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(54) **Determining the average number of localizations per emitter and the average number of emitters associated with one site in localization microscopy.**

(57) A method is described for determining the average number of localizations per site and/or the average number of emitters per site, wherein said method uses a localization microscope which is adapted to generate one or more super-resolution images of emitters in a sample. Said method comprises: measuring the cumulative number of localizations in a super-resolution image captured by said localization microscope and fitting the measured cumulative number of localizations with an exponential function for determining a bleaching rate k_{bl}; determining a spatial correlation parameter Q(t) on the basis of measured localizations in at least one of said one or more super-resolution images and fitting the measured spatial correlation parameter Q(t) with a spatial correlation parameter function $Q(k_{bl}, M_\infty, \mu; t)$ derived from a photo-physical blinking model for modelling the photo-physical behaviour of said emitters, including the effect of photo-switching and photo-bleaching, for determining the asymptotic number of localizations per emitter M_∞ and the stoichiometry parameter μ; determining the average number of emitters per site on the basis of said stoichiometry parameter μ; and/or, determining the average number of localizations per site <M> on the basis of the fitted values of k_{bl}, M_∞ and μ.

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Determining the average number of localizations per emitter and the average number of emitters associated with one site in localization microscopy

Field of the invention

The invention relates to determining the average number of localizations per emitter in localization microscopy and/or and the average number of emitters associated with one site, and, in particular, though not exclusively, to methods and systems for determining the average number of localizations per emitter and/or the average number of emitters associated with one site, an image-processing module using said method, a localization microscopy system comprising such image-processing module and a computer program product for implementing such method.

Background of the invention

The invention relates to the field of microscopy, in particular to the field of super-resolution fluorescence microscopy. In ordinary (optical) microscopy the resolving power is limited to an amount on the order of the ratio between the wavelength of the light that is used in the image formation and the numerical aperture of the microscope. Different techniques for circumventing this so-called diffraction limit ("super-resolution methods") have emerged over the last 10-15 years. An important class of super-resolution microscopy techniques is the family of localization microscopy techniques, such as Photo-Activation Localization Microscopy (PALM, Betzig et al. "Imaging Intracellular Fluorescent Proteins at Nanometer Resolution", Science 313: 1643-1645, 2006) and STochastic Optical Reconstruction Microscopy (STORM, Rust et al., "Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM)", Nature Methods 3: 793-795, 2006).

In these techniques the sample of interest is labeled with a set of fluorescent molecules which can be

stochastically switched between a light emitting state ("on"-state) and a dark state ("off"-state) such that at any instant in time only a sparse subset of all fluorophores is in the on-state. This has the consequence that the individual fluorophores can be individually identified in the camera image. The position of the individual emitters in the plane imaged onto the camera can then be determined with a precision, which is typically on the order of 10 nm depending on photon count, about 10-20 times smaller than the diffraction limit. Repeating this process, i.e. recording many time frames of such sparse images and analysing all frames for the emitter locations, finally gives an image of the sample with details on the order of the localization precision rather than on the order of the diffraction limit.

Typically, fluorescent emitters are bound to the structure of interest by primary and/or secondary antibody labelling to ensure specific labeling via the antibody. On one primary/secondary antibody there are typically a few (~5) binding sites for fluorescent dyes. This results that often many (5-20) fluorescent dye molecules are bound to one binding site (epitope) accessible to an antibody. This stoichiometry is very hard to analyse experimentally as well as to control precisely in the sample preparation phase. The emitters within one epitope or attached to one anti-body are in general not resolvable as their mutual distances are below the localization precision, which is typically 10 nm. The distance between epitopes can vary from 1-100 nm depending on the structure of interest and labelling strategy.

In all of the existing embodiments of this super-resolution technology, the same single fluorescent emitter may contribute many times to the final super-resolution image (even in PALM). A single emitter can go through many on/off cycles during the acquisition process (e.g. Annibale et al., "Identification of clustering artifacts in photoactivated localization microscopy." Nature Methods, 8: 527-528, 2011 or Dempsey et al. "Evaluation of fluorophores for optimal performance in localization-based super-resolution imaging"

Nature Methods, 8: 1027-1031, 2011). Consequently, the reconstructed final super-resolution image shows the density of observed localization events, which does not give a trustworthy representation of the more relevant actual density of emitters or density of epitopes. This is different from traditional imaging (wide-field and confocal microscopy) where there is a known linear relationship between the recorded intensity and the density of emitters, but also to other type of super-resolution imaging such as Stimulated Depletion microscopy (STED). Quantitative knowledge of the density of emitters or density of epitopes is very much desired for studying the sample next to structural details alone. One could obtain this quantitative information if the number of times an emitter is localized would be known.

A number of methods have been disclosed that deal with this problem (e.g. Annibale et al. "Quantitative photo activated localization microscopy: Unraveling the effects of photoblinking" PLoS ONE, 6:e22678, 2011; Veatch et al. "Correlation functions quantify super-resolution images and estimate apparent clustering due to over-counting" PloS ONE, 7:e31457, 2012; Lee et al. "Counting single photoactivatable fluorescent molecules by photoactivated localization microscopy (PALM). Proceedings of the National Academy of Sciences USA, 109:17436-17441, 2012; Nieuwenhuizen et al. "Measuring image resolution in optical nanoscopy" Nature Methods, 10:557-562, 2013).

These methods can be divided into three categories: 1) calibration based techniques (Annibale et al.), 2) Pair-correlation techniques (Veatch et al.) and 3) techniques that employ statistical blinking models. Methods in the first category make use of a calibration measurement that is acquired before the actual acquisition but that is supposed to represent the actual measurement as much as possible. In this prior experiment the blinking statistics, namely the on and off-times of the fluorescent emitters are measured. This is done typically by diluting the dyes sufficiently such that single emitters are visible in a wide-field acquisition and

subsequent analyses of a time-trace of these emitters. Methods in the second category employ post-processing on the set of localized emitters positions such that they cluster emitters location via spatial correlation functions. However, a-prior knowledge about the imaged structure is needed for this method. Methods in the third category employ a photo-physical model on the switching kinetics between the on and off-states of the emitters. This prior knowledge is combined with the evaluation of spatial correlations to estimate the number of emitters from the localizations in the case of STORM type of super-resolution imaging.

The actual counting technology comprised by the three categories of methods is confronted with different problems. Methods in the first category rely on the assumption that the calibration measurement is representative for the actual measurement. This is problematic as for each dye and experimental condition such as illumination power and buffer conditions the switching times can vary, which in turn compromises the certainty of the outcome. For real-time count estimation during acquisition this requires that many calibration experiments are performed prior to the experiment and stored online. Methods in the second category rely on prior knowledge or assumptions on the spatial structure of the imaged sample. If the actual structure does not conform to this assumption, the estimated count of emitters is biased. Methods in the third category rely on a photo-physical switching model. If this model does not describe the actual blinking kinetics the estimate of the number of emitters will be not trustworthy. Currently for STORM-type experiments only a two-state model (on/off) is employed. This neglects the important effect of photo-bleaching, i.e. an irreversible light-induced chemical reaction to a non-fluorescent state. In addition, the uncertainty of estimation of all methods is not well characterized.

Currently there is no method available in the prior art that may be used to reliable determine, or at least reliable estimate, the average number of localizations per

emitter and/or the number of emitters associated with one anti-body binding site or epitope without either a calibration experiment or prior knowledge on the imaged structure. Hence, there is a need in the art for improved methods and system that allows such quantitative analysis in localization microscopy without any prior knowledge of the spatial sample and/or photo-physics of the emitters.

In this disclosure the term "site" defines a spatial domain with the extend of the localization precision of the localization of single emitters. Typically the size of a "site" is smaller than the distance between epitopes and larger than the distance of fluorescent emitters on one anti-body and an epitope itself.

Summary of the invention

As will be appreciated by one skilled in the art, aspects of the present invention may be embodied as a system, method or computer program product. Accordingly, aspects of the present invention may take the form of an entirely hardware embodiment, an entirely software embodiment (including firmware, resident software, micro-code, etc.) or an embodiment combining software and hardware aspects that may all generally be referred to herein as a "circuit," "module" or "system." Functions described in this disclosure may be implemented as an algorithm executed by a microprocessor of a computer. Furthermore, aspects of the present invention may take the form of a computer program product embodied in one or more computer readable medium(s) having computer readable program code embodied, e.g., stored, thereon.

Any combination of one or more computer readable medium(s) may be utilized. The computer readable medium may be a computer readable signal medium or a computer readable storage medium. A computer readable storage medium may be, for example, but not limited to, an electronic, magnetic, optical, electromagnetic, infrared, or semiconductor system, apparatus, or device, or any suitable combination of the foregoing. More

specific examples (a non- exhaustive list) of the computer readable storage medium would include the following: an electrical connection having one or more wires, a portable computer diskette, a hard disk, a random access memory (RAM), a read-only memory (ROM), an erasable programmable read-only memory (EPROM or Flash memory), an optical fiber, a portable compact disc read-only memory (CD-ROM), an optical storage device, a magnetic storage device, or any suitable combination of the foregoing. In the context of this document, a computer readable storage medium may be any tangible medium that can contain, or store a program for use by or in connection with an instruction execution system, apparatus, or device.

A computer readable signal medium may include a propagated data signal with computer readable program code embodied therein, for example, in baseband or as part of a carrier wave. Such a propagated signal may take any of a variety of forms, including, but not limited to, electro-magnetic, optical, or any suitable combination thereof. A computer readable signal medium may be any computer readable medium that is not a computer readable storage medium and that can communicate, propagate, or transport a program for use by or in connection with an instruction execution system, apparatus, or device.

Program code embodied on a computer readable medium may be transmitted using any appropriate medium, including but not limited to wireless, wireline, optical fiber, cable, RF, etc., or any suitable combination of the foregoing. Computer program code for carrying out operations for aspects of the present invention may be written in any combination of one or more programming languages, including an object oriented programming language such as Java(TM), Smalltalk, C++ or the like and conventional procedural programming languages, such as the "C" programming language or similar programming languages. The program code may execute entirely on the user's computer, partly on the user's computer, as a stand-alone software package, partly on the user's computer and partly on a remote computer, or entirely on the remote computer or

server. In the latter scenario, the remote computer may be connected to the user's computer through any type of network, including a local area network (LAN) or a wide area network (WAN), or the connection may be made to an external computer (for example, through the Internet using an Internet Service Provider).

Aspects of the present invention are described below with reference to flowchart illustrations and/or block diagrams of methods, apparatus (systems), and computer program products according to embodiments of the invention. It will be understood that each block of the flowchart illustrations and/or block diagrams, and combinations of blocks in the flowchart illustrations and/or block diagrams, can be implemented by computer program instructions. These computer program instructions may be provided to a processor, in particular a microprocessor or central processing unit (CPU), of a general purpose computer, special purpose computer, or other programmable data processing apparatus to produce a machine, such that the instructions, which execute via the processor of the computer, other programmable data processing apparatus, or other devices create means for implementing the functions/acts specified in the flowchart and/or block diagram block or blocks.

These computer program instructions may also be stored in a computer readable medium that can direct a computer, other programmable data processing apparatus, or other devices to function in a particular manner, such that the instructions stored in the computer readable medium produce an article of manufacture including instructions which implement the function/act specified in the flowchart and/or block diagram block or blocks.

The computer program instructions may also be loaded onto a computer, other programmable data processing apparatus, or other devices to cause a series of operational steps to be performed on the computer, other programmable apparatus or other devices to produce a computer implemented process such that the instructions which execute on the computer or other

programmable apparatus provide processes for implementing the functions/acts specified in the flowchart and/or block diagram block or blocks.

The flowchart and block diagrams in the figures illustrate the architecture, functionality, and operation of possible implementations of systems, methods and computer program products according to various embodiments of the present invention. In this regard, each block in the flowchart or block diagrams may represent a module, segment, or portion of code, which comprises one or more executable instructions for implementing the specified logical function(s). It should also be noted that, in some alternative implementations, the functions noted in the blocks may occur out of the order noted in the figures. For example, two blocks shown in succession may, in fact, be executed substantially concurrently, or the blocks may sometimes be executed in the reverse order, depending upon the functionality involved. It will also be noted that each block of the block diagrams and/or flowchart illustrations, and combinations of blocks in the block diagrams and/or flowchart illustrations, can be implemented by special purpose hardware-based systems that perform the specified functions or acts, or combinations of special purpose hardware and computer instructions.

It is an objective of the invention to reduce or eliminate at least one of the drawbacks known in the prior art. In particular it is an object of the invention to provide a method for measuring the average number of emitters contributing to a localization microscopy image, thus calibrating the linear relationship between the actual emitter density and the visualized intensity derived from the density of localizations. Additionally, it is an object of the invention to use localization microscopy with reversibly switchable fluorophores as a quantitative imaging technique by measuring the average number of activation events per site as a function of time from the build-up of spatial image correlations during image acquisition.

In an aspect the invention may relate to a computer-implemented method for enabling to determine the average number of localizations per site and/or the average number of emitters per site using a localization microscope wherein said localization microscope is adapted to generate one or more super-resolution images of emitters in a sample.

In an embodiment, said method may comprise: measuring the cumulative number of localizations in a super-resolution image captured by said localization microscope and fitting the measured cumulative number of localizations with an exponential function for determining a bleaching rate k_{bl}; and, determining a spatial correlation parameter Q(t) on the basis of measured localizations in at least one of said one or more super-resolution images and fitting the measured spatial correlation parameter Q(t) with a spatial correlation parameter function $Q(k_{bl},M_\infty,\mu;t)$ derived from a photo-physical blinking model for modelling the photo-physical behaviour of said emitters, including the effect of photo-switching and photo-bleaching, for determining the asymptotic number of localizations per emitter M_∞ and the stoichiometry parameter μ.

In an embodiment, the method may comprise determining the average number of emitters per site on the basis of said stoichiometry parameter μ. In another embodiment, the method may comprise determining the average number of localizations per site <M> on the basis of the fitted values of k_{bl}, M_∞ and μ.

Hence, the invention is based on an analysis method of the spatial correlations in a super-resolution image that do not arise from the sample structure itself but rather from having multiple localizations per site. These latter correlations can be characterized by a single parameter Q, which can be measured as a function of time. This measured correlation parameter Q and the cumulative number of localizations as a function of time can be fitted using a photo-physical blinking model including the effects of switching and photo-bleaching. The fitted model may allow both the determination, or at least an accurate estimation, of the

average number of localizations per site as well as a statistical parameter μ indicating the number of emitters from the same site (stoichiometry) may be obtained.

The method according to the invention enables quantitative analysis in localization microscopy without any prior knowledge of the spatial sample and/or photo-physics of the emitters.

In an embodiment, said exponential function may have the form y=a(1-exp(-bt)).

In an embodiment, said spatial correlation parameter function $Q(k_{bl}, M_\infty, \mu; t)$ may be given by:

$$Q = 2(M_\infty - 1)\left(1 - \frac{k_{bl}t}{\exp(k_{bl}t) - 1}\right) + \mu M_\infty (1 - \exp(k_{bl}t))$$

In an embodiment, said stoichiometry parameter μ may be given by:

$$\mu = \frac{\langle S^2 \rangle}{\langle S \rangle} - 1$$

wherein S is the number of emitters per site.

In an embodiment, the average number of localizations per site <M> may be given by:

$$\langle M \rangle = M_\infty \langle S \rangle \left[1 - \exp(-k_{bl}t)\right]$$

The derivation of the spatial correlation function Q for multiple emitters per binding site on the basis of a photo-physical blinking model for modelling the photo-physical behaviour of said emitters, including the effect of photo-switching and photo-bleaching, is not trivial and allows determination of valuable quantitative information of the sample, including the average number of localizations per emitter and the number of emitters associated with one site.

The spatial correlation parameter Q as a function of t is determined by three parameters: the effective bleaching rate k_{bl}, the asymptotic value of the number of activations per

label M_∞ and the stoichiometry parameter μ, which may be easily determined on the basis of information that is derivable from super-resolution images of localizations.

In an embodiment, measuring a spatial correlation parameter Q as a function of time t on the basis of localizations in at least one of said one or more super-resolution images may comprise: determining a set of localizations by measuring localizations in one or more images over a predetermined time; dividing the set of localizations in at least two statistically independent sets f_1 and f_2; determining a spatial correlation as a function of the q by computing:

$$\sum_{|q|=const.} \hat{f}_1(\vec{q})\hat{f}_2(\vec{q})^*$$

wherein is q the spatial frequency and the caret ^ denoting the Fourier transformation of f(q); and, determining Q on the basis of said spatial correlation.

In an embodiment, S = 1 and μ = 0 so that the spatial correlation parameter function Q(t) only depends on the bleaching rate k_{b1} and the asymptotic number of localizations per molecule M_∞.

In an embodiment, a site may comprise one or two emitters and wherein the probability that a site comprises one emitters may be 1-p and the probability that a site comprises two emitters may be p so that <S>=1+p and μ = 2p/(1+p) and so that a one-to-one correspondence between μ and <S> is provided.

In an embodiment S may follow a Poisson-distribution μ=<S> so that both parameters M_∞ and μ may be determined on the basis of said measured spatial correlation parameter Q(t).

In a further aspect, the invention may relate to an image processing module for use in a localization microscope, wherein said image processing module may be configured for determining the average number of localizations per site and/or the average number of emitters per site of localizations and wherein said localization microscope may be

adapted to generate one or more super-resolution images of emitters in a sample.

In an embodiment, said image processing module further being configured for: measuring the cumulative number of localizations in a super-resolution image captured by said localization microscope and fitting the measured cumulative number of localizations with an exponential function, preferably an exponential function of the form y=a(1-exp(-bt)), for determining a bleaching rate k_{bl}; determining a spatial correlation parameter Q(t) on the basis of measured localizations in at least one of said one or more super-resolution images and fitting the measured spatial correlation parameter Q(t) with a spatial correlation parameter function $Q(k_{bl},M_\infty,\mu;t)$ derived from a photo-physical blinking model for modelling the photo-physical behaviour of said emitters, including the effect of photo-switching and photo-bleaching, for determining the asymptotic number of localizations per emitter M_∞ and the stoichiometry parameter μ.

In an embodiment, said image processing module may be configured for determining the average number of emitters per site on the basis of said stoichiometry parameter μ.

In an embodiment, said image processing module may be configured for determining the average number of localizations per site <M> on the basis of the fitted values of k_{bl}, M_∞ and μ.

In an embodiment, said spatial correlation parameter function $Q(k_{bl},M_\infty,\mu;t)$ may be given by:

$$Q = 2(M_\infty - 1)\left(1 - \frac{k_{bl}t}{\exp(k_{bl}t)-1}\right) + \mu M_\infty(1-\exp(k_{bl}t))$$

wherein μ is given by:

$$\mu = \frac{\langle S^2 \rangle}{\langle S \rangle} - 1$$

wherein S is the number of emitters per site; and, wherein the average number of localizations per site <M> may be given by:

$$\langle M \rangle = M_\infty \langle S \rangle \left[1 - \exp\left(-k_{bl} t\right)\right]$$

In an further embodiment, wherein measuring a spatial correlation parameter Q as a function of time t on the basis of localizations in at least one of said one or more super-resolution images by said image-processing module may comprise: determining a set of localizations by measuring localizations in one or more images over a predetermined time; dividing the set of localizations in at least two statistically independent sets f_1 and f_2; determining a spatial correlation as a function of the q by computing:

$$\sum_{|q|=const.} \hat{f}_1(\vec{q}) \hat{f}_2(\vec{q})^*$$

wherein is q the spatial frequency and the caret ^ denoting the Fourier transformation of f(q); and, determining Q on the basis of said spatial correlation.

In another aspect, the invention may relate to the use of the image-processing module as described above in a microscopy system, preferably a localization microscopy system.

In yet another aspect, the invention may relate to a microscopy system comprising an image-processing module according as described above.

The invention may also relate to a computer program product, comprising software code portions configured for, when run in the memory of a computer, executing the method steps according to any of the method steps described above.

The invention will be further illustrated with reference to the attached drawings, which schematically will show embodiments according to the invention. It will be understood that the invention is not in any way restricted to these specific embodiments.

Brief description of the drawings

Fig. 1 depicts a microscopy system using an emitter counter module according to an embodiment of the invention.

Fig. 2 depicts a high-level schematic of a process for forming an image of a sample using localization microscopy;

Fig. 3 depicts the scaled correlation function as a function of the spatial frequency q;

Fig. 4 depicts the experimental validation of the activation-bleaching switching model according to an embodiment of the invention;

Fig. 5 depicts histograms at different points in time of the number of localizations per cluster and the predicted probability distribution of the used photo-physical blinking model;

Fig. 6 is a block diagram illustrating an exemplary data processing system that may be used in systems and methods.

Detailed description

Fig. 1 depicts a microscopy system using an emitter counter module according to an embodiment of the invention. In particular, **Fig. 1** depicts a schematic of a system **100** that may be used for super-resolution microscopy and in particular for localization microscopy. The microscopy system may relate to any type of localization microscopy system including the Photo-Activation Localization Microscopy (PALM) system and STochastic Optical Reconstruction Microscopy (STORM) system.

The system may comprise a microscope objective **106** comprising an optical lens system over a substrate holder (not shown) comprising a substrate **102.** A sample **104** positioned on the substrate may comprise a biological or molecular structure that is selectively labeled with one or more emitters **103,** typically fluorescent molecules such as fluorescent dyes of fluorophores, which can be stochastically switched between a light emitting and dark state such that at any instant in time a sparse subset of all emitters is in the on state. An emitter

may be positioned in the sample at a location (x,y,z), wherein x,y are the coordinates in the plane of the substrate and the axial coordinate z is the coordinate normal to the plane of the substrate.

The optical lens system of the microscope object may be configured to focus light from one or more light sources **108,110** onto the sample and to image light originating from an emitter onto a camera **118**. The first light source may be a switching light source for exposing the sample with light while the second light source may be used to expose the sample with (laser) light for exciting the emitters. In another embodiment, one switching light source may be used that both exposes the sample and excites the emitters. Semi-transparent mirrors may be used for reflecting light from the first and second light source to the microscope objective and transmitting light originating from an emitter to the camera.

In case localization of emitters in the sample is required, the emitted light originating from excited emitters is imaged by the optical system of the microscope objective and one or more further optical elements (not shown) onto the image plane of a digital camera **118**. An image-acquisition and processing module **126** (in short an image-processing module) on a computer **120** that is connected to the camera may instruct the camera to take multiple images (a movie) of the randomly activated light-emitting emitters. The image-processing module may record the images and process the images in order to render an image of all emitters, wherein individual emitters are imaged with a resolution down to the localization precision of typically 10 nm.

Fig. 2 depicts a high-level schematic of a process for forming an image of a sample using localization microscopy. In this process (that may be executed by the image-processing module connected to the camera of the microscope), a sequence of images **202** is taken, wherein each image comprises a subsets of light-emitting emitters (step **202**). Because only a (small) subset of the whole set of emitters is emitting, the individual light-emitting emitters

are visible in each image. These images may be subsequently processed by the image-processing module (step **204**), wherein for each image the individual locations **206**$_{1-4}$ of emitting emitters may be localized. Repeating this process for a large number of images may render precise localization of all emitters. In this way a super-resolution image of localized emitters may be obtained that has details on the order of the localization precision rather than the diffraction limit. As one or more emitters are selectively attached to specific binding sites (so-called epitopes) of the underlying structure (e.g. a macro-molecule, DNA, subcellular structure etc.), the shape of the labelled structure may become visible in the emitter localization image as can be seen in the picture of **Fig. 2** that is obtained on the basis of the STORM imaging technique.

The image-processing module may be further configured to execute a quantitative analysis method on the spatial correlations of the imaged emitters in the super-resolution image that do not arise from the sample structure itself but rather from having multiple localizations, due to repeated activations of the same emitter pertaining to a site and/or having multiple independent emitters per. The spatial correlations of the localizations in the super-resolution image may be characterized by a single correlation parameter Q(t), which can be measured as a function of time. As will be discussed hereunder in detail, the spatial correlation parameter Q that may be measured on the basis of a super-resolution image and the cumulative number of localizations as a function of time may be fitted with a photo-physical blinking model in which the effects of switching and photo-bleaching are taken into account. In this way, a reliable determination, or at least a reliable estimation, of the average number of localizations per site may be obtained as well as a statistical parameter μ that provides an indication of S, the number of emitters associated with the same site (the stoichiometry).

The photo-physical blinking model including the effects of switching and photo-bleaching may be based on the switching between three states, the on-state, the off-state, and the bleached state. The switching between the on-states and off-states is modelled using the Poisson distribution:

$$p_m^{\mathrm{act}} = \frac{r^m}{m!}\exp(-r),$$

where $r = k_{act}t$ wherein t represents time and k_{act} the activation rate, which is related to the lifetimes of the on-states and off-states by $1/k_{act} = \tau_{on} + \tau_{off}$.

The photo-bleaching is governed by a geometric distribution, namely the probability for bleaching at the m-th switching cycle is:

$$p_m^{\mathrm{bl}} = b(1-b)^{m-1}.$$

where b is the probability for bleaching during one cycle. Intuitively, for small time scales the statistics will be close to the activation dominated Poisson-model, whereas for large times it will be close to the bleaching dominated geometric distribution. For intermediate times t, the probability for m activation cycles is the sum of two terms.

The first is the product of the probability pact of having m switching cycles and the probability $(1 - b)^m$ that the emitter has m not bleached in the m switching cycles. The second term is the product of the probability $p_{bl}{}^m$ of bleaching during the m-th switching cycle and the probability of having at least m switching cycles. In mathematical terms (for $m \geq 1$):

$$p_m = (1-b)^m \frac{r^m}{m!}\exp(-r) + b(1-b)^{m-1}\sum_{n=m}^{\infty}\frac{r^n}{n!}\exp(-r).$$

For m = 0 bleaching does not play a role, so the probability is then given by the Poisson term only:

$$p_0 = \exp(-r).$$

Further, it may be verified that:

$$\sum_{m=0}^{\infty} p_m = 1,$$

so that conservation of probability is satisfied.

The probability distribution of the number of activation cycles m is equivalent to the distribution of the minimum of two random variables $m_{Poisson}$ and $mg_{eometric}$, where $m_{Poisson}$ is Poisson distributed with expectation value r and $m_{geometric}$ follows a geometric distribution with expectation value 1/b. The moments of this probability distribution may be calculated from the moment-generating function:

$$\begin{aligned}
G(a) &= \sum_{m=0}^{\infty} p_m \exp(am) \\
&= \exp(-r) + \sum_{m=1}^{\infty} ((1-b)\exp(a))^m \frac{r^m}{m!} \exp(-r) \\
&\quad + b\exp(a) \sum_{m=1}^{\infty} ((1-b)\exp(a))^{m-1} \sum_{n=m}^{\infty} \frac{r^n}{n!} \exp(-r) \\
&= \exp(r(1-b)\exp(a) - r) \\
&\quad + b\exp(a) \sum_{n=1}^{\infty} \frac{1 - ((1-b)\exp(a))^n}{1-(1-b)\exp(a)} \frac{r^n}{n!} \exp(-r) \\
&= \frac{b\exp(a) + (1-\exp(a))\exp(r(1-b)\exp(a) - r)}{1-(1-b)\exp(a)}.
\end{aligned}$$

The moments follow from the derivatives of this function at a = 0:

$$\begin{aligned}
M_1(t) &= \sum_{m=1}^{\infty} m p_m = \left.\frac{dG(a)}{da}\right|_{a=0} \\
&= \frac{1}{b}[1 - \exp(-rb)], \\
M_2(t) &= \sum_{m=1}^{\infty} m^2 p_m = \left.\frac{d^2G(a)}{da^2}\right|_{a=0} \\
&= \frac{1}{b}[1 - \exp(-rb)] + \frac{2(1-b)}{b^2}[1 - \exp(-rb) - rb\exp(-rb)],
\end{aligned}$$

giving the spatial correlation parameter Q as a function of time:

$$\begin{aligned} Q(t) &= \frac{M_2(t) - M_1(t)}{M_1(t)} \\ &= \frac{2(1-b)}{b}\left[1 - \frac{rb}{\exp(rb) - 1}\right]. \end{aligned}$$

If the bleaching rate is defined as $k_{bl} = bk_{ac}$ then the results for the average number of activations and for the correlation parameter Q for a single label (emitter) per site may be written as:

$$\begin{aligned} M_1(t) &= \frac{k_{act}}{k_{bl}}\left[1 - \exp(-k_{bl}t)\right], \\ Q(t) &= 2\left(\frac{k_{act}}{k_{bl}} - 1\right)\left[1 - \frac{k_{bl}t}{\exp(k_{bl}t) - 1}\right]. \end{aligned}$$

The effect of labeling stoichiometry, i.e. multiple labels (emitters) per state, may be determined as follows. Suppose there are K anti-body binding sites with S_i (i=1,2,…,K) fluorescent labels per anti-body which have M_{ij} activations (j=1,2,…,S_i). The number of activations per binding site is then given by:

$$M_i = \sum_{j=1}^{S_i} M_{ij}.$$

The statistics of the number of labels per site are considered to be independent of the site and has moments <S> and <S^2>. Furthermore, the statistics of the number of activations of each label are considered to be independent of label and binding site and gives rise to moments according to the three-state model:

$$\begin{aligned} \langle M_{ij} \rangle &= M_\infty\left[1 - \exp(-k_{bl}t)\right], \\ \langle M_{ij}^2 - M_{ij} \rangle &= 2M_\infty(M_\infty - 1)\left[1 - \exp(-k_{bl}t) - k_{bl}t\exp(-k_{bl}t)\right], \end{aligned}$$

for all i and j and with $M_\infty = k_{act}/k_{bl}$. The spatial correlation parameter Q determined from the spatial correlation analysis is given by:

$$Q = \frac{\langle M_i^2 - M_i \rangle}{\langle M_i \rangle},$$

with:

$$\langle M_i \rangle = \langle S \rangle \langle M_{ij} \rangle,$$

and:

$$\langle M_i^2 \rangle = \langle S(S-1) \rangle \langle M_{ij} \rangle^2 + \langle S \rangle \langle M_{ij}^2 \rangle.$$

Combining all results gives the following analytical expression for the spatial correlation parameter Q:

$$Q = 2(M_\infty - 1)\left[1 - \frac{k_{bl}t}{\exp(k_{bl}t) - 1}\right] + \mu M_\infty \left[1 - \exp(-k_{bl}t)\right],$$

with:

$$\mu = \frac{\langle S^2 \rangle - \langle S \rangle}{\langle S \rangle},$$

Here μ represents the stoichiometry parameter, i.e. a statistical parameter related to S, the number of emitters associated with the same site. The second term of the above expression for Q is now compared to the expression of Q when considering the statistics of a single label. The derivation of the spatial correlation function Q for multiple emitters per binding site is not trivial and - as will be described hereunder in more detail - allows determination of valuable quantitative information of the sample, including the average number of localizations per emitter and the number of emitters associated with one site.

Hence, from the above, it follows that, there are three parameters that determine the spatial correlation parameter Q as a function of t: the effective bleaching rate

k_{bl}, the asymptotic value of the number of activations per label M_∞ and the stoichiometry parameter μ. The expected total number of activations is given by:

$$\begin{aligned}\langle N\rangle &= \sum_{i=1}^{K}\sum_{j=1}^{S_i} M_{ij} = K\langle S\rangle\langle M_{ij}\rangle \\ &= K\langle S\rangle M_\infty\left[1-\exp\left(-k_{\mathrm{bl}}t\right)\right].\end{aligned}$$

These parameters may be obtained by analysis of one or more super-resolution images of localized emitters (a localization image) that are obtained using a localization microscope as described with reference to **Fig. 1** and **2**. The parameters obtained from the analysis may then be used to determine the average number of localizations per emitter and the number of emitters associated with one binding site.

In a first step a set of localizations may be determined by capturing a movie (i.e. multiple images) of the localizations up to a time point t. Then, the set of localizations that is acquired up to a time point t is divided into at least two statistically independent sets f_1 and f_2. In an embodiment, division into two sets may be realized by dividing the acquisition time series into blocks of 500 frames and randomly assigning these blocks to the two image halves. From the sets f_1 and f_2, a spatial correlation may be computed as:

$$\sum_{|q|=const.} \hat{f}_1(\vec{q})\,\hat{f}_2(\vec{q})^*$$

with q the spatial frequency and the caret ^ denoting the Fourier transformation. The spatial correlation is multiplied with a factor $\exp(4\pi^2q^2\sigma^2)$ where σ is the average localization uncertainty. Preferably, a suitably weighted sum of factors $\exp(4\pi^2q^2\sigma^2)$ over the distribution of different σ values may be used. The resulting curve as a function of q comprises a plateau as shown in **Fig. 3**. The value of the plateau of the spatial correlation is equal to the experimentally measured Q,

i.e. the (spatial) image correlation parameter arising from having multiple localizations per emitter.

As already discussed above in detail, the photo-physical model with three states (on, off, bleached) and one label (emitter) per site predicts the relation between Q and the average number of times a fluorescent emitter switches <M> as:

$$\langle M \rangle = M_\infty \left(1 - \exp(-k_{bl} t)\right)$$

$$Q = 2\left(M_\infty - 1\right)\left(1 - \frac{k_{bl} t}{\exp(k_{bl} t) - 1}\right)$$

wherein k_{bl} is the effective rate constant. From this model the effective rate constant k_{bl} may be found from a fit to the cumulative number of localization as a function of time in the form y=a(1-exp(-bt)), where the fit of b gives the effective rate constant k_{bl}. The cumulative number of localization as a function of time may be easily determined by the image-processing module determined during formation of a super-resolution image of localized emitters.

Having this rate constant, the parameter M_∞ may be obtained from the experimentally measured Q as a function of t as described above. Then, <M> (the average number switching cycles per emitter) may be determined on the basis of the measured spatial correlation parameter Q. Dividing the total, cumulative number of localizations by <M> gives the desired average number of emitters contributing to the image.

The above-mentioned analytical relation between Q and the average number of times a fluorescent emitter switches <M> only holds for one emitter per site. As already described above in detail, in case of multiple emitters per site, the analytical expression for the spatial correlation parameter function $Q(k_{bl}, M_\infty, \mu; t)$ derived from the photo-statistical blinking model is given by:

$$Q = 2(M_\infty - 1)\left(1 - \frac{k_{bl} t}{\exp(k_{bl} t) - 1}\right) + \mu M_\infty (1 - \exp(k_{bl} t))$$

wherein μ is a number characterizing the statistics of S, i.e. the number of labels per site (the number of emitters with spatial separation less than the localization uncertainty site) (stoichiometry):

$$\mu = \frac{\langle S^2 \rangle}{\langle S \rangle} - 1$$

Hence, the average number of emitters per binding site may be obtained from the stoichiometry statistics parameter μ. In an embodiment, (the most simple case) that there is only one dye per epitope, so that S = 1 and μ = 0, and only the bleaching rate k_{bl} and the asymptotic number of localizations per molecule M_∞ appear as parameters. In another embodiment, if there are known to be either one or two dyes (with probability 1-p and p, respectively) then <S>=1+p and μ = 2p/(1+p), giving a one-to-one correspondence between μ and <S>. In yet another embodiment, S may follow a Poisson-distribution μ=<S> so that both parameters M_∞ and <S>may be estimated from the measured curve Q(t).

Hence, from the above it follows that both the average number of localizations per emitter and the number of emitters contributing to the localization microscopy image can be obtained on the basis of a three-state model (on, off and bleached state) where the on-off switching is characterized by an activation rate k_{act}, and the photo-bleaching by a bleaching rate k_{bl} and an analysis of the spatial correlations in a super-resolution image that do arise from multiple localizations per site. The analysis process may be performed by the image-processing module of a localization microscope wherein the image-processing module is configured to execute the following steps:

- measuring the cumulative number of localizations in a super-resolution image determined by a localization microscope and fitting the measured cumulative number of localizations with an exponential function, preferably in

the form of $y=a(1-\exp(-bt)$, for determining a bleaching rate k_{bl};

- measuring a spatial correlation parameter Q as a function of time t for at least part of said localizations in said super-resolution image and fitting the measured spatial correlation parameter Q with the spatial correlation parameter Q derived from a photo-physical blinking model that includes the effects of photo-switching and photo-bleaching, for determining the asymptotic number of localizations per molecule M_∞ and the stoichiometry parameter μ;
- determining the average number of emitters per site on the basis of said stoichiometry parameter μ; and/or,
- determining the average number of localizations per binding site <M> on the basis of the fitted values of k_{bl} M_∞ and μ.

The average number of emitters per site may be determined on the basis of μ if the labelling strategy is chosen such that there is an predetermined relationship between the mean and the variance of the distribution of the number of emitters per binding site.

Fig. 4 depicts the experimental validation of the activation-bleaching switching model according to an embodiment of the invention. The model was validated by experiments on isolated (sparsely distributed) clusters of DNA oligomers labelled with single Alexa Fluor 647 dyes on a glass substrate as shown in **Fig. 4(A)**. Clearly recognizable isolated clusters may provide a ground truth for the distribution of localizations per emitter. First order switching kinetics is confirmed by an exponential on-time and off-time distribution giving τ_{on} = 27 ms and τ_{off} = 26 s.

Fig. 4(B) depicts the fitting of the cumulative number of localizations with a single exponential with bleaching rate k_{bl} of 4.8×10^{-3}/s. These data show that the parameter Q from the spatial correlation analysis may be well fitted with the expression for Q giving rise to M_∞ = 10.8 and

with the ground truth value obtained from the cluster analysis.

The prediction for the expected number of localizations per emitter according to the expression of Q agrees well with the ground truth value obtained from the cluster analysis, thus validating the final step of the model. The measured τ_{on} and τ_{off} lead to an activation rate k_{act} = 3.9×10^{-2}/s, which is agreement with the value k_{act} = 5.2×10^{-2}/s obtained from the fitted bleaching rate and asymptotic number of localizations per cluster.

Fig. 5 depicts histograms at different points in time of the number of localizations per cluster and the predicted probability distribution of the used photo-physical blinking model showing that the measured number of localizations match the model with the estimated parameters quite accurately.

The counting method according to the invention assumes constant and uniform rates, which may not apply for varying illumination power or for the depth dependent intensity in TIRF-imaging. These effects may be compensated for by recording the power as a function of time and inclusion of time dependent switching rates into the model.

The counting method according to the invention assumes that three states are sufficient for an effective description of the switching and bleaching behaviour of the emitters. In particular multiple long-lived dark states may alter the relation between the average number of localizations per emitter and the spatial correlation parameter Q.

Fig. 6 is a block diagram illustrating an exemplary data processing system that may be used in systems and methods as described with reference to **Fig. 1-5**. Data processing system **600** may include at least one processor **602** coupled to memory elements **604** through a system bus **606**. As such, the data processing system may store program code within memory elements **604**. Further, processor **602** may execute the program code accessed from memory elements **604** via system bus **606**. In one aspect, data processing system may be implemented as a computer that is suitable for storing and/or executing program

code. It should be appreciated, however, that data processing system **600** may be implemented in the form of any system including a processor and memory that is capable of performing the functions described within this specification.

Memory elements **604** may include one or more physical memory devices such as, for example, local memory **608** and one or more bulk storage devices **610**. Local memory may refer to random access memory or other non-persistent memory device(s) generally used during actual execution of the program code. A bulk storage device may be implemented as a hard drive or other persistent data storage device. The processing system **600** may also include one or more cache memories (not shown) that provide temporary storage of at least some program code in order to reduce the number of times program code must be retrieved from bulk storage device **610** during execution.

Input/output (I/O) devices depicted as input device **612** and output device **614** optionally can be coupled to the data processing system. Examples of input device may include, but are not limited to, for example, a keyboard, a pointing device such as a mouse, or the like. Examples of output device may include, but are not limited to, for example, a monitor or display, speakers, or the like. Input device and/or output device may be coupled to data processing system either directly or through intervening I/O controllers. A network adapter **616** may also be coupled to data processing system to enable it to become coupled to other systems, computer systems, remote network devices, and/or remote storage devices through intervening private or public networks. The network adapter may comprise a data receiver for receiving data that is transmitted by said systems, devices and/or networks to said data and a data transmitter for transmitting data to said systems, devices and/or networks. Modems, cable modems, and Ethernet cards are examples of different types of network adapter that may be used with data processing system **650**.

As pictured in **FIG. 6**, memory elements **604** may store an application **618**. It should be appreciated that data processing system **600** may further execute an operating system

(not shown) that can facilitate execution of the application. Application, being implemented in the form of executable program code, can be executed by data processing system **600**, e.g., by processor **602**. Responsive to executing application, data processing system may be configured to perform one or more operations to be described herein in further detail.

The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the singular forms "a," "an," and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. It will be further understood that the terms "comprises" and/or "comprising," when used in this specification, specify the presence of stated features, integers, steps, operations, elements, and/or components, but do not preclude the presence or addition of one or more other features, integers, steps, operations, elements, components, and/or groups thereof.

The corresponding structures, materials, acts, and equivalents of all means or step plus function elements in the claims below are intended to include any structure, material, or act for performing the function in combination with other claimed elements as specifically claimed. The description of the present invention has been presented for purposes of illustration and description, but is not intended to be exhaustive or limited to the invention in the form disclosed. Many modifications and variations will be apparent to those of ordinary skill in the art without departing from the scope and spirit of the invention. The embodiment was chosen and described in order to best explain the principles of the invention and the practical application, and to enable others of ordinary skill in the art to understand the invention for various embodiments with various modifications as are suited to the particular use contemplated.

CONCLUSIES

1. Werkwijze voor het bepalen van het gemiddeld aantal lokalisaties per locatie en/of het gemiddeld aantal emitters per locatie onder gebruikmaking van een localisatiemicroscoop, waarbij de localisatiemicroscoop is aangepast voor het genereren van één of meerdere superresolutieafbeeldingen van emitters in een monster, de werkwijze omvattende:

het meten van het cumulatieve aantal lokalisaties in een superresolutieafbeelding die door de localisatiemicroscoop is opgenomen en het fitten van het gemeten cumulatief aantal lokalisaties aan een exponentiele functie, bij voorkeur een exponentiele functie van de vorm $y=a(1-\exp(-bt))$, om een bleeksnelheid k_{bl} te bepalen;

het op basis van de gemeten lokalisaties in ten minste één van de één of meerdere superresolutieafbeeldingen bepalen van een spatiele correlatieparameter $Q(t)$ en het fitten van de gemeten spatiele correlatieparameter $Q(t)$ aan een spatiele correlatiefunctie $Q(k_{bl},M_\infty,\mu;t)$, die is afgeleid van een fotofysisch knippermodel voor het modelleren van het fotofysische gedrag van de emitters, inclusief het effect van fotoschakelen en fotobleken, om het asymptotisch aantal lokalisaties per emitter M_∞ en de stoichometrische parameter μ te bepalen;

het bepalen van het gemiddelde aantal van emitters per locatie op basis van de stoichometrische parameter μ; en/of,

het bepalen van het gemiddelde aantal lokalisaties $\langle M \rangle$ per locatie op basis van de gefitte waarden k_{bl}, M_∞ en μ.

2. Werkwijze volgens conclusie 1 waarbij de spatiele correlatieparameterfunctie $Q(k_{bl},M_\infty,\mu;t)$ wordt gegeven door:

$$Q = 2(M_\infty - 1)\left(1 - \frac{k_{bl}t}{\exp(k_{bl}t) - 1}\right) + \mu M_\infty (1 - \exp(k_{bl}t))$$

3. Werkwijze volgens conclusies 1 of 2 waarbij μ wordt gegeven door:

$$\mu = \frac{\langle S^2 \rangle}{\langle S \rangle} - 1$$

waarbij S het aantal emitters per locatie is.

4. Werkwijze volgens één van de conclusies 1-3 waarbij het gemiddeld aantal lokalisaties per locatie <M> wordt gegeven door:

$$\langle M \rangle = M_\infty \langle S \rangle \left[1 - \exp\left(-k_{bl}t\right)\right]$$

5. Werkwijze volgens één van de conclusies 1-4 waarbij het meten van een spatiele correlatieparameter Q als functie van tijd t op basis van lokalisaties in ten minste één van de één of meerdere superresolutieafbeeldingen omvat:

het bepalen van een set van lokalisaties door het voor een bepaalde tijd meten van lokalisaties in één of meerdere afbeeldingen;

het verdelen van de set van lokalisaties in twee statistisch onafhankelijke sets f_1 en f_2;

het bepalen van een spatiele correlatie als een functie van q door het berekenen van:

$$\sum_{|q|=const.} f_1(\vec{q}) f_2(\vec{q})^*$$

waarbij q de spatiele frequentie is en het dakje ^ de Fourier transformatie van f(q) aangeeft; en,

het bepalen van Q op basis van de spatiele correlatie.

6. Werkwijze volgens één van de conclusies 1-5 waarbij S=1 en $\mu = 0$ zodat de spatiele correlatieparameterfunctie Q(t) alleen afhangt van de bleeksnelheid k_{bl} en het asymptotische aantal lokalisaties per molecuul M_∞.

7. Werkwijze volgens één van de conclusies 1-5 waarbij een locatie één of twee emitters omvat en waarbij de waarschijnlijkheid dat een locatie één emitter bevat 1-p is en de waarschijnlijkheid dat een locatie twee emitters bevat p zodat $<S>=1+p$ en $\mu = 2p/(1+p)$ en zodat een één-op-één overeenkomst tussen μ en $<S>$ wordt verkregen.

8. Werkwijze volgens één van de conclusies 1-5 waarbij S een Poissonverdeling volgt zodat beide parameters M_∞ en μ bepaald worden op basis van de gemeten spatiele correlatieparameter Q(t).

9. Een beeldverwerkingsmodule voor gebruik in een localisatiemicroscoop, waarbij de beeldverwerkingsmodule is geconfigureerd voor het bepalen van het gemiddeld aantal lokalisaties per locatie en/of het gemiddeld aantal emitters per locatie, waarbij de localisatiemicroscoop is aangepast voor het genereren van één of meerdere superresolutieafbeeldingen van emitters in een monster, waarbij de beeldverwerkingsmodule is geconfigureerd voor:

het meten van het cumulatieve aantal lokalisaties in een superresolutieafbeelding die door de localisatiemicroscoop

is opgenomen en het fitten van het gemeten cumulatief aantal lokalisaties aan een exponentiele functie, bij voorkeur een exponentiele functie van de vorm y=a(1-exp(-bt)), om een bleeksnelheid k_{bl} te bepalen;

het op basis van de gemeten lokalisaties in ten minste één van de één of meerdere superresolutieafbeeldingen bepalen van een spatiele correlatieparameter Q(t) en het fitten van de gemeten spatiele correlatieparameter Q(t) aan een spatiele correlatiefunctie $Q(k_{bl},M_\infty,\mu;t)$, die is afgeleid van een fotofysisch knippermodel voor het modelleren van het fotofysische gedrag van de emitters, inclusief het effect van fotoschakelen en fotobleken, om het asymptotisch aantal lokalisaties per emitter M_∞ en de stoichometrische parameter μ te bepalen;

het bepalen van het gemiddelde aantal van emitters per locatie op basis van de stoichometrische parameter μ; en/of,

het bepalen van het gemiddelde aantal lokalisaties <M> per locatie op basis van de gefitte waarden k_{bl}, M_∞ en μ.

10. Beeldverwerkingsmodule volgens conclusie 9, waarbij de spatiele correlatieparameterfunctie $Q(k_{bl},M_\infty,\mu;t)$ wordt gegeven door:

$$Q = 2(M_\infty - 1)\left(1 - \frac{k_{bl}t}{\exp(k_{bl}t) - 1}\right) + \mu M_\infty(1 - \exp(k_{bl}t))$$

waarbij μ wordt gegeven door:

$$\mu = \frac{\langle S^2 \rangle}{\langle S \rangle} - 1$$

waarbij S het aantal emitters per locatie is; en,
waarbij het gemiddeld aantal lokalisaties per locatie <M> wordt gegeven door:

$$\langle M \rangle = M_\infty \langle S \rangle \left[1 - \exp\left(-k_{bl} t\right)\right]$$

11. Beeldverwerkingsmodule volgens conclusie 9 of 10, waarbij het meten van een spatiele correlatieparameter Q als functie van tijd t op basis van lokalisaties in ten minste één van de één of meerdere superresolutieafbeeldingen omvat:

het bepalen van een set van lokalisaties door het voor een bepaalde tijd meten van lokalisaties in één of meerdere afbeeldingen;

het verdelen van de set van lokalisaties in twee statistisch onafhankelijke sets f_1 en f_2;

het bepalen van een spatiele correlatie als een functie van q door het berekenen van:

$$\sum_{|q|=const.} \hat{f}_1(\vec{q}) \hat{f}_2(\vec{q})^*$$

waarbij q de spatiele frequentie is en het dakje ^ de Fourier transformatie van f(q) aangeeft; en,

het bepalen van Q op basis van de spatiele correlatie.

12. Gebruik van een beeldverwerkingsmodule volgens één van de conclusies 9-11 in een microscoopsysteem, bij voorkeur een localisatiemicroscoopsysteem.

13. Een microscoopsysteem omvattende een beeldverwerkingsmodule volgens één van de conclusies 9-11.

14. Computerprogrammaproduct omvattende softwarecode onderdelen die geconfigureerd zijn als ze uitgevoerd worden in het geheugen van een computer, de methode stappen volgens één van de conclusies 1-8 executeert.

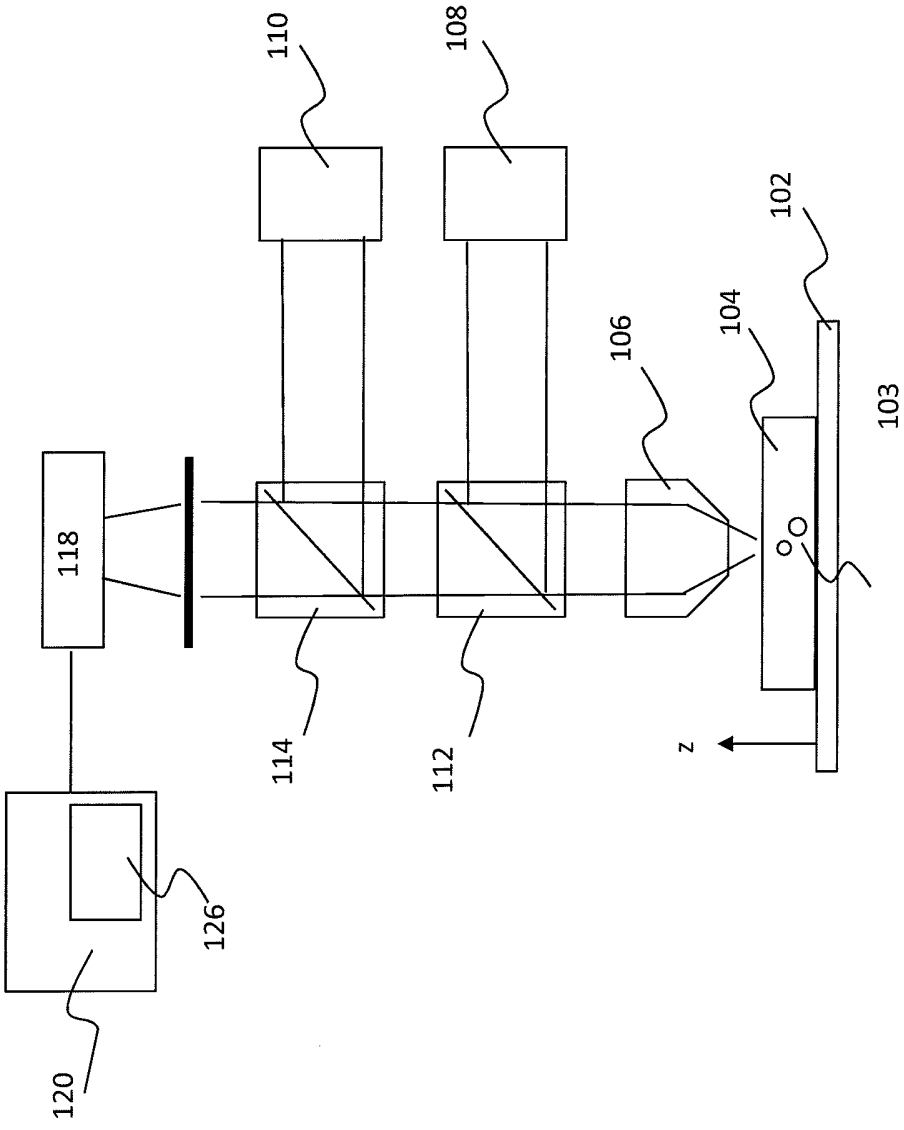

FIG. 1

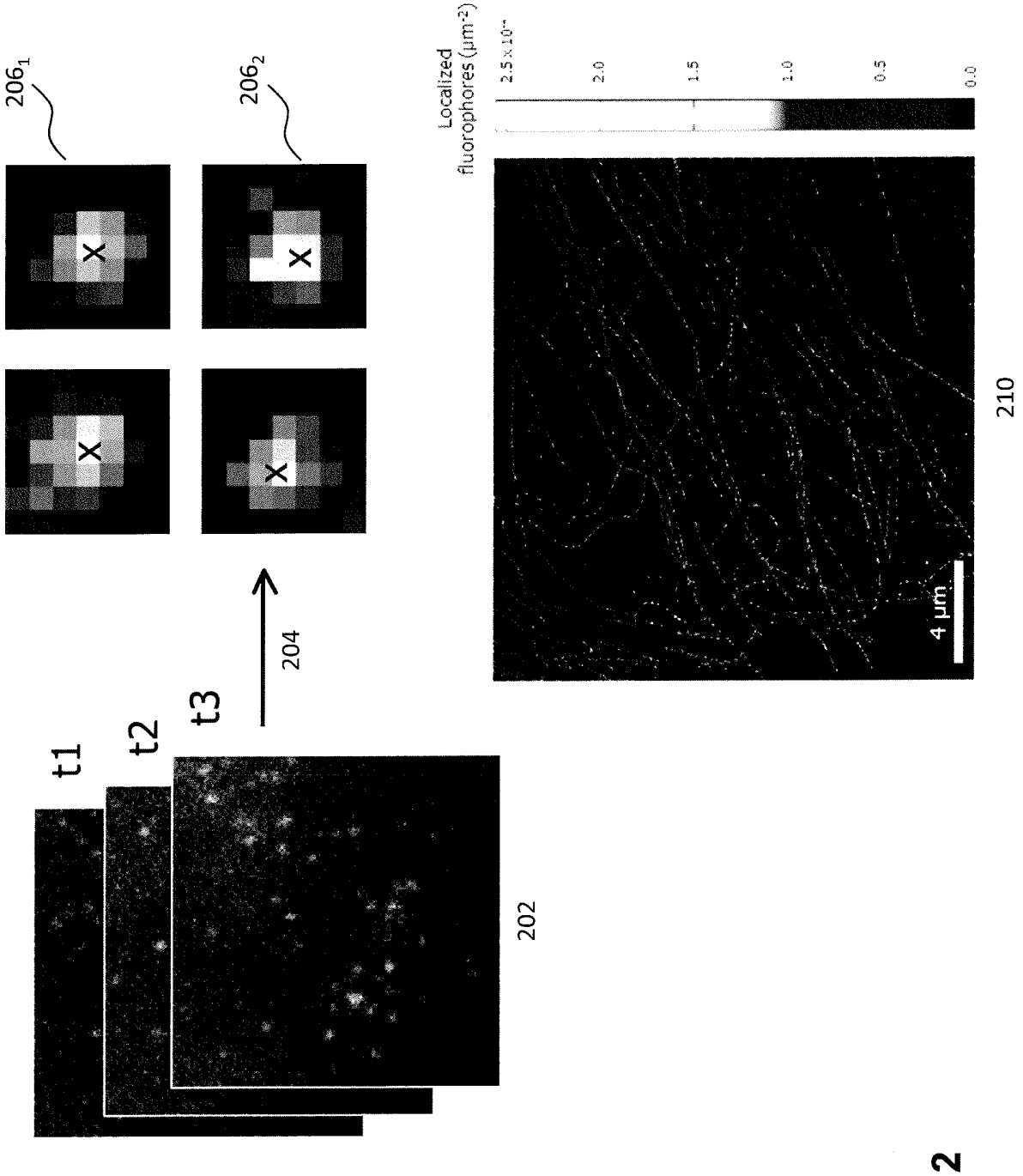
t1
t2
t3
202
204
206_1
206_2
Localized fluorophores (μm^{-2})
2.5 x 10⁻⁴
2.0
1.5
1.0
0.5
0.0
4 µm
210

FIG. 2

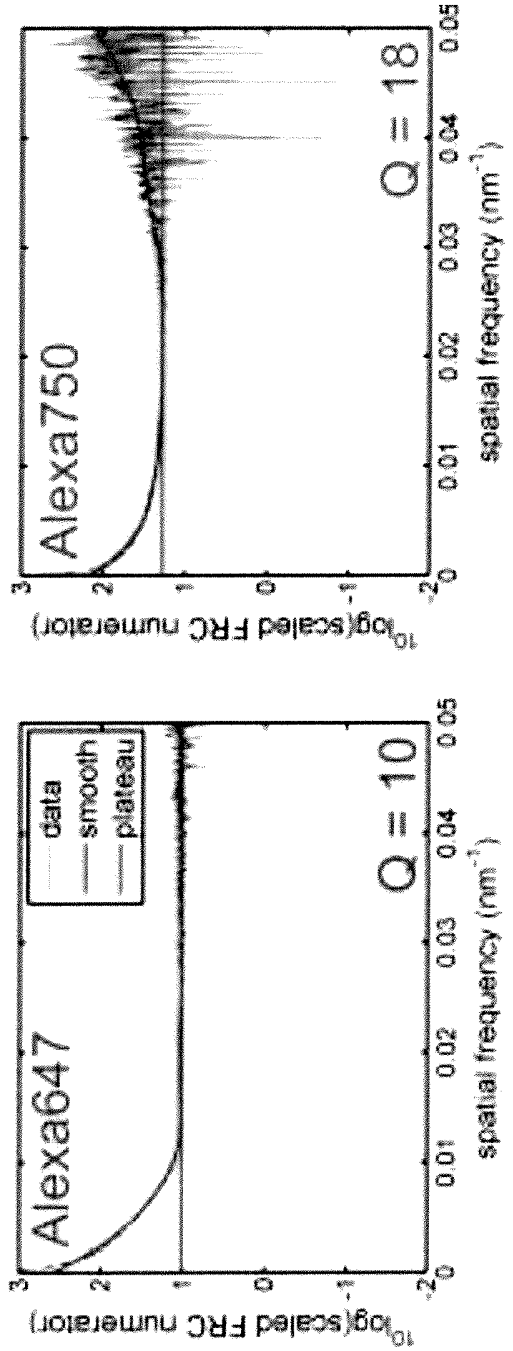
Alexa647
Q = 10
data
smooth
plateau
Alexa750
Q = 18
spatial frequency (nm^{-1})
^{10}log(scaled FRC numerator)

FIG. 3

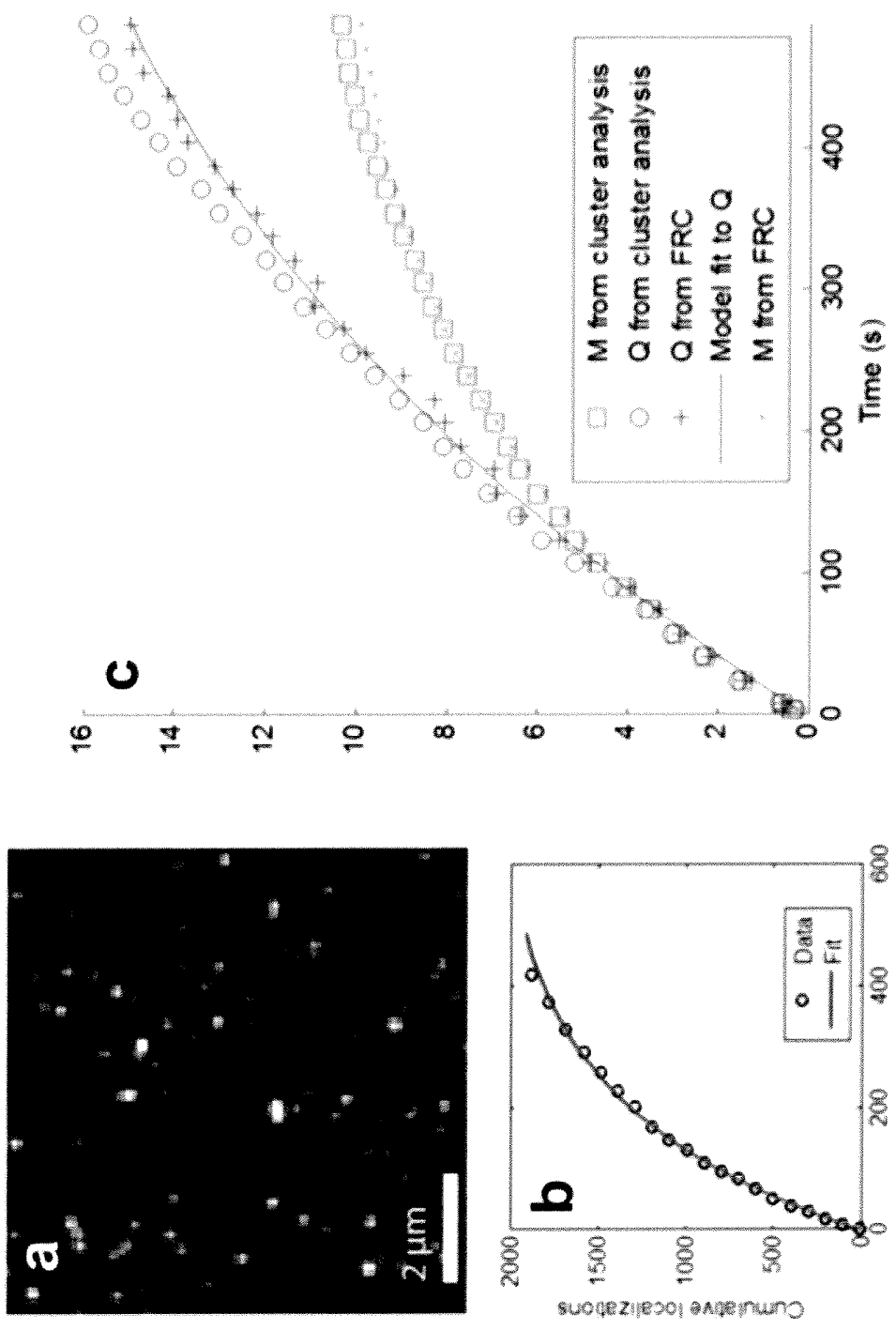
a
2 µm
b
Cumulative localizations
Time (s)
Data
Fit
c
M from cluster analysis
Q from cluster analysis
Q from FRC
Model fit to Q
M from FRC
Time (s)

FIG. 4

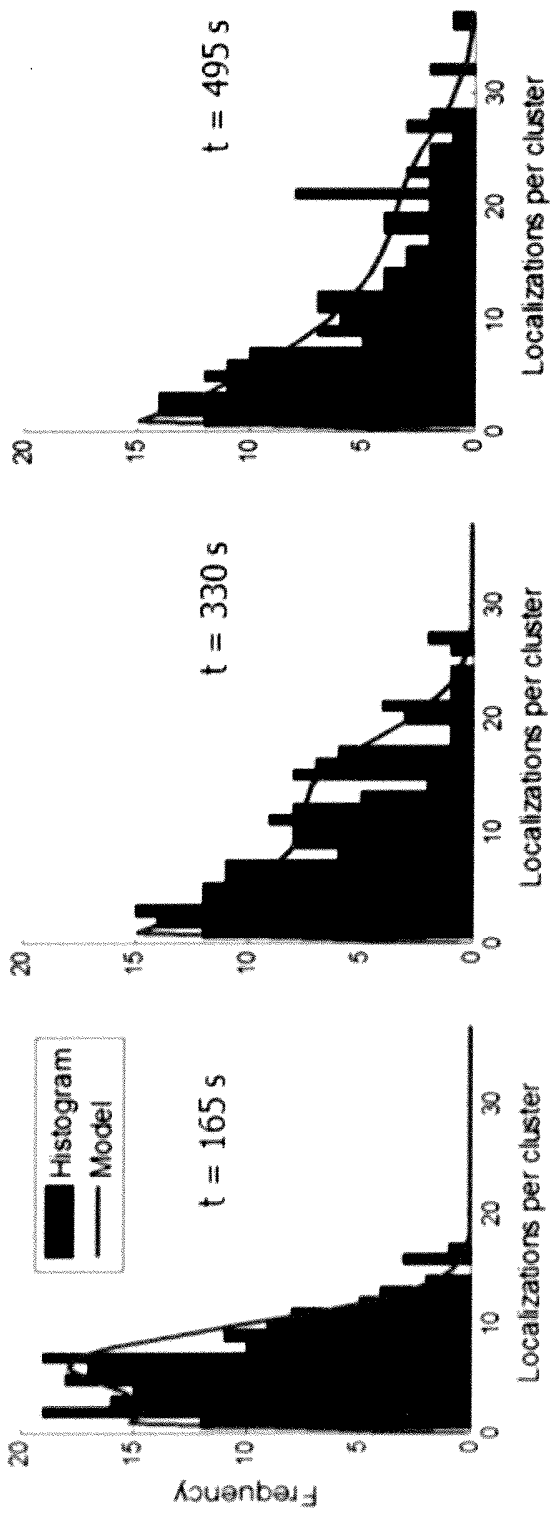
Histogram
Model
t = 165 s
t = 330 s
t = 495 s
Frequency
Localizations per cluster
Localizations per cluster
Localizations per cluster
0
5
10
15
20
0
10
20
30

FIG. 5

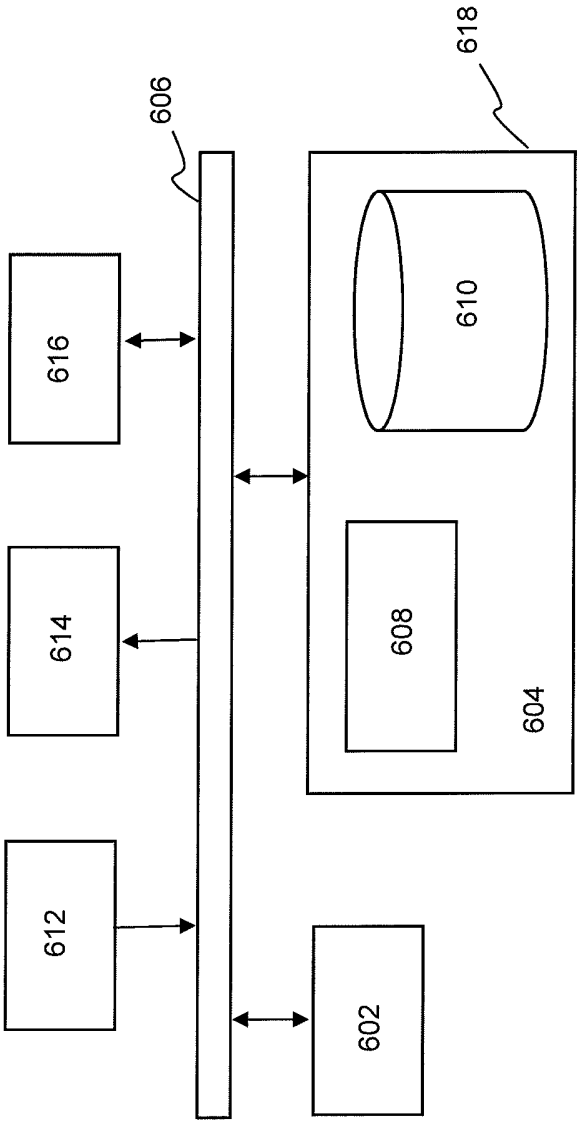

FIG. 6

SAMENWERKINGSVERDRAG (PCT)

RAPPORT BETREFFENDE NIEUWHEIDSONDERZOEK VAN INTERNATIONAAL TYPE

IDENTIFICATIE VAN DE NATIONALE AANVRAGE	KENMERK VAN DE AANVRAGER OF VAN DE GEMACHTIGDE
	NL20342-Vi-td
Nederlands aanvraag nr. **2012169**	Indieningsdatum **30-01-2014**
	Ingeroepen voorrangsdatum
Aanvrager (Naam) **Technische Universiteit Delft, et al**	
Datum van het verzoek voor een onderzoek van internationaal type **19-04-2014**	Door de Instantie voor Internationaal Onderzoek aan het verzoek voor een onderzoek van internationaal type toegekend nr. **SN 61830**

I. CLASSIFICATIE VAN HET ONDERWERP (bij toepassing van verschillende classificaties, alle classificatiesymbolen opgeven)

Volgens de internationale classificatie (IPC)

G01N21/64 G02B21/16 G02B21/36 G02B27/58 G06T3/40

II. ONDERZOCHTE GEBIEDEN VAN DE TECHNIEK

Onderzochte minimumdocumentatie

Classificatiesysteem	Classificatiesymbolen
IPC	**G01N G02B G06T**

Onderzochte andere documentatie dan de minimum documentatie, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

III. ☐ **GEEN ONDERZOEK MOGELIJK VOOR BEPAALDE CONCLUSIES** (opmerkingen op aanvullingsblad)

IV. ☐ **GEBREK AAN EENHEID VAN UITVINDING** (opmerkingen op aanvullingsblad)

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2012169

A. CLASSIFICATIE VAN HET ONDERWERP
INV. G01N21/64 G02B21/16 G02B21/36 G02B27/58 G06T3/40
ADD.

Volgens de Internationale Classificatie van octrooien (IPC) of zowel volgens de nationale classificatie als volgens de IPC.

B. ONDERZOCHTE GEBIEDEN VAN DE TECHNIEK

Onderzochte miminum documentatie (classificatie gevolgd door classificatiesymbolen)
G01N G02B G06T

Onderzochte andere documentatie dan de mimimum documentatie, voor dergelijke documenten, voor zover dergelijke documenten in de onderzochte gebieden zijn opgenomen

Tijdens het onderzoek geraadpleegde elektronische gegevensbestanden (naam van de gegevensbestanden en, waar uitvoerbaar, gebruikte trefwoorden)
EPO-Internal, WPI Data, INSPEC, BIOSIS, COMPENDEX

C. VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
X,D	Robert Nieuwenhuizen ET AL: "Measuring image resolution in optical nanoscopy", Nature methods, 1 juni 2013 (2013-06-01), bladzijde 557, XP055140742, United States DOI: 10.1038/nmeth.2448 Gevonden op het Internet: URL:http://search.proquest.com/docview/1357394150 [gevonden op 2014-09-17] in de aanvraag genoemd * samenvatting * * bladzijde 557, linker kolom - bladzijde 561, linker kolom *	1-14

-/--

[X] Verdere documenten worden vermeld in het vervolg van vak C.

[X] Leden van dezelfde octrooifamilie zijn vermeld in een bijlage

° Speciale categorieën van aangehaalde documenten

"A" niet tot de categorie X of Y behorende literatuur die de stand van de techniek beschrijft

"D" in de octrooiaanvrage vermeld

"E" eerdere octrooi(aanvrage), gepubliceerd op of na de indieningsdatum, waarin dezelfde uitvinding wordt beschreven

"L" om andere redenen vermelde literatuur

"O" niet-schriftelijke stand van de techniek

"P" tussen de voorrangsdatum en de indieningsdatum gepubliceerde literatuur

"T" na de indieningsdatum of de voorrangsdatum gepubliceerde literatuur die niet bezwarend is voor de octrooiaanvrage, maar wordt vermeld ter verheldering van de theorie of het principe dat ten grondslag ligt aan de uitvinding

"X" de conclusie wordt als niet nieuw of niet inventief beschouwd ten opzichte van deze literatuur

"Y" de conclusie wordt als niet inventief beschouwd ten opzichte van de combinatie van deze literatuur met andere geciteerde literatuur van dezelfde categorie, waarbij de combinatie voor de vakman voor de hand liggend wordt geacht

"&" lid van dezelfde octrooifamilie of overeenkomstige octrooipublicatie

Datum waarop het onderzoek naar de stand van de techniek van internationaal type werd voltooid	Verzenddatum van het rapport van het onderzoek naar de stand van de techniek van internationaal type
19 september 2014	

Naam en adres van de instantie	De bevoegde ambtenaar
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Duijs, Eric

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ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2012169

C.(Vervolg). VAN BELANG GEACHTE DOCUMENTEN

Categorie °	Geciteerde documenten, eventueel met aanduiding van speciaal van belang zijnde passages	Van belang voor conclusie nr.
X	ROBERT P. J. NIEUWENHUIZEN ET AL: "Image resolution in optical nanoscopy", PROCEEDINGS OF SPIE, deel 8815, 20 september 2013 (2013-09-20), bladzijde 881508, XP055140750, ISSN: 0277-786X, DOI: 10.1117/12.2025352 * samenvatting * * alineas [001.], [002.], [004.] *	1-14
A	US 6 259 524 B1 (HOFSTRAAT JOHANNES WILLEM [NL] ET AL) 10 juli 2001 (2001-07-10) * kolom 6, regels 51-67 *	1,9
A	US 2009/067458 A1 (JI NA [US] ET AL) 12 maart 2009 (2009-03-12) * alinea [0120]; figuur 12B *	1,9
A	US 2010/303386 A1 (ENDERLEIN JOERG [DE]) 2 december 2010 (2010-12-02) * alineas [0002], [0024] - [0032], [0072], [0082] *	1,9
A	US 2012/313012 A1 (SELVIN PAUL R [US] ET AL) 13 december 2012 (2012-12-13) * het gehele document *	1,9
A	D. T. BURNETTE ET AL: "Bleaching/blinking assisted localization microscopy for superresolution imaging using standard fluorescent molecules", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, deel 108, nr. 52, 13 december 2011 (2011-12-13), bladzijden 21081-21086, XP055141041, ISSN: 0027-8424, DOI: 10.1073/pnas.1117430109 * het gehele document *	1,9

Formulier PCT/ISA/201 (vervolg tweede blad) (Januari 2004)

ONDERZOEKSRAPPORT BETREFFENDE HET RESULTAAT VAN HET ONDERZOEK NAAR DE STAND VAN DE TECHNIEK VAN HET INTERNATIONALE TYPE

Informatie over leden van dezelfde octrooifamilie

Nummer van het verzoek om een onderzoek naar de stand van de techniek
NL 2012169

In het rapport genoemd octrooigeschrift	Datum van publicatie	Overeenkomend(e) geschrift(en)	Datum van publicatie
US 6259524 B1	10-07-2001	AT 244879 T	15-07-2003
		DE 69816289 D1	14-08-2003
		DE 69816289 T2	22-04-2004
		EP 0977981 A1	09-02-2000
		US 6259524 B1	10-07-2001
		WO 9849537 A1	05-11-1998
US 2009067458 A1	12-03-2009	US 2009067458 A1	12-03-2009
		US 2011206075 A1	25-08-2011
		WO 2009035768 A2	19-03-2009
US 2010303386 A1	02-12-2010	EP 2438555 A1	11-04-2012
		JP 2012529119 A	15-11-2012
		US 2010303386 A1	02-12-2010
		US 2014099043 A1	10-04-2014
		WO 2010141608 A1	09-12-2010
US 2012313012 A1	13-12-2012	US 2012313012 A1	13-12-2012
		WO 2011106323 A2	01-09-2011

WRITTEN OPINION

File No.	Filing date *(day/month/year)*	Priority date *(day/month/year)*	Application No.
SN61830	30.01.2014		NL2012169

International Patent Classification (IPC)
INV. G01N21/64 G02B21/16 G02B21/36 G02B27/58 G06T3/40

Applicant
Technische Universiteit Delft, et al

This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☒ Box No. VII Certain defects in the application
- ☒ Box No. VIII Certain observations on the application

	Examiner
	Duijs, Eric

Application number

WRITTEN OPINION

NL2012169

Box No. I Basis of this opinion

1. This opinion has been established on the basis of the latest set of claims filed before the start of the search.

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

   ☐ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☐ on paper

   ☐ in electronic form

   c. time of filing/furnishing:

   ☐ contained in the application as filed.

   ☐ filed together with the application in electronic form.

   ☐ furnished subsequently for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty	Yes:	Claims	1-14
	No:	Claims	
Inventive step	Yes:	Claims	
	No:	Claims	1-14
Industrial applicability	Yes:	Claims	1-14
	No:	Claims	

2. Citations and explanations

see separate sheet

WRITTEN OPINION

Box No. VII Certain defects in the application

see separate sheet

Box No. VIII Certain observations on the application

see separate sheet

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1 Reference is made to the following documents:

D1 Robert Nieuwenhuizen ET AL: "Measuring image resolution in optical nanoscopy", Nature methods, 1 juni 2013 (2013-06-01), bladzijde 557, XP055140742, United States
DOI: 10.1038/nmeth.2448, in de aanvraag genoemd

D2 Robert P. J. Nieuwenhuizen ET AL: "Image resolution in optical nanoscopy", Proceedings of SPIE,
deel 8815, 20 september 2013 (2013-09-20), bladzijde 881508, XP055140750,
ISSN: 0277-786X, DOI: 10.1117/12.2025352

D3 US 6 259 524 B1 (HOFSTRAAT JOHANNES WILLEM [NL] ET AL)

D4 US 2009/067458 A1 (JI NA [US] ET AL)

D5 US 2010/303386 A1 (ENDERLEIN JOERG [DE])

2 **Lack of inventive step**

The present application does not meet the criteria of patentability, because the subject-matter of **claims 1-14** does not involve an inventive step:

2.1 **Independent claim 1**:

D1 is regarded as being the prior art closest to the subject-matter of **claim 1** and discloses a

Werkwijze voor het bepalen van het gemiddeld aantal lokalisaties per locatie en/of het gemiddeld aantal emitters per locatie onder gebruikmaking van een localisatiemicroscoop (abstract; p. 558, left col., l. 5; p. 561, left col., l. 6-7, 20) *, waarbij de localisatiemicroscoop is aangepast voor het genereren van één of meerdere superresolutieafbeeldingen van emitters in een monster, de*

werkwijze omvattende:
-- het meten van het cumulatieve aantal lokalisaties in een superresolutieafbeelding die door de localisatiemicroscoop is opgenomen (text to Fig. 2)*;*
-- het op basis van de gemeten lokalisaties in ten minste één van de één of meerdere superresolutieafbeeldingen bepalen van een spatiele correlatieparameter Q(t) en het fitten van de gemeten spatiele correlatieparameter Q(t) aan een spatiele correlatiefunctie Q, die is afgeleid van een fotofysisch knippermodel voor het modelleren van het fotofysische gedrag van de emitters, inclusief het effect van fotoschakelen en fotobleken (p. 558, right col.; p. 560, left col., par. 2 - p. 561, left col., par. 2)*;*

-- het bepalen van het gemiddelde aantal van emitters per locatie; en/of, het bepalen van het gemiddelde aantal lokalisaties per locatie (p. 558, left col., l. 5; p. 561, left col., l. 6-7, 20).

D2 (abstract; chapters 1. and 2.; p. 6, last par.) also discloses these features.

D1 and **D2** do not specify in detail how the effect of photobleaching and blinking are taken into account when determining Q.

The subject-matter of **claim 1** therefore **differs** from these known methods in that it comprises the step of "*het fitten van het gemeten cumulatief aantal lokalisaties aan een exponentiele functie, om een bleeksnelheid k_{bl} te bepalen", determining "het asymptotisch aantal lokalisaties per emitter M_∞ en de stoichometrische parameter p", and "het bepalen van het gemiddelde aantal van emitters per locatie op basis van de stoichometrische parameter p; en/of, het bepalen van het gemiddelde aantal lokalisaties <M> per locatie op basis van de gefitte waarden k_{bl}, M_∞ en p*".

The **problem** to be solved by the present invention may therefore be regarded as how to provide a method which takes into account the effect of photobleaching and blinking when determining Q.

The **solution** proposed in **claim 1** of the present application cannot be considered as involving an inventive step for the following reasons:

It is explicitly stated in **D1** (p. 558, right col.) that "Careful analysis of the spatiotemporal correlations in the image and the emitter activation statistics (including effects of photobleaching) can provide a way to estimate Q and correct for its effect on image resolution as well as to estimate the number of fluorescent labels contributing to the image".

It is explicitly stated in **D2** (p. 6, last par.) that "In the above we neglected the relevant effect of photobleaching of emitters during the measurement. This introduces a major complication in the analysis; we can no longer assume a Poissionan distribution of the number of localization emitters M . It is clear that it will have an effect on the resolution and its optimum as longer imaging will again increase M but at the same time V *ar*(M) will probably increase stronger. The details depend on the photo physical switching model between the on, off and bleached state, assuming the simplest possible three state model.15,19 We will look into this complicated problem in a future study."

It is generally known that photobleaching can be described by an exponential decay curve, which allows to determine a decay rate (bleaching speed), see for example **D3** (col. 6, l. 51-67), **D4** (FIG. 12B; par. 120), or **D5** (par. 82).

The skilled person of **D1** and/or **D2** working in the field of fluorescence image processing would know how to correct Q to take into account the effect of photobleaching and blinking. Suitable fitting techniques and statistical mathematics are well-known in the field. He would arrive at the subject-matter of **claim 1** without involving an inventive step.

2.2 The same reasoning applies, mutatis mutandis, to the subject-matter of the corresponding **independent claim 9**, which therefore is also considered not inventive.

2.3 Dependent **claims 2-8 and 10-14** do not appear to contain any features which, in combination with the features of any claim to which they refer, meet the requirements of inventive step, see the documents and the passages cited in the search report.

Re Item VII

Certain defects in the application

3 The relevant background art disclosed in **D2-D5** is not mentioned in the description, nor is this document identified therein.

Re Item VIII

Certain observations on the application

4 The statements "may", "can" in the description on p. 9 imply that the subject-matter for which protection is sought may be different to that defined by the claims, thereby resulting in lack of clarity when used to interpret them.