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

by

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**Doctor of Philosophy**


Department of Physics and Astronomy

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# THE PURDUE UNIVERSITY GRADUATE SCHOOL
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# ACKNOWLEDGMENTS

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# LIST OF SYMBOLS

$m$ mass

$v$ velocity

# ABBREVIATIONS

| | |
|---|---|
| abbr | abbreviation |
| bcf | billion cubic feet |
| BMOC | big man on campus |

# 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. Direct STORM approaches leverage the deactivation of standard fluorescent tags, followed by spontaneous or photoinduced reactivation, allowing resolution of fluorophores at distances below the diffraction limit. This basic principle remains one of the method's primary limitations - standard SMLM fitting routines require tight control of activation and reactivation to maintain sparse emitters, presenting a tradeoff between imaging speed and labeling density. Here, I present two parallel projects, which aim to push the current state of the art in SMLM and apply SMLM to the study of gene regulation. The former represents a novel localization technique for dense SMLM, based on deep probabilistic modeling and photon statistics. In the latter, conventional dSTORM is adapted for live cell imaging of chromatin nanodomains, demonstrating that BRD4 protein concentrates in nucleosome depleted regions.

# 1. INTRODUCTION

Single molecule localization microscopy (SMLM) relies on the temporal resolution of fluorophores in the sample whose spatially overlapping point spread functions would otherwise render them unresolvable at the detector. SMLM techniques, such as stochastic optical reconstruction microsscopy (STORM) and photo-activated localization microscopy (PALM) remain desirable for super-resolution imaging of many cellular structures, due to their cost-effective implementation and diffraction unlimited resolution (Schermelleh 2019). 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 direct STORM (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 (Figure 1a). Long dark state lifetimes are commonly used in STORM imaging in order to maintain sparse activation and high resolution.

### 1.0.1 The definition of resolution in SMLM

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 or FRC. Using FRC, one can compute 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 (See Supplement). According to this theory, reducing localization uncertainty while increasing the number of samples, results in an increase in image resolution (Nieuwenhuizen 2013). However, there remains a fundamental limit to the the minimal localization uncertainty which can be obtained.

Localization uncertainty, typically the RMSE of a maximum likelihood or similar statistical estimator, is bounded from below by the inverse of the Fisher information matrix, known as the Cramer-Rao lower bound (Chao 2016). Localization uncertainties in sparse

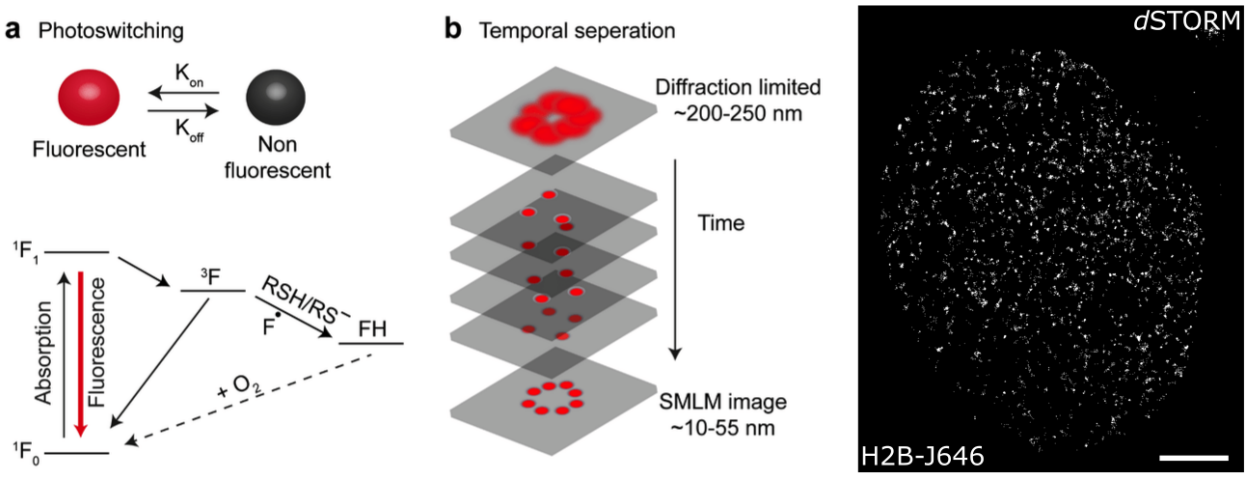


**Figure 1.1. Stochastic optical reconstruction microscopy (STORM)**. (A) Single molecules are resolved by separating their fluorescent emission in time, using fluorophores with multiple photophysical states (B) Example super-resolution image of H2B protein in a living Hela cell nucleus at 37C, 5 percent CO2. Image reconstructed from $10^3$ 10ms frames. Scalebar 5um.

conditions are often tens of nanometers, although recent work on integration of Bayesian priors with modulation enhanced SMLM (meSMLM) or structured illumination with MINFLUX, has reduced spatial resolution below to a few nanometers (Kalisvaart 2022, Gwosh 2020). Nevertheless, managing the increase in localization uncertainty at high labeling density remains a major bottleneck to SMLM. Static uncertainty due to molecular crowding can be partially amelioriated by using pairwise or higher-order temporal correlations within a pixel neighborhood, known as stochastic optical fluctuation imaging or SOFI (Dertinger 2009). 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 SMLM techniques remains unmatched, inciting an effort to increase the resolution of SMLM techniques and explore avenues towards time resolved SMLM.

### 1.0.2 Novel contributions to the field

Here, we present single photon counting enhanced SMLM and a technique for super-resolution imaging of chromatin nanodomains in-vivo. We address dense SMLM by utilizing a high-speed single photon avalanche diode (SPAD) camera. SPAD cameras provide the necessary functionality for both single molecule localization and counting. Then, we present a more conventional dSTORM approach to study spatial organization of nucleosomes in living cells, with a particular focus on the structure of phase separated condensates containing bromodomain protein 4 (BRD4) protein.

# A. CITATIONS AND REFERENCES

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9                 and Ethan Eagle},
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  note = {Editor: Kathryn Hambleton},
  year = {2018},
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  author = {William H. Gerstenmaier},
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  date = {2017-03},
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42   organization = {NASA},
43 }
44 \end{verbatim}
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# B. PHYSICS

### B.0.1 Photoswitching induced spatial coherence

Photoswitching fluorescent molecules are described in the density matrix formalism

$$\rho = \xi \left|\alpha\right\rangle \left\langle\alpha\right| + (1-\xi) \left|0\right\rangle \left\langle 0\right|$$

where $|\alpha\rangle$ is a coherent state with amplitude $\alpha$ i.e., $\langle n\rangle = \langle\alpha| \, n \, |\alpha\rangle = |\alpha^2|$. We consider a simplified model consisting of a single mode field

$$E_0^+ \sim \sum_{\mathrm{j}=1}^{M} \delta(s - s_{\mathrm{j}}) a_{\mathrm{j}} \;\; E^+(r_{\mathrm{i}}) = \int d^2 s E_0^+ h(r-s) = h(r_{\mathrm{i}} - s)\hat{a}$$

$$g_{\mathrm{ij}}^{(2)}(0) = \frac{\langle E^-(r_{\mathrm{i}}) E^-(r_{\mathrm{j}}) E^+(r_{\mathrm{i}}) E^+(r_{\mathrm{j}})\rangle}{\langle E^-(r_{\mathrm{i}}) E^+(r_{\mathrm{i}})\rangle \langle E^-(r_{\mathrm{j}}) E^+(r_{\mathrm{j}})\rangle} = \frac{\mathrm{Tr}(a^\dagger a^\dagger a a \rho)}{\mathrm{Tr}(a^\dagger a \rho)^2}$$

Notice that terms related to point spread function will cancel. Now,

$$\begin{aligned}
\mathrm{Tr}(a^\dagger a^\dagger a a \rho) &= \mathrm{Tr}(a^\dagger a^\dagger a a \left(\xi \left|\alpha\right\rangle \left\langle\alpha\right| + (1-\xi) \left|0\right\rangle \left\langle 0\right|\right)) \\
&= \mathrm{Tr}\left(\xi \mathrm{e}^{-|\alpha|^2} \sum_{n,m}^{\infty} \frac{\alpha^n}{n!} \left|n\right\rangle \left\langle m\right|\right) \\
&= \mathrm{Tr}\left(\xi \mathrm{e}^{-|\alpha|^2} \sum_{n}^{\infty} \frac{|\alpha|^{2n}}{n!} n(n-1)\right) \\
&= \mathrm{Tr}\left(\xi \mathrm{e}^{-|\alpha|^2} \sum_{n=2}^{\infty} \frac{|\alpha|^{2n}}{(n-2)!}\right) \\
&= \xi |\alpha|^4
\end{aligned}$$

The second trace in the denominator proceeds similarly to the first

$$
\begin{aligned}
\mathrm{Tr}(a^\dagger a\rho) &= \mathrm{Tr}(a^\dagger a\left(\xi\left|\alpha\right\rangle\left\langle\alpha\right| + (1-\xi)\left|0\right\rangle\left\langle 0\right|\right)) \\
&= \mathrm{Tr}\left(\xi \mathrm{e}^{-|\alpha|^2}\sum_{n,m}^{\infty}\frac{\alpha^n}{n!}\left|n\right\rangle\left\langle m\right|\right) \\
&= \mathrm{Tr}\left(\xi \mathrm{e}^{-|\alpha|^2}\sum_{n}^{\infty}\frac{|\alpha|^{2n}}{n!}n\right) \\
&= \mathrm{Tr}\left(\xi \mathrm{e}^{-|\alpha|^2}\sum_{n=2}^{\infty}\frac{|\alpha|^{2n}}{(n-1)!}\right) \\
&= \xi|\alpha|^2
\end{aligned}
$$

As expected, this gives $\langle n\rangle$. Putting it all together yields a simple expression for the two-point coherence function

$$
g^{(2)}_{\mathrm{ij}}(0) = \frac{\xi|\alpha|^4}{\xi^2|\alpha|^4} = \frac{1}{\xi}
$$

Notice that as $\xi \to 1$ (always on) we recover the coherent state. As $\xi \to 0$ we observe $g^{(2)}_{\mathrm{ij}}(0) > 1$ i.e., bunching. This is a critical result: photoswitching results in non-trivial correlations between pixels i and j. Introducing more than one photoswitching emitter gives? In practice, we can estimate of $g^{(2)}_{\mathrm{ij}}(0)$ in a finite time interval. I guess that $\langle n_{\mathrm{i}}\rangle = \xi|\alpha|^2\Delta = 0.5$ is reasonable; however this is best addressed by Monte Carlo simulation. The total interval $T$ constrained by the super-resolution frame rate e.g., $T = 10\mathrm{ms}$.

### B.0.2 Details of the Gaussian PSF

We will derive the gradients for the integrated astigmatic Gaussian, since it is the more general case. As before, define $\mathrm{i}_0 = g_k\gamma\Delta t N_0$ such that $\mu'_k = \mathrm{i}_0\lambda_k$

$$
J_{x_0} = \beta_k\lambda_y\frac{\partial\lambda_x}{\partial x_0} \quad J_{y_0} = \beta_k\lambda_x\frac{\partial\lambda_y}{\partial y_0} \quad J_{z_0} = \frac{\partial\mu'_k}{\partial\sigma_x}\frac{\partial\sigma_x}{\partial z_0} + \frac{\partial\mu'_k}{\partial\sigma_y}\frac{\partial\sigma_y}{\partial z_0}
$$

$$
\begin{aligned}
J_{x_0} &= \beta_k \lambda_y \frac{\partial \lambda_x}{\partial x_0} \\
&= \frac{\beta_k \lambda_y}{2} \frac{\partial}{\partial x_0} \left( \operatorname{erf}\left( \frac{x_k + \frac{1}{2} - x_0}{\sqrt{2}\sigma_x} \right) - \operatorname{erf}\left( \frac{x_k - \frac{1}{2} - x_0}{\sqrt{2}\sigma_x} \right) \right) \\
&= \frac{\beta_k \lambda_y}{\sqrt{2\pi}\sigma_x} \left( \exp\left( \frac{(x_k - \frac{1}{2} - x_0)^2}{2\sigma_x^2} \right) - \exp\left( \frac{(x_k + \frac{1}{2} - x_0)^2}{2\sigma_x^2} \right) \right)
\end{aligned}
$$

$$
\begin{aligned}
J_{y_0} &= \beta_k \lambda_x \frac{\partial \lambda_y}{\partial y_0} \\
&= \frac{\beta_k \lambda_x}{2} \frac{\partial}{\partial y_0} \left( \operatorname{erf}\left( \frac{y_k + \frac{1}{2} - y_0}{\sqrt{2}\sigma_y} \right) - \operatorname{erf}\left( \frac{y_k - \frac{1}{2} - y_0}{\sqrt{2}\sigma_y} \right) \right) \\
&= \frac{\beta_k \lambda_x}{\sqrt{2\pi}\sigma_y} \left( \exp\left( \frac{(y_k - \frac{1}{2} - y_0)^2}{2\sigma_y^2} \right) - \exp\left( \frac{(y_k + \frac{1}{2} - y_0)^2}{2\sigma_y^2} \right) \right)
\end{aligned}
$$

$$
\begin{aligned}
J_{\sigma_x} &= \beta_k \lambda_y \frac{\partial \lambda_x}{\partial \sigma_x} \\
&= \frac{\beta_k \lambda_y}{2} \frac{\partial}{\partial \sigma_x} \left( \operatorname{erf}\left( \frac{x_k + \frac{1}{2} - x_0}{\sqrt{2}\sigma_x} \right) - \operatorname{erf}\left( \frac{x_k - \frac{1}{2} - x_0}{\sqrt{2}\sigma_x} \right) \right) \\
&= \frac{\beta_k \lambda_y}{\sqrt{2\pi}} \left( \frac{\left(x - x_0 - \frac{1}{2}\right) \mathrm{e}^{-\frac{\left(x - x_0 - \frac{1}{2}\right)^2}{2\sigma_x^2}}}{\sigma_x^2} - \frac{\left(x - x_0 + \frac{1}{2}\right) \mathrm{e}^{-\frac{\left(x - x_0 + \frac{1}{2}\right)^2}{2\sigma_x^2}}}{\sigma_x^2} \right)
\end{aligned}
$$

$$
\begin{aligned}
J_{\sigma_y} &= \beta_k \lambda_x \frac{\partial \lambda_y}{\partial \sigma_y} \\
&= \frac{\beta_k \lambda_x}{2} \frac{\partial}{\partial \sigma_y} \left( \operatorname{erf}\left( \frac{y_k + \frac{1}{2} - y_0}{\sqrt{2}\sigma_y} \right) - \operatorname{erf}\left( \frac{y_k - \frac{1}{2} - y_0}{\sqrt{2}\sigma_y} \right) \right) \\
&= \frac{\beta_k \lambda_x}{\sqrt{2\pi}} \left( \frac{\left(y - y_0 - \frac{1}{2}\right) \mathrm{e}^{-\frac{\left(y - y_0 - \frac{1}{2}\right)^2}{2\sigma_y^2}}}{\sigma_y^2} - \frac{\left(y - y_0 + \frac{1}{2}\right) \mathrm{e}^{-\frac{\left(y - y_0 + \frac{1}{2}\right)^2}{2\sigma_y^2}}}{\sigma_y^2} \right)
\end{aligned}
$$

Luckily, computing the Hessian matrix for (2.9) is tractable, and is actually quite simple when one takes advantage of the chain rule for Hessian matrices. Looking at (2.9), the

likelihood is a hierarchical function that maps a vector space $\Theta$ to a vector space $\Lambda$ to a scalar value. Formally, we define $T : \Theta \to \Lambda$ and $W : \Lambda \to \mathbb{R}$. The parameter vector $(x_0, y_0, z_0, \sigma_0, N_0) \in \Theta$, the Poisson rate vector $\vec{\lambda} \in \Lambda$ and $\ell \in \mathbb{R}$. Note that we choose to optimize $\sigma_x$ and $\sigma_y$ directly and compute $z_0$ to simplify the computation of the Hessian. To get the Hessian, we need the chain-rule for Hessian matrices, which can be quickly computed in terms of the jacobian and hessian of $T$ and $W$.

$$H_\ell = J_\mu^T H_\ell J_\mu + (J_\ell \otimes I_n) H_\mu$$

where we have used $J_\mu$ to represent the jacobian of $T$ and $J_\ell$ for the jacobian of $W$. Similar notation is used for the corresponding Hessian matrices. In the 3D case, the Hessian matrix is not directly separable since $\mu \propto \lambda_x(x_0, \sigma_0, \sigma_x)\lambda_y(y_0, \sigma_0, \sigma_y)$. To see this, an abstract representation of the Hessian reads

### B.0.3 Fisher information for 2D integrated gaussian

For the 2D integrated gaussian point spread function, the Hessian only contains separable second order derivatives, so the Fisher information matrix takes on a convenient form

$$I_{\mathrm{ij}}(\theta) = \mathop{\mathbb{E}}_{\theta}\left(\frac{\partial \ell}{\partial \theta_{\mathrm{i}}}\frac{\partial \ell}{\partial \theta_{\mathrm{j}}}\right) \tag{B.1}$$

For an arbitrary parameter then we have

$$\begin{aligned}\frac{\partial \ell}{\partial \theta_{\mathrm{i}}} &= \frac{\partial}{\partial \theta_{\mathrm{i}}}\sum_k x_k \log x_k + \mu_k' - x_k \log\left(\mu_k'\right) \\ &= \sum_k \frac{\partial \mu_k'}{\partial \theta_{\mathrm{i}}}\left(\frac{\mu_k' - x_k}{\mu_k'}\right)\end{aligned}$$

$$I_{\mathrm{ij}}(\theta) = \mathop{\mathbb{E}}_{\theta}\left(\sum_k \frac{\partial \mu_k'}{\partial \theta_{\mathrm{i}}}\frac{\partial \mu_k'}{\partial \theta_{\mathrm{j}}}\left(\frac{\mu_k' - x_k}{\mu_k'}\right)^2\right) = \sum_k \frac{1}{\mu_k'}\frac{\partial \mu_k'}{\partial \theta_{\mathrm{i}}}\frac{\partial \mu_k'}{\partial \theta_{\mathrm{j}}}$$

To compute the bound, it turns out all we need is the jacobian $\frac{\partial \mu_k'}{\partial \theta_{\mathrm{j}}}$.

# VITA

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