Localization microscopy (SPDM) facilitates high precision control of lithographically produced nanostructures

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ABSTRACT

Nanoscale resolution in material sciences is usually restricted to scanning electron beam microscopes. Here we present a procedure that allows single molecule resolution of the sample surface with visible light. Highlighting the performance we used electron beam lithography to generate highly regular nanostructures consisting of interconnected cubes. The samples were labeled with Alexa 647 dyes. The spatial organization of the dyes on nanostructured surfaces was localized with single molecule resolution using localization microscopy. This succeeded also in an absolute spatial calibration of the localization method applied (spectral precision distance microscopy/SPDM). The findings will contribute to the field of product control for industrial applications and long-term fluorescence imaging.

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1. Introduction

In numerous fields, the development of innovative technologies requires a refinement and miniaturization of existing systems (Dittrich and Manz, 2006). As a result an increasing requirement for process friendly quality and dimension control for industrial automation emerges, especially for the manufacturing of electrical elements, such as integrated circuits and transistors (Hell, 1996), nanostructured surfaces (Balzarotti and Stefan, 2012) for biosensing applications, research in metamaterials (Valentine et al., 2008), and medical technology. Methods that achieve the desired resolution are for example scanning near field optical microscopy, which provides information on the surface of the sample, as well as neurons, X-ray diffraction and electron microscopy (Reimer, 1998), which require dehydrated preparations and ultra-high vacuum systems. So far, individual controls of surfaces by electron microscopy have been possible only under high vacuum conditions. Due to its facility of operation, far field lightoptical techniques should be highly desirable; however, the use of such methods has been severely limited by the diffraction limit of conventional far field microscopy (Dupas et al., 2007; Cremer, 2012). The substantial improvement of wide-field fluorescence microscopy (Kubitscheck, 2013) towards super-resolution methods such as

4 Pi (Träger, 2012), Structured illumination microscopy (SIM) (Heintzmann and Cremer, 1999) and the various types of localization microscopy, such as spectral precision distance microscopy (SPDM), photoactivated localization microscopy (PALM) (Betzig et al., 2006), stochastic optical reconstruction microscopy (STORM) (Huang et al., 2008; Rust et al., 2006; Bates et al., 2007), presently enables a spatial resolution of the object far beyond the conventional diffraction limit of about 200 nm (Heilemann et al., 2008). In this study, structures were generated by electron beam lithography to illustrate two aspects: the application potential of localization microscopy as an integrated process for quality control in addition to the calibration of fluorescence microscopes. To this end surfaces were patterned by lithographic methods and afterwards metallized with gold nanoparticles, which provide an effective and convenient way to create nanoscale adsorption sites for dye molecules. These surfaces were labeled with Alexa dyes and analyzed with SPDM. The method described is distinguished by very bright, photostable fluorescence, high quantum yield, and ease of storage without evaporation losses.

2. Materials and methods

2.1. Sample preparation and characterization

2.1.1. Lithography

The nanostructures fabricated at Karlsruhe Nano Micro Facility (KNMF) were formed from poly(methyl methacrylate), short
PMMA. Two examples have been investigated. Both consist of interconnected cubes. In sample one the cubes were 600 nm in size, with connecting units of the same height and length, but a width of only 170 nm (Fig. 1). In example two the lithographical structure was downscaled by a factor 0.5. A silicon substrate was employed coated with an adhesion layer of chrome, followed by a gold film of 15 nm thickness. The total size of each patterned surface was 2.5 × 2.5 mm.

2.1.2. Metallization

For SPDM, single molecules were attached to the surface. The structure was coated with a gold nanoparticle layer by deposition of gold seeds and electroless plating from a gold salt solution to generate single molecule adsorption sites (Dahint, 2007). This procedure needed three preparation steps. The first step was the production of charge stabilized colloidal gold nanoparticle seeds. The particles were produced in a redox reaction of tetrachloroauric acid with sodium borohydride and sodium citrate. The starting point was 600 ml of Milli-Q water adding 6 ml of 1% tetrachloroauric acid and 6 ml of 1% sodium citrate solution. The gold salt was reduced by 0.0045 g sodium borohydride, which was dissolved in 6 ml of 1% sodium citrate solution. By this the color of the solution turned red, because the coloring plasmon resonance strongly depends on the particle size and shape. A critical issue for the production of a monodisperse size distribution was strong agitation of the solution at high speed for 60 min.

2.1.3. Attachment

The electrostatic deposition of negatively charged particles with citrate ligand shell required a positively charged functionalization of the substrate. The sample was inserted in a solution of two parts polyethyleneimine solution (2 mg PEI per 1 ml of 0.5 M NaCl) and one part of phosphate buffered saline pH 7.4 (PBS, 1 tablet per 200 ml of Milli-Q water). Unbound polymer layers were rinsed with Milli-Q water. The sample was dried in a stream of nitrogen. For the electrostatic deposition of gold nanoparticles the surface was incubated with colloid solution twice overnight and finally washed with Milli-Q water.

2.1.4. Electroless gold deposition

For growing the gold colloids the samples were subsequently exposed to an aqueous solution of 0.1% tetrachloroauric acid solution by reduction with 0.04 M hydroxyamine solution. The gold ions were reduced by the uptake of electrons, the reducing agent was oxidized. The reaction was stopped by washing with Milli-Q water after 3 min.

2.1.5. Labeling

First the surface was functionalized using fibrinogen (Sigma–Aldrich, Munich, Germany) adsorbed from a 1 mg/ml solution in PBS buffer (Sigma–Aldrich, Munich, Germany) under laboratory conditions for 3 h, washed with Milli-Q water (Millipore GmbH, Eschborn, Germany), and dried. For the production of the reactive dye solution Alexa 647 molecules (A-21676 Component B carboxy acid, succinimidyl ester, Invitrogen, Darmstadt) were used. The dye has a high absorbance coefficient of 239,000 cm−1 M−1, a molecular weight of 1300 g/mol and emits in the visible spectral range (Anon., 2014). Alexa dyes were sequestered over their reactive group selectively with amines of DNA or protein molecules. The dye was freshly solved in DMSO and shortly after diluted with 30 ml of sodium bicarbonate buffer (pH 8.3), mixed in ultrasonic bath and used immediately. The functionalized surface was immersed a few seconds in the dye solution, washed with water and dried. The sample was embedded with Prolong Gold (Invitrogen, Carlsbad, USA). The cover glass was cleaned with glass cleaner (50% water, 25% isopropanol, 25% ammonia).

2.2. Scanning electron microscopy

All scanning electron microscope images were recorded with a Zeiss Gemini Leo 1530 electron microscope at room temperature.
The detection was performed with an in-lens detector. The EHT (electron high tension) was set to 5 kV.

2.3. Localization microscopy (SPDM)

2.3.1. Optical setup

For super resolution light microscopy of the nanostructures, a setup was used, which combines the advantages of single molecule localization microscopy (SMLM) with structured illumination microscopy (SIM) in order to provide high-precision localization and structural information. This allows the high precise measurement of optically isolated molecules (Cremer and Masters, 2013) as well as high density structural analysis (Reymann et al., 2008). The setup has been described in detail in Rossberger et al. (2013). The measurements acquired here were based on photo switching standard dyes by using the single molecule localization microscopy (SMLM) approach SPDMPhymod (Cremer et al., 2011; Baddeley et al., 2009; Lemmer et al., 2008; Gunkel et al., 2009) in the following addressed as SPDM.

2.3.2. Data acquisition

Excitation illumination for imaging was performed with a 671 nm laser line with a laser power of approximately 70 mW in the focal plane (Hartmann, 2013). The fluorophores were transferred into a non-fluorescent state from which they recovered stochastically. An image stack of 10,000 consecutive frames was recorded with an integration time of 227 ms for each individual frame.

2.3.3. Drift correction

The blinking of the single fluorophores that were attached to the structure was relatively poor when compared to other localization microscopy results in biological samples. This fact made the registration of a high number of frames and hence long total acquisition times necessary which induced a considerable mechanical drift accompanying the measurement. It also forbade drift correction of the localized fluorophores found in an individual frame as there was not enough structural information about the sample present in one such frame. However, the majority of the emitting fluorophores were found to reside in the non-blinking state (Fig. 2, left) revealing permanent information of the sample location. The procedure was divided in two parts: The correction of the shift of each frame based on the wide-field image and afterwards the image processing, i.e. the localization of the single molecules in each frame. For drift correction we devised an algorithm to correct for the mechanical drift in the raw image stack. Based on this data we were able to correct for the drift by subtracting the respective shift of every fluorophore (Fig. 2, right).

Every 16th frame was extracted from the raw data as samples. Then the shift of each sample with respect to the first image was calculated in two directions x and y in the space domain using a cross-correlation algorithm yielding sub-pixel accuracy. This shift was not arbitrary but followed a trend which was approximated by a polynomial fit. A two dimensional vectorial-valued 10-order polynomial was then fitted through the thus found shifts to estimate the shift of every single image in x and y (Fig. 3, I). This procedure has proven to be less prone to errors than determining the shift of each image individually. The assumption that the shift can be described with the help of a polynomial leads to a remaining displacement (Fig. 3, II), resulting in a standard derivation smaller than one pixel.

2.3.4. Localization

For the localization image a data stack of 10,000 consecutive image frames indicating blinking of the sample was recorded (Kaufmann et al., 2009). The localization image was then acquired.
Fig. 3. Drift correction. Analysis of the mechanical drifts along the x-coordinate (red) and y-coordinate (blue) as a function of single frames based on fluorescent events (I). The displacement after drift correction in x-direction (II) and y-direction (III). One Pixel corresponds to 64.5 nm in the sample. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

by determining the center of gravity of every single blinking event using the fastSPDM algorithm described by (Grull et al., 2011). It basically computes the difference image of every frame. Local blinking events were identified as such if a pixel value exceeded the mean of the difference image by more than 4 \( \sigma \) (standard deviation). A region of interest (ROI) was drawn around the events thus detected, wherein the center of gravity \( \bar{r}_x \)

\[
\bar{r}_x = \frac{1}{\sum_{k} I_k f_k(i) \sum_{i} I_k f_k(i)} \sum_{i} I_k f_k(i) \bar{r}_{ROI}(i)
\]

Fig. 4. Fluorescence microscopy image of the sample. Wide-field overview of the structure (left) and a horizontal intensity scan along the x-coordinate of the image (right). The Gaussian intensity distribution of the wide-field image is modulated according to the spacing of next neighbor connection units (1.2 \( \mu \)m). An SPDM image of the insert (white box) is shown in Fig. 5b.
for the pixel \((k,l)\) (Kirchgessner, 2011) is determined. The index \(i\)
denotes the respective fluorophore.

2.3.5. Image processing

The structural details were determined based on both, the
conventional wide-field image and the localization image. Both images
were integrated along one grating direction. This was achieved by
computing the sum \(I_k\) over the intensity values \(I_k = \sum_j I_j\) of all pix-
els in the \(y\)-direction. The alignment was optimized by maximizing
the amplitude of \(I_k\) under small variation of the rotation angle of
the image.

3. Results

The high numerical aperture wide field image shown in Fig. 4
(left) shows the high quality of the pattern. To confirm the
structural pattern, its dimensions at three different lateral \(x\)-,
\(y\)-positions were measured. On average one period of the highly
periodic structure was mapped correctly. This becomes clear from
the illumination profile across the sample, which is due to the
TEM00 mode of the laser Gaussian (Fig. 4, right). The detected inten-
sity is modulated corresponding to the distance between centers of
connection units (1.2 \(\mu\)m).

The conventional wide field resolution was not enough to
resolve the width of the connection units, but the localization image
evaluation indicated a localization accuracy (Fig. 5) down to 14 nm
and facilitated the precision control of dyes deposited on the inter-
connected cubes with a density of detected events of 41 \(\pm\) 1 \(\mu\)m\(^{-2}\).
The detected photon number averaged over the single events was
\((4.2 \pm 0.3) \times 10^3\) photons.

The most striking effect observed was that the lattice structure
can be reconstructed using the SPDM approach. An independent

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**Fig. 5.** Localization microscopy of grating structure. (a) Localization image (SPDM) alone and (b) overlay from the insert (white box) in Fig. 4 from the wide-field (green) and localization image (red) of the labeled structures; (c) enlarged SPDM (red) and conventional wide field image (green) of the insert (white box) in (b). The individual molecule positions were Gaussian blurred with the localization accuracy. Right: Histogram of the localization accuracy of the individual fluorescent events. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

**Fig. 6.** SEM and SPDM of a downscaled structure. (I) SEM image of the downscaled structure acquired with LEO1530 Gemini (Zeiss, Hamburg, Germany). (II) Wide-field image and (III) localization image (SPDM) of the labeled nanostructure. The regions marked by red and blue boxes. (IV) An enlarged SPDM images of the insert (red box) in III. (V) An enlarged SPDM image of the insert (blue box) in IV. Scales: 200 nm (I); 500 nm (II); 1 \(\mu\)m (III); 100 nm (IV); and 97 nm (V). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
confirmation with a second structure size downscaled by a factor of 2 is shown in Fig. 6. The cube is 600 nm high and 276 ± 7 nm in width and length. Connection units are equally high but only 94 ± 5 nm wide. We defined the lattice constant as the length of the connection unit plus the width of the squares and measure 615 ± 7 nm in the SEM image (Fig. 6 I). In the wide-field image the lattice constant was determined to be 600 ± 30 nm, which was close to the values taken by SEM, however, in the conventional wide field image the interconnection units were resolved diffraction-limited. It is interesting to note that most signals were detected on the cubes.

For a better readout the intensity distribution along the x-coordinate of both the wide-field and localization images, were plotted in Fig. 7. The structure size and lattice constant were analyzed with high precision using the Gaussian modulations of the intensity distribution corresponding to the sum of fluorescence events on cubes as a function of the x-coordinate. The full width at half maximum of the modulation gives an upper limit of the structure size. The peak to peak distance equals the center to center distance of next neighbor unit cells. The values determined with the help of the localization image (SPDM) were substantially more exact than the values obtained by the diffraction limited wide-field method.

4. Discussion

In this report, we demonstrate an optical analysis of protein patterns on lithographically fabricated nanostructures, using spectral precision distance microscopy (SPDM), a variant of localization microscopy. The high contrast observed in the localization images was a result of the fact that few or no fluorescent signals were detected in the space between the nanostructures. There are several explanations for this rather surprising behavior of the sample. The inhomogeneous distribution of the wet chemically deposited nanoparticles (Fig. 1, d) upon incubation for 48 h could provide an important indication. Due to the depth of the structure at a contact angle of more than 100°, the number of nanoparticles per unit area on the substrate was reduced. In addition, the adsorption of proteins depends on the nanostructure. Theoretical calculations (Körtge et al., 2013) showed an increased adsorption probability of topographical corners. A basic assumption of this is the consideration of protein–protein-interactions within the framework of DLVO-theory (Oberholzer et al., 1997) and the numerical description of the protein–nanostructure-interaction according to the Poisson–Boltzmann-equation (Paul Labute, 1997). The result of a shorter incubation period of a few seconds in the dye solution may lead to an insufficient diffusion of the Alexa molecules and hence most of them might be unable to reach the lower area. Thus the localization microscopy method allows an estimate of the wettability of the surface or adhesion of molecules on the nanoscale. This is increasingly important based on lotus effects observed in the plant kingdom in the field of biomedical applications, environmental issues, flow profiles in air or water and self-cleaning surfaces. Another explanation for the absence of 'blinking' events in lower areas might be the quenching due to localized plasmonic excitations observed in the spectral range of the fluorescence emission (Willets, 2007). All molecules adsorbed on the substrate are located on the bottom of a well of which the sidewalls are populated with gold nanoparticles. As the illumination and detection takes place along the sample normal, the molecules deposited on top of the connection units (upper plateau of the well walls) are less quenched. Furthermore, the detection was investigated under different incidence angles of the laser radiation. The blinking behavior was not affected. Hence, the shadowing effect as a result of the topography does not play a major role. In conclusion, localization microscopy approaches, such as SPDM, improve substantially the optical resolution of such nanostructures compared to conventional fluorescence microscopy. Using long time imaging, a quantitative SPDM analysis of the structure morphology provides a nondestructive way for shape control. Compared with other superresolution localization microscopy techniques like PALM (Betzig et al., 2006) or STORM (Huang et al., 2008; Rust et al., 2006; Bates et al., 2007), SPDM may be applied with standard fluorophores in combination with standard preparation conditions and single line laser excitation. This will be especially useful for routine applications of superresolution light microscopy in nanostructure control.

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Fig. 7. Intensity plot of the nanostructure in Fig. 6. The intensity distributions of the wide-field image I and the localization image II are modulated according to the spacing of next connection units. The full width at half maximum (FWHM) of the intensity peaks in the localization microscopy data (217 ± 29 nm) was about two times smaller than the corresponding value in the wide-field image (401 ± 84 nm). The variation of the FWHM values (standard deviation) of the intensity peaks was about three times smaller for the SPDM data than for the wide field intensity plots. The peak to peak distance in the intensity plot I (wide-field) was 590.0 ± 9.4 nm and in the intensity plot II (SPDM) it was 603.3 ± 9.8 nm.
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