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Localization microscopy of DNA in situ using Vybrant® DyeCycle™ Violet Fluorescent Probe: a new approach to study nuclear nanostructure at single molecule resolution

Dominika Żurek-Biesiadaa, Aleksander T. Szczurekb, Kirti Prakashbc, Giriram K. Mohana, Hyun-Keun Legbd, Jean-Yves Rognantb, Udo Birkbd, Jurek W. Dobruckia,*, Christoph Cremerb,c,d,*

aLaboratory of Cell Biophysics, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Gronostajowa 7, 30-387, Kraków, Poland,
bInstitute of Molecular Biology (IMB), Ackermannweg 4, 55128, Mainz, Germany,
cInstitute for Pharmacy and Molecular Biotechnology (IPMB), University of Heidelberg, Im Neuenheimer Feld 364, D-69120, Heidelberg, Germany,
dDepartment of Physics, University of Mainz (JGU), Staudingerweg 7, 55128, Mainz, Germany.
*Corresponding authors: c.cremer@imb-mainz.de; +49 (0) 6131-39-21518; jerzy.dobrucki@uj.edu.pl; +48 12 664-63-82;

Abstract

Higher order chromatin structure is not only required to compact and spatially arrange long chromatids within a nucleus, but have also important functional roles, including control of gene expression and DNA processing. However, studies of chromatin nanostructures cannot be performed using conventional widefield and confocal microscopy because of the limited optical resolution. Various methods of superresolution microscopy have been described to overcome this difficulty, like structured illumination and single molecule localization microscopy. We report here that the standard DNA dye Vybrant® DyeCycle™ Violet can be used to provide Single Molecule Localization Microscopy (SMLM) images of DNA in nuclei of fixed mammalian cells. This SMLM method enabled optical isolation and localization of large numbers of DNA-bound molecules, usually in excess of $10^6$ signals in one cell nucleus. The technique yielded high-quality images of nuclear DNA density, revealing subdiffraction chromatin structures of the size in the order of 100 nm; the interchromatin compartment was visualized at unprecedented optical resolution. The approach offers several advantages over previously described high resolution DNA imaging methods, including high specificity, an ability to record images using a single wavelength excitation, and a higher density of single molecule signals than reported in previous SMLM studies. The method is compatible with DNA/multicolor SMLM imaging which employs simple staining methods suited also for conventional optical microscopy.

Key words: DNA dyes; dSTORM; SMLM; photoconversion; Vybrant DyeCycle Violet; chromatin structure
Introduction

During the last decade, impressive progress has been made in studies of functional implications of DNA methylation, histone modifications, and chromatin remodeling events related to gene regulation (Cremer & Cremer, 2010; Bell et al., 2011). However, it has also become obvious that decoding the chromatin language does not suffice to fully understand the formation of the different epigenomes present in various cell types of a multicellular organism. Different epigenomes and their functional implications depend on differences in higher order chromatin organization and nuclear architecture (Cremer & Cremer, 2001; Rouquette et al., 2010). The connections between structural and functional changes of cell nuclei during development and differentiation must be counted among the great, unresolved problems of cell biology (Popken et al. 2014). For a comprehensive understanding of such epigenetic nuclear structure-function relationships it is of utmost importance to decipher the rules of a higher order nuclear organization, including detailed information on the nuclear nanostructure. Until the advent of super-resolution fluorescence microscopy techniques (for review see Cremer & Masters, 2013) this has been extremely difficult to achieve, due to the limited resolution of conventional light microscopy.

Recently developed approaches of structured illumination (SIM) and single molecule localization microscopy (SMLM) of fluorescently labeled cellular subdiffraction structures provide an optical resolution much better than conventional fluorescence widefield or confocal microscopy. While SIM methods (Heintzmann & Cremer 1999; Gustafsson 2000) achieve an enhanced resolution around 100 nm (Heintzmann et al. 2009), SMLM approaches presently allow an enhanced resolution of cellular structures in the 20 nm range (Huang et al., 2008) giving similar results to STED microscopy (Persson et al., 2011).

Using SIM, various chromatin subdiffraction structures were described in mammalian cell nuclei including light microscopy evidence for the existence of an interchromatin compartment (Schermelleh et al., 2008; Markaki et al. 2010; Popken et al. 2014). These studies indicated that a still higher spatial resolution should be highly useful to study the nuclear landscape in more detail. Such a further enhanced resolution has become possible by approaches of localization microscopy. Generally, this method is based on the optical isolation and highly precise localization of fluorescent point emitters (e.g. molecules) by appropriate ‘spectral signatures’, e.g. absorption/emission, fluorescence lifetimes, etc. (Betzig, 1995; Cremer et al., 1996; Esa et al., 2000; Heilemann et al., 2002). Recently, Single Molecule Localization Microscopy approaches using stochastic photoswitching (blinking) schemes for the optical isolation of single point emitters (Rust et al., 2006; Betzig et al., 2006; Reymann et al., 2008; Lemmer et al., 2008; Heilemann et al., 2008) have made it possible to discriminate hundreds or even thousands of individual molecules per µm², and localize them with a precision down to a few nm. In addition to the enhanced optical resolution (i.e. the capability to localize two adjacent point emitters), such ‘single molecule localization microscopy’ (SMLM) techniques allowed enhanced structural resolution (i.e. the capability to resolve nanostructures composed of a large number of adjacent molecules). The combination of blinking based localization with previous localization microscopy schemes based on differences in the absorption/emission spectrum made possible multicolor
The spatial resolution improvement provided by the new high resolution optical microscopy methods yields spectacular results, especially in the case of relatively sparsely arranged structures of a shape, which is known a priori, like microtubules. Achieving high resolution imaging of DNA molecules in cell nuclei is more difficult, due to a high packing density of this polymer and an unknown higher order spatial arrangement of chromatin fibers on the nanoscale (Misteli, 2013). So far SMLM was used in attempts to 'nano-image' isolated DNA in vitro (with fluorescent stains YOYO-1 and PicoGreen (Flors et al., 2009; Schoen et al., 2011)), and the density of DNA in situ in fixed and live cells (with fluorescent stains TOPRO (Flors, 2010) and PicoGreen (Benke and Manley, 2012)), as well as immuno-fluorescently labeled histones in a cell nucleus (Matsuda et al., 2010; Cremer et al., 2011; Ricci et al., 2015). Recently we have demonstrated that photoconversion of Hoechst 33258, Hoechst 33342 and DAPI provides an opportunity to image DNA in situ at a high spatial resolution using localization microscopy (Żurek-Biesiada et al., 2013; Żurek-Biesiada et al., 2014; Szczurek et al., 2014). In this report we present a new high resolution DNA SMLM approach using Vybrant® DyeCycle™ Violet DNA dye. Localization microscopy based on detection of blinking of photoconverted VdcV can be achieved with a single excitation wavelength only (blue light, UV/405 nm is not required). Vybrant® DyeCycle™ Violet is therefore a good candidate for the enhanced resolution analysis of DNA/multicolor super-resolution imaging of fixed specimens.

Materials and Methods

Cell culture and sample preparation

Vero-B4 African green monkey kidney fibroblast-like cells were grown on 0.17 mm thick coverslips in 6-well plates filled with RPMI 1640 medium supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences, Germany), 100 U/ml penicillin and 0.1 mg/ml streptomycin in 37°C, high humidity and 5% CO₂. After an 8 min fixation with 4% formaldehyde and following permeabilization using 1% Triton X-100 (5 minutes), the cells were washed with PBS and incubated with a 1 ml aqueous solution of RNase A (0.2 mg/ml, 37°C) for at least one hour in order to hydrolize RNA. Afterwards, the cells were incubated for 30 minutes with 1 ml water solution of Vybrant® DyeCycle™ Violet (VdcV) stain (Life Technologies, Poland) (added directly to the 6-well plate) at the concentration range 10 nM - 5 µM. Cells were washed with PBS and embedded in an imaging buffer containing enzymatic oxygen scavenging system, 90% glycerol + 10% of a stock (comprising 5 mg/ml glucose oxidase, 0.4 mg/ml catalase) + 10% (w/v) glucose in PBS), and immediately sealed with nail polish. This medium was chosen in a process of optimization (see next sections); it was already used in our recent work (Szczurek et al., 2014) and had been inspired by a previous recipe presented in (Heilemann et al., 2008). In confocal microscopy measurements of the dye immobilized on the polymer surface, sample preparation followed a protocol described previously in (Kędziora et al., 2011).

Drosophila melanogaster w^1118 wandering third instar larvae were used for preparation of polytene chromosome squashes as described in (James, 2005), with some modifications. The third instar larvae were dissected in 0.1% Triton X-100 in PBS (pH 7.5), salivary glands were removed and fixed for 30 seconds in 4% formaldehyde, 1% Triton X-100 in PBS (pH 7.5). Next, the samples were incubated for 2 minutes in 4% formaldehyde and 50% acetic acid in H₂O. Subsequently salivary glands were placed on a coverslip (24 x 24 mm) and
picked up onto a poly-L-lysine coated coverslip (24 x 50 mm). Using a pencil, corners of the 24 x 24 mm coverslip were tapped and a hard press on salivary glands was exerted. Good quality polytene chromosome squashes (after inspection under a phase contrast microscope) were frozen in liquid nitrogen and the 24 x 24 mm coverslip was flicked off with razor blade. Poly-L-lysine coated coverslips carrying polytene chromosome squashes were kept in PBS at room temperature for 10 minutes and subsequently used for staining: incubation was performed for 12 h with 1:500 rabbit primary antibody against RNA polymerase II phosphorylated at serine 2 (Pol II Ser2P) (Abcam, ab5095) and washed with KCM buffer (120 mM KCl, 20 mM NaCl, 10 mM Tris/HCL pH 8, 0.5 mM EDTA, 0.1% v/v Triton X-100). Next, the samples were incubated at room temperature for 1.5 h with 1:5000 anti-rabbit antibody conjugated with Alexa Fluor 555 (Life Technologies, Germany). Samples were washed with PBS, stained for 30 minutes with 250 nM VdcV, and embedded in the same imaging buffer as indicated above. Samples were sealed with nail polish. All reagents were purchased from Sigma-Aldrich, Germany, if not indicated otherwise.

Microscopy setup and data analysis
All experiments were performed on a custom-built Vertico-SPDM setup (Reymann et al., 2008). The microscopy setup and the algorithm used for reconstruction of the SMLM images are described in (Szczurek et al., 2014). The average photon number was estimated using the ThunderSTORM software with appropriate camera input parameters (Ovesný et al., 2014). To obtain SMLM images of the green-emitting form of Vybrant® DyeCycle™ Violet under the conditions used, it is necessary to perform bleaching prior to image acquisition. This allows to eliminate the overlapping signals of the simultaneously fluorescing neighboring molecules within the same observation volume as given by the conventional optical resolution limit (ca. 200 nm laterally, 600 nm axially). Afterwards, typically 20,000 frames (nuclei) or 40,000 frames (chromosomes) were acquired with an integration time of 50 ms, and 0.525 - 1.5 kW/cm² object plane intensity of a 491 nm emitting laser (Cobolt AB, Sweden). For investigation of photoconversion and its influence on the density of single molecule localizations additional 2 W/cm² 405 nm was applied and 20,000 frames with 50 ms exposure time were acquired. The density of localizations was calculated by measuring the number of points within a given area of a binary SMLM reconstruction. The illumination laser beam was collimated in the sample plane. Intensity of the excitation light has been calculated from the laser power measured in front of the objective lens, and from the illumination spot size (Gaussian fit to the detected signal reflected from an empty sample). For two color experiments, a drift was corrected based on autocorrelation of underlying structures for both channels separately with subsequent manual overlay of both channels. Chromatic aberrations of the microscope optics were measured separately using multi-color stained samples (microtubules). In experiments using photoconverted VdcV, the spectral detection bands for both channels were identical. In this way it was possible to eliminate chromatic aberrations in detection.

Signals were determined from the raw multi-frame acquisitions as follows (Gruell et al., 2011): noise has been assumed to follow a Poisson distribution, i.e. the standard deviation of the noise (STD) is given by STD = sqrt (background). Initially, the background was calculated by averaging 8 initial frames. Via an iterative approach, background has been subtracted from each of the images resulting in difference images. Smoothing with 3 x 3 kernel has been applied to the difference image, and the initial positions of the peaks were estimated with precision of pixel position based on the criterion that only peaks higher or equal to 2*STD are considered. A region of interest (ROI) of 7x7 pixels centered at the initial position estimation was used later in the difference image (no smoothing was applied) to fit the position of the signal with sub-pixel precision. Sub-pixel precision estimates of the signal positions (x,y) were performed using a center of gravity algorithm, yielding additionally the error $\sigma_{loc}$ (standard deviation) in the estimated positions (Thompson et al., 2002).
Subsequently, the difference image was utilized for background correction of the next image in a multi-frame acquisition as described in detail by (Gruell et al., 2011). Signals appearing in subsequent frames were combined if their lateral distance was less than 2.5 $<\sigma_{loc}>$, with $<\sigma_{loc}>$ being the mean localization error.

Microscope and software have been validated using calibration samples to indicate the best achievable resolution (for details see Supplementary Figure S14).

Results

Vybrant® DyeCycle™ Violet (VdcV) is a fluorescent dye with a predominant affinity for DNA, and a weaker affinity for RNA (Supplementary Fig S4) without disclosed mode of binding. While bound to DNA in nuclei of fixed cells, VdcV has an excitation maximum within the near UV-range and emits fluorescence in the blue region of the visible spectrum (abs./em. 369/437 nm). A continued exposure to UV or to 405 nm light resulted in a decrease of fluorescence intensity due to photobleaching, and generation of a form of the dye (Fig. 1A) with absorption and emission maxima shifted towards longer wavelength (Żurek-Biesiada et al., 2013). This photoprodut of VdcV was excited optimally with blue light ($\lambda_{exc} = 491$ nm), and emitted green luminescence ($\lambda_{em} = 540$ nm) while bound to cellular DNA (Fig. 1C). Interestingly, the photoprodut was also generated when VdcV was immobilized in a polymer (Fig. 1B) demonstrating that DNA was not required for phototransform. In cell samples prepared under these experimental conditions, without prior UV illumination, a small fraction of the green-emitting form of VdcV already existed (Żurek-Biesiada et al., 2013; Żurek-Biesiada et al., 2014), while the majority of the probe exhibited the typical spectral characteristics of the blue emitting form (exc./em. 369/437 nm) (Fig. 1 and Materials and Methods). The spectral properties of VdcV changed as a result of exposure to hydrogen peroxide and low pH (Supplementary Fig. S5) in a manner similar to a minor-groove binding Hoechst 33258 (Cosa et al., 2001; Żurek-Biesiada et al., 2013; Żurek-Biesiada et al., 2014). In both cases the intensity of fluorescence of the green-emitting form increased. We found that a red-shift (the difference between emission maxima of both forms of VdcV) was 103 nm. This is significantly more than the value of this parameter reported for Hoechst dyes (76 nm) (Szczurek et al., 2014). Such large shifts in the wavelength facilitate easier separation of the detected signals of both forms, thus providing higher signal-to-noise ratio and better quality single molecule data based on blinking of the photoprodut.
Figure 1. Conversion of Vybrant® DyeCycle™ Violet fluorescent probe to its green emitting form.

A – Images of the blue-emitting form (a) and the green-emitting form (b, c) of VdcV (conc. 50 nM) prior to (a, b) and after (c) a short UV illumination (405 nm laser beam, 290 µW, 15s). Fluorescence of the green-emitting form increased 2.6 times following exposure to UV. Widefield fluorescence microscopy. a – exc. 405 nm (450 µW), em. 450 - 490 nm, b, c – exc. 488 nm (0.05 kW/cm²), em. 585 - 675 nm. For more details see (Żurek-Biesiada et al., 2013).

B – Photoconversion of VdcV (10 µM) immobilized in a polymer block. As in the case of Hoechst 33258 (Żurek-Biesiada et al., 2013) photoconversion of VdcV is independent of the presence of DNA.

C – Fluorescence spectra of the blue (exc. 405 nm) and green (exc. 458 nm) emissions of VdcV (1 µM) recorded in fixed HeLa cells, using spectrally resolved confocal microscopy.

D – Bleaching curve of the green-emitting form of VdcV (exc. 458 nm, em. 480 - 600 nm), generated by exposure of cells in PBS to UV. Bleaching of the green-emitting product was less pronounced as compared to Hoechst dyes (Żurek-Biesiada et al., 2013) indicating higher photostability - collecting 50 images resulted in a 20% decrease of the intensity of fluorescence.

E – A comparison of the green-emission spectra of VdcV (1 µM) stained cells in neutral pH (PBS), pH = 3.7, or H₂O₂ (30%). Exc. 458 nm.
When excited with a high intensity 405 nm light (~0.5 kW/cm²), the predominant, blueemitting form of VdcV did not undergo the process of blinking required for SMLM. However, we noticed that upon illumination with high intensity 491 nm laser light (0.525 - 1.125 kW/cm²) the signal of the green-emitting form ($\lambda_{\text{em}}^{\text{max}} = 540$ nm) averaged over the entire cell nucleus diminished to a low level, and single green emitting molecule fluorescent bursts appeared (see Supplementary Fig. S2). This observation indicates that the 491 nm laser beam induced the formation of a nonemitting ‘OFF-state’ in the vast majority of molecules of the green-emitting form of VdcV, i.e. they were no longer excited to emit green fluorescence ($\lambda_{\text{em}}^{\text{max}} = 540$ nm) by 491 nm illumination, while at any given time a few individual molecules were not subjected to this transition. These remaining ones, sparsely distributed (and thus optically isolated) molecules were capable of absorbing blue light and emitting green fluorescence (abs./em. 491/540 nm), if only for a short time until they were transferred to the ‘OFF-state’ again or bleached out permanently. The recorded multi-frame image sets contained stochastically appearing bright single molecule (SM) signals at different sites over the time-course of an experiment; i.e. the VdcV molecules were first transferred by the 491nm illumination into the ‘OFF-state’ (i.e. not detectable in the green-yellow detection band) from which these molecules were subsequently stochastically switched back to the green emitting fluorescent state and then bleached, in a similar way as described previously for a variety of other types of molecules. This enabled the optical isolation of a large number of VdcV molecules per µm². The subsequent incorporation of individual molecule positions into a joint localization map enabled reconstruction of SMLM images of nuclear DNA distribution in a way which yielded a substantially enhanced structural resolution.

Detecting fluorescence of single molecules of VdcV required optimization of the embedding medium, according to the principles of dSTORM (Heilemann et al., 2008) and SPDM_PhyMod (Lemmer et al., 2008). Fluorescent bursts of individual optically isolated molecules can be recorded with the appropriate efficiency only in a specific chemical environment, which favors blinking and suppresses irreversible bleaching. Thus, we first carefully adjusted the components of the buffer, in which the sample was immersed, in order to provide appropriate conditions for photoconversion and detection of blinking of the green-emitting form of VdcV. Interestingly, we found that an imaging medium based on glycerol with an addition of the standard enzymatic oxygen scavenging system fulfilled this requirement. Addition of this component to the glycerol resulted in roughly 200% increase in the number of the detected blinking molecules. The same medium was used previously in studies of Hoechst dyes (Szczurek et al., 2014). Optimization the imaging conditions and behavior in the presence of a broad range of chemical environments for VdcV is described in detail in Supplementary Materials. Using finally optimized conditions we found that Vybrant DyeCycle Violet molecules have a very long OFF-duty cycle time. Within a 30,000 frame acquisition (50 ms/frame) they typically re-appear approximately 4 times (Supplementary Figure S6). Such a property is very beneficial for imaging of the cell nucleus, since the total DNA content within an individual optical plane is overwhelming and the number of molecules detected in one frame may be too high for optical isolation. In order to acquire clearly isolated single molecule signals the dye molecule needs to spend a vast fraction of the time in a non-fluorescent ‘OFF-state’. This is not required for previously reported blinking DNA dyes, like YOYO-1 which was successfully used to image single DNA fibers (Schoen et al., 2011). In this case, however, the amount of DNA embraced by one field of view was significantly lower than the amount of DNA within one imaging plane across the cell nucleus. The repetitive binding and unbinding of YOYO-1 resulted in a very short OFF-duty cycle time, and enabled imaging of DNA in vitro within a reasonable time of acquisition. This was not possible with VdcV and DNA in vitro due to a long OFF-duty cycle time (data not shown).
As presently we do not possess the knowledge required to manipulate the ‘OFF-state’ residence time of VdcV, we decided to investigate the influence of the concentration of the dye loaded onto cells in order to yield maximal density of localizations in the final SMLM reconstruction. When fixed cells were stained with VdcV at low concentrations (10 - 200 nM), only a relatively low amount of the green-emitting form (abs./em. 491/540 nm) of the dye was detected. Under these conditions, due to irreversible photobleaching and long OFF-duty cycle time, the signal (averaged over the area of the nucleus) of the green-emitting form rapidly decreased and the number of signals required for image reconstruction became insufficient (Supplementary Fig. S7). We also tested higher concentrations of VdcV (above 1 µM) and observed an increased density of single molecule signals of the green-emitting form, rendering it impractical for SMLM (in particular when an additional photoconverting 405 nm laser light was applied along with the 491 nm illumination) as the overlapping signals introduce additional error in the estimates of localization of a single molecule of the fluorophore. However, even at low concentrations of VdcV (10 - 200 nM), at the beginning of the experiment, the high signal density precluded detection of optically isolated single molecule fluorescent signals. Only after the majority of the green-emitting molecules had been bleached or transferred to the ‘OFF-state’, the detection of single fluorescent bursts became possible. Therefore, at the initial stage of the imaging experiment, we used a continuous exposure to high intensity exciting light (0.5 kW/cm² of λexc = 491 nm) to cause gradual ‘pre-bleaching’ of the green emitting form in order to induce a transition of the vast majority of the green-emitting molecules to a ‘OFF-state’. Under these conditions, the averaged signal originating from the green-yellow detection channel (λem,max = 540 nm) eventually decreased to the level, which was sufficiently low to detect individual, stochastically occurring fluorescence bursts of the green-emitting form. Depending on the concentration of VdcV, the time of pre-bleaching varied from minutes to hours. At this step the image acquisition was commenced. Worth noticing is the fact that the detection rate of single molecule signals per time unit with a reasonable approximation was constant. This indicates that possible differences in the time of pre-bleaching from cell to cell should not disable comparative studies between independent measurements (Supplementary Fig. S8). This observation suggests that quantitative chromatin studies based on SMLM data obtained with VdcV may be possible. In summary, the initial concentration of VdcV influenced the number of the single green emitting molecules that could be detected and optically isolated in the final image (Supplementary Fig. S7).

As an example of SMLM imaging under a successfully optimized protocol, Figure 2 presents an image of an equatorial slice of a nucleus of a Vero-B4 cell stained with VdcV (500 nM). The pre-bleaching of the green emitting fluorescence of the DNA bound VdcV lasted for 15 minutes. The resulting image consists of more than 10⁸ precisely localized individual single molecule fluorescent bursts, which (apart from the highly enhanced optical and structural resolution) corresponds well with the widefield image; in addition, it reveals nanostructures that were not resolved by standard optical microscopy (Fig. 2, for more details see Supplementary Fig. S11). Next, we binned the Vybrant Dye Cycle Violet density map in to square grid of 40 x 40 nm, and we found that the density of DNA-associated signal varied in the order of >10 fold (Supplementary Fig. S12). Low-density regions (<1000 molecules per µm²) likely represent interchromatin compartments and high-density regions (~20,000 localizations per µm²) can be attributed to heterochromatin (Cremer and Cremer, 2010; Markaki et al., 2010; Popken et al., 2014).

The mean localization precision (standard deviation) achieved in this experiment was
approximately $\sigma_{loc} = 19$ nm with an average photon count of 2409 (for histograms see Supplementary Fig. S9). Assuming a normal distribution of the localization precision $\sigma_{loc}$, the sample drift after correction ($\sigma_{drift} = 10$ nm), and the sampling ($\sigma_{sampling} = 2/\sqrt{5000} \mu m$ for a density of 5000 signals/$\mu m^2$, see Figure 4), an average optical (two point) resolution (as estimated by the Full-Width-at-Half-Maximum/FWHM) of $2.35\sigma = 2.35 (\sigma_{loc}^2 + \sigma_{drift}^2 + \sigma_{sampling}^2)^{0.5} = 83$ nm was obtained. Since the underlying (theoretical) assumptions may not be valid for DNA data with high out-of-focus contributions to the overall signal in the cell nucleus, we also calculated the structural image resolution by means of Fourier Ring Correlation (Nieuwenhuizen et al., 2013) and obtained a value of $103 \pm 3$ nm. In order to verify the value for the structural resolution obtained experimentally, we extracted and analyzed profiles across a heterochromatin layer at the nuclear envelope (Supplementary Fig. S3). The FWHM measured in these profiles reached $108 \pm 19$ nm, a value well below the conventional diffraction limit of ~250 nm.

Figure 2. Super-resolution image of an optical slice of a nucleus of a Vero-B4 cell stained with 500 nM Vybrant® DyeCycle™ Violet. Bottom right - an image of the same cell nucleus acquired in a widefield microscope (exc. 405 nm, 450 $\mu$W, em. 450 - 490 nm). In the enlarged part of the SMLM image interchromatin compartments can be discerned (highlighted by dotted lines). The second inset (bottom) presents a periphery of the nucleolus with perinucleolar heterochromatin. Super-resolution image: exc. 491 nm, 0.525 kW/cm², em. 585 - 675 nm. 20,000 frames were collected during the measurement with 50 ms integration time per frame. The signal density was 5010 SM/$\mu$m². For more information and detailed analysis of the image see Supplementary Figures S10 - S12.
Two SMLM imaging strategies to obtain high structural resolution of VdcV stained nuclear DNA have been explored. The first one was based on increasing the concentration of the dye in order to provide a sufficient concentration of the green-emitting form of VdcV bound to DNA. In this case the 491 nm excitation was used to reversibly bleach and excite molecules of the VdcV photoproduct after the stochastic return from the reversibly bleached ‘OFF-state’. The second strategy employed a simultaneous exposure to low intensity 405 nm light to constantly repopulate the pool of the green-emitting form of the dye by recruitment from the standard blue-emitting form; also in this case 491 nm light was used to bring to an ‘OFF-state’ and excite the green-emitting form of VdcV. In our hands, using high concentrations of VdcV and 491 nm excitation alone yielded images of a higher quality than the strategy, which employed two excitation wavelengths and a lower dye concentration (Fig. 2, Fig. 3). Another advantage of the monochromatic excitation protocol with 491 nm alone was the elimination of damage inflicted on objective lenses by the 405 nm irradiation focused onto the lens.
We applied our image acquisition protocol to study the structure of mitotic chromosomes. Figure 4 presents an image of chromosomes in anaphase, when the sister chromatids are being pulled apart to the opposite poles of the cell by the spindle apparatus. Localization microscopy facilitated visualization of local DNA densities within the chromosomes and provided an image of significantly better quality than typical wide-field or confocal images of chromosomes in cell division (Dobrucki and Darzynkiewicz, 2001).
Figure 5. Dual color single molecule localization microscopy of Drosophila polytene chromosome stained with VdcV (green) and Pol II Ser2P-Alexa Fluor 555 (red).

A - a widefield image of a fragment of a polytene chromosome.

B - SMLM dual color reconstruction based on blinking of Alexa Fluor 555 and of VdcV photoproduct acquired subsequently. Arrows indicate empty region inside the polytene chromosome.

C - an enlarged fragment of the image shown in B, embracing dense heterochromatic and lower density euchromatic regions of DNA.

D - intensity profiles for widefield and localization microscopy images of VdcV-
DNA integrated in a rectangular areas A2 and B2 as indicated in (C). Localization microscopy of DNA provides insight into inaccessible information about chromosome banding.

E - Emission spectra of VdcV photoproduct and Alexa Fluor 555. Emission bandpass filter (585 - 675 nm) used for registration of blinking is marked as a grey rectangle.

Figure 5 presents a polytene chromosome from salivary glands of Drosophila Melanogaster. It consists of many homologous chromatids which are synapsed together without undergoing proper cell division. This arrangement results in specific dark and light bands in quinacrine-stained chromosomes examined by light microscopy (Hochstrasser and Sedat, 1987). The distinct banding pattern of polytene chromosomes, combined with molecular techniques like immunostaining, facilitates studies of the distribution of chromosome binding proteins, and understanding of epigenetic modifications and transcription patterns (Johansen et al., 2009). A high resolution of the banding pattern is essential to obtain precise structural information encoded in patterns of immunostaining of polytene chromosome squashes. Electron microscopy provided an extensive knowledge about the structure of polytene chromosomes (Alanen, 1981). Here we demonstrate that SMLM can capitalize on a high specificity of fluorescent DNA dyes, and may provide new insights into DNA arrangements in polytene chromosomes. Such an approach enabled resolving additional bands in a polytene chromosome, as compared to standard microscopy (Fig. 5D). Figure 5 presents an SMLM image of a detail of a polytene chromosome with a characteristic banding pattern. The image demonstrates the feasibility of two-color localization microscopy, after staining DNA with VdcV, and fluorescently labeling the second target. As a proof of principle, we stained immunofluorescently RNA polymerase II phosphorylated on serine 2, using Alexa Fluor 555 conjugated antibody (other fluorescent probes can also be efficiently used, for details see Supplementary Table 2). In a variety of studies using various microscopy techniques including electron microscopy and axially/laterally structured illumination (Martin et al. 2004; Markaki et al. 2010), these RNA polymerase II molecules have been shown to form small clusters in the size range below 100 nm. Figure 5 indicates a similar size also in polytene chromosomes.

The type of dual-color localization microscopy used here has several advantages - the same detection channel (585 - 675 nm filter) was used for the two different excitation wavelengths, 561 nm was used to induce blinking of Alexa Fluor 555, and subsequently 491 nm to induce blinking of the green-emitting form of VdcV, while Alexa Fluor 555 was already bleached. Fig. 5E demonstrates the simplicity of this approach - there is no necessity to correct for chromatic shifts between the two reconstructed images since only one detection channel is used.

Alexa Fluor 555 performs exceptionally well in the buffer optimized for VdcV. Typically 4875 SM/µm² Alexa Fluor 555 localizations were detected in euchromatic bands with an average of 8646 photons per single molecule burst (corresponding to σ_loc of roughly 11 nm and hence a FWHM-based resolution estimated at 25 nm). Pol II Ser2P is a known marker of active transcription which occurs primarily within euchromatic bands of polytene chromosomes, that is in regions of low DNA density (Johansen et al., 2009), in agreement with our SMLM images. Additionally, the known toroidal structure (Sorsa, 1983) of the polytene chromosome could be visualized due to the sectioning capability of SMLM reconstructions (Fig. 5B, arrows). This usually cannot be observed by means of widefield or confocal microscopy.
Discussion

Several approaches have been described to fluorescently label and image DNA and chromatin in situ at a single-molecule level. These superresolution microscopy techniques have been based on detecting optically isolated, fluorescently tagged anti-histone antibodies, fluorescently labeled DNA precursor analogs, or fluorescent dyes bound to DNA. In the previous report (Szczurek et al., 2014), we introduced a single molecule localization microscopy (SMLM) method for high resolution imaging of DNA in intact cell nuclei, using DAPI, Hoechst 33258 and Hoechst 33342 dyes binding directly to DNA. Up to 400,000 DNA sites were localized in a single optical section through a HeLa cell nucleus. In the present report, we describe a further improvement of single molecule localization microscopy of the nuclear genome, using the standard DNA specific dye Vybrant® DyeCycle™ Violet (VdcV), to realize a substantially higher spatial resolution of DNA both in cell nuclei and in mitotic chromosomes. Here, the number of single molecule signals detected in one optical nuclear section usually exceeded $10^6$ signals. This approach allowed us to visualize spatial distribution of the DNA and the interchromatin compartment at an unprecedented level of spatial resolution. The chromosome territory – interchromatin compartment model (Cremer & Cremer 2001, 2010; Rouquette et al. 2010; Markaki et al. 2010) predicts a higher order chromatin organization based on a 3D network of chromatin domain clusters (CDCs) and a co-aligned network of almostDNA free IC-channels, representing a sponge-like structure with major constraints on the diffusion of proteins involved in replication, transcription, splicing, and repair processes. While localization microscopy using DAPI and Hoechst dyes so far allowed us to visualize directly large heterogeneity of the DNA nanoscale distribution, as predicted by the interchromatin compartment model, it was not possible to clearly distinguish small chromatin domain clusters and the IC channels separating them. The results obtained with VdcV-based single molecule localization microscopy presented here are fully compatible with the general features of this model and provide a basis for future detailed superresolution studies.

The method of SMLM/DNA imaging presented here has several advantages over previously presented methods. As the Vybrant Dye Cycle Violet has only a low affinity to RNA, additional RNase treatment is not necessary prior to SMLM imaging (as would be a case when using YOYO-1). Moreover, Vybrant Dye Cycle Violet is a subject to single molecule overcounting only to a low degree (Supplementary Figure S6). As a consequence the density of localizations closely reflects the density of fluorophore molecules. The previously described methods of imaging of the chromosomal DNA take advantage of either standard STORM fluorophores that typically undergo a process of switching even two dozen times (Zessin et al., 2012), or are based on repetitive binding and unbinding of a DNA dye molecule to single binding sites, making it impossible to determine how many times an individual binding site of a particular accessibility in the cell nucleus was "sampled" (Schoen et al., 2011). In addition, a photoproduct of Vybrant Dye Cycle Violet is imaged using a single excitation wavelength only, unlike the previously reported photoconvertible Hoechst dyes (Szczurek et al., 2014).

Here, for the first time, this approach was applied to VdcV, and optimized based on the switching buffers described previously for dSTORM (direct Stochastic Optical Reconstruction Microscopy) technique (Heilemann et al., 2008). Moreover, we present here the first chromatic aberration-free dual color localization microscopy of a DNA dye and single Alexa Fluor molecules. Even premium corrected objective lenses of high numerical aperture (NA) have object plane chromatic aberrations in the range of tens of nanometers, depending on
the detection wavelengths and the position within the field of view. While such shifts are usually not noticed in conventional microscopy (due to the optical resolution of about 250 nm), they have to be taken into consideration at the resolution level achieved in localization microscopy. These shifts can be reduced to a few nm by appropriate calibration procedures (Manders, 1997; Bornfleth. et al., 1998; Edelmann et al., 1999; Esa et al., 2000) or eliminated by using suitable pairs of molecules where the position is obtained from the same type of reporter molecule (Bates et al., 2007). Compared with these relatively complex methods, the approach to eliminate chromatic aberrations in the detection pathway described in this report is simple and easy to implement. Moreover, according to the data given in Supplementary Table 2, adding the third label emitting in red (e.g. Alexa647) should also be possible.

The application example chosen for this type of dual color SMLM shows the positions of individual molecules of one selected protein (polymerase II) versus the high structural resolution image of local DNA density. These SMLM images clearly indicate that, in contrast to the conventional wide field microscopy image, the Pol II molecules in the inter-band regions are not distributed in large stretches but form a limited number of small distinct clusters with an apparent size lower than 100 nm. In addition, they were found in areas of apparently very low DNA density, mostly adjacent to areas of substantially higher DNA density. These data also support models of nuclear organization postulating an interchromatin domain compartment where transcription and other essential nuclear processes take place (Rouquette et al., 2010; Cremer and Cremer, 2010). Interchromatin domain compartment regions have also been observed in SMLM images of Vero-B4 nuclei (compare Fig. 2) where they surround small chromatin domain clusters. The 'channels' of very low DNA density detected here have an apparent diameter of several hundred nm and are connected with the nuclear envelope. Similar interchromatin 'channels' were also observed in super-resolution images of DAPI-stained C2C12 cell nuclei obtained with structured illumination microscopy (Schermelleh et al., 2008). In the SMLM images reported here the spatial resolution was further improved to the single molecule detection level. This allows analyses of the spatial relationships between interchromatin compartments and chromatin domains in an intact fixed cell nuclei with molecular detail. The imaging approach described here can be developed further to allow multicolor imaging of eukaryotic nuclei with respective components targeted by means of immunofluorescence, click chemistry, SNAP technology, CRISPR, and other labeling methods, such as fluorescence in situ hybridization with sequence specific DNA probes.

An important advantage of the approach reported here is the ability to use only one excitation wavelength for photoswitching and excitation of fluorescence. Many other similar localization imaging strategies, including our own recently published method (Szczurek et al., 2014), require the use of two excitation wavelengths, like in the original STORM (Rust et al., 2006), PALM (Betzig et al., 2006), and FPALM (Hess et al., 2006) approaches. A need to use two wavelengths (one for photoconversion, another for induction of blinking) is costly and impractical in attempts to realize multi-color localization microscopy. In the case of dyes excited by 405 nm, the use of a high intensity laser beam focused into a high NA objective lens may lead to damage inflicted on the objective. Also, an extensive use of 405 nm precludes any noninvasive imaging of live cells. Therefore, single wavelength excitation of VdcV by blue light with a longer wavelength (491 nm) constitutes an important practical advantage, and makes this method of high resolution imaging of DNA potentially much more accessible to routine use in biological studies. Moreover, this novel staining outperforms previous methods of DNA imaging in localization microscopy of the cell nucleus in terms of single molecule signal density. Here we report the typical density of signals exceeding 5,000...
SM/µm², whereas previous studies reported values of densities in the range of 3,500 SM/µm² with PicoGreen (Benke and Manley, 2012) and 4,000 SM/µm² with Hoechst 33258 (Szczurek et al., 2014).

Naturally, as with other dyes, the stability of VdcV is another important issue to take into account. However, in this case the stability and possible transitions of the forms with different emission spectra need to be considered. It is possible that the blue-excited, green-emitting form of the dye is formed in the stock solution over time. This could explain the fact that we detected the presence of this form even without exposing the stained samples to UV or visible light. Generation of the green-emitting form of VdcV at acidic pH or by applying hydrogen peroxide (Fig. 1G) is also of interest. However, we did not explore the applicability of these conditions as they were likely to influence the enzymatic activity of glucose oxidase and catalase, which were used to enhance conditions promoting blinking by oxygen scavenging. A similar blinking behavior of Hoechst dyes and VdcV, and the fact that we used the same imaging buffers to induce blinking, suggest that the photophysical mechanism underlying the conversion of the blue-emitting to the green-emitting form of VdcV may be protonation, as it was demonstrated for bisbenzimide dyes (Żurek-Biesiada et al., 2013). Additional chemical reactions, however, may underlie the process of blinking of the green-emitting form and simple switching between blue- and green-emitting forms may be insufficient for a full explanation. Nevertheless, studies of binding mechanism as well as stability, photoconversion, oxidation and other possible reactions of the dye are required as they may help to improve the staining and imaging protocols. This is particularly important in order to implement 3D SMLM of the cell nucleus. However, in a typical 3D SMLM approach, based on introduction of astigmatic aberration to point spread functions, only the dyes with the highest photon counts are of use and none of DNA dyes fulfills this requirement. Thus, only the approaches employing the brightest STORM fluorophores to label specific DNA base or histones (Zessin et al., 2012; Ricci et al., 2015) are applicable to 3D SMLM of chromatin.

The data shown here are based on fixed cell samples only. However, the imaging protocol was optimized with respect to light exposure. A major goal of super-resolution light microscopy is to perform also live cell imaging. While SMLM ‘snapshots’ of unfixed cells have been reported (Betzig et al., 2006; van de Linde et al., 2011), prolonged SMLM imaging of live cells has been impractical, either due to the high light intensities needed, or due to the toxicity of switching buffers. According to the manufacturer (Life Technologies, Carlsbad, CA, USA), VdcV has been used successfully in sorting of live cells, indicating low alteration to the DNA structure. In this report we demonstrate that the minimal laser intensity necessary to induce blinking of VdcV falls in the order of 0.5 kW/cm². However, DNA strand breaks might be induced during live cell experiments, as DNA dyes could act as photosensitizers. Damage rates are higher upon exposure to UV/blue light, thus absorption of such dyes in the green-red spectrum is favorable. Altogether, in our opinion live cell SMLM imaging should be explored but we have concerns about live-cell imaging of genomic DNA itself due to aforementioned issues.

To summarize, we present a novel framework for investigations of the chromatin distribution in the eukaryotic cell nucleus with unprecedented resolution for optical microscopy with least stringent sample preparations required. We anticipate that such single molecule localization microscopy methods to study the cellular DNA distribution at the high density single molecule level will contribute to unravel the still not well understood processes involving global and local changes in the chromatin nanostructure related to cell differentiation or to sensing environmental conditions, such as effects of ionizing radiation or chemical substances. We
suggest that localization microscopy of chromatin labeled with directly bound DNA dyes constitute a feasible complementary method for visualizing the chromatin, as compared to its alternatives such as 3D structured illumination microscopy or electron microscopy.

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Authors' Contributions

D.Ż-B. and A.S. planned the experiments. D.Ż-B. and A.S. performed the experiments, drafted and revised the manuscript, D.Ż-B., K.P. and A.S. performed image data reconstruction. G.K.M. prepared polytene chromosome samples, J.Y. R., U.B., J.D., C.C. supervised the work and contributed to writing the manuscript.

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Highlights:

- Super-resolution imaging of nuclear DNA with Vybrant Violet and blue excitation
- 90 nm resolution images of DNA structures in optically thick eukaryotic nuclei
- Enhanced resolution confirms the existence of DNA-free regions inside the nucleus
- Optimised imaging conditions enable multicolour super-resolution imaging