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Sub-10 nm Imaging of Nucleic Acids Using Spectroscopic Intrinsic-Contrast Photon-Localization Optical Nanoscopy (SICLON)

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Abstract

Elucidating chromatin structure *in vitro* requires resolution below 10 nm to visualize the mononucleosome and has been an ongoing challenge. In this work, we achieve sub-10 nm imaging of nucleic acids via Spectroscopic Intrinsic-Contrast photon-Localization Optical Nanoscopy (SICLON) without the use of external labels. SICLON leverages two key innovations: using endogenous nucleotides as the emission source, and a custom-made imaging system that can simultaneously record the position and optical spectra of emitting molecules. With a novel spectral-regression algorithm that identifies the spectroscopic fingerprints of neighboring molecules that were previously indistinguishable, we demonstrate the utility of SICLON by visualizing unlabeled polynucleotides and linear single stranded DNA fibers with a resolution of 6.2 nm.

Keywords

(100.6640) Superresolution; (170.6280) Spectroscopy; fluorescence and luminescence; (110.3010) Image reconstruction techniques; (220.4830) Systems design

The nanoarchitecture of chromatin underlies and regulates essentially all genetic machinery with a complex organization ranging from individual macromolecules that are a few nanometers in size (e.g. DNA), to macromolecular assemblies that may span tens of

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nanometers (e.g. nucleosome, chromatin fiber), to micron-scale structures forming topologically associating domains (TAD) and chromatin compartments [1]. The major scientific challenge is to understand this heterogeneous chromatin structure at all length scales in non-perturbed states. Tremendous advances in techniques such as neutron scattering, small angle x-ray scattering, electron microscopy, super-resolution microscopy, etc. have greatly enriched our knowledge of chromatin nanostructure in the past decades [2–5]. Super-resolution microscopy, specifically photon localization microscopy (PLM), is capable of resolving chromatin at subdiffractional length scales ($<200\text{nm}$) with a micron-scale field of view. PLM has been employed to visualize the second order (e.g. chromatin fiber) and the higher order chromatin structure (e.g. TAD) [6, 7]. However, to visualize even finer details of chromatin such as a single nucleosome or DNA (length scales $<10\text{ nm}$), using conventional PLM is challenging. The resolution of conventional PLM is limited by two key factors: labeling density and the number of photons from each emission (localization uncertainty). High label density is required in order to resolve structures according to the Nyquist criterion. Even with sufficiently high labeling density, the precision of fitting a point-spread function (PSF) during image reconstruction in PLM is dependent on the number of photons in each emission event [8]. Specifically, the spatial resolution of the PSF fit after reconstruction is proportional to $1/\sqrt{n}$, where n is the number of emitted photons. Together, these two factors have limited the resolution of conventional PLM techniques to approximately 20 nm . To improve the resolution of PLM, we describe spectroscopic intrinsic-contrast photon-localization optical nanoscopy (SICLON), a superresolution microscopy technique to overcome both of these obstacles. Using SICLON, we demonstrate the ability to visualize DNA with sub- 10 nm resolution.

To date, the methods for directly imaging chromatin generally require staining, such as the fluorescence dye in superresolution microscopy (e.g. PALM/STORM) [9] and combined fluorescence dye and heavy metal stain in electron microscopy (e.g. ChromEMT) [4]. However, the use of exogenous dyes has fundamental limitations: the uptake, diffusion, and localization of dyes depends non-linearly on the local environment and could render the chromatin image difficult to interpret, especially for the high label densities required to satisfy the Nyquist criterion for sub- 10 nm resolution; and with spacing smaller than 30 nm , steric hindrance and epitope accessibility for most fluorophore probes becomes a significant issue as the labels are nearly the size of the molecules of interest. Additionally, use of DNA intercalating dyes have the drawbacks of potentially distorting and even damaging DNA structure [10]. Due to these challenges, an alternative approach using label-free contrast for direct imaging has considerable advantages.

To tackle the labeling density issue and eliminate the potential artifacts introduced by labels, we utilized a newly discovered phenomenon: DNA can fluoresce under visible light, and the nucleotides of DNA itself used as the source of photon emission. While DNA had previously been considered “dark” in the visible spectral range, recently it was shown to exhibit photoswitchable autofluorescence when illuminated by visible light using ground-state depletion (GSD) with dark-state shelving and stochastic return [11]. Dong *et al.* previously investigated the photochemical properties of DNA autofluorescence in detail [11, 12]. By leveraging GSD, direct nanoscopic imaging of nucleic acids using their intrinsic

fluorescence for contrast has been demonstrated. Under visible light illumination, unmodified DNA has the capacity to stochastically emit photons allowing for their use in photon localization microscopy. While nucleic acids have weak absorption within the visible range, they have a remarkably high quantum efficiency, a long-lived dark state, and comparably high photon emission counts [11]. Furthermore, as the base unit of chromatin is nucleic acids, their use for PLM completely bypasses the labeling density limits presented by exogenous approaches and the non-linear effects of local macromolecular density would have on molecular mobility and binding affinities. Using the intrinsic stochastic fluorescence of nuclear acids, the capacity to image chromatin without exogenous fluorophores with ~20 nm resolution has been demonstrated [12].

To measure spatial information and spectra of fluorescent molecules (e.g., nucleotides) simultaneously, we built the SICLON instrument shown in Fig. 1. Continuous wave laser illumination is focused onto the back focal plane of a high numerical aperture (NA) objective to allow collimated laser light to exit the objective. The position and angle of the laser can be controlled to achieve total internal reflection fluorescence (TIRF) illumination to excite the fluorescent molecules into long-lived dark states and subsequently recover them by stochastic photo-switching. The emission image is collected by the high NA objective, and coupled into a Czerny–Turner monochromator (SP2150, Princeton Instruments) via a matched tube lens. The emission image is split into its zeroth-order and spectrally dispersed, first-order image via a blazed dispersive grating (150 grooves per mm) and projected onto a high sensitivity EMCCD (proEM, Princeton Instruments) with a resolution of 0.63 nm. This configuration allows simultaneous collections of spatial (zeroth-order) and spectral (first-order) information from each emission event. It should be noted that only ~1/4 of the light is sent to the zero-order, we see a decrease in localization precision equal to approximately $\sqrt{4} = 2$ times. Experimentally we have measured an average localization uncertainty of approximately 40 nm on average, indicating that our theoretical resolution should be 20 nm, comparable to traditional label-based STORM techniques.

As mentioned previously, the precision of image reconstruction in most photon localization microscopy techniques like STORM/PALM relies on fitting the PSF to emission events. The resolution of the localization of any emission event is proportional to $1/\sqrt{n}$. In order to combine emission events from the same emitter and increase the number of photons, conventional reconstruction algorithms consider all emissions occurring in the same resolution limited pixels in consecutive time frames from the “same” molecule [13]. However, this approach has two shortcomings: 1) It might lead to poor localization precision due to inappropriate merging of unique emitters and 2) it does not consider multiple emission events from the same emitter which are not in temporally neighboring frames. To overcome this we have developed a spectral regression algorithm to more accurately merge recurrent emission events from a single emitter[8].

The principle of the spectral regression algorithm is that the emission spectra of the same molecule from different blinking events remain consistent compared to the variations of the spectra from other molecules in close proximity [8]. For different nucleotides, we found that the nucleic acid emission has spectroscopic specificity, meaning that the peak position and frequency of the emission spectra depends on the nucleotide residue. Figure 2(a) shows that

20 base pair (bp) DNA molecules (Integrated DNA Technologies, USA) with different molecular makeups (different nucleotides) have unique spectral properties. Fig. 2(b) shows the heterogeneity of DNA emission spectra extracted from salmon (Sigma-Aldrich, USA). We were able to differentiate distinct molecules by comparing the average standard deviation (SD) of the spectral centroids of recurrent emissions. For single DNA emitters, we calculated the average SD of the spectral centroid from recurrent blinking events to be 3.25 nm. For comparison, the SD of the spectral centroid of emissions from unique DNA emitters was 19.84 nm. Thus, the spectra from a given emitter are consistently unique and can be differentiated from the spectra of other molecules.

The probability that a different molecule generates a spectrum with the same spectral centroid as a target molecule (α) can be estimated from the error function (erf) and the standard deviation of spectral centroids from the same molecules (σ_{single}) and different molecules (σ_{multiple}) using the equation:

$$\alpha \sim \sqrt{\frac{2}{\pi}} \int_0^{\infty} \left\{ \operatorname{erf} \left(\frac{x + \frac{\sigma_{\text{single}}}{\sigma_{\text{multiple}}}}{\sqrt{2}} \right) - \operatorname{erf} \left(\frac{x}{\sqrt{2}} \right) \right\} \exp \left(-\frac{x^2}{2} \right) dx$$

In the particular case in Fig. 2(b-d), the added relative localization uncertainty is negligible at $\sim \alpha/2 \approx 4\%$. Under the key principle, we can safely rely on the centroid wavelength to determine whether two blinking events within the resolution limit of conventional STORM originate from the same molecule. The working principles of the spectral regression algorithm are shown in Fig. 3. To implement the algorithm, the neighborhood around each blinking event (within the localization uncertainty) in all frames will be searched to yield candidate events that can be categorized as repeat emissions. Based on the position of the spectral centroid, we determine whether multiple emissions belong to the same molecule. All emissions corresponding to the same molecule were merged by calculating the average of their localized positions. This process was repeated until all unique molecules in the dataset have had their repeated emissions combined and new centroids [8]. Molecules with few repeated emission events were filtered out to remove false blinking events and ensure sufficiently high localization precision of all molecules.

To improve imaging resolution, the intrinsic fluorescence contrast from nucleic acids that form chromatin completely bypasses the labeling density limits presented by exogenous approaches for high resolution imaging, and the spectral regression algorithm significantly improves the localization quality by precisely classifying neighboring emissions and identifying recurrent emissions from the same molecule. It is possible to push the resolution further down to the sub-10 nm range by taking advantage of both techniques. Using the new nanoscale imaging technology combining time-resolved visible-light stochastic photon localization with simultaneous spectroscopic molecular-signature-carrying intrinsic fluorescence detection, referred to as SICLON, we achieve a lateral resolution of 6.2 nm in both linear single-stranded DNA fibers and unmodified poly-nucleotide sequences. The

resolution of SICLON is confirmed by AFM imaging of similar linear single-stranded DNA fibers prepared in parallel.

As demonstrated in Fig. 4(a), isolated 20mer poly-DNA complexes deposited on glass coverslips have weak, but observable fluorescence under wide-field illumination. By using SICLON and analyzing the zero-order emissions, resolution can be improved to 40 nm (Fig. 4(b & c)). As the zero-order source is composed of distinct emission spectra owing to conformational and molecular variations, each molecule can be identified utilizing spectral regression (Fig. 4(c)). Significantly, even for molecules that are less than 20 nm apart (theoretical resolution limit), spectral regression allows clear delineation of molecular features down to 6.2 nm, as measured by the full-width-half-max (FWHM) of the nucleotide source. With this resolution, SICLON can fully differentiate two molecules that are 16 nm apart (Fig. 4(d)).

To test the improved resolution for unmodified nucleotide samples, we imaged isolated single stranded salmon sperm DNA (Fig. 5). The single stranded DNA was spin coated on a glass coverslip to produce easily identifiable linear DNA fibers [14]. To confirm the molecular origin of these fibers was nucleic acids, we utilized Hoechst 33342 wide-field fluorescent imaging using a 405 nm source (Fig. 5(a)). Next, we performed SICLON imaging of the isolated fibers using 532 nm excitation to produce a traditional PLM spatial image (0th order image) of the DNA (Fig. 5(b & d)). As we have previously demonstrated, we observed co-localization between the PLM image and wide-field fluorescent Hoechst 33342 imaging, thus confirming the DNA origin of the structure. Next, we utilized SICLON collection of the 0th order emission and the 1st order spectra from the fiber to apply spectral regression and emission integration (Fig. 5(c & e)). Using SICLON, we observe an average of approximately 2100 photons recorded from each emitter merged in the zero-order image, resulting in a minimal FWHM of the DNA fiber samples of 8.5 nm (Fig. 5(g)). To compare this resolution with the ground truth of the fiber, we performed Atomic Force Microscopy (AFM) of DNA samples spin-coated in parallel on freshly cleaved mica at 0.98 nm resolution (Fig. 5(f)). With AFM, we observe a minimal FWHM of 4.1 nm of isolated fibers with similar geometry and prepared using the same method as those observed with SICLON in Fig. 5(a-e). Through convolution of the minimal FWHM of 4.1 nm observed with AFM and the 6.2 nm resolution observed in SICLON (Fig. 4) yields a fiber with an observable FWHM of 8.5 nm which matches the SICLON result as shown in Fig. 5(g-h). This further confirms the estimated lateral resolution of SICLON as 6.2 nm.

In this work, we present a nanoscopic imaging technique, SICLON, to directly image nucleic acids with the resolution required to fully image the organizational structure of chromatin down to the single nucleosomal level without the need for extrinsic dyes. Using the intrinsic contrast produced by the stochastic emission of nucleic acids and spectral regression, we demonstrate in isolated nucleotides and single stranded DNA a lateral resolution of 6.2 nm. This resolution surpasses the capabilities presented *ex-vivo* with DNA-PAINT [15] or dual-objective STORM [16]. SICLON can also be paired with these enabling technologies to further increase resolution and provide molecular-specific information about the *in-vitro* structure of chromatin. We envision that subsequent work with SICLON will provide direct mapping of nucleosomal occupancy in fixed cells using complementary

hybridization to demarcate genes of interest. Importantly, as we observe small but detectable differences in the spectral emission from each nucleotide, it may be possible in the future to utilize spectral regression to both identify the molecular composition (molecular fingerprint) and structure of chromatin without exogenous labels. We believe the potential of SICLON to fully map the structure and chemistry of chromatin *in-vitro* will greatly expand our understanding of how chromatin topology influences essentially all genetic machinery.

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Fig. 1. The system schematic. Fluorescent images were collected by a high numerical aperture objective lens and sent to a Czerny–Turner monochromator where the zero- and first-order images are read separately and simultaneously with an EMCCD.

Fig. 2.

(a) Photon counts and emission spectra centroids associated with 100 blinking events for unlabeled 20bp sequences of poly-G, poly-A, poly-T and poly-C. (b) Spectral heterogeneity of unlabeled poly-DNA molecules. (c) & (d) Emission spectra of individual stochastic localization at two neighboring spots indicated by blue and red dots in (b). The emission events can be separated clearly according to their emission properties, indicating they are from two different emitters.

Fig. 3. Flow chart of spectral regression algorithm. Conventional PLM reconstruction was performed in ThunderSTORM. Using spectral regression algorithm, emission events that belong to the same molecule were added (merged) to improve the accuracy of emitter localization.

Fig. 4.

(a) Wide field image of 20mer poly-DNA, (b) STORM reconstruction of 20mer poly-DNA, (c) enlarged view of two 20mer poly-DNA molecules, (d) after SR the two 20mer poly-DNA molecules are separable, and (e) the FWHM of each individual molecule is shown to be 6.2 nm, and the molecules are shown to be spaced 16 nm apart.

Fig. 5.

(a) Wide field fluorescence image of Hoechst stained DNA fiber, (b) STORM reconstruction of DNA fiber using 532 nm illumination, (c) STORM reconstruction of DNA fiber after applying SR algorithm, (d) enlarged view of selection shown in b, (e) enlarged view of selection shown in c, (f) an image acquired by AFM of a separate single DNA fiber, (g) comparison of resolution between traditional PLM and SICLON with SR shows a 4X resolution enhancement, and (h) AFM reveals the ground truth width of the fiber is estimated to be 4.1 nm.