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Dna origami beads for fluorescence quantification in microfluidics

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Publication date:
2019

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):
Selnihhin, D., Simonsen, J. B., & Pedersen, F. S. (2019). Dna origami beads for fluorescence quantification in microfluidics. (Patent No. *WO2019149932*).

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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
08 August 2019 (08.08.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/149932 A1

(51) International Patent Classification:
G01N 33/53 (2006.01) *G01N 33/543* (2006.01)
G01F 25/00 (2006.01) *G01N 33/58* (2006.01)

(21) International Application Number:
PCT/EP2019/052640

(22) International Filing Date:
04 February 2019 (04.02.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
18154868.6 02 February 2018 (02.02.2018) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *of inventorship (Rule 4.17(iv))*

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: DNA ORIGAMI BEADS FOR FLUORESCENCE QUANTIFICATION IN MICROFLUIDICS

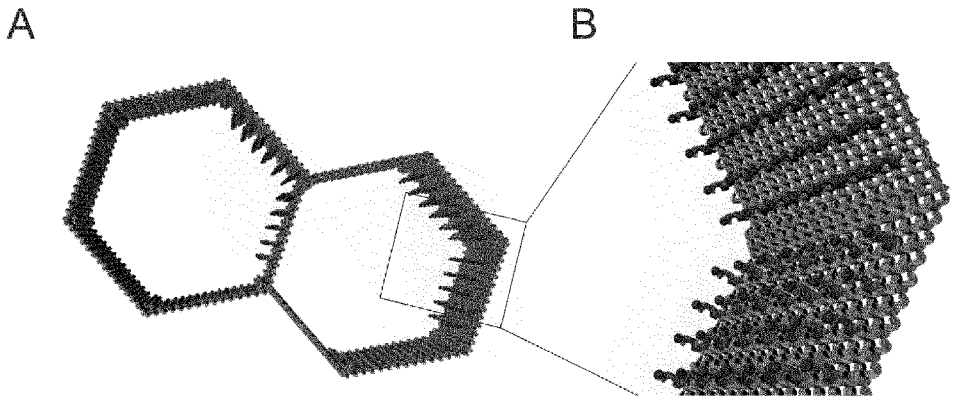

Figure 2

(57) Abstract: A method is provided for calibrating a microfluidic system comprising, which method is based on using beads having a structure based on DNA origami, wherein said structure comprises a predetermined number of fluorophores. Furthermore, methods for determining the level of an antigen using microfluidics calibrated using fluorescent microbeads as provided herein. Also, methods are provided for determining the presence or state of a haematological disease by calibrated a microfluidics system.

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DNA origami beads for fluorescence quantification in microfluidics

Technical field

The present invention discloses beads comprising at least one structure based on DNA origami and fluorophores for use in microfluidics, such as flow cytometry.

Background

The commercial calibrators on the market today that are used to quantify numbers of fluorophores per particle(cell) from the flow cytometric fluorescence intensity are associated with several drawbacks: (a) The current calibrator beads are made of plastic and thus exhibit a high auto-fluorescence corresponding to about 200-2.000 fluorophores per bead. (b) The degree of fluorophore-labelling of commercial beads is associated with a large variation, (c) These calibrations are based on thousands of fluorophores per bead (including high auto-fluorescence) and thus the extrapolation into the lower range (50-2.000 fluorophores is associated with very high uncertainty).

DNA-origami-based standards for microscopy have been described in US 20140057805 A1.

Summary

The present invention provides beads comprising a structure based on DNA-origami, wherein said structure comprises a predetermined number of fluorophores for microfluidic applications, such as flow cytometry. The bead has several advantages over the prior art. One advantage is that the bead exhibits no or very little auto-fluorescence. Moreover, the number of fluorophores attached to the structure based on DNA-origami is known and can be precisely controlled so that the bead can be labelled with a distinct number of fluorophores down to as few as one fluorophore. Thus, calibration with the methods of the present disclosure allows quantification of antigens present in a sample at a very low level.

One advantage of the present invention lies in that the origami structure allows fluorescence quantification in microfluidics, where the signal is obtained differently compared to fluorescence microscopy. In microscopy, the object is stationary and thus the fluorophores attached to the object have more excitation exposure and emission time to generate the signal, while in microfluidics, such as flow cytometry, the beads

are moving rapidly past the detector, which substantially limits the exposure and detection time. The calibration of flow cytometry systems may require beads with specific number of fluorophores, up to 1500-3000 fluorophores.

One aspect of the present disclosure relates to a method of calibrating a microfluidics system comprising:

a. providing at least one bead having a structure based on DNA origami, wherein said structure comprises a predetermined number of fluorophores;
b. measuring the fluorescence of said at least one bead; and
c. calibrating the microfluidics system on the basis of the fluorescence measurement of b.

thereby obtaining a calibration curve of the microfluidics system

Calibration of a microfluidics systems, such as flow cytometry setups, using calibration beads as defined herein allows precise biochemical analysis of small biological particles e.g. extracellular vesicles, viruses and bacteria. Thus, the provided methods for calibrating microfluidics systems can advantageously be used in academic, industrial as well as for clinical analyses, including diagnostic methods based on microfluidics, such as flow cytometry.

Another aspect of the present disclosure relates to a method for determining the level of an antigen in a sample by a microfluidics system comprising

a. calibrating a microfluidics system according to the method of any one of the preceding claims, thereby obtaining a calibration curve;
b. providing a sample comprising at least one antigen, wherein said antigen is attached to a cell or to a vesicle or to a virus-like particle or a viral particle or to an extracellular vesicle;
c. contacting the sample with at least one ligand binding molecule coupled to a fluorescent label, wherein said ligand binds the at least one antigen;
d. measuring the fluorescence of said fluorescence label;
e. scoring the fluorescence of said fluorescence label based on the calibration curve, and
f. determining the level of said at least one antigen in the sample.

Another aspect of the present disclosure relates to a method for determining the presence or state of a haematological disease in an individual by a microfluidics system, such as a flow cytometry system, the method comprising

a. calibrating a microfluidics system according to the method disclosed herein, thereby obtaining a calibration curve;
b. providing a blood sample from said individual comprising at least one blood cell;
c. contacting the sample with at least one ligand binding molecule coupled to a fluorescent label, wherein said ligand is associated with the presence or state of said haematological disease;
d. measuring the fluorescence of said fluorescent label;
e. scoring the fluorescence of said fluorescent label based on the calibration curve; and
f. determining the level and/or concentration of said at least one antigen associated with the presence or state of said haematological disease.

Another aspect of the present disclosure relates to a kit for calibration of microfluidic instruments comprising a bead having a structure based on DNA origami wherein said structure comprises a predetermined number of fluorophores.

Description of drawings

Figure 1. Example of a possible design of flow cytometry calibration bead. A: Side view of a DNA-based monomer bead. B: Front view of monomer. C: Dimerization of a whole particle. D: Dimer bead. E: TEM characterization of dimer beads. TEM images are 200*200 nm. F: Positioning of fluorophores in a dimer bead. G: Flow cytometry data and analysis of dimer beads labelled with 0, 120 and 242 Cy5 molecules by Cytoflex flow cytometry system. H: Alternative positioning of the fluorophores on outer surface of the bead.

Figure 2. Proposition of a possible bead design based on 51kb scaffold. A. Front view of number 8 shaped bead B. Zoom in on fluorophore strand attachment to a complementary toehold protruding from the structure.

Detailed description

Definitions

The term “structure based on a DNA origami” as used herein refers to a DNA origami formed from a scaffold DNA strand and shorter DNA segments, also called staple strands, which form a predetermined structure of the scaffold DNA strand. The scaffold DNA strand is usually a long single-stranded DNA molecule. The length of the scaffold DNA strand depends on the system used for its production. Scaffold DNA strands of approximately 7.000 nucleotides may be produced using for example M13mp18 phage. Scaffold DNA strands of approximately 50.000 nucleotides may be produced using for example a λ/M13 hybrid virus as described in Marchi et al. (2014). The structure based on a DNA strand can comprise further components such as dyes, plasmonic structures, biological molecules such as proteins, enzymes, nanoparticles, and small molecules such as biotin. Alternatively, the structure based on a DNA origami can also be constructed solely from short DNA segments, as recently described by Ong et al. (2017). Constructing a structure based on a DNA origami solely from short DNA segments is also possible for large structures, such as structures in the gigadalton range.

The term “DNA” as used herein, is understood to mean not only strands of deoxyribonucleic acid, but also analogous structures, such as strands of ribonucleic acids (RNA), peptide nucleic acid (PNA), as well as hybrid structures.

The term “shorter DNA segments” as used herein refers to the nucleotide molecules which are also referred to as “staple strands” or “staple DNA strands” and which have a sequence complementary to a sequence of the scaffold DNA strand or another shorter DNA segment. Furthermore, said shorter DNA segments can be used to fold the long DNA strand into the predetermined structure. Alternatively, the shorter DNA segments can be those which hybridize with the DNA scaffold strand of the DNA origami in predetermined regions. The shorter DNA segments may comprise a sequence that does not hybridize to the DNA scaffold strand, called “sticky end” herein, which can be used for decorating the structure with fluorophores. The shorter DNA segments can also be used to bind/stick individual DNA origami structures together into large DNA origami oligomers in a controllable manner.

The term “calibration” is understood here to mean quantifying a measured variable on the basis of one or more reference samples or determining the properties of an apparatus, such as the sensitivity and resolution. For example, the methods disclosed herein may be used for calibration of a flow cytometer.

Structure based on DNA origami

In one aspect the present disclosure relates to a method of calibrating a microfluidics system, such as a flow cytometer, comprising providing at least one bead having a structure based on DNA origami, wherein said structure comprises a predetermined number of fluorophores.

The structure based on DNA origami usually comprises a single stranded DNA molecule, also called DNA scaffold strand, which is folded into a predetermined structure with the help of a plurality of staple strands. Each staple strand hybridizes to specific parts of sequence on the single stranded DNA molecule thereby determining how the single stranded DNA molecule folds.

Depending on the length of the DNA scaffold strand, a certain number of staple DNA strands will be required for folding the scaffold strand into the predesigned structure.

Thus, in one specific embodiment the structure comprises a predetermined number of fluorophores, a single stranded DNA molecule and staple DNA strands, wherein the number of staple DNA strands (Ns) is as in the formula below:

$$Ns = Lss/n,$$

wherein Lss is the length of the single stranded DNA molecule, and
wherein n is an integer number comprised between 26 and 34.

In another specific embodiment, the structure comprises a predetermined number of fluorophores, a single stranded DNA molecule and staple DNA strands, wherein the number of staple DNA strands (Ns) is as in the formula below:

$$Ns = Lss/n,$$

wherein Lss is the length of the single stranded DNA molecule, and
wherein n is an integer number comprised between 28 and 32.

However, the present invention is not limited to embodiments meeting the above equation. Indeed, it is possible to produce many different DNA origami structures, which do not follow this equation.

The length of the staple DNA strands may differ from one staple DNA to the other. The minimum length is a length that allows the staple DNA strand to be stable at room temperature.

In one embodiment, the length of the staple DNA strands is in the range between 12 and 60 nucleotides, preferably between 15 and 50 nucleotides.

Thus, in one embodiment the present disclosure relates to a method of calibrating a flow cytometer comprising providing at least one bead having a structure based on DNA origami, wherein said structure comprises a predetermined number of fluorophores, a single stranded DNA molecule and staple DNA strands.

The length of the single stranded DNA molecule is important for the size of the bead as well as for the number of fluorophores that the bead can accommodate.

Thus, the single stranded DNA molecule or DNA scaffold strand may have a length in a range between 1.500-nucleotides and 70.000-nucleotides, such as between 2.000-nucleotides and 68.000-nucleotides, such as between 5.000-nucleotides and 65.000-nucleotides, for example between 10.000-nucleotides and 60.000-nucleotides, such as between 15.000-nucleotides and 55.000-nucleotides, preferably between 20.000-nucleotides and 55.000-nucleotides, such as between 50.000 and 52.000-nucleotides. Thus, the single stranded DNA molecule or DNA scaffold strand may have a length of 51.466-nucleotides.

The single stranded DNA molecule may have a length in a range between 5.000-nucleotides and 70.000-nucleotides, for example between 7.000-nucleotides and 70.000-nucleotides, such as between 10.000-nucleotides and 70.000-nucleotides, preferably between 13.000-nucleotides and 70.000-nucleotides, such as between 15.000-nucleotides and 70.000-nucleotides, for example between 18.000-nucleotides and 70.000-nucleotides, such as between 20.000-nucleotides and 70.000-nucleotides, preferably between 23.000-nucleotides and 70.000-nucleotides, such as between 25.000-nucleotides and 70.000-nucleotides, for example between 28.000-nucleotides

and 70.000-nucleotides, such as between 30.000-nucleotides and 70.000-nucleotides, preferably between 33.000-nucleotides and 70.000-nucleotides, such as between 35.000-nucleotides and 70.000-nucleotides, for example between 38.000-nucleotides and 70.000-nucleotides, such as between 40.000-nucleotides and 70.000-nucleotides, preferably between 43.000-nucleotides and 70.000-nucleotides, such as between 45.000-nucleotides and 70.000-nucleotides, for example between 48.000-nucleotides and 70.000-nucleotides, such as between 50.000-nucleotides and 70.000-nucleotides, preferably between 53.000-nucleotides and 70.000-nucleotides, such as between 55.000-nucleotides and 70.000-nucleotides, for example between 58.000-nucleotides and 70.000-nucleotides, such as between 60.000-nucleotides and 70.000-nucleotides, preferably between 63.000-nucleotides and 70.000-nucleotides, such as between 65.000-nucleotides and 70.000-nucleotides, for example between 5.000-nucleotides and 65.000-nucleotides, such as between 5.000-nucleotides and 60.000-nucleotides, preferably between 5.000-nucleotides and 55.000-nucleotides, such as between 5.000-nucleotides and 50.000-nucleotides, for example between 5.000-nucleotides and 45.000-nucleotides, such as between 5.000-nucleotides and 40.000-nucleotides, preferably between 5.000-nucleotides and 35.000-nucleotides, such as between 5.000-nucleotides and 30.000-nucleotides, for example between 5.000-nucleotides and 25.000-nucleotides, such as between 5.000-nucleotides and 20.000-nucleotides, preferably between 5.000-nucleotides and 15.000-nucleotides, such as between 5.000-nucleotides and 10.000-nucleotides, for example between 5.000-nucleotides and 7.000-nucleotides.

The single stranded DNA molecule may have a length of at least 5.000-nucleotides, for example at least 7.000-nucleotides, preferably at least 10.000-nucleotides, such as at least 15.000-nucleotides, for example at least 20.000-nucleotides, preferably at least 25.000-nucleotides, such as at least 30.000-nucleotides, for example at least 35.000-nucleotides, preferably at least 40.000-nucleotides, such as at least 45.000-nucleotides, for example at least 50.000-nucleotides.

In one embodiment the single stranded DNA molecule may be produced using a phage and has a length in a range between 5.000-nucleotides and 10.000 nucleotides.

In one embodiment the single stranded DNA molecule may be produced using a hybrid virus and has a length in a range between 5.000-nucleotides and 55.000 nucleotides.

It may be beneficial to produce long single stranded DNA molecules, such as single stranded DNA molecules having a length of at least 30.000-nucleotides as these long molecules can carry a higher number of fluorophores compared to single stranded DNA molecules having a length in the range of between 5.000 and 10.000-nucleotides, such as at the most 30.000-nucleotides.

For example, a bead comprising a single stranded DNA molecule of 7.000 to 12.000-nucleotides may accommodate 1 to 200 fluorophores; a bead comprising a single stranded DNA molecule of 40.000 to 50.000-nucleotides may accommodate 1 to 1500 fluorophores.

Another way to increase the number of fluorophores comprised in a bead is the production of dimeric, such as trimeric structures through hydrophobic interactions and hydrogen bond interactions.

Thus, in one embodiment the bead comprises a dimeric structure based on DNA origami. In another embodiment, the bead comprises a trimeric structure based on DNA origami. However, it is also possible to produce larger oligomer structures such as tetrameric, pentameric, hexameric, heptameric and/or larger multiple oligomeric structures.

The structure based on DNA origami may have any shape and it may be flat or curved. A certain geometry may be preferred over other geometries. For example a geometry that hides the fluorophores inside the structure to prevent undesired oligomerization of structures through possible hydrophobic interactions between the fluorophores may be preferred. Thus, in one embodiment the structure based on DNA origami may be a sphere, an hexagon, a barrel, a tube or a combination of these structures.

In one embodiment the structure based on DNA origami may be a sphere.

In one embodiment the structure based on DNA origami may be a barrel or a tube.

In one embodiment the structure based on DNA origami may be an hexagon.

In one embodiment, the structure based on DNA origami may be a combination of the proposed geometrical structures.

In one embodiment, the structure based on DNA origami comprises a positioning domain ,a fluorescent domain and/or triggering domain

A positioning domain is a domain where a fluorophore is placed on the structure. It can be one of the staple strands that are fully complementary to the scaffold strand or it can have a single stranded overhang sequence that is not complementary to scaffold strand. The overhang is used to attach a fluorophore labelled oligo that is complementary to the overhang.

The fluorescent domain is the domain where the fluorophores are attached. Thus, the fluorescent domain comprises the predetermined number of fluorophores. In addition, each bead may comprise more than one different type of fluorophore, for example, each bead may comprise two or more different fluorophores or fluorescent domains, such as 2, 3, 4, 5, 6, 7, 8, 9 or 10 different fluorophores or fluorescent domains. In a preferred embodiment, the beads comprises 1-4 different fluorophores or fluorescent domain.

In another embodiment, the DNA based origami structure comprises a triggering domain.

The triggering domain is a domain where a different type of fluorophore or nanoparticle can be attached. The nanoparticle *e.g.* gold or silver nanoparticle can efficiently spread light. Thereby, these fluorophores or nanoparticles in the triggering domain can be used to trigger detection of a calibration bead.

An advantage of using beads comprising a structure based on DNA origami is that a precise and known number of fluorophores can be attached on the structure, and therefore accurate calibration of the measuring instrument, in this case of the flow cytometer, is possible.

In one embodiment the fluorescent domain comprises sticky ends and the fluorophores are conjugated to a DNA fragment complementary to said sticky ends, so that the fluorophores are attached to said sticky ends via said complementary DNA fragment.

A bead of the present disclosure comprises a predetermined number of fluorophores. Based on the length and the design of the structure based on DNA origami, the bead may comprise a certain maximum number of fluorophores.

In one embodiment the structure based on DNA origami comprises between 1 and 50 fluorophores, such as between 1 and 80 fluorophores, such as between 1 and 100 fluorophores, such as between 1 and 150 fluorophores, such as between 1 and 200 fluorophores, such as between 1 and 300 fluorophores, such as between 1 and 400 fluorophores, such as between 1 and 500 fluorophores, such as between 1 and 600 fluorophores, such as between 1 and 700 fluorophores, such as between 1 and 800 fluorophores, such as between 1 and 900 fluorophores, preferably at the most 1000 fluorophores, such as at the most 1200 fluorophores, such as at the most 1500 fluorophores, such as at the most 1800 fluorophores, such as at the most 2000 fluorophores, such as at the most 2200 fluorophores, such as at the most 2500 fluorophores, such as at the most 2700 fluorophores, such as at the most 3000 fluorophores.

In one embodiment the bead comprises a dimeric or trimeric structure based on DNA origami, and each monomer comprises between 1 and 1800 fluorophores.

In one embodiment the bead comprises a monomeric structure based on DNA origami and said structure comprises between 1 and 900 fluorophores, such as between 1 and 1500 fluorophores, preferably between 1 and 1800 fluorophores, such as between 1 and 2700 fluorophores, preferably at the most 3000 fluorophores.

In one embodiment the bead comprises a structure based on DNA origami, wherein said structure comprises a single stranded DNA molecule of at least 30.000-nucleotides and comprising between 1 and 1000 fluorophores.

In one embodiment the bead comprises a structure based on DNA origami, wherein said structure comprises a single stranded DNA molecule of at least 50.000-nucleotides and comprising between 1 and 1800 fluorophores.

Any fluorophore suitable for flow cytometry can be used. However, the preferred fluorophores are selected from small organic molecules.

In one embodiment, the small organic molecules are smaller than 5 nm.

In one embodiment, the fluorophore is selected from a group consisting of Quasar® 566 nm (Cy 3), Quasar® 670 nm (Cy 5), and fluorescein isothiocyanate (FITC).

In one embodiment the structure based on DNA origami comprises 120 fluorophores, for example 120 Cy5 fluorescence molecules.

In one embodiment the structure based on DNA origami comprises 242 fluorophores, for example 242 Cy5 fluorescence molecules.

Suitable fluorophores are known to the skilled person and include Alexa Fluor® 488, Alexa Fluor® 532, Alexa Fluor® 647, ATTO 488 and ATTO 532 and 5/6 carboxy-tetramethyl rhodamine (TMR), 6-carboxyfluorescein (6-FAM), Alexa Fluor® 350, DY-415, ATTO 425, ATTO 465, Bodipy® FL, fluorescein isothiocyanate, Oregon Green® 488, Oregon Green® 514, Rhodamine Green™, 5'-Tetrachloro-Fluorescein, ATTO 520, 6-carboxy-4',5'-dichloro-2',7'-dimethoxyfluoresceine, Yakima Yellow™ dyes, Bodipy® 530/550, hexachloro-fluorescein, Alexa Fluor® 555, DY-549, Bodipy® TMR-X, cyanine phosphoramidites (cyanine 3, cyanine 3.5, cyanine 5, cyanine 5.5), ATTO 550, Rhodamine Red™, ATTO 565, Carboxy-X-Rhodamine, Texas Red (Sulforhodamine 101 acid chloride), LightCycler® Red 610, ATTO 594, DY-480-XL, DY-610, ATTO 610, LightCycler® Red 640, Bodipy 630/650, ATTO 633, Bodipy 650/665, ATTO 647N, DY-649, LightCycler® Red 670, ATTO 680, LightCycler® Red 705, DY-682, ATTO 700, ATTO 740, DY-782, IRD 700 and IRD 800, CAL Fluor® Gold 540 nm, CAL Fluor® Gold 522 nm, CAL Fluor® Gold 544 nm , CAL Fluor® Orange 560 nm, CAL Fluor® Orange 538 nm, CAL Fluor® Orange 559 nm, CAL Fluor® Red 590 nm, CAL Fluor® Red 569 nm, CAL Fluor® Red 591 nm, CAL Fluor® Red 610 nm, CAL Fluor® Red 590 nm, CAL Fluor® Red 610 nm, CAL Fluor® Red 635 nm, Quasar® 570 nm, Quasar® 548 nm,

Quasar® 566 nm (Cy 3), Quasar® 670 nm, Quasar® 647 nm, Quasar® 670 nm (Cy 5), Quasar® 705 nm, Quasar® 690 nm, Quasar® 705 nm (Cy 5.5), Pulsar® 650 Dyes, SuperRox® Dyes.

In one embodiment the structure based on DNA origami comprises a DNA scaffold of a length between 50.000 and 52.000-nucleotides, for example of a length of 51.466-nucleotides; a number of staple strands between 1000 and 2000, for example between 1500 and 1600, preferably 1550; and between 50 and 1800 fluorophores.

In one embodiment the structure based on DNA origami comprises a DNA scaffold of a length between 50.000 and 52.000-nucleotides, for example of a length of 51.466-nucleotides; a number of staple strands between 1000 and 2000, for example between 1500 and 1600, preferably 1550; and between 50 and 900 fluorophores.

In one embodiment the structure based on DNA origami comprises a DNA scaffold of a length between 50.000 and 52.000-nucleotides, for example of a length of 51.466-nucleotides; a number of staple strands between 1000 and 2000, for example between 1500 and 1600, preferably 1550; and between 50 and 1800 fluorophores, for example 80 fluorophores, for example 160 fluorophores, such as 900 fluorophores, such as 1800 fluorophores.

In one embodiment the structure based on DNA origami comprises a DNA scaffold of a length between 50.000 and 52.000-nucleotides, for example of a length of 51.466-nucleotides; a number of staple strands between 1000 and 2000, for example between 1500 and 1600, preferably 1550; and 80 fluorophores, for example 80 Cy5 fluorescence molecules.

In one embodiment the structure based on DNA origami comprises a DNA scaffold of a length between 50.000 and 52.000-nucleotides, for example of a length of 51.466-nucleotides; a number of staple strands between 1000 and 2000, for example between 1500 and 1600, preferably 1550; and 160 fluorophores, for example 160 Cy5 fluorescence molecules.

Method of calibrating a microfluidics system

Microfluidic systems involves setups with very small volumes of fluids, and microfluidic devices utilises the principles of microfluidics and can thereby exploit the physical and chemical properties of liquids and gases at the microscale. Many operations can be executed at the same time thanks to their compact size and the shortened experiment time while they still offer an excellent data quality.

The current invention relates to methods for using DNA origami in calibration of a microfluidics system.

So, one aspect the present disclosure relates to a method of calibrating a microfluidic device comprising:

a. providing at least one bead having a structure based on DNA origami, wherein said structure comprises a predetermined number of fluorophores;
b. measuring the fluorescence of said at least one bead; and
c. calibrating the microfluidic device on the basis of the fluorescence measurement of b.
thereby obtaining a calibration curve of the microfluidic device.

Another aspect of the present disclosure relates to a method for determining the level of an antigen in a sample by a microfluidic technology comprising

a. calibrating a microfluidic device according to the method disclosed herein, thereby obtaining a calibration curve;
b. providing a sample comprising at least one antigen, wherein said antigen is attached to a cell or to a vesicle or to a virus-like particle or a viral particle or to an extracellular vesicle;
c. contacting the sample with at least one ligand binding molecule coupled to a fluorescent label, wherein said ligand binds the at least one antigen;
d. measuring the fluorescence of said fluorescence label;
e. scoring the fluorescence of said fluorescence label based on the calibration curve, and
f. determining the level of said at least one antigen in the sample.

In a preferred embodiment, the microfluidic system is a flow cytometer system, where structures based on DNA origami, wherein said structures comprise a predetermined

number of fluorophores, are used for calibration; cf. herein below. However, any specific embodiment described herein below in respect of flow cytometer systems can also be applied to microfluidic systems in general.

Method of calibrating a flow cytometer

In one aspect the present disclosure relates to a method of calibrating a flow cytometer comprising:

a. providing at least one bead having a structure based on DNA origami, wherein said structure comprises a predetermined number of fluorophores;
b. measuring the fluorescence of said at least one bead; and
c. calibrating the flow cytometer on the basis of the fluorescence measurement of b.

In one embodiment the method of calibrating a flow cytometer comprises providing a series of beads each comprising a different predetermined number of fluorophores. For example at least two beads may be provided, such as at least three beads, preferably at least four beads, even more preferably at least five beads may be provided, each bead comprising a different predetermined number of fluorophores.

For example, a series of 2 beads comprising 50 and 100 fluorophores, respectively, may be provided; preferably a series of 3 beads comprising 50, 100, 200 fluorophores, respectively, may be provided; preferably a series of 4 beads comprising 50, 100, 200 and 500 fluorophores, respectively, may be provided; preferably a series of 5 beads comprising 50, 100, 200, 500 and 1000 fluorophores, respectively, may be provided; for example a series of 6 beads comprising 50, 100, 200, 500, 1000 and 1500 fluorophores, respectively, may be provided.

Thus, the fluorescence of each bead will be measured according to step b. of the method above.

Although it is possible to calibrate a flow cytometer with the methods of the present disclosure by using only two beads having a structure based on DNA origami as disclosed herein, it is preferable to use more than two beads. In fact, the precision of the calibration will increase with the number of beads used.

When the method of calibrating a flow cytometer comprises providing a series of beads each comprising a different predetermined number of fluorophores, it may be beneficial that at least one bead comprises a number of fluorophores as close as possible to the sensitivity of the method.

For example, a first bead may comprise a number of fluorophores between 1 and 50; a second bead may comprise a number of fluorophores between 51 and 100; a third bead may comprise a number of fluorophores between 101 and 150; a fourth bead may comprise a number of fluorophores between 151 and 300; a fifth bead may comprise a number of fluorophores between 301 and 500. If a series of beads comprising a number of fluorophores as in this example are used, the data for a higher number of fluorophores may easily be extrapolated.

For example, a first bead may comprise a number of fluorophores between 1 and 50; a second bead may comprise a number of fluorophores between 51 and 100; a third bead may comprise a number of fluorophores between 101 and 300; a fourth bead may comprise a number of fluorophores between 301 and 750; a fifth bead may comprise a number of fluorophores between 751 and 1500; a sixth bead may comprise a number of fluorophores between 1501 and 2700.

In one embodiment the method of calibrating a flow cytometer comprises measuring the fluorescence of the at least two beads provided, such as of the at least three beads provided, preferably of the at least four beads provided, even more preferably of the at least five beads provided.

In one embodiment the method of calibrating a flow cytometer comprises calibrating the flow cytometer on the basis of the fluorescence measurements of the at least two beads provided, such as of the at least three beads provided, preferably of the at least four beads provided, even more preferably of the at least five beads provided.

The calibration of a flow cytometer according to the methods disclosed herein allows precise determination of the sensitivity of the instrument and generation of a calibration curve. The calibration curve allow us to correlate fluorescence intensity with the number of fluorescent molecules (fluorophores) on the entity of interest for a given fluorophore.

One advantage of the calibration method of the present disclosure is that, thanks to the use of beads comprising a structure based on DNA origami and a predetermined number of fluorophores, fluorescence can be determined with precision and a linear correlation between fluorescence and number of fluorophores is determined, allowing the establishment of a zero-point. The zero-point is a reference value, which functions as a measurement of the background fluorescence e.g. the auto-fluorescence of the utilized buffer.

Thanks to this zero-point, measurements performed with different flow cytometer systems can be compared. The zero-point, together with the calibration curve, also allows precise extrapolation of values based on the fluorescence signal.

Method for determining the level of an antigen

The calibration of a flow cytometer setup performed using calibration beads as described herein will allow precise biochemical analysis of small biological particles e.g. extracellular vesicles, viruses and bacteria. Such analyses can be applied in academic, industrial as well as for clinical settings

Extracellular vesicles includes exosomes and microvesicles and are secreted by almost all cells. Extracellular vesicles are generally difficult to characterize at single particle level because of their small size. Exosomes are the smallest entities with a size of 30-150 nm. These are formed by inward budding of the endosomal membrane during maturation of large multivesicular endosomes. Fusion of multivesicular endosomes with the plasma membrane results in the release of exosomes into the extracellular space. Microvesicles are larger with a size of 50-500 nm. These are released into the extracellular space by outward budding directly from the plasma membrane. Extracellular vesicle cargo is very heterogeneous and can include nucleic acids, proteins and lipids. These extracellular vesicles play an important role mediating intercellular communication. The surface proteins embedded in these exosomes can reveal their subcellular origin, producer and recipient cell type. Characterization of these vesicles at single particle level will help to understand the intercellular communication code but also allow diagnosing e.g. tumor development and progression.

One aspect of the present disclosure relates to a method for determining the level of an antigen in a sample by a microfluidic system, such as flow cytometry comprising

a. calibrating said microfluidic system, such as flow cytometer according to the method disclosed herein, thereby obtaining a calibration curve;
b. providing a sample comprising at least one antigen, wherein said antigen is attached to a cell or to a vesicle or to a virus-like particle or a viral particle or an exosome;
c. contacting the sample with at least one ligand binding molecule coupled to a fluorescent label, wherein said ligand binds the at least one antigen;
d. measuring the fluorescence of said fluorescence label;
e. scoring the fluorescence of said fluorescence label based on the calibration curve, and
f. determining the level of said at least one antigen in the sample.

In one embodiment, the method for determining the level of an antigen in a sample by flow cytometry as disclosed herein can detect as few as one single antigen in the sample provided.

In one embodiment, the sample comprises at least one antigen, such as at least 10 antigens, for example at least 25 antigens, preferably at least 50 antigens, wherein said antigen or said antigens are attached to one or more cells and/or vesicles and/or virus-like particles and/or viral particles and/or exosomes.

Method for diagnosis of an haematological disease

One aspect of the present disclosure relates to a method for determining the presence or state of a haematological disease in an individual by a microfluidic system, such as flow cytometry, the method comprising

a. calibrating said microfluidic system, such as flow cytometer according to the method disclosed herein, thereby obtaining a calibration curve;
b. providing a fluid sample from said individual comprising at least one blood cell;
c. contacting the sample with at least one ligand binding molecule coupled to a fluorescent label, wherein said ligand is associated with the presence or state of said haematological disease;
d. measuring the fluorescence of said fluorescent label;

e. scoring the fluorescence of said fluorescent label based on the calibration curve; and
f. determining the level and/or concentration of said at least one antigen associated with the presence or state of said haematological disease.

In one embodiment, the fluid is selected from a group consisting of blood, bone marrow, spinal fluid, bronchoalveolar lavage and serous effusions.

In one embodiment, the individual is human.

In one embodiment the method for diagnosis of a haematological disease by flow cytometry further comprises a step of comparing said level and/or concentration of said at least one antigen on the blood cell with a cut-off value,
wherein said cut-off value is determined from the concentration range of said at least one antigen in healthy individuals, such as individuals who do not present with a haematological disease, and
wherein a level and/or concentration that is lower or greater than the cut-off value indicates the presence or absence of said haematological disease.

The fluid sample can be a blood sample, and the blood sample can be full blood, but more preferably plasma, serum, cell-free or a sample comprising at least one blood cell or blood cell derived microvesicle.

For example the methods disclosed herein can be used for diagnosis of leukemia or lymphoma by performing an immunophenotyping of a fluid sample, such as by determining the level of certain markers expressed on cells of any of the above mentioned fluid samples.

For example the following leukemias and lymphomas may be diagnosed using the methods disclosed herein: acute myelogenous leukemia (or acute myeloid leukemia), acute lymphoblastic leukemia, chronic lymphocytic or myelocytic leukemias, B-cell and T-cell non-Hodgkin lymphomas, erythroleukemia (RBC leukemia), megaloblastic leukemia (platelets), and multiple myeloma.

In one embodiment, the level of any one of the following antigens may be determined: ZAP-70, HLA-DR, TdT, CD34, CD38, CD117, CD19, CD20, CD22, CD79a, immunoglobulin heavy (gamma, alpha, mu or delta) and light chains (kappa or lambda), CD10 (pre-B cell), CD2, CD3, CD5, CD7, CD4, CD8, MPO (myeloperoxidase), CD11, CD13, CD15, CD16b, CD33, CD66, CD11b, CD16, CD56, CD31, CD36, CD41, CD42, CD61, CD235a, CD14, CD33, CD64, CD68, CD11c, and CD103. The skilled person will know which antigens are expressed by the different types of blood cells and which levels are expected in an individual affected by a leukemia or lymphoma; see for example Morice et al. 2004; Jaffe 1987; Hanson et al. 1999; Langerak et al. 2001.

Kit for calibration of flow cytometry instruments

One aspect of the present disclosure relates to a kit for calibration of microfluidic instruments, such as flow cytometry instruments comprising at least one bead having a structure based on DNA origami wherein said structure comprises a predetermined number of fluorophores.

In one embodiment of the present disclosure, the at least one bead is as disclosed herein in the section “Structure based on DNA origami”.

In one embodiment of the present disclosure, the kit comprises at least two beads, for example at least three beads, such as at least 4 beads, preferably at least 5 beads, wherein each bead comprises a different predetermined number of fluorophores, as described in the section “Structure based on DNA origami”.

Examples

Example 1. Hexagonal double barrel.

Assembly of the calibration beads

Calibration beads are self-assembled into designed structure a circular single stranded scaffold strand (8064 bp) and 211 shorter single-stranded staple DNA strands. The scaffold strand is bought from Tilibit nanosystems and staple strands are produced chemically by Integrated DNA technologies. Staples strands bind to scaffold strand parts of the scaffold strand making it fold into a predesigned structure as described in (Rothemund 2006).

In the present case, the designed bead is assembled into a hexagonally shaped barrel (Figure 1A and B) that has a self-complementary side (Figure 1A) that governs assembly of a homodimer structure (Figure 1C) resulting in a longer hexagonal barrel with (Figure 1D and E). The structure has a size of approximately 42*45*70 nm. The size of transmission electron micrographs is 200*200 nm.

Some of the staple strands are labelled with fluorophores (Figure 1F). To avoid oligomerization the structure is designed with fluorophores residing inside the barrel thus fluorophores are shielded from the environment by the structure and preventing undesired oligomerization. Analysis of beads with 0, 120 and 242 Cy5 fluorophores using Cytoflex (Beckman Coulter) flow cytometry system (Figure 1G) shows that structures not labelled with any fluorophores do not exhibit any significant fluorescence compared with buffer alone. Addition of 120 and 242 Cy5 resulted in incremental increase in fluorescence intensity.

Example 2. Eight-shaped DNA origami structure

Assembly of the calibration beads

Calibration beads are self-assembled into designed structure from a circular single-stranded DNA scaffold strand (51.466 bp) and 1550 shorter single-stranded staple DNA strands. The scaffold strand is produced biologically as described in (Marchi, Saaem et al. 2014) while staple strands are produced chemically. Staple strands bind to complementary parts of the scaffold strand making it fold into a predesigned structure as described in (Rothemund 2006).

In the present case, the designed bead is assembled into two hexagonally shaped structures forming an 8 number (Figure 2A). The structure had a size of approximately 128*220*60 nm.

Alternative way of labelling structures: some of the staple strands contain additional DNA sequences without complementarity to the scaffold strand, so called sticky ends which serve as a docking sequence for fluorophore labelled DNA strands. Hence, fluorophore labelled strands have a complementary sequence to sticky ends and are labelled with a fluorophore (Figure 2B).

Fluorophores attachment

Fluorophore labelled strands are produced by chemically attaching a fluorophore to a DNA strand and subsequently purifying the conjugate using high pressure liquid

chromatography. The number of strands in the structure containing sticky ends determines the number of annealed fluorophore strands which equals the number of fluorophores on a bead and can range from 1 to 4000. Labelled fluorophore strands dock onto complementary sticky ends that point into the structure (Figure 2B). This way the structure screens the fluorophores from the environment and prevents undesired oligomerization of several beads through hydrophobic fluorophore interactions, which would lead to oligomers with non-defined number of fluorophores.
Alternatively, instead of sticky ends the strands in the structure can be conjugated directly to a fluorophore of interest.

Assembly of the structure.

Design and assembly of DNA origami calibration beads. CadNano software (http://cadnano.org/) was used for generation of staple strand sequences AutoCAD Maya (http://www.autodesk.com/) was used for evaluation of the 3D shape and designing shape complementary domain for dimerization into fully assembled bead. The self-assembly of DNA origami structures was performed by mixing Single-stranded Scaffold DNA (p8064, tilibit nanosystems) with 10x excess of staple strands in 100 µL TAE/Mg2+ buffer (40 mM Trisacetate, 1 mM EDTA, pH = 8.3, 12 mM Mg2+). Annealing of staple strands was done using a following ramp: 65°C for 5 min, followed by a temperature rapid decrease to 50°C and slow cooling for 200min for each 1°C from 50-43°C then rapid cooling to 20°C. In structures assembled with fluorophores, fluorophore strands were conjugated with a fluorophore in 5'end. Assembled structures were purified from excess of staple strands gel extraction. Purified structures were characterized by transmission electron microscopy and used directly for flow cytometry analysis.

Results

A Cytoflex (Beckman Coulter) cytometry system was used to test calibration beads assembled according to the description of example 1 above and labelled with none, 120 and 242 Cy5 fluorescence molecules. Cytoflex system showed a significant difference between the obtained fluorescence signal coming from unlabelled, labelled with 120 Cy5 fluorescence molecules and labelled with 242 Cy5 fluorescence molecules, respectively. Remarkably, the unlabeled calibration beads do not have any autofluorescence and the obtained fluorescence signal is equal to the signal of buffer. This allows to define a "0" point as a real point without any need for down extrapolation

of the dataset. This allows the making of a calibration curve for flow cytometry systems that enables a precise quantification of flow cytometry output signals of low abundant antigens.

Bigger beads compared to the one of Example 1 can be assembled either by using a longer scaffold strand as described in Example 2 or by organizing the beads in dimers or trimers through hydrophobic interactions. This allows each bead to accommodate a larger number of fluorophores, such as up to 1500, 1800, 2000, 2500, 2700 fluorophores, and so a more diverse pool of beads to choose from for the calibration.

The quality of the synthesized beads is evaluated by cryo-TEM and agarose gel.

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Tables

Table 1. An example of a DNA origami scaffold sequence (SEQ ID NO: 1)

GGCAATGACCTGATAGCCTTTGTAGATCTCTCAAAAATAGCTACCCTCTCCGGCATTAATTTATC
AGCTAGAACGGTTGAATATCATATTGATGGTGATTTGACTGTCTCCGGCCTTTCTCACCCTTTTG
AATCTTTACCTACACATTACTCAGGCATTGCATTTAAAATATATGAGGGTTCTAAAAATTTTTATCC
TTGCGTTGAAATAAAGGCTTCTCCCGCAAAAGTATTACAGGGTCATAATGTTTTTGGTACAACCG
ATTTAGCTTTATGCTCTGAGGCTTTATTGCTTAATTTTGCTAATTCTTTGCCTTGCCTGTATGATTT
ATTGGATGTTAATGCTACTACTATTAGTAGAATTGATGCCACCTTTTCAGCTCGCGCCCCAAATG
AAAATATAGCTAAACAGGTTATTGACCATTTGCGAAATGTATCTAATGGTCAAACTAAATCTACTC
GTTCGCAGAATTGGGAATCAACTGTTATATGGAATGAAACTTCCAGACACCGTACTTTAGTTGCA
TATTTAAAACATGTTGAGCTACAGCATTATATTCAGCAATTAAGCTCTAAGCCATCCGCAAAAATG
ACCTCTTATCAAAAGGAGCAATTAAAGGTACTCTCTAATCCTGACCTGTTGGAGTTTGCTTCCGG
TCTGGTTCGCTTTGAAGCTCGAATTAAAACGCGATATTTGAAGTCTTTCGGGCTTCCTCTTAATC
TTTTTGATGCAATCCGCTTTGCTTCTGACTATAATAGTCAGGGTAAAGACCTGATTTTTGATTTAT
GGTCATTCTCGTTTTCTGAACTGTTTAAAGCATTTGAGGGGGATTCAATGAATATTTATGACGATT
CCGCAGTATTGGACGCTATCCAGTCTAAACATTTTACTATTACCCCCTCTGGCAAAACTTCTTTT
GCAAAAGCCTCTCGCTATTTTGGTTTTTATCGTCGTCTGGTAAACGAGGGTTATGATAGTGTTGC
TCTTACTATGCCTCGTAATTCCTTTTGGCGTTATGTATCTGCATTAGTTGAATGTGGTATTCCTAA
ATCTCAACTGATGAATCTTTCTACCTGTAATAATGTTGTTCCGTTAGTTCGTTTTATTAACGTAGA
TTTTTCTTCCCAACGTCCTGACTGGTATAATGAGCCAGTTCTTAAAATCGCATAAGGTAATTCACA
ATGATTAAAGTTGAAATTAAACCATCTCAAGCCCAATTTACTACTCGTTCTGGTGTTTCTCGTCAG
GGCAAGCCTTATTCACTGAATGAGCAGCTTTGTTACGTTGATTTGGGTAATGAATATCCGGTTCT
TGTCAAGATTACTCTTGATGAAGGTCAGCCAGCCTATGCGCCTGGTCTGTACACCGTTCATCTG
TCCTCTTTCAAAGTTGGTCAGTTCGGTTCCCTTATGATTGACCGTCTGCGCCTCGTTCCGGCTAA
GTAACATGGAGCAGGTCGCGGATTTCGACACAATTTATCAGGCGATGATACAAATCTCCGTTGT
ACTTTGTTTCGCGCTTGGTATAATCGCTGGGGGTCAAAGATGAGTGTTTTAGTGTATTCTTTTGC
CTCTTTCGTTTTAGGTTGGTGCCTTCGTAGTGGCATTACGTATTTTACCCGTTTAATGGAAACTTC
CTCATGAAAAAGTCTTTAGTCCTCAAAGCCTCTGTAGCCGTTGCTACCCTCGTTCCGATGCTGTC
TTTCGCTGCTGAGGGTGACGATCCCGCAAAAGCGGCCTTTAACTCCCTGCAAGCCTCAGCGAC
CGAATATATCGGTTATGCGTGGGCGATGGTTGTTGTCATTGTCGGCGCAACTATCGGTATCAAG
CTGTTTAAGAAATTCACCTCGAAAGCAAGCTGATAAACCGATACAATTAAAGGCTCCTTTTGGAG
CCTTTTTTTTGGAGATTTTCAACGTGAAAAAATTATTATTCGCAATTCCTTTAGTTGTTCCTTTCTA
TTCTCACTCCGCTGAAACTGTTGAAAGTTGTTTAGCAAAATCCCATACAGAAAATTCATTTACTAA
CGTCTGGAAAGACGACAAAACTTTAGATCGTTACGCTAACTATGAGGGCTGTCTGTGGAATGCT
ACAGGCGTTGTAGTTTGTACTGGTGACGAAACTCAGTGTTACGGTACATGGGTTCCTATTGGGC
TTGCTATCCCTGAAAATGAGGGTGGTGGCTCTGAGGGTGGCGGTTCTGAGGGTGGCGGTTCTG
AGGGTGGCGGTACTAAACCTCCTGAGTACGGTGATACACCTATTCCGGGCTATACTTATATCAA
CCCTCTCGACGGCACTTATCCGCCTGGTACTGAGCAAAACCCCGCTAATCCTAATCCTTCTCTT
GAGGAGTCTCAGCCTCTTAATACTTTCATGTTTCAGAATAATAGGTTCCGAAATAGGCAGGGGG
CATTAACTGTTTATACGGGCACTGTTACTCAAGGCACTGACCCCGTTAAAACTTATTACCAGTAC
ACTCCTGTATCATCAAAAGCCATGTATGACGCTTACTGGAACGGTAAATTCAGAGACTGCGCTTT
CCATTCTGGCTTTAATGAGGATTTATTTGTTTGTGAATATCAAGGCCAATCGTCTGACCTGCCTC
AACCTCCTGTCAATGCTGGCGGCGGCTCTGGTGGTGGTTCTGGTGGCGGCTCTGAGGGTGGT
GGCTCTGAGGGTGGCGGTTCTGAGGGTGGCGGCTCTGAGGGAGGCGGTTCCGGTGGTGGCT
CTGGTTCCGGTGATTTTGATTATGAAAAGATGGCAAACGCTAATAAGGGGGCTATGACCGAAAA
TGCCGATGAAAACGCGCTACAGTCTGACGCTAAAGGCAAACTTGATTCTGTCGCTACTGATTAC
GGTGCTGCTATCGATGGTTTCATTGGTGACGTTTCCGGCCTTGCTAATGGTAATGGTGCTACTG
GTGATTTTGCTGGCTCTAATTCCCAAATGGCTCAAGTCGGTGACGGTGATAATTCACCTTTAATG
AATAATTTCCGTCAATATTTACCTTCCCTCCCTCAATCGGTTGAATGTCGCCCTTTTGTCTTTGGC
GCTGGTAAACCATATGAATTTTCTATTGATTGTGACAAAATAAACTTATTCCGTGGTGTCTTTGCG
TTTCTTTTATATGTTGCCACCTTTATGTATGTATTTTCTACGTTTGCTAACATACTGCGTAATAAGG
AGTCTTAATCATGCCAGTTCTTTTGGGTATTCCGTTATTATTGCGTTTCCTCGGTTTCCTTCTGGT
AACTTTGTTCGGCTATCTGCTTACTTTTCTTAAAAAGGGCTTCGGTAAGATAGCTATTGCTATTTC
ATTGTTTCTTGCTCTTATTATTGGGCTTAACTCAATTCTTGTGGGTTATCTCTCTGATATTAGCGC
TCAATTACCCTCTGACTTTGTTCAGGGTGTTCAGTTAATTCTCCCGTCTAATGCGCTTCCCTGTTT
TTATGTTATTCTCTCTGTAAAGGCTGCTATTTTCATTTTTGACGTTAAACAAAAAATCGTTTCTTAT
TTGGATTGGGATAAATAATATGGCTGTTTATTTTGTAACTGGCAAATTAGGCTCTGGAAAGACGC
TCGTTAGCGTTGGTAAGATTCAGGATAAAATTGTAGCTGGGTGCAAAATAGCAACTAATCTTGAT

TTAAGGCTTCAAAACCTCCCGCAAGTCGGGAGGTTCGCTAAAACGCCTCGCGTTCTTAGAATAC
CGGATAAGCCTTCTATATCTGATTTGCTTGCTATTGGGCGCGGTAATGATTCCTACGATGAAAAT
AAAAACGGCTTGCTTGTTCTCGATGAGTGCGGTACTTGGTTTAATACCCGTTCTTGGAATGATAA
GGAAAGACAGCCGATTATTGATTGGTTTCTACATGCTCGTAAATTAGGATGGGATATTATTTTTCT
TGTTCAGGACTTATCTATTGTTGATAAACAGGCGCGTTCTGCATTAGCTGAACATGTTGTTTATT
GTCGTCGTCTGGACAGAATTACTTTACCTTTTGTCGGTACTTTATATTCTCTTATTACTGGCTCGA
AAATGCCTCTGCCTAAATTACATGTTGGCGTTGTTAAATATGGCGATTCTCAATTAAGCCCTACT
GTTGAGCGTTGGCTTTATACTGGTAAGAATTTGTATAACGCATATGATACTAAACAGGCTTTTTCT
AGTAATTATGATTCCGGTGTTTATTCTTATTTAACGCCTTATTTATCACACGGTCGGTATTTCAAA
CCATTAAATTTAGGTCAGAAGATGAAATTAACTAAAATATATTTGAAAAAGTTTTCTCGCGTTCTTT
GTCTTGCGATTGGATTTGCATCAGCATTTACATATAGTTATATAACCCAACCTAAGCCGGAGGTT
AAAAAGGTAGTCTCTCAGACCTATGATTTTGATAAATTCACTATTGACTCTTCTCAGCGTCTTAAT
CTAAGCTATCGCTATGTTTTCAAGGATTCTAAGGGAAAATTAATTAATAGCGACGATTTACAGAA
GCAAGGTTATTCACTCACATATATTGATTTATGTACTGTTTCCATTAAAAAAGGTAATTCAAATGA
AATTGTTAAATGTAATTAATTTTGTTTTCTTGATGTTTGTTTCATCATCTTCTTTTGCTCAGGTAATT
GAAATGAATAATTCGCCTCTGCGCGATTTTGTAACTTGGTATTCAAAGCAATCAGGCGAATCCGT
TATTGTTTCTCCCGATGTAAAAGGTACTGTTACTGTATATTCATCTGACGTTAAACCTGAAAATCT
ACGCAATTTCTTTATTTCTGTTTTACGTGCAAATAATTTTGATATGGTAGGTTCTAACCCTTCCATT
ATTCAGAAGTATAATCCAAACAATCAGGATTATATTGATGAATTGCCATCATCTGATAATCAGGAA
TATGATGATAATTCCGCTCCTTCTGGTGGTTTCTTTGTTCCGCAAAATGATAATGTTACTCAAACT
TTTAAAATTAATAACGTTCGGGCAAAGGATTTAATACGAGTTGTCGAATTGTTTGTAAAGTCTAAT
ACTTCTAAATCCTCAAATGTATTATCTATTGACGGCTCTAATCTATTAGTTGTTAGTGCTCCTAAA
GATATTTTAGATAACCTTCCTCAATTCCTTTCAACTGTTGATTTGCCAACTGACCAGATATTGATT
GAGGGTTTGATATTTGAGGTTCAGCAAGGTGATGCTTTAGATTTTTCATTTGCTGCTGGCTCTCA
GCGTGGCACTGTTGCAGGCGGTGTTAATACTGACCGCCTCACCTCTGTTTTATCTTCTGCTGGT
GGTTCGTTCGGTATTTTTAATGGCGATGTTTTAGGGCTATCAGTTCGCGCATTAAAGACTAATAG
CCATTCAAAAATATTGTCTGTGCCACGTATTCTTACGCTTTCAGGTCAGAAGGGTTCTATCTCTG
TTGGCCAGAATGTCCCTTTTATTACTGGTCGTGTGACTGGTGAATCTGCCAATGTAAATAATCCA
TTTCAGACGATTGAGCGTCAAAATGTAGGTATTTCCATGAGCGTTTTTCCTGTTGCAATGGCTGG
CGGTAATATTGTTCTGGATATTACCAGCAAGGCCGATAGTTTGAGTTCTTCTACTCAGGCAAGTG
ATGTTATTACTAATCAAAGAAGTATTGCTACAACGGTTAATTTGCGTGATGGACAGACTCTTTTAC
TCGGTGGCCTCACTGATTATAAAAACACTTCTCAGGATTCTGGCGTACCGTTCCTGTCTAAAATC
CCTTTAATCGGCCTCCTGTTTAGCTCCCGCTCTGATTCTAACGAGGAAAGCACGTTATACGTGCT
CGTCAAAGCAACCATAGTACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTA
CGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTT
CCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTT
CCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTTGGGTGATGGTTCACGTAGT
GGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTG
GACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGGCTATTCTTTTGATTTATAAGGG
ATTTTGCCGATTTCGGAACCACCATCAAACAGGATTTTCGCCTGCTGGGGCAAACCAGCGTGGA
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TTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAA
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CGAGCTCGGTACCCGGGGATCCTCAACTGTGAGGAGGCTCACGGACGCGAAGAACAGGCACG
CGTGCTGGCAGAAACCCCCGGTATGACCGTGAAAACGGCCCGCCGCATTCTGGCCGCAGCAC
CACAGAGTGCACAGGCGCGCAGTGACACTGCGCTGGATCGTCTGATGCAGGGGGCACCGGCA
CCGCTGGCTGCAGGTAACCCGGCATCTGATGCCGTTAACGATTTGCTGAACACACCAGTGTAA
GGGATGTTTATGACGAGCAAAGAAACCTTTACCCATTACCAGCCGCAGGGCAACAGTGACCCG
GCTCATACCGCAACCGCGCCCGGCGGATTGAGTGCGAAAGCGCCTGCAATGACCCCGCTGAT
GCTGGACACCTCCAGCCGTAAGCTGGTTGCGTGGGATGGCACCACCGACGGTGCTGCCGTTG
GCATTCTTGCGGTTGCTGCTGACCAGACCAGCACCACGCTGACGTTCTACAAGTCCGGCACGT
TCCGTTATGAGGATGTGCTCTGGCCGGAGGCTGCCAGCGACGAGACGAAAAAACGGACCGCG
TTTGCCGGAACGGCAATCAGCATCGTTTAACTTTACCCTTCATCACTAAAGGCCGCCTGTGCGG
CTTTTTTTACGGGATTTTTTTATGTCGATGTACACAACCGCCCAACTGCTGGCGGCAAATGAGCA
GAAATTTAAGTTTGATCCGCTGTTTCTGCGTCTCTTTTTCCGTGAGAGCTATCCCTTCACCACGG
AGAAAGTCTATCTCTCACAAATTCCGGGACTGGTAAACATGGCGCTGTACGTTTCGCCGATTGT
TTCCGGTGAGGTTATCCGTTCCCGTGGCGGCTCCACCTCTGAAAGCTTGGCACTGGCCGTCGT
TTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCC
CCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGC

AGCCTGAATGGCGAATGGCGCTTTGCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTG
GCTGGAGTGCGATCTTCCTGAGGCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCACGG
TTACGATGCGCCCATCTACACCAACGTGACCTATCCCATTACGGTCAATCCGCCGTTTGTTCCC
ACGGAGAATCCGACGGGTTGTTACTCGCTCACATTTAATGTTGATGAAAGCTGGCTACAGGAAG
GCCAGACGCGAATTATTTTTGATGGCGTTCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATT
TAATGCGAATTTTAACAAAATATTAACGTTTACAATTTAAATATTTGCTTATACAATCTTCCTGTTTT
TGGGGCTTTTCTGATTATCAACCGGGGTACATATGATTGACATGCTAGTTTTACGATTACCGTTC
ATCGATTCTCTTGTTTGCTCCAGACTCTCA

Table 2. Examples of staple strand sequences suitable for hexagonal barrel structure

Flurophore strand sequence	SEG ID NO
ATACAGGAATGCCTGAGTAATATCAATA	2
CTCAGAGGCAAGGATAAAAATGGAGAGG	3
TTCCCAAGAGTAGATTTAGTTCTAAATC	4
CTGAATAAAAAGGTGGCATCATAGTAGT	5
TAAAGTACAATAACCTGTTTAAAGAATT	6
AAATCAGATTGCTCCTTTTGAGTAGCTC	7
GGATTGCCGGAAGCAAACTCCTGGAAGT	8
GGCGAAGTTTTGCCAGAAGATTAA	9
GTGTCCCCCTCAAATGCTCAGAAA	10
ACCATAGCGTCCAATACCCCTGAC	11
GGTTGTAGGGAGAAGCCTTTAGAGATCT	12
AGCATTATAAAGATTCAAAAGGCCGGAG	13
AGCAAAAACCCTCATATATTTTCTAGCT	14
AACATGTTTTCATTTGGGGCGATAAATC	15
TTCATTCTAGATACATTTCGCATAAAGC	16
GAGGAAGGAGCTTCAAAGCGACAGTTGA	17
ACGAGAATCATTTTTGCGGATTTAATTG	18
TATTATACAGGATTAGAGAGTATGCAAC	19
ATAATAGTAAAATGTTTGCAAAGC	20
CCCTGACGGTTCGTCATAAATATTAAATCAA	21
ACATAACTTGGGAAGGCAAAAAGGAAGTTTCCATTGCATAAC	22
GCGATTATACGCAACGGCTACAGAAAGGCCG	23
CAACCATATTTCTTAAACAGCGTTAGTA	24
CAGCAGCTTTTTTCACGTTGAAGGAATT	25
CTTGCAGAGCCTTTAATTGTATTCAACA	26
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Claims

1. A method of calibrating a microfluidic system comprising:
   a. providing at least one bead having a structure based on DNA origami, wherein said structure comprises a predetermined number of fluorophores;
   b. measuring the fluorescence of said at least one bead; and
   c. calibrating the microfluidic system on the basis of the fluorescence measurement of b.
   thereby obtaining a calibration curve of the microfluidic system.

2. The method according to claim 1, wherein said microfluidic system is a flow cytometer

3. The method according to any one of the preceding claims, wherein the structure comprises a predetermined number of fluorophores, a single stranded DNA molecule and staple DNA strands, wherein the number of staple DNA strands (Ns) is as in formula I:
   Ns = Lss/n,
   wherein Lss is the length of the single stranded DNA molecule, and
   wherein n is an integer number comprised between 26 and 34.

4. The method according to any one of the preceding claims, wherein the single stranded DNA molecule has a length in a range between 1.500-nucleotides and 70.000-nucleotides, preferably between 2000- nucleotides and 68.000-nucleotides, such as between 5.000 and 65.000-nucleotides, for example between 10.000-nucleotides and 60.000-nucleotides, such as between 15.000-nucleotides and 55.000-nucleotides, preferably between 20.000-nucleotides and 55.000-nucleotides.

5. The method according to any one of the preceding claims, wherein the single stranded DNA molecule has a length of at least 2.000-nucleotides, such as at least 5.000- nucleotides, such as at least 7.000-nucleotides, such as at least 10.000-nucleotides, such as at least 15.000-nucleotides, such as at least 20.000-nucleotides, such as at least 25.000-nucleotides, such as at least 30.000-nucleotides, such as at least 35.000-nucleotides, such as at least

40.000-nucleotides, such as at least 45.000-nucleotides, such as at least 50.000-nucleotides.

6. The method according to any one of the preceding claims, wherein each staple DNA strand has a length in a range between 12 and 60 nucleotides.

7. The method according to any one of the preceding claims, wherein the structure comprises:
    a. a positioning domain,
    b. a fluorescent domain and/or
    c. . a triggering domain;

8. The method according to any one of the preceding claims, wherein the fluorescent domain comprises the predetermined number of fluorophores.

9. The method according to any one of the preceding claims, wherein the fluorophores are attached to the staple DNA strands.

10. The method according to any one of the preceding claims, wherein at least a part of the staple DNA strands comprises sticky ends, wherein the fluorophores are conjugated to a DNA fragment complementary to said sticky ends, and wherein the fluorophores are attached to said sticky ends via said complementary DNA fragment.

11. The method according to any one of the preceding claims, wherein the bead comprises a dimeric structure based on DNA origami.

12. The method according to any one of the preceding claims, wherein the bead comprises a trimeric structure based on DNA origami.

13. The method according to any one of the preceding claims, wherein the structure is a sphere, a barrel, a tube, a hexagon, or a combination thereof.

14. The method according to any one of the preceding claims, wherein the structure comprises at least 1 fluorophore, such as at least 50 fluorophores, such as at

least 100 fluorophores, such as at least 200 fluorophores, such as at least 300 fluorophores, such as at least 400 fluorophores, such as at least 900 fluorophores, such as at least 1200 fluorophores, such as at least 1500 fluorophores, such as at least 1800 fluorophores, such as at least 2000 fluorophores, such as at least 2700 fluorophores.

15. The method according to any one of the preceding claims, wherein each bead comprises two or more different types of fluorophores or fluorescent domains.

16. The method according to any one of the preceding claims, wherein the fluorophore is selected from a group consisting of Quasar® 566 nm (Cy 3), Quasar® 670 nm (Cy 5), and fluorescein isothiocyanate (FITC).

17. The method according to any one of the preceding claims, wherein at least two beads, preferably at least three beads, are provided, and wherein each bead comprises a different predetermined number of fluorophores.

18. The method according to any one of the preceding claims, comprising measuring the fluorescence of the at least two, preferably at least three beads.

19. The method according to any one of the preceding claims, comprising calibrating the microfluidics system such as a flow cytometer on the basis of the fluorescence measurements of the at least two, preferably at least three beads.

20. A method for determining the level of an antigen in a sample by a microfluidics system comprising

a. calibrating a microfluidics system according to the method of any one of the preceding claims, thereby obtaining a calibration curve;
b. providing a sample comprising at least one antigen, wherein said antigen is attached to a cell or to a vesicle or to a virus-like particle or a viral particle or an extracellular vesicle;
c. contacting the sample with at least one ligand binding molecule coupled to a fluorescent label, wherein said ligand binds the at least one antigen;
d. measuring the fluorescence of said fluorescence label;

e. scoring the fluorescence of said fluorescence label based on the calibration curve, and
f. determining the level of said at least one antigen in the sample.

21. The method according to claim 20, wherein the level of an antigen is determined by flow cytometry.

22. The method according to any one of claims 20 and 21, wherein the sample comprises at least one antigen, such as at least 10 antigens, for example at least 25 antigens, preferably at least 50 antigens, wherein said antigen or said antigens are attached to one or more cells and/or vesicles and/or virus-like particles and/or viral particles and/or exosomes.

23. A method for determining the presence or state of a haematological disease in an individual by a microfluidics system, the method comprising
a. calibrating a microfluidics system according to the method disclosed herein, thereby obtaining a calibration curve;
b. providing a fluid sample from said individual comprising at least one blood cell;
c. contacting the sample with at least one ligand binding molecule coupled to a fluorescent label, wherein said ligand is associated with the presence or state of said haematological disease;
d. measuring the fluorescence of said fluorescent label;
e. scoring the fluorescence of said fluorescent label based on the calibration curve; and
f. determining the level and/or concentration of said at least one antigen associated with the presence or state of said haematological disease.

24. The method of claim 23, wherein the fluid sample is selected from a group consisting of blood, bone marrow, spinal fluid, bronchoalveolar lavage and serous effusions.

25. The method of any one of claims 23 and 24, comprising the further step of comparing said level and/or concentration of said at least one antigen with a cut-off value,

wherein said cut-off value is determined from the concentration range of said at least one antigen in healthy individuals, such as individuals who do not present with a haematological disease, and

wherein a level and/or concentration that is lower or greater than the cut-off value indicates the presence or state of said haematological disease.

26. A kit for calibration of microfluidic instruments comprising a bead having a structure based on DNA origami wherein said structure comprises a predetermined number of fluorophores.

27. The kit of claim 26, wherein said bead is as defined in anyone of claims 2 to 14.

28. The kit according to any one of claims 26 and 27, wherein the kit comprises at least two beads, for example at least three beads, such as at least 4 beads, preferably at least 5 beads, wherein each bead comprises a different predetermined number of fluorophores.

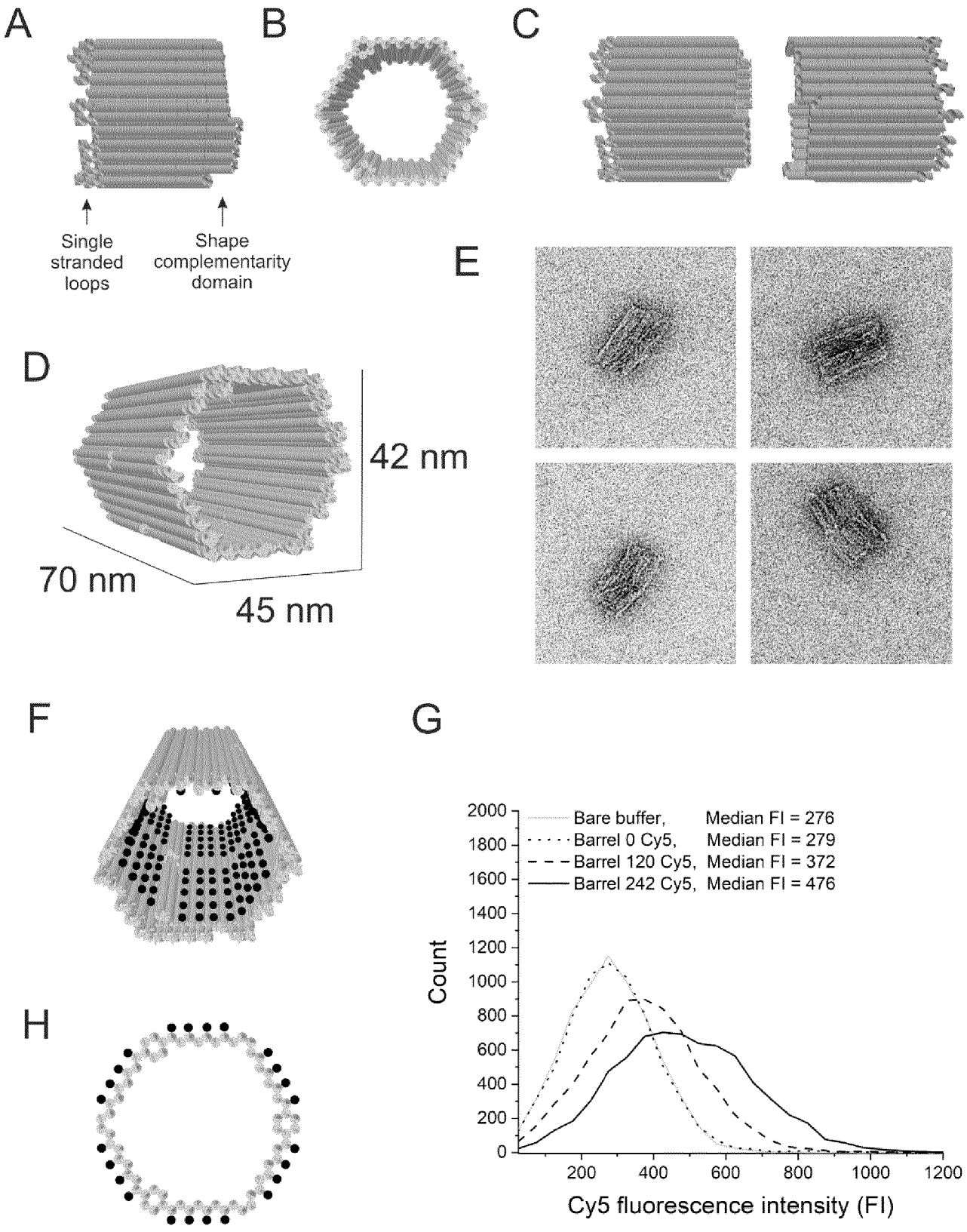
A
B
C
Single stranded loops
Shape complementarity domain
D
42 nm
70 nm
45 nm
E
F
G
Bare buffer, Median FI = 276
Barrel 0 Cy5, Median FI = 279
Barrel 120 Cy5, Median FI = 372
Barrel 242 Cy5, Median FI = 476
Count
Cy5 fluorescence intensity (FI)
H

Figure 1

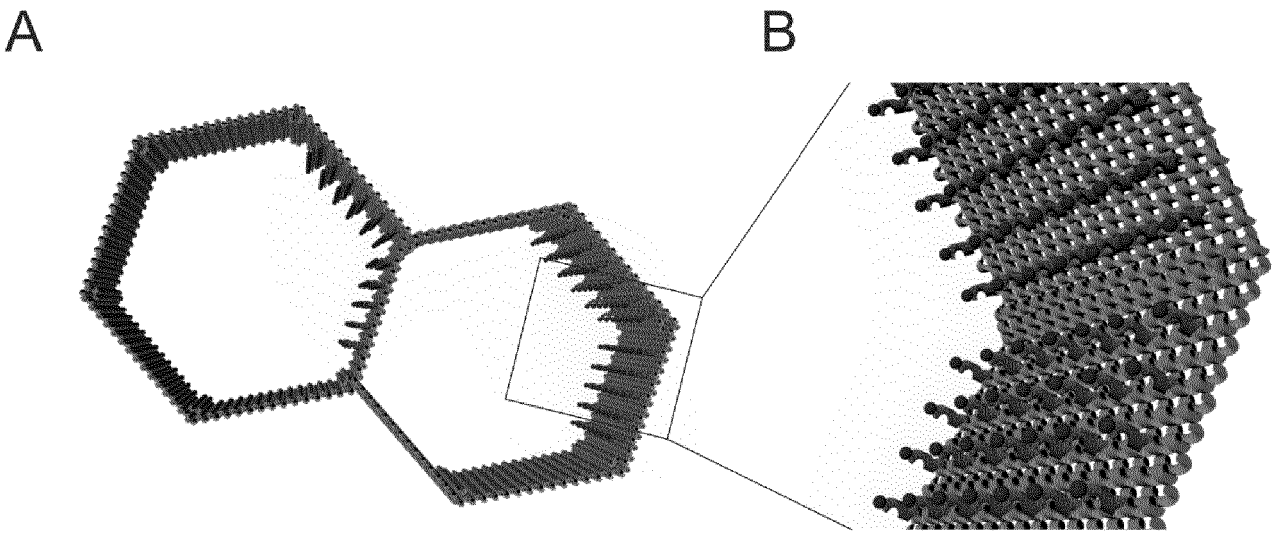

Figure 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/052640

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/53 G01F25/00 G01N33/543 G01N33/58
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
G01N G01F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LIN WANG ET AL: "Fluorescence calibration research of microspheres in a microfluidic chip", DESIGN FOR MANUFACTURABILITY THROUGH DESIGN-PROCESS INTEGRATION III, vol. 5718, 22 January 2005 (2005-01-22), page 272, XP55575505, 1000 20th St. Bellingham WA 98225-6705 USA ISSN: 0277-786X, DOI: 10.1117/12.591610 ISBN: 978-1-5106-2781-9 the whole document In particular: Title; Abstract; Experimental section; Conclusion. ----- -/--	1-25

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 March 2019	04/04/2019

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARIO RAAB ET AL: "Using DNA origami nanorulers as traceable distance measurement standards and nanoscopic benchmark structures", SCIENTIFIC REPORTS, vol. 8, no. 1, 29 January 2018 (2018-01-29), XP55575522, DOI: 10.1038/s41598-018-19905-x	26-28
Y	the whole document In particular: Title; Abstract; Figures 1-4; Methods.	1-25

X	VIRPI KORPELAINEN ET AL: "DNA origami structures as calibration standards for nanometrology", MEASUREMENT SCIENCE AND TECHNOLOGY, IOP, BRISTOL, GB, vol. 28, no. 3, 23 January 2017 (2017-01-23), page 34001, XP020313856, ISSN: 0957-0233, DOI: 10.1088/1361-6501/28/3/034001 [retrieved on 2017-01-23]	26-28
Y	the whole document In particular: Title; Abstract; Samples; Figures 1-6.	1-25

X	US 2014/057805 A1 (TINNEFELD PHILIP [DE] ET AL) 27 February 2014 (2014-02-27) cited in the application	26-28
Y	the whole document In particular: Figures 1-3b; Paragraphs 1 and 58-63; Claims 1-21.	1-25

X	INGO H. STEIN ET AL: "Single-Molecule FRET Ruler Based on Rigid DNA Origami Blocks", CHEMPHYSCHEM - A EUROPEAN JOURNAL OF CHEMICAL PHYSICS & PHYSICALCHEMISTRY., vol. 12, no. 3, 25 February 2011 (2011-02-25), pages 689-695, XP55574486, DE ISSN: 1439-4235, DOI: 10.1002/cphc.201000781 the whole document In particular: Title; Abstract; Figures 1-3; Experimental section.	26-28

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2019/052640

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2014057805 A1	27-02-2014	DE 102012107719 A1	27-02-2014
		US 2014057805 A1	27-02-2014