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DNA origami nanorobot fiber optic genosensor to TMV

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There is always a need to find non-PCR DNA hybridization methods enabling rapid and sensitive detection of target analytes. We present herein the combination of a molecular self-assembly scaffolded DNA origami technique with optical fiber transducer which enables to create a novel hybrid system, in which 3D DNA origami nanorobots are immobilized on glass optical fiber tips. Purified DNA origami can be successfully deposited on glass substrates using aminopropyltriethoxysilane and capture probes. Immobilized DNA nanorobots with a switchable flap can then be actuated by a sample containing specific target DNA. Thus in the presence of target DNA, DNA nanorobots expose a hemin/G-quadruplex DNAzyme which then catalyzes the generation of chemiluminescence once the specific fiber probes are immersed in a luminol-based solution. Integrating organic nanorobots to inorganic fiber optics creates a hybrid system that has potential applications in a wide range of fields, including diagnostics and cellular *in vivo* biosensing when using ultrathin fiber optic probes.

Introduction

A chemiluminescent based DNA origami-optical fiber genosensor has been developed for the detection of a model TMV target DNA sequence. Biosensor devices couple an immobilized biospecific recognition element entity to the surface of a transducer, which 'transduces' a molecular recognition event into a measurable signal, pinpointing the presence of the target measurand. Optical fiber sensors are ideal transducers governed by the Snell's law, having several advantages that make them attractive options for use in sensing applications^[1]. In the present study, we couple a switchable DNA origami intimately to the surface of a glass optical fiber, thereby obtaining a chemiluminescence signal collection with a portable transducer once a target is detected.

DNA origami technology enables increased control in self-assembly of 2D^[2,3,4,5] and 3D nanoscale complex structures^[6,7,8,9,10,11] of purpose designed shaped nanomaterials exhibiting high precision and specificity^[12]. The method uses hundreds of short oligonucleotide 'staple' strands to direct the folding of a long single 'scaffold' strand of DNA into a pre-programmed arrangement^[2].

To date, self-assembled DNA nanostructures have demonstrated a great potential in a wide range of innovative applications due to programmable control of shape and size, precise spatial addressability, easy and high-yield preparation, and biocompatibility^[13]. Previous applications with DNA origami were found in photonics, therapeutics, nanofabrication and nanomechanics^[14,15,16,17,18]. Many of these applications are not only dependent on structural design, but also on the adhesion of the DNA origami nanostructures to surfaces^[19]. The immobilization of DNA nanostructures on a glass surface is a key step for the integration of DNA nanorobot on optical fiber tips for sensing applications.

Little work exists on deposition methods of 3D purpose DNA that involve immobilization on glass surfaces in order to obtain a platform for biomolecular immobilization^[20], nanoantennas^[21], nanopores^[22,23] and wafer pattern^[24]. DNA nanostructures can be selectively guided and anchored on glass capillaries by trapping them when a positive voltage is applied to a reservoir^[22,23]. Recently, it has been shown that DNA origami can be uniformly deposited by a spray-coating technique without using pre-treatment of the substrate^[24]. Nevertheless, the aforementioned methods can anchor the nanostructure on glass in a way that could inhibit the actuation mechanism of immobilized 3D DNA nanorobots, thereby changing its conformation from a closed state to an open one. Herein we utilized a DNA origami as a functional smart molecular device without losing its switchable feature despite solid-phase immobilization. In order to allow the immobilization of DNA nanorobots, we extended a DNA origami staple strand thus introducing an anchor sequence that allows its hybridization with the complementary capture sequence previously deposited on the silanized glass.

After DNA origami immobilization on optical fiber tips, we demonstrate its rationale use in detecting a model target sequence. A specific hybridization event target-probe induces the flap opening of the immobilized DNA nanorobot resulting in the peroxidase activity of a DNAzyme to oxidize a luminol/H₂O₂ solution^[11] producing a signal light that couples into a photomultiplier tube. We are the first, to the best of our knowledge to conjugate DNA origami to an optical fiber transducer thereby producing a DNA origami genosensor. This new

system could find use in many applications, such as diagnostics or water monitoring for pathogens.

Results and Discussion

Rationale

The aim of this work was to build an organic-inorganic hybrid system in which glass optical fiber tips has been functionalized with DNA nanorobot origami. Recently, a cylindrical nanorobot with a switchable flap had been designed, produced, characterized and tested^[11]. In this paper we went a step further, showing the possibility to form a hybrid system DNA origami-optical fiber enabled by its immobilization to a glass via silanization. We then demonstrated its application in detecting a model tobacco mosaic virus (TMV) target sequence thereby opening way to the addressability and versatility of DNA nanorobot genosensors to real life applications.

Device fabrication

Fibers are a convenient material for optical sensor design because they can be inexpensive and provide easy and efficient signal delivery. Several fiber-based technologies have been developed that take advantage of optical fiber's cost effectiveness and its waveguiding capabilities^[25,26]. Indeed optical fiber sensors have unique properties such as a small-size, very light weight, solid phase exhibiting of molecular recognition bioreceptors, and flexibility but also robustness, which make them an attractive alternative with respect to other sensing technologies. Optical fibers have been employed in medical diagnosis^[27,28], DNA detection^[29], single cell measurement^[30], virus detection^[31,32].

The motivation to combine the undisputed potential of DNA origami (i.e. addressability, versatility and loading capabilities) to the unique advantages of optical fiber transducers has been the key factor at the base of our new organic-inorganic hybrid system.

Glass optical fibers were coated with an outer protective coating called jacket that serves to add extra support and strength to the delicate fibre. The higher refractive index of the fibre core enables total internal reflection of the coupled signal as it is surrounded by the lower refractive index cladding. The light therefore is propagated through the fibre length. Once the signals reached the photomultiplier tube (PMT), the reading is analyzed by a computer. DNA nanorobots are immobilized at the endface interfacial surface of the core itself acting as the transducing element of the genosensor (Fig. 1).

The immobilized DNA origami is able to respond to an external molecular target stimulus and actuates by a physical switch from a disarmed to an armed configuration. In the presence of a small amount of TMV target DNA, the robot moves a flap promoting the exposition of its cargo DNA and the self-assembly of a stable hemin/G-quadruplex horseradish peroxidase (HRP)-mimicking a DNAzyme^[11]. DNA origami-optical fiber sensor was first immersed in a solution containing TMV target sequences enabling molecular recognition and hybridization and then the test probe is dipped in a luminol-based solution for confirmation of the presence of the target in the test sample. The well-folded DNAzyme catalyzes the H₂O₂-mediated oxidation of luminol to yield chemiluminescence. The light emitted is collected by the optical fiber and then a measurementis made by a PMT module and analyzed by a computer.

DNA nanorobot origami

DNA nanorobots were prepared by folding the XmnI digested M13mp18 scaffold strand with staple strands, as previously reported^[11]. In order to immobilize DNA nanostructures onto the glass surface and verify their attachment using fluorescence microscopy, we used a protruding anchor sequence complementary to a capture sequence and Alexa 488 labeled staple strands at six sites on the origami structure. The selective hybridization of anchor and capture sequences was previously demonstrated using fluorescein-labeled oligonucleotides^[33]. Agarose gel electrophoresis and TEM images of a well-folded DNA origami are provided in Fig. 2 and Fig. 3.

DNA nanorobot immobilization on cover glass and optical fiber tips

Optical fibers have many advantages, but it is necessary to modify their surface to immobilize active biological recognition molecules using the procedure of silanization. Silane coatings for adhesion promotion applications should offer a robust and internally cross-linked layer of reproducible tickness^[34]. It has also been demonstrated that the monolayer of oligonucleotides obtained by silane grafting is stable^[35,36]. Many studies suggested the use of APTES for the immobilization of different entities with biological interest, including oligonucleotides, on various substrates^[37,38,39]. Taking this into account, we linked short oligonucleotide capture sequences to a glass support by electrostatic adsorption onto positevely charged glass surfaces obtained after silanization. The immobilization method consists of three main steps: silane grafting of the glass surface of the optical fiber tips using 3-aminopropyltriethoxysilane (APTES), deposition of capture polynucleotide sequences dispersed in a water solution for immobilization onto the cationic aminosilanized surface, and finally, hybridization between the adsorbed capture sequence and the DNA nanorobot

protruding anchor sequence as developed earlier.

Silanized optical fibers were characterized by Field Emission SEM (Fig. 4 and Fig. 5). The silane layer over the optical fiber surface, as compared to that of a bare fiber was observed. Deposition of APTES from water resulted in a uniform monolayer with occasional aggregates, as previously reported^[34,40]. In the presence of aggregates, the visualization of DNA nanostructures on silanized glass using AFM is difficult: for this reason fluorescence microscopy was used here instead of AFM in order to demonstrate the feasibility of our method. After silanization, capture sequence attachment was obtained by the application of a capture oligonucleotide solution onto amino-silanized glass. The interaction between the positively charged amine of the silane molecules and the negatively charged phosphate backbone of the DNA helps the nucleic acid to be stably immobilized on the slide surface^[35]. After washing steps, Alexa 488 labeled DNA origami nanorobots were incubated with modified glass surfaces (cover glasses or glass optical fibers). To verify the successful silanization and immobilization of the DNA origami structure on silanized glass due to hybridization captureanchor sequences, optical fluorescence microscopy was used. Fig. 6 shows the green fluorescence microscopic images of both the cover glass and optical fibers with and without DNA origami when excited at 501 nm: only functionalized labeled DNA nanorobot surfaces presented a high fluorescence intensity. Using silanization and capture sequences, DNA origami were successfully deposited on glass surfaces in a simple way. Our results indicated not only that DNA nanorobots were bonded to silanized optical fibers, but also that APTES was immobilized on the optical fibers. In a similar way, other studies provided evidence that APTES was present onto the glass surface by fluorescence microscopy using fluorochromes or other fluorescent molecules^[41,42].

Recently, fluorescence microscopy was used to demonstrate the spray large-scale deposition of 3D DNA origami on glass^[24]. Their method does not require washing steps or optional pre-treatments of the substrates and enables the deposition and patterning of DNA origami. However the spray-coating techniques may affect the opening mechanism of dynamic DNA origami with precisely controlled motions. Here we present a simple approach to immobilize DNA origami on glass surfaces which permits the unrestricted actuation mechanism of 3D nanostructures, requiring fewer steps and being less expensive compared to other methods based on biotin-streptavidin linkage^[20,21]. Thus in this paper, we demonstrated for the first time to our knowledge the functionalization of an optical fiber with a DNA origami.

DNA nanorobot-optical fiber genosensor

The immobilized DNA nanorobots can be dynamically manipulated by an external DNA sequence. When the functionalized optical fibers were immersed in a DNA target solution, the robot moved a flap promoting the exposition of a DNAzyme and the self-assembly of a stable hemin/G-quadruplex. The subsequent incorporation of hemin via the formation of intramolecular guanine quadruplexes defined a cofactor-utilizing nucleic acid able to catalyze the oxidation of luminol by H₂O₂. As shown in Fig. 7, the activation of the flap movement with 12 pmol of target DNA was detected using a PMT module. This result was in agreement with our previous data: to confirm the movement of the flap in an open position, an assay based on the generation of chemiluminescence in the presence of luminol/H₂O₂ was reported. In this previous work all the experiments were conducted using micro cuvettes containing H₂O₂ and actuated DNA origami: the luminol solution was injected into a micro cuvette and the DNA origami opening was measured immediately with a CCD detector analyzing the light emission intensity^[11]. Here we go a step further introducing a portable genosensor. In summary, at first we couple DNA origami intimately to the surface of a glass optical fiber. The detection scheme combines the intrinsic recognition abilities of the immobilized switchable DNA nanorobots with chemiluminescence signal collection with that of a portable transducer. Therefore, we constructed a fully functional and self-contained sensing device for viral detection of TMV without the need for a lab as it may be dispatched with our proprietary portable photodetector.

Conclusion

Due to the self-recognition properties of DNA molecules, it is possible to create numerous artificial DNA nanostructures with well-defined structures and DNA nanodevices with precisely controlled motions^[38]. Recently a DNA nanorobot triggered by target DNA strand was designed, produced, characterized and tested^[11]. Here we proposed a biosensor based on a surface functionalized optical fiber tips for a specific DNA sequence detection. The bio-recognition scheme was achieved by surface functionalization of the bare optical fiber with a monolayer of APTES and capture probe ssDNA sequences that permit the anchor of a dynamic 3D DNA origami. Using fluorescence imaging, we demonstrated the immobilization of DNA nanorobot at the tip of an optical fiber. We showed that switchable DNA origami can be succefully deposited on glass without loss of its functionality. The present study demonstrated the use of our genosensor for the detection of picomol amounts of a DNA target. This DNA nanorobot-optical fiber system combines the intrinsic characteristics of an optical fiber and the vast detection possibilities of DNA origami nanotechnology: such a system could find many applications, such as diagnostics and *in vivo* biosensing.

Methods

Materials and reagents. Hemin was purchased from Porphyrin Products (Logan, UT) and used without other purification. A hemin stock solution (5 mM) was prepared in DMSO and stored in the dark at - 20°C. Oligonucleotides without any modification and with Alexa 488 label at the 5' end were purchased from Integrated DNA Technologies Pte Ltd (Singapore) and were resuspended in sterile MilliQ water to give stock solutions of 100 μM. Capture sequence, anchor sequence and labeled nucleotide sequences are shown in Table 1. In the 3D DNA origami used in this work 13 previously published sequences^[11] were labeled with Alexa 488 and one sequence (anchor sequence) was elongated in order to permit the attachment of the complementary capture sequence. M13mp18 was purchased from Bayou Biolabs, LA, USA. *Xmn*I enzyme and bovine serum albumin molecular biology grade were purchased from New England Biolabs, Singapore. All other chemicals were purchased from Sigma-Aldrich Pte Ltd (Singapore).

3D DNA origami design. To modify and assemble the 3D origami object, the single-stranded M13mp18 genome was digested at the two *Xmn*l restriction sites after the hybridization of ssDNA with *Xmn*l cut-site oligonucleotides: the resulting 2289 nts scaffold strand was incorporated into the 3D DNA origami structure^[11]. The square-lattice version of the caDNAno software from Douglas *et al.* (2009) was used.

3D DNA assembly and Purification. 3D DNA origami was synthetized and purified as previously described^[11].

Nucleic Acids Visualization. Folded and purified DNA-origami were run on a 0.7% (w/v) agarose gel (SeaKem Agarose, Lonza): gels were previously added with 0.5x Gel Red[™] nucleic acid stain (Biotium, Hayward, CA) and run in 1x TAE (for 200 ml of a 50x buffer solution: 400 mM Tris-HCl, 11.42 ml acetic acid, 20 ml EDTA 0.5 M, pH 8.0) at 75 V for 45 minutes and then visualized and photographed under UV light (Molecular Imager Gel Doc[™] XR+ Imaging System, Biorad). The 1 kb DNA ladder (Sigma-Aldrich, Singapore) was used as a molecular weight marker.

TEM imaging. For TEM imaging 10 µl of the samples were adsorbed for 3 minutes on carbon-coated copper grids and imaged with a JEOL JEM 1011 transmission electron microscope (Tokyo, Japan) operating at 100 kV. The samples were stained for 2 minutes with a 2% (w/v) uranyl acetate solution and washed in milliQ water for three times.

Silanization of the glass slides, capture probe binding and DNA origami attachment. As initial solid phase support, microscope cover glasses were used. The glass silanization protocol used was described by Manzano *et al.*, 2015 and Marcello *et al.*, 2013 and followed here with some modifications. The cover

glasses were treated with 10% (w/v) NaOH at room temperature for 1 h, rinsed with deionized water and treated with 0.1 M HCl for 15 min. After a washing step with deionized water, the glass slides were rinsed in acetone and dried at 50 °C for a few minutes, and immersed in a 0.5% (v/v) APTES solution in deionized water for 30 min at room temperature. Slides were then rinsed three times in deionized water, dried at 160 °C for 1 h and cooled at room temperature for 30 min. 10 µl drops of the capture probe at 100 ng/µl were deposited in triplicate on each glass slide and incubated at 4 °C overnight to conjugate the microscope cover glass surface.

The glass slides with the bound capture probe were washed twice in deionized water prior to utilization. Then, 1 µl or 10 µl of the 3D DNA origami samples was spotted on each slide. The microscope cover glasses were incubated at room temperature for 2 h in the dark and they were washed twice in sterile deionized water to eliminate the unbound DNA nanostructures.

Silanization of the optical fiber tips and capture probe binding. SFS400/440B Superguide G UV-Vis silica fibres (Fiberguide Industries, Stirling, USA) were used for all experiments. The fibers had an original numerical aperture (NA) of 0.22, a core diameter of 400 µm (refractive index of 1.457 at 633 nm) and a surrounding silica cladding with a width of 40 µm (refractive index of 1.44 at 633 nm), in addition to a 150µm-thick silicon buffer and a 210-µm-thick black Tefzel[®] jacket. The length of a single fibre used in the experiments was 20 cm. The black Tefzel[®] jacket and silicon buffer were mechanically stripped away using a fibre stripping tool (Micro-Strip[®], from Micro-Electronics Inc., USA) to expose a 2 mm naked optical fibre core tip.

In preparation for the silanisation procedure, fibres were soaked in a 1:1 methanol/37% (v/v) HCl solution for 10 min to purify the newly exposed fibre core from micro-contaminants. After sonication for 10 minutes, the fibre tips were dried under N_2 and dipped in Piranha solution ($35\% H_2O_2$: $96\% H_2SO_4$ in the ratio 3:7) for 15 min at 90 °C to enhance the exposure of the hydroxyl groups on the silica surface. After a washing step with deionized water, the glass tips were rinsed in acetone and dried at 40 °C for a few minutes, and immersed in a 0.5% APTES solution in sterile deionized water for 30 min at room temperature. In the presence of water, the hydrolysis of ethoxy groups of APTES and their condensation into siloxane (Si-O-Si) occur, leading to the formation of a silane layer. The amino group it self is believed to act as a catalyst and enhance the adsorption rate of APTES molecules on glass

surfaces^[34]. Fiber tips were then rinsed three times in deionized water and acetone. APTES films were

cured in an oven at 80 °C for 3 hours: heat treatment helps to inter-crosslink the silane molecules on the surface though the elimination of water molecules forming a relatively more robust silane layer. Curing at high temperature in the presence of surface water increased the number of siloxane bonds in APTES film. During heat curing, silanes condensed rapidly to siloxanes^[40]. After incubation at room temperature for 30 min, fibers were incubated in 25 µl of the capture probe at 100 ng/µl at 4 °C overnight. The fibers with the bound capture probe were washed twice in deionized water prior to utilization.

Field Emission SEM. Silanized optical fibers were visualized using Supra 55 Field Emission SEM, Carl Zeiss. Since the surface was non-conducting, the operating gun voltage and detectors (In-Lens or SE) were chosen in a manner to get clear images of the silanized surface.

Fluorescence microscopy. Glass slides and optical fibers with fluorescently labelled 3D DNA origami were imaged using a Leica fluorescent microscope AF6000 equipped with a 10x and 20× magnification objectives. The external light source was a Leica EL6000 lamp (HXP 120 W 45C VIS-Osram, Metal Halogenide bulb). The Alexa 488 labeled DNA nanostructures were excited at 501 nm and the fluorescence emission at 521 nm in wavelength was collected.

3D DNA origami actuation and attachment to fiber tips. Opening of the closed 3D DNA origami was induced using 0.5 µM (12 pmol) of nucleic acid target and allowed to react for 1 hour and 30 minutes (reaction volume: 21.5 µl). In order to ensure the formation and availability of G-guadruplex structures through the opening of the origami, the mixture was heated at 40 °C for 3 minutes. After 5 minutes, the washed fiber tips were immersed in the actuated DNA origami solution and incubated at 25 °C for 30 minutes. Then, hemin (1.6 µM) was added and the solution was incubated for 2 hours and 30 minutes at 25 °C to form the respective hemin/G-quadruplex structures and to guarantee the 3D DNA origami attachment to the glass surface trough the protruding anchor probe sequence complementary to the capture probe. **Chemiluminescence assay.** Chemiluminescence measurements were conducted using a Hamamatsu HC135-01 Photo Multiplier Tube (PMT) Sensor Module, combining the sensitivity of a photomultiplier tube with the intelligence of a microcontroller. The instrument was placed in a light-tight box equipped with a manual shutter (71430, Oriel) in front of the detector, and a custom-made lever outside the box was used to move the slide shutter of the photon counting unit inside. The far end of each fibre was held by a fibre holder (FPH-DJ, Newport) and placed into an adjustable single-fibre mount (77837, Oriel). The assays were performed in a solution containing 1.2 mM luminol and 74 mM H₂O₂. Briefly, the fibre tip surfaces were washed twice in sterile deionized water to eliminate the unbound DNA nanostructures and then dipped in a

solution containing 6.6 μ l of 5 mM luminol and 20 μ l of H₂O₂ solution (98 mM). DNA origami opening was measured immediately analyzing the light emission intensity as the mean value of the photon counts for a period of 15 seconds: each measurement is presented as the mean of 3 fibers set and reported in arbitrary unit (a.u.).

Safety considerations

Piranha solution is highly toxic, corrosive and reactive and can become extremely hot when prepared; it is corrosive and irritating to the eyes, skin, and respiratory tract. When working with Piranha, it is highly recommended to use only glass containers. During Piranha solutions preparation, it is compulsory to add peroxide solution to acid very slowly in a ventilated fume hood wearing gloves, a lab coat, and goggles.

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Author contributions

R.S.M. and E.T. designed the research. E.T. performed the experiments. M.M. performed silanization. S.K.S. guided sample preparation and FE SEM imaging. R.S.M. and E.T. analyzed data and prepared manuscript. All authors discussed the results.

Additional information

Competing financial interest: The authors declare no competing financial interests.

Figure Legends

Figure 1. Schematic of DNA origami-optical fiber genosensor. The glass optical fibers are coated with an outer protective coating called jacket that is mechanically stripped away using a fibre stripping tool to expose a 2 mm fibre core tip (A). The biorecognition elements (DNA nanorobot) are immobilized at the interface surface (core) that is connected to the fiber optic (transducing element), using a capture sequence (green) and an anchor sequence (purple) (B). Closed 3D DNA origami with the unreacted probe (light blue) (1). The 3D DNA origami during the hybridization probe (light blue) / target (red) (2). Actuated 3D DNA origami armed with hemin/G-quadruplex DNAzyme complex (3); the probe (light blue) hybridized to target (red), the flap opened and a DNAzyme (pale grey) was pulled out of the cylinder and exposed to hemin (black dots)^[11]. After the actuation of the DNA origami and the generation of a chemiluminescence in the presence of luminol/H₂O₂, the signals arrive at a photomultiplier tube (PMT): once the signals have passed through the detector/sensor module, they are sent to a computer.

Figure 2. Agarose gel electrophoresis of well-folded nanorobot. The assembly reactions of the DNA origami were run on a standard agarose gel GelRed[™] stained and imaged under UV light. Lane from left to right: 1) 1 Kb DNA ladder; 2) M13mp18 single stranded; 3) *Xmn*l digested M13mp18 DNA, used as negative

control: the lower band is the 2289 nts fragment used as scaffold and incorporated into the three dimensional DNA origami; the upper band is the remaining M13mp18 single stranded; 4) well-folded DNA nanorobot. The arrow shows the folded structure.

Figure 3. TEM images of 3D DNA origami nanorobot.

Figure 4. FE SEM micrograph of silanized (left) and bare (right) optical fiber tip (lateral side).

Figure 5. FE SEM micrograph of silanized optical fiber tip (bottom).

Figure 6. Fluorescence images of the cover glass after immobilization of Alexa 488 labeled DNA nanorobots at different magnitude (a, b), the optical fiber tips functionalized with Alexa 488 labeled DNA nanorobots (c) and the bare fiber (d).

Figure 7. Light emission intensity (expressed in a.u.). From left to right: background, hemin/G-quadruplex DNAzyme standard solution, hemin/G-quadruplex DNAzyme complex in the absence of the target sequence (closed DNA nanorobot) or in the presence of the target sequence at a final concentration of 0.5 μ M (actuated DNA nanorobot). (mean ± standard deviation, n=3).

3'.

Oligo name	Sequence (5'-3')	Length
0[55]	Alexa 488-GCCCGGAACCGCCACCCTCAGAGCATAGGAAC	32
0[119]	Alexa 488-GATTAGCGAGTTAATGCCCCCTGCATGGCTTT	32
4[158]	Alexa 488-CCCTCAGAACCGCCACCCTCAGAGCCGCCATCTGAAT	37
11[17]	Alexa 488-GAACGAGGGTAGCAAAGAGGCGAAAGAATA	30
12[158]	Alexa 488-GGACGTTGGGAAGAATCATTATACCAGTCAAACACTA	37
15[72]	Alexa488-CGCATAGGGTCGAAATCCGCGACCACAAAGTA	32
Anchor sequence- 3[17]	TGTGGTGGTGGTGGTGGTGTTTT- TACAACGCCTGTAGCTGCTAAACAACTTTC	53













