To remove the unbound secondary antibodies, as the final step, samples should be washed four times with the aforementioned washing buffer in total for twenty minutes.
5.3.3 Two-Dimensional (2D) and Three-Dimensional (3D) dSTORM setup

After labeling RNAP II with photo-switching Alexa-647, we adhered the coverslip, on which cells were seeded, with nail polish to the concavity microscope slide (ThermoFisher) in which we drilled two holes in advance (see Figure 27). We then injected dSTORM imaging buffer into the chamber. The imaging buffer we used for this experiment consisted of Phosphate Buffered Saline (PBS), pH 7.6, 10% glycerol, 10% glucose, 28 mM MEA (mercaptoethylamine) and an oxygen scavenging system which was 1 mg/ml glucose oxidase and 40 µg/ml catalase (GLOXY)\textsuperscript{15}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure27.png}
\caption{Imaging Chamber used To Study Mammalian Cells Adhered to the Coverslip}
\end{figure}

There are two drilled holes within the glass microscope slide with concavity and the coverslip, that mammalian cells were seeded on top, is adhered to the slide with nail polish.

After injecting the imaging buffer, we performed dSTORM upon an inverted Olympus IX81 microscope controlled by the open-source software \textit{μManager}\textsuperscript{43,44} and equipped with a TIRF 60X oil immersion objective (Olympus, APON 60XOTIRF, N.A. 1.49). We employed 637 nm (World Star Tech, NRC B-2) and 405 nm (Coherent, CUBE) laser light to excite and activate Alexa-647 fluorophores, respectively. The power of these wavelengths was tuned with an acousto-optical tunable filter (AOTF) such that the intensity of 637 nm was \(\approx1.5\) KW/cm\(^2\) and the intensity of 405 nm was \(\approx1\) W/cm\(^2\) in our region of interest. We recorded about 20,000 to 30,000 image frames at a frame rate of 20 Hz with an electron multiplying charge-coupled device (EMCCD, Andor iXon3) camera. Owing to the thickness of the mammalian cells we studied (\(\approx10\) µm), we performed our imaging with the highly inclined and laminated optical sheet (HILO) microscopy\textsuperscript{11}.
As a note, we conducted both two-dimensional (2D) and three-dimensional (3D) dSTORM with the same adjustments. However, to perform three-dimensional dSTORM as mentioned in the method chapter we inserted a 0.5 m cylindrical lens within the final imaging telescope to generate an astigmatism and make the PSFs of the fluorophores elliptical. From the ellipticity of PSFs, we could determine the axial position of the fluorophores. Therefore, before 3D imaging, we performed a calibration experiment in which we imaged a single fluorophore at various axial planes. In this experiment, first, we found the sharpest focus of the fluorophore defined as zero μm, then by employing a piezo stage, we changed the axial position within the range of -1 μm to 1 μm with the increment of 20 nm and at each axial plane we imaged the fluorophore. Using this calibration data, we could determine the axial position of the fluorophores.

5.4 dSTORM Images Results

5.4.1 Two-Dimensional (2D) Imaging Results

After data acquisition for 2D dSTORM, we applied a temporal median filter available as a plugin in ImageJ (and developed within our lab) to remove the background that is generated by autofluorescence within the cells. After removing background, we analyzed the stack of images with the open-source software rapidSTORM. We obtained the localization table by fitting a 2D Gaussian function with a fixed-width 300 nm PSF and 10,000 counts (≈500 photons) as the threshold of this fit to the fluorescent fluorophores. After retrieving the localization table, we corrected the drift, which is introduced into the images during our acquisition, by employing a cross-correlation analysis, which we explained earlier. After drift-correction, we reconstructed the final super-resolved image by assigning a 2D Gaussian function with a standard deviation of 20 nm to each localization.

Here we present the dSTORM images of RNAPII molecules within the nucleus of mouse cortex cells and embryonic stem cells (Figure 28, B, and D). Furthermore, we display the classical fluorescence microscopy images of the same regions of interest (Figure 28, A, and C).
5.4.2 Three-Dimensional (3D) Imaging Results

As mentioned above, for 3D imaging, we should also provide a calibration curve, which displays the PSF ellipticity at multiple z (axial)-planes (see Materials and Methods). Having this calibration
measurement and raw dSTORM data, since rapidSTORM does not provide 3D data rendering, we used an open-source single-molecule localization software package called ThunderSTORM, which is an interactive plug-in for ImageJ, to both obtain the table of localizations (by fitting an elliptical 2D Gaussian function to the PSF) and to visualize the super-resolved image\textsuperscript{79}. Note that before obtaining the localization table, first we removed the background from the raw images (consists of 30,000 frames) by a temporal median filter. Then, we obtained the spatial coordinates for the fluorophores located within $-500 \text{ nm} < z < 500 \text{ nm}$, corrected the drift using cross-correlation analysis and merged the temporal successive localizations located within 20 nm of each other. We performed all of these post-processing analyses and rendered 3D images (using Gaussian rendering, 20 nm standard deviation) with ThunderSTORM. We illustrate the 3D dSTORM image of RNAPII molecules within a 1 $\mu$m-thick section of the nucleus of a mouse cortex cell in Figure 29.

![Figure 29] Three-Dimensional (3D) dSTORM Images of RNAPII (tagged with Alexa-647) Spatial Distribution within a Mouse Cortex Cell.

Images represent a 1 $\mu$m section of the nucleus. Image A is the side view of the nucleus and image B refers to 30$^\circ$ of view. Scale bars are 3 $\mu$m.
5.5 Concluding Remarks

In conclusion, we could successfully label RNAPII molecules through indirect immunofluorescence labeling with Alexa-647 fluorophores. We could map the spatial organization of active RNAPII molecules within the nucleus of mouse embryonic cells and mouse cortex cells. We processed raw data with post-processing image tools developed in chapter 2. Moreover, we conducted 3D dSTORM to image RNAPII molecules within a 1 μm thick section of nucleus. This 3D SMLM data was utilized as test cases for clustering algorithms developed in our lab.

To understand the underlying transactional RNAPII mechanisms that determine the fate of the stem cells, one should count and measure the size of the active transcription factories in the stem cell and correlate the results with the developmental stage of the cells. However, to obtain reliable statistics, one should take images of dozens of cells. More importantly, the nuclei of the mammalian cells are typically very large (10-20 μm) and one could only image a (1 μm) slice of it at a time. Considering the slow throughput of SMLM, imaging the whole nucleus of the cell would be a very slow procedure. In addition, imaging regions of interest (ROI) deep within the nucleus is very difficult because of the noise coming from the other RNAPII molecules labelled fluorophores. Therefore, according to these complications, in the next chapter, we shifted to another biological system E.coli and studied another type of protein H-NS using SMLM PALM\(^7\) and another super-resolved technique SRRF\(^8\).
Chapter 6: Spatial Redistribution of the Global Transcriptional Regulator H-NS in E. coli under Osmotic Stress*

*This chapter is based upon our article in preparation “Growth phase-dependent chromosome condensation and H-NS protein redistribution in E. coli under osmotic stress”. Contributions: Dr. Cordovan at Prof. Navarre’s Lab (University of Toronto) performed H-NS cloning to fluorescent protein mEos3.2 and Nafiseh Rafiei conducted all the data acquisition, image-analysis and sample preparations.

6.1 Summary

The heat-stable nucleoid structuring (H-NS) protein is an abundant histone-like DNA-binding protein in gram-negative bacteria such as E. coli, which preferentially binds to AT-rich domains of DNA and affects both DNA packaging and gene regulation. More precisely, H-NS globally coordinates the regulation of over 200 genes in E. coli by altering the DNA architecture.

In this study, by applying a combination of super-resolved microscopy techniques, we investigated how the intracellular, spatial organization of H-NS proteins in E. coli alters when bacterial cells are exposed to osmotic stress. We found that the resulting intracellular distribution of H-NS is very sensitive to the growth phase. We observed that for osmotically stressed cells in early stationary phase H-NS markedly alters its intracellular organization detaching from a tightly condensed chromosome and moving toward the periphery of the cell. Notably, we did not see this response by H-NS in exponential phase despite a similar condensation of the chromosomal DNA. Remarkably, we noticed that in exponential phase such a response could be induced by exposing the bacterial cells to the antibiotic coumermycin, which inhibits DNA gyrase, a protein that induces supercoiling. This observation suggests that the level of supercoiling in E. coli may help coordinate the osmotic stress response.

6.2 Introduction

Bacterial cells have evolved to sense changes that occur in their external environment and to respond correspondingly. Two of the extreme environmental stresses that enteric bacteria such
as E. coli and Salmonella regularly encounter are osmolarity and temperature changes when bacteria enter and exit from the intestine of the host. Bacteria are exposed to a temperature of 37°C and high osmotic pressure inside the large intestine of humans, and as soon as they are outside the host, they are exposed to a lower osmolarity and temperature drop (cold-shock). In addition, they undergo other environmental shifts such as pH changes, nutrient deprivation, and oxidative stresses through their lifetime. The mechanisms that enable bacteria to survive in different environmental niches are not well understood.

The heat-stable nucleoid structuring (H-NS) protein, one of the central nucleoid-associated proteins (NAPs) in gram-negative bacteria, for decades has been thought to play a key role in the adaptation of cells to environmental stresses such as pH, thermal and osmotic shifts. This small protein is approximately 15 kDa and abundant (>20,000 copies per cell). It negatively regulates its own gene and more than 200 genes (~5% of genes in E. coli) many related to adaptation to environmental changes. H-NS forms an oligomeric construct along the DNA and inhibits transcription either by preventing RNA polymerase from binding to the promoter or by trapping this molecule in a loop. The abundance of H-NS is relevantly constant through the bacteria’s growth cycle, although it is reported at early stationary phase that there is a small increase. H-NS intrinsically recognizes minor grooves of DNA that are usually associated with promoters, however, it preferentially binds to adenine and thymine rich (AT-rich) domains of DNA. This protein plays a dual role as a nucleoid organizer and global gene regulator. It impacts both DNA compaction and globally represses genes. Because of the similarity of this protein’s function in condensing DNA in prokaryotic cells to the function of histone proteins in eukaryotic cells, H-NS is also referred to as the histone-like nucleoid structuring protein.

One effective way to enable bacteria to survive and adapt to new environments is by acquiring new genetic traits from foreign DNA segments (xenogeneic) that integrate into the cells via horizontal gene transfer (HGT). However, for bacteria, it is very metabolically costly to express most horizontally acquired genes. Therefore, many enteric bacteria have evolved to reduce the costs of HGT by using H-NS proteins, which bind to and silence foreign DNA.
When bacteria face an osmolarity up-shift or down-shift, osmotic pressure differences at both sides of the semi-permeable cell-membrane lead to water transport through aquaporins (water channels).\textsuperscript{94} Downshift in osmotic pressure results in water influx into the cell producing cell swelling and even cell lysis.\textsuperscript{94} Likewise, an increase in the external osmolarity causes water efflux from the cell that results in cell dehydration and significant cytoplasmic volume shrinkage known as plasmolysis.\textsuperscript{94,95} These changes in cytoplasmic water volume perturb cellular function, for instance, plasmolysis can inhibit biological processes such as DNA replication.\textsuperscript{95} Therefore, in order to survive in these unfavorable conditions, bacteria have developed adaptation strategies.

An immediate osmoadaptation mechanism employed by bacteria, occurring within the first few seconds of shock, is to change the concentration of free ionic solutes such as K\textsuperscript{+} within the cytoplasm.\textsuperscript{96} In high-osmotic pressure, which is the focus of this study, bacteria take K\textsuperscript{+} from the growth media, and due to cytoplasmic K\textsuperscript{+} accumulation, the net charge of the cytoplasm becomes positive.\textsuperscript{83} To preserve electroneutrality, bacteria transport or synthesize de novo the solute (-1 charged) glutamate, as the K\textsuperscript{+} counter ionic solute, or export (+2 charged) putrescine.\textsuperscript{83} In contrast to glutamate that does not impair cellular function, K\textsuperscript{+} is not compatible with biological processes such as cell growth. Therefore, this mechanism can protect bacteria only for a short period, and bacteria require a secondary procedure for osmotic protection.\textsuperscript{98}

The secondary response of bacteria to osmotic stress occurs at the level of gene expression, transcription/translation, and in a time frame of several minutes to hours.\textsuperscript{99} At this phase of the response, the excess of K\textsuperscript{+} is replaced by compatible solutes referred to as osmolytes or long-term osmoprotectants.\textsuperscript{97} Osmolytes are organic molecules such as proline (amino acids), trehalose (polyol), and glycine betaine (methyl-amines) and their accumulation within the cell does not hinder cell function.\textsuperscript{97,100} In contrast to K\textsuperscript{+}, which should be only imported into the cell in response to high osmotic stress, osmolytes are biosynthesized and/or transported to the cell.\textsuperscript{97} The abundance of these molecules is mediated by multiple enzymes, channel proteins, and transporters within the cell.\textsuperscript{99}

In particular, glycine betaine, which is vital for E. coli proliferation under high external osmolarity, and proline are transported through a transport system that is encoded by the \textit{proU} operon.\textsuperscript{99,100}
This operon is inhibited by H-NS in normal growing condition but in the presence of high osmolarity this inhibitor dissociates from the promoter region, and the genes for the glycine betaine and proline uptake system are expressed\(^99\). However, it has been shown that chromosomal DNA supercoiling impacts transcription of the \textit{proU} operon, and decreasing superhelicity reduces the expression level of this operon\(^{99,101}\).

It is known that DNA structure dynamically changes in response to environmental stresses such as osmolarity and temperature shifts\(^{102,103}\). This alteration of DNA topology in response to environmental stress may signal virulence genes to express in the correct niches\(^{102}\). At lower temperature, DNA becomes more negatively supercoiled\(^{104}\) and H-NS’s affinity to DNA increases\(^{81}\). However, for osmoadaptation, there is a contradiction on reports about the role of superhelicity. There are reports on an enhancement of supercoiling in response to osmotic shock\(^{99,101}\), and in contradiction, it is reported that under osmotic stress (0.3 M NaCl) DNA curvature can be decreased or removed completely\(^{89}\). The latter observation suggests the release of H-NS from curved DNA\(^{89}\). This postulation is confirmed by other findings that display H-NS dissociates from the nucleoid in response to an osmolarity up-shift\(^{83}\), and H-NS’s affinity to purified bacteriophage \(\lambda\)-DNA also decreases upon osmotic shock\(^{89}\).

Although the underlying mechanism that causes the dissociation of H-NS from the nucleoid under high osmolarity is not understood\(^{98}\), it has important regulatory effects. Apart from osmotically inducible genes encoded by the \textit{proU} operon regulated by H-NS, many other genes are regulated directly or indirectly by H-NS and expressed in response to osmotic shock. One of the primary proteins that is expressed in \textit{E. coli} in response to osmotic stress is the \(\sigma\)-factor, \(\sigma^5\) (or, \(\sigma^{38}\)) encoded by \textit{rpoS} gene\(^{105,106}\). This sigma factor, in particular, is expressed under conditions of starvation (i.e., stationary phase) and regulates more than 30 genes when bacteria enter stationary phase\(^{105}\). However, it is reported that even in exponential phase the level of \(\sigma^5\) expression increases under osmotic shock\(^{105}\). It has been shown that H-NS triggers degradation of \(\sigma^5\) and in the strains where the \textit{hns} gene was depleted, this sigma factor is stabilized and its expression level is enhanced\(^{106}\). These observations about the induction of \(\sigma^5\) and the \textit{proU} operon, in response to high osmolarity, agree with dissociation of H-NS from the nucleoid since...
they act as a repressor of these genes. However, it is not very well studied how H-NS regulates osmoadaptation in different phases of growth or how superhelicity influences this response depending on the phase. In order to get insight into this mechanism, in this study, by employing a combination of super-resolved microscopy techniques (PALM and SRRF), we probed the spatial-temporal redistribution of H-NS proteins in E. coli under high osmolarity at both exponential phase and stationary phase.

### 6.3 Materials and Methods

#### 6.3.1 Labeling H-NS proteins with fluorescent protein mEos3.2 in E. coli

To enable us to conduct PALM, the *hns* gene was fused to the monomeric, green-to-red photo-activated fluorescent protein mEos3.2. At the beginning of this study, we utilized E. coli K12 strains that had the *hns* gene knocked from the chromosome. Instead, these bacterial strains were transfected with a low-copy number plasmid that encoded an auto-regulated *hns* gene tagged with mEoS3.2. However, we were concerned that H-NS may still be overproduced and that this may lead to artifacts. Therefore, to assure that what we observed as the intracellular reorganization of H-NS molecules is not an artificial effect introduced by the plasmid, to continue this study, we used the E. coli K12 strains in which the *hns* gene was fused to mEos3.2 in the chromosome and not on a plasmid.

#### 6.3.2 Stressing the Bacterial Cells and Fixation

We grew bacterial strains in 37°C M9GTC media within a sterile 500 ml Erlenmeyer flask shaking at 220 rpm. As a note, M9GTC media is minimal media M9 (BioShop) supplemented with 2 mM MgSO₄, 0.1 mM CaCl₂, 0.01% (w/v) thiamine, 0.1% (w/v) casamino acids, and 1% (w/v) glucose. We grew cells until they reached the growth phase of interest (i.e., exponential phase or stationary phase). To determine the phase of growth, we measured the OD₆₀₀ of culture (1 ml) as an indication of the number of cells within the media. To reach exponential phase, OD₆₀₀ should be in the range of 0.2-0.5 (takes ~ 3 hours) and to reach stationary phase, OD₆₀₀ should be about 2 (takes ~ 6 hours).
After reaching the appropriate growth phase, to apply osmotic shock, we added 6.2 M NaCl solution to the culture and diluted 20.6x to obtain 300 mM NaCl as a final concentration$^{102}$. Then, to examine how the type of salt would affect the bacterial response, we substituted KCl for NaCl. We added 4.6 M KCl solution to the culture, diluted 15.2x and obtained a final concentration of 300 mM to shock the bacterial cells with the same level of osmolarity as 300 mM NaCl (i.e., ~0.6 Osm/L). Note, we did not observe any differences in the subcellular response to osmotic stress by substituting KCl for NaCl as the source of osmotic shock. For all parts of this study other than the time-course experiments, we exposed bacteria for 30 minutes to osmotic shock. In the time-course experiments, we shocked the cells for various durations: 5, 10, 20, 30, 45 and 60 minutes. In all our experiments, to prepare non-shocked cells as a control, we simply did not add any salt solution to the culture, instead we just added the same amount of autoclaved pre-warmed 37°C H₂O to the culture and we fixed control cells and osmotic-shocked cells simultaneously.

In all our experiments, after subjecting the culture to the shock, we fixed the cells utilizing 36.5-38% formaldehyde (Sigma-Aldrich, includes 10-15% methanol) that was 10X diluted in H₂O (i.e., final formaldehyde concentration is 3.8% to 4%). Note, we did not observe any effect on our results by employing 4X diluted 16% methanol-free formaldehyde (ThermoFisher). To fix the cells, we incubated the bacteria with formaldehyde for 30 minutes at room temperature while shaking using an orbital shaker. After incubation with formaldehyde, we centrifuged the cells at 1000 g at room temperature for 15 minutes, then discarded the supernatant, added 1 ml PBS to the pellet and mixed the solution manually/with an orbital shaker for 5 minutes at room temperature. After this washing step, again we centrifuged the solution for 10 minutes at 1000 g at room temperature. We repeated 10-minute centrifugation followed by 5-minute PBS wash three times. After the last washing step, the sample is ready for microscopy or we can store it at 4°C to be utilized within one week.

### 6.3.3 Labeling Chromosome with DAPI

To enable us to image the chromosome, we post-stained fixed cells with a blue-fluorescent DNA stain, DAPI (4’, 6-diamidino-2-phenylindole)$^{39}$. We added this stain (already dissolved in PBS) to the culture up to a final concentration of 0.1 μg/ml and let it incubate at room temperature for
5 minutes. Then, as the washing step, we centrifuged the sample at 5000 g at room temperature for 1 minute, discarded the supernatant and resuspended the pellet in 500 μl PBS. We repeated the washing step in total three times to remove excess DAPI.

### 6.3.4 Mounting the Sample

To mount the cells on the coverslip, we put 3 μl of sample on top of a thin 2 mm by 2 mm, 1.5% agarose pad that we prepared in advance. We flipped over the agarose pad onto a coverslip (Fisher scientific, 0.17 to 0.25 mm) that we cleaned by sonicating for 30 minutes first in 99% Ethanol and then in 3 M KOH, rinsing with dH₂O three times after each sonication. After immobilizing the cells between the agarose pad and the coverslip, the coverslip was glued to a glass microscope slide (Fisher scientific, 1 mm) to which we already adhered a CoverWell spacer with a 2.5 mm thickness (Sigma-Aldrich).

### 6.4 Fluorescence Microscopy Methods

For this study, we conducted all of our microscopy techniques upon the same microscope, using the same detection path and EMCCD camera that we utilized for the experiments discussed before. However, in this study, in comparison to our previous measurements, the only change we made was the insertion of a telescope into the detection path to magnify the image of the bacterial cells 1.6X in addition to the magnification we to obtain (136.5X), which leads to an effective pixel size of ~73 nm.

#### 6.4.1 Super-Resolution Microscopy Methods

Since observing the organization of H-NS by conventional light microscopy within bacteria would be very difficult, we started this study by employing PALM.

After observing the pronounced intracellular reorganization of H-NS proteins in response to osmotic shock, we realized that the ultra-fine detail resolved by PALM was not necessary to visualize this response. Therefore, we changed our microscopy technique from PALM to a post-processing super-resolved technique SRRF (super-resolution radial fluctuations) that could
achieve a resolution of under 150 nm\textsuperscript{8}, sufficient to investigate the spatial, intracellular organization of H-NS proteins in E. coli under osmotic stress.

In the following subsections, we explain how we applied both of these techniques in this study.

6.4.1.1  Photo-Activated Localization Microscopy (PALM)

To conduct PALM and activate only a sparse subset of mEos3.2 fluorescent proteins, we applied a 561 nm laser (CUBE, Coherent) as the excitation laser with a very high intensity (~1 kW/cm\textsuperscript{2}) and a 405 nm laser (CUBE, Coherent) as the activation laser with a much lower intensity (~1 W/cm\textsuperscript{2}). We imaged mEos3.2 in the red channel using the relevant dichroic (Chroma, ZT561rdc) in the microscope filter cube as well as utilizing a 600/50 band-pass filter in the high-speed filter wheel. Using an EMCCD camera at a frame rate of 20 Hz, we acquired 12,000 to 25,000 frames. As in our previous analysis, after acquiring the stack of images, we used rapidSTORM\textsuperscript{45} to obtain a localization table. However, to build this table, we set the threshold at 16,000 counts (~ 800 photons) and a 300 nm FWHM width for the 2D Gaussian fit to each localization. Then, to correct the lateral drift that was introduced into the images during the acquisition because of thermal and mechanical fluctuations\textsuperscript{46}, we processed the localization table with the cross-correlation drift-correction software we developed before (in MATLAB). Finally, to visualize the super-resolved PALM image, we employed the Gaussian rendering method we established in MATLAB. For each localization, we assigned a 2D Gaussian function with a standard deviation of 20 nm (which is on the order of the localization precision), mapped the PSFs onto a 2D grid that is binned at 20 nm and summed up the overlapping PSFs enclosed by each bin to reconstruct the final image\textsuperscript{8}.

6.4.1.2  Super-Resolution Radial Fluctuations (SRRF)

To obtain super-resolved SRRF images, we simply conducted wide-field fluorescence microscopy. We imaged mEos3.2 molecules, this time in the green channel, applying a 488 nm laser (Coherent, OBIS) with a low intensity (~8 W/cm\textsuperscript{2}), using an appropriate dichroic (ZT514rdc) and emission band-pass filter 520/35 nm (Chroma) in the microscope filter cube and emission band-pass filter 540/40 (Chroma) in the high-speed filter wheel. We collected 100 frames with an EMCCD camera at a frame rate of 20 Hz followed by processing the images with the SRRF
algorithm available as an ImageJ plugin\textsuperscript{8} in an open source software ImageJ. As we explained earlier, the SRRF algorithm consists of a spatial analysis (that generates the radiality map of each single frame) followed by a temporal analysis\textsuperscript{8}. To apply SRRF, we are required to input a ring radius for the radiality computation. However, as we discussed before, to evaluate how accurate this parameter was, we varied the value of the ring radius and produced the corresponding SRRF image from the same stack of raw images. Then, on each generated SRRF image, we employed SQUIRREL analysis to evaluate the error introduced to the SRRF image and chose the ring radius value such that this error was minimized\textsuperscript{33}.

6.4.2 DAPI-stained Nucleoid Imaging

To image the DAPI-stained nucleoid of the bacterial cells, we employed an arc lamp (X-cite, series 120 Q, 120 W), using an excitation band-pass filter 325/50 and an emission band-pass filter 447/60 inside the microscope filter cube. Using an EMCCD camera, we acquired a stack of 100 frames at the frame rate of 20 Hz.

6.4.3 Image-Processing and Analysis

To evaluate the response of H-NS and the chromosomal DNA to osmotic stress, we developed analytical tools in MATLAB and ImageJ\textsuperscript{107} and analyzed more than \textasciitilde100 cells for each experiment. Using these tools, we quantified the spatial distribution of the fluorescence intensity coming from mEos3.2 fused to H-NS as well as the size of the chromosome within the bacterial cells. However, the first step in quantifying the intracellular response is to segment the bacteria enclosed by each region of interest (ROI) in each image. This way we may extract the intensity coordinates and chromosome area for each cell.

Initially, we tried to develop a tool that could automatically perform the segmentation. We applied various standard methods for cell segmentation: watershed analysis, edge finding, pattern matching, etc., as well as employing the open source cell segmentation package MorphoLibJ\textsuperscript{108}. However, the segmentation turned out to be much trickier than we expected because the bacteria are tightly compacted into clusters and they are quite small in stationary phase. Therefore, it became very difficult to determine the boundary of individual bacteria. One
method that was promising was to segment the bacteria by using the outline of the DAPI stained nucleus as a seed and to expand its outline until it overlaps with the contour of the cell. This method is similar to the ‘seeded watershed’ approach. Unfortunately, while this method could segment most of the cells, there remained cells that could not be separated (see Figure 30).

Eventually we resorted to manually selecting cells in ImageJ and saved the boundaries of the cells, as well as the coordinates and intensity of all pixels enclosed by each cell. Note, during the segmentation process, we avoided the cells that seemed to be dividing (by excluding cells of length > 3 μm).

6.4.3.1 Spatial Analysis of Protein Distribution

As we explained above, using ImageJ, we manually extracted the intensities of all pixels enclosed by each individual bacteria cell and their corresponding coordinates (see Figure 31, A). Since the bacteria are oriented in different directions, employing software we developed in MATLAB, we first rotated the cells such that all of them lie along their long axis. After rotating all of the bacteria, we normalized the size of the cells along the minor (short) and major (long) axis. We then considered two types of cross-sections in an attempt to best quantify the spatial variation of the protein distribution throughout the cellular interior: either a 1) single-pixel slice along the major or minor axis, or 2) a weighted cross-section along each axis. When considering a single-pixel cross-section, we assessed how the intensity per pixel varied along the minor and major axis of each cell, and when considering a weighted cross-section, we studied how the average of the
pixel intensities varied along both axes (see Figure 31, B). After obtaining the cross-sections for all the individual cells, we averaged data over all the cells to obtain the collective response.

6.4.3.2 Quantification of Chromosome Response

To assess chromosome response to osmotic stress, first using ImageJ, we obtained the average intensity image of a stack of 100 frames. Then, we analyzed chromosome compaction in two ways. In the first approach, we computed the absolute value of the chromosome area enclosed by each individual bacterial cell, and as a second method, we calculated the chromosome area relative to the size of the cell to which it belongs. In both methods, it is essential to segregate the cells and define the boundaries in the fluorescence images. From the H-NS (tagged with mEos3.2) fluorescence images, we manually (in ImageJ) segmented the cells and saved the outlines of the bacteria. Then, using ImageJ, we converted the fluorescence image of the chromosome (labeled with DAPI) to a binary image and overlapped the outlines of the cells to this image (see Figure 32). Using ImageJ, we then calculated the area of the chromosome enclosed by each individual cell as well as the fraction of the bacterial cell that is occupied by the chromosome. We
performed this analysis for all of the individual cells and we averaged over them. While we were determining the outlines of the cells in the fluorescence images, we attempted to avoid the dividing cells.

**Figure 32: Chromosome Area Quantification**

*Binary image of the chromosome is generated in ImageJ from the DAPI fluorescence image. White areas represent the chromosomes of the bacterial cells. Yellow ellipses display the boundaries of the bacterial cells.*

6.5 Results

6.5.1 Intracellular Reorganization of H-NS under Osmotic Stress at Different Phases of Bacterial Growth

Applying PALM and imaging the bacteria strains deficient of *hns* gene in the chromosome and transfected with a plasmid (encoded *hns* gene fused to mEos3.2), we observed the profound redistribution of H-NS in response to osmotic stress only when bacterial cells are in the stationary phase of growth (no response was observed in exponential phase). Here we witnessed H-NS molecules clearly clustering at the center and around the periphery of the cells (see **Figure 33**).
After detecting such a clear response, which could even be observed with conventional light microscopy, we realized we did not need the enhanced spatial resolution given by PALM. Still, we wanted to apply a super-resolved technique to resolve the response better for accurate further analysis. Furthermore, PALM experiments typically take 15-20 minutes for each acquisition and several hours of post-processing analysis. Therefore, we shifted our microscopy from PALM to classical light microscopy followed by SRRF for the rest of this study.

In addition, as mentioned earlier, hns was auto-regulated on the plasmid. Therefore, there was a concern that what we observed as clusters were artificial and possibly coming from overproduction of H-NS molecules. Our concern proved to be warranted. In cells where the hns gene is fused to mEos3.2 and directly inserted into the chromosome, we observed that the bright foci often detected in the centre of the cells vanished. However, the redistribution of H-NS to the periphery of the cells remained (see Figure 34).

Figure 33) PALM Imaging: Intracellular Reorganization of H-NS in E. coli under Osmotic Stress in Stationary Phase of Growth

In this strain, hns gene is knocked out from the chromosome and hns gene fused to mEos3.2 in the plasmid. Image (A) displays non-shocked cells and image (B) illustrates osmotic-shocked cells. To apply osmotic stress, 300 mM NaCl was added to the growth culture for 30 minutes. In the stationary phase (OD₆₀₀ = 2) under osmotic stress, H-NS proteins tend to cluster at the centre and periphery of the cells. Scale bar displays 500 nm.
To quantify our observation on H-NS molecules clustering around the periphery, we used ImageJ and the analytical tools we developed in MATLAB. We obtained the single-pixel and weighted cross-section intensity of H-NS molecules for 40 bacterial cells that are osmotically shocked for 30 minutes. By averaging over all the cells, we obtained a two peak distribution displaying the exclusion of H-NS from the center of the cell (see Figure 35).

Figure 34) Intracellular Reorganization of H-NS in E. coli under Osmotic Stress in Stationary Phase and Artefacts from Transfecting Bacteria with Plasmid that Encodes hns Gene (fused to mEos3.2)

A) hns gene is knocked out from the chromosome and it is fused to mEos3.2 in the plasmid followed by transfecting the cells. H-NS molecules cluster at the centre and the periphery of the cells. B) hns gene is fused to mEos3.2 in the chromosome. H-NS molecules cluster around the periphery of the cells. Scale bar is 2 μm.
However, the question about how the chromosome (as a substrate to which H-NS tightly binds) redistributes under the same conditions as H-NS molecules remained. We hypothesized that since H-NS binds to AT-rich domains of DNA, the chromosome should also cluster around the periphery. To examine this hypothesis, we first imaged H-NS molecules fused to mEos3.2 in the green channel and then without changing the ROI, we imaged the DAPI-stained chromosome in the blue channel. Subsequently, we processed H-NS images stack with SRRF analysis and obtained the average image of the DAPI-stained chromosome. We then overlapped these two images by merging the blue and green channels. What we observed is that, in contrast to our hypothesis, it seems that H-NS molecules and the chromosome are excluded from each other during stationary phase bacteria post osmotic shock (see Figure 36).

**Figure 35** Axial Intensity of H-NS molecules in E. coli under Osmotic Stress at Stationary Phase

*E. coli cells were grown up to stationary phase followed by exposing to osmotic shock for 30 minutes. Image A and Image B in respect show single-pixel and weighted cross-section intensity along normalized major axis. Each blue line associates to one bacteria cell and the blue squares represent the average over 40 bacteria cells. Clearly there are two peaks in each graph which indicate there is a cluster of H-NS molecules around the periphery of the cells.*
6.5.2 Intracellular Reorganization of DNA-binding HU protein in E. coli in Response to Osmotic Stress

Apart from H-NS, we explored the intracellular reorganization of HU proteins, another nucleoid associated protein, in response to osmotic shock in E. coli. HU is known to introduce negative supercoiling into the bacterial chromosome and unlike H-NS, which only binds to the specific (i.e., AT-rich) regions of DNA, HU binds non-specifically to DNA\textsuperscript{109}. 

\textbf{Figure 36} H-NS and Chromosome Redistribution in E. coli in Response to Osmotic Stress

\textit{E. coli is at the stationary phase of growth and exposed to osmotic shock for 30 minutes. Image A illustrates H-NS molecules fused to mEos3.2 and image B displays DAPI-stained chromosome. H-NS molecules redistribute towards the periphery of the cells. Upon image C that shows the overlap of images of H-NS and chromosome, it seems H-NS and chromosome are excluded from each other. Images D, E and F are the magnified images of the squared regions in the corresponding images above. Scale bar is 2\textmu m.}
Similar to our H-NS experiments, we examined the response of HU molecules to osmotic shock (30 minutes) in exponential phase and stationary phase. To image HU, this molecule was similarly labeled with the photo-activated monomeric protein mEos3.2 and inserted into the E. coli chromosome. We grew bacterial cells exactly under the same conditions as we grew strains for the H-NS experiments. We did not observe any response in the exponential phase and that the HU molecules reorganize in stationary phase in response to osmotic stress. In addition, by imaging HU followed by imaging the chromosome, we observed that HU proteins also appear to be excluded from chromosome post osmotic stress (see Figure 37). Surprisingly, HU does not exhibit the same level of exclusion as we witnessed by H-NS, and many molecules appear to remain distributed throughout the central region of the cell.

**Figure 37** HU and Chromosome Redistribution in E. coli in Response to Osmotic Stress

Image A represents HU fused to mEos3.2, image B displays DAPI-stained chromosome and image C shows the overlap of images A and B. Under osmotic stress and in stationary phase, HU cluster around the periphery of the cell (image A) and it seems HU and chromosome are excluded from each other. Images D, E and F are the magnified images of the squared regions in the associated images above. Scale bar is 2 μm.
As a side note, HU, which is known to non-specifically bind across the chromosome, is often used as an indicator of the arrangement of bacterial DNA. These results show that this is not always a reliable strategy.

6.5.3 Spatial Organization Dynamics of Chromosome and H-NS in Response to Osmotic Stress

Next, by conducting series of time-course experiments, we studied how the intracellular organization of H-NS and the chromosomal DNA dynamically evolve after osmotic shock in both exponential phase and stationary phase. We grew cells until the growth phase of interest (exponential or stationary), then exposed the cells to osmotic stress for different incubation times: 5, 10, 20, 30, 45 and 60 minutes. After inducing osmotic stress for each of these incubation periods, we fixed the cells with (10X diluted 38%-40%) formaldehyde followed by treating with DAPI to post-stain the chromosome. Afterwards, we conducted two-color imaging to visualize H-NS and the chromosome. In the following subsections, we explain our results in both phases of growth.

6.5.3.1 H-NS and Chromosome Dynamics in Response to Osmotic Stress in Exponential Phase

We conducted time-course experiments to probe H-NS and chromosome dynamics in response to osmotic stress when the bacterial cells are in the exponential phase. In Figure 38, we only display our results for bacteria at 5-, 10-, and 45-minute post osmotic induction as well as non-osmotically shocked bacteria (i.e., control). We did not observe any remarkable/dynamic change in the distribution of H-NS in exponential phase under osmotic stress. However, chromosomal DNA displayed a dynamic compaction followed by expansion. We observed that the chromosome shrinks after roughly 5 minutes exposure to osmotic stress and expands again after nearly 10 minutes. This observation is in agreement with recent results found in the literature\textsuperscript{83}.
To investigate H-NS and chromosome dynamics in response to osmotic stress in stationary phase, we performed a similar series of time-course experiments followed by two-color imaging. Again, we only display the results for the control and after 5, 10, and 45 minutes of osmotic induction (see Figure 39). We observed that after 5 minutes of exposure to osmotic stress, H-NS starts to migrate toward the membrane and by 10 minutes almost all of the proteins are excluded from the center of the cells. In addition, it seems that the chromosome also slightly compacts after 5 minutes.
minutes of exposure to osmotic stress. However, we did not observe any additional dynamics for both H-NS and chromosomal DNA after 10 minutes. We then overlapped the image of H-NS, which we took in the red channel and the image of the chromosome taken in the blue channel (see Figure 39, I-L). In the figure, as we expected, it is clear that H-NS and chromosome are excluded from each other.

Figure 39) Dynamics of Intracellular Reorganization of H-NS and Chromosome in E. coli under Osmotic Stress in “Stationary Phase”

From left to right: non-osmotic shocked bacteria (control), t=5 min, t=10 min and t=45 min. The images A to D show distribution of H-NS molecules (fused to mEos3.2) and the images E to H present DAPI-stained chromosome distribution. The images I to L are generated by overlapping images of chromosome and H-NS. The images I to L are magnified images of squared regions shown in the corresponding images above. Scale bars represent 2 μm.
We quantified the chromosomal DNA dynamics in more than 80 bacterial cells under osmotic stress in both exponential and stationary phase. We quantified the average chromosome area as well as the average fractional area of the chromosome over the time-course of 60 minutes in Figure 40. Our quantification agrees with our observation just discussed.

### 6.5.4 How DNA Gyrase Inhibition Alters Chromosome/H-NS Response to Osmotic Stress

We next studied how the subcellular organization of H-NS molecules in E. coli would alter if we treated bacterial cells with coumermycin before applying osmotic shock. As we mentioned earlier, coumermycin is an antibiotic that is known to inhibit DNA gyrase, a protein that causes negative supercoiling in DNA to facilitate transcription by releasing torsional stress in DNA. Note the chromosome in E. coli on average is negatively supercoiled. However, the overall level of supercoiling (as well as transcriptional activity) decreases when bacteria go from exponential phase to stationary phase. By treating cells with coumermycin, we attempted to alter the overall level of supercoiling in both phases to investigate how this change would influence the reorganization of H-NS under osmotic stress.

**Figure 40** Quantification of H-NS and Chromosome Dynamics in Response to Osmotic Stress for Exponential and Stationary Phases

Blue and pink bars in respect represent exponential phase and stationary phase. Left image presents the average of chromosome area values for different osmotic shock exposure times. Right image displays the evolution of “fractional” chromosome area. Error bars indicate the standard error. (*** P-value<0.001)
Therefore, when bacteria reached the growth phase of interest (exponential/stationary), we added coumermycin to the culture to a final concentration of 5 μg/ml and incubated the culture with this antibiotic for 30 minutes. Then, we exposed the cells to osmotic stress for 30 minutes followed by fixing them with (10X diluted 38%-40%) formaldehyde. We post stained the cells with DAPI and performed two-color imaging.

Notably, by pre-treating cells in exponential phase with coumermycin before applying osmotic shock, the chromosome similarly compacts as in stationary phase, and H-NS proteins are excluded from the chromosome. A response we had never detected before in that phase. However, it seems that this treatment does not affect our results in the stationary phase (see Figure 41 and Figure 42).

*Figure 41* **Intracellular Reorganization of H-NS Molecules under Osmotic Stress in Coumermycin Pretreated E. coli**

E. coli cells are pretreated with coumermycin for 30 minutes and then they are exposed to osmotic stress for 30 minutes. Images from left to right are H-NS molecules (fused to mEo3.2) distribution, DAPI-stained chromosome and overlay. Images at the first row (A, B, C) and second row (D, E, F) in respect display exponential phase and stationary phase. Scale bars represent 2 μm.
Figure 42) Coumermycin Effect on Chromosome Area Compaction in E. coli

From left to right: Bacterial that are not treated with coumermycin and not osmotically shocked (control), treated with 5 µg/ml coumermycin for 30 minutes and bacteria that are first treated with coumermycin followed by osmotic shock (30 min). First row images correspond to exponential phase and the second row images present stationary phase. Scale bars are 2 µm.

Figure 43) Quantification of Coumermycin Effect on Compaction of Chromosome in E. coli at Both Phases of Growth.

A) Control (no treatment with coumermycin, no osmotic shock), B) Osmotically shocked cells, and C) Coumermycin Pretreated bacterial cells followed by 30-minute osmotic shock. The y axis indicates the average of chromosome area fraction over 80 cells. The error bars represent standard deviation of the mean values. (*** P-value<0.001)
We have quantified our observations and analyzed chromosome area size for 80-110 cells for both phases of growth. Our analysis agrees with our observations (see Figure 43).

To conclude, inhibiting DNA gyrase and inducing a similar level of chromosomal supercoiling in both growth phases led to a similar osmotic response. This observation suggests that the difference in the level of supercoiling in both phases, which is mediated by the level of activity of DNA gyrase, causes the osmotic response to be growth-dependent. However, we did not observe any effect by DNA gyrase inhibition in stationary phase, and this can because the chromosome might already reach its limit for condensation in this phase. Even though, it appears the chromosome compacts and H-NS is knocked off the DNA. It is not clear that the chromosome compaction to this extent causes the proteins are pushed aside, or H-NS’s dissociation from the chromosome leads to this level of condensation. In addition, by relaxing supercoiling, we intuitively expected the chromosome expanded. Instead, our observation completely indicates a contradiction, and we do not know why this inhibition caused compaction of the chromosome. However, one explanation could be that perhaps other proteins, which we did not consider, bind to the chromosome and make it denser.

6.6 Concluding Remarks

To conclude, we found that the bacteria osmotic stress response is phase-dependent and that in stationary phase the H-NS proteins are repelled from the chromosome towards the periphery of the cells. This behavior is absent in exponential phase.

Based on our observations, the intracellular organization of H-NS alters dynamically in response to osmotic stress in stationary phase. After 5 minutes of exposure to osmotic stress, H-NS proteins are expelled from the chromosome and start to translocate to the membrane of the cells. By 10 minutes, almost all proteins are excluded from the chromosome and cluster around the periphery of the cells. The organization of the chromosome itself was observed to change dynamically in both exponential and stationary phase. In exponential phase, the chromosome tends to collapse and expand, while, in exponential phase, the chromosome tends to condense and remains tightly condensed. The chromosome, notably, is much more condensed in stationary phase in comparison to exponential phase.
In addition, we observed that after treatment with coumermycin followed by osmotic shock, the chromosome, remarkably, condenses in exponential phase as well. H-NS proteins then start to relocate to the periphery of the cells as observed previously in stationary phase.

As a summary, in Figure 44, we depict the schematic of E. coli response to 30-minute osmotic shock at both growth phases with and without coumermycin treatment.

![Figure 44](image)

*Figure 44* Schematic of E. coli Response (H-NS Spatial Redistribution) to 30-minute Osmotic Stress at Exponential Phase and Stationary Phase.

The red circles represent H-NS molecules. Left image displays the response when the cells are not treated with coumermycin. Right image illustrates the response of the cells that are pre-treated with coumermycin (i.e., DNA gyrase is inhibited). Upon DNA gyrase inhibition, overall negative supercoiling is reduced in both phases and it seems in this condition, H-NS molecules in exponential phase show the response that is similar to the response in stationary phase.

### 6.7 Future Directions

Although our images show the exclusion of H-NS proteins from the chromosome under osmotic stress, we are not sure that these proteins are entirely off the chromosomal DNA. By performing chromatin immunoprecipitation (ChIP) experiments in E. coli under exactly the same conditions we performed our experiments, we can probe whether there are some genes that H-NS still binds to. If this becomes true, then it suggests that the chromosome reorganizes in
response to osmotic stress such that the genes that are regulated by H-NS should be located at the edge of the chromosome. Hence, we can obtain insight about the chromosome architecture.
Chapter 7: Conclusions and Future Directions

To conclude, as the crucial first step, in chapter 2, we developed our optical setup for conducting accurate PALM and dSTORM for a range of photo-switching fluorophores such as Alexa-647, Atto-532, and mEos3.2. We installed relevant excitation lasers (532 nm, 561 nm) into our setup (647 nm laser line was already implemented). Additionally, we placed the necessary optics into the excitation path and introduced a high-speed filter wheel into the detection path. Using an AOTF, we adjusted the intensity of the excitation lasers to the highest values and activation laser (405 nm) to the lowest magnitude. We modified our setup to be able to perform all three modes of microscopy (i.e., wide-field, HILO and TIRF) and apply one of these modes depending on the system of study. Furthermore, we adjusted our setup to allow for three-dimensional (3D) SMLM by integrating a piezo-stage into our microscope and inserting a cylindrical lens into the detection path, and we calibrated for 3D microscopy.

After developing the experimental setup for conducting proper 2D or 3D one- or two-color SMLM, as the second essential step to reconstruct accurate super-resolved images, we developed image-processing methods for analyzing SMLM data. We established analytical techniques that discard noise or out-of-focus localizations, correct for lateral drift introduced to the images during the acquisition, merge temporal consecutive localizations emerged from one fluorophore into one blink and as the final analysis, visualize the localizations by assigning a 2D Gaussian function that has a standard deviation on the order of ten nanometers.

Following the construction of the optical setup for performing SMLM and developing the required analytical techniques to analyze SMLM raw data, we studied the photo-physics of Alexa-647 and Atto-532 in vitro by employing dSTORM. We measured the number of photons emitted by these photo-switching fluorophores and collected by EMCCD camera in each blinking event. In our experimental conditions, we detected about 3600 photons for Alexa-647 and about 4500 photons emitted for Atto-532 per blink. We also measured the duty cycle of these fluorophores to be about $10^{-5}$. This sufficient low value for the duty cycle and the high number of detected
photons per blink ensured us that these fluorophores are very suitable to achieve a high-precision one- (used individually) or two- (utilized as a pair) color dSTORM.

Furthermore, we employed these experimental parameters together with our experimental settings (such as frame rate) explicitly into the SMLM simulation we developed. In our lab, a mathematical theory was developed that can extract the number of molecules, which are labeled with photo-switchable fluorophores under the conditions required for SMLM experiments, from the number of blinks. To test the accuracy of this theory, we established two simulations, one as the calibration platform and the other as the main simulation. In the calibration simulation, we simulated an array of sparse photo-switching fluorophores (such as Alexa-647) that undergo switching between dark and fluorescent states according to a geometric distribution. From this calibration in-silico experiment, we could extract the characteristic number of blinks ($\lambda$) within that acquisition time. In the main simulation, we set the acquisition time to be the same as the one in the calibration. We simulated an array of molecules that are binomially labeled with Alexa-647 fluorophores. These molecules are distributed in the field of view sparsely, and their tagged fluorophores undergo blinking with a geometric distribution (like the calibration experiment).

To validate the molecular counting theory, we measured the total number of blinks from our SMLM simulation and inserting this parameter together with $\lambda$ within the theory, we obtained the estimated number of molecules. This value turned out to be in excellent agreement with the actual number of target molecules implemented in the simulation.

Next, we applied SMLM experimental and analytical tools we optimized or developed up to then into two different biological systems. We studied the spatial distribution of two kinds of proteins: RNAPII within the nuclei of mouse cortex cells and mouse embryonic stem cells, and H-NS proteins within E. coli.

To be able to explore the spatial organization of active RNAPII by applying dSTORM, we successfully labelled these proteins with Alexa-647 through an indirect immunofluorescence labeling. To understand how this spatial distribution could influence the fate of stem cells, we probed both mouse embryonic stem cells and a differentiated cell such as a mouse cortex cell. We conducted dSTORM and applying the image-processing tools developed, we mapped RNAPII
proteins within the nucleus of both cells. Furthermore, by adding a 0.5 m cylindrical lens within the telescope before the camera and using the axial-image plane calibration we obtained before, we conducted 3D dSTORM and could image RNAPII proteins within a 1µm thick section of the nuclei of mouse cortex cells. Our 2D dSTORM data was used as a test platform to examine clustering algorithms such as (2D) FOCAL already developed and published, and 3D dSTORM data was used to test 3D FOCAL under development in our lab.

In the future, we can improve our understanding of the underlying transcriptional mechanism that defines the direction of cell differentiation by studying the interaction of RNAPII with another protein of interest such as transcription factors (TFs) that play crucial roles in pluripotency of stem cells (i.e., Oct3/4, Sox2, Klf4 or Nanog). Alternatively, one can explore the spatial organization of active RNAPII proteins and inactive ones within the nucleus of the embryonic stem cells and differentiated cells and correlate the results within both cells. To study the interaction of two kinds of proteins of interest (i.e., RNAPII and TF) or to differentiate active RNAPII from inactive one, two-color SMLM could be a technique of choice. However, to conduct an accurate two-color SMLM, one should first correct for chromatic aberration. We facilitated this part of the study by performing this correction. As the fiducial markers, we designed a Nano-whole array, which was fabricated at the Toronto Nanofabrication Centre (TNFC) and applied this micro-array to map the aberrations. We analyzed the errors introduced into our two-color measurements and found that, we could register images of green channel to images of the red channel with 3.6 nm precision.

This project had to be halted there due to the prolonged procedure of applying dSTORM and subsequent image-processing methods for this kind of study. At the time, we could only image 1 µm thick sections of the nuclei. The nuclei of mammalian cells are typically about 10-20 µm. Note, each dSTORM imaging experiment is followed by image-processing analysis which takes two to three hours. Hence, imaging the entire nucleus would be a very slow procedure. In addition, to obtain reliable statistics, we should image dozens of cells. In addition, to understand the underlying transcriptional mechanism, results in the embryonic stem cells should be correlated to the results within differentiated cells. Therefore, all the imaging experiments should be
conducted in both types of cells. Collectively, these slow procedures made us realize that this study could be beyond the scope of this thesis timeline.

Therefore, we shifted the biological system of our study to E. coli (cells that we could easily maintain in our lab), and we studied intracellular re-structure of H-NS proteins (fused to photo-switching green-to-red fluorescent protein mEos3.2) in response to osmotic shock. We explored this response employing PALM\textsuperscript{7} in combination with another super-resolved technique SRRF\textsuperscript{8}. Initially, applying PALM, we probed the intracellular reorganization of H-NS proteins under 30 minutes of osmotic stress in both phases of growth (i.e., exponential phase and stationary phase). We observed that H-NS proteins under osmotic stress are translocated towards the periphery of the cells in stationary phase only. This response was so noticeable that we could observe it with other microscopy techniques than PALM, such as SRRF that achieves a resolution lower than PALM but is much faster. Hence, we carried on this study applying classical light microscopy followed by the post-processing super-resolved SRRF technique.

We determined the intracellular response of H-NS to osmotic shock is phase-dependent and proteins are excluded from the chromosome and cluster around the membrane of the cell only in stationary phase and not in exponential phase. Up to then, we only explored the static osmotic response (i.e., 30-minute shock). Next, we probed the dynamics of this response for the chromosome and H-NS proteins in exponential phase and stationary phase. We conducted a series of time-course experiments in both phases. We exposed cells to osmotic shock for variable durations of stress incubation. We did not notice a dynamical response in H-NS proteins in exponential phase. However, we observed H-NS proteins distribute dynamically within the cells in stationary phase. It appears after 5 minutes being exposed to the shock, H-NS proteins start to repel from the chromosome, and after a 10-minute incubation, almost all of the proteins are found around the membrane. Remarkably, the chromosome displays a dynamical response to osmotic shock in both growth phases. In exponential phase, the chromosome condenses after a 5-minute osmotic incubation, and by 10 minutes of incubation, it expands back. In stationary phase, the chromosome collapses after a 5-minute exposure to stress, but it remains condensed for the rest of the experiment. Notably, as we expected for all the time points, the chromosome is much more compact in stationary phase compared to exponential phase.
Up to then, we only observed the osmotic response of H-NS proteins in stationary phase, and this response was always absent in exponential phase. Therefore, to uncover the underlying mechanism that causes this phase-dependent response, we investigated the effect of reducing the overall level of chromosomal DNA supercoiling. The reason we explored this feature was that in exponential phase the level of transcription and correspondingly the overall level of supercoiling is much higher than stationary phase. We hypothesized that possibly this difference in the overall level of supercoiling could lead to the phase-dependent response. Hence, we inhibited DNA gyrase (an enzyme that introduces negative supercoiling in DNA) by applying the antibiotic coumermycin, and we probed the reorganization of H-NS and the chromosome under 30 minutes of osmotic shock in both phases.

Remarkably, we observed after treatment with coumermycin in exponential phase, the chromosome compacts as much as in stationary phase and H-NS proteins are excluded from the chromosome and repelled towards the periphery of the cells. This was the same response that was previously only detected in stationary phase. We quantified our observation by analyzing chromosome compaction in about 100 bacterial cells for each condition (i.e., control, osmotic shock, and coumermycin pretreated followed by osmotic stress). Our results show that by treating the cells with coumermycin in exponential phase followed by osmotic stress, the chromosome becomes as compact as in stationary phase. This observation could justify why we detected the intracellular response of H-NS to osmotic stress only in stationary phase. We hypothesize that perhaps since in stationary phase the chromosome becomes so condensed in response to osmotic stress, it repels H-NS proteins towards the membrane. However, it is possible that H-Ns proteins are not entirely excluded from the chromosome and there are still some genes on the chromosome around the periphery that are regulated by H-NS. As a future direction, this hypothesis can be tested by conducting ChIP experiments to explore the genes that are still bound to H-NS proteins in the bacterial cells.
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