Research proposal

1 Title of the project

Functionalized nanostructures for super-resolution microscopy and single molecule studies.

2 Abstract

Superresolution microscopy generates significant research interest. In this research we aim to make calibration samples for superresolution miscoscopy applications. Such a calibration sample or "nanoruler" must contain features and inter-feature spacings of sizes below the diffraction limit of visible light. The samples are made using glass cover slides, coated with a protective carbon coating. The protective carbon coating is locally removed by means of a focused ion beam, resulting in the exposure of the underlying glass. The exposed glass can be functionalized and used for the selective attachment of fluorescent molecules, while the surrounding carbon coating is inert. The locally attached layer of fluorescent molecules can be resolved using superresolution microscopy.

3 Applicants

K. Evers
Dr. W. F. van Dorp
Prof. Dr. J.T.M. de Hosson
Dr E.C plötz
Dr T.M Cordes

4 Key publications of the applicant

E. Ploetz, B. Visser, W. Slingenbergh, K. Evers, D. Martinez-Martinez,Y. T. Pei,B. L. Feringa,J. Th. M. De Hosson,T. Cordes and W. F. van Dorp, Mater. Chem. B ,2014,2,2606

5 FOM-research group

N/A

6 Institute

Materials science group
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7 Duration of the project

The project will start on September 1ˢᵗ 2014 and will continue until August 31ᵗʰ 2018.

8 Personnel

Name	Function	Time
Dr. W. F. van Dorp (Senior scientist)	Supervision and management	10%
Dr E.C plötz (Senior scientist)	Supervision and management	10%
Dr T.Cordes (Senior scientist)	Supervision and management	5%
V. Krasnikov (Senior scientist/technician)	Technical support and experiment	30%
K. Evers (Junior scientist)	Experiment and analysis	100%

9 Cost estimates

The funding is requested for one PhD position. Furthermore, all required research materials such as substrates, fluorophores, proteins and other chemicals are included in the total sum. Equipment needed for the manufacturing of nanostructures, as well as several electron based and optical microscopes are excluded, due to their availablility in both the materials science and single molecule biophysics groups at the Zernike intitute for advanced materials campus.

9.1 Personnel positions

One PhD position for four years.

€204.000 (Conform oio norm FOM)

9.2 Running budget

€15.000 / Year

9.3 equipment

No new equipment will be purchased using the requested budget.

9.4 other support

This project will be also supported by the available facilities at the Zernike Institute for Advanced materials and the Rijksuniversiteit Groningen. One technician position will be supported by the Single molecule biophysics (SMB) group, part of the Zernike Institute for Advanced materials.

9.5 budget summary

	2014	2015	2016	2017	2018	TOTAL
personnel (positions): PhD students	1	1	1	1	1	1
postdocs	-	-	-	-	-	-
technicians	-	-	-	-	-	-
guests	-	-	-	-	-	-
personnel (costs)	€14.500	€46.000	€51.000	€54.000	€38.500	€204.000
running budget	€5.000	€15.000	€15.000	€15.000	€10.000	€60.000
equipment	-	-	-	-	-	-
TOTAL (requested from FOM)	€19.500	€61.000	€66.000	€69.000	€48.500	€264.000

10 Research programme

10.1 Introduction

Over the past several decades, fluorescence microscopy has become an essential tool for examining a wide variety of biological molecules, such as proteins and DNA, as well as cells and tissue. Fluorescent imaging is used because it is non-invasive and therefore compatible with cells that are maintained in a culture. One of the challenges in this field however is the resolution limit of this technique. Fluorescent imaging can resolve to about 0.2μm in the lateral direction (in the image plane) and about 0.6μm in the axial (focus) direction. To surpass this resolution limit, several techniques have been developed. Competing techniques in the field are stochastic optical reconstruction microscopy (STORM) [1], photoactivated localization microscopy (PALM) [2], stimulated emission depletion (STED) [3] and reversible saturable opticaL fluorescence (RESOLFT) [4] collectively called "localization-based superresolution microscopy". The techniques differ in the fact that STORM and PALM rely on stochastic/random switching of fluorophores, while with RESOLFT and STED use a targeted and coordinated approach to selectively switch a certain area of fluorophores. All these techniques however rely on a separation in time of the signals, making it possible distinguish the signals of individual fluorescent molecules within an otherwise unresolvable distance. Assuming each individual molecule is a point source, photons which are emitted from each individual molecule have a 2D spatial Gaussian distribution on the detector due to the lenses of the optical imaging system. Therefore, the location of the fluorescent molecule can be pinpointed by using the maximum of the 2D Gaussian curve.[5] This technique enables us to determine the position of the molecule with sub-diffraction limit accuracy.
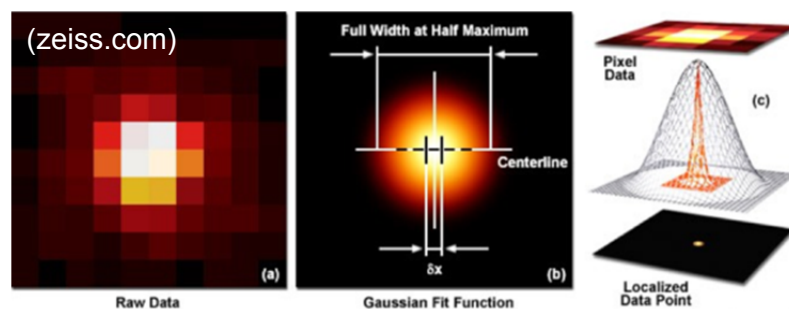

Figure 1 *Principle of localization-based superresolution microscopy.*

Next to localization-based microscopy techniques, efforts have been made to resolve non-separated fluorescent signals. One promising method is superresolution optical fluctuation imaging (SOFI) [6]. This algorithm is available since 2009 but still undergoing improvement [7]. This technique is able to reconstruct individual fluorophores by cross-correlation of pixel value fluctuations.

Another radically different superresolution method, which works without objective lenses, is scanning near-field optical microscopy (SNOM)[8]. With this technique a probe (usually a glass fiber) is brought very close to the sample, within the distance of the wavelength of the emitted light. The aperture in this probe, instead of the wavelength of the light, is the limiting factor for obtainable resolution of the image. Therefore sub-diffraction limited features can be observed. The SNOM method is known both for its high-resolution power as well as its fragility and low transmitting efficiency [9] The major limiting factor for biological purposes however, is that only surface structures can be imaged [10].

Regardless of the used technique, there is a high demand to validate and test the used microcope and microscopy method. This requires well-defined samples that can be used as a standard. Such samples can be used in experiments to allow the researcher and/or microscope's manufacturer to distinguish instrument-specific problems from specimen-specific problems.

Moreover, such well-defined samples serve as a nanoruler with which calibrations can be made. For this purpose such a sample has distances beyond the resolution limit of light that are resolved after reconstruction. These samples are used to optimize photochemical properties by optimizing parameters such as laser power and buffer composition, and verify optical magnification.

10.2 Current state of the art

Currently resolution is usually determined by looking at cross-sections at arbitrary positions where two filaments (Usually of the Actin protein) are arranged in parallel. Unfortunately, measurement noise in super-resolution images render individual cross-sections not meaningful for quantifying microscope resolution, and the labeling density is often not well defined. Another method is the use of DNA origami, which has shown recently to be able to resolve distances up to 31 nm[11] However, in the case of DNA origami there is a tradeoff between length or area and stiffness of the structure. Another calibration method is the use of nuclear pore complexes (NPC) [12]. Because nuclear pore complexes are highly symmetric they can be model structures to test the capability and reliability of super-resolution imaging methods. Distances up to 35 nm were resolved [12] However, nuclear core complexes up to 152 nm were observed. The uncertainty in diameter, however, makes the complexes less suitable for distance calibration. Loschberger et al [12] state that they are mostly suitable to "control the efficiency of chromatic aberration corrections in multicolor super-resolution imaging experiments" We conclude that until now 100% reliable calibration samples superresolution microscopy, which are rigid and well-known before imaging for could not be made.

10.3 Aim of the research

The study on functionalized nanostructures can make a great contribution in the superresolution microscopy field by providing a calibration sample that is well defined and trustworthy. Users of superresolution techniques can characterize their system and fluorophores to get reliable results. Moreover, manufacturers of super-resolution microscopes need demonstration samples to show users that the setup is working correctly. Current methods to make such samples are not suitable for large-scale production, and difficult to tackle with other techniques.

We aim to make nanostructures with well-defined dimensions, which will act as a calibration ruler. Those structures should be written with features beyond the resolution limit of light. Functionalizing the nanostructures with fluorescent dyes suitable for STORM and SOFI gives us the reference that superresolution microscopy users need, and provide ideal samples to study how the attainable imaging resolution depends on the imaging conditions.

Furthermore we aim to use functionalized nanostructures to localize molecules, by efficiently monitoring and studying them in nanosized dots. Using an array of localized molecules, interactions can be measured with nanometer precision. We aim to provide single molecule control. Specifically for single single molecular motors, proteins that convert chemical energy to mechanical force, which are essentially responsible for all active biological motions [13]. Functionalising nanostructures with those proteins can give us a comprehensive understanding of the microscopic mechanism of molecular motors, which so far has proved challenging. Such an understanding is invaluable for the design and application molecular motors in future applications, such as in bio-nanorobots, nanodevices and nanomedicine[14].

10.5 research methods

Creation of functionalized nanostructures

In our research a carbon coating will be deposited on glass slides through plasma assisted vapor deposition (PECVD). We aim to use hydrogenated DLC, known for its high hardness, chemical inertness, biological compatibility and smoothness. The etching is done with a focused ion beam (FIB) of gallium ions. A bottom-up approach is then used to functionalize the structures with fluorophores of cyanine derivatives. In this approach, the nanostructures are first silinized, followed by a passivation step (PEG or BSA). As a last step the fluorophores are bound specifically to the structures using a covalent protein interaction widely used in biophysics, namely the biotin-avidin interaction.
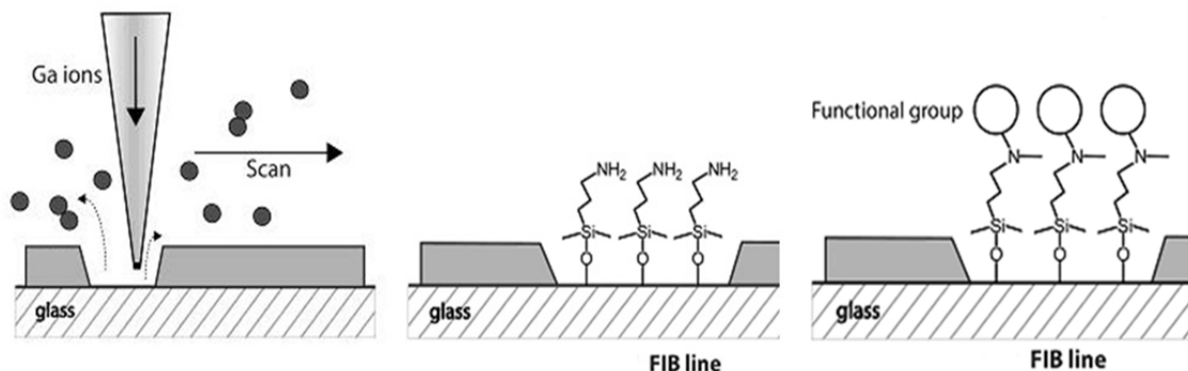

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Figure 2 *creation of functionalized nanostructures. [15]*

Imaging

Dyes used for imaging can be of any kind that can be excited with present lasers. We will investigate the properties and compatibility of cyanine dyes such as cy5, cy7 and cy3 and of the alexa and rhodamine families, such as Alexa488 and RhodamineB. Imaging of the structures is done using a microscope in a total internal reflection fluorescence imaging (TIRF) mode. Using TIRF, the background is severely reduced because fluorophores are only excited using a evanescent wave originating from the liquid/interface surface.

Movies that are recorded using a CCD camera with pixels of 80x80 nm, are processed using superresolution software. The switching behavior can be modified by adjusting the switching buffer and laser power.

10.6 Current work

We are currently succesful in functionalizing nanostructures and resolving distances beyond the diffraction limit. Our current research shows that by using SOFI on cy5-functionalized structures, we can resolve distances below 175 nm. Blinking behavior of another dye, Cy3B, is very promising for STORM applications. Interactions with the used carbon-based coating are currently not well understood, and need to be investigated, and etching needs to be perfected to a point in which we can make reliable, well-defined nanostructures.
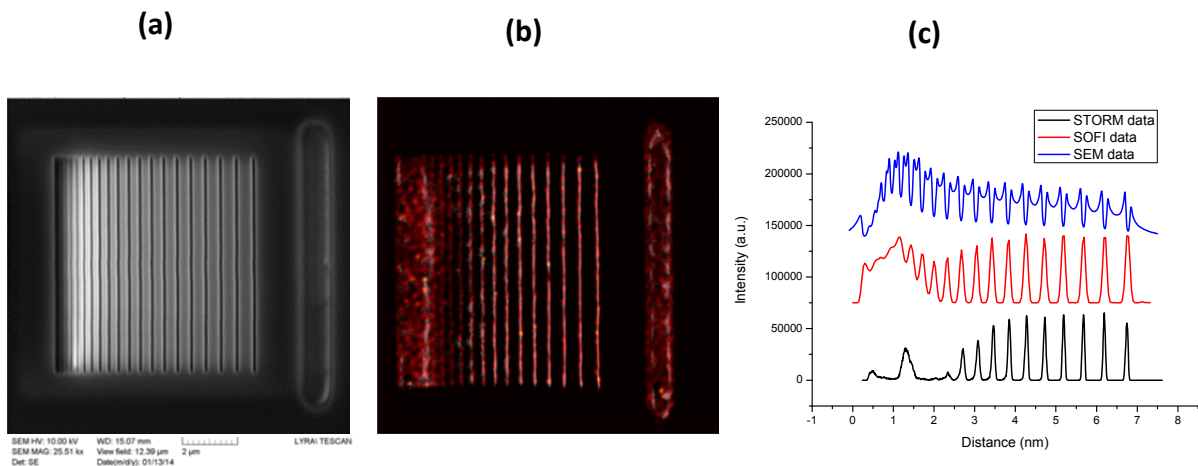

Figure 3 Through our *research we are successful in functionalizing nanostructures and resolving distances beyond the diffraction limit. a) SEM image of lines with varying spacing, coming closer together b) overlaid STORM and SOFI reconstructions and c) Fitted line profiles showing SOFI superiority using our current approach.*

10.5 research plan

The project will take 48 months in total. A time schedule is given below :

Time	Research Task
1st year	PECVD and FIB process study and optimization, investigation of alternative lithography processes. An effort will be made to etch reliable nanostructures below 30 nm.
2nd year	Further optimization of the functionalization procedure. Modification of fluorophore switching behavior according to the chosen method, determining perfect imaging parameters for chosen fluorophores. Establishing a calibration sample for superresolution microscopy.
3th year	Investigation in single-molecule control, demonstration of single molecular motor confinement. Localisation and observation of molecular motor movement in realtime. Create an understanding of underlying mechanisms.
4th year	Study on the statistics of chemical reactions for productional catalysis, providing a hole matrix sample with relyable single-molecule applications, study on the improvement and stabilization of confined molecules. Providing an extensive and useful tool for single-molecule biophysics.

11 Infrastructure

The project will be carried out in both the MK and SMB group at the Zernike Institute of Advanced materials. Experimental setups are available at both groups for this project.

The MK group, headed by prof. dr. J.T.M de Hosson, has sufficient technical staff (3 in total), and among others deposition equipment such as a PECVD setup, a dual beam system for lithography, AFM, STM, TEM and different SEM systems are available. The SMB group, headed by prof. dr. A. van Oijen, is equipped has 2 technicians and is equipped with a laboratory for chemical cleaning procedures, the preparation of buffers and other (bio)chemical applications. Present in the lab are also an oxygen plasma cleaner, purification systems and storage systems such as dessicators, as well as incubators and a fume hood for safe preparation of reactive chemicals. The microscopes in this group are capable of TIRF imaging, as well as wide field and confocal optical imaging techniques, with tested and working superresolution software available for use.

12 application perspective

Miniaturization is a driving force in information industry. Therefore lithography at nanometer scale is required. The application of selectively functionalized nanostructures that can be activated by laser illumination, is very useful as a calibration sample but has applications beyond the realm of superresolution.

Functionalisation of molecular motors on patterned nanostructures as proposed here, can lead to fundamental understanding of their microscopic mechanisms. When understood and applied to nanodevices, this can lead to revolutionary results for nanodevices. Through molecular motors, nanodevices can react to the environment. Nanodevices could use molecular motors as a motion component, allowing them to move linear, by rotation or in a more complex three-dimensional manner. More advanced features are transport and device transformation.

13 FOM subfield classification

This project is classified as "Nano imaging celprocessen"

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