

**ADVANCING SUPER RESOLUTION MICROSCOPY FOR
QUANTITATIVE IN-VIVO IMAGING OF CHROMATIN
NANODOMAINS**

by

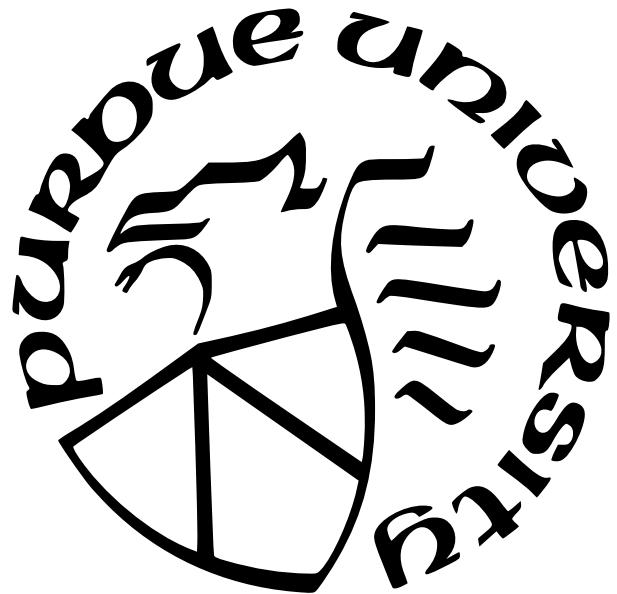
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I dedicate this thesis to Alexandra, who has steadily encouraged my pursuit of a doctoral degree. I am forever indebted for your patience, understanding, and proofreading.

What we observe is not nature itself, but nature exposed to our method of questioning

Werner Heisenberg

To deal with a 14-dimensional space, visualize a 3-dimensional space and say “fourteen” to yourself very loudly. Everyone does it

Geoffrey Hinton

Information is the resolution of uncertainty

Claude Shannon

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LIST OF SYMBOLS

\mathbf{x}	An image at base resolution
\mathbf{y}	An image at higher resolution
δ	Pixel lateral width
k	Pixel or mode index
θ	A parameter
u, v	Cartesian coordinates in two-dimensions
g_k	Pixel-wise gain
o_k	Pixel-wise offset
w_k	Pixel-wise readout noise standard deviation
μ_k	Pixel-wise expected value
s_k	Pixel-wise measured signal
ξ_k	Pixel-wise measured readout noise
O	Point spread function
erf	Error function
$\sigma_{\mathbf{x}}$	Gaussian PSF width
$\sigma_{\mathbf{y}}$	Kernel width for kernel density estimate
ϵ	An image of pure Gaussian noise
β	Diffusion model noise variance
SNR	Diffusion model signal to noise ratio
ψ	Diffusion model parameters
\mathcal{L}	An objective function
D_{KL}	KL-divergence
p	A discrete or continuous probability distribution
\mathbb{E}_p	Expectation with respect to a distribution p
I	Fisher information matrix
λ	Expected number of background counts per frame
d	Lateral dimension of a region of interest
ζ	Photon detection probability

τ	Delay time
$g^{(2)}(\tau)$	Second order coherence function
$G^{(2)}(m)$	Measured number of signal-signal coincidences at lag time m
M	Number of frames
N	Number of active fluorescent emitters
N^*	Maximum a posteriori estimate of number of active emitters
n	Number of photon counts in a frame
ℓ	Log-likelihood
\hat{a}	Ladder operator
ρ	Number density
$L(r)$	Besag's L-function
$K(r)$	Ripley's K-function
$G(r)$	Nearest neighbor distribution function
ξ	A delta-correlated Gaussian noise
k_B	Boltzmann's constant
T	Temperature
U	Potential energy
D	Diffusion coefficient
r_0	Harmonic bond equilibrium length
R_0	Binder potential equilibrium length

ABSTRACT

Single molecule localization microscopy (SMLM) techniques, such as direct stochastic optical reconstruction microscopy (dSTORM), can be used to produce a pointillist representation of fluorescently-labeled biological structures at diffraction-unlimited precision. This class of techniques permits localization of fluorescent molecules in the cell with nanometer precision and thus is commonly referred to as *nanoscopy*. Conventional nanoscopy utilizes the deactivation of standard fluorescent tags, followed by spontaneous or photoinduced reactivation, to resolve fluorophores at distances below the diffraction limit. While powerful, this approach has limited throughput and requires localization in sparse scenes. This dissertation introduces fluorescence nanoscopy and covers its innovation and application as discussed in the following papers:

Quantum enhanced localization microscopy with a single photon avalanche diode array [1] leverages recent advancements in single photon avalanche diode array technology to count fluorescent emitters using a widefield microscope.

Uncertainty-aware localization microscopy by variational diffusion [2] describes a novel algorithm that applies a diffusion model in order to model a posterior distribution on high resolution localization microscopy images, given low resolution inputs.

BRD4 phosphorylation regulates structure of chromatin nanodomains [3] describes the role of the BRD4 phosphoswitch in the maintenance of chromatin nanodomains via super resolution microscopy and molecular dynamics simulation. We demonstrate that BRD4 phosphorylation regulates chromatin packing and mobility in mammalian nuclei.

1. SINGLE MOLECULE LOCALIZATION MICROSCOPY

1.1 Introduction

1.1.1 Breaking the diffraction barrier

In the quest to understand cellular function, biologists aim to directly observe the processes enabling cells to maintain homeostasis and respond dynamically to internal and environmental cues at the molecular level. However, the inherent limitations imposed by diffraction have historically constrained the resolution achievable with conventional light microscopy. The diffraction limit, first described by Ernst Abbe in the 19th century, dictates that the resolution of a microscope is fundamentally limited by the wavelength of light used for imaging. Objects closer than approximately half the wavelength of light cannot be distinguished as separate entities. For visible light, this translates to a resolution limit of about 200-250 nanometers, which is insufficient for resolving many subcellular structures and molecular complexes.

Super-resolution (SR) microscopy techniques have emerged as a pathway to observing subcellular structures and dynamics with enhanced resolution, surpassing the classical Abbe diffraction limit. Fluorescence microscopy techniques continually push the resolution boundary towards nanometer scales, facilitating imaging of cellular structures with a level of detail previously achievable only with electron microscopy. Concurrently, SR techniques retain the advantages of optical microscopy in biological experiments, including sample preservation, imaging flexibility, and target specificity. SR enables extraction of quantitative information on spatial distributions and often absolute numbers of proteins, nucleic acids, or other macromolecules within subcellular compartments.

A host of SR methods have been developed in recent years, which fundamentally differ in how fluorescently labeled samples are excited and how the emitted photons are detected. Here, we focus on a particular technique referred to as single-molecule localization microscopy or nanoscopy. This class of diffraction-unlimited SR methods leverage fluorescence intermittency to resolve fluorophores which would otherwise be unresolvable at the detector (Figure 1.1). Nanoscopy approaches, such as direct-STORM (dSTORM), have become quite popular because they can be implemented at low cost on conventional wide-field setups, shifting the

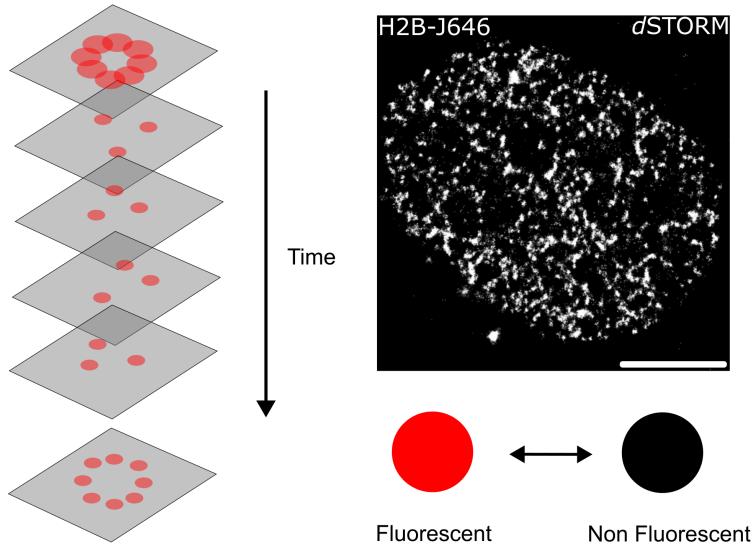


Figure 1.1. Stochastic optical reconstruction microscopy (STORM)
 Single molecules are resolved by separating their fluorescent emission in time using fluorophores with multiple photophysical states. Example super-resolution image of H2B protein in a living HeLa cell nucleus at 37C, 5 percent CO₂. Image reconstructed from 1k 10ms frames. Scalebar 5μm.

complexity to biological sample preparation and image post-processing. Common strategies for the temporal separation of molecules involve transient intramolecular rearrangements to switch from dark to fluorescent states, or the exploitation of non-emitting molecular radicals. For example, in dSTORM, rhodamine derivatives can undergo intersystem crossing to a triplet state, which can be reduced by thiols to form a dark radical species. The dark state can then be quenched by oxidative processes, driving the fluorophore back to its ground state.

In nanoscopy applications, we seek the position and intensity of isolated fluorophores as well as estimate the accuracy and precision of these parameters. Accuracy is a measure of the systematic error or bias, and precision is a measure of the statistical error of an estimator. Both of these metrics are crucial in nanoscopy, as they determine the quality of SR reconstructions and maximum achievable resolution of the method, respectively. Importantly, a SR image in nanoscopy is simply a kernel density estimate (KDE) of the located fluorescent

emitters, using an isotropic Gaussian kernel. The width of one such placed Gaussian kernel, σ is given by the precision of the fluorophore position. The value of σ will depend on experimental factors such as the signal-to-noise ratio (SNR). A fundamental lower bound on σ is discussed in Section 1.2.1.

1.1.2 Biological discovery with fluorescence nanoscopy

Using fluorescence nanoscopy, scientists can now explore the nanoscale organization of cells and their components, leading to groundbreaking discoveries and new insights into cellular processes. In immunology, nanoscopy has been employed to study the spatiotemporal dynamics of T cell antigen receptor (TCR) complexes and linker for activation of T cells (LAT), an important adaptor molecule in the TCR signaling pathway [4]. Others used nanoscopy to demonstrate that clustering patterns of the tyrosine kinase Lck were controlled by the conformational states of Lck, with the open, active conformation inducing clustering and the closed, inactive conformation preventing clustering [5]. Nanoscopy has also proven invaluable in virology. In this field, researchers have utilized nanoscopy techniques to visualize Human immunodeficiency virus type 1 (HIV-1) assembly and budding at the plasma membrane of living host CD4+ T cells by tracking the viral membrane Gag proteins and its derivatives from the host cell plasma membrane [6]. This has led to a deeper understanding of the molecular mechanisms underlying viral infections and has provided insights into the structure of viral capsids and replication mechanisms.

The ability to study cellular structures at the nanoscale has also advanced our understanding of molecular machines and complexes within cells. For instance, detailed imaging of the nuclear pore complex, a crucial structure for nucleocytoplasmic transport, has revealed the precise arrangement of its constituent proteins [7]. This has enhanced our knowledge of how the nuclear pore complex regulates the passage of molecules between the nucleus and cytoplasm, a process essential for cellular function. In epigenetics, nanoscopy has facilitated the investigation of fundamental nature of chromatin structure at the nanoscale [8]. The effects of histone modifications or the assembly of transcriptional complexes can be directly visualized and quantitatively measured with spatial resolution [8]–[10].

As nanoscopy continues to evolve, future advancements are likely to enhance both the resolution and speed of imaging, perhaps allowing researchers to capture dynamic processes within living cells. New techniques and improvements in instrumentation will expand the capabilities of nanoscopy, enabling even more detailed studies of cellular functions and interactions. Towards this aim, we will now derive a fundamental statistical description of fluorophore detection in nanoscopy. This description is necessarily simplified. For example, the emission rate of chemically identical fluorophores can vary, owing to effects such as uneven illumination profile, dipole orientation, or different optical path lengths.

1.2 Likelihood of localization microscopy images

It is common to describe the optical impulse response of a microscope as a two-dimensional isotropic Gaussian [11]. This is an approximation to the more rigorous diffraction models given by [12], [13]. Over a continuous domain, the impulse response reads

$$O(u, v) = \frac{1}{2\pi\sigma^2} e^{-\frac{(u-\theta_u)^2+(v-\theta_v)^2}{2\sigma^2}} \quad (1.1)$$

for a fluorescent emitter located at $\theta = (\theta_u, \theta_v)$. The above expression can be loosely interpreted as a probability distribution over locations where a photon can be detected. For discrete detectors, such as a camera, we bin this expression by integrating over pixels. The number of photon arrivals at a particular pixel k will follow Poisson statistics [14], [15], with expected value

$$\mu_k = i_0 \left(\int_{u_k - \delta/2}^{u_k + \delta/2} O(u; \theta_u) du \right) \left(\int_{v_k - \delta/2}^{v_k + \delta/2} O(v; \theta_v) dv \right) \quad (1.2)$$

The scalar quantity i_0 represents the amplitude of the signal, which is proportional the quantum efficiency of a pixel η , the duration of exposure, Δ , and the expected number of photons emitted by a fluorescent molecule per unit time ζ . With no loss of generality, $\Delta = \eta = 1$ and there is a single free parameter ζ . Terms above in parentheses are simply integrals of Gaussian functions and can be evaluated analytically. We define them as Γ_u, Γ_v , respectively. For example,

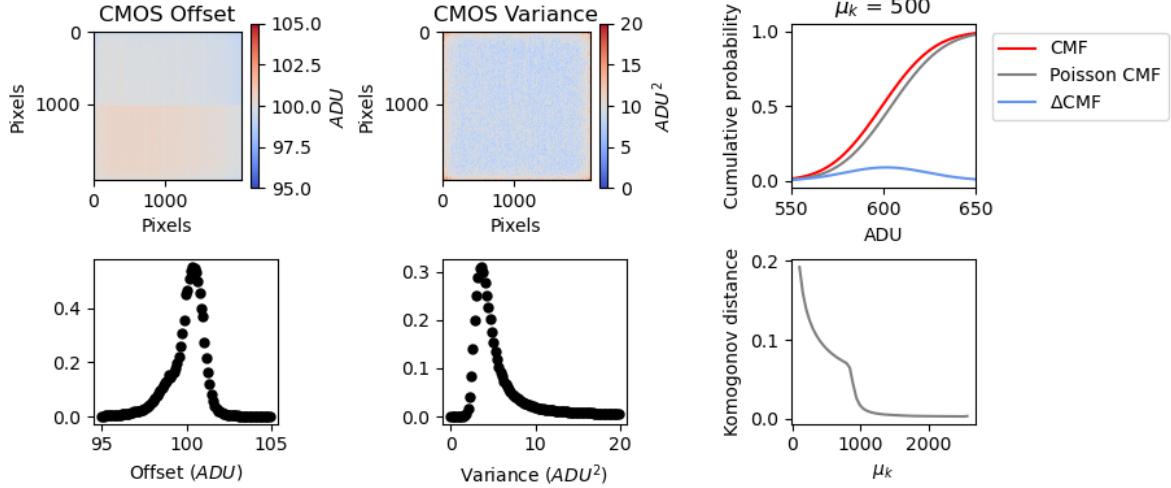


Figure 1.2. Noise calibration for a CMOS camera used for maximum-likelihood localization (left column) CMOS offset for zero incident photons (middle column) CMOS variance for zero incident photons (upper right) Cumulative mass function for the convolution distribution and its Poisson approximation for rate parameter $\mu_k = 500$ counts (lower right) Komogonov distance measured as a function of rate parameter μ_k

$$\begin{aligned}\Gamma_u &:= \int_0^{u_k + \delta/2 - \theta_u} O(u) du - \int_0^{u_k - \delta/2 - \theta_u} O(u) du \\ &= \frac{1}{2} \left(\operatorname{erf} \left(\frac{u_k + \frac{\delta}{2} - \theta_u}{\sqrt{2}\sigma_x} \right) - \operatorname{erf} \left(\frac{u_k - \frac{\delta}{2} - \theta_u}{\sqrt{2}\sigma_x} \right) \right)\end{aligned}$$

where we have used the common definition, $\operatorname{erf}(z) = \frac{2}{\sqrt{\pi}} \int_0^z e^{-t^2} dt$. Recall the central objective of localization microscopy is to infer a set of molecular coordinates $\theta = (\theta_u, \theta_v)$ from measured low resolution images \mathbf{x} . In general, the likelihood on a particular pixel $p(\mathbf{x}_k | \theta)$ is taken to be a convolution of Poisson and Gaussian distributions, due to shot noise $p(s_k) = \text{Poisson}(\mu_k)$ and sensor readout noise $p(\xi_k) = \mathcal{N}(o_k, w_k^2)$. Characterization of the offset o_k and variance w_k^2 of the readout noise of the imaging sensor is a critical step in parameterizing localization algorithms used in nanoscopy (Figure 1.2). Then one defines the likelihood as a convolution distribution:

$$p(\mathbf{x}_k|\theta) = A \sum_{q=0}^{\infty} \frac{1}{q!} e^{-\mu_k} \mu_k^q \frac{1}{\sqrt{2\pi w_k}} e^{-\frac{(\mathbf{x}_k - g_k q - o_k)^2}{2w_k^2}} \approx \text{Poisson}(\mu'_k) \quad (1.3)$$

where A is some normalization constant. For the sake of generality, we include a per-pixel gain factor g_k [ADU/photon], which is often unity. Sampling from $p(\mathbf{x}_k|\theta)$ is trivial; however, for computation of a lower bound on uncertainty in θ , the summation can be difficult to work with. Therefore, we choose to use a Poisson approximation for simplification, valid under a range of experimental conditions [15]. The quality of this approximation will depend on the signal level, with higher signal levels or higher exposure times leading to a reduced Komogorov distance between the convolution distribution and the approximation (Figure 1.2). After subtraction of the offset o_k of the pixel array, the variance of the Poisson likelihood per pixel becomes $\mu'_k = \mu_k + w_k^2$. Ultimately, the model negative log-likelihood is

$$\ell(\mathbf{x}|\theta) = -\log \prod_k \frac{e^{-(\mu'_k)} (\mu'_k)^{n_k}}{n_k!} = \sum_k \log n_k! + \mu'_k - n_k \log(\mu'_k) \quad (1.4)$$

where n_k is the number of ADU measured at pixel k . Localization then proceeds by optimization of $\ell(\mathbf{x}|\theta)$ to obtain to maximum likelihood estimate (MLE) of the coordinates.

1.2.1 Cramer-Rao lower bound

Reliable inference of θ from \mathbf{x} in general requires performance metrics for model selection. Here, we use the Fisher information as an information theoretic criteria to assess the discrepancy between theoretical minimum precision with respect to the root mean squared error (RMSE) of our predictions of θ , i.e. localization uncertainty [16]. The Poisson log-likelihood $\ell(\mathbf{x}|\theta)$ is convenient for computing the Fisher information matrix [14] and thus, the Cramer-Rao lower bound (CRLB). This bounds the variance of a statistical estimator of θ , from below: $\text{var}(\hat{\theta}) \geq I^{-1}(\theta)$. The Fisher information is given by the expression

$$I_{ij}(\theta) = \mathbb{E}_{\theta} \left(\frac{\partial \ell}{\partial \theta_i} \frac{\partial \ell}{\partial \theta_j} \right) \quad (1.5)$$

For an arbitrary parameter, we find that, for a Poisson log-likelihood ℓ :

$$\begin{aligned}\frac{\partial \ell}{\partial \theta_i} &= \frac{\partial}{\partial \theta_i} \sum_k \log n_k! + \omega'_k - n_k \log(\omega'_k) \\ &= \sum_k \frac{\partial \omega'_k}{\partial \theta_i} \left(\frac{\omega'_k - n_k}{\omega'_k} \right)\end{aligned}$$

Using this result, we can compute the Fisher information matrix $I(\theta)$

$$I_{ij}(\theta) = \mathbb{E}_{\theta} \left(\sum_k \frac{\partial \omega'_k}{\partial \theta_i} \frac{\partial \omega'_k}{\partial \theta_j} \left(\frac{\omega'_k - n_k}{\omega'_k} \right)^2 \right) = \sum_k \frac{1}{\omega'_k} \frac{\partial \omega'_k}{\partial \theta_i} \frac{\partial \omega'_k}{\partial \theta_j} \quad (1.6)$$

A fundamental lower bound on the localization uncertainty in our estimates of θ then is found from its inverse: CRLB = $I^{-1}(\theta)$.

1.2.2 Maximization of density, minimization of error

The distribution of a particular biomolecule in the cell can be described as a probability density over a two-dimensional space, casting super-resolution as a density estimation problem. Intuitively, the spatial resolution of SMLM images then increases as we draw more samples from this density, a concept which is made mathematically precise by the so-called Fourier ring correlation (FRC) [17]. Using FRC, one can define image resolution as the spatial frequency at which a correlation function in the frequency domain drops below a threshold, typically taken to be 1/7 (Figure 1.3). This correlation function is defined for two concentric rings in the frequency domain:

$$\text{FRC}(q) := \frac{\sum_{\vec{q} \in \text{ring}} \tilde{f}_1(\vec{q}) \tilde{f}_2(\vec{q})^*}{\sqrt{\sum_{\vec{q} \in \text{ring}} |f_1(\vec{q})|^2} \sqrt{\sum_{\vec{q} \in \text{ring}} |f_2(\vec{q})|^2}} \quad (1.7)$$

According to this theory, reducing localization uncertainty while increasing the number of samples, results in an increase in image resolution [17]. However, typical nanoscopies favor fewer samples and lower localization uncertainty as few tools to deal with localization in dense scenes are available. Indeed, localization uncertainties in sparse conditions are often tens of nanometers and managing the increase in localization uncertainty at high labeling density remains a major bottleneck. Static uncertainty due to molecular crowding

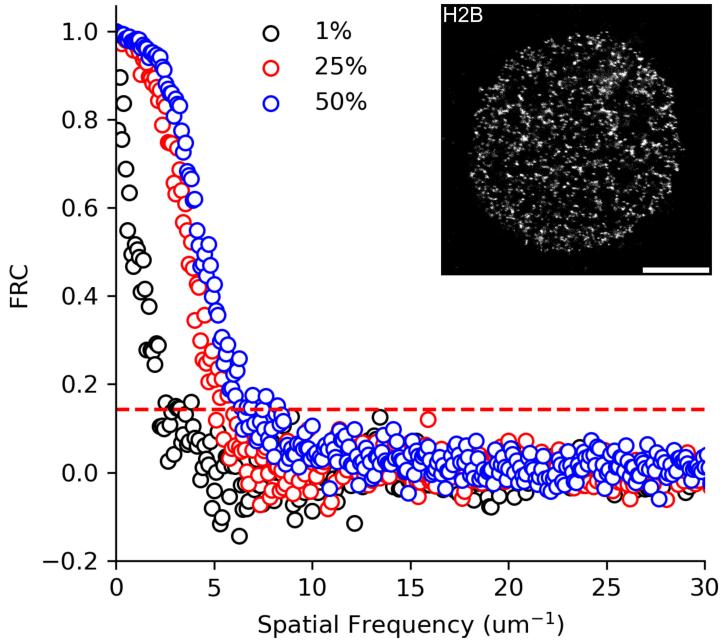


Figure 1.3. Resolution in nanoscopy depends on sampling density
Fourier ring correlation (FRC) for different sampling ratios relative to an approximate total 200k localizations (inset) Example kernel density estimate of H2B-HaloTag in a living HeLa cell nucleus. Scalebar approximately 5 μm

can be partially ameliorated by using pairwise or higher-order temporal correlations within a pixel neighborhood, known as stochastic optical fluctuation imaging (SOFI) [18]. Other approaches such as stimulated emission and depletion (STED) imaging bring control over the photophysical state of a chosen subset of the sample, yet the need for laser scanning prevents widespread application in live-cell studies.

The spatial resolution and relative simplicity of nanoscopy techniques has incited an effort to increase their resolution, precision, and explore avenues towards time-resolved nanoscopy. In the next two chapters, we develop novel approaches to these issues which address localization in dense scenes as an inference problem in a high dimensional parameter space. In Chapter 2, we leverage single photon avalanche diode (SPAD) arrays to count active fluorescent emitters which may lead to constrained multi-emitter localization using the image likelihood. SPAD cameras, with their high temporal resolution and single photon sensitiv-

ity, can potentially enable precise localization in non-sparse scenes. In Chapter 3, we design a generative modeling framework for kernel density estimation in localization microscopy, using variational diffusion. This approach not only accelerates super-resolution imaging but also provides a way to estimate uncertainty in the results. These two methods, one focusing on computational models and the other on cutting-edge hardware, offer complementary solutions to the high-dimensional inference problem in super-resolution microscopy. Using them separately or in tandem, we can achieve more accurate and reliable imaging of biological structures at the nanoscale. Finally, in Chapter 4, we apply a more conventional form of localization microscopy to the study of chromatin organization in living mammalian cells.

2. QUANTUM ENHANCED LOCALIZATION MICROSCOPY WITH A SINGLE PHOTON AVALANCHE DIODE ARRAY

2.1 Background

2.1.1 A brief introduction to quantum optics

Experimental techniques have surfaced which take advantage of the quantum nature of light to enhance imaging methods. The advent of fast and sensitive detectors allows us to measure electromagnetic fields at a timescale where quantum effects can be seen. We often speak of methods such as photon counting or photon statistics, and it is prudent to briefly define the photon and how it can be measured with this technology.

Quantization of the electromagnetic field generally begins by writing a Fourier expansion of solutions to Maxwell's equations called *modes* and identifying the canonical position and momentum variables. This procedure is out of the scope of this thesis. Instead, we start by stating the major result of quantization, in which each mode of the field is quantized as a quantum harmonic oscillator. As such, the Hamiltonian for one particular mode has energy eigenvalues $E_k = \hbar\omega_k(n + \frac{1}{2})$ for mode k with frequency ω_k . Since there may be infinitely many modes, the wavefunction is $|\psi\rangle = |n_1, n_2, \dots\rangle$ where n_1 is the number of photons in the first mode, n_2 is the number of photons in the second mode, and so on. The number operator for the k -th mode gives $\hat{n}_k |\psi\rangle = n_k |\psi\rangle$.

The number operator $\hat{n}_k = \hat{a}_k^\dagger \hat{a}_k$ is defined in terms of operators \hat{a}_k and \hat{a}_k^\dagger which are photon annihilation and creation operators for the k -th mode, respectively. These operators satisfy the commutation relations $[\hat{a}_k, \hat{a}_{k'}^\dagger] = \delta_{kk'}$ and $[\hat{a}_k, \hat{a}_{k'}] = [\hat{a}_k^\dagger, \hat{a}_{k'}^\dagger] = 0$. The action of the annihilation and creation operators on the joint number states is:

$$\hat{a}_k |n_1, n_2, \dots\rangle = \sqrt{n_k} |n_1, n_2, \dots\rangle \quad \hat{a}_k^\dagger |n_1, n_2, \dots\rangle = \sqrt{n_k + 1} |n_1, n_2, \dots\rangle$$

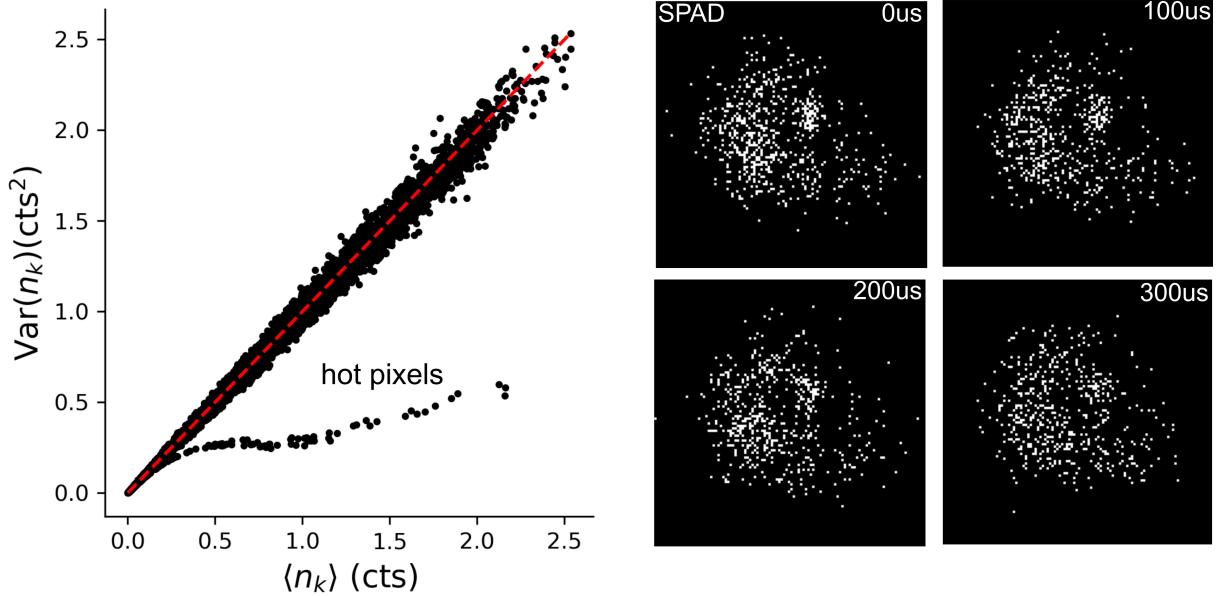


Figure 2.1. Poissonian photon statistics of a Gaussian laser spot
 (left) Fano factor plot of pixel-wise variance in photon counts with respect to average photon counts, for 100us exposures of a Gaussian beam pulsed at 10MHz. Equal mean and variance (Poisson statistics) showed as a dashed red line. (right) Example images taken in sequence with the SPAD array.

2.1.2 Photon statistics

There are certain states $|\psi\rangle$ which have special properties, such as the coherent state. Coherent states resemble classical oscillations of the EM field. A coherent state $|\alpha\rangle$ is defined as the eigenstate of the annihilation operator \hat{a} :

$$\hat{a} |\alpha\rangle = \alpha |\alpha\rangle$$

where α is a complex number. The coherent state for a single mode field is given by:

$$|\alpha\rangle = e^{-\frac{|\alpha|^2}{2}} \sum_{n=0}^{\infty} \frac{\alpha^n}{n!} |n\rangle$$

It turns out that if we measure a number of photons in this state, we would find that the number of photons has Poisson statistics. To see this, we calculate the probability $p(n)$ of finding n photons in a coherent state $|\alpha\rangle$:

$$p(n) = |\langle n | \alpha \rangle|^2 = |e^{-\frac{|\alpha|^2}{2}} \frac{\alpha^n}{n!}|^2 = e^{-|\alpha|^2} \frac{|\alpha|^{2n}}{n!}$$

This is simply the Poisson distribution with mean $\langle \hat{n} \rangle = |\alpha|^2$. The variance of the photon number distribution in a coherent state is also $|\alpha|^2$, characteristic of Poisson statistics, where the mean and variance are equal. Poissonian fluctuations of a Gaussian beam can be spatially resolved using a single photon avalanche diode (SPAD) array (Figure 2.1).

In contrast, single photon states, such as $|1\rangle$, do not necessarily follow Poisson statistics. This state could be prepared by an isolated single photon source such as a fluorescent dye molecule, quantum dot, or nitrogen vacancy, which can produce only a single photon at a time. This phenomenon is referred to as *fluorescence antibunching* where photons tend to be detected as isolated events rather than in bursts or bunches. Single photon sources have a fluorescence lifetime, typically on the order of nanoseconds, which results in a sequence of photon arrivals with a more regular structure than would be expected under Poisson statistics. Note that for such a single photon source the single-mode field can be in state $|1\rangle$ but not state $|2\rangle$ at any given time. If more single photon sources are present or multi-level relaxations occur, states beyond $|1\rangle$ are possible. This has led to the introduction of binomial states of the quantized field [19]. This fact is leveraged in the following sections, to count the number of single photon sources in a region of interest using a SPAD array.

2.2 Second-order coherence

The second-order coherence function, $g^{(2)}(\tau)$, is a fundamental tool in quantum optics, providing insight into photon statistics. It can be expressed in terms of photon counts $n(t)$ as:

$$g^{(2)}(\tau) = \frac{\langle n(t)n(t+\tau) \rangle}{\langle n(t) \rangle^2} \quad (2.1)$$

This formulation highlights how the intensity correlations between photons detected at times t and $t + \tau$ are normalized by the square of the mean photon count rate, providing a dimensionless measure of the correlation. The value of $g^{(2)}(\tau)$ and its dependence on τ reveal essential characteristics of the light source. For sub-Poissonian statistics, which

is synonymous with photon antibunching, $g^{(2)}(0) < 1$ and the likelihood of detecting two photons in quick succession is reduced. This behavior is closely related to the fluorescence lifetime of the emitters; after emitting a photon, a fluorophore requires a characteristic time to re-enter the excited state, leading to a dip in $g^{(2)}(\tau)$ at short τ . Coherent light, discussed in the previous section, shows no correlation between photon arrival times, resulting in $g^{(2)}(\tau) = 1$ for all τ . Super-Poissonian statistics, seen in thermal light, exhibit photon bunching, where $g^{(2)}(\tau)$ starts high at $\tau = 0$ and decays to 1 as τ increases, indicating a higher probability of photons arriving close together in time.

Techniques such as Hanbury Brown and Twiss (HBT) interferometry are commonly employed to measure $g^{(2)}(\tau)$, using two single photon sensitive detectors and a beam splitter. In the following sections, we will incorporate an empirical estimate of $g^{(2)}(\tau)$ into a framework for inference of the number of fluorescent emitters in a diffraction limited region.

2.3 Quantum-enhanced localization microscopy

Single photon avalanche diodes (SPADs) have long been the detector of choice for highly sensitive biological imaging and sensing, such as single molecule imaging and single molecule spectroscopy. Its fast temporal response and low background noise allow for the registration of a single photon with a temporal resolution as precise as several picoseconds [20]. However, standard SPAD detectors lack spatial resolution and are typically integrated in laser scanning microscopes for biological imaging, which significantly limits the imaging speed. Recently developed SPAD arrays removed this limitation and extended the high spatiotemporal imaging to widefield microscopy. SPAD arrays can achieve orders of magnitude higher temporal resolutions than standard cameras while preserving the SPAD detectors original signatures, such as single photon sensitivity, low dark count rates, and time-gated photon collection. Furthermore, the reduced readout noise and large fill-factor of recently commercialized SPAD arrays suggests their use for precision widefield bioimaging for single molecule studies. Such technologies have also attracted considerable attention in the bioimaging community for various applications including widefield fluorescence lifetime imaging [21]–[23].

The spatial configuration of the SPAD array can also function as a two-dimensional and on-chip Hanbury-Brown and Twiss setup that measures the antibunching effect, which is a purely quantum optical phenomenon [24]–[27] that reports the single photon emission signature of a quantum emitter. A simplified SPAD array has been previously discussed for localization microscopy in non-sparse scenes [28] to image quantum dots beyond the diffraction limit. However, this imaging modality was challenged by a general issue encountered in fluorescent labeling, i.e., how to quantify multiple fluorophores in a diffraction-limited spot?

The antibunching property is manifest in both the second-order coherence of photon arrivals as well as the photon counting histogram (PCH) [29], [30] i.e., the distribution of the number of photons collected from a diffraction-limited volume. Importantly, the number of photons detected during high-speed imaging provides evidence for the number of single photon sources present in the imaged region. Previous studies have used the PCH to quantify the number of active fluorescent emitters by using conventional SPAD detectors in confocal setups. However, using a SPAD array, the PCH can be measured over a large field of view with spatial resolution by fast acquisition of binary images synchronized with pulsed laser excitation.

Here, we demonstrate that the PCH can be parameterized by the number of active fluorescent emitters in a region of interest (ROI) and their associated molecular brightness. Then, by integrating the PCH into a Bayesian inference scheme, the relative probability of various numbers of fluorescent emitters can be compared, while expressing uncertainty in the molecular brightness. We experimentally demonstrate the model by counting quantum dots and varying numbers of fluorescent dye molecules attached to DNA origamis. In parallel, we theoretically examine second-order coherence of single-molecule fluorescence as a validation metric in both background-free environments and in the presence of a coherent background signal. Our findings indicate that sensitivity of the second order coherence to background conditions limits its effectiveness to scenarios with high signal-to-background ratios.

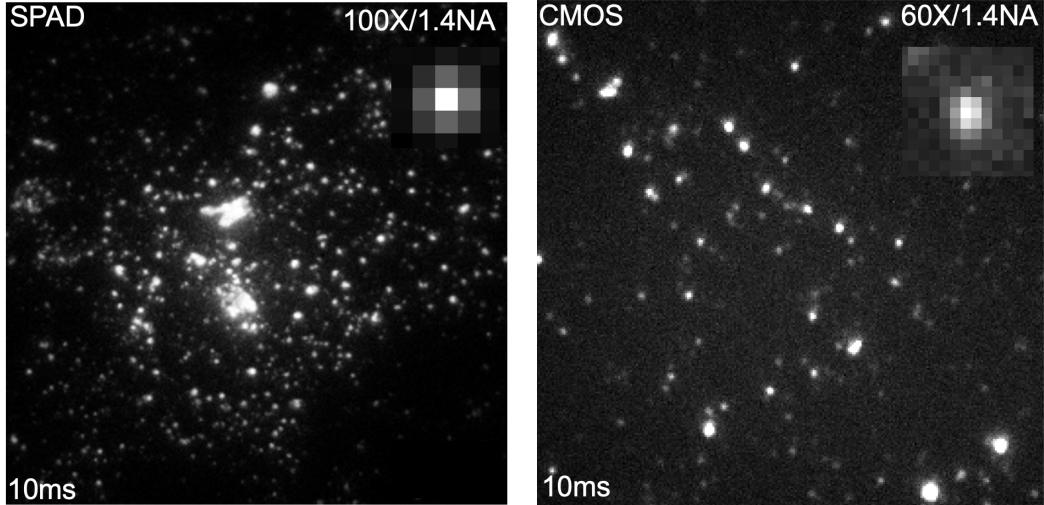


Figure 2.2. Comparison of quantum dot images between CMOS and SPAD cameras (left) SPAD image of Qdot655 coated on a glass coverslip using a 100X/1.4NA oil-immersion objective and a 10ms exposure time (right) CMOS image of Qdot655 using a 60X/1.4NA oil-immersion objective and a 10ms exposure time. Both use continuous-wave 640nm excitation

2.3.1 Results

Model

Consider a simplified description of widefield photon counting for a single photon source in the object plane labeled by a continuous-valued coordinate $\theta = (\theta_u, \theta_v)$. The spatial profile O of the field in image space is again presumed to have a Gaussian shape [11]–[13].

$$I(t) = \frac{W(t)}{2\pi\sigma^2} e^{-\frac{(u-\theta_u)^2 + (v-\theta_v)^2}{2\sigma^2}} \quad (2.2)$$

In general, fluorescent emission is a doubly stochastic process [29], due to temporal fluctuations in the intensity $W(t)$ as well as the quantum nature of light. We therefore define the molecular brightness $\zeta(t) \propto W(t)$ as the probability a photon is detected from a fluorescent emitter at a time t . All factors that affect the photon count rate, such as laser power, absorption cross section, fluorescence quantum yield, detector efficiency, etc., are absorbed into $\zeta(t)$. Under stationary conditions in which $W(t)$ and $\zeta(t)$ are constants,

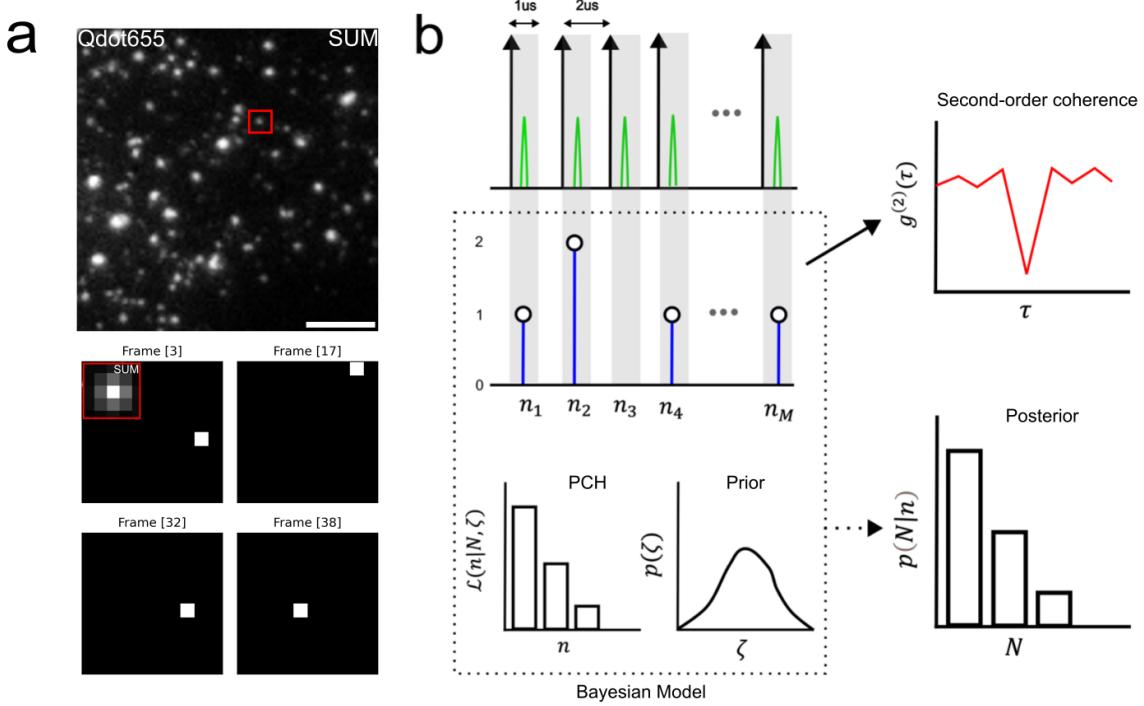


Figure 2.3. Single photon counting with a SPAD array (a) Simplified diagram of the widefield photon counting setup (b) Single photon imaging scheme using 1us exposures containing a picosecond laser pulse (c) Sum of photon counts over a 5x5 region of interest (ROI), taken with $N_{\text{frames}} = 5 \times 10^5$

we write $\zeta(t) = \zeta$. In this case, for an isolated single photon source, the PCH will follow a Bernoulli distribution:

$$\mathcal{L}_{\text{signal}}^{(1)}(n_{\text{signal}}|\zeta) = \text{Bernoulli}(\zeta) \quad (2.3)$$

If N fluorescent emitters are present in the ROI, the PCH will be the N -th convolution of the single emitter PCH with itself [29]

$$\mathcal{L}_{\text{signal}}(n_{\text{signal}}) = \mathcal{L}_{\text{signal}}^{(1)}(\zeta_1) \otimes \mathcal{L}_{\text{signal}}^{(2)}(\zeta_2) \otimes \dots \otimes \mathcal{L}_{\text{signal}}^{(N)}(\zeta_N) \quad (2.4)$$

Therefore, for N identical fluorophores emitting photons which can be detected within a ROI of the array, the number of signal photons measured n_{signal} following a single excitation pulse will have Binomial statistics $n_{\text{signal}} \sim \text{Binom}(N, \zeta)$. Photon pile-up at a single detector

element can be safely neglected in this model due to its relatively low likelihood. Coherent background signal will follow Poissonian statistics $n_{\text{bg}} \sim \text{Poisson}(\lambda)$ for an expected number λ of background counts in the ROI per frame. The total number of counts $n = n_{\text{signal}} + n_{\text{bg}}$ detected in the region of interest following a single pulse is then distributed by the PCH:

$$\mathcal{L}(n | N, \zeta, \lambda) = \mathcal{L}_{\text{signal}}(n_{\text{signal}} | N, \zeta) \otimes \mathcal{L}_{\text{bg}}(n_{\text{bg}} | \lambda) = \sum_{i=0}^{\infty} \binom{N}{i} \zeta^i (1 - \zeta)^{N-i} \frac{\lambda^{n-i}}{(n-i)!} e^{-\lambda} \quad (2.5)$$

The expression above represents a convolution of Poisson and Binomial probability mass functions. This result is the primary means of inference of the number of active emitters N in a ROI as well as theoretical analysis of the second-order coherence.

Empirical estimation of second-order coherence

To measure fluorescence antibunching in experiments, the second order coherence function $g^{(2)}(m)$ is used [28]. An empirical estimate of $g^{(2)}(m)$ is made, based on the number of coincidences $G^{(2)}(m)$ at a lag time m

$$g^{(2)}(m) = \frac{G^{(2)}(m)}{\langle G^{(2)}(m) \rangle} \quad (2.6)$$

The function $G^{(2)}(m)$ counts the number of coincidences at a lag time m and is normalized by its expectation $\langle G^{(2)}(m) \rangle$. It is well known that, for coherent (Poissonian) light, the second order coherence function $g^{(2)}(m)$ is approximately unity for all lags m . This is not necessarily the case, however, for binomial states [19] of the quantized radiation field. To investigate the properties of the $g^{(2)}(0)$ dip for binomial photon statistics, we compared the expected number of coincidences at zero-lag $G^{(2)}(0)$ as well as the $g^{(2)}(0)$ value for purely binomial or Poissonian photon statistics, with the same mean. As expected, we found that binomial statistics gave a dip below $g^{(2)}(0) = 0.5$ for small fluorophore numbers while Poissonian statistics gave a value of $g^{(2)}(0) = 0.5$ (Figure 2.8a). This result was also seen for pure binomial statistics (zero background signal) over a range of ζ values (Figure 2.8b). For nonzero background signal, we find that the signal to background ratio becomes relevant, with decreasing ζ values raising the expected dip to $g^{(2)}(0) = 0.5$ (Figure 2.8c).

Bayesian inference of the number of active emitters

When the number of fluorescent emitters N is unknown, the PCH (likelihood function) can be used in a Bayesian inference scheme to construct a posterior distribution on the binomial parameters:

$$p(N, \zeta|n) \propto p(n|N, \zeta)p(\zeta) \quad (2.7)$$

We use a Gaussian prior on ζ , i.e. $p(\zeta) = \mathcal{N}(\mu_\zeta, \sigma_\zeta^2)$. Prior uncertainty in the value of ζ stems from fluorophores with potentially complex photophysical properties. This posterior can be integrated over ζ to produce a posterior distribution on the fluorophore number N . That is, $p(N = N'|n) \propto \int_0^1 \mathcal{L}(n|N', \zeta)p(\zeta)d\zeta$ for $\mathcal{L}(n|N', \zeta) = \prod_{j=1}^{N_{\text{frames}}} p(n_j|N', \zeta)$. The likelihood is made tractable by a log-sum-exponential trick: $\mathcal{L}(n|N', \zeta) = e^{\left(\sum_j \ell(n_j|N', \zeta) + C\right)}$, where C is an arbitrary constant determined empirically. This same constant C is used for all N' . Monte Carlo integration is then employed to integrate out ζ . This involves sampling many ζ values from the Gaussian prior and, for each sampled ζ , the Poisson-Binomial likelihood $\mathcal{L}(n|N', \zeta)$ is computed. These likelihood values are then weighted by the prior probabilities $p(\zeta_i)$. The final result is obtained by averaging these weighted likelihoods over all sampled ζ , which approximates the integral:

$$p(N = N'|n) = \int_0^1 \mathcal{L}(n|N', \zeta)p(\zeta) d\zeta \approx \frac{1}{S} \sum_{i=1}^S \mathcal{L}(n|N', \zeta_i)p(\zeta_i) \quad (2.8)$$

where S is the number of samples from the prior. This method provides a powerful way to handle the integration of complex or high-dimensional functions, especially when analytical solutions are intractable. The final posterior can then be estimated by minibatching the data and averaging the posterior $p(N|n)$ over minibatches. An estimate of the fluorophore number N within each ROI is found by the maximum a posteriori (MAP) estimate N^* given by this distribution.

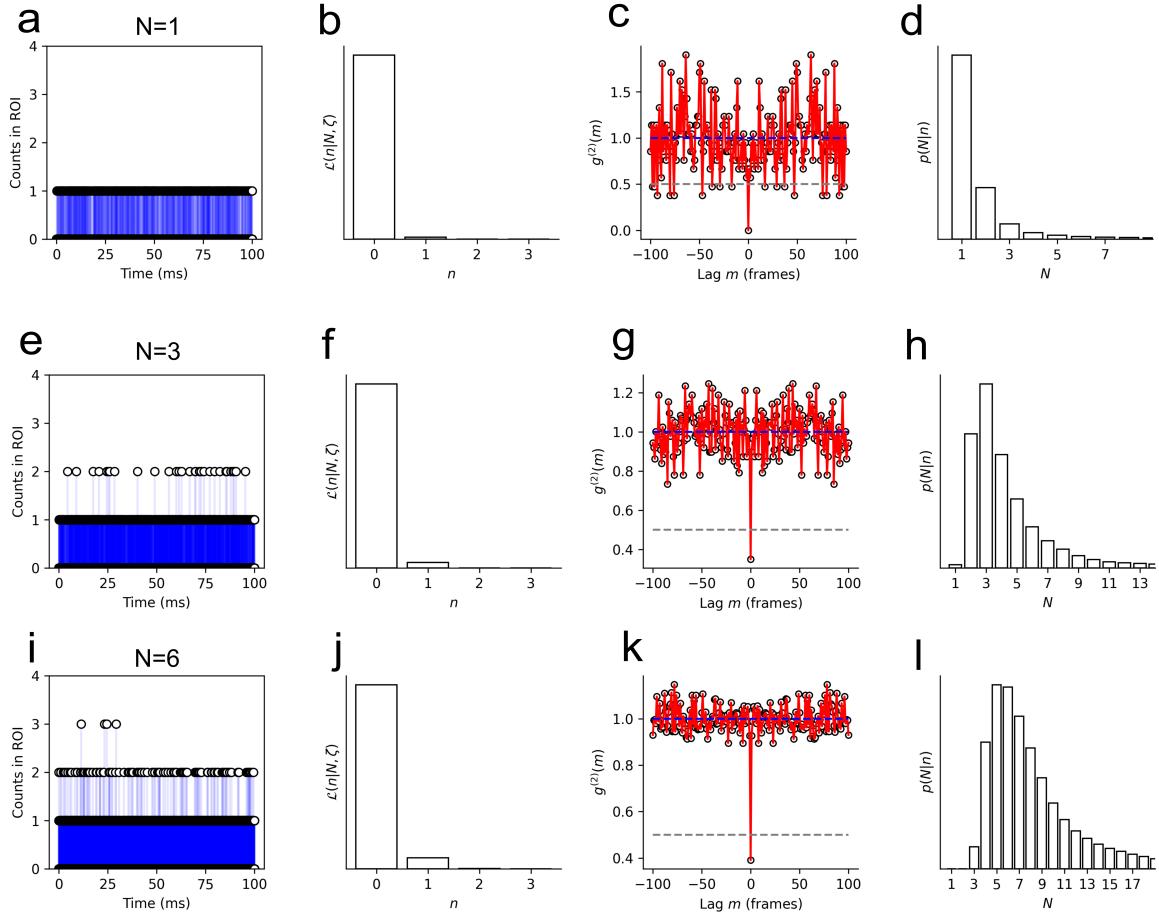


Figure 2.4. Photon counting histogram and analysis on zero-background simulations (a) Simulated photon counts in 1us exposure for $N = 1$ and $\zeta = 0.01$ (b) Photon counting histogram for counts in (a) (c) Second-order coherence function for data in (a) (d) Posterior distribution for data in (a) (e) Simulated photon counts in 1us exposure for $N = 1$ and $\zeta = 0.01$ (f) Photon counting histogram for counts in (e) (g) Second-order coherence function for data in (e) (h) Posterior distribution for data in (e) (i) Simulated photon counts in 1us exposure for $N = 1$ and $\zeta = 0.01$ (j) Photon counting histogram for counts in (i) (k) Second-order coherence function for data in (i) (l) Posterior distribution for data in (i) Inference in (d,h,l) use $\mu_\zeta = 0.01, \sigma_\zeta = 0.001$

Simulations

To validate our model, we apply it on the simulated photon emissions from single and multiple quantum emitters. Simulated photon count time-series were generating by sampling from the Poisson-Binomial PCH described using variable values for N and $\zeta = 0.01$ (Figure 2.4a,e,i). As expected, we found that $g^{(2)}(0) < 0.5$ while for increasing values of N , $g^{(2)}(0)$ approached $g^{(2)}(0) = 0.5$ (Figure 2.4c,g,k). Analysis of the posterior distribution on N successfully recovered the value of N used to parameterize simulations (Figure 2.4d,h,l).

Distinguishing single quantum dots from assemblies

A custom widefield fluorescence microscope was built for widefield photon counting by synchronization of laser pulses with 1-bit exposures of a SPAD array. This excitation scheme permits the computation of the second order coherence function $g^{(2)}(m)$ and visualization of the spatial intensity distribution by summing photon counts over time. The acquisition scheme and analytical methods were then applied in two experimental contexts. First, we examined the posterior distribution and $g^{(2)}(m)$ function for putatively isolated quantum dots and then examined quantum dot aggregates. Quantum dots exhibit temporally heterogeneous photoluminescence (PL) due to nonradiative transitions of electrons in the conduction band giving rise to a phenomenon known as blinking [19], [31]. Quantum dots showing clear two-state PL fluctuations exhibited $g^{(2)}(0) < 0.5$ and a posterior distribution maximized at $N = 1$ (Figure 2.5a-d). Aggregates of quantum dots with more complex PL fluctuations showed $g^{(2)}(0) = 0.5$ and a posterior distributed around larger values of N (Figure 2.5e-h)

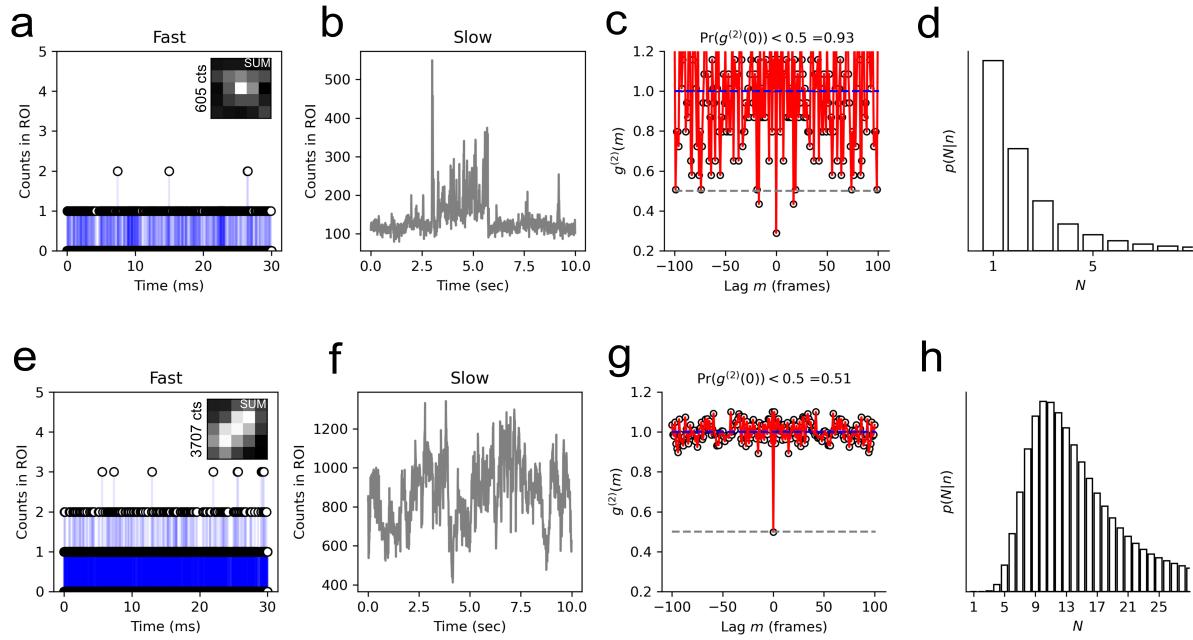


Figure 2.5. Distinguishing single and multiple quantum dots (a) Photon counts in 1 μ s exposure using 532nm pulsed excitation of a putatively isolated QD (b) Photon counts in 10ms exposure using 488nm continuous-wave excitation (c) Second-order coherence function for data in (a) (d) Posterior distribution for data in (a) (e) Photon counts in 1 μ s exposure using 532nm pulsed excitation of a QD aggregate (f) Photon counts in 10ms exposure using 488nm continuous-wave excitation (g) Second-order coherence function for data in (e) (h) Posterior distribution for data in (e). Inference parameters can be found in Table 1.

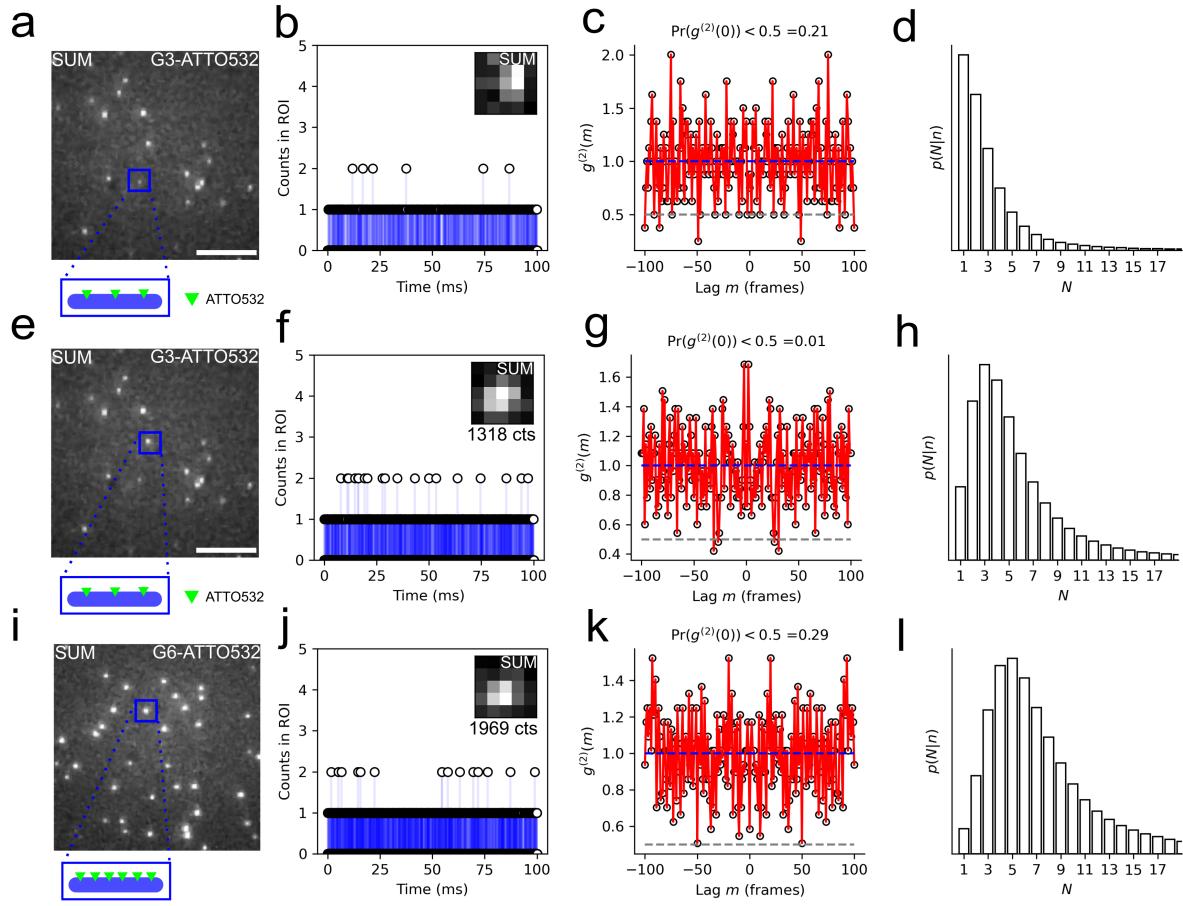


Figure 2.6. Counting ATTO532 dye bound to DNA origamis (a) Example DNA origami with three ATTO532 binding sites (b) Photon counts in 1us exposure using 532nm pulsed excitation of G3 sample and sum of counts (inset) (c) Second order coherence for the spot in (b) (d) Posterior distribution for the spot in (b) (e) Example DNA origami with three ATTO532 binding sites (f) Photon counts in 1us exposure using 532nm pulsed excitation of G3 sample and sum of counts (inset) (g) Second order coherence for the spot in (f) (h) Posterior distribution for the spot in (f) (i) Example DNA origami with six ATTO532 binding sites (G6 sample) (j) Photon counts in 1us exposure using 532nm pulsed excitation of G6 sample and sum of counts (inset) (k) Second order coherence for the spot in (j) (l) Posterior distribution for the spot in (j).

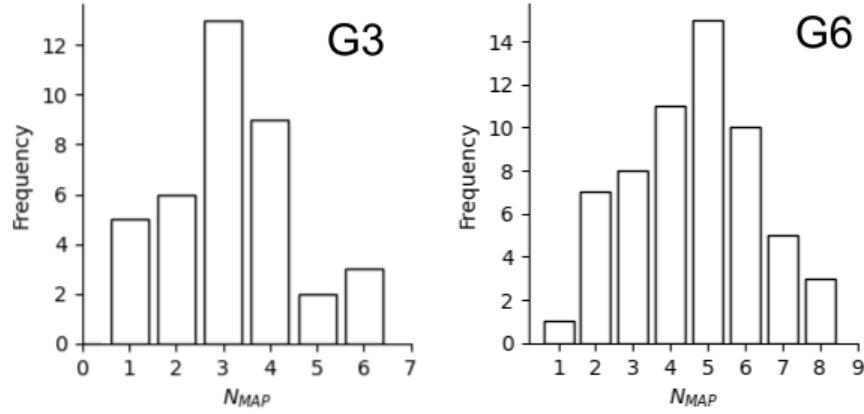


Figure 2.7. Distributions of the maximum a posteriori estimate of N (left) Distribution of estimate of N for DNA origamis with three ATTO532 binding sites (G3 sample) (right) Distribution of estimate of N for DNA origamis with six ATTO532 binding sites (G6 sample). Inference parameters can be found in Table 1.

Counting fluorophores bound to DNA origamis

In a second series of experiments, we examined the posterior distribution and $g^{(2)}(m)$ function for DNA origamis which can bind up to $N = 3$ or $N = 6$ ATTO532 fluorescent dye molecules, referred to as G3 and G6, respectively (Figure 2.6a,f,j) For origamis which can bind up to $N = 3$ ATTO532 dyes, no clear $g^{(2)}(0)$ dip was observed, due to the low signal to background ratio at this signal level (Figure 2.6c,g). For origamis which can bind up to $N = 6$ ATTO532 dyes, no clear $g^{(2)}(0)$ dip was observed, due to the large N character of the $g^{(2)}(0)$ dip predicted by the theory (Figure 2.6k). We conclude that the $g^{(2)}(0)$ dip may lead to ambiguous interpretations at low signal levels. However, the posterior distribution can recover the known N value from the photon count distribution for G3 (Figure 2.6d,h) and G6 (Figure 2.6l). Maximum a posteriori (MAP) estimates of the fluorophore number showed consistency with the known value of N for G3 and G6 (Figure 2.7).

Table 1 - Posterior Parameters

Sample	μ_ζ	σ_ζ	λ	Samples	Batches
Qdot655	0.01	0.005	0.008	100	50
ATTO532	0.002	0.001	0.006	100	50

2.3.2 Materials and Methods

Quantum dots and DNA origamis preparation

Fluorescent samples used here were either Quantum dots (Qdot655, ThermoFisher) coated on a glass coverslip, or ATTO532 tagged DNA origamis (GATTAAquant). Fluorophores in a region with quasi-uniform laser power were selected for analysis to simplify the prior distribution on the molecular brightness. DNA origami samples contained origamis with either three or six ATTO532 binding sites for testing the Bayesian inference scheme and second-order coherence analysis.

Single molecule imaging with the SPAD array

A 512x512 Fluorescent SPAD512 array (Pi Imaging Technologies) was connected to a custom single molecule imaging system (ASI). A picosecond 532nm pulsed laser (Picoquant) was triggered at 500 KHz as the excitation source. Laser power was set at 300uW at the back focal plane of the microscope objective for all experiments. Emission light was collected using an oil-immersion 100X/1.4NA objective (Nikon). The emission signal was filtered to exclude the laser line (Semrock) and projected onto the SPAD512 sensor using a tube lens. The acquisition of the SPAD assay is synchronized with the pulsed laser frequency. Each acquisition consists of a series of 1-bit frames, using a 1us exposure per frame. For quantum dot imaging, 30ms total exposure was used (30k frames). For DNA origami imaging, 100ms total exposure was used (100k frames). To obtain time-course data of photon counts, we (i) summed binary images over the entire acquisition (ii) estimated spot centroids using the Laplacian of Gaussian (LoG) detection algorithm, and (iii) extracted total counts in a 5x5 pixel region of interest around each detected spot.

Computation of the second order coherence

In practice, we compute the second order coherence at zero lag using

$$G^{(2)}(0) = \sum_t \mathbb{I}(n_t > 1)$$

where \mathbb{I} is an indicator function. The value of this function at nonzero lag m is given by

$$G^{(2)}(m) = \sum_t \mathbb{I}(n_t n_{t+m} \geq 1)$$

The second order coherence $g^{(2)}(m)$ is then computed over a range $-m_{min} \leq m \leq m_{max}$. Theoretical estimates of $g^{(2)}(0)$ can be obtained by determining the generating model of $G^{(2)}(m)$. We find that for a sequence of M frames,

$$G^{(2)}(0) \sim \text{Binomial}(M, p) \quad p = \sum_{n \geq 2} \mathcal{L}(n|N, \zeta)$$

$$G^{(2)}(m) \sim \text{Binomial}(M, q) \quad q = \left(\sum_{n \geq 1} \mathcal{L}(n|N, \zeta) \right)^2$$

Fluorescence antibunching is conventionally characterized by a dip in the $g^{(2)}(m)$ function for $m = 0$, indicating a reduced coincidence probability. Error in the estimate of $g^{(2)}(0)$ are found by the expression

$$\sigma = \text{RMSE}[g^{(2)}(0)] = \sqrt{\left[\frac{\partial g^{(2)}(0)}{\partial \langle G^{(2)}(m) \rangle} \delta \langle G^{(2)}(m) \rangle \right]^2 + \left[\frac{\partial g^{(2)}(0)}{\partial G^{(2)}(0)} \delta G^{(2)}(0) \right]^2} \quad (2.9)$$

One can then integrate a Gaussian with this σ below a threshold e.g, $g^{(2)}(0) = 0.5$ to obtain the confidence interval. Lastly, $G^{(2)}(0), G^{(2)}(m)$ will have Binomial statistics, which we approximate as Poisson for simplicity. Error estimates are then $\delta G^2(0) = \sqrt{G^2(0)}, \delta \langle G^2(m) \rangle = \sqrt{\frac{\langle G^2(m) \rangle}{M}}$. This expression simplifies considerably in the case that $\delta \langle G^{(2)}(m) \rangle$ i.e., certainty in $G^{(2)}(m)$.

$$\sigma_{g^{(2)}(0)} = \sqrt{\frac{p(1-p)}{Mq^2}} \quad (2.10)$$

The value of $\sigma_{g^{(2)}(0)}$ is therefore a function of ζ as well as the number of frames in the acquisition M .

2.3.3 Discussion

Here, we leveraged the recently developed SPAD array to spatially resolve the photon counting histogram with a widefield microscope. The PCH was integrated into a Bayesian inference scheme to extract the number of fluorescent emitters throughout the field of view and measure the second order coherence function. We demonstrated accurate counting of fluorescent quantum dots and fluorescent dyes bound to DNA origami, suggesting that this is a capable method for quantitative widefield fluorescent microscopy. Future work may assess more complex fluorescent imaging scenarios.

For example, fluorescence intermittency or photobleaching leads to a $\zeta(t)$ which is not constant and can be fluorophore specific. This may affect the observed photon-number fluctuations and the PCH function $\mathcal{L}_{\text{signal}}(n_{\text{signal}})$ used here may no longer be stationary. Therefore, due to the unique dynamics of $\zeta(t)$ for each fluorophore, this PCH is no longer appropriate and one can expect more complex behavior of $g^{(2)}(0)$. As an example, challenges may arise in distinguishing one homogeneously emitting fluorophore and several blinking fluorophores. If the effect of censoring photons by blinking can be accounted for, the technique used here may be compatible with common nanoscopy methods which rely on fluorescence intermittency. However, neither of these effects need be considered here. The tested quantum dots are photostable and photobleaching of ATTO532 could be mitigated under the experimental conditions used. Moreover, the on and off state lifetime of tested quantum dots and are significantly longer than the acquisition sequence, making fluorescence intermittency negligible.

The acquisition times necessary to obtain sufficient photon counts for computing the necessary statistics can potentially be very short. Most fluorophores have relaxation times in the nanosecond range and thus photons can be collected at a rate of tens of millions of excitation pulses per second. These rates are currently difficult to obtain, however, due to limitations

in detector throughput. Moreover, the data volume can quickly become intractable due to the need for several thousands of frames for a millisecond-scale exposure time.

Theoretical and experimental estimation of $g^{(2)}(0)$ demonstrate that the suitability of this measure can depend on experimental conditions. Collection of thousands of photons is necessary to reduce the error in the $g^{(2)}(0)$ value to its minimal value. Furthermore, we showed experimentally that low signal to background ratios results in loss of the binomial character of the data. This suggests the compatibility of bright fluorophores (such as quantum dots) for antibunching-based measurements. On the other hand, the Bayesian inference scheme of the fluorophore number will not be as sensitive to the fluorophore brightness in this way, as the PCH can effectively model various ζ values.

Lastly, the method proposed here may lead to significant advances in localization microscopy. Many of these schemes utilize the concept of precise localization of fluorescent emitters to produce super-resolved images [32], [33]. However, an inherent problem with such methods is the assumption that fluorescent emitters are isolated, which can lead to undercounting and localization errors. Various models for multi-emitter localization have been developed to approach this issue statistically [34]–[37], which necessarily treat the number of fluorescent emitters as an unknown. The approach proposed here provides a physical means to quantifying active fluorescent emitters based on widefield photon counting.

In conclusion, we propose a single molecule imaging technique that allows for counting of fluorescent molecules by modeling the quantum properties of fluorescence emission. The technique does not require a nonclassical light source and is designed to supplement standard single molecule localization microscopy techniques. The proposed method can be implemented with a standard widefield fluorescence microscope.

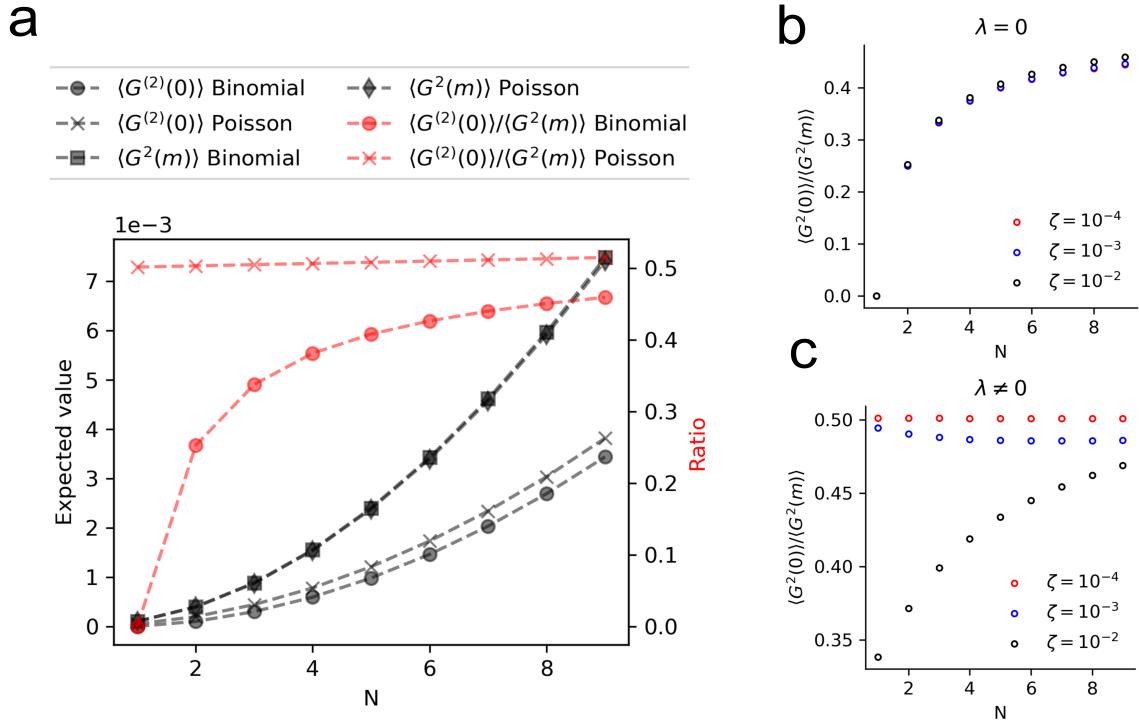


Figure 2.8. Theoretical scaling of the second-order coherence with the fluorophore number (a) Expected number of coincidences at zero-lag (black) and the $g^{(2)}(0)$ ratio (red) in the case of pure binomial or pure Poisson photon statistics, as a function of the fluorophore number. Poisson and Binomial data are assumed to have an identical mean of 0.01 (b) $g^{(2)}(0)$ as a function of the fluorophore number for zero background conditions (pure binomial statistics) and various ζ values (c) $g^{(2)}(0)$ as a function of the fluorophore number for nonzero background conditions (Poisson-Binomial statistics, $\lambda = 0.0075$) and various ζ values.

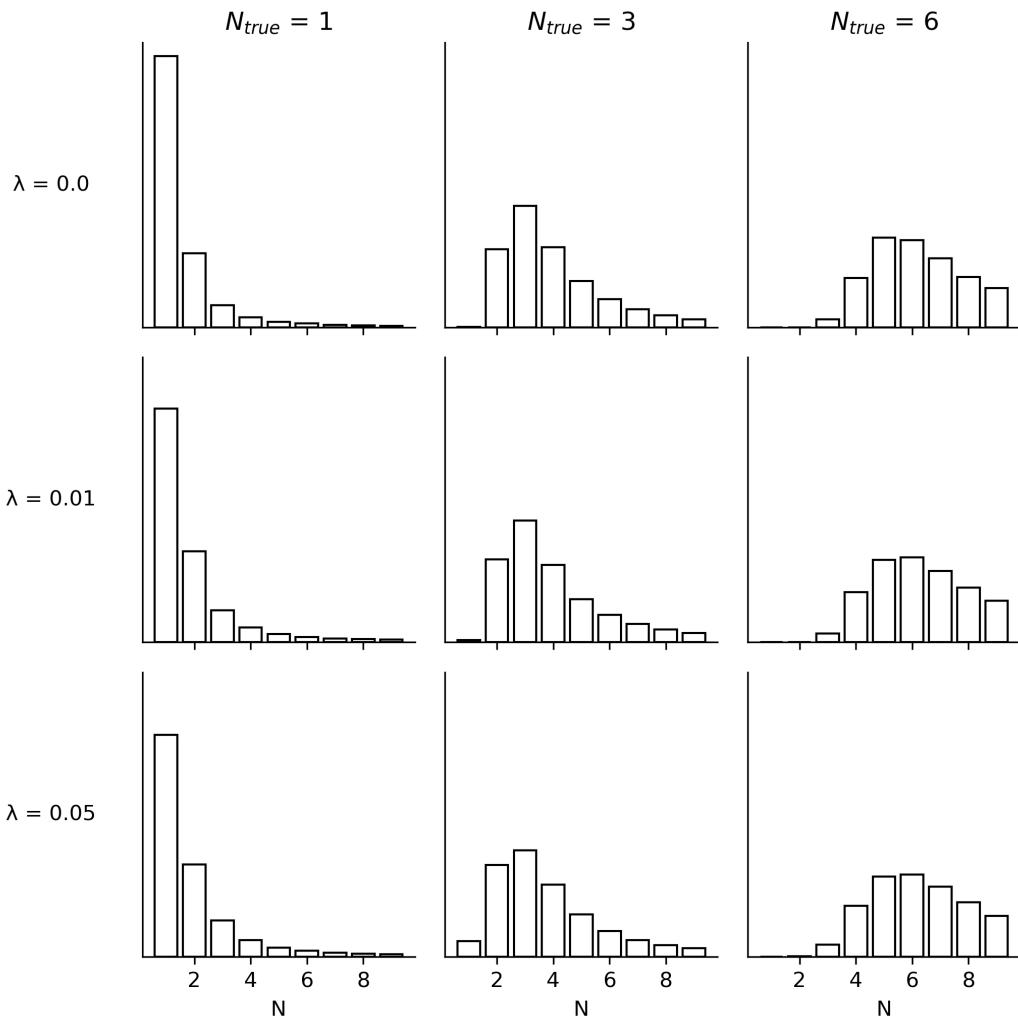


Figure 2.9. Posterior distributions of the fluorophore number Samples from the Poisson-Binomial convolution distribution using $\zeta = 0.01$ for various values of λ and $N = 1, 3, 5$ were simulated. The variable ζ was integrated out by Monte Carlo integration, sampling 1000 ζ values from the prior distribution (see main text for details)

2.4 Future directions: quantum illumination strategies

In recent years, novel quantum illumination strategies have leveraged spatial correlations between downconverted photon pairs to demonstrate the first quantum illumination full-field imaging protocol. These spatial quantum correlations are generated by a nonlinear optical process referred to as spontaneous parametric downconversion (SPDC). SPDC is typically realized using a nonlinear crystal such as Beta Barium Borate (BBO) or $\beta\text{-BaB}_2\text{O}_4$. BBO crystals are highly anisotropic and exhibit nonlinear optical properties, which means that polarization of the crystal varies nonlinearly with electric field strength. This nonlinear behavior can lead to a number of useful effects such as second harmonic generation, third harmonic generation, or SPDC [38]. In the SPDC process, a pump photon typically in the ultraviolet range is downconverted into a pair of lower-energy photons, with wavelengths in the visible range. These photons are known as signal and idler photons. This process is a purely quantum mechanical one, and the daughter photons born in this process possess useful quantum mechanical properties. Indeed, physical principles such as conservation of energy and momentum require that the daughter photons born in this nonlinear process are spatially entangled in several degrees of freedom [38].

A relatively straightforward application of SPDC is prediction of photon arrival times of the signal beam by measurement of the idler beam with a single photon sensitive detector. For example, this has been applied to improve the signal-to-noise ratio for measuring the absorption of objects through subshot-noise measurements [39] or imaging through detector noise and background signal [40], [41]. In such applications, twin beams produced through the SPDC process are directed to different regions of a spatially resolved detector such as a CCD camera or single-photon avalanche diode (SPAD) array detector. By performing a pixel-by-pixel AND-operation between two regions of the array detector containing the SPDC beams, we can preferentially select correlated photon-pair events and reject uncorrelated background light and sensor noise events. Heralding photons using the signal and idler beams involves detecting one photon (either the signal or idler) and using this detection to infer the presence of its entangled counterpart. This method enhances the precision of photon detection and significantly improves the signal-to-noise ratio in quantum imaging.

This methodology has yet to be fully demonstrated in single molecule imaging or localization microscopy, which is generally quite sensitive to background signal or detector noise. Moreover, typical CCD technologies do not possess the time resolution of novel SPAD arrays, which may permit spatially resolved coincidence detection of photon pairs with nanosecond time resolution. Incorporating this technique into fluorescence imaging may further enhance the imaging quality and localization precision. By utilizing the known fluorescence lifetime of a single photon source such as a fluorescent dye molecule, one can possibly herald a fluorescence photon by measurement of the idler beam.

3. UNCERTAINTY AWARE LOCALIZATION MICROSCOPY BY VARIATIONAL DIFFUSION

3.1 Background

3.1.1 The curse of dimensionality

Dimensionality refers to the number of variables or features in a dataset. In conventional fluorescence nanoscopy, an image generated by (1.1) containing N fluorophores has $2N$ parameters. Similarly, the image itself is a high dimensional variable where the number of pixels is the number of dimensions. As the number of dimensions in the parameter space increases, it becomes increasingly difficult to infer the parameters from the data. The curse of dimensionality is a term coined to describe various phenomena that arise when working with high-dimensional data, making statistical analysis and machine learning more challenging. Mathematically, the curse of dimensionality can be described through the following issues.

As dimensionality increases, the volume of the space grows exponentially. For instance, the volume of a hypercube with side length l in d -dimensional space is given by l^d . This rapid increase in volume means that data points become sparser, making it difficult to estimate densities and find meaningful patterns. Furthermore, in high-dimensional spaces, the concept of distance becomes less informative. The difference between the minimum and maximum distance between data points tends to shrink as dimensionality increases, which can make clustering and nearest-neighbor algorithms less effective. With a large number of dimensions, models can easily become overly complex, capturing noise instead of the underlying signal. This leads to overfitting, where the model performs well on training data but poorly on unseen data.

Deep models have captured the attention of many researchers, as they can perform inference tasks in high dimensional spaces without an excessive computational burden. These models are not entirely immune to the curse of dimensionality or the complexity of data distributions, however, and overfitting is still an important problem. Simultaneously, many deep models are deterministic neural networks, which give a single output given an input. This is in sharp contrast to classical statistical inference methodologies such as Bayesian inference, which model a distribution on their outputs, allowing one to express uncertain-

ties during inference. Bayesian methods such as Markov Chain Monte Carlo (MCMC) or variational inference lack the scaling of deep learning but maintain a scientific value [42].

3.1.2 The Bayesian calculation

Bayesian inference provides a rigorous framework for updating beliefs about the world in light of new data. It leverages the principles of probability theory to combine prior knowledge with empirical evidence, resulting in updated, posterior beliefs. In parametric modeling, we assume that the data x are generated from a distribution with a set of parameters y . Parametric models simplify the problem by assuming a specific form for the underlying distribution of the data, characterized by a finite set of parameters. This approach allows us to use mathematical functions to describe complex systems and make inferences about the parameters based on observed data.

At the heart of Bayesian inference is Bayes' rule, which allows us to update our beliefs based on new evidence. Bayes' rule is derived from the definition of conditional probability. The conditional probability of y given x is defined as $p(y|x) = \frac{p(x,y)}{p(y)}$ provided that $p(y) > 0$. Similarly, the conditional probability of x given y is $p(x|y) = \frac{p(x,y)}{p(x)}$. Rearranging these equations and solving for $p(y|x)$ gives us Bayes' rule:

$$p(y|x) = \frac{p(x|y)p(y)}{p(x)}.$$

Here, $p(y|x)$ is the called the posterior distribution, representing our updated beliefs about the parameters after observing the data. $p(x|y)$ is the likelihood, the probability of the observed data given the parameters. $p(y)$ is the prior distribution, representing our beliefs about the parameters before observing the data. $p(x)$ is the marginal likelihood or evidence, which normalizes the posterior distribution and ensures it sums to one.

One of the main challenges in Bayesian inference is the computation of the posterior distribution. The denominator in Bayes' rule, $p(x)$, involves an integral over all possible values of the parameters:

$$p(x) = \int p(x|y)p(y) dy.$$

In many practical applications, this integral is intractable due to the high dimensionality of the parameter space. To address this challenge, various approximation methods have been developed. One such method is Markov Chain Monte Carlo (MCMC), which generates samples from the posterior distribution by constructing a Markov chain that has the desired distribution as its equilibrium distribution. MCMC algorithms were originally developed for applications in statistical physics but were eventually adapted to a broader range of statistical problems. Well known MCMC algorithms include the Metropolis-Hastings algorithm, Gibbs sampler [43], and gradient-based algorithms inspired by Langevin dynamics. In general, MCMC is asymptotically exact but can be computationally expensive for large datasets and high dimensional parameter spaces. Variational inference is an alternative approach, which is not exact, but reduces the computational burden in these scenarios.

3.1.3 Variational inference

Variational inference involves approximating the true posterior distribution $q(y|x)$ with a simpler, parameterized distribution $p_\psi(y)$ by minimizing the Kullback-Leibler (KL) divergence between them. The KL divergence $D_{KL}(q(y|x)||p_\psi(y))$ measures how one probability distribution diverges from a second probability distribution. To minimize the KL divergence, we first express it as

$$D_{KL}(q(y|x)||p_\psi(y)) = \mathbb{E}_{q(y|x)} \left[\log \frac{q(y|x)}{p_\psi(y)} \right] \quad (3.1)$$

This can be rewritten using Bayes' rule as

$$\begin{aligned} D_{KL}(q(y|x)||p_\psi(y|x)) &= \mathbb{E}_{q(y|x)} \left[\log \frac{q(y|x)}{p_\psi(y|x)} \right] \\ &= \mathbb{E}_{q(y|x)} \left[\log \frac{q(y|x)p_\psi(x)}{p_\psi(x,y)} \right] \\ &= \mathbb{E}_{q(y|x)} \left[\log \frac{q(y|x)}{p_\psi(x,y)} \right] + \mathbb{E}_{q(y|x)} [\log p_\psi(x)] \end{aligned}$$

Defining $\mathcal{L} = -\mathbb{E}_{q(y|x)} \left[\log \frac{q(y|x)}{p_\psi(x,y)} \right] = \mathbb{E}_{q(y|x)} \left[\log \frac{p_\psi(x,y)}{q(y|x)} \right]$, we can write,

$$\mathbb{E}_{q(y|x)} [\log p_\psi(x)] = D_{KL}(q(y|x)||p_\psi(y|x)) + \mathcal{L} \geq \mathcal{L} \quad (3.2)$$

which is often called a variational objective. Given that $\log p_\psi(x)$ is constant, minimizing $D_{KL}(q(y|x)||p_\psi(y|x))$ is equivalent to maximizing \mathcal{L} . For practical reasons, we often instead solve a minimization problem with respect to ψ

$$\mathbb{E}_{q(y|x)} [-\log p_\psi(x)] \leq -\mathcal{L} = \mathbb{E}_{q(y|x)} \left[-\log \frac{p_\psi(x,y)}{q(y|x)} \right] \quad (3.3)$$

This approach provides a practical way to perform Bayesian inference, especially in high-dimensional settings. It is worth noting that this derivation often begins by taking the expectation with respect to the model distribution, since the true distribution is unknown. However, for the following application, the target distribution is known and thus the objective is formulated in this way.

3.2 Uncertainty-aware localization microscopy

Deep models have attracted tremendous attention from researchers in the natural sciences, with several foundational applications arising in microscopy [44], [45]. In particular, the application of deep models for image restoration in single-molecule localization microscopy (SMLM) has received considerable interest. The use of deep models in localization microscopy has been proposed as an alternative to traditional localization algorithms to increase imaging speed and enhance spatial resolution. For example, localization in dense scenes has been performed using traditional convolutional networks to reduce the total acquisition time to generate a super-resolution image [34], [35].

Localization in dense scenes is a difficult inverse problem, and a single measurement can underdetermine the number of spots and their respective locations. Since this problem is ill-posed, we choose to model the uncertainty directly. Therefore, we approach localization in dense scenes by predicting high-resolution kernel density (KD) estimates from low resolution images using a diffusion model. Diffusion models can model the conditional probability distribution on high-resolution images given low-resolution inputs. This contrasts with tradi-

tional methods based on convolutional networks, which provide point estimates and preclude computation of uncertainty at test time under a fixed model.

Our implementation is based on the conditional variational diffusion model (CVDM) – a type of continuous-time diffusion model developed in the context of image super-resolution [46]. CVDM is inspired by recent variational perspectives on diffusion [47]–[50]. Variational diffusion models provide a mechanism for scalable inference, which can be trained using a variational bound written in terms of the signal-to-noise ratio of the diffused data, and a simple noise estimation loss. In the remainder of this paper, we introduce a generative model on dense localization microscopy images and use it to simulate data for training and validation of CVDM. The model is then tested on experimental images of the nuclear pore complex protein 96 (Nup96) tagged with mMaple.

3.2.1 Approach

Direct optimization of the image likelihood from observations \mathbf{x} alone is challenging when fluorescent emitters are dense within the field of view and fluorescent signals significantly overlap. However, CNNs have recently proven to be powerful tools fluorescence microscopy to extract parameters describing fluorescent emitters such as color, emitter orientation, z -coordinate, and background signal [51]–[53]. For localization tasks, CNNs typically employ upsampling layers to reconstruct Bernoulli probabilities of emitter occupancy [34], [35]. We choose to instead infer KDEs, denoted by \mathbf{y} , which are latent in the low-resolution data \mathbf{x} . KDEs are a common data structure used in nanoscopy, and can be easily generated from molecular coordinates, alongside observations \mathbf{x} .

To go beyond point estimation of the KDE given the low-resolution data, we seek a generative model for the posterior. Unfortunately, the posterior can be intractable, since molecules cannot be easily resolved and therefore its dimensionality is not well-defined. To address this issue, we instead model a conditional distribution on the latent \mathbf{y} : $p(\mathbf{y}|\mathbf{x})$, where \mathbf{y} is of known dimensionality. We choose to model $p(\mathbf{y}|\mathbf{x})$ with a diffusion model, given that the distribution $p(\mathbf{y}|\mathbf{x})$ is expensive to compute.

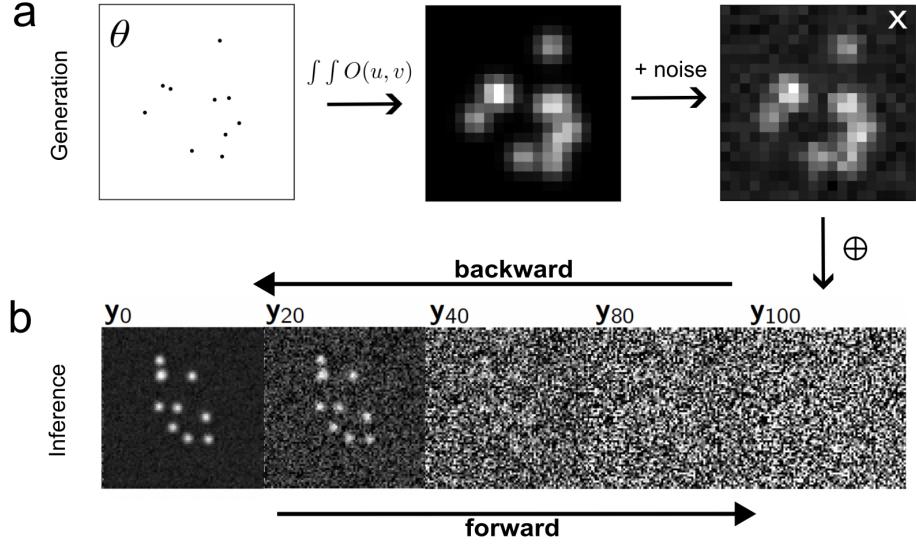


Figure 3.1. Generation of localization microscopy images and diffusion-based inference to enhance resolution (a) Pipeline of generation of low-resolution localization microscopy image \mathbf{x} using the point spread function model $O(u, v)$ and noise model (b) Concatenation of 4x interpolated low-resolution images with Gaussian noise and application of the conditional variational diffusion model for inference of a high-resolution image \mathbf{y}_0 via the reverse process.

3.2.2 Variational diffusion

Diffusion models [54], [55] are a class of generative models originally inspired by nonequilibrium statistical physics, which slowly destroy structure in a data distribution via a fixed Markov chain referred to as the *forward process*, which is characterized by a variance schedule $\gamma(t, \mathbf{x})$:

$$q(\mathbf{y}(t)|\mathbf{y}_0) = \mathcal{N}(\sqrt{\gamma(t, \mathbf{x})}\mathbf{y}_0, (1 - \gamma(t, \mathbf{x}))I)$$

Note that the continuous-time variance schedule $\gamma(t, \mathbf{x})$ is analogous to the discrete-time product of variances used in discrete models [54]. However, the relationship between the variance schedule $\gamma(t, \mathbf{x})$ and the variance between fixed time points is more complex in the continuous-time formalism. Suppose times runs over the continuous interval $[0,1]$ and the

diffusion process is sampled at T time steps in intervals Δt . We define the *stepped forward process* as:

$$q(\mathbf{y}(t + \Delta t) | \mathbf{y}(t)) = \mathcal{N}(\sqrt{1 - \beta_T(t, \mathbf{x})}\mathbf{y}_0, \beta_T(t, \mathbf{x})I)$$

where $\beta_T(t, \mathbf{x}) = 1 - \frac{\gamma(t + \Delta t, \mathbf{x})}{\gamma(t, \mathbf{x})}$. The value of $\beta_T(t, \mathbf{x})$ controls the variance of the transition of $\mathbf{y}(t)$ over a period of time Δt . The *stepped reverse process* reads:

$$p(\mathbf{y}(t) | \mathbf{y}(t + \Delta t)) = \mathcal{N}\left(\boldsymbol{\mu}_\psi(\mathbf{y}(t + \Delta t)), \beta_T(t, \mathbf{x}) \frac{1 - \gamma(t, \mathbf{x})}{1 - \gamma(t + \Delta t, \mathbf{x})} I\right),$$

where $\boldsymbol{\mu}_\psi(\mathbf{y}(t + \Delta t))$ is the estimated expected value of the transition distribution by a neural network ψ . This expectation or drift can be seen as the central component to the diffusion model. However, it is not estimated directly. First, notice that by the definition of the forward process, it can be reparametrized in terms of Gaussian noise with zero mean and unit variance: $\mathbf{y}(t + \Delta t) = \sqrt{\gamma(t + \Delta t, \mathbf{x})}\mathbf{y}_0 + \sqrt{1 - \gamma(t + \Delta t, \mathbf{x})}\boldsymbol{\epsilon}$ where $\boldsymbol{\epsilon} \sim \mathcal{N}(0, \mathbf{I})$. Then, we can estimate \mathbf{y}_0 by rearranging this expression and using a neural network to estimate the noise $\hat{\boldsymbol{\epsilon}}$ i.e., $\hat{\mathbf{y}}_0 = \frac{1}{\sqrt{\gamma(t + \Delta t, \mathbf{x})}} (\mathbf{y}(t + \Delta t) - \sqrt{1 - \gamma(t + \Delta t, \mathbf{x})}\hat{\boldsymbol{\epsilon}})$.

Finally, to sample from the reverse process, we can instead sample from the forward process starting at $\hat{\mathbf{y}}_0$. We find that $\boldsymbol{\mu}_\psi(\mathbf{y}(t + \Delta t))$ can be computed from the noise estimate:

$$\boldsymbol{\mu}_\psi(\mathbf{y}(t + \Delta t)) = \sqrt{\gamma(t, \mathbf{x})}\hat{\mathbf{y}}_0 = \frac{1}{\sqrt{1 - \beta_T(t, \mathbf{x})}} (\mathbf{y}(t + \Delta t) - \sqrt{1 - \gamma(t + \Delta t, \mathbf{x})}\hat{\boldsymbol{\epsilon}})$$

Repeated application of this process permits sampling from the reverse process over several time steps. Here, we will use 200 time steps for all experiments.

3.2.3 Results

Localization error

To verify the localizations made by the diffusion model, we simulated localization microscopy datasets with various densities (Figure 3.2). For a KDE predicted by the diffusion model, objects are detected using the Laplacian of Gaussian (LoG) detection algorithm,

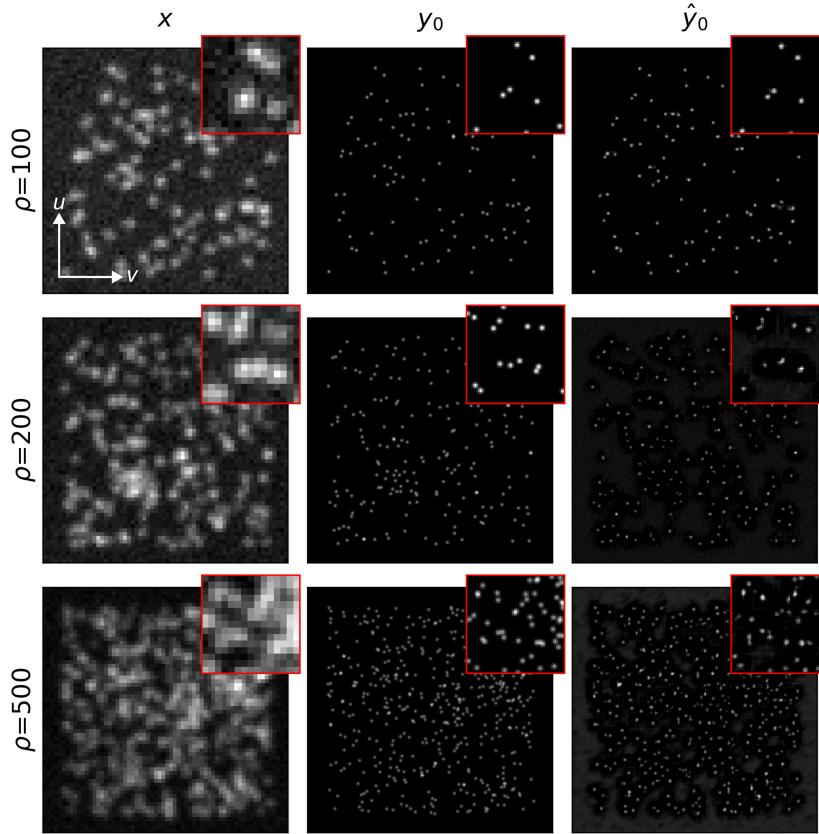


Figure 3.2. Performance of conditional variational diffusion on simulated data (left) Example low resolution, (middle) high resolution, and (right) estimated high resolution localization microscopy images at fluorophore numbers ρ in the image.

which permits more direct computation of localization error, compared to image similarity measures. Moreover, localization with sub-pixel precision can be easily implemented by identification of local maxima in the predicted KDE [34]. A particular LoG localization in the KDE is paired to the nearest ground truth localization and is unpaired if a localization is not within 5 KDE pixels (125nm) of any ground truth localization. As expected, we find a density dependence to localization error in the u and v directions, over the tested signal range (Figure 3.3). In addition to localization error, we measure a density-dependent precision $P = TP/(TP+FP)$ and recall $R = TP/(TP+FN)$ where TP denotes true positive localizations,

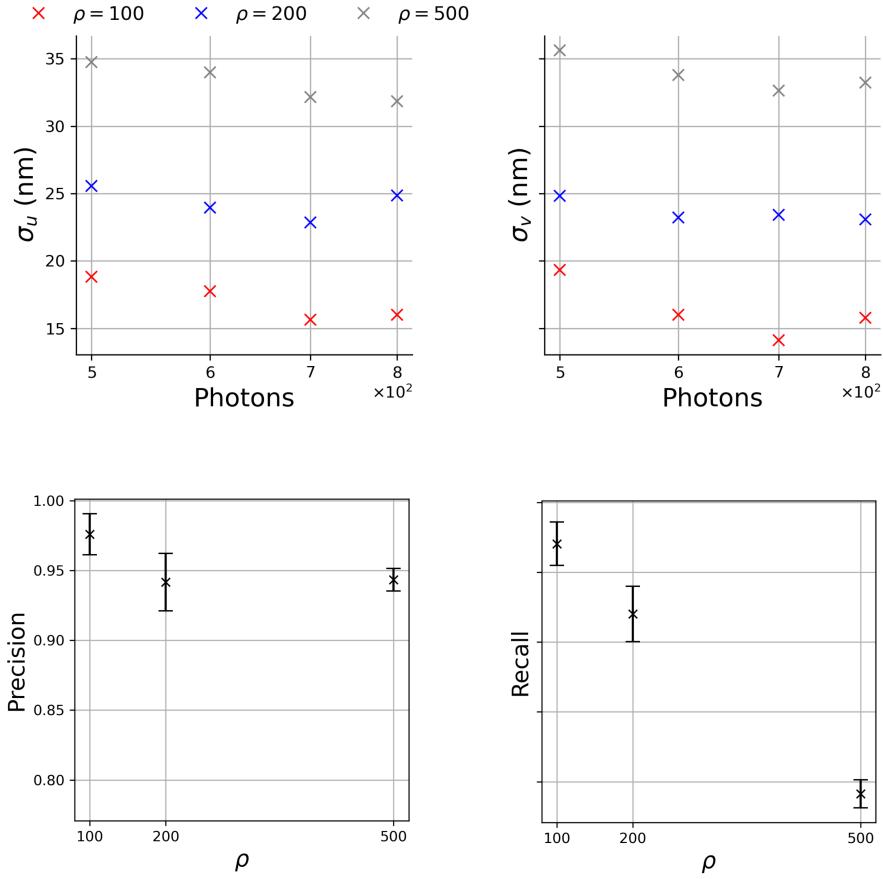


Figure 3.3. Performance of conditional variational diffusion on simulated data (top) Localization uncertainty in the u and v directions as a function of incident photon count, for various densities (bottom) Precision and recall of localized spots as a function of ρ . Error bars represent standard deviations over nine batches of data.

FP denotes false positive localizations, and FN denotes false negative localizations (Figure 3.3).

Sample variation for simulated localization microscopy data

Leveraging the ability of a diffusion process to model a distribution on high-resolution KDEs $p(\mathbf{y}_0|\mathbf{x})$ as opposed to a deterministic point estimate of \mathbf{y}_0 , we assess the sample variation on simulated localization microscopy data. Upon inspection of samples from the

diffusion model, we find that computing a pixel-wise average and standard deviation can summarize model predictions (Figure 3.4a). However, careful inspection of the individual samples can reveal the appearance/disappearance of predicted spots in the KDE across samples, expressing uncertainty in the number of spots in the KDE due to the many to one nature of image restoration tasks (Figure 3.4b). Furthermore, variation in the predicted locations of spots in the KDE is apparent, with more variation in higher density samples (Figure 3.4c).

Application to Nup96 localization microscopy data

To validate the model on experimental localization microscopy data, we create dense images by summing five consecutive frames of sparsely labeled nuclear pore complex protein 96 (Nup96) tagged with mMaple [35] (Figure 3.5a). Scattered localizations predicted by the CVDM model showed strong agreement with standard maximum likelihood estimation (MLE) based fitting of single low-resolution frames (Figure 3.5b). An obvious resolution enhancement was observed in CVDM predictions compared to low-resolution inputs (Figure 3.5c). By matching CVDM predicted coordinates to MLE localizations using a search radius of 50nm, we find that over 99 percent of CVDM localizations match to a MLE coordinate. Moreover, 60 percent of MLE coordinates were recovered, which is an acceptable result since MLE often detects the same fluorescent emitter multiple times with localization differences far below the minimum resolvable distance of CVDM.

3.2.4 Discussion

Application of the CVDM model to localization microscopy showed that the model is capable of not only enhancing the resolution of fluorescence images but also performing localization tasks. Indeed the model can maintain approximately 30nm localization error and high precision/recall on dense images. This result suggests that this model could be used to speed up localization microscopy acquisitions and enhance their resolution, while expressing uncertainty in the predicted outputs.

Importantly, the principles underlying this method resonate across various fields, suggesting its potential applicability in domains beyond localization. For instance, the extension of similar techniques to general image processing tasks highlights the potential to address uncertainty in a wide range of applications in bioimaging or medical imaging. Moreover, the utilization of diffusion models for uncertainty estimation aligns with a broader trend in leveraging probabilistic frameworks for enhancing deep learning applications. By bridging these interdisciplinary boundaries, this method not only addresses a critical need in localization microscopy but also contributes to the advancement of uncertainty-aware deep learning methodologies.

Appendix

3.2.5 Loss derivation

The complete loss given in [46] reads

$$\mathcal{L} = \mathcal{L}_{\text{prior}} + \mathcal{L}_\infty + \alpha \mathcal{L}_\gamma + \mathcal{L}_\beta \quad (3.4)$$

where α is a constant controlling the contribution of \mathcal{L}_γ to the total loss. We now derive the first two terms and discuss the latter two terms in the following section. Similar derivations can be found in [46], [48], [49]. The approach starts with writing the ELBO for a discrete-time diffusion model, and then taking the limit $T \rightarrow \infty$. In the discrete-time formalism time is indexed as $t \in \{1, 2, \dots, T\}$. The ELBO reads

$$\begin{aligned} -\log p(\mathbf{y}_0) &\leq -\mathbb{E}_{q(\mathbf{y}_{1:T}|\mathbf{y}_0)} \log \frac{p(\mathbf{y}_{0:T})}{q(\mathbf{y}_{1:T}|\mathbf{y}_0)} \\ &= -\mathbb{E}_{q(\mathbf{y}_{1:T}|\mathbf{y}_0)} \log \frac{p(\mathbf{y}_T)p(\mathbf{y}_0|\mathbf{y}_1)\prod_{t=2}^T p(\mathbf{y}_{t-1}|\mathbf{y}_t)}{q(\mathbf{y}_T|\mathbf{y}_0)\prod_{t=2}^T q(\mathbf{y}_{t-1}|\mathbf{y}_t, \mathbf{y}_0)} \\ &= -\mathbb{E}_{q(\mathbf{y}_{1:T}|\mathbf{y}_0)} \left[p(\mathbf{y}_0|\mathbf{y}_1) + \log \frac{p(\mathbf{y}_T)}{q(\mathbf{y}_T|\mathbf{y}_0)} + \sum_{t=2}^T \log \frac{p(\mathbf{y}_{t-1}|\mathbf{y}_t)}{q(\mathbf{y}_{t-1}|\mathbf{y}_t, \mathbf{y}_0)} \right] \\ &= -\mathbb{E}_{q(\mathbf{y}_{1:T}|\mathbf{y}_0)} [p(\mathbf{y}_0|\mathbf{y}_1)] + D_{KL}(q(\mathbf{y}_T|\mathbf{y}_0)||p(\mathbf{y}_T)) \\ &\quad + \sum_{t=2}^T \mathbb{E}_{q(\mathbf{y}_t|\mathbf{y}_0)} D_{KL}(q(\mathbf{y}_{t-1}|\mathbf{y}_t, \mathbf{y}_0)||p(\mathbf{y}_{t-1}|\mathbf{y}_t)) \end{aligned}$$

As before, we omit conditioning on \mathbf{x} to simplify the notation. The first term is typically ignored, as it does not contribute meaningfully to the loss [48]. Therefore we are left two terms, called the prior loss $\mathcal{L}_{\text{prior}}$ and diffusion loss \mathcal{L}_T . The former is straightforward to optimize using analytical expressions [46]. Furthermore, define β_t as the variance at time step t , $\alpha_t = 1 - \beta_t$ and $\gamma_t = \prod_t \alpha_t$. The latter KL-divergence of q and p is between two Gaussians with identical variances $\sigma^2 = \frac{(1-\gamma_{t-1})(1-\alpha_t)}{1-\gamma_t}$, and expectations

$$\boldsymbol{\mu} = \frac{\sqrt{\gamma_{t-1}}(1-\alpha_t)}{1-\gamma_t} \mathbf{y}_0 + \frac{\sqrt{\alpha_t}(1-\gamma_{t-1})}{1-\gamma_t} \mathbf{y}_t \quad \boldsymbol{\mu}_\psi = \frac{\sqrt{\gamma_{t-1}}(1-\alpha_t)}{1-\gamma_t} \hat{\mathbf{y}}_0 + \frac{\sqrt{\alpha_t}(1-\gamma_{t-1})}{1-\gamma_t} \mathbf{y}_t$$

for a fixed noise schedule [56]. Therefore, we have

$$\begin{aligned} D_{KL}(q(\mathbf{y}_{t-1}|\mathbf{y}_t, \mathbf{y}_0) || p(\mathbf{y}_{t-1}|\mathbf{y}_t)) &= \frac{1}{2\sigma^2} \|\boldsymbol{\mu} - \boldsymbol{\mu}_\psi\|_2^2 \\ &= \frac{1}{2} \frac{\gamma_{t-1}(1-\alpha_t)}{(1-\gamma_{t-1})(1-\gamma_t)} \|\mathbf{y}_0 - \hat{\mathbf{y}}_0\|_2^2 \\ &= \frac{1}{2} \frac{\gamma_{t-1}((1-\gamma_t) - \alpha_t(1-\gamma_{t-1}))}{(1-\gamma_{t-1})(1-\gamma_t)} \|\mathbf{y}_0 - \hat{\mathbf{y}}_0\|_2^2 \\ &= \frac{1}{2} \frac{\gamma_{t-1}((1-\gamma_t) - \frac{\gamma_t}{\gamma_{t-1}}(1-\gamma_{t-1}))}{(1-\gamma_{t-1})(1-\gamma_t)} \|\mathbf{y}_0 - \hat{\mathbf{y}}_0\|_2^2 \\ &= \frac{1}{2} \left(\frac{\gamma_{t-1}}{1-\gamma_{t-1}} - \frac{\gamma_t}{1-\gamma_t} \right) \|\mathbf{y}_0 - \hat{\mathbf{y}}_0\|_2^2 \\ &= \frac{1}{2} (\text{SNR}_{t-1} - \text{SNR}_t) \|\mathbf{y}_0 - \hat{\mathbf{y}}_0\|_2^2 \end{aligned}$$

where we have defined $\text{SNR}_t = \frac{\gamma_t}{1-\gamma_t}$. Putting it all together gives that

$$\mathcal{L}_T = \frac{1}{2} \sum_{t=2}^T \mathbb{E}_{q(\mathbf{y}_t|\mathbf{y}_0)} (\text{SNR}_{t-1} - \text{SNR}_t) \|\mathbf{y}_0 - \hat{\mathbf{y}}_0\|_2^2 \quad (3.5)$$

Using a Monte Carlo estimate of \mathcal{L}_T [50] which optimizes random terms of the summation to avoid calculating all terms simultaneously, we arrive at the objective written in the main text (8)

$$\mathcal{L}_T = \frac{T-1}{2} \mathbb{E}_{\epsilon \sim \mathcal{N}(0,I), t \sim U(1,T)} (\text{SNR}_{t-1} - \text{SNR}_t) \|\mathbf{y}_0 - \hat{\mathbf{y}}_0\|_2^2 \quad (3.6)$$

Taking the limit $T \rightarrow \infty$, this becomes:

$$\begin{aligned}\mathcal{L}_\infty &= -\frac{1}{2} \mathbb{E}_{\epsilon \sim \mathcal{N}(0, I)} \int_0^1 \text{SNR}'(t) \|\mathbf{y}_0 - \hat{\mathbf{y}}_0\|_2^2 dt \\ &= -\frac{1}{2} \mathbb{E}_{\epsilon \sim \mathcal{N}(0, I), t \sim U([0, 1])} [\text{SNR}'(t) \|\mathbf{y}_0 - \hat{\mathbf{y}}_0\|_2^2]\end{aligned}$$

Moving to continuous time is a critical step, as it has been shown that increasing the number of time steps T should reduce to diffusion loss [50]. This diffusion loss is often recast as a noise estimation loss by making use of the relationship given in (3.9). Indeed, after some algebra we find that

$$\mathcal{L}_\infty = \frac{1}{2} \mathbb{E}_{\epsilon \sim \mathcal{N}(0, I), t \sim U([0, 1])} \|\boldsymbol{\epsilon} - \hat{\boldsymbol{\epsilon}}\|_2^2$$

which gives us the continuous-time diffusion loss used in the complete objective \mathcal{L} . Note that in the continuous-time framework, time is instead uniformly sampled over an interval $[0, 1]$.

Regularization of the noise schedule

The second two terms of \mathcal{L} , namely \mathcal{L}_γ and \mathcal{L}_β , act to constrain the learned noise schedule. Their full mathematical justification is out of the scope of this thesis and the following is a brief introduction to the full argument given in [46]. In the continuous-time framework the noise schedule is given now by a continuous function $\gamma(t, \mathbf{x})$. A first constraint is placed on $\gamma(t, \mathbf{x})$ in order to preserve the similarity between discrete and continuous time implementations

$$\mathcal{L}_\gamma = \|\gamma''(t, \mathbf{x})\|_2^2$$

Furthermore, in their generalization to continuous time, [46] introduced the continuous function $\beta(t, \mathbf{x})$ such that

$$\frac{\partial \gamma(t, \mathbf{x})}{\partial t} = -\beta(t, \mathbf{x})\gamma(t, \mathbf{x})$$

where $\beta(t, \mathbf{x})$ is a separable function of time and space: $\beta(t, \mathbf{x}) = \tau_\Theta(t)\lambda_\Phi(\mathbf{x})$, which are computed by neural networks Θ and Φ , respectively. The equation above acts as a constraint on the noise schedule, ensuring the diffusion process is smooth and free of sudden jumps

$$\mathcal{L}_\beta = \mathbb{E}_{t \sim U([0,1])} \left[\left\| \frac{\partial \gamma(t, \mathbf{x})}{\partial t} + \beta(t, \mathbf{x}) \gamma(t, \mathbf{x}) \right\|_2^2 + \|\gamma(0, \mathbf{x}) - 1\|_2^2 + \|\gamma(1, \mathbf{x}) - 0\|_2^2 \right]$$

The last two terms codify the soft constraints $\gamma(0, \mathbf{x}) = 1$ and $\gamma(1, \mathbf{x}) = 0$, which help to ensure that the forward process starts at \mathbf{y}_0 and ends in a standard Gaussian variable [46].

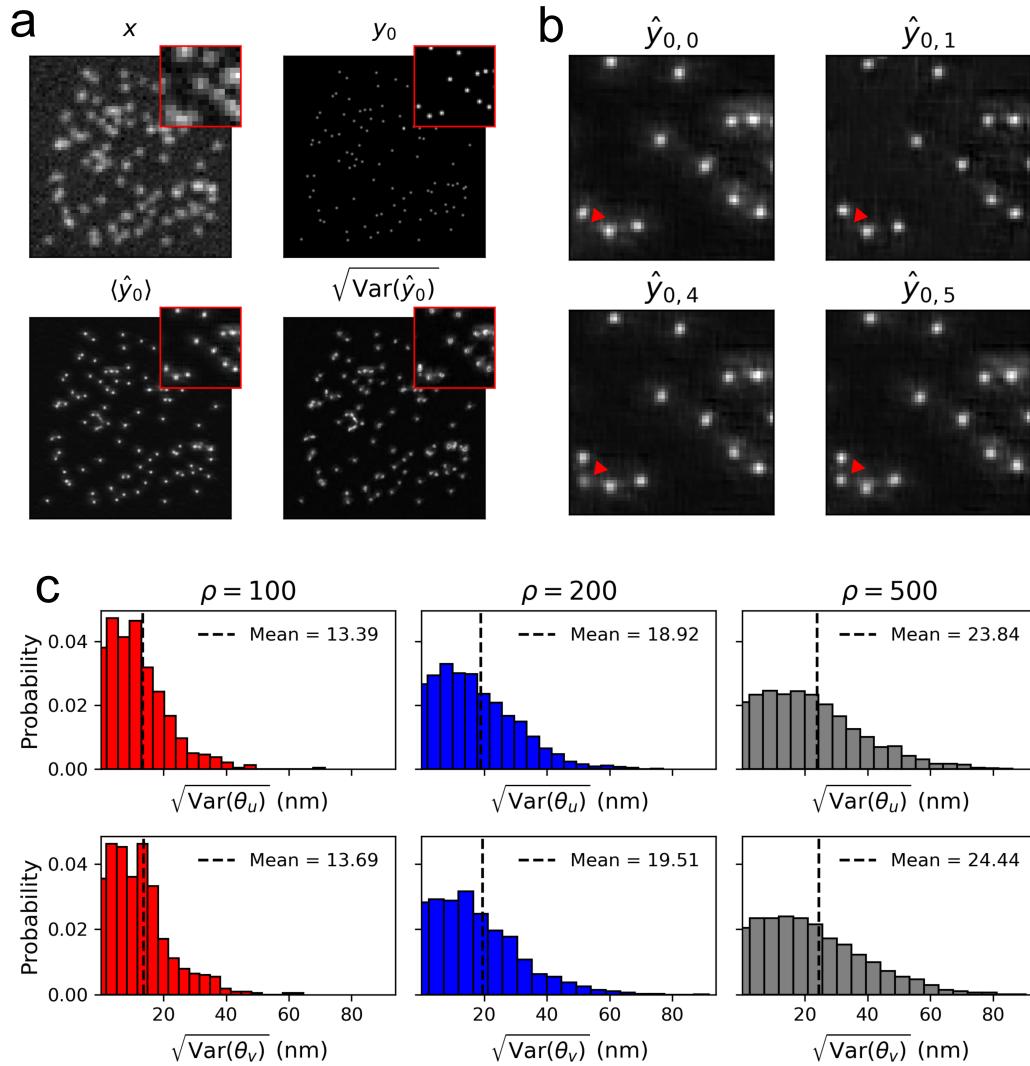


Figure 3.4. Sample variation for simulated localization microscopy data (a) Low-resolution, high resolution target, pixel-wise averaged prediction, and pixel-wise standard deviation (b) Samples from the diffusion model on high resolution images (c) Distribution of the standard deviation in emitter locations for various densities. Spots with zero variation are removed for visualization.

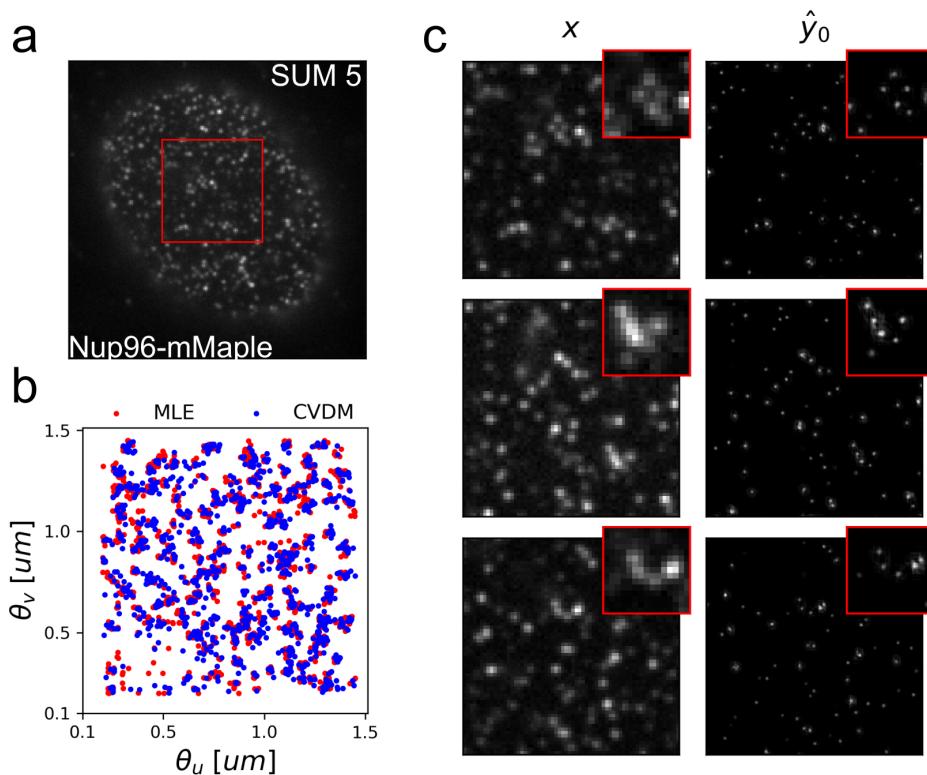


Figure 3.5. Super resolution of Nup96-mMaple (a) Low-resolution frame of Nup96-mMaple obtained by summing five consecutive images (SUM 5) (b) Scattered coordinates found by maximum likelihood estimation on individual frames and the CVDM model on SUM 5 data (c) Zoomed in regions from the data shown in (a) and corresponding samples from the model on high-resolution images

4. FLUORESCENCE NANOSCOPY IN THE STUDY OF CHROMATIN ORGANIZATION

4.1 Background

Chromatin is a complex and dynamic structure that packages eukaryotic DNA with histones. The higher-order structure of chromatin is highly non-random and plays an important role in regulation of genomic functions such as gene expression, DNA synthesis, and DNA repair. Chromatin structure is strictly controlled and constantly remodeled as cells differentiate, divide, and respond to genomic insults [57]–[61].

While DNA linearly encodes genetic information, serving as a template for RNA and protein production, the spatial and temporal organization of chromatin plays a fundamental role in determining intranuclear activities and gene stability [63], [64]. For example, local structural fluctuations in nucleosomes on microsecond to second timescales transiently expose buried DNA sites, thus providing temporary access to interaction sites [65]. Similarly, chromatin fibers are subject to rapid conformational dynamics [66]. This intrinsic motion of chromatin is closely linked to the underlying polymeric structure and directly affects molecular interactions at the local level by dictating the accessibility of DNA for various epigenetic effectors, chromatin regulators, and transcription factors (TFs). Indeed, the nanoscale spatiotemporal profile of chromatin may modulate the interaction of DNA with regulatory molecules, impacting the global patterns of gene expression [10], [67]–[69]. The dynamics of chromatin are best described by anomalous diffusion models [70], [71].

Most of what we know about local chromatin motion in chromatin remodeling has been derived from ensemble measurements, which provide a picture of biochemical access to chromatin over large populations of cells. Recently, advances using localization microscopy approaches have enabled the direct observation of the dynamics of individual molecules and the structure of chromatin domains in a single cell nucleus. These techniques, when paired with functional bioassays, permit the use of spatiotemporal localizations to make functional conclusions about genomic activities.

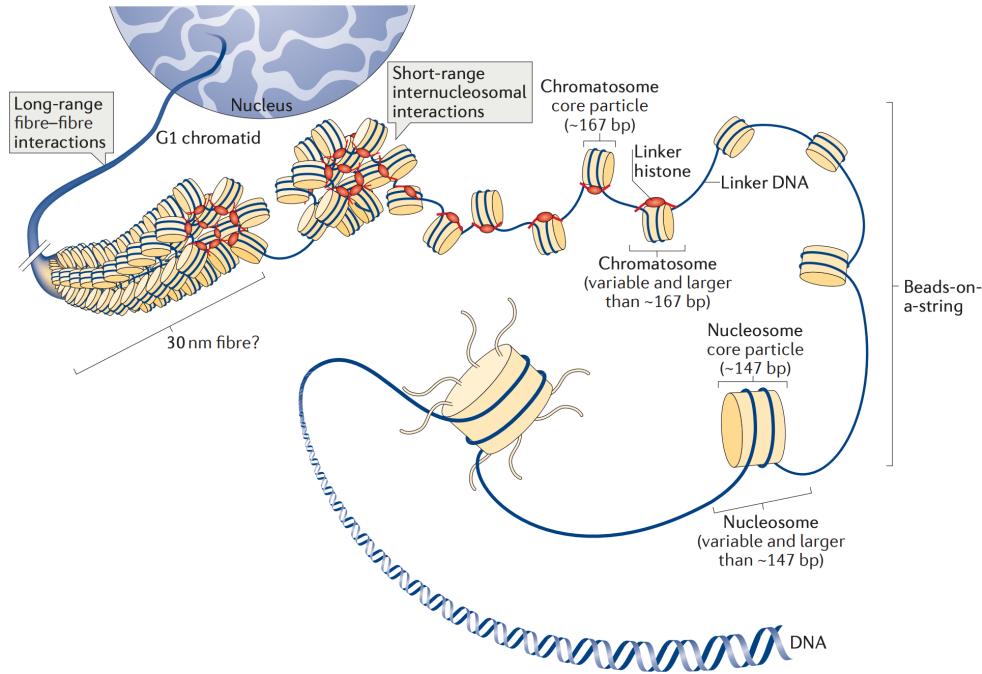


Figure 4.1. The hierarchical structure of chromatin DNA compaction within the interphase nucleus occurs through multiple levels of histone-dependent interactions. This includes the formation of the nucleosome core particle, strings of nucleosomes, the chromatosome core particle, the debated 30nm fibers, and the association of individual fibers, which eventually produces tertiary structures. Retrieved from [62].

4.1.1 Chromatin-associated-protein based labeling strategies

Core histones (H2A, H2B, H3, and H4), the fundamental units of chromatin tightly wrapped by DNA molecules, are common targets for imaging. In fixed cells, chromatin-associated proteins can be visualized through immunostaining with antibodies [8], [69], [72]. In live cells, histones can be directly fused with fluorescent proteins (FPs) [73]–[75]. Photo-activatable fluorescent proteins have been developed, allowing real-time control and quantitative characterization of protein clustering. Such strategies can be combined with other technical advancements such as single-molecule tracking and super-resolution imaging [9], [76], [77]. Alternatively, histone labeling in live cells can utilize prevalent self-label tags [78], [79], such as Halo Tag, Snap Tag, CLIP tag, and TMP Tag. These offer advantages

like small size, brightness, photostability, monomerization, and adjustable fluorescent dye concentration, demonstrating superior performance in single-molecule imaging [9], [80], [81]. Other genomic elements can also be fluorescently labeled to assess structure and dynamics, including telomeres [82], centromeres [82], [83], H-NS or HU in *E. coli* [84], heterochromatin proteins [85], and transcription factors (TFs) [86], [87].

4.1.2 Sequence specific labeling strategies

For fixed cell applications, fluorescence in situ hybridization (FISH) can detect and locate sequence-specific DNA or RNA in fixed cells using probes complementary to the target sequence [88], [89]. Primarily due to the need for cell permeabilization and sensitive hybridization conditions, FISH is limited to fixed cell samples.

Locus-specific labeling in live cells remains highly sought after. This can be achieved by either inserting artificial DNA sequences next to target genes, such as the repressoroperator array system (Lac operator (LacO) and Tet operator (TetO) systems), or by using modified genome-editing tools with inactive nucleases like zinc finger proteins (ZFPs), transcription activator-like effectors (TALEs), and clustered regularly interspaced short palindromic repeats (CRISPRs). The LacO-Laci-FP and TetO-TetI-FP systems are derived from the lactose and tetracycline operons of *E. coli*, respectively. Lac and Tet repressor proteins, fused to FPs, serve as tracking foci by recognizing repressor tandem repeat sequences inserted next to the position of interest [90], [91]. Multiple systems and repressor segments can be utilized concurrently within a single cell to increase system multiplicity and fluorescent amplification [92]–[94].

Point accumulation for imaging in nanoscale topography (PAINT) techniques are appealing for localization microscopy due to their lack of photon budget restrictions. DNA-based PAINT [95] has been explored and refined over the past decade. Variants like Quantitative PAINT (qPAINT), Förster resonance energy transfer PAINT (FRET-PAINT) [95], and Exchange PAINT have been developed to generalize the use of DNA origami for revealing cellular interactions. However, any intrusive DNA insertion can potentially disrupt function and alter chromatin locus position and mobility.

Non-intrusive methods, such as ZFPs, TALEs, and CRISPR-dCas9, avoid these constraints by operating without artificial DNA insertion [96]–[98]. These systems rely on modular proteins with specific DNA recognition, with endonuclease-deficient proteins typically fused with FPs to serve as detectable signals. Among these strategies, CRISPR imaging systems are gaining considerable attention. Chen et al. re-engineered the type II system to visualize both repetitive elements in telomeres and the non-repetitive MUC4 [99]. For multicolor imaging within the CRISPR system, one strategy involves using fluorescent Cas9 orthologs from different bacterial species simultaneously, such as *Streptococcus pyogenes* (SpCas9), *Neisseria meningitidis* (NmCas9), and *Streptococcus thermophilus* (St1Cas9) [100]. Another strategy involves engineering single-guide RNA (sgRNA) into a scaffold RNA (scRNA) to encode information about the gene of interest and multiple fluorescent reporters [101]. Recent modifications to CRISPR imaging systems, such as CRISPR-display [102] and CRISPR-rainbow [103], indicate promising applications for investigating chromatin organization and visualizing genome instability and rearrangement [96].

4.1.3 Instrumentation for chromatin imaging

Spatial and temporal resolution of nuclear imaging strategies are critical experimental conditions which dominate the reliability of downstream biophysical analysis [104]. Therefore, for most reported work in intranucleus chromatin imaging, objectives with high numerical aperture are employed. For many imaging experiments, an epi-illumination fluorescent microscope equipped with a modern camera (EMCCD or sCMOS) can provide time-course recording of live cells. Additionally, emerging LED light sources are replacing laser excitation in these microscopes, which significantly reduces the cost of the microscope [105]–[107]. Confocal microscopy is another widely applicable imaging system for chromatin imaging, which offers quality z-sectioning capability to reduce out-of-focus background. However, the applicability of confocal microscopy for the study of chromatin structure can be limited due to the reduced frame rate [71]. Confocal microscopy suffers from intrinsic limitations such as photo-bleaching/photo-toxicity and poor temporal resolution, which restrict their applications for sensitive chromatin imaging at the single-molecule level.

Light sheet illumination offers solutions to the aforementioned challenges and achieves a balance among spatiotemporal resolution, photo-bleaching effects, and background reduction. The technique has garnered tremendous attention for its advantages in reducing phototoxicity, enhancing sectioning capability, and enabling live-cell three-dimensional (3D) imaging. Early iterations of light sheet microscopy has shown great performance for 3D imaging of tissues, embryos, and organs [108]–[111]. However, its application has been limited by the geometric hindrance of using two objectives. Single objective live sheet methods such as the oblique plane microscope [112], as well as light-sheet analogues which generate a highly inclined and laminated optical sheet (HILO) for single-molecule imaging [113]. An AFM cantilever was initially used to reflect the illumination light sheet by 90 degrees to bypass geometric hindrance [87]. A similar idea was proposed using a microfabricated reflecting chip next to the sample reservoir [114]. Furthermore, the lattice light sheet (LLS) microscope, which generates the light sheet with a Bessel beam, significantly reduces the thickness of the light sheet to 300 nm [115]. The LLS has demonstrated superior performance in terms of spatial resolution, temporal resolution, phototoxicity, and sensitivity [116].

It is worth noting that a significant portion of studies address chromatin structure and dynamics in only two dimensions. While two-dimensional measurements (i.e., positions or trajectories projected in a single plane) are practical and generally well-suited for flat nuclei in monolayer cell cultures, 3D measurements are necessary to improve accuracy, particularly for round nuclei where the relationship between 2D and 3D distances deteriorates, and for short distances (<5 um) where the average 2D/3D discrepancy is 30 percent [117]. Moreover, nuclear inhomogeneity in all 3 dimensions must be considered. For motion measurements, the 2D/3D discrepancy of 30 percent might not be very significant, but considering local fluctuations of 50 nm, the 3D nature cannot be ignored.

Advances in super-resolution imaging will also benefit intranuclear chromatin imaging. For example, aspherical optics were initially utilized in super-resolution imaging to convert the z-axis position of a single molecule into a distorted point spread function (PSF) on the x-y plane using a cylindrical lens [118]. This method was further developed in single-molecule imaging/tracking to register the 3D position of a moving molecule through a rotational PSF [119]–[122]. Additionally, applying super-resolution imaging to live-cell single-molecule imag-

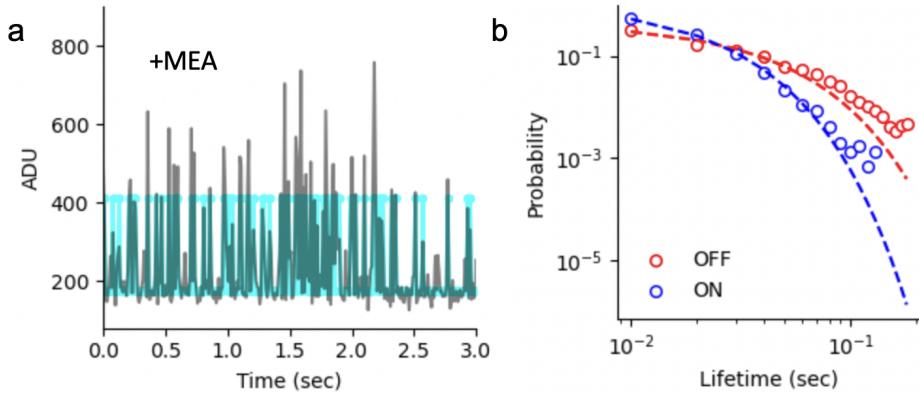


Figure 4.2. Photoswitching of JF646 bound to H2B-HaloTag (a) Peak intensity fluctuations of a putatively isolated JF646 molecule excited at 640nm and imaged with 10ms exposure time in the presence of 100mM MEA buffer. Fit of a Hidden Markov Model is shown in cyan (b) ON and OFF state lifetime distributions found by pooling data from 16 JF646 spots in a single HeLa cell nucleus. Single component exponential fits shown as dashed lines.

ing produces high-density trajectories of molecules, enabling the integration of biophysical analysis such as stochastic models of nonequilibrium motions to recover forces, subcellular organizations, diffusion kinetics, and other biophysical features at unprecedented spatiotemporal resolution [123]–[125].

4.2 The structure of chromatin nanodomains

Super-resolved nucleosome organization has been studied extensively in fixed cells to reveal segregated nanodomains hundreds of nanometers in size, including dispersed nanodomains and compact large aggregates. Nucleosomes are now known to assemble into heterogeneous clusters of variable sizes, interspersed with nucleosome-depleted regions [8]. However, our current understanding of nucleosome patterning in the nucleus is limited by the need for fixation. Chemical fixation with paraformaldehyde, glutaraldehyde, methanol, or other chemical fixatives may alter the structure of chromatin, introducing error in our measurements [126]. Live cell imaging carries its own issues, particularly due to local and long-range chromatin motions in the nucleus. At the nanoscale, local chromatin motion ap-

pears to be isotropic and driven by thermal fluctuation [126]. Therefore, we presume that for sufficiently brief imaging duration (tens of seconds) nanodomain structure is approximately static. As a consequence, vibrations of single nucleosomes are, in effect, an addition of low amplitude noise to the localization dataset.

To perform live cell super-resolution imaging of chromatin nanodomains, we adopt the HaloTag system, a modified haloalkane dehalogenase designed to covalently bind to synthetic ligands (Los 2008). The HaloTag protein is fused to nucleosome H2B and is then bound by a rhodamine-derived fluorescent ligand, JF646. JF646 undergoes fluorescence intermittency (blinking) in the presence of a cysteamine (MEA) containing buffer (Figure 4.2). Once precise positions of the fluorophores are obtained, Besags L-function $L(r)$ is used to analyze the clustering. The L-function $L(r) = \sqrt{\frac{K(r)}{\pi}}$ is a transformation of Ripley's K-function $K(r)$. The function $K(r)$ is designed such that $K(r)$ is the number of localizations within a radius r of a randomly chosen localization. Importantly, in the case of complete spatial randomness, $L(r) = r$. Thus, in general, to quantify the degree of clustering, one uses $L(r) - r$, which measures the deviation of a point pattern from CSR. To demonstrate this, example point patterns have been generated using complete spatial randomness (Figure 4.3) or a Thomas point process (Figure 4.4). Note that $\sigma = 0$ is used to denote CSR. The Thomas process is parameterized by the standard deviation of a point from its cluster center σ . under CSR and a Thomas point process.

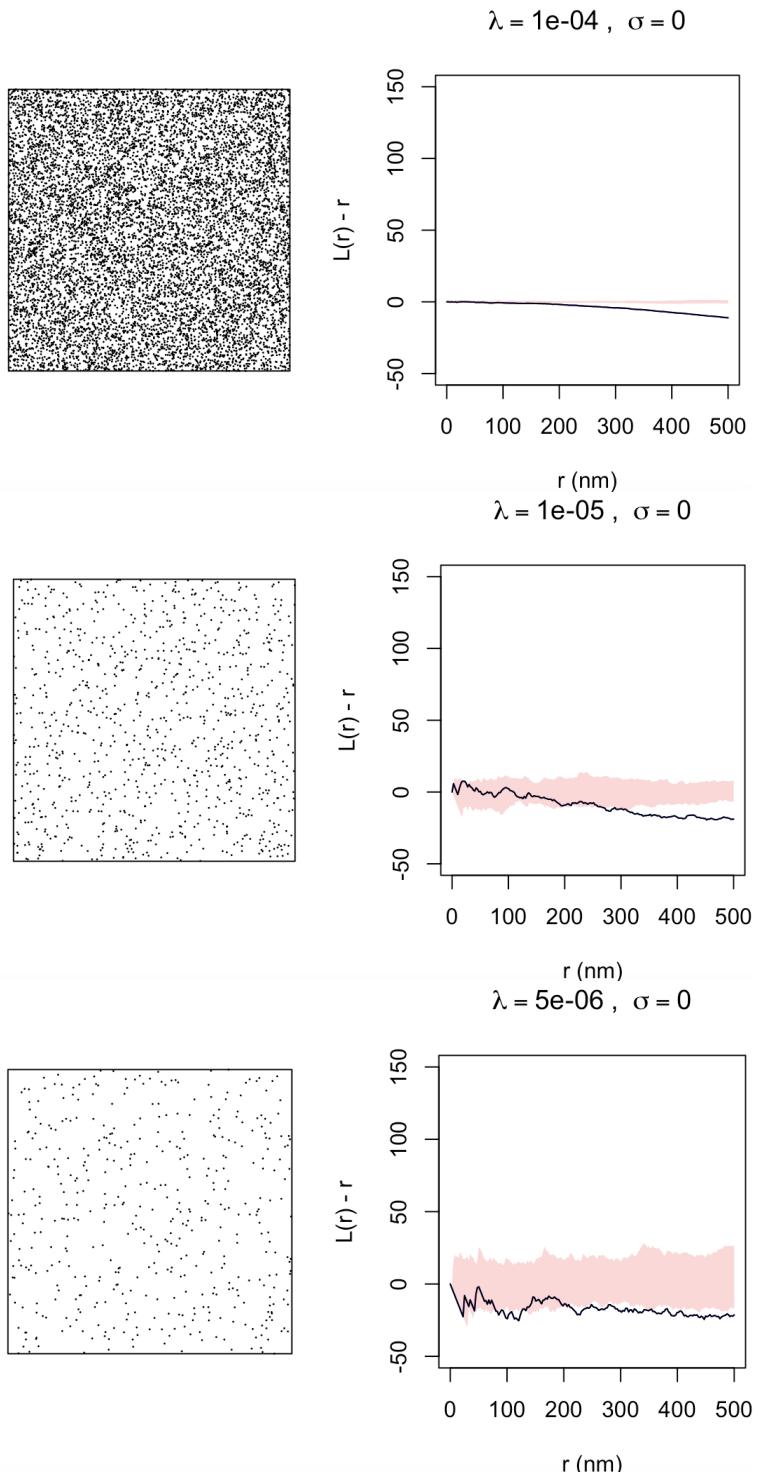


Figure 4.3. Besag's L-function under complete spatial randomness (CSR) Point pattern simulations for CSR for various intensities of the point process. Simulation envelopes are shown in pink, which represent the significance band of the estimated $L(r) - r$

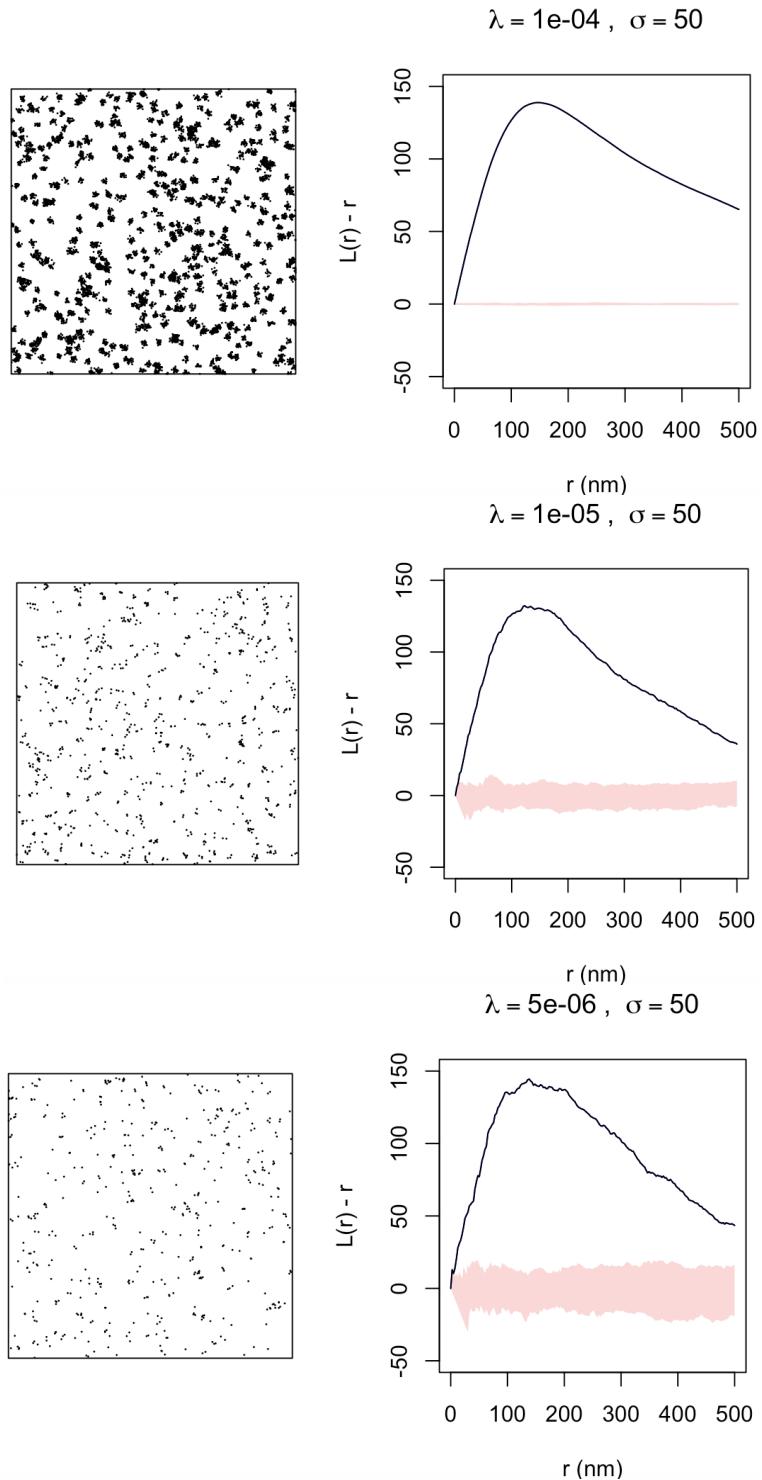


Figure 4.4. Besag's L-function for a Thomas process Point pattern simulations for the Thomas process ($\sigma = 50\text{nm}$) for various intensities of the point process. Simulation envelopes are shown in pink, which represent the significance band of the estimated $L(r) - r$

4.3 Polymer models and chromatin dynamics

By accumulating single molecule localizations over a time series of frames, quantitative information regarding the motion of the particle can be extracted and related to experimental conditions. It is important to note that the chromatin structure is highly complex and cannot be exactly represented by the diffusion of individual particles. However, by treating each fluorescent spot as a point mapped in space and time, we can extract parameters of the diffusion and classify tracks as sub-diffusive, Brownian, or super diffusive.

The well-known diffusion equation is a special case of the more general Kramers-Moyal expansion, which describes the dynamics of the probability density of a stochastic process $P(x, t)$ [127]. Pure diffusion is the case where transitions in the process are drawn from a distribution with zero mean (zero drift) and constant variance.

$$\frac{\partial P(x, t)}{\partial t} = D \frac{\partial^2 P(x, t)}{\partial x^2} \quad (4.1)$$

The diffusion coefficient D is in fact just half of the variance of the transition distribution $D = \sigma^2/2$. We would like to solve the above equation, but it is a PDE which usually require some tricks to solve e.g., integral transforms. This particular PDE can be solved by Fourier transformation. For brevity, I simply state the result

$$P(x, t) = \frac{1}{\sqrt{2Dt}} \exp\left(-\frac{(x - x_0)^2}{4Dt}\right) \quad (4.2)$$

which is a Gaussian distribution with time-dependent variance $\sigma = \sqrt{4Dt}$, given originally by Einstein in his famous paper on Brownian motion in 1905. The most common method to analyze single particle trajectories is to calculate the mean square displacement (MSD), which can be used to determine the diffusion coefficient D .

$$\text{MSD} = \frac{1}{N} \sum_{i=1}^N [\mathbf{r}(t + \tau) - \mathbf{r}(t)]^2 \quad (4.3)$$

The sampling time given by τ is interpreted as the frame rate of the camera. Certain studies have shown that the observed motion varies with different values of τ [128], [129]. It is not difficult to see that the solution to the diffusion equation has a MSD which is

linear: $\text{MSD}(\tau) = 4Dt$. A linear MSD is therefore a defining characteristic of Brownian motion. Sub-diffusive and super-diffusive diffusion can be classified by use of the more general anomalous diffusion model:

$$\text{MSD}(\tau) = 2mD\tau^\alpha \quad (4.4)$$

where m is the number of dimensions. For trajectories with $\alpha = 1$, the motion is considered to be Brownian motion; when $\alpha > 1$, it is said to be superdiffusive; and $\alpha < 1$ is classified as subdiffusive. Importantly, different parameter values allow for further interpretation and classification of different diffusion modes of particles [130], [131].

By comparing the D values of all particles within the nucleus, spatial heterogeneity can be clarified. While the calculation of D and α is particularly reliant on the length of the trajectory and the number of data points, recent investigations have built diffusion color maps of fluorescently labeled histones by computing vast amounts of super-resolution trajectories [9], [128], [132].

4.3.1 Trajectory linking

A diverse set of particle tracking algorithms utilize probabilistic models of particle motion in order to add detected particles to existing tracks. Perhaps the most fundamental particle tracking method in this category is the nearest neighbor linking algorithm first introduced by Crocker and Grier [133]. The algorithm constructs particle trajectories by assuming that the ensemble consists of non-interacting indistinguishable particles undergoing Brownian motion. As a result, the displacement of each particle follows a Gaussian distribution, parameterized by the diffusion coefficient and the time-resolution of the sequence. The most probable assignment of detected particles to existing tracks can then be found by maximizing the product of several Gaussian distributions [133]. An attractive feature of this algorithm is that it is relatively simple to implement; however, assumptions that underlie the method impose limitations. In particular, if a typical displacement δ in one time step is comparable to the typical inter-particle spacing, tracking becomes highly error-prone [133]. In other words, when particles are simultaneously densely distributed and undergoing fast

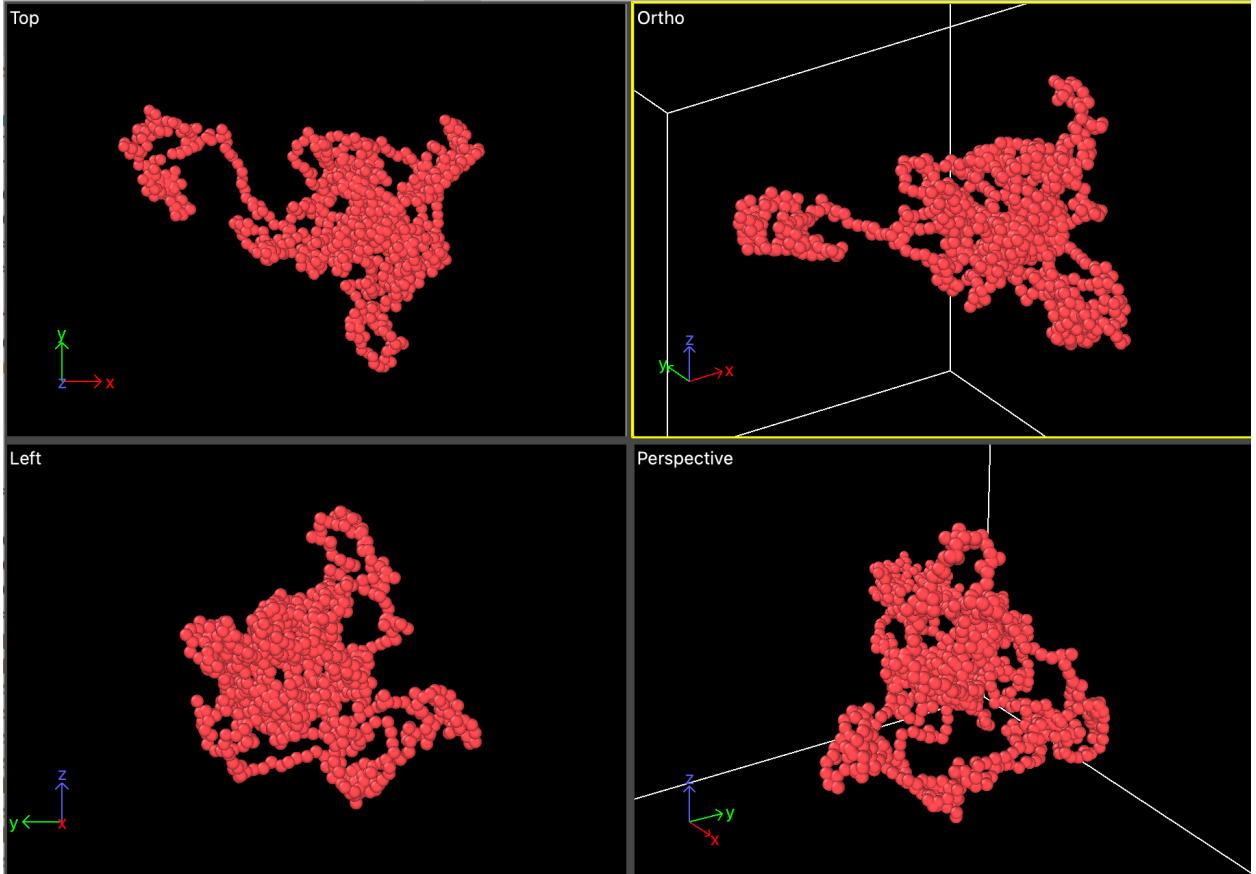


Figure 4.5. Snapshots of the Rouse polymer Four perspectives of the Rouse polymer generated with $N = 2000$ beads connected with harmonic bonds with equilibrium length 200nm. Polymer is initialized with all harmonic bonds at equilibrium

diffusion, the algorithm can fail to build accurate trajectories due to the ambiguity introduced by overlapping trajectories. This also applies to the limit of low frame rates when small displacements are not recorded by the sensor. Importantly, the method alone cannot handle cases where particles disappear permanently, temporarily disappear due to blinking, photo-bleaching, or missed localization [134].

4.3.2 Chromatin polymer models

It is well-known that the DNA double helix structure consists of millions of base pairs chained together by the sugar-phosphate backbone. DNA, being a molecule built from

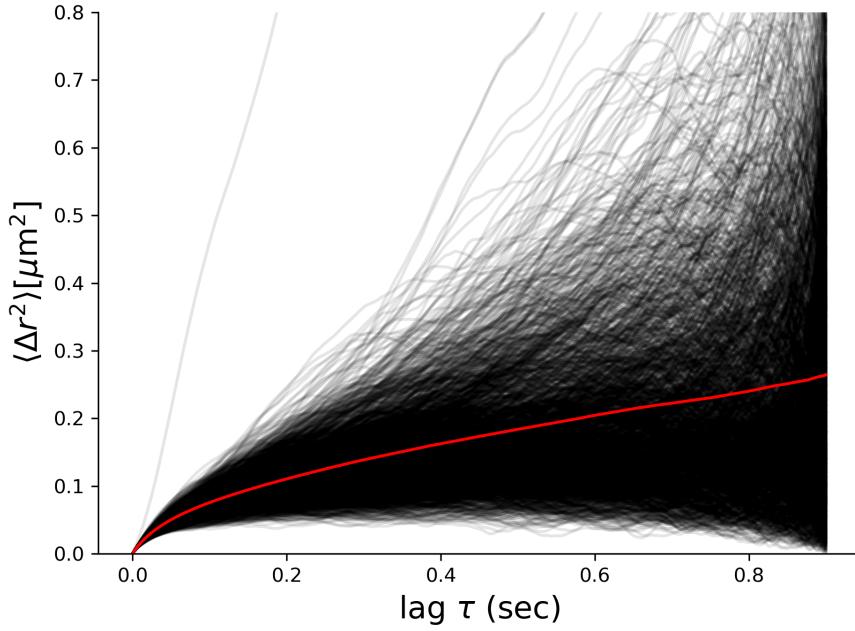


Figure 4.6. Subdiffusive dynamics of a Rouse polymer Mean squared displacement (MSD) of 2k beads shown in black, average MSD shown in red.

many similar monomers bonded together, is naturally analyzed using a polymer model. The simplest polymer model for chromatin is the Rouse polymer model, which utilizes monomers connected by springs, resembling the string-of-beads structure observed under the electron microscope (Figure 4.5). Forces acting on a bead by neighboring beads results in confinement and subdiffusive dynamics (Figure 4.6). To refine the modeling of chromatin properties according to the exponent α , the β -polymer model was introduced. This model accounts for mid-range and long-range interactions between monomers. Indeed, the β -polymer model can be selected to address cases where α is in the range of 0 to 0.5 [128], [135], [136].

Furthermore, as the polymer model usually folds at different spatial scales and generates various sizes of loops, a simulation model adding connectors between randomly chosen non-nearest neighbor monomer pairs, known as the randomly cross-linked (RCL) polymer model, was presented. In this model, α is reduced to below 0.5 by adding connectors. Recent evidence suggests that the number and distribution of connectors impact physical parameters

Parameter	Value
Temperature (T)	310 K
Bond Length (σ)	200×10^{-9} m
Cutoff Distance	500×10^{-9} m
Spring Constant (κ)	$90.0 k_B T / \sigma^2$
Neighbor Distance	500×10^{-9} m
Damping Coefficient (γ)	10^{-4}
Time Step	1s
Number of Time Steps	10^4
Number of Atoms	2000

Table 4.1. Rouse polymer parameters

in various ways, but this domain remains underexplored. Notably, the RCL polymer model may be crucial for studying dynamics in processes such as CTCF and cohesion regulating chromatin loop stability [137].

4.4 BRD4 phosphorylation regulates the structure of chromatin nanodomains

The cell nucleus is a densely packed environment with chromatin comprising a dominant component. The compartmentalization of chromatin with other intranuclear components by phase separation is therefore an efficient strategy to ensure precise spatial and temporal coordination of complex dynamics. A growing number of phase separated nuclear bodies have been identified, including transcriptional condensates [138], [139], nuclear speckles [140], and DNA damage repair foci [7]; however, the interplay of phase separated condensates with the underlying chromatin structure remains poorly understood. Transcriptional condensates have been identified as an ideal model to study the kinetic and thermodynamic contributions of chromatin substrate binding, as the ability of transcriptional activators to both condense and bind chromatin is well established [138], [141]–[144]. Here, we extend this effort by investigating the regulation of chromatin structure by phase separated transcriptional condensates. We focus on the BRD4 protein - a well-studied transcriptional activator that localizes to acetylated chromatin sites [145], recruits pTEF-b, and initiates transcription of key genes involved in signal response, immunity, and oncogenesis [146].

The BRD4 long isoform is characterized by structured N-terminal tandem acetyl-lysine binding bromodomains and an extra-terminal domain, connected by intrinsically disordered regions [147]. Perhaps the most fundamental of BRD4 functions is the ability to bind to acetylated chromatin through bromodomain 1 (BD1) and bromodomain 2 (BD2) that are in tandem within the N-terminal part of the protein (Figure 4.7). BRD4 inhibitors such as (+)-JQ1 competitively bind to the acetyl-binding pocket of BRD4, displacing BRD4 from chromatin [148]. It is also well known that BRD4 association with acetylated chromatin is enhanced by casein kinase II (CK2)-mediated phosphorylation of seven N-terminus phosphorylation sites (NPS), followed by intramolecular rearrangement of BRD4 protein and/or BRD4 dimerization [149], [150].

Recent studies have demonstrated that BRD4 is present in discrete nuclear bodies that occur at super-enhancers, which exhibit properties of other well-studied biomolecular condensates, including rapid recovery of fluorescence after photobleaching and sensitivity to 1,6-hexanediol, which disrupts liquid-like condensates [138]. Both BRD4 long and short

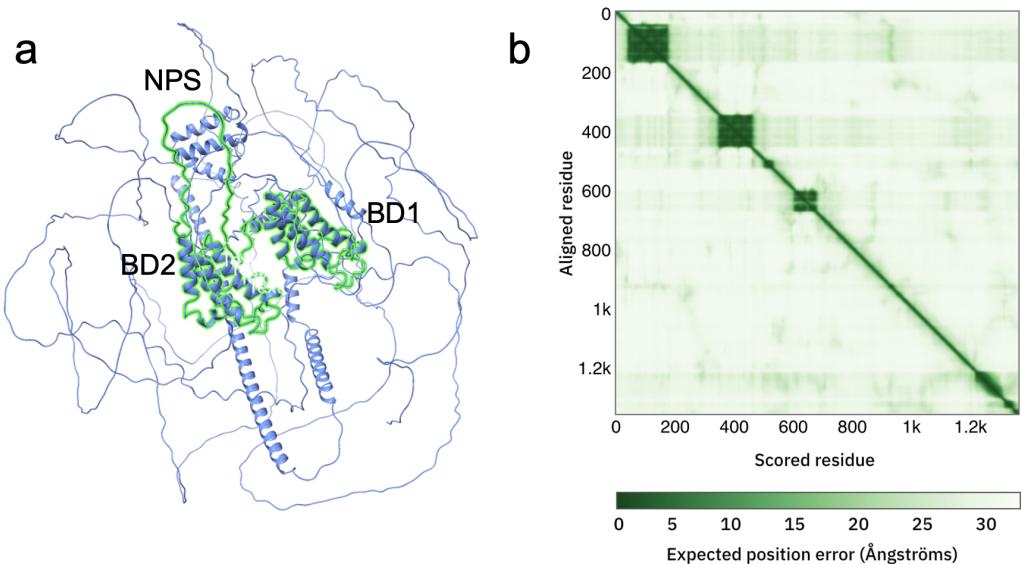


Figure 4.7. Bromodomain protein 4 (BRD4) structure prediction
 (a) AlphaFold predicted structure of BRD4 long isoform (blue) with bromodomain 1 (BD1) aa58-163, bromodomain 2 (BD2) aa349-456, and N-terminus phosphorylation sites (NPS) aa462-506 highlighted in green (b) Heat map of expected position error in AlphaFold prediction

isoform are found in phase separated condensates in the nucleus and are associated with active gene transcription. Importantly, CK2-mediated NPS phosphorylation state regulates chromatin binding activity of BRD4 as well as BRD4 phase separation [147]. This has led to the conclusion that phosphorylation of BRD4 inhibits interaction with chromatin and reduces phase separation, while remaining necessary for active gene transcription. Moreover, phosphorylated and unphosphorylated BRD4 form different molecular associations transient polyvalent associations of unphosphorylated BRD4 contrast with the stable dimeric interaction and chromatin binding of phosphorylated BRD4 [150]. Therefore, we speculated that BRD4 phosphorylation can modulate a multivalent binding interaction between the transcriptional condensate with NNs, making BRD4 phosphorylation necessary for the maintenance of NN structure and dynamics.

4.4.1 Results

Colocalization of BRD4 mutants with nucleosome nanodomains

To address the role of BRD4 binding and phase separation on chromatin structure, we express FLAG-tagged BRD4 mutants with NPS or bromodomain mutations in HeLa cells and measure their effects on chromatin organization. In particular, we express a constitutively phosphorylated (7D mutant), constitutively unphosphorylated (7A mutant), and bromodomain-deactivated (BD mutant) protein (Figure 4.8 a,b,c). Colocalization analysis of BRD4 mutants with NNs using nearest neighbor distance distribution showed an obvious colocalization of these mutants with NNs. The 7D mutant showed the closest proximity to NNs relative to wild type, followed by BD and 7A mutants (Figure 4.8 d). This result is consistent with known dependence of BRD4 chromatin binding on phosphorylation state.

BRD4 condensates regulate chromatin structure and dynamics

To assess the functional role of BRD4 in maintaining the NN environment, we interrogated the dynamics of NNs, as well as their structure, in the presence of BRD4 mutants. Histone H2B was tagged with HaloTag [151] (H2B-Halo), to which a fluorescent ligand JaneliaFluor646 (JF646) can bind specifically in a living cell. Low concentrations of JF646 were used to obtain sparse labeling of nucleosomes for single-nucleosome imaging (Figure 4.11 a,b). JF646-labeled nucleosomes in HeLa cells were recorded at 10fps (200 frames, 20 s total) and a reduced diffusion coefficient was measured in cells expressing 7A, 7D, and BD mutants, with respect to cells expressing the wild-type BRD4 protein (Figure 4.11 c). HeLa cells exposed to (+)-JQ1 in DMSO for 8h showed an increase in nucleosome dynamics with respect to DMSO alone (Figure 4.11 d). We then conducted super resolution imaging of NN using direct stochastic optical reconstruction microscopy (dSTORM) by promoting JF646 fluorescence intermittency with a cysteamine buffer (Figure 4.10 a,b). JF646 is known to exhibit a transient fluorescent state lasting tens to hundreds of milliseconds and stable dark state lasting hundreds of milliseconds to seconds [152]. Two color imaging of H2B-Halo-JF646 and GFP-tagged BRD4 shows that BRD4 and NNs form complementary biomolecular condensates.

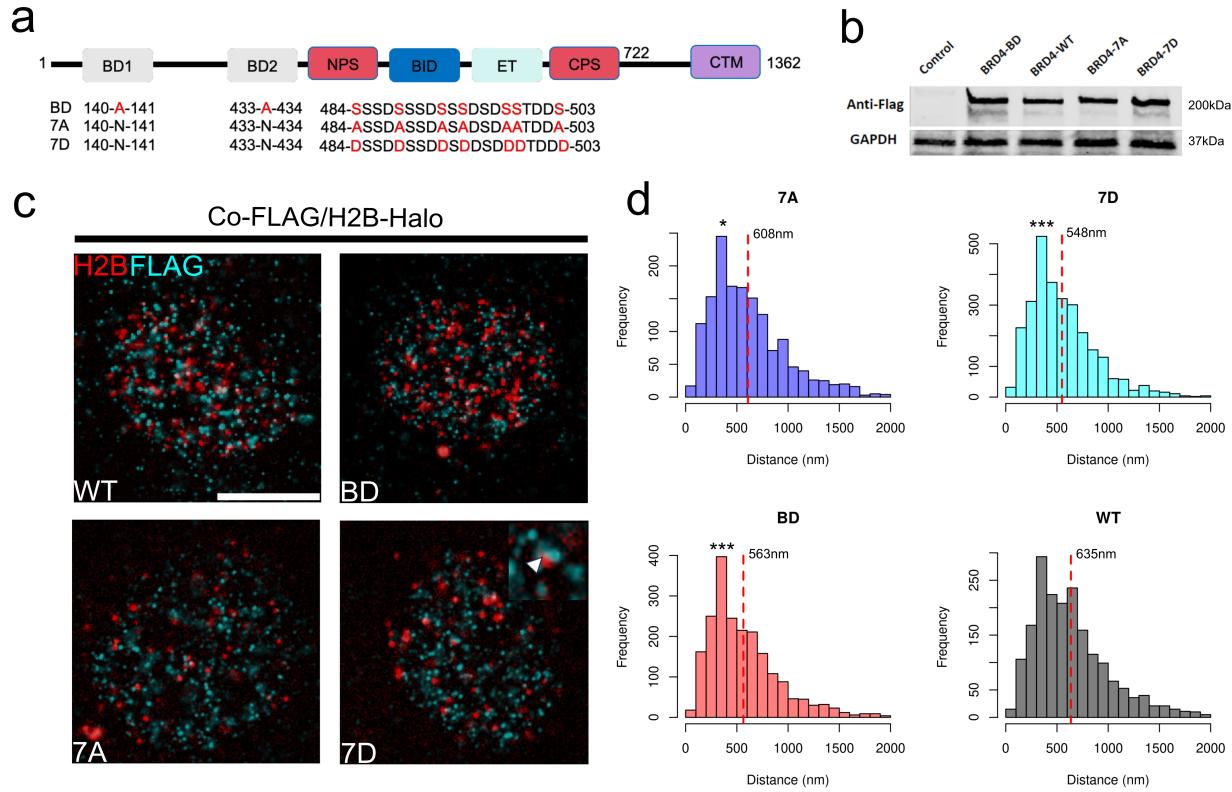


Figure 4.8. BRD4 mutants colocalize with nucleosome nanodomains
 (a) Schematic of the BRD4 protein sequence and mutations shown in red. (b) Anti-FLAG western blot of bulk expression of BRD4 mutants (c) Combined immunofluorescence of FLAG-tagged BRD4 mutants with JF646-tagged H2B-Halo (d) Histograms of nearest neighbor distances of H2B puncta to FLAG puncta over 10 cells for each mutant. Red dashed lines are drawn at the mean, statistical significance determined by Welch t-test for each mutant relative to wild-type (* p<0.05, *** p<0.001)

sates in the nucleus, consistent with current models of BRD4 chromatin reading mechanism (Figure 4.10 c). Ensemble averages of Besags L-function showed an increase in nucleosome clustering in cells expressing the 7D BRD4 mutants, while all other groups were consistently indistinguishable from WT cells (Figure 4.10 e). HeLa cells exposed to (+)-JQ1 in DMSO for 8h showed a reduction in nucleosome clustering with respect to DMSO alone (Figure 4.10 f).

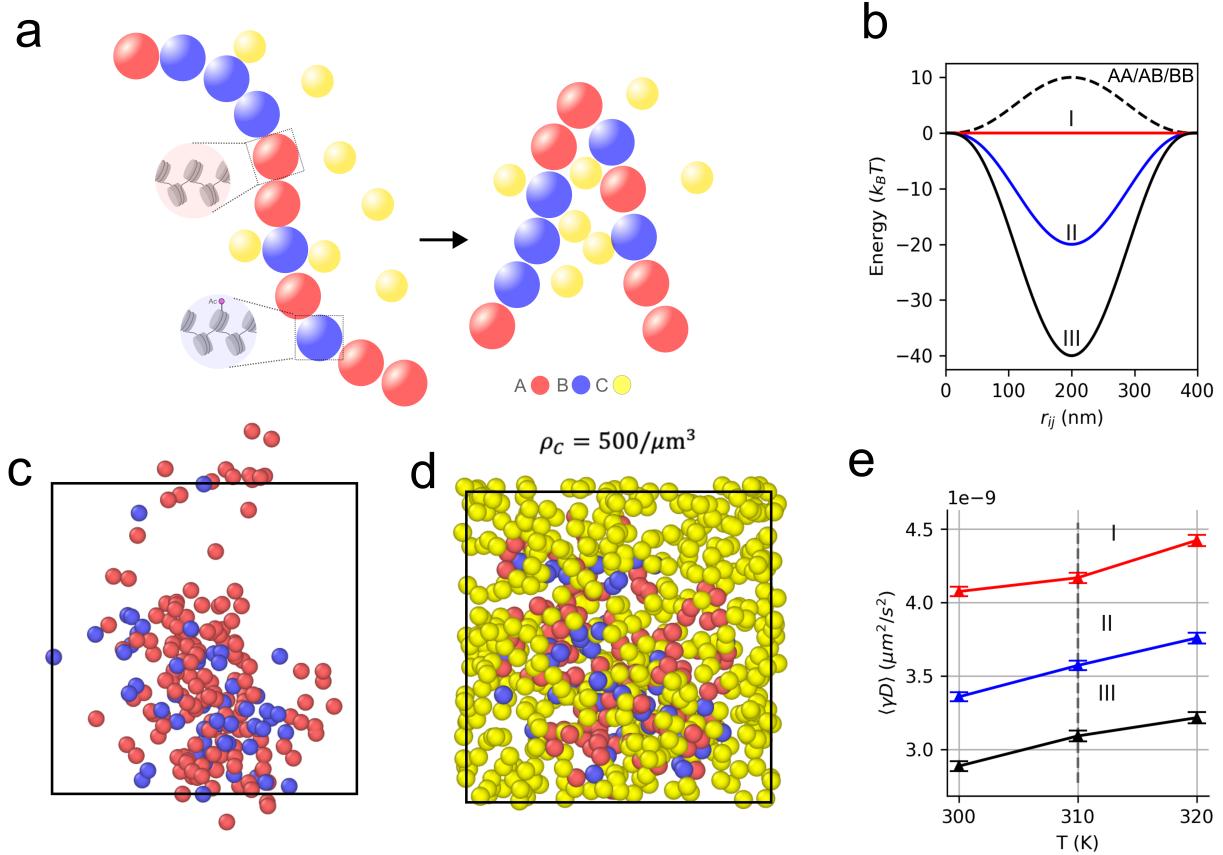


Figure 4.9. Strong multivalent chromatin binders reduce diffusion of nucleosome nanodomains (a) Heteropolymer model of chromatin consisting of A-type, B-type, and C-type particles (b) Interaction potentials $U_{BC}(r_{ij})$ of multivalent chromatin binders with B-type chromatin beads (c,d) Example free heteropolymer and heteropolymer with a number density of C-type particles of $\rho = 500/\mu\text{m}^3$ in a $10\mu\text{m}$ periodic box (e) Scaled diffusion coefficient D for various chromatin binding energies of C-type particles, averaged over ten independent simulations, with burn-in discarded.

Heteropolymer model

To interpret our experimental findings, we adopt a heteropolymer chromatin model to capture the interaction of chromatin with multivalent BRD4-like binders (Figure 4.9 a). The heteropolymer consists of a coarse-grained bead-and-spring chain composed of $N_b = 200$ beads, connected by harmonic bonds with equilibrium length r_0 whose energy is dened as

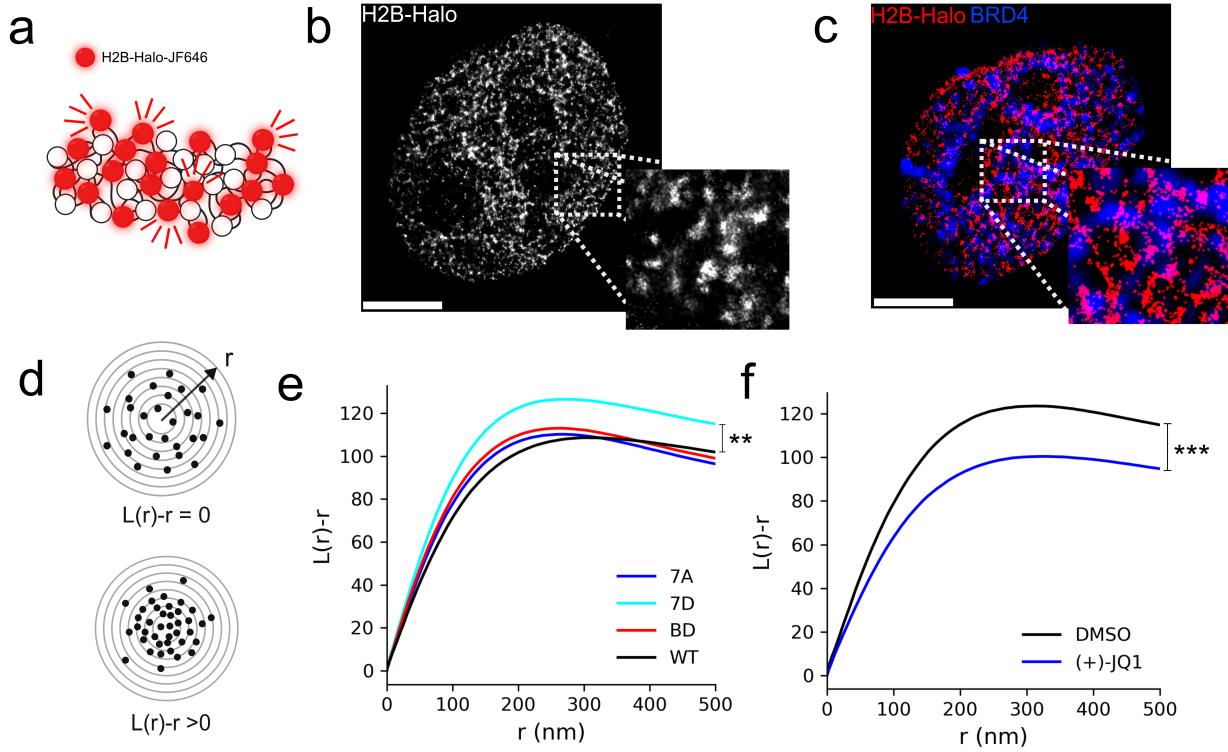


Figure 4.10. Expression of constitutively phosphorylated BRD4 compacts nucleosome nanodomains (a,b) Direct stochastic optical reconstruction microscopy (dSTORM) imaging strategy of single nucleosomes and an example super-resolution image (c) Two-color image of super-resolved H2B-JF646 with diffraction-limited GFP-tagged BRD4 (d) Quantification of degree of clustering using the L-function (e) L-function for BRD4 mutants (f) L-function after 8h exposure to 1uM (+)-JQ1 in DMSO and DMSO only. All scalebars 3um. ** p<0.01, *** p<0.001

$$U_{AB}(r_{ij}) = \frac{\kappa}{2}(|r_{ij}| - r_0)^2 \quad (4.5)$$

where r_{ij} is a vector connecting the center of a bead of type i to a bead of type j and $i, j \in (A, B)$. In all simulations, we assume $\kappa = 90k_B Tr_0^2$ where k_B is the Boltzmann constant and $r_0 = 200\text{nm}$. Random beads in the chain are selected to represent locally unacetylated (A-type particles) and acetylated chromatin (B-type particles). B-type particles undergo multivalent interactions with a third group of C-type particles, which can promote cross-linking of the polymer. We presume a Bernoulli probability of $p = 0.3$ for any given bead to

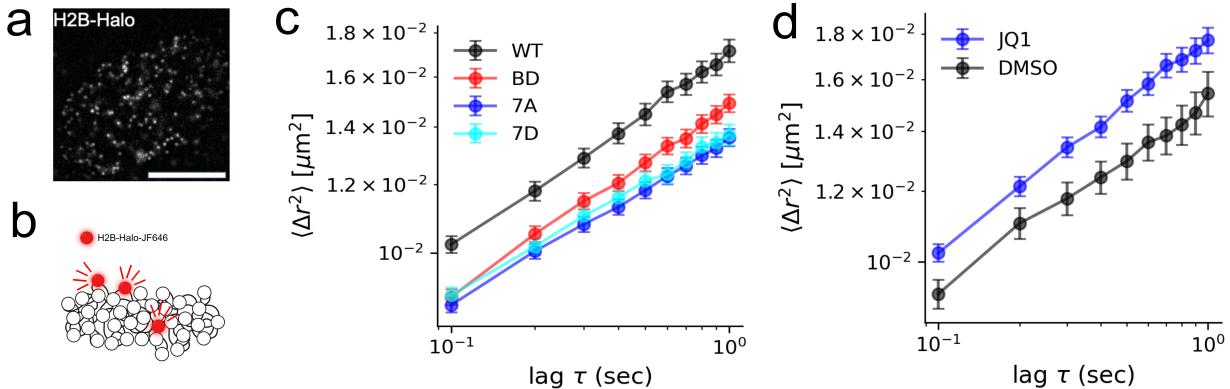


Figure 4.11. BRD4 mutants reduce single nucleosome dynamics in living cells (a,b) Sparse labeling of single nucleosomes in a living HeLa cell (scalebar 5um) (c) Average mean squared displacement (MSD) of single nucleosomes for wild-type and mutated BRD4, error bars represent the standard error of the mean (d) Average MSD of single nucleosomes after 8h exposure to 1uM (+)-JQ1 in DMSO and DMSO only, error bars represent the standard error of the mean

be in an acetylated-like state. Interaction of multivalent chromatin binders with chromatin beads are then mediated by the following potential:

$$U_{BC}(r_{ij}) = \epsilon \left(1 - \left(\frac{|r_{ij}|}{R_0} \right)^2 \right)^3 \quad (4.6)$$

where $R_0 = 200\text{nm}$. The potential U_{BC} is considered over a domain $0 \leq |r_{ij}| \leq 2R_0$. In all simulations, ten replicates were run for each condition tested. A and B type particles within the chromatin polymer have repulsive interactions with $\epsilon = +10k_BT$. Binding energy of the acetylated beads with binders was varied with $\epsilon_I = 0k_BT, \epsilon_{II} = -20k_BT, \epsilon_{III} = -40k_BT$ (Figure 4.9 a). The dynamics of chromatin chains are approximated by Brownian dynamics within a cubic box with side length of 10um and periodic boundary conditions. Brownian dynamics follows the stochastic differential equation.

$$\dot{\mathbf{r}} = \gamma^{-1} \nabla U(\mathbf{r}) + \sqrt{2k_BT} \gamma^{-1/2} \xi(t) \quad (4.7)$$

Parameter	Value
Bond Length (σ)	200×10^{-9} m
Cutoff Distance	500×10^{-9} m
Spring Constant (κ)	$90.0 k_B T / \sigma^2$
Neighbor Distance	500×10^{-9} m
Damping Coefficient (γ)	10^{-7}
Time Step	10^{-4} s
Number of Time Steps	10^4
Number of Atoms	200

Table 4.2. Multivalent binder model parameters

where γ is a diagonal friction tensor and $\xi(t)$ is a three-dimensional delta-correlated white noise $\langle \xi(t) \xi(t + \tau) \rangle = \delta(t, t + \tau)$. Integrating the Brownian dynamics showed an overall reduction of the variance of the diffusion coefficient D of single beads, and quasi-linear scaling of the diffusion coefficient with respect to temperature (Figure 4.9 e).

4.4.2 Discussion

Our data support the model that phosphorylated and unphosphorylated BRD4 form different molecular associations in the nucleus. BRD4 nuclear localization is only weakly affected by bromodomain inhibition with monovalent BET inhibitors, while exposure to phase separation inhibitors such as 1,6 Hexanediol have a significant effect on BRD4 localization in the nucleus. Colocalization activity of 7A/7D mutants relative to wild type measured here is consistent with this model of BRD4 localization to NNs. Strong colocalization of the BD mutant relative to the wild type suggests a possible link between bromodomain structure with localization of BRD4.

Concurrently, reduced diffusivity of single nucleosomes with all mutants tested suggests a relationship between elevated colocalization and the degree of confinement of nanodomain diffusion. At the same time, increased nucleosome diffusivity and reduced nucleosome clustering after (+)-JQ1 exposure indicate BRD4 condensate formation is necessary in maintaining the nanodomain architecture. We further hypothesize effects of BRD4 localization on the viscosity of the nanodomain environment and potentially increased cross-linking of nan-

odomains. Indeed, we find this to be a natural result of molecular crowding resulting from overexpression of a constitutively phase separating protein, capable of multivalent interactions. Substantial colocalization of the 7D mutant with nanodomains along with increased chromatin compaction points towards a BRD4-mediated cross-linking mechanism of nucleosomes within the nanodomain. This result of increased affinity can also be seen in coarse grained simulations of chromatin interactions with multivalent binders.

We conclude that nascent BRD4 condensates are likely seeded by promiscuous interactions of BRD4 with cofactors, followed by phosphorylation followed by chromatin interactions mediated by the phosphorylated form. The stable dimeric interaction and binding of phosphorylated BRD4 to acetylated chromatin would then mediate control of the chromatin architecture by promoting cross-linking of the chromatin fiber and acting a molecular bridge between transcriptional condensates with chromatin.

4.4.3 Materials and Methods

Cell lines, cell culture conditions, and transfection

HeLa cells were cultured in DMEM supplemented with 10 percent fetal bovine serum (Gibco) at 37C 5 percent CO₂ in a humidified incubator. Cultures were tested routinely for mycoplasma contamination; all tests were negative. For super-resolution experiments, cells were seeded in a 35mm FluoroDish (WPI), and transiently transfected using Lipofectamine 3000 with pBREBACK-H2BHalo plasmid (Addgene plasmid 91564) (ThermoFisher), pcDNA5-Flag-BRD4-7A (Addgene Plasmid 90006), pcDNA5-Flag-BRD4-7D (Addgene Plasmid 90007), pCDNA5-Flag-BRD4-BD (Addgene Plasmid 90005), pcDNA5-Flag-BRD4-WT (Addgene Plasmid 90331)

4.4.4 Plasmid DNA purification

Plasmids were transformed in E. coli at 4C and selected using an antibiotic agar plate. A single colony from the plate was selected and placed into sterile antibiotic LB Broth followed by incubation with shaking at 37C for 12h. After amplification, DNA was purified using a Miniprep kit (Promega). Following extraction, the concentration and purity were measured

using the NanoDrop 2000 software. Plasmids were stored with optimal concentration and purity at -20C.

Super-resolution imaging of nucleosome nanodomains in living cells

After transient transfection, H2B-Halotag HeLa cells were incubated with 3pM JF646 HaloTag ligand overnight. Cells were imaged in a dSTORM photoswitching buffer containing 100mM MEA, 50 ug/ml Glucose Oxidase, and 3.4 mg/ml Catalase (Sigma). Buffer pH was adjusted to 8 using HCl. Movies were collected using a custom Olympus IX83 microscope body equipped with an Olympus 60X 1.25NA oil-immersion objective. During imaging cells were maintained at 37C and 5 percent CO₂ in a stage top incubator (Tokai Hit). Images were projected onto an ORCA-Fusion sCMOS camera (Hamamatsu) and 2000 frames were captured at 100fps. The microscope was controlled using Micromanager software. HaloTag-JF646 molecules were imaged using oblique illumination with a 640nm laser (Excelitas) held at 20mW, as measured at the back focal plane of the objective. Super resolution reconstructions were obtained using the ThunderSTORM ImageJ plugin. Background signal was subtracted using a rolling ball filter with radius of 10 pixels. Spots were fit using an integrated Gaussian point spread function model with maximum likelihood estimation [14], [15]. Experimental conditions for single molecule tracking are nearly identical. However, H2B-Halotag HeLa cells were incubated with 3pM JF646 HaloTag ligand. HaloTag-JF646 molecules were illuminated at 10mW, 100 frames were captured at 10fps.

Colocalization of BRD4 mutants with nucleosome nanodomains

We colocalize FLAG-tagged BRD4 mutants with NNs by simultaneous FLAG immunofluorescence with imaging of sparsely labeled of H2B-JF646. Puncta were detected in both channels using the Laplacian of Gaussian (LoG) detection algorithm to generate a multi-type point pattern. We then computed the nearest neighbor distance distribution as the distance from a random H2B-JF646 puncta to the nearest BRD4-FLAG puncta. Nearest distances values were pooled over 10 cells for each mutant.

Single molecule tracking

Nucleosomes were localized using an integrated Gaussian point spread function model with maximum likelihood estimation [14], [15] and tracked using TrackPy Python software. Trajectories lasting less than 80 frames were removed from further analysis. The individual mean squared displacement (MSD) is computed as $\langle \Delta r^2 \rangle = \frac{1}{|S_\tau|} \sum_{\Delta r \in S_\tau} (\Delta r)^2$ where S_τ is the set of all displacements in a time interval τ . The diffusion coefficient for both simulations as well as experimental data was computed by linear regression of the formula $\log \langle \Delta r^2 \rangle = \log 4D + \alpha \log \tau$

Immunofluorescence

Cells grown in 35mm dishes were fixed with Formaldehyde in 1xPBS at 37C incubator for 20 minutes, and then permeabilized with 0.3 percent (v/v) Triton-X100 (Sigma-Aldrich) in PBS and blocked for 1h in 5 percent (w/v) nonfat dry milk at 4C. Cells were incubated overnight at 4C using primary antibodies anti-FLAG (Cell Signaling, clone M2; 1:1000), and anti-BRD4 (Cell Signaling, clone E2A7X; 1:1000) in blocker. Secondary antibodies for BRD4 (Cell Signaling Anti-Mouse IgG-Alexa488, 1:1000) were used.

Immunoblotting

Cells were washed and lysis buffer added (RIPA buffer: PMSF: protease inhibitor cocktail: orthovanadate=100:1:2:1). Cells were then scraped and sonicated for 15 seconds using an ultrasonic homogenizer. Lysate was centrifuged at high speed (13200r/min) for 15 minutes at 4C to pellet the cellular debris. Total protein concentration was determined by a BCA Protein Assay Kit (Pierce). For electrophoresis, protein samples were prepared according to a protein-4x loading buffer (containing DTT) ratio of 3:1, 4x loading buffer containing DTT was diluted with 3 aliquots of protein sample. The sample was mixed and heated at 95C for 5 min, followed by vortex and centrifuge. After running the gel, it was removed from the cassette and assembled inside the Trans-Blot Turbo Transfer System cassette. Transfer was run at 2.5A and 25V for 7mins. The sample was then blocked for at least 1 hour using

5 percent skim milk blocking solution prepared with PBS in RT. Primary FLAG antibody was diluted in PBST with 3 percent skim milk (1:500) and incubated at 4C overnight. The secondary antibody (Licor Anti-Mouse IgG- IRDye 800CW) was diluted in PBST with 3 percent skim milk (1:5000) and placed on a rocker and incubated at RT for 45min. Western blots on Nitrocellulose membranes were scanned using the Odyssey fluorescence scanning system software.

4.5 A potential application of sequence specific SR in immune evasion

Chromatin imaging strategies discussed so far in this chapter are capable tools for genome-wide structural analyses. Recent developments in labeling technologies have extended this approach further, making sequence specific super-resolution approaches possible. For example, OligoSTORM [153] is a super-resolution imaging strategy that reveals the distribution of nucleosomes within specific genes, through the simultaneous visualization of DNA and histones. OligoSTORM is based on in-situ hybridization, taking advantage of array-derived oligonucleotide (oligo) probes. OligoSTORM reconstructs the trajectory of a genomic region of interest by tiling the region with approximately 100 to 100,000 or more species of oligos, each carrying either a fluorophore or a binding site for a fluorescently labeled secondary oligo [10], [153]. Custom oligo probes can now be amplified from a dsDNA library and fluorescently labeled at relatively low cost. In this process, an unlabeled dsDNA oligo library up to approximately 100nt in length can be purchased from a commercial vendor, followed by T7 in-vitro transcription to ssRNA followed by addition of fluorescently modified primers and reverse transcription [154], [155]. This method may allow us to link local chromatin structures with cellular states, for example resistant states of cancer cells to immune recognition.

Immune evasion involves numerous mechanisms at molecular and cellular levels, including genetic and epigenetic heterogeneities. A primary mechanism by which tumor cells suppress the immune assault is by enhanced expression of PD-L1 ligand for the PD-1 immune inhibiting checkpoint. Binding of PD-L1 to its receptor PD-1 on activated T cells inhibits anti-tumor immunity by counteracting T cell-activating signals, thereby suppressing

antitumor immune activity. Antibody-based ICB treatments utilize PD-1-PD-L1 inhibitors to target this mechanism and can induce durable tumor remissions in patients in various cancers. However, the development of resistance to ICB treatment raises important questions regarding the fundamental determinants of PD-L1 expression. Furthermore, IFN- γ , a soluble cytokine and prominent inducer of PD-L1 expression, has surfaced as an important mediator for adaptive resistance to ICB by facilitating nuclear translocation and phase separation of key markers of immune resistance and tumor progression. Indeed, IFN- γ has been shown to promote the expression of Yes-associated protein (YAP) - a key component of phase-separated droplets in the nucleus which can influence PD-L1 expression. Interestingly, PD-L1 expression itself is regulated by YAP and BRD4, both key components of transcriptional condensates, alongside a super-enhancer (SE) PD-L1L2-SE. I speculate that IFN- γ signaling promotes PD-L1 expression by way of BRD4 and YAP-dependent phase separation of PD-L1, PD-L2, and PD-L1L2-SE loci and associated proteins.

Elevated PD-L1 expression in the tumor microenvironment is one of several avenues towards immune evasion, yet there remain many unsolved problems related to how elevated expression is achieved. In general, the development of tumor tolerance is frequently regulated by epigenetic mechanisms. DNA methylation downregulates antigen processing and presentation molecules, leading to immune escape and a reduction in sensitivity of the tumor cells to immunotherapy [156]. Transient histone demethylation can also allow cells to survive drug exposure, that is, the tolerant state is transient and reversible. Gene editing recently revealed that transcription of PD-L1 and PD-L2 gene expression is regulated by a novel super-enhancer PD-L1L2-SE [157]. Super-enhancers are complexes made of multiple enhancer elements, transcription factors, and mediating structural proteins which phase separate in so-called transcriptional condensates to stimulate amplified expression of genes defining cell identity. For this reason, SEs are of considerable interest in cancer and are now known to be a tool for tumor progression and adaptive resistance [157]. Given these findings, I further speculate that IFN- γ exposure induces structural variants of PD-L1/PD-L2/PD-L1L2-SE loci, driven in part by phase separation of YAP and BRD4. Both are key components of transcriptional condensates and have been implicated in PD-L1 expression; however, whether they act directly or indirectly is largely unknown [157], [158].

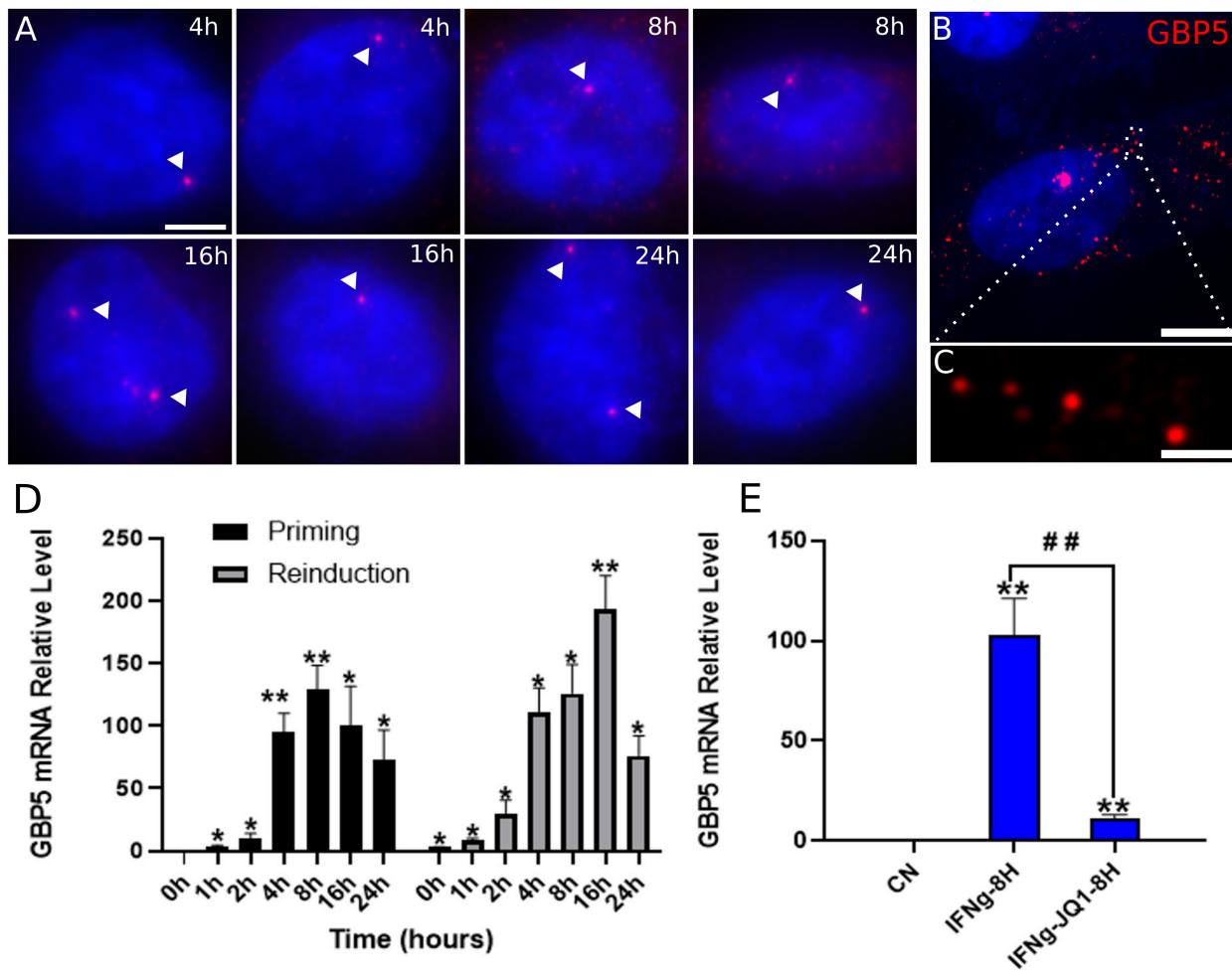


Figure 4.12. Example knockdown of a BRD4 regulated gene with (+)-JQ1 (a) Transcriptional site identification by in-situ hybridization (b,c) Example image of GBP5 induced gene expression by IFN- γ (d-e) qPCR time-series of IFN- γ induced GBP5 expression and its knockdown 8h after IFN- γ exposure with 1uM (+)-JQ1

Mechanisms contributing to PD-L1 expression are also of high importance for the clinical development of immune checkpoint blockade therapies. Chemotherapeutic treatment with (+)-JQ1 has been demonstrated to specifically inhibit super-enhancer component BRD4, dramatically decreased the expression of PD-L1 and PD-L2 in SUM-159 and MDA-MB-231 cells [157]. However, it remains unclear whether PD-L1 inhibition with JQ1 is indirect and what the role of BRD4 is, as SE structural modifications have not been directly observed.

Furthermore, other potential IFN- γ inducible SEs have also appeared in the literature, as observed by YAP puncta formation following IFN- γ induction [158]. Phase separation has been directly observed for the Interferon-inducible guanylate binding proteins (GBPs) - a group interferon-inducible GTPases [159]. The GBP locus has been colocalized with SE components MED1 and BRD4 with the GBP gene cluster in murine macrophages after infection with *Mycobacterium tuberculosis* [160]. Interestingly, inhibition of BRD4 chromatin binding activity after (+)-JQ1 exposure suppresses IFN- γ induced expression of a member of the guanylate binding protein gene cluster (Figure 4.12). Taken together, these results make several genomic loci, including PD-L1/PD-L2/PD-L1L2-SE, interesting candidates for super-resolution imaging and correlation of epigenetic states with IFN- γ exposure.

In summary, there remain many potentially correlated factors which have not yet been demonstrated: (i) direct dependence of PD-L1/PD-L2/PD-L1L2-SE structure on the presence of BRD4 and YAP, (ii) the dependence of PD-L1/PD-L2/PD-L1L2-SE structure on IFN- γ priming (iii) an association between PD-L1 transcriptional activity and PD-L1/PD-L2/PD-L1L2-SE structure. Potential conceptual innovations include: (i) A novel role for IFN- γ signaling pathways inducing immune evasion by phase separation (ii) Phase separation as an important biophysical mechanism during the immune response. In terms of methodology, this work would provide the first application of super-resolution-based reconstruction of chromatin architecture in the context of the PD-L1 axis and phase separation. This project also will continue ongoing development of novel chromatin architecture reconstruction algorithms to be shared with the scientific community.

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