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High Resolution Single Molecule Optical Localization of Multiple Fluorophores on DNA Origami Constructs Fluorophores on DNA Origami Constructs

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TITLE

High Resolution Single Molecule Optical Localization of Multiple Fluorophores on DNA Origami Constructs

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ABSTRACT

An ongoing challenge in the development of nanoelectronics and nanophotonics is the nondestructive, high-resolution localization in space of single molecules and multi-molecular assemblies. The apparent barrier to the use of optical microscopy at the sub-100 nm scale is the well known Abbe Limit, the diffraction limit to resolution. This laboratory has adapted a technique called Single-molecule high resolution imaging with photobleaching (SHRIMP). We have developed methods for utilizing SHRIMP for the determination of the separation of two fluorophores in single DNA origami constructs.

DNA Origami is extremely useful because it can address nanocomponents down to 2nm separation. It should be possible to prepare standards for the measurement of distances on the order of 10-200nm, between light emitting particles in these DNA self-assembled nanoconstructs, by incorporating two fluorophores of the same color or different colors.

Particularly in this work, 1) two channel fluorescence imaging has been implemented using an Optosplit equipped Nikon microscope; 2) four types of DNA origami test objects have been constructed and characterized; 3) a highly effective system to remove adventitious fluorescent contamination from substrates and from buffer solutions has been constructed, because a pre-requisite for single molecule microscopy is the production and maintenance of coverslips and solutions with essentially no fluorescent contamination; 4) a software based protocol for Gaussian fitting single molecule images to find the center of fluorophores and to determine the distance between the fluorophores has been developed. The application of this software to an Origami construct with two rhodamine labels is presented.

ACKNOWLEDGEMENTS

It would be my pleasure to thank all persons and organizations who facilitated the completion of this project. First of all, I would like to dedicate this work to my parents, Thatha Rao Rajulapati and Vijayalakshmi Rajulapati. It is their motivation and support which made me succeed in my academic endeavors.

I would like to express my heartfelt thanks to my advisor, mentor and one of the best persons I have ever known, Dr. Michael L Norton. Without his support, my ambition to study abroad could hardly have been realized. His guidance, support, and encouragement have made this scholarly journey, such a rewarding experience and his thoroughness and dedication have made this research what it is. I hope everyone gets to be blessed with the kind of advisor that I have had him. My grateful thanks to my committee members, Dr. Bin Wang and Dr. Brian Scott Day for their timely suggestions and remarks. I deeply express my thanks to my colleagues in Norton nanolaboratories David Neff, Masudur Rahman, Hong Zhong, Dawn Nicholas, Maryna Lvovska, Wanqiu Shen, Nathaniel Crow, Rusty Parrot and Anne for their timely suggestions and help in understanding the research puzzles. Without their help I couldn't have succeeded in finishing this research. At last but not the least, I would like to thank all my Huntington friends for their moral support.

LIST OF ABBREVIATIONS

AFM	Atomic force microscope
CCD	Charge coupled device
CdSe	Cadmium Selenide
ddH_2O	Double distilled water
DM	Dichroic mirror
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded Deoxyribonucleic acid
EGFP	Enhanced green fluorescent protein
EMCCD	Electron multiplying charge coupled device
FIONA	Fluorescence imaging with one nanometer accuracy
FRET	Fluorescence Resonance Energy Transfer
G3	Generation 3
H_2O	Water
Hg	Mercury
HPLC	High performance liquid chromatography
Mg	Magnesium
$MgCl_2$	Magnesium Chloride
N_2	Nitrogen
NA	Numerical aperture

NALMS	Nanometer localized multiple single molecule
NHS	N-Hydroxysuccinimide
O_2	Oxygen
PAINT	Point accumulation for imaging in nanoscale topography
PALM	Photoactivatable localization microscopy
PCR	Polymerase chain reaction
PSF	Point spread function
Qdot	Quantum dot
RFP	Red fluorescent protein
SHREC	Single Molecule High Resolution Colocalization
SHRIMP	Single-molecule high resolution imaging with photobleaching
SPDM	Spectral position determination microscopy
SSMCC	Sulfo-4-(N-maleimidomethyl)cyclohexane-1-carboxylic 3- sulfo-n-hydroxysuccinimide ester
STORM	Stochastic Optical Reconstruction Microscopy
TAE	Tris-acetate-EDTA
TEMED	Tetramethylethylenediamine
UV	Ultraviolet

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PROJECT OVERVIEW

A. Purpose:

Research on nanostructure formation requires a nondestructive, rapid, water compatible, high resolution method for the determination of absolute and relative placement of molecules within those structures. The purpose of this research was to construct an instrument capable of performing these functions with these constraints, developing appropriate software and testing the system.

B. Specific Aim:

To measure the distances between light emitting particles linked to DNA based nanostructures at a nominal separation of 51.2 nm with ~10nm accuracy. This will be performed by fitting the image of each of the fluorophores using a two dimensional Gaussian function to determine the center of intensity. A graphical representation of the objective is shown in Figure 1. A 100nm DNA origami construct is smaller than the region represented by a single pixel, even at very high magnification, each pixel represents ≈155nm x155nm. In this work two objects (fluorophores, shown in red in Figure 1) on the surface of the origami are to be localized, and the distance between them determined.

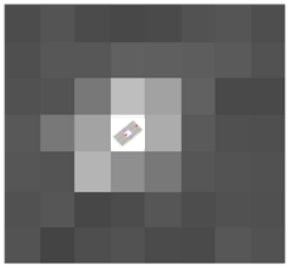


Figure 1: Image of Origami with two fluorophores as projected onto CCD camera chip by the optical system of the microscope

C. Hypothesis:

It is possible to measure the distance between two fluorophores linked to DNA based nanostructures with high resolution (uncertainty of position $\leq$ 5nm) using optical microscopy.

SINGLE MOLECULE STUDIES

A. Background:

At the end of the 19th century, many physicists and chemists had started to recognize accumulated indications of the existence of atoms and molecules. They obtained strong proof at the beginning of the 20th century, with the quantization of charge, Brownian motion and most importantly the diffraction of X-rays by crystals. In 1911, Jean Perrin attempted to observe single fluorescent molecules, using the optical microscope. He implemented his idea with black soap films, which gave very low background scattering (Rayleigh and Raman), with very low concentrations of fluorescent molecules [(1)]. Due to the lack of sensitivity of the human eye, the poor quality of sources (spectral lamps) and filters (colored glass), and the limited durability of the fluorescent dyes, it was difficult to see single molecules. Success was made possible by using today's microscopes, lasers, filters and dyes. In 1976 Hirschfeld succeeded in observing the location of individual molecules visually under the optical microscope by observing a single antibody molecule attached to 80-100 fluorophores [(2)]. Later during the 1980s, Keller and his group developed detection schemes for small numbers of fluorescent molecules in solution [(3, 4)]. By 1989 Moerner & Orrit performed optical detection of single fluorophores and spectroscopy of single fluorophores at room temperature in biologically relevant environments [(5)]. In the early 2000's, detection of single molecules or single luminescent objects (nanocrystals, quantum dots, metal particles, color centers, etc.) in condensed matter was enabled by further progress in sources, optics and detectors. This research is of interest for the nanoscience field. The optical detection and study of single molecules at room-temperature allowed optical microscopes to investigate problems in molecular biochemistry and biophysics, for example the study of the dynamics of single molecules and tracking of single molecules. In 2003 Yildiz *et al.*,

demonstrated localization of single fluorophores with 1-2 nm precision by using a two-dimensional Gaussian function to fit the intensity data in images of single fluorophores and fluorophores of two different colors [6].

Two important factors which play a crucial role in the detection of single molecules via optical microscopy are interference and photobleaching. Strong optical background signal from Rayleigh and Raman scattering from condensed matter on the surfaces is one source of optical interference. Another source is fluorescent impurities in the sample, which may easily dominate the fluorescence from a single molecule. Therefore, the purity of the sample is crucial. When imaging large samples such as cultured cells, researchers have generally not been concerned with contamination of cover-slips and solutions by fluorescent molecules. This type of sample typically contains many fluorescent molecules that have been concentrated on discrete structures as labels. That is, the sample is much brighter than the background. However when imaging single molecules, as in the present work, the detector (camera) must be very sensitive. Imaging with a detector of this type allows one to observe any fluorescent material that has contaminated the substrate surface. Second, fluorophores are subject to irreversible transformations called photobleaching, which renders observation of fluorophores under the microscope for long periods impossible. The signal from a single molecule is limited to very short times due to photobleaching. In this photochemical process, a single molecule excited to a triplet state may react with oxygen, yielding a nonfluorescent product. Photobleaching occurs about 4 times faster in air than in nitrogen in Qdots, indicating the formation of non-radiative recombination centers during photooxidation[7]. Even with highly photostable fluorophores, the observation time of an individual molecule may be limited to 10–100 s on a surface due to photobleaching [8].

The signal-to-noise ratio plays a key role in successful single-molecule detection. Background noise can make it difficult to detect single fluorescent molecules at room temperature [(9)]. When working with single molecules, some important principles need to be considered including signal size, background issues, signal-to-noise ratio and signal to background ratio. The fluorescent signal arises from the fluorescent photons emitted by the single molecule. For one molecule the number of photons emitted per second is far less than the number of photons in the incident light (mercury emission light in our case) [(10)].

Light from a laser or mercury lamp source can excite the single molecule efficiently. The process is shown schematically in Figure 2. Photons of energy hv (blue arrow) excite the molecules to an upper vibrational level of the dipole allowed singlet-singlet transition followed by rapid relaxation to the lowest vibrational state of S_1 through non-radiative processes (black arrow). From S_1, the molecule may return to S_0 through the emission of fluorescence. The molecule may also undergo intersystem crossing from S_1 into a triplet state (T_1) from which it can then return to the ground state at rate Kt (Figure 2[(11)]) [(10, 12)]. Dotted arrows indicate fluorescence emission, and intersystem crossing and triplet decay are shown with gray arrows [(12)].

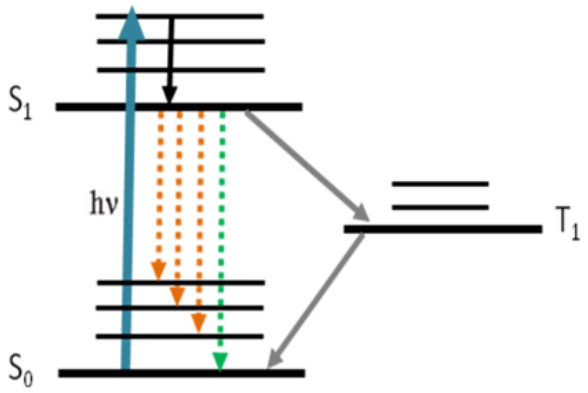


Figure 2: Energy level scheme for a single-molecule flourophore

In order to achieve maximum signal and minimum background (background refers to photons that may arrive at the detector from any source other than the single molecule of interest), fluorophores must be selected with high efficiency of fluorescence (quantum yield) [(10)], and noise from unwanted sources must be identified and removed. All single-molecule fluorescence experiments are background and shot noise limited, therefore it is important to optimize the signal-to-noise of the spectral feature. Background may result from the experimental apparatus, such as residual fluorescence from optical parts, i.e. from colored glass filters or from the microscope objective itself. It is also possible to have residual emission from the excitation source in the red-shifted spectral range where fluorescence is detected. A smaller pixel size will also contribute toward improving the signal-to-noise ratio [(13)]. However, background photons arising from the sample matrix itself are more difficult to suppress. By using ultra clean substrates of the highest quality, background from this source can be minimized.

Single-molecule experiments on biological molecules have recently become experimentally possible and represent a big shift in the way one can study and visualize molecular processes [14]. Working at the single molecule level has a number of advantages over working with ensembles. The variation, heterogeneity, in a particular property as well as the average can be measured from single molecule experiments. Examples include the conformations of the biomolecule, the different pathways from one conformation to another, the variation in the rate of individual enzymes and how this rate varies with time [15]. Single-molecule techniques can measure intermediates and follow time-dependent pathways of chemical reactions and folding mechanisms that are difficult or impossible to synchronize at the ensemble level. Thus, using single-molecule fluorescence in combination with advanced microscopes and manipulation techniques, one can make "molecular movies" of biological processes that help one propose and understand the underlying molecular mechanism [16]. One can perform experiments on small amounts of materials, which is particularly important with biomolecules which are often isolated in small amounts [17]. Single molecules are useful in nanodevices, in which they can perform very well defined functions, including transfer of information from the macroscopic world to the nanoworld [18].

Fluorescence is a good method for the study of single molecules in solutions and on surfaces at time scales ranging from nanoseconds to seconds. Recent advances in single molecule fluorescence microscopy include super resolution techniques for breaking the diffraction limit [19]. Studying single molecules became easier due to the use of photostable and bright fluorophores. For single molecule studies, fluorophores should not only have high photostability, but also less probability to go into dark states which produces blinking. The linker between the biomolecule and the fluorophore should be rigid enough to eliminate the contribution of linker motion to any

observed dynamics. The linker also must not quench the fluorophore by interacting with the molecule to which it is attached [20].

B. Current Fluorescence Methods for Single Molecule Studies

Ernst Abbe (1873) discovered that "the traditional light microscope cannot resolve special structures that are smaller than about half the wavelength of the focused light" [21]. The Far-field (the region where the angular field distribution is essentially independent of distance from the source) diffraction limit (a physical limit to how focused a beam of light can be) of light is approximately λ/2NA, where λ is the wavelength and NA (for a microscope, the maximum NA is <1.65) [22] is the numerical aperture of the collecting lens. Two spots emitting in the visible wavelength range, cannot be separated if they are closer than ~200nm (λ=600nm, NA= 1.49) because the point spread function (PSF) of the two spots will strongly overlap each other. If those two spots are separated farther than 200nm apart, one can resolve the two spots individually with a normal microscope because the PSF's do not overlap [23]. Super resolution techniques in fluorescence microscopy allow one to resolve the position of an individual fluorescent emitter with precision greater than described by Abbe's law [24]. The position or center of the molecule can be obtained by fitting a Gaussian function to the measured fluorescent intensity profile of the spot.

There are several methods, in current use, to resolve two dyes on a single molecule, with nanometer precision. From the Rayleigh criterion, the resolution of the conventional light microscope is limited to separations > 200nm [25]. Spectroscopic techniques such as fluorescence resonance energy transfer (FRET) cannot be used to measure distances >10 nm. This leaves a ''gap'' in the ability of optical techniques to measure distances on the 10- to 200-nm scale [20].

FRET (Fluorescence Resonance Energy Transfer) can measure the exact distance between two single molecules with sub nanometer resolution [26]. FRET is a distance dependent interaction between a donor and an acceptor. The donor, initially in its electronic excited state, may transfer energy to an acceptor chromophore through a nonradiative dipole-dipole coupling (Figure 3[26]) [27]. If two fluorophores are very close to each other (typically 10-100 Å), FRET will occur. The efficiency of energy transfer drops to 50% at the Förster distance (R_o in Figure 3c) [28].

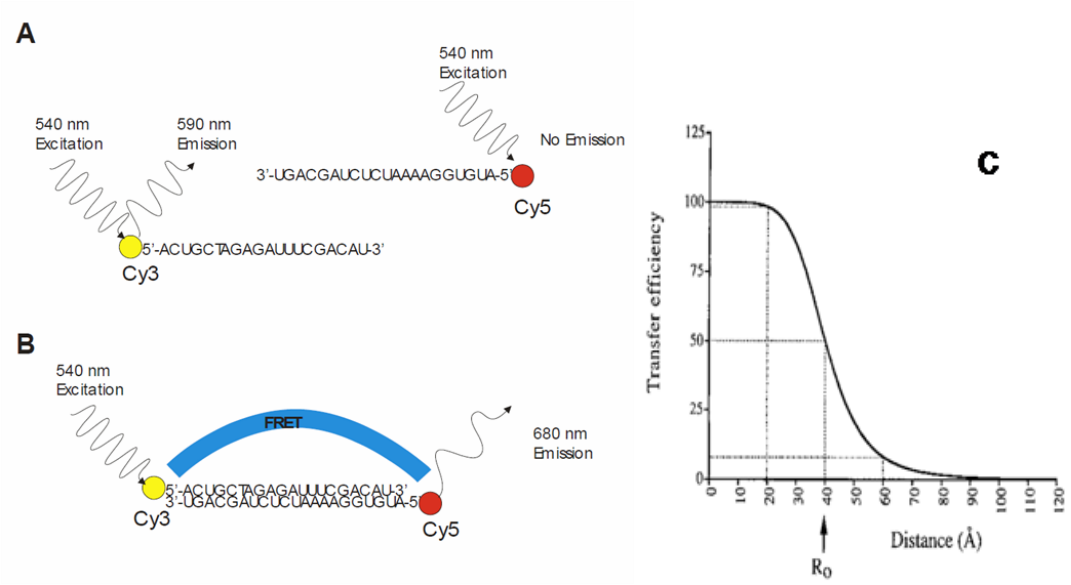


Figure 3: Schematic representation of FRET (A) two complementary oligonucleotides are labeled with Cy3 and Cy5 respectively. When these labeled molecules are not hybridized, FRET does not occur. (B) After hybridization, the close proximity of the molecules allows for FRET transfer to occur, which results in the emission of light at 680nm when the annealed molecule is excited with 540nm light. (C) The solid curve represents the r^6 relationship that describes the relationship between the efficiency of

the fluorescence resonance energy transfer and the distance separating the donor and acceptor. R_0 is defined as the distance separating a donor and acceptor pair where the transfer efficiency is 50%

The most common super resolution methods are introduced below.

I. FIONA:

In 2003 Yildiz *et al.,* introduced Fluorescence Imaging with One Nanometer Accuracy (FIONA). Fiona is a technique to locate the center of a fluorophore with ~1 nanometer accuracy within the x-y plane of the image. If a single fluorescent molecule forms an image of width ~200nm (Rayleigh limit) [25] with visible light, the center of the fluorophore can be located with high accuracy and precision by collecting a sufficient number of photons (higher signal-to-noise ratio). FIONA has been demonstrated to attain 1-2nm accuracy [29, 30]. FIONA is performed simply by taking the point spread function of a single fluorophore excited with wide field illumination. The emission intensity distribution [$I(x, y)$] of a fluorophore on the CCD imaging plane is fit to the following 2D Gaussian function (eq 1) in order to determine the lateral position of the fluorophore in the specimen plane [31].

$$f(x,y) = Ae^{-\left(\frac{(x-x_0)^2}{2\sigma_x^2}\right)+\left(\frac{(y-y_0)^2}{2\sigma_y^2}\right)} \quad (1)$$

Where, A is amplitude,

x_0 and y_0 are the coordinates of the center,

σ_x and σ_y are standard deviations of the distribution in each direction.

II. SHRIMP:

To resolve two dyes, high resolution variants of FIONA can be used [32]. Although the resolution (distance at which two fluorophores can be distinguished from each other) for a normal microscope is 200 - 250nm, Single-molecule high resolution imaging with Photobleaching (SHRIMP) resolves two different dyes that are very close to each other and within the same sample, by exciting both and waiting until one photobleaches, and then determining the position of the remaining dye molecule using the FIONA approach. The position of the photobleached fluorophore is determined by subtracting the image of the unbleached fluorophore from the image of the two dye molecules before photobleaching. The resulting image of the photobleached fluorophore can be fit with a gaussian function, enabling the position of each dye molecule to be determined with nanometer accuracy. Fitting the two individual spots to a 2D-Gaussian function enables the intermolecular distance to be determined with a high degree of precision. This technique requires use of fluorescence microscopy, an intense excitation source, deoxygenation, high quantum yield fluorophores and a low noise detector. SHRIMP is useful for immobilized molecules on dry surfaces. Inherent optical problems such as chromatic aberrations do not occur with SHRIMP [20].

III. SHREC:

Another alternative method is called two color FIONA, and also called Single Molecule High Resolution Co-localization (SHREC). This method is based on having two fluorophores that have well separated emission peaks [22], which are imaged on different parts of the same CCD camera. Each dye is excited with light that matches its excitation peak (a Hg source is used in this research project), and the resulting emissions are split with the appropriate dichroic mirrors and emission filters. After obtaining the CCD images of both fluorophores, one can

apply a mapping function to co-localize the dye positions. Often two-color experiments have the difficulty of finding a sharp focal plane for both colors simultaneously because of the chromatic aberrations of the microscope objective [33].

IV. STORM:

Stochastic Optical Reconstruction Microscopy (STORM) was invented by Zhuang[34] and his co-workers to localize photoswitchable fluorophores with high accuracy. STORM is one of the new high resolution techniques for imaging objects to ±20nm. If one object has multiple, special, fluorophores, all fluorophores are excited with a red laser pulse until they all enter a nonfluorescent state, then a green laser pulse is used to switch a fraction of the fluorophores to their emitting state. In one set of experiments, optically switched fluorophores were attached to short double stranded DNA molecules. With only a few of the fluorophores in the field of view switched on, a Gaussian fit is used to find the centers of the emitters. The image is reconstructed by combining all of the localized fluorophores [35].

V. Other Techniques:

Many methods based on FIONA have been reported (with similarly creative acronyms). These include nanometer localized multiple single molecule analysis (NALMS) [36], point accumulation for imaging in nanoscale topography (PAINT) [37], photoactivatable localization microscopy (PALM) [38, 39] and spectral position determination microscopy (SPDM) [31]. Recent reviews have highlighted the usefulness of such tools to study complex biological processes [40-43].

C. DNA Origami:

DNA Origami is DNA folded to create designed two and three dimensional shapes at the nanoscale. The specificity of the interactions between complementary base pairs make DNA a useful construction material through design of its base sequences. This process involves the folding of a long single strand of viral genomic DNA with the assistance of multiple short designed staple strands. Depending on their complementary sequences the shorter DNA strands will bind the longer DNA in specific locations, resulting in the desired shape. DNA Origami is extremely useful because it can be used to address nanocomponents to specific locations with a resolution of ~2nm [(44)]. It should be possible to create standard test samples with distances between light emitting particles on the order of 10-200nm, using these DNA self-assembled nanoconstructs by incorporating two fluorophores of the same or different colors. To incorporate a fluorophore into the Origami, one would select the specific location (staple) within the Origami and replace that specific DNA strand with a DNA oligomer that is modified with a fluorophore.

INSTRUMENT CONFIGURATION AND REPORTERS: BACKGROUND

A. Optosplit Microscope Configuration:

The advantage of employing an optosplit microscope over the conventional fluorescence microscope is its capability to acquire simultaneous images at two different emission wavelengths. A high intensity light source (100W Hg lamp) is used for excitation of the sample. This light is passed through a dichroic filter cube containing a fluorescence band pass excitation filter (it will select specific wavelengths of light for excitation of the specimen by filtering all other unwanted wavelengths out). Light emitted from the fluorescent species, is transmitted through the beam splitter (optical device that splits an incident beam of light into two directions

depending on their wavelengths (long/short)). In this research project, the optical microscope is connected to an Optosplit II splitter (Cairn Research Limited). The Cairn Optosplit image splitter is a simple device enabling a single camera to record images simultaneously at two different optical wavelengths. The Image splitter is connected to a high sensitivity camera with a special CCD chip called an EMCCD (electron multiplying charge coupled device). This type of chip amplifies the light to charge conversion signal that normally occurs in a digital camera chip.

Fluorescence images were acquired with a Rolera-MGi Plus back-illuminated EMCCD camera (QImaging, Surrey, BC, Canada) mounted on a Nikon ECLIPSE TE200 inverted microscope. In most cases a Nikon 100 X oil immersion objective was used. Excitation light was produced by a 100 W Hg lamp (CHIU Technical Corp) coupled to a filter cube (Chroma technology corp.) delivering the light to the microscope through an excitation filter. Excitation filters (Chroma technology corp.) were 470/40 nm for fluorescein or EGFP and 550/20 for rhodamine. A dichroic filter (catalog no: 59004x, Chroma technology corp.) was placed to selectively pass light emitted from the sample while reflecting other. Emitted fluorescence reached the Optosplit II image splitter fitted with a T540LPXR (205080, Chroma Technology corp.) beam splitter, and two emission filters, one at 515/30(Chroma Technology corp.) for Fluorescein and EGFP and another at 595/50 nm (Chroma Technology corp.), for Rhodamine. For each excitation wavelength, the image splitter allowed capture of the two images (through the 515/30 nm and 595/50 nm emission filters) in the same frame on the EMCCD chip (Figure 4), allowing continuous imaging with an image capture frequency of 6.89 Hz. The spectral characteristics of the system are shown in Figure 5.

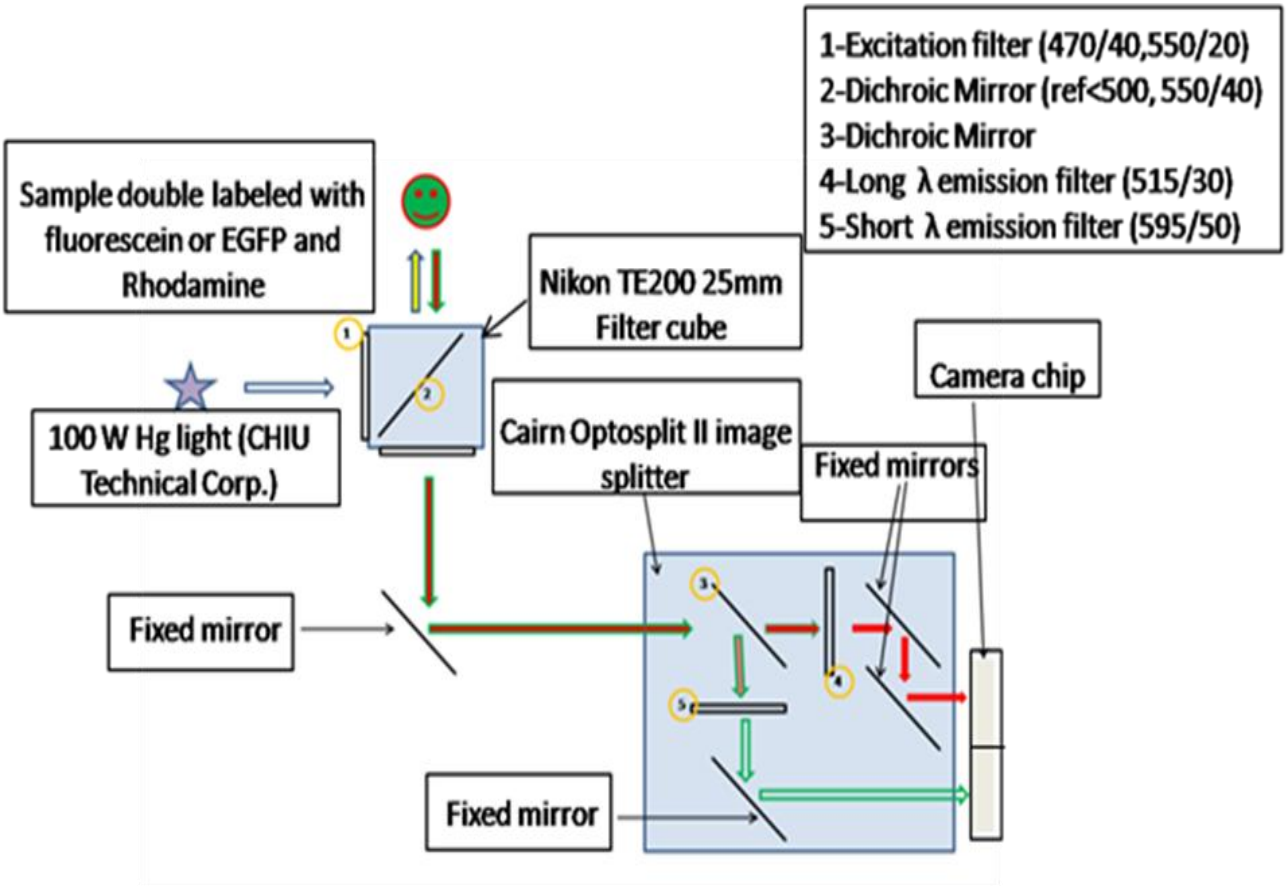


Figure 4: Optosplit II Microscope setup to form two images

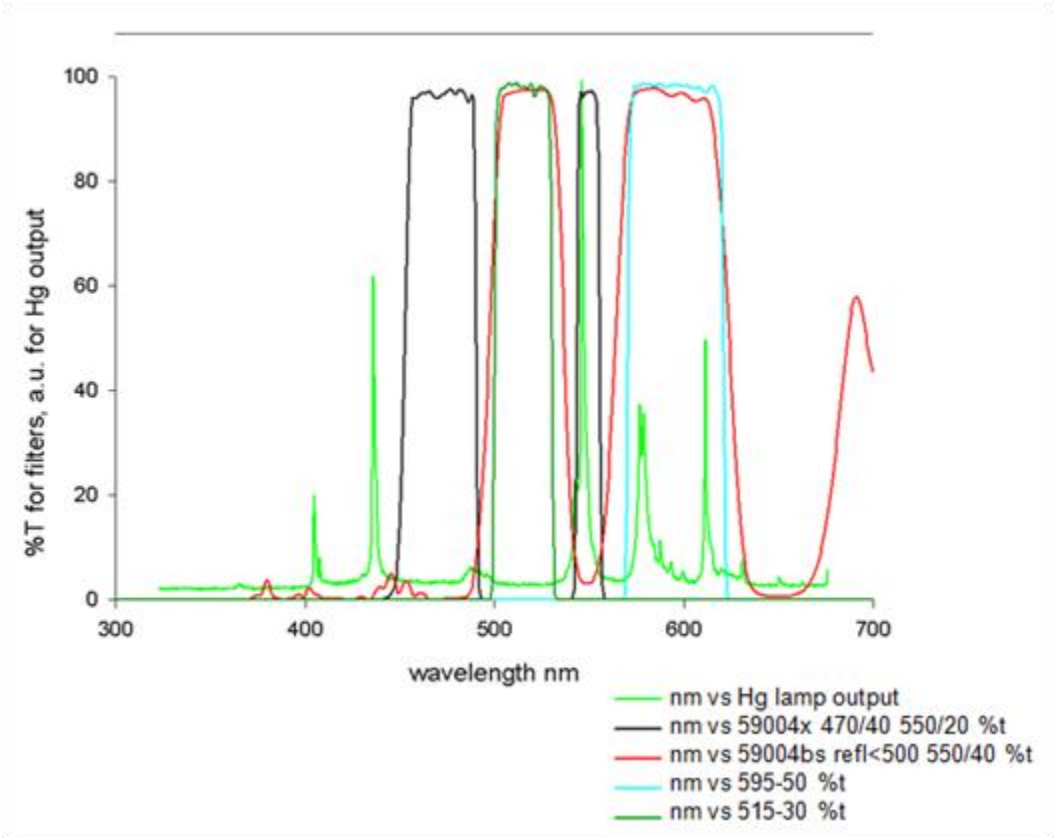


Figure 5: Optosplit microscope spectral characteristics

B. Reporters:

I. Quantum Dots (Qdot):

Qdots are nanometer sized crystalline particles of semiconductor materials, inorganic fluorophores with unique optical and spectroscopic properties. Qdots are brightly fluorescent and very photostable potentially yielding a high signal-to-noise ratio. Because of their photostability, broad absorption (Figures 6 [45] & 7 [46]) and well-defined, sharp emission spectra (Figures 6 & 7), they are very useful in single molecule detection at high repetition rates over long time periods. Biotin functionalized DNA oligomers and Streptavidin coated Qdots may be very helpful in nanoelectronics and nanophotonics. The blinking property (intermittent fluorescence) of Qdots

helps in the recognition of individual quantum dots. Although the absorption only weakly dependent on the dimensions of the Qdot, the emission wavelength is sensitively dependent on the size of the Qdot. Small size Qdots emit blue, larger ones emit red light [40]. Qdots have quantum yields of 0.65-0.85, with very high molar absorption coefficients of 10^5-10^6 $M^{-1}cm^{-1}$ and fluorescence lifetimes of more than 10 nsec [47].

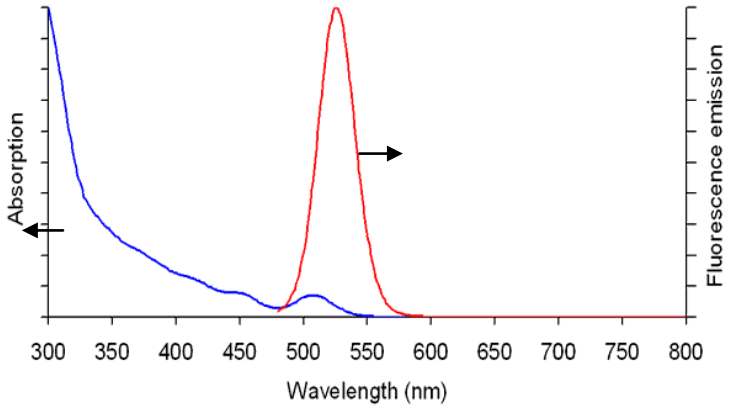


Figure 6: Absorption and fluorescence emission spectra of Qdot 525 streptavidin conjugate in pH 7.2 buffer

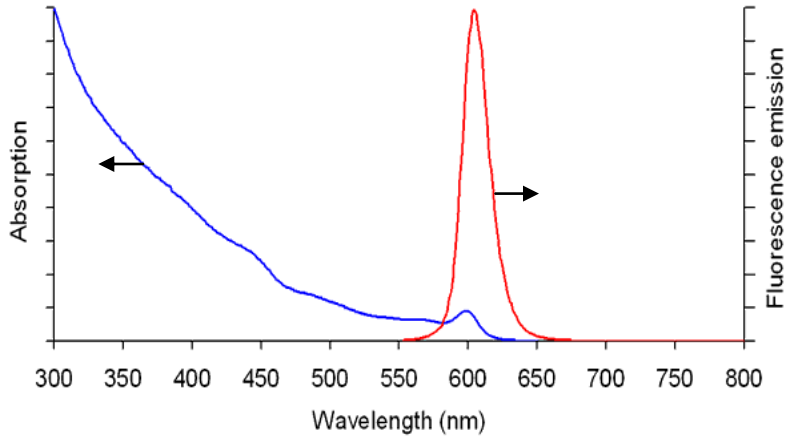


Figure 7: Absorption and fluorescence emission spectra of Qdot 605 streptavidin conjugate in pH 7.2 buffer

A schematic structure of a Qdot used in our study is shown in Figure 8. The heavy metal chalcogenide core is responsible for the fluorescent property of the Qdot. The nonemissive shell stabilizes the core, whereas the coating layer provides anchor sites for organic and biological ligands such as antibodies, peptides, and other organic molecules [34].

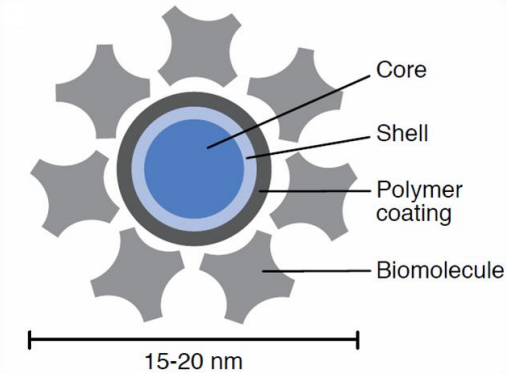


Figure 8: Structure of a semiconductor fluorescent Qdot construct

II. EGFP:

Enhanced green fluorescent protein (EGFP) is one of the red-shifted variants of wild-type GFP extracted from the jellyfish *Aquorea Victoria.* EGFP is a small protein with a barrel shape with a width of 2nm and a height of 4nm [48, 49] (Figure 9[50]). Through energy transfer, it converts the blue chemiluminescence of the protein *aequorin* into green fluorescent light. EGFP is a very stable protein containing 265 amino acids with excitation and emission peaks at 488 nm and 507 nm (Figure 10[42]) [41]. EGFP has a quantum yield of 0.6 [42], with a molar absorption coefficient of 49.550 $M^{-1}cm^{-1}$ and a lifetime of more than 2.51 nsecs [51].

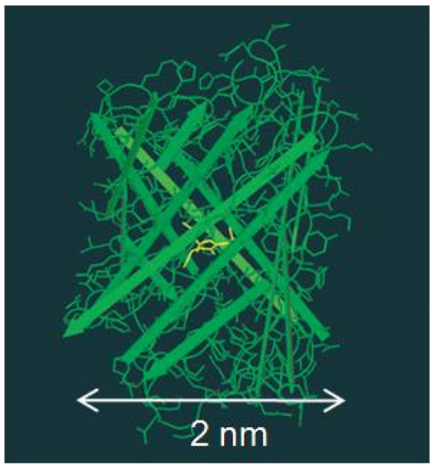


Figure 9: Structure of EGFP

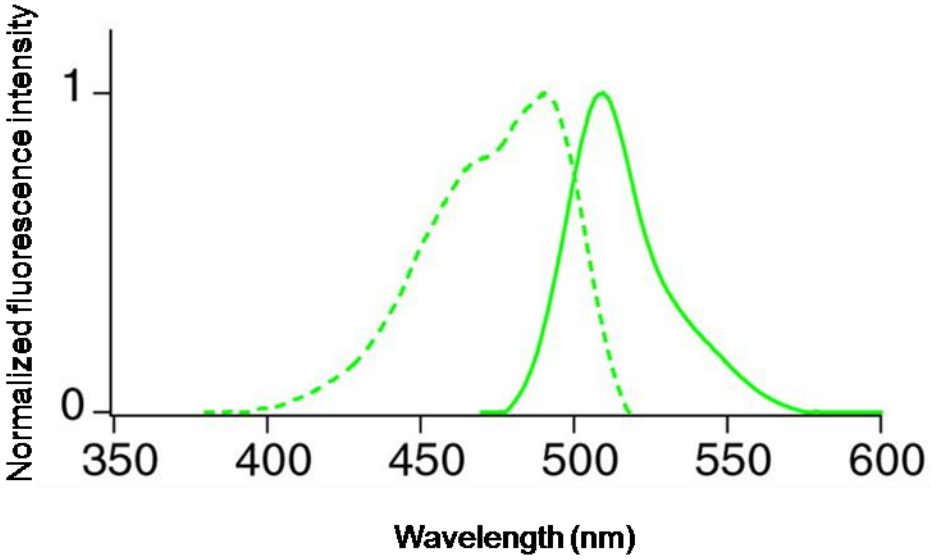


Figure 10: The excitation and emission spectra of EGFP with excitation and emission peaks at 488 nm and at 509 nm

III. Camera Calibration Standard:

In order to register (superimpose) the two different color channels, a fluorescent standard simultaneously visible in both channels was identified. These are polystyrene spheres with carboxylic acid surface activation, stained by swelling/impregnation with Surf green dye by Bang's labs. (Catalog no: FC02F/6484, 210nm diameter, Bangs Laboratories, Inc.) and used to produce calibration images. An example is provided in Figure 11. The spectrum of Surf Green dye is shown in Figure 12[(52)].

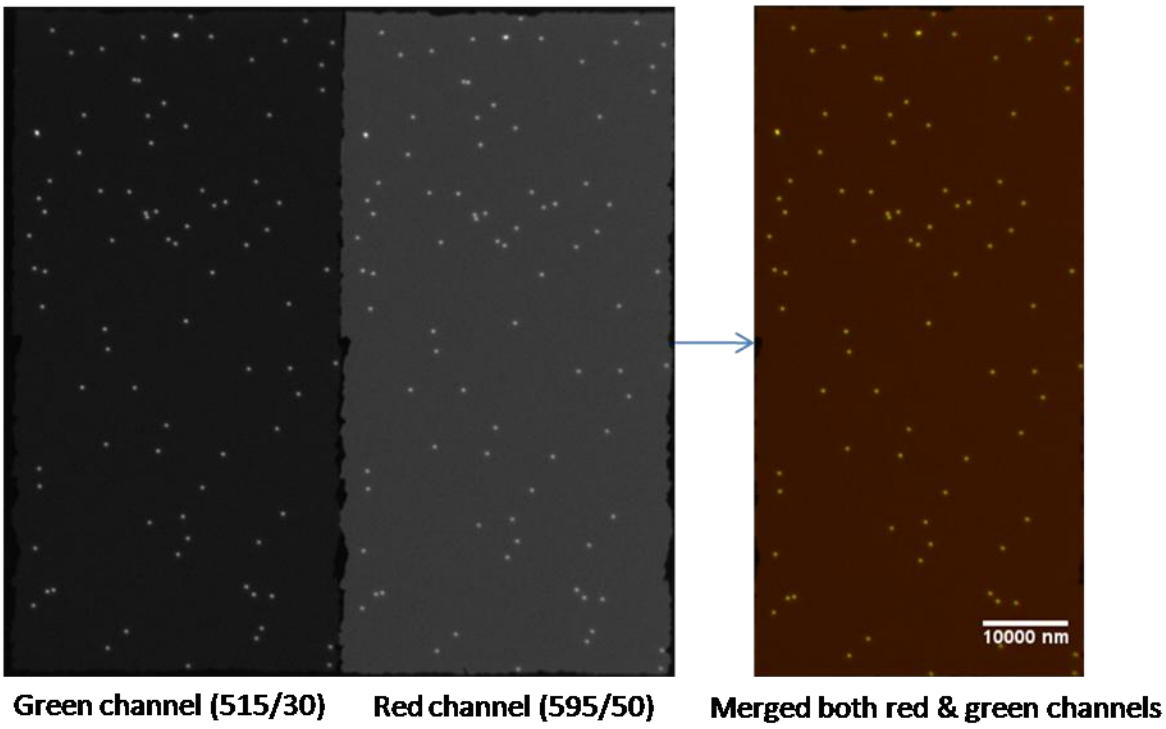


Figure 11: Two channel optosplit image of Surf Green (470/525) (left) and with both channels merged (right)

The boundaries of both red and green channels are cropped in the merged image, because the physical boundary of each image window is not well defined.

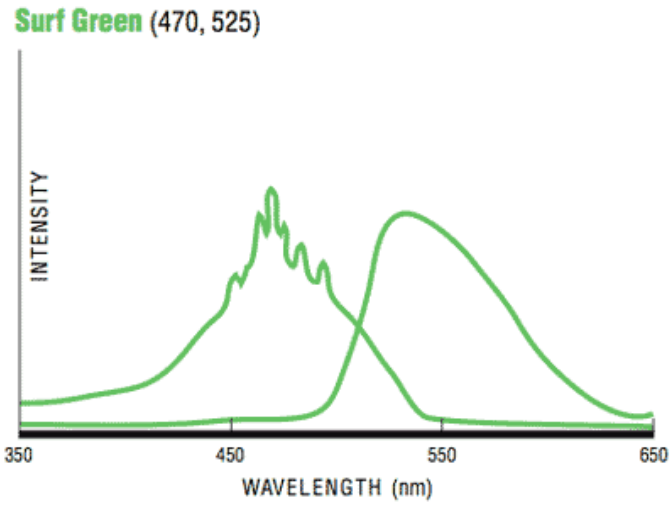


Figure 12: The excitation and emission spectra of Surf Green dye

IV. Organic Small Molecules:

Rhodamine is a succinimidyl ester of lissamine rhodamine B-labeled aminohexanoic acid (Figure 13A [53]). Rhodamine Red-X conjugate are small fluorescent organic molecules with absorption and emission maxima at 570nm and 590nm, respectively (Figure 13B [54]). Rhodamines are less intense compared to fluorescein because of a lower quantum yield, but more photostable and pH insensitive [43]. Rhodamine has a life time of 4 nsec with a quantum yield of 0.950 [55].

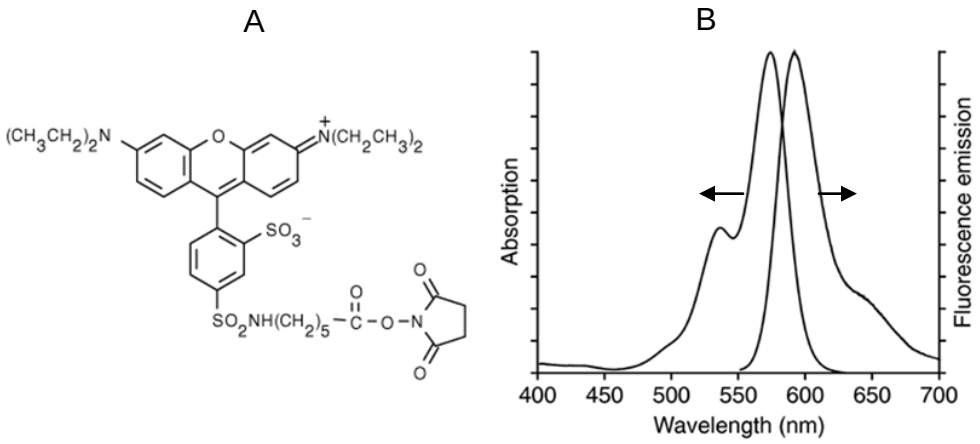


Figure 13: A) Chemical structure B) Absorption and fluorescence emission spectra of Rhodamine Red-X succinimidyl ester

NHS-Rhodamine is an amine-reactive fluorescent probe that contains a carboxy-succinimidyl ester group which reacts with primary amine groups (in G3 Dendrimer) at pH 7-9 (Invitrogen, Thermo scientific) with an excitation/emission of 544/576 nm, ε of 60,000 $M^{-1}Cm^{-1}$.

NHS-Fluorescein [5-(and 6-)carboxyfluorescein, succinimidyl ester]has excitation/emission of 450 to 500 nm /518 nm (Figure 14), ε of 68,000 $M^{-1}cm^{-1}$ [56], with quantum yield of 0.925 and life time of 4 nsecs [55] are also amine-reactive fluorescent probes that contain a carboxy-succinimidyl ester group which reacts with primary amine groups (in G3 Dendrimer) at pH 7-9 (Invitrogen, Thermo scientific).

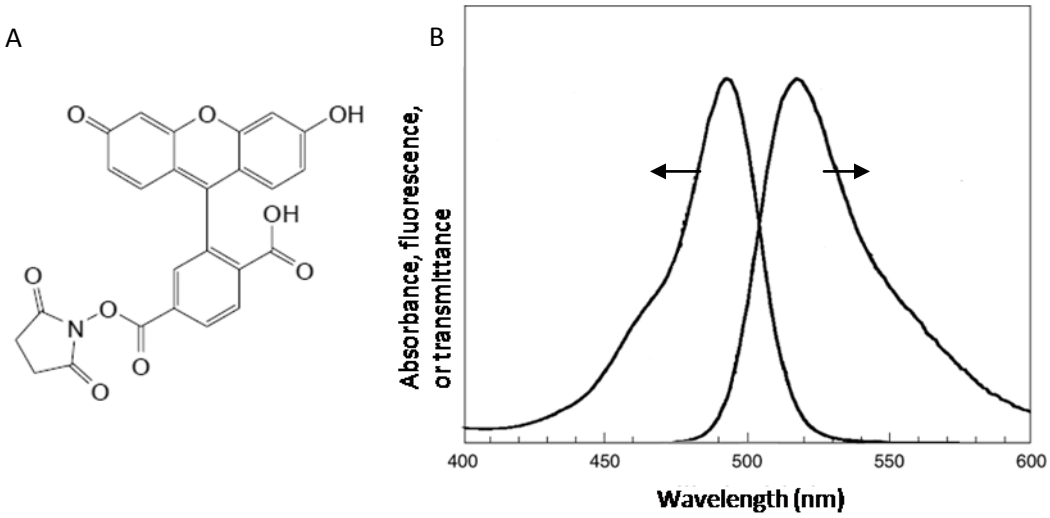


Figure 14: A) Chemical structure B) Absorption and fluorescence emission spectra of NHS-Fluorescein

MATERIALS AND METHODS

A. Substrate cleaning method:

Place Fisher brand premium coverslip (#1.5~170um) in a Teflon coverslip holder and place the holder in a very clean 50ml beaker (washed with acetone and exposed to UV light for 10min) containing 40ml of Acetone (HPLC grade, Sigma Aldrich). Coverslips are sonicated for 30 minutes by placing in a sonicator (FS20, Fisher scientific), rinsed with water (H_2O prepared as described in next section), and dried with flowing N_2 gas. To eliminate remaining fluorescent species, the coverslips were exposed to a low pressure UV lamp (SEN LIGHT CORP., Japan, UVL20US-60) for 10min (Figure 15). Distance from lamp to coverslip was ~2cm for a dosage power of 125mW/cm^2.

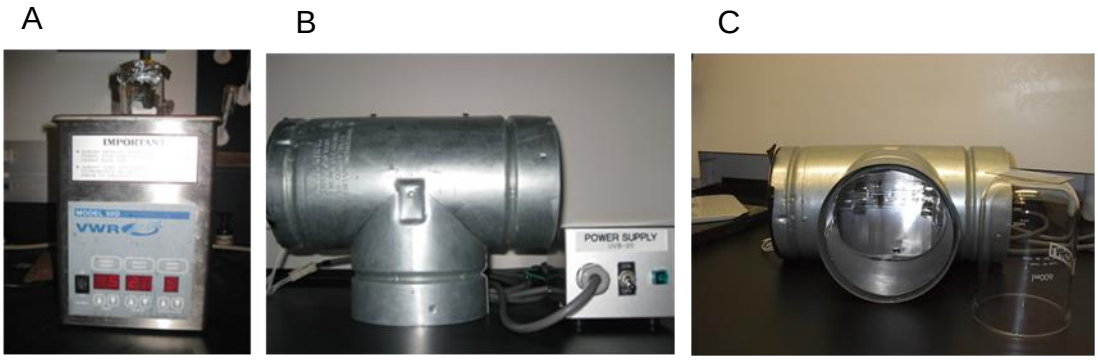


Figure 15: Sonicator and UV exposure system for substrate cleaning A) Sonicator, B) UV coverslip treatment chamber. C) UV bulb is at top of chamber, cover slips are raised to within 2 cm of source on platform within enclosure

B. Water Preparation:

House water (from MU BBSC D.I. tap, water deionized by reverse osmosis) is further purified by glass distillation. This water still contains fluorescent contamination. Water distillation is followed by UV light exposure using a high intensity UV chamber (Minipure by

Atlantic Ultraviolet Corporation, dosage ~ 120mW/cm^2) to photo bleach these contaminants. Components of this setup are shown in Figure 16.Note that the receiver is emptied into the UV treatment chamber in a batch process. 200ml are UV treated at one time.

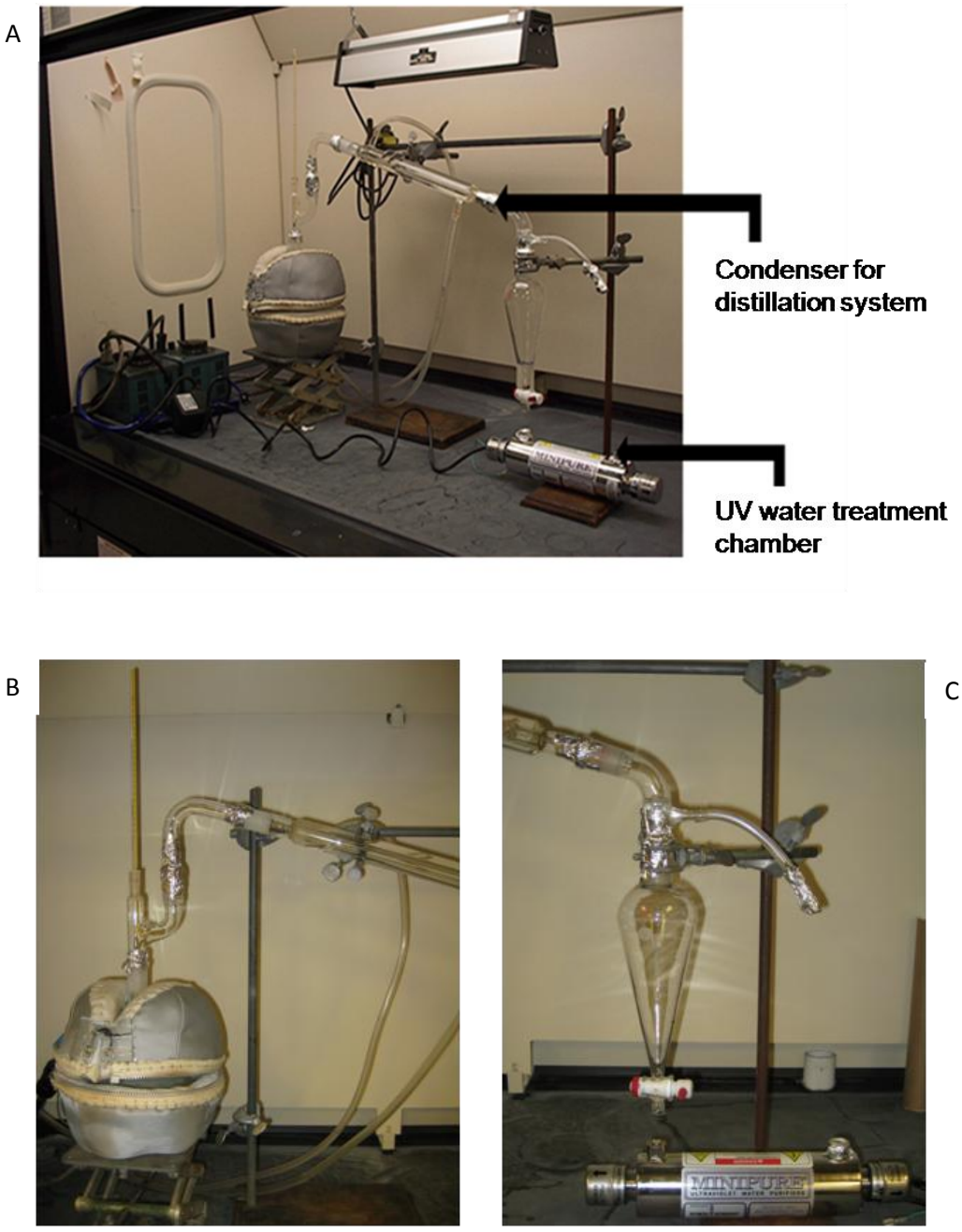


Figure 16: A) System used to prepare UV treated glass distilled water B) Closeup of distillation apparatus C) Closeup of receiver and UV treatment chamber.

C. Preparation of Triangular Origami with Qdots:

The folding diagram for the triangular Origami construct with two Qdots is shown in Figure 17.

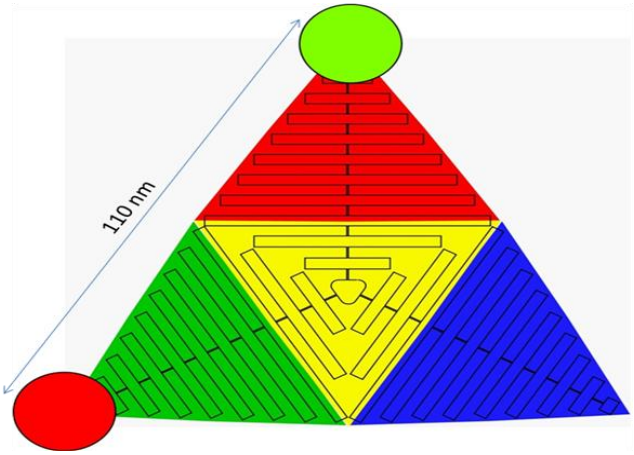


Figure 17: Schematic of Triangular Origami with 525 nm (green) and 605 nm (red) Qdots

(Designed by Hong Zhong)

a) Synthesis:

The single stranded M13mp18 viral DNA backbone plasmid was purchased from New England Biolabs. The DNA staple strands were purchased from Integrated DNA Technologies, Inc, HPLC grade. Before use, all strands are diluted in fluorophore free water to a concentration of 1µM. A molar ratio of 1:100 was used between the long M13mp18 DNA and the staple strands. 50µl of all staple strands, 5µl of 0.1µM M13mp18, 50µl of 10XTAE-100mM Mg^{2+} buffer and make up with fluorophore water to 500µl, and divided into 0.2ml PCR tube. Origami nanoconstruct hybridization was performed in a 1x TAE buffer with 10mM $MgCl_2$ (pH 7.8) by cooling slowly from 90ºC to 20 ºC at 1 ºC per minute rate in the PCR machine (part no: PCR 96^{plus}, MWG BIOTECH). For example, to prepare 500ul of 0.2 nM Triangular Origami with 525 nm Qdot (Cat. No. Q10041MP, Invitrogen) and 605 nm Qdot (Cat. No. Q10001MP, invitrogen) sample, incubate 20µl of 20 nM biotinylated DNA staple (red)

(5Biosg/TTAAAACCAAAATATT;T6-001-B1-T1) with 20µl of 20 nM red Qdots and 20µl of 20 nM biotinylated DNA staple (green) (TTCCAGCGCCAAAGTT/3Bio/;T6-002-B1-T1) with 20µl of 20 nM green Qdots for 3 hrs. Add both these staples with all other DNA strands (appendix under Triangular Origami sequences) with1:1 ratio. Add 5ul of 1 nM single stranded M13mp18 viral DNA to a 1.5 µl centrifuge tube, add 50ul of 10x TAE buffer with 10 mM $MgCl_2$ then add 50ul of 10 nM all DNA strands then make up with ddH_2O. Then keep it in PCR machine, after annealing incubate in 4^oC refrigerator overnight.

b) Procedure for using YM-100 filter (Millipore) to purify origami:

This process removes most single stranded DNA from the origami product.

1. Add 200 µl Origami with 500µl 1xTAE 10 mM Mg2+ buffer into the Microcon® Centrifugal filter unit (Catalog no: YM100, Millipore).
2. Centrifuge for 3.5 min at 6000rpm in microcentrifuge (Model no:SD110, Clover laboratories)
3. Add 1XTAE mM Mg^{++} buffer to the unit until the whole volume reaches 700µl
4. Repeat steps 2 to 3, 2-4 times
5. Invert the Microcon® Centrifugal filter unit into a 1.5ml autoclaved centrifuge tube and collect the sample.

D. Preparation of Circular Origami with EGFP:

The folding diagram for the circular Origami construct with two EGFP molecules is shown in Figure 18 [57]. EGFP molecules are associated with DNA strands using an NTA / His tag linker, as described in reference 57.

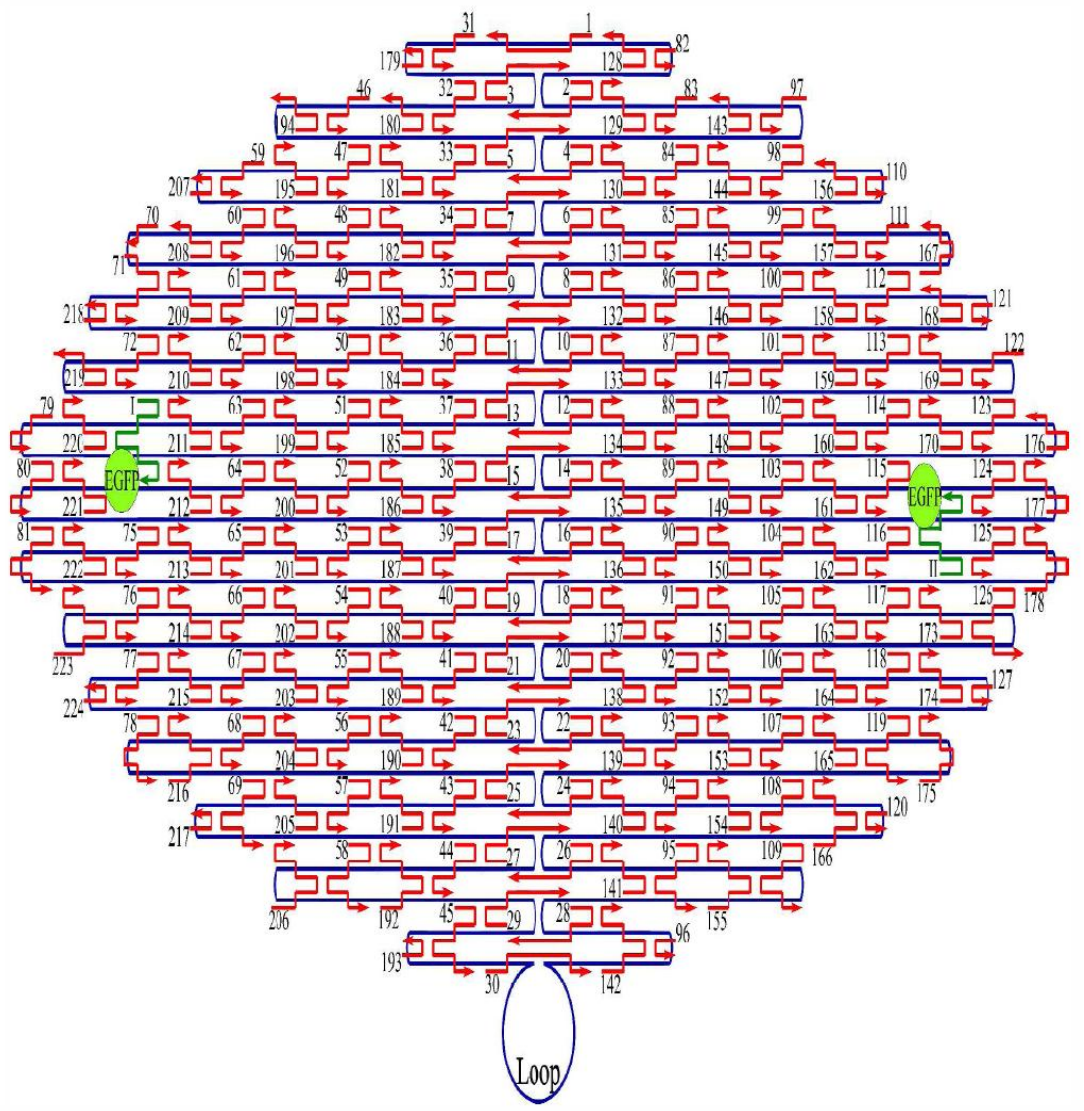


Figure 18: Schematic showing the design of circular DNA Origami

The figure displays each of the two EGFP attachment positions. Modified strand I replaces original strands 73 and 74, and modified strand II replaces original strands 171 and 172, (see Appendix-B, Table 3 for the sequences of these two modified strands I and II).

Wanqiu Shen of our laboratory prepared the sample on mica and this Origami system is described in detail in Reference 57.

E. Preparation of Fluorescently labeled Dendrimers on Triangular Origami:

The folding diagram for the triangular Origami construct with two different Dendrimer labels is shown in Figure 19.

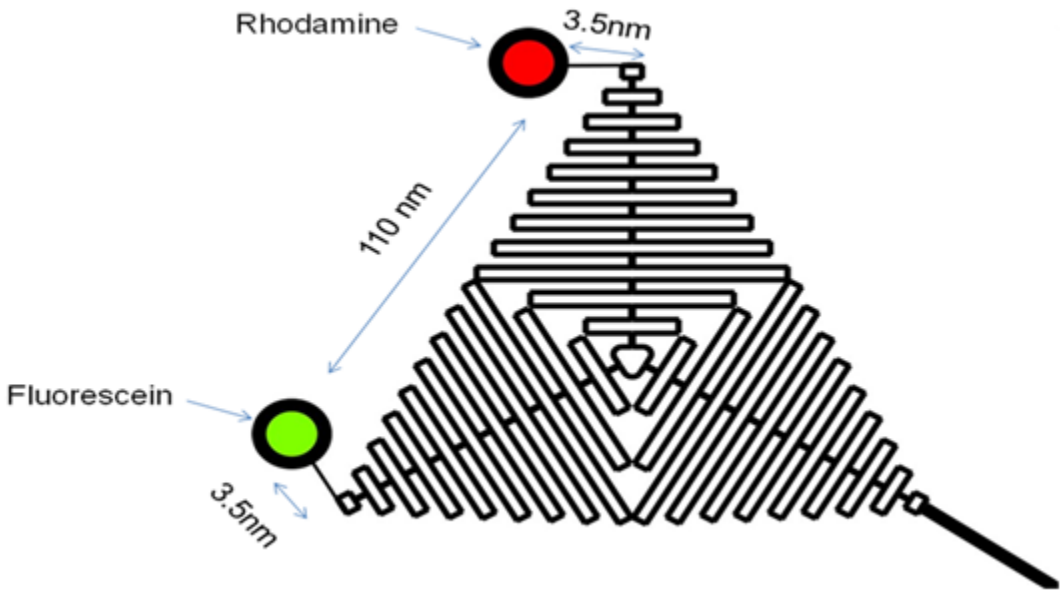


Figure 19: Triangular Origami construct with NHS- Rhodamine and NHS-Fluorescein labeled dendrimers

(Designed by Hong Zhong)

Synthesis of components of the construct fluorescently labeled dendrimers on Triangular Origami:

This is a three part procedure. First, two fluorophore – dendrimer – DNA macromolecules are prepared. After preparation of the Fluorescein – Dendrimer - DNA Complex (Figure 20) and the Rhodamine – Dendrimer - DNA Complex (Figure 21), they are added to the single stranded M13mp18 viral DNA and the DNA staple strands (for sequence information see Appendix C under Triangular Origami sequences). A molar ratio of 1:100 was used between the long M13mp18 DNA and the staple strands, Origami nanoconstruct hybridization was performed in a 1x TAE buffer with 10mM $MgCl_2$ (pH 7.8) by cooling slowly from 90ºC to 20 ºC at a 1 ºC per minute rate in a PCR machine (catalog no: Primus 96 plus, MWG Biotech).

I. Synthesis of the Fluorescein – Dendrimer - DNA Complex:

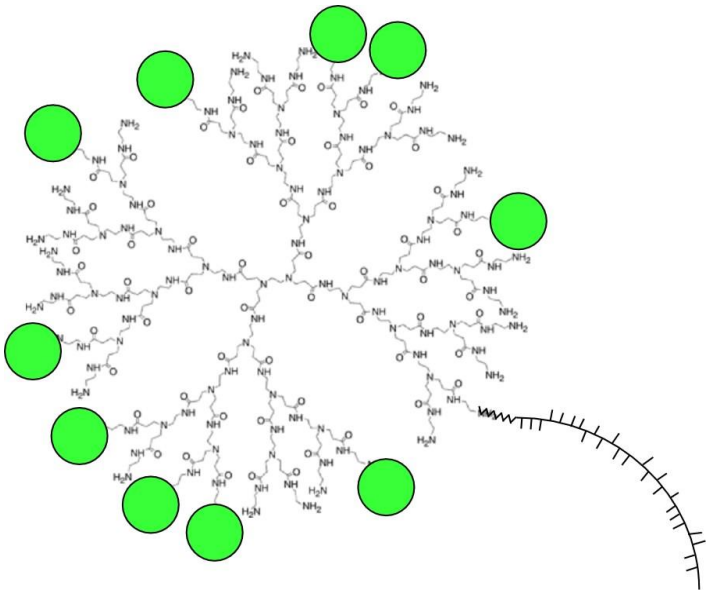


Figure 20: G3 Dendrimer with 10 Fluorescein molecules attached to DNA through a spacer molecule

This procedure is for attaching 10:1 ratio of Fluorescein: dendrimer to SH-DNA. By combining dendrimer with SSMCC in a 1:2 ratio & NHS- Fluorescein in a 1:10 ratio (Dendrimer: NHS-Fluorescein).

1. Weigh 0.001 g of SSMCC (Sulfo-SMCC, Catalog no:22322, Pierce Biotechnology) and transfer it to a 1.5ml centrifuge tube. Weigh 0.005gm of NHS-Fluorescein (Catalog no: 53029 , Thermo Scientific) and transfer it to the same tube that has SSMCC. Add 100µl DMF (N, N Dimethylformamide, Catalog no: 270547, Sigma Aldrich) and 900µl of phosphate buffer, vortex them for 10 seconds and add 50µl of 25 mM G3 dendrimer (Catalog no:412422-5G, Sigma Aldrich), vortex it again then incubate for 40min at room temperature.

2. After incubation period run the above solution through a PD10 column to remove any unbound or extra NHS-Fluorescein molecules.

3. Mix 50µl of 25 mM dendrimer solution with HS-DNA (5ThioMC6-D/TTTAAAACCAAAATATTTGCGAGAACGATA, IDT DNA) at 50:1 (50 dendrimer: 1 DNA) ratio and incubate it overnight in a -4^{o}C freezer.

4. Dialyze the sample with a 7k MW cutoff filter for overnight and concentrate the sample using the concentrator (part name: concentrator 5301, Eppendorf) and purify the samples by running through a 8 % Polyacrylamide gel using the procedure below.

5. Prepare 8% native polyacrylamide gel

   40% acrylamide (catalog no; BP1406, Fisher Bioreagents) – 6ml

   10x TAE (catalog no; BP1335, Fisher Bioreagents) – 3ml

10% APS – 250 µl

TEMED (catalog no; T9281, Sigma Aldrich) - 20 µl, ddH_20 – 21ml

6. Run gel at 260 volts for 2 hours after loading DNA samples into wells with 1 kb DNA ladder(catalog no: G5711,Promega) in another well.

7. Using UV gel doc cut the separated band.

II. Synthesis of the Rhodamine – Dendrimer - DNA Complex :

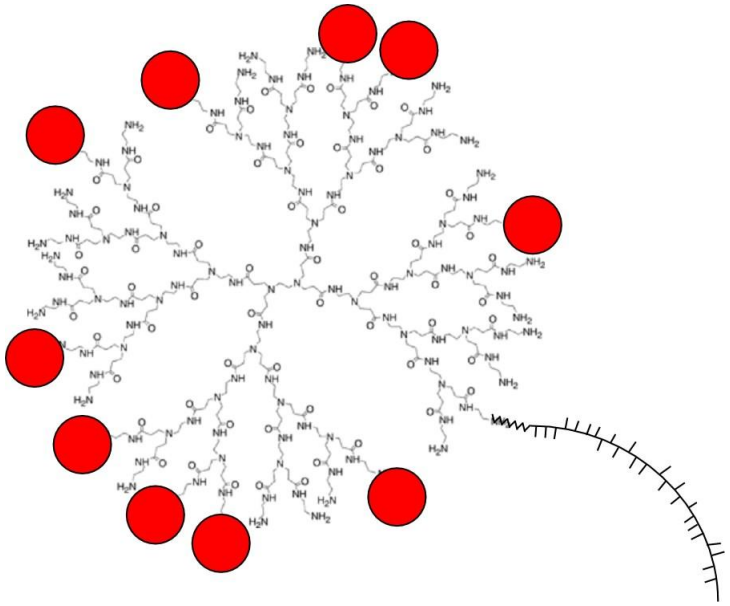


Figure 21: G3 Dendrimer with 10 Rhodamine molecules attached to DNA through a spacer molecule

This procedure is for attaching 10:1 ratio of Rhodamine: dendrimer to SH-DNA by combining dendrimer with SSMCC in a 1:2 ratio & NHS- Rhodamine in a 1:10 ratio (Dendrimer: NHS-Rhodamine).

1. Weigh 0.001 g of SSMCC (Sulfo-SMCC, Catalog no:22322, Pierce Biotechnology) and transfer it to a 1.5ml centrifuge tube. Weigh 0.005gm of NHS-Rhodamine (Catalog no: 40406, Thermo Scientific) and transfer it to the same tube that has SSMCC. Add 100µl DMF (N, N Dimethylformamide,Catalog no: 270547, Sigma Aldrich) and 900µl of phosphate buffer, vortex them for 10 seconds and add 50µl of 25 mM G3 dendrimer (Catalog no:412422-5G, Sigma Aldrich), vortex it again then incubate for 40min at room temperature.

2. After incubation period run the above solution through a PD10 column to remove any unbound or extra NHS-Rhodamine molecules.

3. Mix 50µl of 25 mM dendrimer solution with HS-DNA (5ThioMC6D/TTTCCAGCGCCAAAGTTTA CAAAAGGTTTA, IDT DNA) at 50:1 (50 dendrimer: 1 DNA) ratio and incubate it overnight in a -4^{o} freezer.

4. Dialyze the sample with a 7k MW cutoff filter for overnight and concentrate the sample using the concentrator (part name: concentrator 5301, Eppendorf) and purify the samples by running through a 10% Polyacrylamide gel as explained above.

F. Preparation of Rectangular Origami with two Rhodamine molecule labels:

The folding diagram for the rectangular Origami construct with two rhodamine labels is shown in Figure 22.

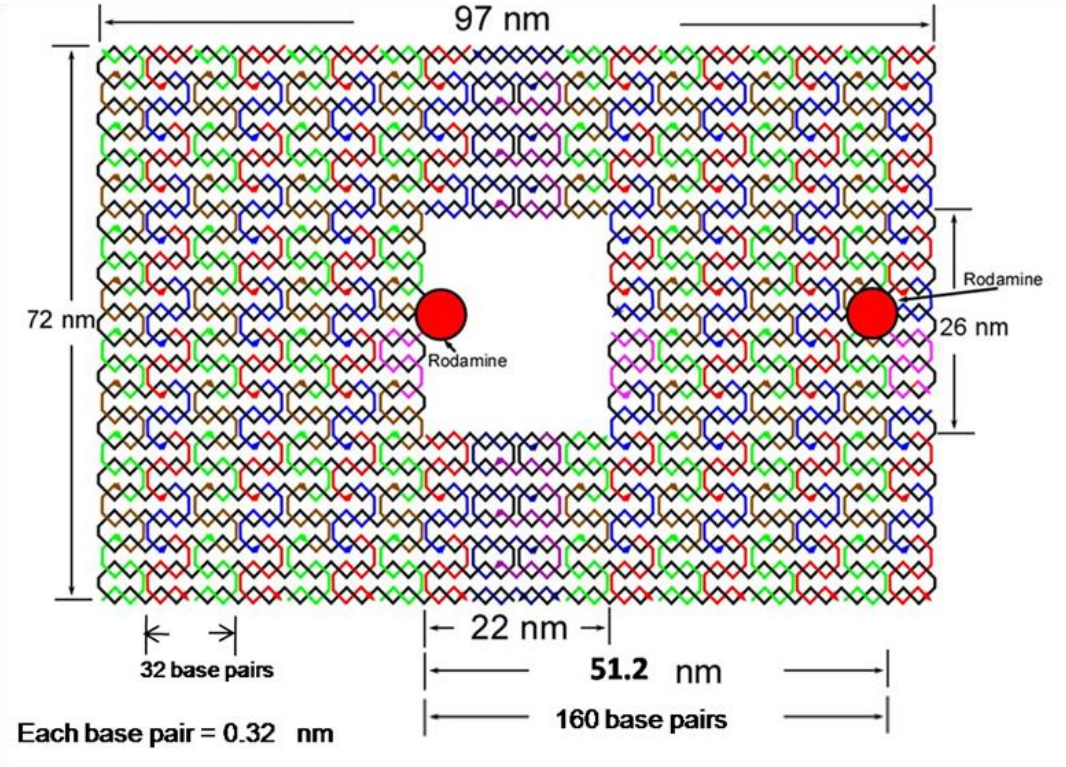


Figure 22: Rectangular Origami with Two Rhodamine labels

Mix all DNA staples (Appendix-A, Tabel 2) with M13MP18 at a 100:1 ratio. (Including modified DNA with rhodamine; **5**-RhoRXN/CTGCGGAATCGTCATATGGATAGCGTCC AATATTTTATTATTACAGGTAGAAGAACTAAC-3' rhodamine modification 60mer for the aperture of the rectangular Origami and 5-RhoR-XN/TCCCGACTTGCGGGAGAATTTACG

AGCATGTAAATAATATCCCATCCT-3' rhodamine modification 48mer for the edge of the rectangular Origami). To prepare 500 µl of rectangular origami with two rhodamine labels, Add 5µl of 1 nM single stranded M13mp18 viral DNA to a 1.5 µl centrifuge tube, add 50µl of 10x TAE-100mM $Mg(OAc)_2$ buffer pH 7.8 then add 50 µl of 10 nM all DNA strands then add 50µl of modified DNA with rhodamine then make up with ddH_2O. Then anneal it in Thermalcycler machine, after annealing incubate in 4^oC refrigerator overnight.

Anneal the mixture from 90^{o} C to 20^{o} C at 1 degree/minute cooling rate. Incubate the mixture at 4^{o} C for at least 30 min afterwards and do purification as mentioned above using a YM-100(Millipore) centrifugal filter as explained above under the triangular Origami discussion.

G. Sample preparation on coverslip:

The protocol for the preparation of the sample on the coverslip is the same for all DNA constructs. Begin with a clean coverslip. Make a scratch on the coverslip with a diamond scribe. This scratch provides an object on the top surface of the coverslip to focus on during white light imaging with the microscope. Clean the coverslip (clean according to the method described under substrate cleaning). Treat with 10µl of 25 mM $MgCl_2$ (solution should be UV photobleached) for 10 min and then wash coverslip with photobleached, fluorophore free H_2O and then dry with flowing N_2 gas. After preparation of the DNA Origami with 2 rhodamine molecule labels, as mentioned above, put 15µl of sample on the clean $MgCl_2$ pretreated coverslips. Incubate at room temperature for 10 min and then wash coverslip with photobleached water and then dry with flowing N_2 gas. To obtain images of well separated origami with a sufficient number of Origami per square centimeter of surface area of the coverslip, spread 15µl of a 20 fM DNA Origami solution on ~ 1 cm^2 of the cleaned coverslip using the pipette tip as the spreader.

H. Kinetics of Photobleaching studies:

To decrease the rate of photobleaching of fluorophores during imaging a small enclosure was constructed to surround the sample. Room air was displaced with N_2 by flowing N_2 gas from a gas cylinder through the protective enclosure. The % oxygen at the sample surface was determined by using an O_2 gas sensor (O_2-BTA, Vernier). Note that this sensor must be maintained in an upright configuration. % oxygen data was collected as a function of time using

a Lab Quest display module (order code: LABQ, Vernier Software & Technology). Because covering the sample with the small enclosure could cause reflection of the excitation light from the objective and produce additional background fluorescence signal, the inside of the enclosure was covered with Black Non-Adhesive Flock paper (Catalog no: NT54-571, EDMUND OPTICS). A picture of the microscope with the O_2 sensor and the small enclosure on the stage is shown in Figure 23.

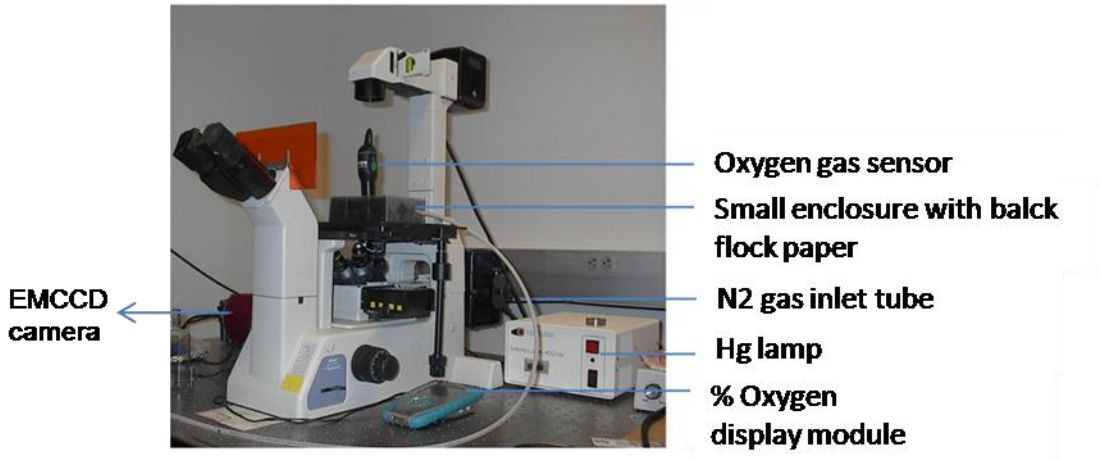


Figure 23: Photograph of modification used for controlled atmosphere tests

I. Data Collection:

In order to allow the system to equilibrate, the camera and excitation lamp were turned on one hour before acquiring images. After preparation of the samples on the coverslip as explained above, samples were mounted on the inverted microscope and the sample was covered with the small enclosure. Nitrogen gas flow through the enclosure was activated until the display module registered a % oxygen reading of less than 0.2%. Open the QImaging software on the remotely located computer monitor to capture videos. Click the preview button for fine focus adjustment

and hit snap. The software panel images below contain all of the settings normally used for acquiring images (Figure 24).

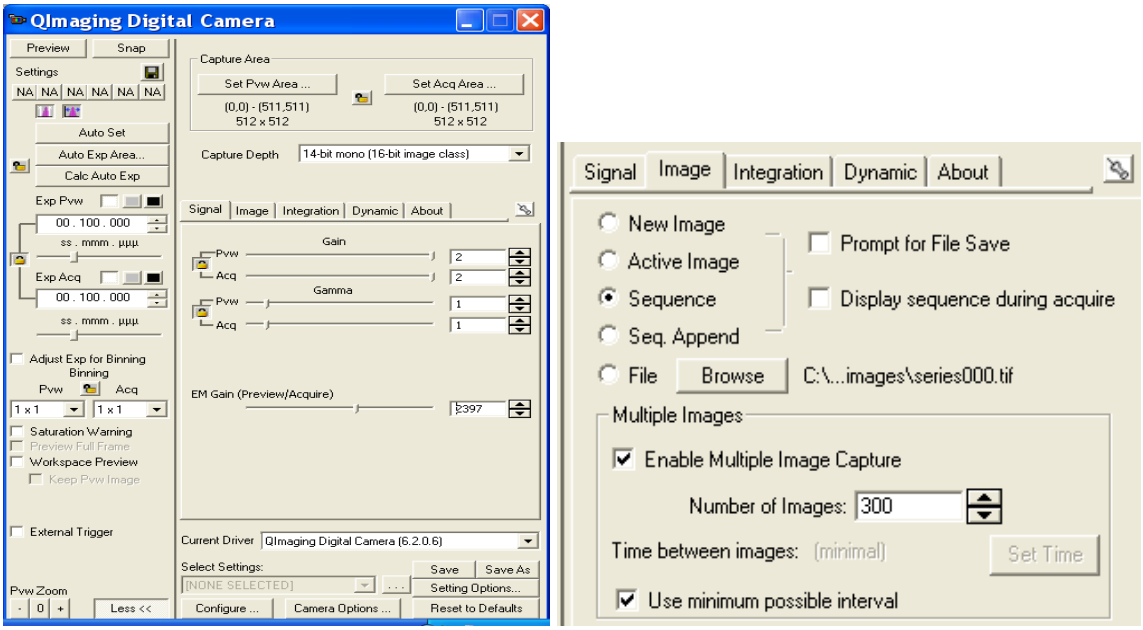


Figure 24: QCapture software panel settings

Exposure time is usually set to 100 milliseconds. Transfer time adds approximately 40 msecs to 45 msecs to the total frame time, yielding an approximate frame rate of 6.89 Hz. Each frame is time stamped with the real time for documentation purposes.

J. Data Analysis and processing:

I. Image Registration:

The MATLAB Image processing tool box (The Math Works, Natick, Massachusetts) will accurately find the local weighted mean center of fluorophores. This is a three step process, finding the fiducial data (data coming from one channel of the microscope image), transforming

real data onto the fiducial data and calculating the distance between fluorophores. Collection of fiducial data (surf green images, Figure 11) is important for the calculation of the mapping function from one channel to another channel. The position of each bead in each channel must be determined. Control points are points with known positions for each member of the pair in the image. Six control points close to each other in the first channel are selected. Using these control points from channel 1 along with their counterparts in channel 2, the second order polynomial transform is determined using a least squares fit. By measuring the distance from the selected control point to the furthest of the six surrounding control points in channel 1, it is possible to determine the radius of influence that this polynomial will have in the final transformation. The "Cp2tform" command of Matlab will do all these calculations at the same time. The error can be evaluated by sequentially leaving out a pair of control points and calculating local weighted mean mapping for this pair using the remaining pairs of control points. In Matlab the "Imtransform" function will perform this process. Once the transformation has been determined, it can be applied to various samples to find the location of fluorophores [58].

II. Developing and implementing SHRIMP method:

Because of programming difficulties with MATLAB, the software currently used is comprised of MATLAB and LabVIEW, developed by the research group Anuradha Rajulapati, Nathaniel Crow, Rusty Parrott, Alessandro Giusti (Varese, Italy) and David Neff. The software is composed of 3 modules, video processing, spot selection and gaussian fitting.

i. Video Processing:

After acquiring fluorescence images with the EMCCD camera, videos in sequence file format (~500 frames with an image capture frequency of 6.89 Hz.) are converted into avi files using ImageJ (1.42 version)[59].

ii. Observation of 2 step Photobleaching by using software written in MATLAB:

The data analysis will be explained by taking one image as an example (Figure 25).

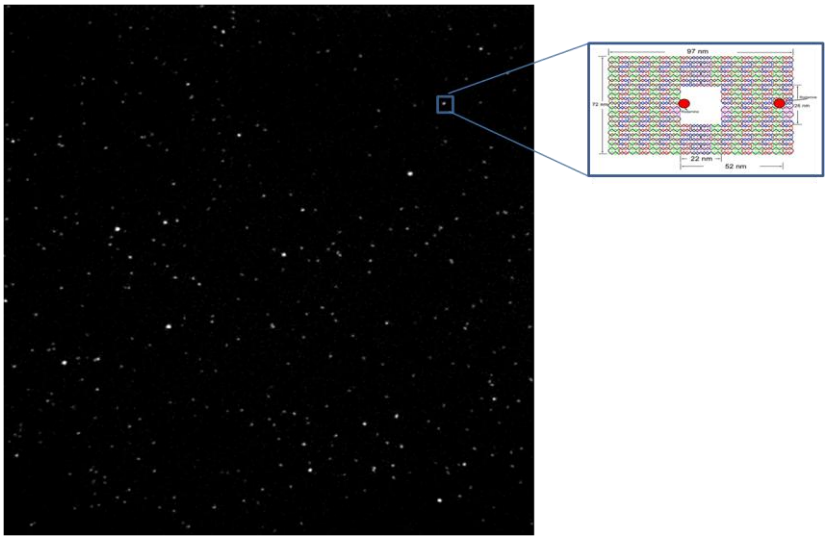


Figure 25: Fluorescence image of Rectangular DNA Origami with 2 rhodamine molecules on coverslip taken with the microscope with a 100 x objective (155nm/pixel) (without splitter)

Due to variability in the fluorescence intensity among molecules, it is not possible to determine when photobleaching events occurred for some observed molecules. In the image shown above (Figure 25), the intensity of a few spots is significantly higher than that of the other spots. The spots that are less intense are more likely to be single molecules (each corresponding to an Origami with 2 rhodamine molecules), while spots that are brighter may indicate clustering of 2, 3 or more Origami. To increase throughput, labeled DNA Origami images were analyzed by using software written in MATLAB (Appendix-D, Table 5). Each image frame contains several DNA Origami molecules. The software picks individual, well-separated spots, then plots a graph of intensity (in arbitrary units on the Y-axis) vs. Frame number (on the X-axis) for the

spots picked. These plots are displayed in a serial order with respective numbering related to the location of the object in a composite image. From these outputs, spots which display clean, two-step intensity profiles as shown in Figure 26 are manually selected from these outputs.

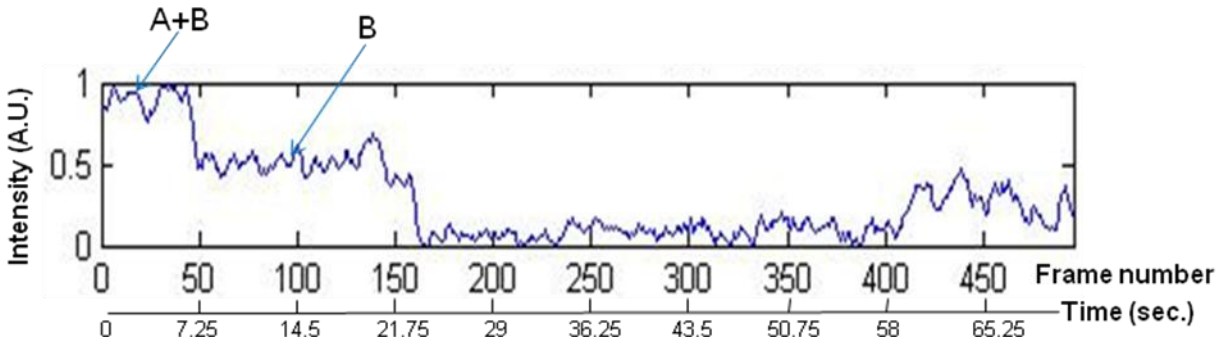


Figure 26: Plot of Intensity versus frame number for two closely spaced rhodamine molecules in rectangular Origami, showing two-step photobleaching behavior

iii. Performing a two-dimensional Gaussian fit (Lab VIEW):

For a single spot that is displaying 2 step photobleaching, the maximum number of frames that are common to both A+B and B time domains are cropped (8x8 pixel region) and averaged with MATLAB code (Appendix-D,Table 6). The Lab VIEW code will then process images output by MATLAB and will determine the centers of the 2 single fluorophores by fitting each to a 2 dimensional Gaussian function. The separation between the two single fluorophores is computed using the distance formula by: d= $\sqrt{(X_1 - X_2)^2 + (Y_1 - Y_2)^2}$). The Gaussian peaks for individual fluorophores will appear as shown in Figure 27. The resulting full screen image of the software panel displaying 3 gaussian peaks along with distance information is presented in the Figure 28.

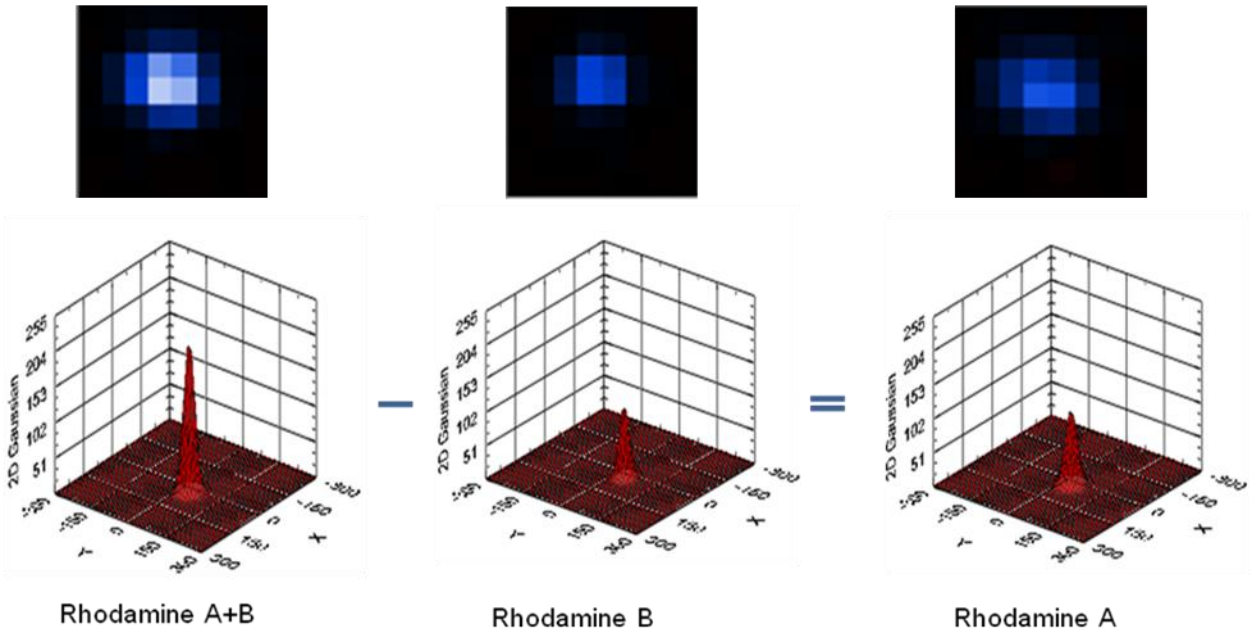


Figure 27: Gaussian curves for before (Rhodamine A+B) and after photobleaching (Rhodamine B) and the subtraction of those two (Rhodamine A)

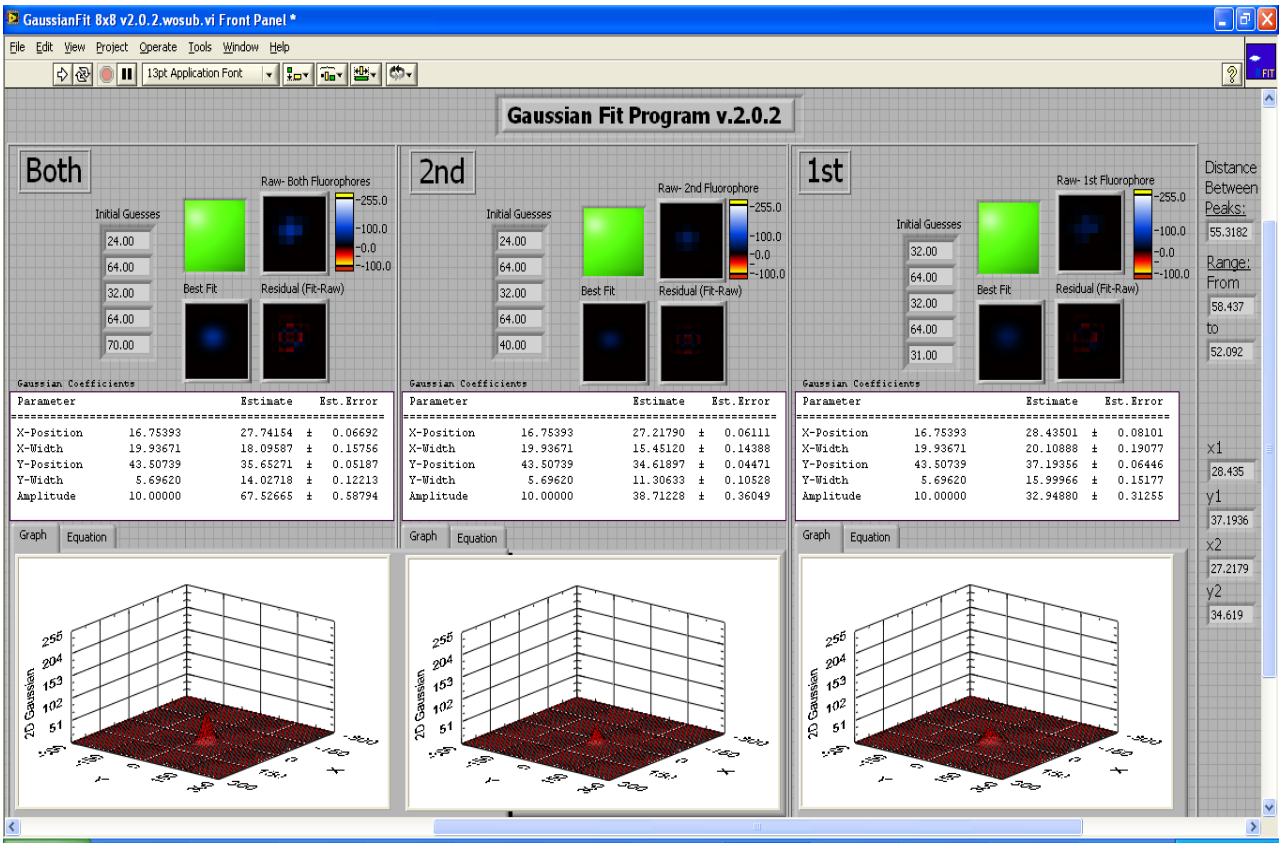


Figure 28: Lab VIEW software panel showing 3 individual Gaussian peaks and calculated distance information

RESULTS AND DISCUSSION

A. Substrate Cleaning:

When imaging large samples such as cultured cells, researchers have generally not been concerned with contamination of cover-slips and solutions by fluorescent molecules because the sample is much brighter than the background. However when imaging single molecules as in the present work, the CCD detector reveals any fluorescent material that has contaminated the coverglass surface (Figure 29A). With background contamination this severe, one cannot be sure whether they are imaging sample molecules or contamination molecules. For that reason, all coverslips and solutions are treated as described under cleaning procedures to increase the signal-to-background ratio (Figure 29B). Light itself does not destroy the molecules. They are being photo-oxidized by reacting excited state molecules with oxygen molecules, leading to oxidation. Removing any protective oils on the surface of the glass by rinsing with acetone and then exposing the surface to UV light is a gentle method to destroy the fluorophores (Figure 29C).

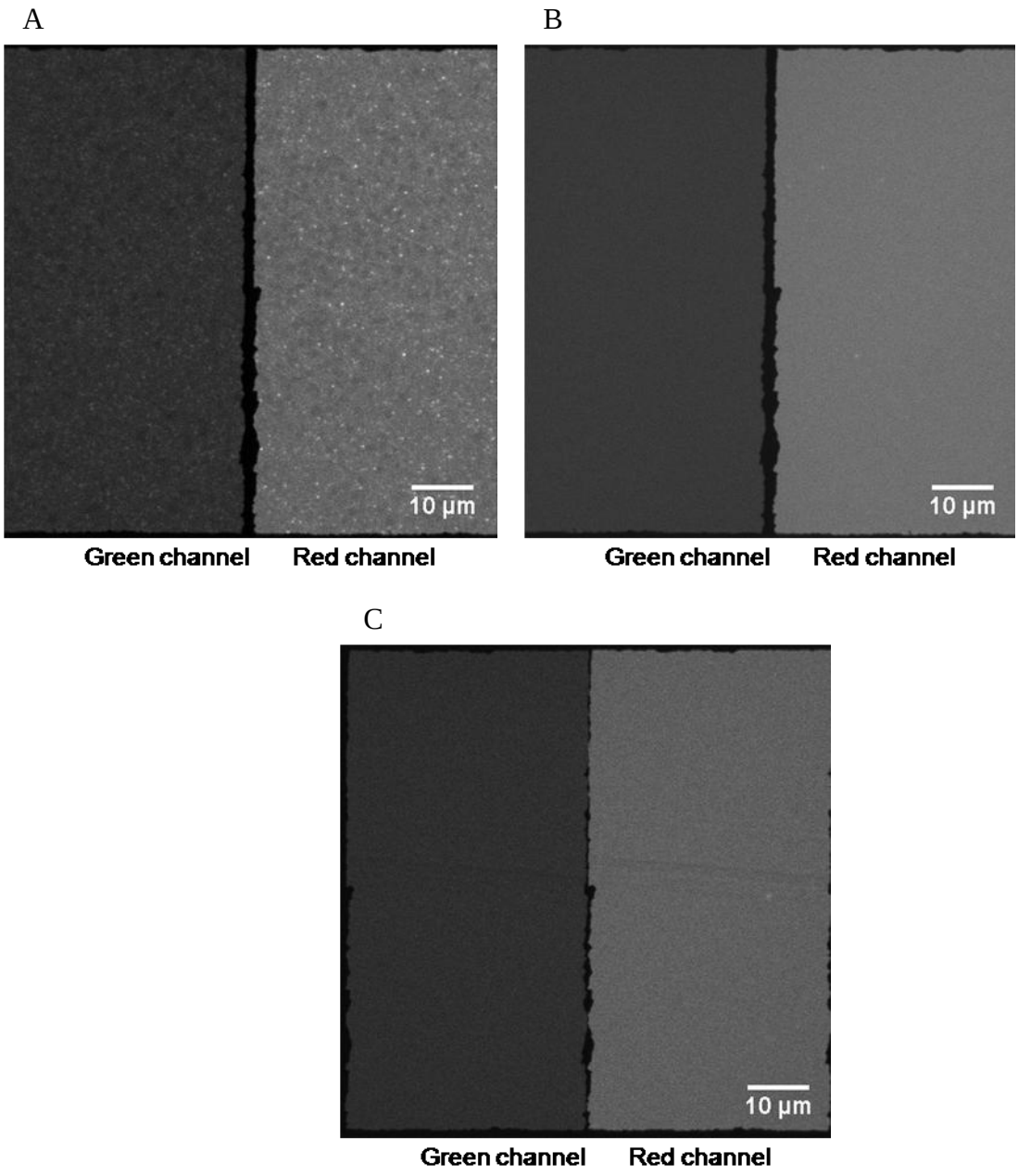


Figure 29: Comparison of coverslip contamination A) as received, B) after cleaning with acetone, and C) after UV treatment.

Figure 29 A) represents a fluorescent image of an untreated coverslip directly from the box, which shows significant fluorescent contamination in both the red and the green channels. Figure 29 B) Shows a coverslip after 30min sonication with acetone at room temperature. Comparing

the untreated and acetone-treated coverslips, it is clear that a significant reduction in background particles has been achieved, although a few fluorescent contaminants remain. Figure 29 C) shows the fluorescence image of a coverslip, fully UV treated as explained above, with virtually no fluorescent contamination in either the red or green channels.

In contrast to one possible alternative, oxygen plasma treatment, the UV treatment is a very low energy one on the order of one bond energy and it will not re-distribute materials. Plasma treatment appeared to significantly increase the roughness of the coverslip surface.

B. Water contamination and purification:

After careful coverslip preparation and subsequent application and evaporation of 10 µl of distilled water (house water or HPLC double distilled from Fisher) and drying with flowing nitrogen gas, some fluorescent contamination is still observed in Figure 30(A). In Figure 30(B), we see a fully prepared coverslip that was treated with water in the same manner as the surface in A. However, in this case, we used water that was treated as described in the procedures section above (including UV exposure). Almost no fluorescent contamination is observed in either the red or the green channels.

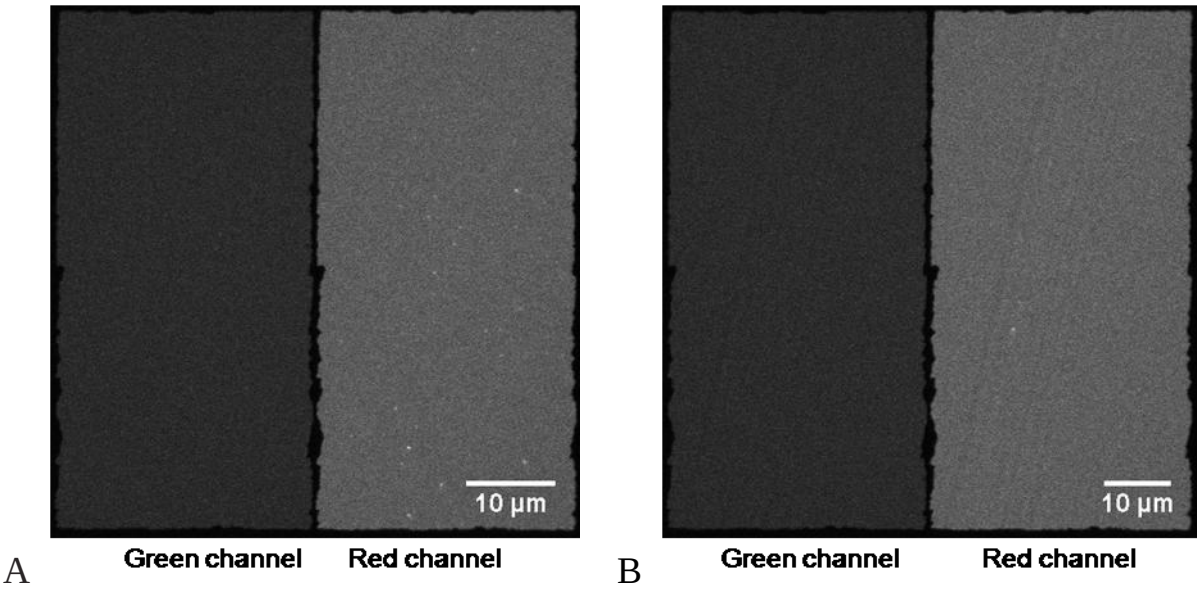


Figure 30: Photomicrograph demonstrating water contamination and purification A) Double distilled water and B) photobleached double distilled water

In single molecule studies, background contamination can still enter through either buffer or substrate solutions. By exposing the buffers to UV light, fluorophores in the buffer will be photobleached.

C. Fluorescently labeled Dendrimers on Triangular Origami:

Figure 31 presents an optosplit image of a surface coated with the fluorescently labeled triangular origami constructs.

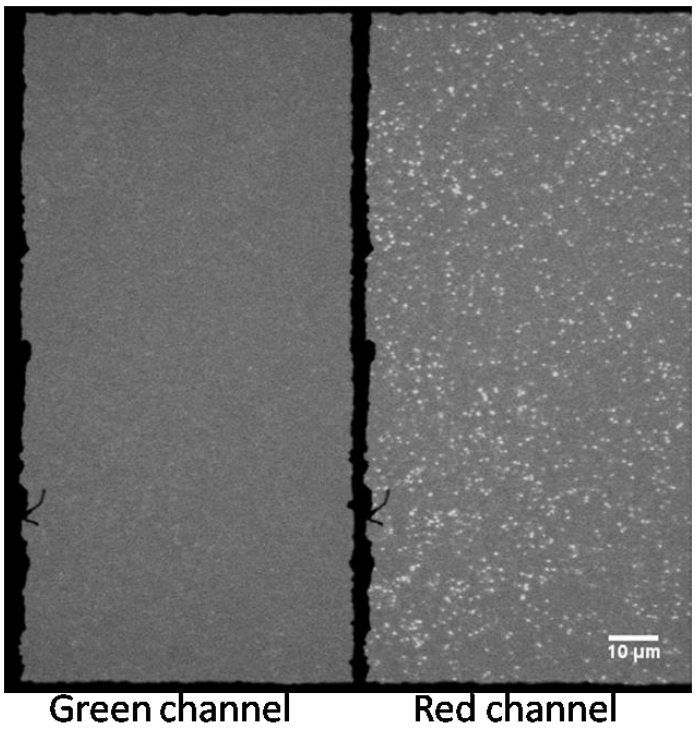


Figure 31: Fluorescence photomicrograph of triangular Origami sample with

10 Rhodamine-dendrimer and 10 Fluorescein-dendrimer modifications

Notice that the signal from the fluorescein labeled molecules (left side of Figure 31) is much weaker than the rhodamine signal (right side Figure 31). According to the design, the two types of fluorophore constructs should appear in both channels with approximately equal intensity, but they do not. This is believed to be a result of insufficient intensity at the excitation energy for fluorescein. This hypothesis was verified by matching the output of our Hg lamp (green line seen in Figure 32) with our excitation filters (black lines in Figure 32).

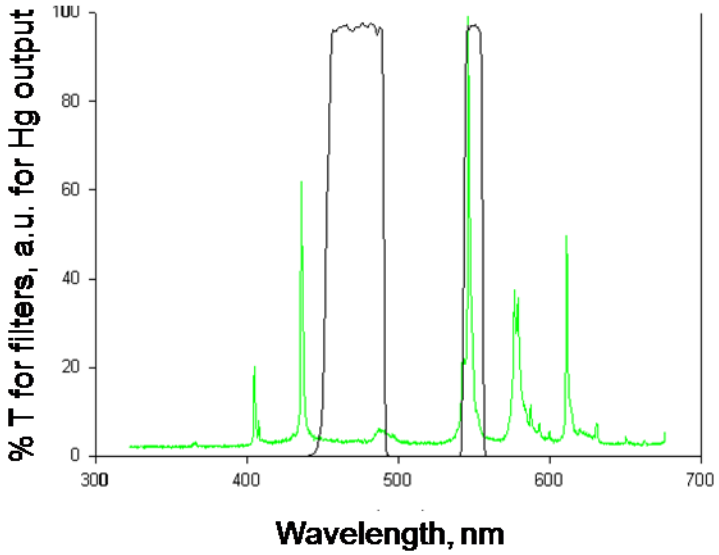


Figure 32: Representation of Hg lamp excitation wavelengths passed by the filter configuration (green= Hg lamp output, black= pass bands of filters)

A spectrum of the excitation light as it came from the microscope was taken using a spectrometer (part no: spectra pro 2500i, Acton Research Corp.). This spectrum is shown in Figure 33 as the blue tracing. It shows a significant lamp emission peak at ~550nm and much lower, intensity at shorter λ. This excitation power will sufficiently excite only rhodamine (absorption shown as red arrow in Figure 33) not fluorescein (absorption shown as green arrow in Figure 33).

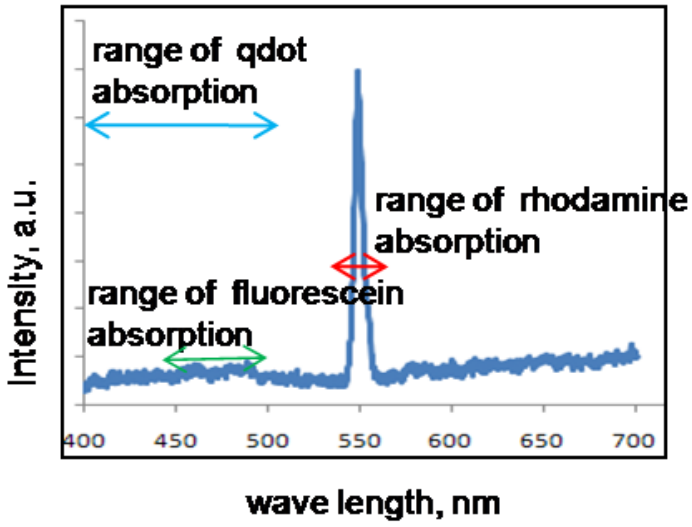


Figure 33: Absorption ranges for Qdot, Fluorescein and Rhodamine

This choice of filter sets largely accounts for the fact that fluorescein does not appear with significant intensity in these experimental results (Figure 30, left side 'green' channel).

Organic molecular fluorophores such as rhodamine are known to photobleach; that is to absorb energy and then enter a state that is reactive with other species (often oxygen). Once a molecule bleaches, it is unlikely to recover to an emitting state. Rhodamine photobleaching is demonstrated in Figure 34. Bleaching of the system was quantified as ~90% decrease in fluorescence intensity within 40 seconds in air at room temperature.

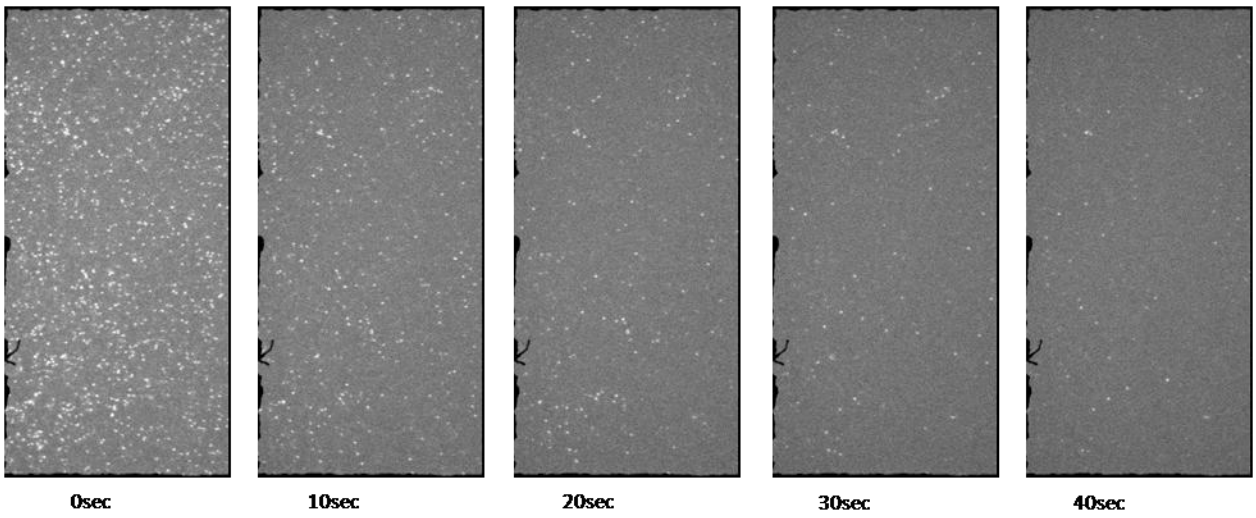


Figure 34: Photo bleaching of Triangular Origami with 10 Rhodamine - Dendrimer complex

Fluorescein and Rhodamine are very small in size (~ 0.5 nm). These two fluorophores are very bright (intense) and chemically stable but not photostable. These fluorophores will photobleach rapidly. The fluorescent reporter location would be well defined because of the small size and high intensity of these fluorophores. The fluorescently labeled dendrimers on Triangular Origami constructs are very complex structures. They did not purify well.

Interestingly, some of these fluorophores were observed to blink. While observing the videos, on the red channel side, some of the fluorescent molecules suddenly changed from a dark state to a very bright state then back to a dark state.

Because of the lack of sufficient fluorescein excitation intensity, the uncertainty in the number of labels on each Dendrimer and the large size of the rhodamine Dendrimer constructs, a new DNA construct with two Qdots was designed and fabricated.

D. Qdots on Triangular Origami:

Figure 35 provides an image acquired with 60x oil objective (266.4nm/pixel), showing triangular Origami with Qdot 525 emission on the left side and Qdot 605 emission on the right side of the image.

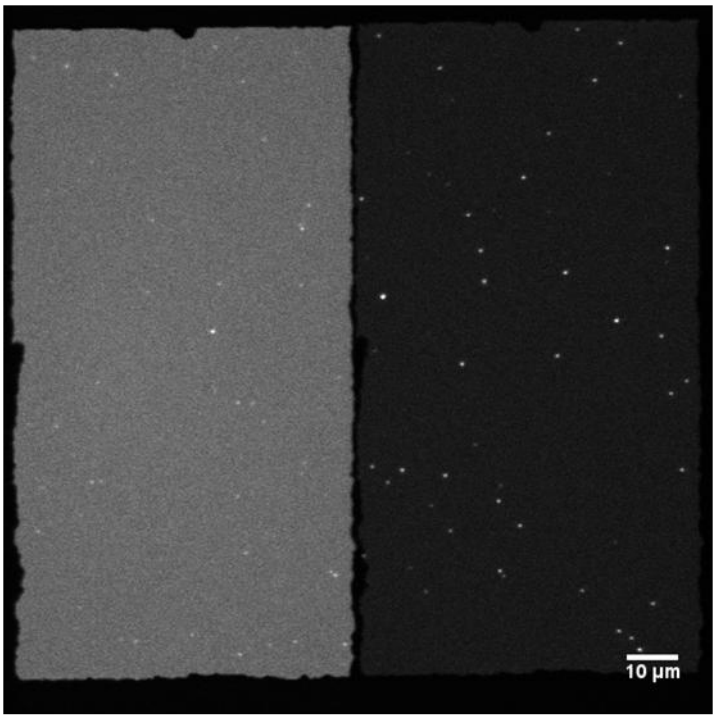


Figure 35: Triangular Origami sample with 525 & 605 emission Qdots

Qdots are useful in optical studies because of their high quantum yield. However, because of their large size they occupy significantly more space on the Origami surface than other fluorophores. These particles have to be conjugated with proteins, oligonucleotides, etc. for immobilization. Despite their size and shape problems Qdots can act as well separated single molecules on the Origami surface with high resistance to photobleaching, making Qdots excellent bright fluorescent probes (Figure 35). Although inorganic fluorophores (example, CdSe

Qdots) generally do not photobleach, they will blink. Blinking is a phenomenon in which a fluorophore enters a metastable state in which it temporarily cannot fluoresce but can recover from blinking and reenter an emissive state. Blinking of quantum dots can cause some difficulty in quantifying emission over time, which is important in localizing fluorophores by finding multiple centers. If a Qdot blinks out for 50% of an image exposure, it will appear half as bright in the final image. The blinking property of Qdots is illustrated in Figure 36.

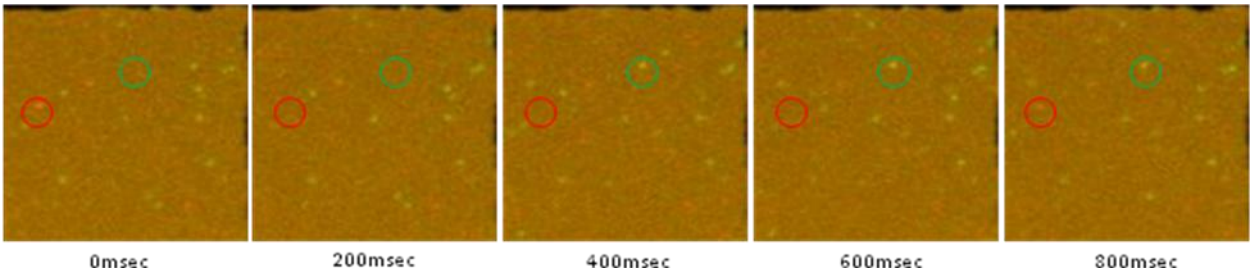
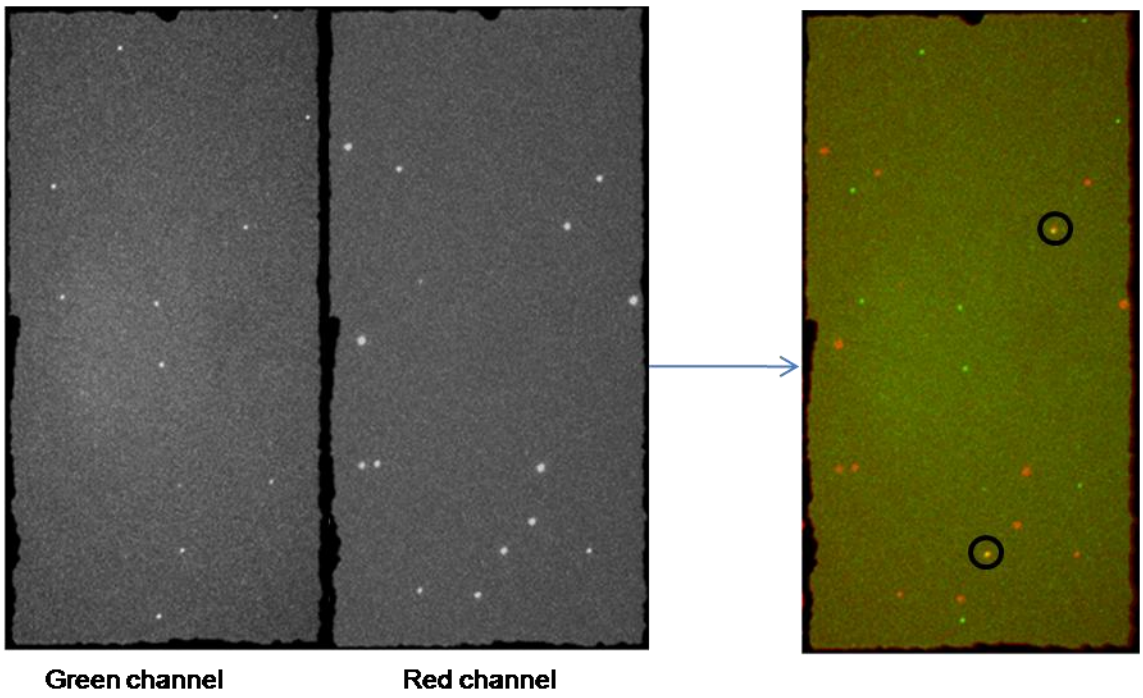


Figure 36: Frames from video illustrating blinking

In Figure 36 frames from a time series of images show blinking in a mixture of red and green Qdots (no DNA Origami here) on a glass surface. Notice that these particles 'blink' on, off, and on again (specific particles are indicated in the red and green circles).

Optical co-localization experiments indicated that the yield of Origami with two Qdots co-localized was very low (Figure 37).



(a) (b)

Figure 37: (a) Triangular Origami sample with 525 & 605nm emission Qdots (b) Merged image of the two grey scale channels (green & red). Black circles indicate co-localization of green & red emitting Qdots

This merge was not performed with FIONA accuracy, it was performed manually to demonstrate that only two Origami provided Qdot co-localization out of 24 potential Origami observed in the merged image.

Because of the large size, blinking and low sample yield, another DNA construct with a protein reporter was designed, constructed and characterized.

E. EGFP on Circular Origami:

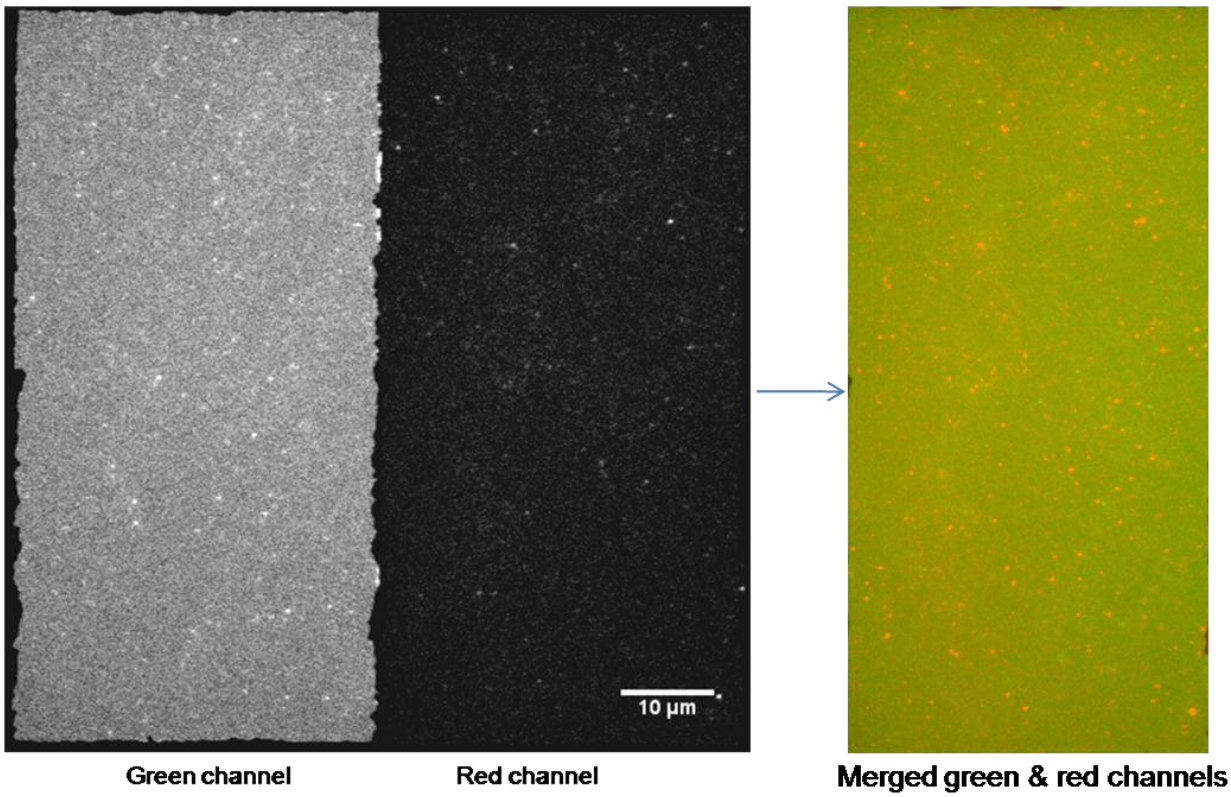


Figure 38: Optosplit image of sample containing EGFP on circular Origami constructs

Compared to other fluorophores, EGFP is a medium sized, relatively intense reporter with very good photostability. The molecules appear to be well defined in images (e.g. composite of 300 images, shown in Figure 38). Some molecules unexpectedly also appear in the red channel. One explanation is that EGFP can undergo photoconversion to a red fluorescent species (emits at 600 nm) under rigorously anaerobic conditions [(41)]. It is also possible that this might be inhomogeneous broadening of the sample. Alternatively, the fluorescence could arise in the red channel from a RFP (red fluorescent protein) impurity in a small fraction. Hydration and pH can affect the EGFP signal as well. Any one of the above reasons might be the cause of the fluorescence observed in both channels. Because of this complexity in the sample, another DNA construct was designed, constructed and characterized.

F. Rhodamine on Rectangular Fenestrated Origami:

Figure 39 presents a representative image of the construct best fitting the sample requirements of this project. The photobleaching kinetics are first discussed, followed by a treatment of the results of distance determinations.

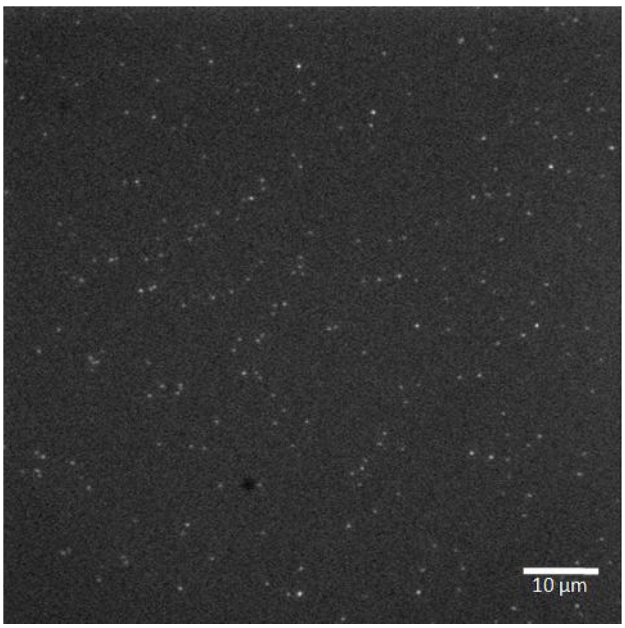


Figure 39: Fluorescence image of a sample of Fenestrated Rectangular Origami with two rhodamine molecules with a separation of 51.2 nm

I. **Kinetics of Photobleaching**:

Organic molecular fluorophores such as rhodamine are known to photobleach. In order to extend the observation time, a series of experiments were performed to determine the contributions to photobleaching. When data for experiments performed under N_2 and room air (21% O_2) were analyzed (Figure 40, 41), R^2 values for zero, first and second order fits indicated that the first order function best fits the O_2 data. $t_{1/2}$ for the first order reaction was calculated using this equation $t_{1/2}= 0.693/k$. This experiment was performed twice (trial 1, trial 2). Fits to

zero, first and second order reactions for trial 1 and trial 2 are shown in Figures 40 and 41 (raw data values in Appendix - E Table 7). The photobleaching follows different kinetics in the N_2 atmospheres, indicating another photobleaching mechanism is active.

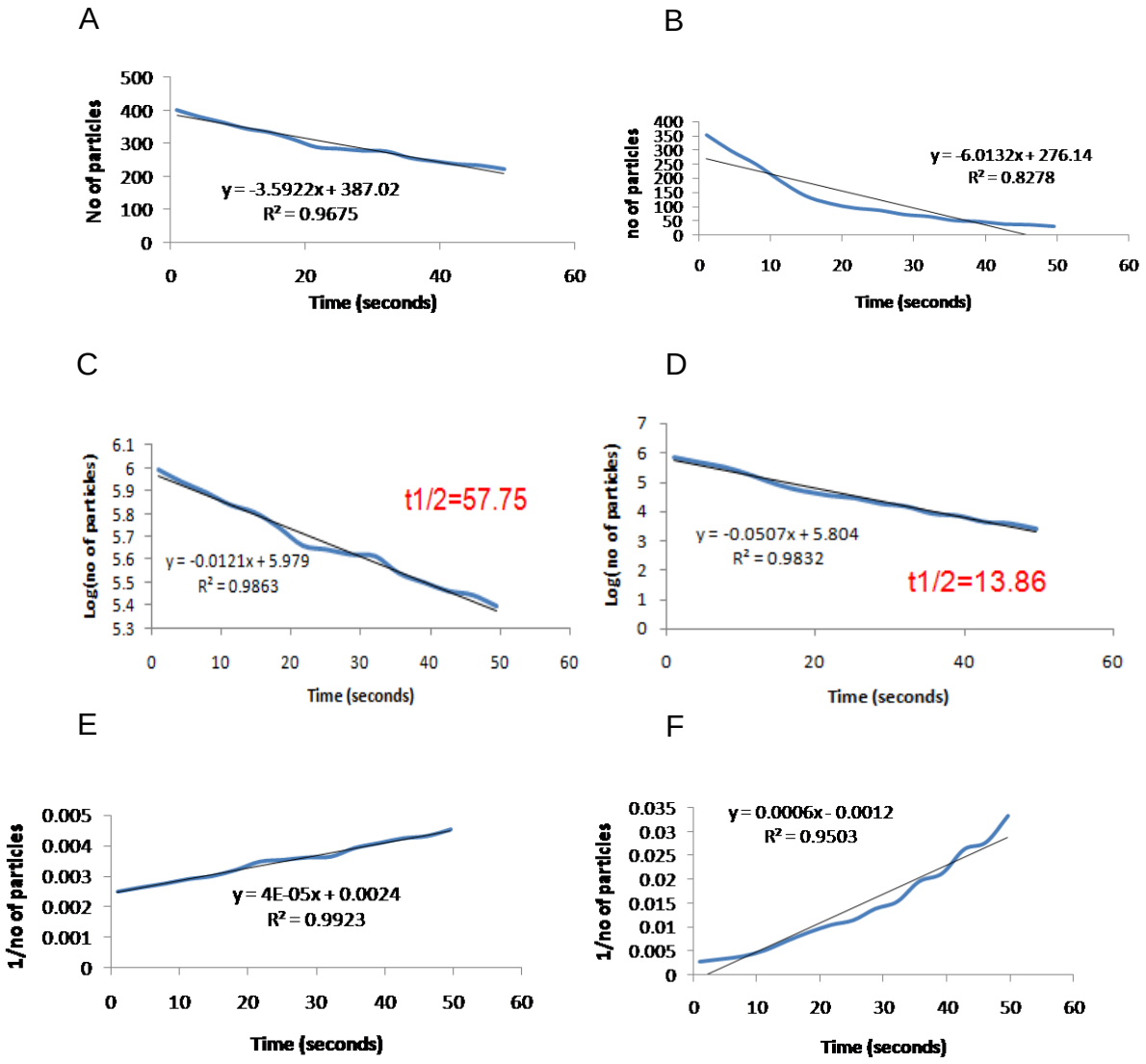


Figure 40: Zero, first, and second order fits to photobleaching data for Rhodamine, under N_2 (A, C, E) and room air (21% O_2) (B, D, and F) (trial 1)

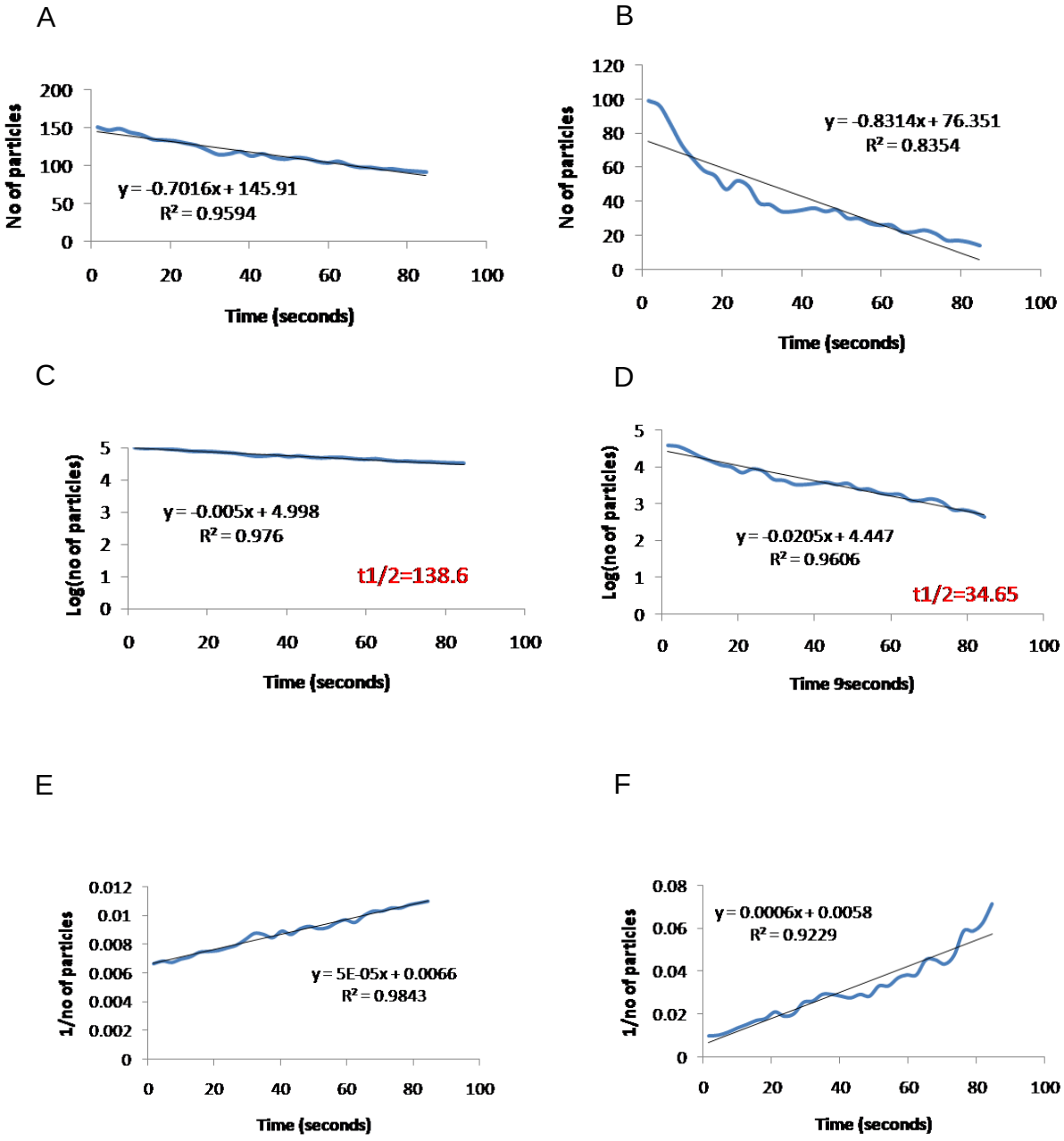


Figure 41: Zero, first, and second order fits to photobleaching data for Rhodamine, under N_2 (A, C, E) and room air (21% O_2) (B, D, and F) (trial 2)

Photobleaching occurs about 4.05 times faster in air than in nitrogen, providing significantly higher observation times. To minimize the photobleaching and increase the useful life of the fluorophores, all experiments are now performed under N_2 gas. A similar change in stability has been reported for Qdots [57].

II. Intermolecular distance determination using SHRIMP:

After determining the species for analysis by observing two step photobleaching of fluorophores, the centers of the fluorophores are located by fitting the composite images to a Gaussian function with two spatial dimensions.

Most of the spots display one step photobleaching, perhaps because one of the fluorophores has photobleached in the time lapse between fine focus adjustment of the sample and initiating image acquisition (hitting snap button in the data collection software). One example spot displaying two step photobleaching is shown in Figure 42.

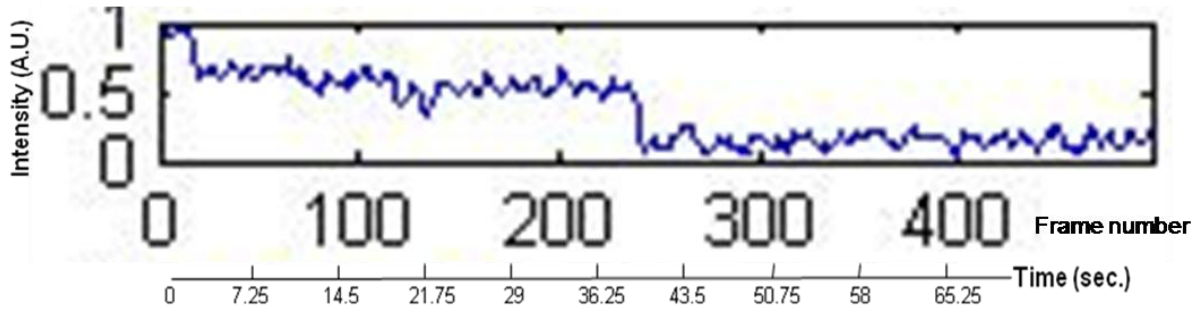


Figure 42: Spot showing two step photobleaching

Distances for evaluated spots are tabulated in Table 1 and the histogram of number of observations vs. range in distance was plotted (Figure 43). The average distance was found to be 51.68 nm.

Table 1: Tabulated distance between fluorophores for 18 analyzed spots

Image name	Distance (nm)	Estimated error ¥	Nominal error$	# frames per spot*
S1#58	48.16	+/- 1.38	- 2.837	120
S1#48	19.01	+/- 2.03	- 31.99	50
S1#39	45.24	+/- 2.1	- 5.76	100
S1#57	40.14	+/- 6.2	- 10.86	20
S2#13	50.07	+/- 3.05	- 0.93	30
S2#16	68	+/- 3.49	+ 17	100
S2#23	62.70	+/- 3.35	+ 11.7	15
S3#7	40.43	+/- 2.89	- 10.57	40
S6#9	51.47	+/- 5.185	+ 0.4736	50
S13#45	55.31	+/- 2.34	+ 4.3182	40
S618	41.92	+/- 1.34	- 9.08	20
S9#4	55.83	+/- 5.11	+ 4.83	10
S9#3	44.72	+/- 0.325	- 6.28	15
S10#21	69.78	+/- 2.13	+ 18.78	100
S7#15	93.81	+/- 3.92	+ 42	24
S5#4	16.02	+/- 1.96	- 34.98	51
S5#6	35.6	+/- 2.28	- 15.4	30
S4#5	85.01	+/- 8.97	+ 34.01	7

¥ = Error based on quality of gaussian fit

$ = Difference between nominal distance determined using gaussian fit

* = The number of frames used to compose the composite images A+B and B

The Z test and Q test were applied in order to determine whether the outliers could be eliminated from the data set. The highest and lowest values are, however, within three standard deviations and were therefore maintained in the data set. The value of the mean distance of separation is calculated to be 51.2 ± 8.47 at the 95% confidence level, using the student's t test. This is in good agreement with the nominal value of 51.2nm. An analysis of possible contributions to the error in these determinations is provided in the next section.

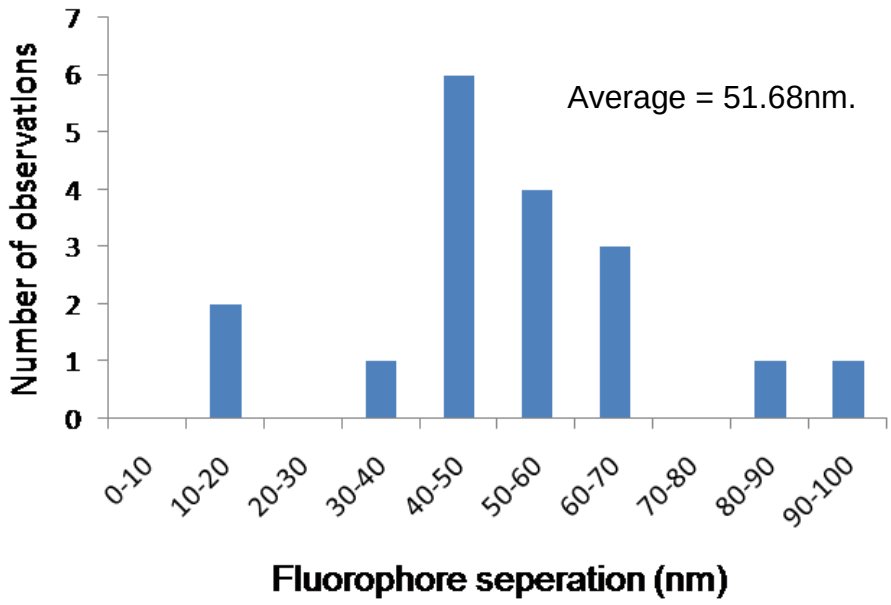


Figure 43: Histogram of observed separation distances.

n = 18

Mean = 51.68

Standard deviation = 17.24094

III. Error Analysis:

Although the nominal error is small, when we observe individual distances, some of them differ from the nominal distance considerably more than our estimated errors would predict. Several factors contribute independently to variations in the nominal separation. These are listed and discussed below. They include, but are not limited to: (1) double stranded DNA elasticity, (2) linker length and alignment of linkers on DNA Origami, (3) sample preparation and (4) fluorophore dipole orientation. Another source of even larger variations in fluorophore separation would be the random distribution of fluorophores on the coverslip surface.

1. Double stranded DNA elasticity: According to Michalet etal. [55] dsDNA stretching elasticity results in the standard deviation of end-to-end distance which scales linearly with the DNA strand length, with a factor of 0.21 Å per base pair. In our case this would add a standard deviation of 3.466 nm to the 160 base pairs

2. Linker length and alignment of linkers on DNA Origami: Fluorophores are attached to DNA through spacers. In our case, these spacers (C_6, $C_6ON_2H_{12}$) are ~ 2 nm in length. Our nominal distance between fluorophores in an Origami is 51.2 nm, this is only true when both spacers are perpendicular to the Origami. The spacers can also adopt a wide range of orientations, yielding a variety of distances ranging from 47.2 to 55.2 nm as depicted in Figure 44.

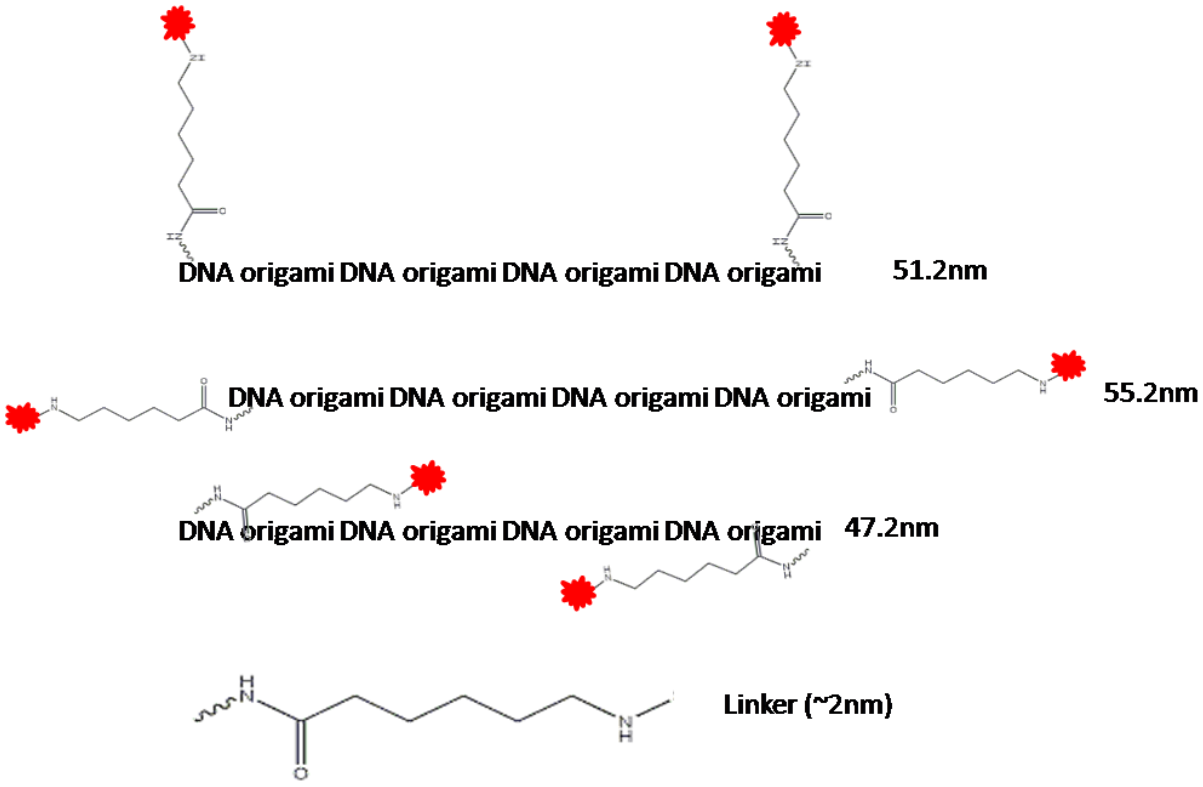


Figure 44: Depiction of range of separations enabled by use of a flexible linker

3. Sample preparation: The Origami constructs were deposited on glass from an aqueous buffer solution with fluid rinsing or flow, which may distort the origami structure beyond the value predicted based upon intrinsic elasticity. The crosslinking of double DNA strands in Origami does allow for some flexibility in the structure. Although this flexibility has not been measured experimentally, it may be estimated to be on the order of 4%.

4. Fluorophore Dipole orientation: The assumption of a gaussian emission distribution assumes that the dipole is pointing perpendicular to the origami surface. This is statistically unlikely in any individual case.

CONCLUSIONS

A DNA Origami based distance standard suitable for SHRIMP calibration has been developed.

Multiple fluorophores can be optically located with high special precision, on Origami nanostructures. It does not appear that the glass or DNA properties significantly interfere with this optical characterization method.

Several potential problems with the distance standards have been identified and must be addressed in order for experiment plots to probe for fine effects, related to the local composition and properties of the fluorophore environment as well as fluorophore dipole mutual orientations.

FUTURE DIRECTIONS

We would like to conduct the same experiment by changing the position of the rhodamine reporters in the Origami. So that the distance between the final two fluorophores will be 30 nm. This experiment will further test the software.

The studies of distance measurement should be extended to the determination of more than two fluorophores (Figure 45) in a single DNA Origami molecule.

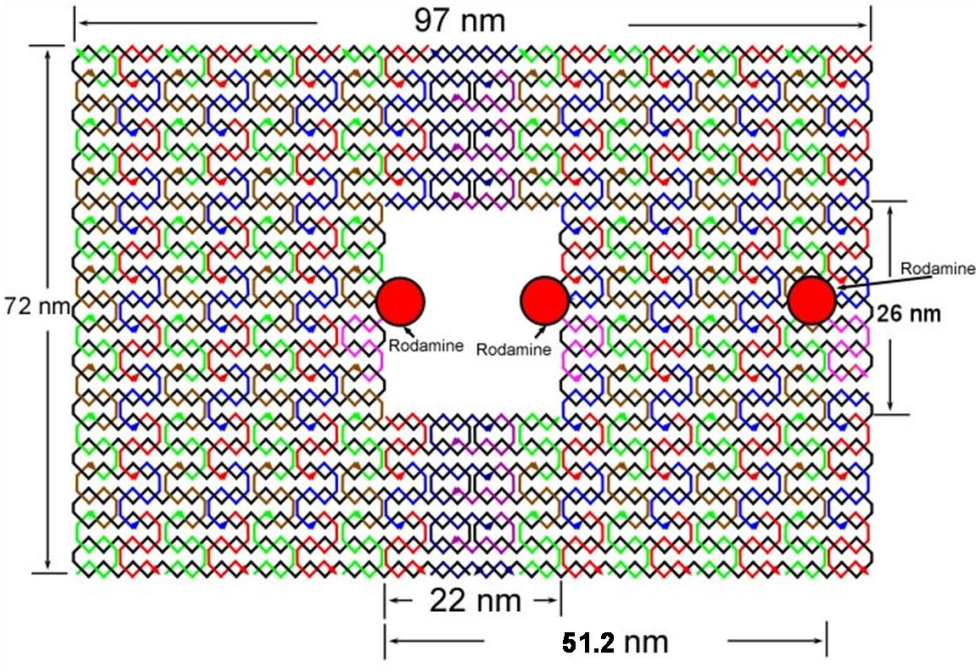


Figure 45: Rectangular Origami with three rhodamine molecules

REFERENCES

1. http://www.molphys.leidenuniv.nl/monos/students/pdf/smon01.pdf; Michel Orrit 2004; (accessed 11/04/09).
2. T. Hirschfeld. *Applied Optics*, 1976, 15, 2965-2966.
3. N. J. Dovichi, J. C. Martin, J. H. Jett, R. A. Keller. *Science* 1983, 219, 845-847.
4. D. C. Nguyen, R. A. Keller, M. Trkula. *J. Opt. Soc. Am*. 1987, 4, 138.
5. W. E. Moerner and L. Kador. *Anal. Chem*. 1989, 61, 21, 1217-1223.
6. Ahmet Yildiz, Joseph N. Forkey, Sean A. McKinney, Taekjip Ha, Yale E. Goldman, Paul R. Selvin. *Science* 2003, 300, 2061-2065.
7. Wilfried G. J. H. M. van Sark, Patrick L. T. M. Frederix, Dave J. Van den Heuvel, and Hans C.Gerritsen. *J.Phys. Chem*. 2001, 105, 35, 8281-8284.
8. Bustamante, C., Macosko, J.C. nad Wuite. *Nat. Rev. Mol. Cell Biol*. 2000, 1, 130-136.
9. K. Perronet, P. Bouyer, N. Westbrook, N. Soler, D. Fourmy and S. Yoshizawa , K. Perronet. *Journal of Luminescence* 2007, 127, 264-268.
10. W.E. Moernera and David P. Fromm. *Rev. Sci. Instrum*. 2003, 74, 8, 3597-3619.
11. W.E. Moerner. *J.Phys. Chem*. 2002, 106, 910-927.
12. Katherine Willets, Robert Twieg, and W.E. Moerner, *SPIE Newsroom. New J. Phys*. 2004, 88, 13-15.
13. Thompson RE, Larson DR,Webb WW. *Biophys. J*. 2002, 82, 2775-2783.
14. F Ritort. J. *Phys*.: Condens. Matter, 2006, 32, 18, R531-R583.
15. H. Li, L. Ying, X. Ren, S. Balasubramanian and D. Klenerman. *Biochemical Society Transactions* 2004, 32, 5, 753-756.
16. http://www.st-andrews.ac.uk/~singlemol/singlemol.html; (accessed 11/09/09)

17. Shimon Weiss. *Science* 1999, 283, 5408, 1676-1683.

18. Florian Kulzer and Michel Orrit. *Ann. Rev. Phys. Chem*. 2004, 55, 585-611.

19. Moerner, W. E.. *Proc. Natl. Acad. Sci*. 2007, 104, 12596-12602.

20. Matthew P. Gordon, Taekjip Ha, and Paul R. Selvin. *PNAS* 2004, 101, 6462-6465.

21. Mats GL Gustafsson. *Curr. Opin. Struct. Biol.* 1999, 9, 627-634.

22. Erdal Toprak and Paul R. Selvin. *Annu. Rev. Biophys. Biomol. Struct*. 2007, 36, 349-69.

23. Stefan W Hell. *Nature Methods* 2009, 6, 24-32.

24. Mark Bates, Bo Huang, and Xiaowei Zhuang. *Curr. Opin. Struct. Biol.* 2008, 12, 5, 505-514.

25. Sripad Ram, E. Sally Ward, and Raimund J. Ober. *PNAS* 2006, 103, 4457-4462.

26. http://www.biotek.com/resources/docs/Gene_Blazer_Light_120307_kl_v4.pdf; Paul Held 2007; (accessed 11/07/09).

27. Kinosita K Jr, Itoh H, Ishiwata S, Hirano K, Nishizaka T, Hayakawa T. *J. Cell Biol*. 1991, 115, 67-73.

28. Dos Remedios CG, Moens PD, *J. Struct Biol*. 1995, 115, 175-85.

29. Yildiz A., Forkey J, N., McKinney S.A., Ha T., Goldman Y.E., and Selvin P.R.. *Science* 2003, 300, 2061-2065.

30. Chirlmin Joo, Hamza Balci, Yuji Ishitsuka,Chittanon Buranachai, and Taekjip Ha. *Annu. Rev. Biochem*. 2008, 77, 51-76.

31. Paul R. Selvin, Tyler Lougheed, Melinda Tonks Hoffman, Hyokeun Park, Hamza Balci, Benjamin H. Blehm, and Erdal Toprak. Fluorescence Imaging with One-Nanometer Accuracy (FIONA). *Cold Spring Harb. Protoc*. 2007.

32. Xiaohui Qu, David Wu, Laurens Mets, and Norbert F. Scherer. *PNAS* 2004, 101, 31, 11298-11303.
33. Churchman LS, Okten Z, Rock RS, Dawson JF, Spudich JA. *Proc. Natl. Acad. Sci*. 2005, 102, 1419-1423.
34. http://www.invitrogen.com/etc/medialib/en/filelibrary/cell_tissue_analysis/pdfs.Par.44459.File.dat/O-073210_QDot_AppNote_LRF.pdf; (accessed 11/09/09).
35. Bo Huang, Wenqin Wang, Mark Bates, Xiaowei Zhuang. *Science* 2008, 319, 810-813.
36. Qu, X., D. Wu, L. Mets, and N. F. Scherer. *Proc. Natl. Acad. Sci*. 2004, 101, 11298-11303.
37. Sharonov, A., and R. M. Hochstrasser. *Proc. Natl. Acad. Sci*. 2006, 103, 18911-18916.
38. Betzig, E., G. H. Patterson, R. Sougrat, O. W. Lindwasser, S. Olenych. *Science*. 2006, 313, 1642-1645.
39. Biteen, J. S., M. A. Thompson, N. K. Tselentis, G. R. Bowman, L. Shapiro, *Nat Methods* 2008**,** 5, 947-949.
40. Smita Pathak, Elizabeth Cao, Marie C. Davidson, Sungho Jin, and Gabriel A. Silva. *Journal of Neuroscience* 2006, 26, 7, 1893-1895.
41. Roger Y. Tsien. *Annu. Rev. Biochem.* 1998, 67, 509-44.
42. Satoshi Karasawa, Toshio Araki, Miki Yamamoto-Hino, and Atsushi Miyawaki. *The Journal of Biological Chemistry* 2003, 278, 36, 34167-34171.
43. Brelje, T.C., M.W Wessendrof, R.L. Sorenso. *Methods Cell Biol*. 1993, 38, 97-181.
44. Paul W. K. Rothemund. *Nature* 2006, 440, 297-302.
45. http://www.invitrogen.com/site/us/en/home/support/Product-Technical-Resources/Product-Spectra.QD525SA.html; (accessed 11/06/09).

46. http://www.invitrogen.com/site/us/en/home/support/Product-Technical-Resources/Product-Spectra.QD605SA.html; (accessed 11/06/09).
47. Ute Resch-Genger, Markus Grabolle, Sara Cavaliere-Jaricot, Roland Nitschke and Thomas Nann. *Nature Methods* 2008, 5, 763-775.
48. Johannes A. Schmid and Hannah Neumeier. *ChemBioChem* 2005, 6, 7, 1149-1156.
49. Roger Y. Tsien. *Annual Review of Biochemistry* 1998, 67, 509-544.
50. http://www3.interscience.wiley.com/cgi-bin/fulltext/110513258/PDFSTART; (accessed 11/06/09).
51. Ye chen1 and Ammasi periasamy. *Microsc. Res. Tech*. 2004, 63, 72-80.
52. http://www.bangslabs.com/learning/spectra_information; (accessed 11/06/09).
53. http://www.invitrogen.com/site/us/en/home/support/Product-Technical-Resources/Product-Structures.-06160.html; (accessed 11/06/09).
54. http://images.google.com/imgres?imgurl=http://probes.invitrogen.com/media/spectra; (accessed 11/06/09).
55. Douglas Magde, Roger Wong, Paul G. Seybold. *Photochemistry and Photobiology* 2002, 75, 4, 327-334.
56. http://www.piercenet.com/files/0367as4.pdf; (accessed 11/06/09).
57. Wilfried G. J. H. M. van Sark, Patrick L. T. M. Frederix, Dave J. Van den Heuvel, and Hans C. Gerritsen. *J. Phys. Chem. B* 2001, 105, 35, 8281-8284.
58. Josh Antelman, Connie Wilking-Chang, Shimon Weiss, and Xavier Michalet. *Nano Lett*., 2004, 9, 5, 2199-2205.
59. http://rsbweb.nih.gov/ij/download.html; (accessed 11/06/09).

APPENDIX

APPENDIX-A

Rectangular Origami:

The image illustrates how DNA strands folded into the designed shape

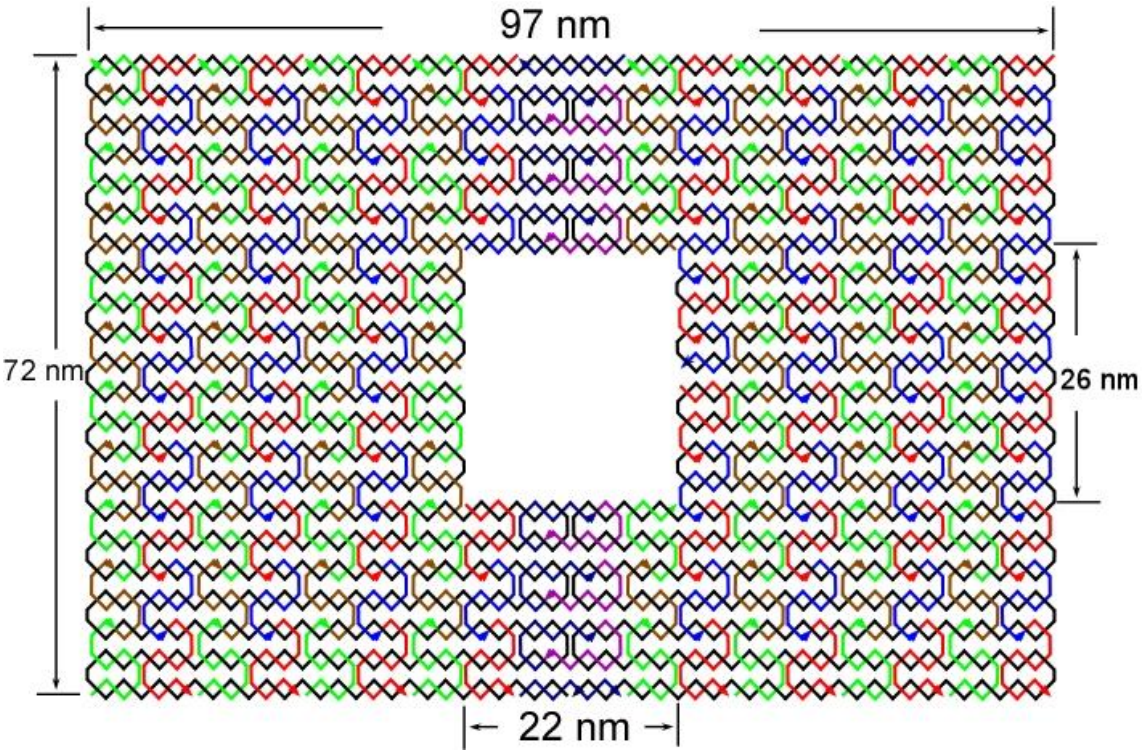


Figure 46: The structure of Rectangular Origami with an aperture

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Table 2: DNA sequences for all the DNA staple strands for the rectangular Origami.

Listed below are all of the DNA staple sequences that are used in the design. Staples are named according to their positions on the blueprint. (in HPLC purified form from Integrated DNA Technologies, Inc.)

BROWN-X1-Y1;TCAGAGCCCATTGACAGGAGGTTGGAAACATG
GREEN-X1-Y2;ATTTGGGATACCATTAGCAAGGCCCACCACCC
BROWN-X1-Y2;GAGGAAACATGATTAAGACTCCTTCTTGAGCC
GREEN-X1-Y3;AGAATAACTTAACTGAACACCCTGAGGAAACC
BROWN-X1-Y3;AGCGAACCTCAAGATTAGTTGCTATTACAGAG
GREEN-X1-Y4;ACAAGAAAGAAACCAATCAATAATGGCGTTTT
BROWN-X1-Y4;AATTCTTATAATTGAGAATCGCCAAGTCCTGA
GREEN-X1-Y5;GAGAAGAGAGAGACTACCTTTTTAGTTATACA
BROWN-X1-Y5;GGGAGAAACAAGTTACAAAATCGCAAGACGCT
BROWN-X2-Y1;CCTCAGAATGGCCTTGATATTCACTGCCCCCT
GREEN-X2-Y2;AAGGTGAAAAACCATCGATAGCAGGCCGCCAC
BROWN-X2-Y2;AGATAGCCGCAAACGTAGAAAATAATTCATTA
GREEN-X2-Y3;CGTCAAAAAATTGAGCGCTAATATAAGTAAGC
BROWN-X2-Y3;TTATCCGGAATTTTATCCTGAATCTTGTTTAA
GREEN-X2-Y4;GCGCCTGTTCATTCCAAGAACGGGTAGAAGGC
BROWN-X2-Y4;AAAAGCCTCATGTAATTTAGGCAGTGCAGAAC
GREEN-X2-Y5;GAAAACATGGGTTATATAACTATATTACTAGA
BROWN-X2-Y5;GATGAATACATTTCAATTACCTGAGAATCCTT

BROWN-X3-Y1;ACCACCGGATTAAAGCCAGAATGGCCTTGAGT

GREEN-X3-Y2;GGAAGGTAGACAGAATCAAGTTTGCCAGAGCC

BROWN-X3-Y2;TCTTACCGAACATATAAAAGAAACTGAGGGAG

GREEN-X3-Y3;CCAATCCAAAGAATTGAGTTAAGCAATAGCTA

BROWN-X3-Y3;GCCCAATAGCGTCTTTCCAGAGCCTATTTATC

GREEN-X3-Y4;AGAATAAAGCACTCATCGAGAACAATTACCGC

BROWN-X3-Y4;TAAGAATAGTAATAAGAGAATATAAGACGACG

GREEN-X3-Y5;TCGCTATTAATCCAATCGCAAGACGCGTTAAA

BROWN-X3-Y5;AGAAATTGAAACAAACATCAAGAAGTAAATCG

BROWN-X4-Y1;TTTTCATAATTTACCGTTCCAGTATGTACTGG

GREEN-X4-Y2;ACAAAAGGGTAGCGCGTTTTCATCTTGCCATC

BROWN-X4-Y2;ATAAGTTTATTTTGTCCGCCAAAG

GREEN-X4-Y5;ATGTGAGTCTTTTTGAAATATATTTGGTTTGAAATACCGA

BROWN-X4-Y5;ATCAAAATTTTAACAATTTCATTTATCAATAT

BROWN-X6-Y1;AGGCTTTGAGCCTTTAATTGTATCGAATAGAA

GREEN-X6-Y2;GGACAGATAAGGCACCAACCTAAAGGCTACAG

BROWN-X6-Y2;GGAACAACCCGGATATTCATTACCTTGAAAGA

GREEN-X6-Y3L;GTCCAATATTTTATTATTACAGGTAGAAGAACTAAC

BROWN-X6-Y3L;CCAACAGGTTTTCTGCGGAATCGTCATATGGATAGC

GREEN-X6-Y4L;TCAATAACTTTTTCAGGATTAGAGAGTAAGCAAACT

BROWN-X6-Y4;ATAAAAATCTGTTTAGCTATATTTGCAAATGG

GREEN-X6-Y5;CAAAAACAGTGAGAAAGGCCGGAGACGCAAGG

BROWN-X6-Y5;ACGACGACTTTTTGTTAAATCAGCAAAAGCCC

BROWN-X7-Y1;CAGCATCGTTCGAGGTGAATTTCTCTAAACAA

GREEN-X7-Y2;ATAAGGGAAATACACTAAAACACTGCGAAAGA

BROWN-X7-Y2;AAGAAAAAAGCTGCTCATTCAGTGGGTCAATC

GREEN-X7-Y3;GGGGTAATATTTAGGAATACCACAACGTTGGG

BROWN-X7-Y3;AGCTTCAACCCTCAAATGCTTTAATGCCAGAG

GREEN-X7-Y4;TAGTTTGATTGATAAGAGGTCATTTTAATTCG

BROWN-X7-Y4;TTTGCGGGCTGAAAAGGTGGCATCAGTAGATT

GREEN-X7-Y5;ATATGTACCAATATGATATTCAACGTAATACT

BROWN-X7-Y5;CATCGTAAAGGAACGCCATCAAAATGTCAATC

BROWN-X8-Y1;TTTGCGGGGATAGTTGCGCCGACAGTTAGTAA

GREEN-X8-Y2;ACTTAGCCCGATTATACCAAGCGCAGGCCGCT

BROWN-X8-Y2;AACTGGCTACGAGAACACCAGAACTCCATGTT

GREEN-X8-Y3;GAGAGGCTACATAACGCCAAAAGGATTTTAAG

BROWN-X8-Y3;CCGAAAGAGAATGACCATAAATCAAAAATAGC

GREEN-X8-Y4;AGTTGATTAGCTTAATTGCTGAATGAGGAAGC

BROWN-X8-Y4;TTGTACCAGTAGCATTAACATCCACATGTAAC

GREEN-X8-Y5;TGAACGGTTTAATGCCGGAGAGGGTAAATCGG

BROWN-X8-Y5;AATGGGATCTTCCTGTAGCCAGCTAGAATCGA

RED-X2-Y1;ATTATTCTAGGCAGGTCAGACGATCCGCCACC

BLUE-X2-Y1;CTCAGAGCGGAAACGTCACCAATGTTATCACC

RED-X2-Y2;GTCACCGAATTACGCAGTATGTTAGAACAAAG

BLUE-X2-Y2;TTACCAGAAACAAAGTCAGAGGGTATGAAAAT

RED-X2-Y3;AGCAGCCTTTTTGCACCCAGCTACTATTCTAA

BLUE-X2-Y3;GAACGCGACGGCTGTCTTTCCTTATTATCAAC

RED-X2-Y4;AATAGATATATTTAACAACGCCAAGTTTAGTA

BROWN-X7-Y2;AAGAAAAAAGCTGCTCATTCAGTGGGTCAATC

GREEN-X7-Y3;GGGGTAATATTTAGGAATACCACAACGTTGGG

BROWN-X7-Y3;AGCTTCAACCCTCAAATGCTTTAATGCCAGAG

GREEN-X7-Y4;TAGTTTGATTGATAAGAGGTCATTTTAATTCG

BROWN-X7-Y4;TTTGCGGGCTGAAAAGGTGGCATCAGTAGATT

GREEN-X7-Y5;ATATGTACCAATATGATATTCAACGTAATACT

BROWN-X7-Y5;CATCGTAAAGGAACGCCATCAAAATGTCAATC

BROWN-X8-Y1;TTTGCGGGGATAGTTGCGCCGACAGTTAGTAA

GREEN-X8-Y2;ACTTAGCCCGATTATACCAAGCGCAGGCCGCT

BROWN-X8-Y2;AACTGGCTACGAGAACACCAGAACTCCATGTT

GREEN-X8-Y3;GAGAGGCTACATAACGCCAAAAGGATTTTAAG

BROWN-X8-Y3;CCGAAAGAGAATGACCATAAATCAAAAATAGC

GREEN-X8-Y4;AGTTGATTAGCTTAATTGCTGAATGAGGAAGC

BROWN-X8-Y4;TTGTACCAGTAGCATTAACATCCACATGTAAC

GREEN-X8-Y5;TGAACGGTTTAATGCCGGAGAGGGTAAATCGG

BROWN-X8-Y5;AATGGGATCTTCCTGTAGCCAGCTAGAATCGA

RED-X2-Y1;ATTATTCTAGGCAGGTCAGACGATCCGCCACC

BLUE-X2-Y1;CTCAGAGCGGAAACGTCACCAATGTTATCACC

RED-X2-Y2;GTCACCGAATTACGCAGTATGTTAGAACAAAG

BLUE-X2-Y2;TTACCAGAAACAAAGTCAGAGGGTATGAAAAT

RED-X2-Y3;AGCAGCCTTTTTGCACCCAGCTACTATTCTAA

BLUE-X2-Y3;GAACGCGACGGCTGTCTTTCCTTATTATCAAC

RED-X2-Y4;AATAGATATATTTAACAACGCCAAGTTTAGTA

BLUE-X2-Y4;TCATATGCACCTCCGGCTTAGGTTAGCGATAG

RED-X2-Y5;CTTAGATTGCAGAGGCGAATTATTTACAGTAA

BLUE-X2-Y5;CAGTACCTTTTGCGGAACAAAGAACTTTACAA

RED-X3-Y1;ACAGTTAAAAACGAATGGATCTTCAACCGCCT

BLUE-X3-Y1;CCCTCAGACACCGTAATCAGTAGCAATATTGA

RED-X3-Y2;CGGAAATTCATACATAAAGGTGGCAAGCCCTT

BLUE-X3-Y2;TTTAAGAACAGAGAGATAACCCACAATAAGAA

RED-X3-Y3;ACGATTTTTTACCAACGCTAACGAGCAAGCAA

BLUE-X3-Y3;ATCAGATATATTAAACCAAGTACCCAACATGT

RED-X3-Y4;TCAGCTAAAGGCATTTTCGAGCCAAACACCGG

BLUE-X3-Y4;AATCATAATGTAAATGCTGATGCAAATTAATT

RED-X3-Y5;TTCCCTTAGCAAAAGAAGATGATGCGTAGATT

BLUE-X3-Y5;TTCAGGTTGAATTATCATCATATTGATAATAC

RED-X4-Y1;GGTCAGTGAAAGCGCAGTCTCTGAATCAAAAT

BLUE-X4-Y1;CACCGGAACCTTTAGCGTCAGACTGCGACATT

RED-X4-Y2;CAACCGATGCAAAGACACCACGGAGAAACAAT

BLUE-X4-Y2;GAAATAGCCCAATAATAAGAGCAATTTTAAATAAAC

RED-X4-Y3;AGCCATATTAATTTGCCAGTTACATTTTTTTCATCG

BLUE-X4-Y3;TAGGAATCAGCAAGCCGTTTTTATTTTTTAAAGTAA

RED-X4-Y4;TTCTGTCCAAGTACCGACAAAAGGCCGTGTGA

BLUE-X4-Y4;TAAATAAGAAAGAACGCGAGAAAAGAATAACC

RED-X4-Y5;TTGCTTCTAACAAAATTAATTACATATTAGCA

BLUE-X4-Y5;CGTAAAACTGGCAATTCATCAATACACTAACA

RED-X6-Y1;ATTGCGAAAAAAGGCTCCAAAAGGAGGACTAA

BLUE-X6-Y1;AGACTTTTACGTAATGCCACTACGGAACGGTG

RED-X6-Y2;TACAGACCTAATCTTCGACAAGAA

BLUE-X6-Y4;TTTTAGAACCCTCATAGTAAAGATTCAAAAGGGGAAGATT

RED-X6-Y5;GTATAAGCTAAAATTCGCGTTAAACGTATCGG

BLUE-X6-Y5;CCTCAGGATTCTGGTGCCGGAAACATTCGTAA

RED-X7-Y1;CGGAGTGAGGTTTATCAGCTTGCTGAACGAGG

BLUE-X7-Y1;GTAGCAACACGAAAGAGGCGAAAGACCGAACT

RED-X7-Y2;GACCAACTCAAATCAACGTAACAATCTACGTT

BLUE-X7-Y2;AATAAAACAGATTCATCAGTTGAGAGTAAAAT

RED-X7-Y3;GTTTAGACAATATTCATTGAATCCAGCGAACC

BLUE-X7-Y3;AGACCGGACCTTTAATTGCTCCTTCCATTAGA

RED-X7-Y4;TACATTTCTCATTTGGGGCGCGAGAGAAGCCT

BLUE-X7-Y4;TTATTTCAACAGTCAAATCACCATCCCGGTTG

RED-X7-Y5;ATAATCAGTCATTTTTTAACCAATCCGTGCAT

BLUE-X7-Y5;CTGCCAGTCGCCATTCAGGCTGCGGACTCTAG

RED-X8-Y1;GGATTTTGTAAACAGCTTGATACCATCGTCAC

BLUE-X8-Y1;CCTCAGCACATCTTTGACCCCCAGGGAACGAG

RED-X8-Y2;GCGCAGACAATAAGGCTTGCCCTGCATTATAC

BLUE-X8-Y2;CAGTCAGGTTCAACTAATGCAGATTTTGCAAA

RED-X8-Y3;AGAAGTTTACAGTTCAGAAAACGACTTCAAAT

BLUE-X8-Y3;ATCGCGTTTTTGCGGATGGCTTAGCCCAATTC

RED-X8-Y4;TGCGAACGAATTCTACTAATAGTAAAAACATT

BLUE-X8-Y4;ATGACCCTCGTTCTAGCTGATAAAAATCGTAA

RED-X8-Y5;AACTAGCAATAATTCGCGTCTGGCAGGTTACG

BLUE-X8-Y5;TTGGTGTACGATCGGTGCGGGCCTCGGCCAGT

RED-X9-Y1;TTCCAGACATGACAACAACCATCGGCTTGCAG

BLUE-X9-Y1;GGAGTTAAGAAACAAAGTACAACGGAAATCCG

RED-X9-Y2;CGACCTGCGAGTAGTAAATTGGGCTGAATTAC

BLUE-X9-Y2;CTTATGCGAATTACGAGGCATAGTGACGACGA

RED-X9-Y3;TAAAAACCAAAATCAGGTCTTTACGCATCAAA

BLUE-X9-Y3;AAGATTAACTGGTGCTGTAGCTCATCTGGAAG

RED-X9-Y4;TTTCATTCATAAATCATACAGGCACCTCAGAG

BLUE-X9-Y4;CATAAAGCTAGCTATTTTTGAGAGGTCTGGAG

RED-X9-Y5;CAAACAAGTTCATCAACATTAAATGGGAACAA

BLUE-X9-Y5;ACGGCGGAGCTGGCGAAAGGGGGAAGGGTTT

SEAM-BLACK-1;CTCCAAAATAATAATTTTTTCACGTTTGATGA

SEAM-BLACK-2;GGTAAAATTCATGAGGAAGTTTCCGCCCCCTT

SEAM-BLACK-3;ATCAAGAGAGGCGCATAGGCTGGCTCATATGG

SEAM-BLACK-4;ATGTGTAGTATTTTAAATGCAATGTCTGACCT

SEAM-BLACK-5;TATTTTGTAAATATTTAAATTGTATGGAAACA

SEAM-BLACK-6;GGCACCGCAGATCGCACTCCAGCCTATGGAAG

SEAM-PURPLE-1;TACAGGAGAGCGTCATACATGGCTTTGAAAAT

SEAM-PURPLE-2;ATTAGCGTGGCATTTTCGGTCATAATTAAACG

SEAM-PURPLE-3;TTTACCAGACAATCAATAGAAAATTGACCTTC

SEAM-PURPLE-4;AAATTTAATTAGTTAATTTCATCTCCTGAGTA

SEAM-PURPLE-5;GTACATAAGAATTACCTTTTTTAAAACGTTAA

SEAM-PURPLE-6;GAATTGAAGATTATACTTCTGAATAGCTTTCC

DBLUE-1-p;TCAAGAGAAGGATTAGAAGAGGCT

DBLUE-2-p;CTCAGTACCAGGCGGACGGAACCT

DBLUE-3-p;GGTTGATATAAGTATACCGTATAA

DBLUE-4-p;TCACCGTACTCAGGAGTTTAACGG

DBLUE-5-p;CTCAGAACCGCCACCCTCAGAACCGCCACCCT

DBLUE-6-p;CAGAGCCACCACCCTCCTAAAGGA

DBLUE-7-p;AGCCCAATAGGAACCCACTTTCAG

DBLUE-8-p;AGTTTCGTCACCAGTATCTGTATG

DBLUE-9-p;TAGCATTCCACAGACATGTCGTCT

GREEN-X1-Y1;AAAGTATTGATTAGCGGGGTTTTG

GREEN-X2-Y1;GCCTATTTTAAGTGCCGTCGAGAG

GREEN-X3-Y1;AACAGTGCGCCCGGAATAGGTGTA

GREEN-X4-Y1;TAATAAGTGTTTAGTACCGCCACC

GREEN-X6-Y1;AGGAACAAATTTTCAGGGATAGCA

GREEN-X7-Y1;CTTTCAACATGTACCGTAACACTG

GREEN-X8-Y1;ATGAATTTCAAACTACAACGCCTG

GREEN-X9-Y1;TAAAGTTTGCCCTCATAGTTAGCG

GREEN-X1Y6;ACAACTCGTATTAAATTGAGTAACATTATCATTTTACATC

GREEN-X2Y6;ATTTAGAAGTATTAGAACCACCAGAAGGAGCGTAACGTCA

GREEN-X3-Y6;ATTAGAGCCGTCAATACCTGATTATCAGATGAAGAAATAA

GREEN-X4-Y6;AAAATATCTTTAGGTGTAATCCTGATTGTTTGCCAACCAT

GREEN-X5-Y6;ATAGCTGTTTCCTGTGTTGAGGAA

GREENX6Y6;CGGGTACCGAGCTCGACAGGCAAAGCGCCATTTTGAGGGG

GREENX7Y6;TGCATGCCTGCAGCTCCAACTGTTCCCAAGGGGATGGGCG

GREEN-X8-Y6;CGACGTTGTAAAACGACTTCGCTATTACGCCATTGACCGT

GREEN-X9Y6;TAAGTTGGGTAACGCCTGTGCTGCAAGGCGATCCGTCGGA

BROWN-X9-Y1;TCGCTGAGCCCACGCATAACCGATTAACGATC

GREEN-X9-Y2;ATTGTGTCGAGATTTGTATCATCGATATTCGG

BROWN-X9-Y2;AATCATTGTTGAGATGGTTTAATTCCTGATAA

GREEN-X9-Y3;GTTTACCAAAGAGCAACACTATCATCAACTTT

BROWN-X9-Y3;AGCGGATTCCTGACTATTATAGTCTAACCCTC

GREEN-X9-Y4;GTACGGTGACATGTTTTAAATATGAGAAGCAA

BROWN-X9-Y4;CAATAAAGAGGCAAAGAATTAGCACAACTAAA

GREEN-X9-Y5;GCTGAGAATCTACAAAGGCTATCAAATTAAG

BROWN-X9-Y5;TTCTCCGTGTGAGCGAGTAACAACAGGTCATT

RED-X1-Y1;GAGACTCCCCAGAGCCGCCGCCAGGCCACCAG

BLUE-X1-Y1;AACCACCATCACCAGTAGCACCATATTAGAGC

RED-X1-Y2;CAGCAAAAACCCAAAAGAACTGGCGCAATAAT

BLUE-X1-Y2;AACGGAATGCATTAGACGGGAGAAATAAAAAC

RED-X1-Y3;AGGGAAGCGTTTTGAAGCCTTAAATCCCGACT

BLUE-X1-Y3;TGCGGGAGAATTTACGAGCATGTAAATAATAT

RED-X1-Y4;CCCATCCTGCTCAACAGTAGGGCTCCAGTATA

BLUE-X1-Y4;AAGCCAACCAAAATCATAGGTCTGTCAATAGT

HZ2-RED-X1-Y5;GAATTTATTGATTGCTTTGAATACCAATAACG

HZ2-BLUE-X1-Y5;GATTCGCCATTAATTTTAAAAGTTCCTTTGCC

APPENDIX-B

Circular Origami with two EGFP

Figure 47 illustrates how DNA staples folded into circular Origami with two EGFP

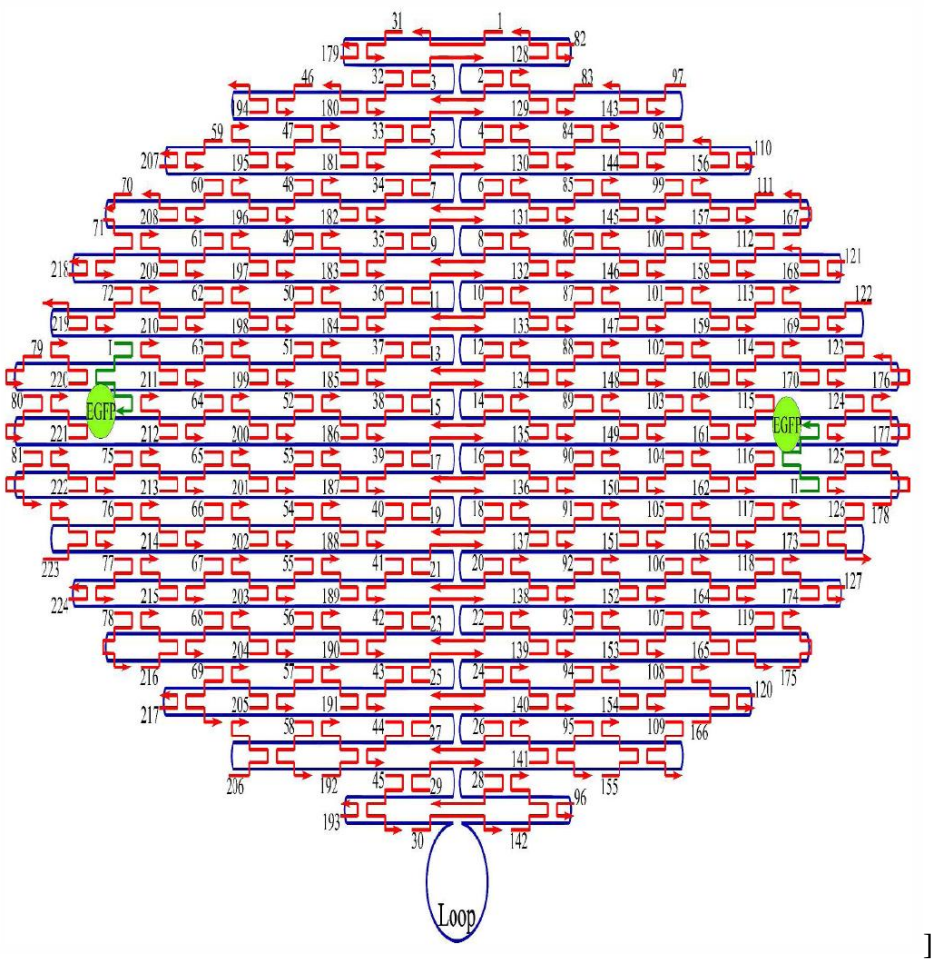

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Figure 47: Schematic showing the design of DNA Origami and displaying each of the two EGFP attachment positions

Modified strand I replaces original strands 73 and 74, Modified strand II replaces original strands 171 and 172, also see Table 3 for the sequences of these two modified strands I and II.

Table 3: DNA sequences for all staple strands folded into the circular Origami with two EGFP

DNA sequences (in HPLC purified form from Integrated DNA Technologies, Inc.) for all the staple strands including the two thio modified strands, Strand I and Strand II. The strand numbers in this table correspond to the numbers in Figure 47, thio modified Strand I replaces strands 73 and 74, thio modified Strand II replaces strands 171 and 172 in Figure 47.

Number Sequences for staple strands including the two replacing strands (Left to right: 5' to 3')
I GTTGGGAAGGTAATAGTAAAATGTCCCTCAAATGCTTTAATTTGCGGATG-S-C3
II AGACAAAGAATAAGGCGTTAAATAGAGCCAGTAATAAGAGAATAATATCC-S-C3
1 GCCCGGAATAGGTGTATCACCGTACTCAGGAG
2 TTTGCTCAGAGAAGGATTAGGATTCCTCATTT
3 TCAGGGATCCCTCAGAGCCACCACAGCGGGGT
4 GGAGTGTATCATACATGGCTTTTGATTTTCTG
5 TATGGGATAGACGTTAGTAAATGAATGATACA
6 CCGCCACCCCCTCAGAGCCGCCACCGCCGACA
7 ATGACAACTTGATACCGATAGTTGCCTCAGAA
8 GCAAAATCTGAGCCATTTGGGAATAGAATACA
9 CTAAAACAAAACGAAAGAGGCAAATAGAGCCA
10 GCATGATTATAACGGAATACCCAATTACCCAA
11 ATCAACGTAAGAACCGGATATTCAAAGAACTG
12 AAATGAAAAAACGATTTTTTGTTTAAAAGGAA
13 TTACGAGGGCAGATACATAACGCCAACGTCAA

14 CGAGAACATATTAAACCAAGTACCCTTCAAAT

15 ATCGCGTTGAGGAAGCCCGAAAGAGCACTCAT

16 TCAACAGTTTCTTACCAGTATAAAGGGGCGCG

17 AGCTGAAAAGCTATATTTTCATTTGCCAACGC

18 AGAGTCAAATAGCTTAGATTAAGACAACCGTT

19 CTAGCTGACCATCAATATGATATTCGCTGAGA

20 GAAACAATGTAACAGTACCTTTTAAAAAATAA

21 TTCGCGTCCAATAGGAACGCCATCCATCGGGA

22 ATTAATTTTATTAAATCCTTTGCCAGGCTGCG

23 CAACTGTTGCGCCATTCGCCATTCCGAACGTT

24 GCCAGCAGGCCTGCAACAGTGCCAGCTCACAA

25 TTCCACACTGTGAAATTGTTATCCCGCTGAGA

26 GCCAACAGACCAGTAATAAAAGGGTCACCAGT

27 GAGACGGGAGGGTGGTTTTTCTTTACATTCTG

28 CGGCCTTGCCTGAGTAGAAGAACTATAGGGTT

29 GAGTGTTGAAAAGAATAGCCCGAGCAAACTAT

30 AACGTCAAAGGGCGAAGTCCATCACGCAAATT

31 GTTTAGTACCGCCACCACCCTCAG

32 AACCGCCAAGCAAGCCCAATAGGAGTTTTGTC

33 GTCTTTCCTTTGCTAAACAACTTTGAATTTCT

34 TAAACAGCAACCATCGCCCACGCACGAAGGCA

35 CCAACCTACTCATCTTTGACCCCCCAAGAGTA

36 ATCTTGACAACAAAGCTGCTCATTACCACATT

37 CAACTAATCATAGTAAGAGCAACAGCATCAAA

38 AAGATTAATTAATTCGAGCTTCAAGGTCAATA

39 ACCTGTTTAGGTGGCATCAATTCTGGAGACAG

40 TCAAATCATAAATTAATGCCGGAGCAGCTCAT

41 TTTTTAACTGGCCTTCCTGTAGCCCCGGAAAC

42 CAGGCAAAGGGAAGGGCGATCGGTTCATAGCT

43 GTTTCCTGAACATACGAGCCGGAATTGCGTAT

44 TGGGCGCCCAACAGCTGATTGCCCAAATCCCT

45 TATAAATCTTCCAGTTTGGAACAAAAAGAACGTGGACTCC

46 ACTGAGTTTCGTCACCGACAGCCC

47 TCATAGTTGTGAGAATAGAAAGGAATTGTATC

48 GGTTTATCTCGCTGAGGCTTGCAGCCATTAAA

49 CGGGTAAAGCGAAACAAAGTACAACAGACCAG

50 GCGCATAGCCCTGACGAGAAACACGTAGAAAG

51 ATTCATCATTACCAGACGACGATACCTGACTA

52 TTATAGTCAGCAAACTCCAACAGGTTTAGTTT

53 GACCATTATTAACATCCAATAAATGTAGGTAA

54 AGATTCAAAGAGATCTACAAAGGCTTGTTAAA

55 ATTCGCATAAATGTGAGCGAGTAACTCCAGCC

56 AGCTTTCCTTACGCCAGCTGGCGACCCGGGTA

57 CCGAGCTCCTGGGGTGCCTAATGAAATGAATC

58 GGCCAACGGAGAGAGTTGCAGCAAAATCCTGTTTGATGGT

59 CGAATAATAATTTTTTCTCCAAAA

60 AAAAGGCTTTTGCGGGATCGTCACTGAGGACT

61 AAAGACTTCGCCTGATAAATTGTGCCAACTTT

62 GAAAGAGGTTGGGCTTGAGATGGTTAAAACGA

63 ACTAACGGGAGGCTTTTGCAAAAGGAATGACC

64 ATAAATCACCTTTAATTGCTCCTTACAGTTGA

65 TTCCCAATAGAATTAGCAAAATTACATATATT

66 TTAAATGCGAGAGTCTGGAGCAAAAAGCAAAT

67 ATTTAAATCCGTGGGAACAAACGGACGACGAC

68 AGTATCGGAAGGCGATTAAGTTGGCTTGCATG

69 CCTGCAGGTAATTGCGTTGCGCTCCCAGTCGGGAAACCTG

70 CAGCATCGGAA

71 CGAGGGTAGCAGCTCCATGTTACTTAGGCAGACGG

72 TCAATCATTCATTGTGAATTACCTGTCAGGAC

73 GTTGGGAAGGTAATAGTAAAATGTCCCTCAAA

74 TGCTTTAATTTGCGGATGGCTTAGAAGTACGG

75 TGTCTGGAAGCATAAAGCTAAATCTTCAACGC

76 AAGGATAACGGTAATCGTAAAACTTCAGAAAA

77 GCCCCAAAGGATAGGTCACGTTGGCATCGTAA

78 CCGTGCATCCCAGTCACGATTTTGCTTGTAAAAC

79 CCAATACTGCGTTTTGAA

80 TCGTCATAATAATGCTGTATTTTGCT

81 CAACATGTTTATGACCCTGTTTTTAATACTTTTG

82 TTCGAGAGTGCCGTTT

83 CATGAAAGTATTAAGAACGGGGTC

84 AGTGCCTTCGCAGTCTCTGAATTTTCAGAGCC

85 GCCACCAGCACCGGAACCAGAGCCCCATTAGC

86 AAGGCCGGTCATTAAAGGTGAATTCAGTATGT

87 TAGCAAACAGTTACCAGAAGGAAAAGAGAATA

88 ACATAAAAACAGCCATATTATTTATTTCATCG

89 TAGGAATCCGGCTGTCTTTCCTTATCGCCATA

90 TTTAACAAAAGCCTGTTTAGTATCATCATAGG

91 TCTGAGAGAATTTTCCCTTAGAATTGCTTTGA

92 ATACCAAGGATTTTCAGGTTTAACATTATCAT

93 TTTGCGGAGTATTAGACTTTACAAAGCATCAC

94 CTTGCTGAAAAACAGAGGTGAGGCACCTGAAA

95 GCGTAAGATTATTTACATTGGCAGCAATATTACCGCCAGC

96 TTTGATTACTTCTTTT

97 TTCCCTGCCTATTTCGGAATAAACAG

98 TTAATGCCAAATAAATCCTCATTAGCCGCCAG

99 CATTGACAATTAGCGTTTGCCATCACCATCGA

100 TAGCAGCAAGGGAGGGAAGGTAAATAAAGGTG

101 GCAACATATTTTTAAGAAAAGTAAGACGGGAG

102 AATTAACTTTCCAGAGCCTAATTTGCAAGCAA

103 ATCAGATAAATTTACGAGCATGTAAGGCAGAG

104 GCATTTTCAGAATAAACACCGGAACCGGCTTA

105 GGTTGGGTAACCTTGCTTCTGTAAAGGCGAAT

106 TATTCATTTGCACGTAAAACAGAAAAGGAGCG

107 GAATTATCCGTCAATAGATAATACCCTCAATC

108 AATATCTGTAAAAATACCGAACGAATATTTTT

109 GAATGGCTCTACATTTTGACGCTCACGCTCATGGAAATAC

110 TTATTGGCCAGACGTT

111 TTTTCATCGGCATTTTCAGAATCA

112 AGTTTGCCCCAAAGACAAAAGGGCACACCACG

113 GAATAAGTATGAAATAGCAATAGCGTCAGAGG

114 GTAATTGAATCCTGAATCTTACCATATTCTAA

115 GAACGCGAAGTCCTGAACAAGAAAAATATAAA

116 GTACCGACTACCGACCGTGTGATAAATGCTGA

117 TGCAAATCAACAGTACATAAATCAAAGAAGAT

118 GATGAAACGAAGGGTTAGAACCTATCAGATGA

119 TGGCAATTTTTAGGAGCACTAACAACAGTTGAAAGGAATT

120 TTGATAGCCGAACTTT

121 TTAAATTCAATAGATT

122 TTTGAGTTAAGCCCAATAGATAACCC

123 ACAAGAATTTAGTTGCTATTTTGCTCCCGACT

124 TGCGGGAGTGCAGAACGCGCCTGTCTGTCCAG

125 ACGACGACTCATCTTCTGACCTAAAACGCGAG

126 AAAACTTTACAATTTCATTTGAATAAATTAATTACATTTATT

127 TTTGGATTATTGTTTT

128 CGGATAAGGGTTGATATAAGTATA

129 AAGTTTTAGGCTGAGACTCCTCAAGTACCAGG

130 CACCACCCACCGTTCCAGTAAGCGCTGGTAAT

131 CACCATTAACCACCGGAACCGCCTCTCAGAGC

132 TTATTACGATCACCGTCACCGACTACCAGTAG

133 CTTTACAGCCGAGGAAACGCAATAAAGACTCC

134 GTTTTTATTCCCAATCCAAATAAGATAGCAGC

135 ATTGAGAATCATTCCAAGAACGGGAGCAAGCC

136 TTATCAAAATATGCGTTATACAAAAGGGCTTA

137 CGCCTGATCCTTGAAAACATAGCGTAGTGAAT

138 TGAGTAACGTCAGATGAATATACAAACGGATT

139 AAATCTAAACAATTCGACAACTCGTAAAAGTT

140 CCCTTCTGGGTCAGTATTAACACCCAAATGAA

141 ATCCAGAAATTCACCAGTCACACGAGATAGAA

142 AACCGTTGTAGCAATAGTAATAACATCACTTGCTGGTAAT

143 GTGCCCGTACCTATTATTCTGAAA

144 CCAGAGCCAAGCCAGAATGGAAAGGAGTAACA

145 CCAATGAATTTTCATAATCAAAATAACCACCA

146 TACATACATATTGACGGAAATTATAAACGTCA

147 GCGCATTAGCAGATAGCCGAACAAGTAGAAAA

148 GCCCAATAGCCAGTTACAAAATAAACAGGGAA

149 TGTAATTTGAAACCAATCAATAATATTACCGC

150 TTTAACCTTCATAATTACTAGAAACGCCAACA

151 TCGCGCAGATCGTCGCTATTAATTACTACCTT

152 ACCACCAGATAAAGAAATTGCGTATTACAAAA

153 TATCAAACATTTGAGGATTTAGAAACAAAGAA

154 CACAGACAACCACCAGCAGAAGATACCTCAAA

155 CATTGCAACAGGAAAAAATCGTCTGAAATGGAATACGTGG

156 AGGCAGGTCTTGATATACACAAAC

157 AGTAGCGACGGTCATAGCCCCCTTGGAGGTTG

158 ACGCAAAGGACATTCAACCGATTGCCGTAATC

159 TGAACAAATATCTTACCGAAGCCCTAAAAGAA

160 TTATCCGGACGCTAACGAGCGTCTGAACACCC

161 AATAAGAGAATAATATCCCATCCTTAGAAGGC

162 TATATGTAAATAAGGCGTTAAATAGAGCCAGT

163 CTGAGCAAATATATGTGAGTGAATTATATAAC

164 CCTGATTACCATATCAAAATTATTTCAATTAC

165 GCAAATCAACTAATAGATTAGAGCATCATATT

166 ATTAGTCTTTAATGCGCCTAAAACATCGCCATGTCAGTTG

167 TTTAGCGTCAGTTTTACTGTAGCGCG

168 TCACAATCATATGGTTTACCAGCG

169 ATCAGAGAATAAGAGCAAGAAACATTATTTTG

170 AGCGAACCACCCAGCTACAATTTTGCGCTAAT

171 AAGTAATTTTATCAACAATAGATAGGCGTTTT

172 AGACAAAGATTTAATGGTTTGAAAAAAAGGTA

173 AGAAAACATACCTTTTTTAATGGACAATCGCA

174 TAATCCTGATACTTCTGAATAATGAAACATCA

175 GAGGAAGGTTATTTTTCTAAAATATCCATCAATA

176 GCCTTTTTTAAATCAAGA

177 ACATTTTTGTTCAGCTAAGTTTTGAA

178 TTCAAATATATTTTTTTTAGTTAATTAATAAACA

179 TTACCGCCCTCAGATT

180 GATCTAAAACCCATGTACCGTAAC

181 TTCGAGGTCAACAGTTTCAGCGGAAGCGTAAC

182 TGCCACTATAACCGATATATTCGGAGCTTGCT

183 ACCTTCATAGCGATTATACCAAGCATACGTAA

184 TTAGGAATCAGTGAATAAGGCTTGGCTGGCTG

185 AGCGGATTCTATCATAACCCTCGTGTTGAGAT

186 TCGCAAATAGCGAACCAGACCGGAAGAAGCAA

187 GAAAGGCCACTAATAGTAGTAGCAGATACATT

188 TGTTAAATAGGGTAGCTATTTTTGAAGGGTGA

189 TTCTGGTGAGCTTTCATCAACATTTAAATTTT

190 AATCATGGGCGGGCCTCTTCGCTAGGCACCGC

191 GAGGCGGTGCATAAAGTGTAAAGCGAATTCGT

192 GGTTCCGAAATCGGCATTCACCGCCTGGCCCTCGCGGGGA

193 TTACTATTGAGTCCTT

194 ATTCCACAAGTACAAACTACAACGTT

195 AGCCTTTAACAACTAAAGGAATTGCCTGTAGC

196 GGAAGTTTGGAGTTAAAGGCCGCTCCAAAAGG

197 ACGGTGTACGGAGATTTGTATCATTTTCATGA

198 TATTACAGCAGAACGAGTAGTAAAACAGATGA

199 GTCTTTACAAAACCAAAATAGCGAAACAACAT

200 CGAGTAGATCAGGATTAGAGAGTAAAAATCAG

201 AGTAATGTCATACAGGCAAGGCAATCTGCGAA

202 TTAATATTTATCAGGTCATTGCCTAATGCCTG

203 AGATCGCACAACCCGTCGGATTCTTGTAAACG

204 AGAGGATCAAGGGGGATGTGCTGCCCTCAGGA

205 GCTGCATTGTGAGCTAACTCACATTCGACTCT

206 TTTGCCCCAGCAGGCGAAGCGGTCCACGCTGGTTTCGTGCCA

207 TTGAAAATCACGTTTT

208 AGAGGCTTCCTCAGCAGCGAAAGA

209 CGAACTGATCGAAATCCGCGACCTACGGCTAC

210 TACGTTAATTAATTTCAACTTTAAAAGGGAAC

211 GAAAACGAAAGTTTTGCCAGAGGGGAAAAATC

212 TCCATATATTGATAAGAGGTCATTACAGTTCA

213 AGAACCCTAGCAATAAAGCCTCAGAGTTTCAT

214 GATTGTATCAAGAGAATCGATGAAAAATTTTT

215 TTGAGGGGCGGATTGACCGTAATGAACAGGAA

216 GACGGCCAGTGCCAAGGTAACGCCAGGGTTTTCTGCCAGT

217 TTCGCTTTACTGCCTT

218 TTCGAGGCCCGGAATT

219 TTATACCATATGCGATTTTAAGAATT

220 TTGAATCCTTAGACTGGATAGCGTCTGGCTCA

221 TGCAACTAAGCTTAATTGCTGAATAATATTCA

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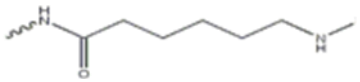
224 TTTGGGCGTGTAGATT



C_6 Linker

APPENDIX-C

Triangular Origami

Table 4: DNA sequences for all DNA staple strands used to produce Triangular Origami.

Here below are the all DNA staple sequences (in HPLC purified form from Intergrated DNA Technologies, Inc.) that are used for design along with linkers DNA to attach fluorophore to DNA.

TATATTTTAAATTTT;Linker-L-1P-T1
TACCTTTAATTTTTT;Linker-L-2P-T1
AGACTGGATAGTTTT;Linker-L-3P-T1
CAAAAGAATTTTTTT;Linker-L-4P-T1
CCCTCAGCAGCTTTT;Linker-R-1P-T1
ATTCAGTGAATTTTT;Linker-R-2P-T1
CAAAAGGAATTTTTT;Linker-R-3P-T1
CCTCGTTTTTTTTTT;Linker-R-4P-T1
ATTTTTAGAACCCTCACAATAAAT;BLUE-X1-Y1-T1
CATACAGGTAGTAGCATTAACATC;RED-X1-Y1-T1
TTTGTTGATAACATT;BLUE-X1-Y2-T1

AGAATTAGGAACGCAAGGATAAAA;BROWN-X1-Y1-T1

TCTGCGAATCAATTCTACTAATAGCAAGGCAA;GREEN-X1-Y1-T1

GCTCCTTTTGAGAAGTTTCATTCCATATTCCCAAT;BROWN-X1-Y2-T1

GGAGAAGCCTTTATTTCAAAATTA;BLUE-X2-Y1-T1

AGCAATAAAGCTGAAAAGGTGGCACGAGTAGA;RED-X2-Y1-T1

TTTAGTTTTAAAGTACGGTGTCTGTAAGAGGT;BLUE-X2-Y2-T1

CATTTTTGGGTCAGGATTAGAGAGAAGAGGAA;RED-X2-Y2-T1

GCCCGAAATTGCATCAAAAAGATT;BLUE-X2-Y3-T1

TTGAAAACAGTTCAT;RED-X2-Y3-T1

AGCATAAACTGTAATACTTTTGCG;BROWN-X2-Y1-T1

GATACATTTTTCATTTGGGGCGCGAGCCTCAG;GREEN-X2-Y1-T1

TTAGAGCTGTTTTAAATATGCAACGACCATTA;BROWN-X2-Y2-T1

ATATCGCGGAAGCAAACTCCAACACGGATGGC;GREEN-X2-Y2-T1

CCATAAATTCAGAAGCAAAGCGGAGACTTCAA;BROWN-X2-Y3-T1

CGTCCAATACTCTCAAATGCTTTAAACGAGAATGA;GREEN-X2-Y3-T1

TAAAATACATGAGGAAGTTTCCATCAAAAACA;SEAM-1-T1

TTATGACCGCTAAATCGGTTGTACTAAACGGG;SEAM-2-T1

ACCTGCTCTGATAAATTGTGTCGAACCTGTTT;SEAM-3-T1

AGCTATATTCGCAAATGGTCAATAAATCCGCG;SEAM-4-T1

CTTGACAATGGCTGACCTTCATCATGCTGTAG;SEAM-5-T1

CTCAACATTAATTGCTGAATATAAAGAGTAAT;SEAM-6-T1

TTAATTTCGTAGTAAATTGGGCTTAAAGCGAA;SEAM-7-T1

CCAGACCGTTTTAATTCGAGCTTCGAGATGGT;SEAM-8-T1

TAAAACGAGTTGGGAAGAAAAATCACCCTGAC;SEAM-9-T1

TATTATAGCAAAAATCAGGTCTTTTACGTTAA;SEAM-10-T1

TAATGCAGAGATTTAGGAATACCATATTCATT;SEAM-11-T1

GAATCCCCGCGGAATCGTCATAAACATTCAAC;SEAM-12-T1

ATCATAACACGAGGCATAGTAAGATAATAGTA;SEAM-13-T1

AAATGTTTGTTTTGCCAGAGGGGGGCAACACT;SEAM-14-T1

ACCAGACGGGCTTTTG;SEAM-15-T1

TTGCGAGAACGATAT;SEAM-16-T1

TAAAACCAAAATATT;SEAM-17-T1

GACTAAAGACTTTTTCGTAATGCC;BLUE-X4-Y1-T1

ACTACGAAGATTTGTATCATCGCCCATGTTAC;RED-X4-Y1-T1

TTAGCCGGGACCAGGCGCATAGGCGAACCGGA;BLUE-X4-Y2-T1

TATTCATTAGAAACACCAGAACGAAACTTTAA;RED-X4-Y2-T1

TCATTGTGTTATACCAGTCAGGACACTAACGG;BLUE-X4-Y3-T1

AACAACATAAAGATTCATCAGTTGATACATAACGC;RED-X4-Y3-T1

CCTAAAACCTACAGAGGCTTTGAG;BROWN-X4-Y1-T1

GCAGACGGAACAAAGTACAACGGAGGCACCAA;GREEN-X4-Y1-T1

CAAGCTAAAGATGAACGGTGTACAAACGAGGC;BROWN-X4-Y2-T1

TATGCGATAAGGCTTGCCCTGACGACCCAAAT;GREEN-X4-Y2-T1

TTTAAGAACTGGCTCAAATTACCT;BROWN-X4-Y3-T1

TAGGTAGTATTACTT;GREEN-X4-Y3-T1

ACGAGGGTAGCAACGGGAAAGAGG;BLUE-X5-Y1-T1

CAAAAGAAATTATACCAAGCGCGATCAATCAT;RED-X5-Y1-T1

AAGGGAACAACTTTGAAAGAGGACCAAAGCTGCTC;BLUE-X5-Y2-T1

AACACTCAGAAAGACAGCATCGGA;BROWN-X5-Y1-T1

TCTTTGACCCCCAGCGTACACTAA;GREEN-X5-Y1-T1

TTTGACCCGAACTTT;BROWN-X5-Y2-T1

GTGAGAATAGATTTTTTTTCAGACGTTAGT;Linker-12-1-T2

CCTTTAATTGTTTTTTTCGTCACCAGTA;Linker-12-2-T2

TTTGCGGGATCTTTTAGCCCGGAATA;Linker-12-3-T2

CATATTTAACATTTTTTTTTGCGTTATACA;Linker-23-1-T2

GTAATTCTGTCTTTTTTAAATACCGACC;Linker-23-2-T2

TCCTTATCATTTTTTGAGACTACCTT;Linker-23-3-T2

TTTTGTTAAAATTTTTTTTTAATCAGAAAA;Linker-31-1-T2

CTTCCTGTAGCTTTTTTTCATTGCCTGA;Linker-31-2-T2

GGACGACGACATTTTATGCCTGAGTA;Linker-31-3-T2

TTTATTTAAAAGCCATTT;Seam-3-1-T2

AATTCTTACCAGTATAATTGTAAACGTTAATA;Seam-3-2-T2

TAACCAATTTTTTGTTAAATCAGCACTAGAAA;Seam-3-3-T2

AAGCCTGTCACCGGAATCATAATTTCATTTTT;Seam-3-4-T2

GATTGACCGATTCTCCGTGGGAACAAAACTTT;Seam-3-5-T2

TTCAAATAAGACAAAGAACGCGAGAAACGGCG;Seam-3-6-T2

TTTGTAGGGTGGGATTTT;Seam-2-1-T2

AAATGAATTTTCTGTACTTAATTGAGAATCGC;Seam-2-2-T2

GCCAGTAATAATTTAGGCAGAGGCTAACGATC;Seam-2-3-T2

TAAAGTTTGCCCTCATAGTTAGCGATTTTCGA;Seam-2-4-T2

TCCTGAACGCCTGTTTATCAACAAACCCTCAT;Seam-2-5-T2

TTTCAGGGCACCCTCAGAGCCACCTAGATAAG;Seam-2-6-T2

TTTAACTTTGATTGTTTT;Seam-1-1-T2

GCCCCAAAAACAGGAACAACAGTTTCAGCGGA;Seam-1-2-T2

TTCACGTTAAAGGAATTGCGAATACATGTCAA;Seam-1-3-T2

TCATATGTGTAATCGTAAAACTAGATAATTTT;Seam-1-4-T2

ATGACAACTTGATACCGATAGTTGCTGATAAA;Seam-1-5-T2

TTAATGCCTATTCAACCGTTCTAGCGCCGACA;Seam-1-6-T2

GTGAGAATAGATTTTTTTTCAGACGTTAGT;Linker-12-1-T3

CCTTTAATTGTTTTTTTCGTCACCAGTA;Linker-12-2-T3

TTTGCGGGATCTTTTAGCCCGGAATA;Linker-12-3-T3

CATATTTAACATTTTTTTTTGCGTTATACA;Linker-23-1-T3

GTAATTCTGTCTTTTTTAAATACCGACC;Linker-23-2-T3

TCCTTATCATTTTTTGAGACTACCTT;Linker-23-3-T3

TTTTGTTAAAATTTTTTTTTAATCAGAAAA;Linker-31-1-T3

CTTCCTGTAGCTTTTTTTCATTGCCTGA;Linker-31-2-T3

GGACGACGACATTTTATGCCTGAGTA;Linker-31-3-T3

TTTATTTAAAAGCCATTT;Seam-3-1-T3

AATTCTTACCAGTATAATTGTAAACGTTAATA;Seam-3-2-T3

TAACCAATTTTTTGTTAAATCAGCACTAGAAA;Seam-3-3-T3

AAGCCTGTCACCGGAATCATAATTTCATTTTT;Seam-3-4-T3

GATTGACCGATTCTCCGTGGGAACAAAACTTT;Seam-3-5-T3

TTCAAATAAGACAAAGAACGCGAGAAACGGCG;Seam-3-6-T3

TTTGTAGGGTGGGATTTT;Seam-2-1-T3

AAATGAATTTTCTGTACTTAATTGAGAATCGC;Seam-2-2-T3

GCCAGTAATAATTTAGGCAGAGGCTAACGATC;Seam-2-3-T3

TAAAGTTTGCCCTCATAGTTAGCGATTTTCGA;Seam-2-4-T3

TCCTGAACGCCTGTTTATCAACAAACCCTCAT;Seam-2-5-T3

TTTCAGGGCACCCTCAGAGCCACCTAGATAAG;Seam-2-6-T3

TTTAACTTTGATTGTTTT;Seam-1-1-T3

GCCCCAAAAACAGGAACAACAGTTTCAGCGGA;Seam-1-2-T3

TTCACGTTAAAGGAATTGCGAATACATGTCAA;Seam-1-3-T3

TCATATGTGTAATCGTAAAACTAGATAATTTT;Seam-1-4-T3

ATGACAACTTGATACCGATAGTTGCTGATAAA;Seam-1-5-T3

TTAATGCCTATTCAACCGTTCTAGCGCCGACA;Seam-1-6-T3

ATGTGTAGGTAAAGAT;Blue-31x1y1-T3

TCAAAAGGGTGAGAAAAGGCTATCAGG;Brown-31x1y1-T3

ATCTACAAGGCCGGAGACAGTCAA;Blue-31x2y1-T3

GAGTCTGGAGCAAACATTTGAGAG;Red-31x2y1-T3

ATCACCATCAATATGAGGAGAGGG;Brown-31x2y1-T3

TAGCTATTAGAGAATCGATGAACGACCCCGGTTGA;Green-31x2y1-T3

TAAACAGCAACCATCGCCCACGCA;Blue-31x4y1-T3

AAGGAACAACTGAAAATCTCCAAAAAAGAATTTCT;Red-31x4y1-T3

TAACCGATATATTCGGAGCTTGCT;Brown-31x4y1-T3

TTCGAGGTAAGGCTCCAAAAGGAG;Green-31x4y1-T3

ATCGGTTTATCTCGCTGAGGCTTGCAG;Blue-31x5y1-T3

GGAGTTAAAGGCCGCT;Brown-31x5y1-T3

GGTGTATCACCGTACT;Blue-32x1y1-T3

CAGGAGGTTTAGTACCACACTGAGTTT;Brown-32x1y1-T3

GTACCGTAGCCACCCTCAGAACCG;Blue-32x2y1-T3

CAAACTACAACGCCTGGAACCCAT;Red-32x2y1-T3

CCACCCTCAGAACCGCATAGCAAG;Brown-32x2y1-T3

CCCAATAGTAGCATTCCACAGACATGTCGTCTTTC;Green-32x2y1-T3

CAGAACGCAAGAAAAATAATATCC;Blue-32x4y1-T3

ACGCCAACATGTAAGAGAATATAAAGTAGCTAATG;Red-32x4y1-T3

CATCCTAATTTACGAGAATAAACA;Brown-32x4y1-T3

ACATGTTCACCGACAAAAGGTAAA;Green-32x4y1-T3

CAGACGACGACCATGTAGAAACCAATC;Blue-32x5y1-T3

AATAATCGGCTGTCTT;Brown-32x5y1-T3

TTTAACCTCCGGCTTA;Blue-33x1y1-T3

GGTTGGGTTATATAACTTAATGGTTTG;Brown-33x1y1-T3

ACCTAAATTATATGTAAATGCTGA;Blue-33x2y1-T3

GTGTGATAAATAAGGCATCTTCTG;Red-33x2y1-T3

TGCAAATCCAATCGCATATTTTAG;Brown-33x2y1-T3

TTAATTTCGTTAAATAAGAATAAATTAGTATCATA;Green-33x2y1-T3

ACCCGTCGGTAATGGGATAGGTCA;Blue-33x4y1-T3

TTCGCATTAAAAGGAACGCCATCAAAAGAGTAACA;Red-33x4y1-T3

CGTTGGTGTAGATGGGAACATTAA;Brown-33x4y1-T3

ATGTGAGCATAATTCGCGTCTGGC;Green-33x4y1-T3

CAGCTTTCATCCGCATCGTAACCGTGC;Blue-33x5y1-T3

ATCTGCCAGTTTGAGG;Brown-33x5y1-T3

AAAATATCTTTTTTT;Linker-L-1P-T4

TTATACTTCTGTTTT;Linker-L-2P-T4

AAATCATAGGTTTTT;Linker-L-3P-T4

TTTTTTGTTCCAGTT;Linker-R-1P-T4

TTTTACCTGTCGTGC;Linker-R-2P-T4

TTTTCGGCCTCAGGA;Linker-R-3P-T4

AATAGTGAATTTATCAATTACCTT;BLUE-X1-Y1-T4

TTTTAATGTAACAATTTCATTTGA;RED-X1-Y1-T4

TTTTAACAGGGAGTT;BLUE-X1-Y2-T4

ACATAAATGACGCTGAGAAGAGTC;BROWN-X1-Y1-T4

TTCGCCTGCAAAATTAATTACATTGAAACAGT;GREEN-X1-Y1-T4

TTTTAATAATGGAAGCAGTACCTTTTACATCATAACGGA;BROWN-X1-Y2-T4

CGATAGCTTAGATTAACAATATAT;BLUE-X2-Y1-T4

GTGAGTGAACAAACATCAAGAAAAATTGCTTT;RED-X2-Y1-T4

GAATACCAGATGAATATACAGTAAGGTTAGAA;BLUE-X2-Y2-T4

CCTACCATATCCTGATTGTTTGGATGCGGAAC;RED-X2-Y2-T4

AAAGAAACAGTAACATTATCATTT;BLUE-X2-Y3-T4

TTTTTAGACTAAGTATTT;RED-X2-Y3-T4

GCTTCTGTATCCTTGAAAACATAG;BROWN-X2-Y1-T4

AATCGCGCAAAAGAAGATGATGAAATAACCTT;GREEN-X2-Y1-T4

TATTTGCATTCAGGTTTAACGTCAAGTTACAA;BROWN-X2-Y2-T4

GGAGCGGAGCAATTCATCAATATAATCAAAAT;GREEN-X2-Y2-T4

AATTCGACTAATTTTAAAAGTTTGCACCAGAA;BROWN-X2-Y3-T4

TTAGGAGCACTAAACATTTGAGGATTTAGTTACAAAC;GREEN-X2-Y3-T4

TTACCGTCTCAATCTT;SEAM-1-T4

TTTTAATATCCGAAAATTTT;SEAM-2-T4

CTCCAACGTCAAAGGGTGGTCAGTTGGCAAAT;SEAM-3-T4

GGTTATCTCAACAGTTGAAAGGAATATTAAAG;SEAM-4-T4

AACGTGGATGGAACAAGAGTCCACTTGAGGAA;SEAM-5-T4

TAGATAATCAACTAATAGATTAGAAAGAATAG;SEAM-6-T4

CCCGAGATATCCCTTATAAATCAAGCCGTCAA;SEAM-7-T4

AACGTTATAACTCGTATTAAATCCCACGCTGG;SEAM-8-T4

TTTGCCCCGTTGCAGCAAGCGGTCTTTGCCCG;SEAM-9-T4

AGATGATGATTATCATCATATTCCATTGGGCG;SEAM-10-T4

CCAGGGTGGAGAGGCGGTTTGCGTTGATTATC;SEAM-11-T4

CGTAGATTCGTAAAACAGAAATAAATGAGTGA;SEAM-12-T4

GCTAACTCAAGCCTGGGGTGCCTAAGAAATTG;SEAM-13-T4

ACCTGAGCAGAGGCGAATTATTCAGACTCTAG;SEAM-14-T4

AGGATCCCTGCATGCCTGCAGGTCTTTCAATT;SEAM-15-T4

CCCTTAGAAAATCGTCGCTATTAACAACTGTT;SEAM-16-T4

GGGAAGGGCGCCATTCAGGCTGCGTTAATTTT;SEAM-17-T4

CAGGCAAAGCGCCATTCGATCGGT;BLUE-X4-Y1-T4

GCGGGCCTCGGCCAGTGCCAAGCTCGGGTACC;RED-X4-Y1-T4

GAGCTCGAGGAAGCATAAAGTGTAACATTAAT;BLUE-X4-Y2-T4

TGCGTTGCTCGGCCAACGCGCGGGGTTTTTCT;RED-X4-Y2-T4

TTTCACCAGCCTGGCCCTGAGAGAAGCAGGCG;BLUE-X4-Y3-T4

AAAATCCTTTCCGAAATCGGCAAAAGGGTTGAGTGTTTT;RED-X4-Y3-T4

TTACGCCATTCTGGTGCCGGAAAC;BROWN-X4-Y1-T4

TCATGGTCCGACGTTGTAAAACGACTTCGCTA;GREEN-X4-Y1-T4

CCCGCTTTACACAACATACGAGCCATTCGTAA;BROWN-X4-Y2-T4

GGCAACAGCAGCTGCATTAATGAAGCTCACTG;GREEN-X4-Y2-T4

CTGATTGCCCTTCACCGTGAGACG;BROWN-X4-Y3-T4

TTTGGTGGGTTTGATTTT;GREEN-X4-Y3-T4

AGCTTTCCGGCACCGCGCTGGCGA;BLUE-X5-Y1-T4

AAGGTGGAAGGGTTTTCCCAGTCAATAGCTGT;RED-X5-Y1-T4

TTCCTGTGATCCGCTCACAATTCCCCAGTCGGGAATTTT;BLUE-X5-Y2-T4

AAGGCGATAGATCGCACTCCAGCC;BROWN-X5-Y1-T4

TAAGTTGGGTAACGCCTGTGCTGC;GREEN-X5-Y1-T4

TTTGTTTGAAATTTT;BROWN-X5-Y2-T4

TGCTGAACCTCAAATATCAAACAGGGCGATGGCCCACTACGT;e1-T4

GAACCATCACCCAAATCAAGTGAAAAATCTAAAGCATCACCT;e2-T4

CTGAGAGCCAGCAGCAAATTTTTTGGGGTCGAGGTGCC;e3-T4

AAAGCACTAAATCGGAACCACCGCCTGCAACAGTGCCA;e4-T4

GGTGAGGCGGTCAGTATTAACCTAAAGGGAGCCCTCGATTTA;e5-T4

GAGCTTGACGGGGAAAGCCGGACCAGCAGAAGATAAAACAGA;e6-T4

APPENDIX-D

Table 5: Written software code in MATLAB for observation of 2 step photobleaching

```
%% Read avi and make 3D uint8 matrix
a=aviread('2_1_n2.avi');
a=cat(3,a.cdata);
imshow(a(:,:,1)); title('Original video: first frame');


%% Crop right part of video and trim black borders
a=a(:,1:end,:);
a=a(25:end-25,256:end-25,:);
imshow(a(:,:,1)); title('Cropped video: first frame');


%% Find location of spots
% Perform temporal averaging with a 10-frame MA filter in the first part of
% the video
ata=convn(a(:,:,1:150),ones(1,1,10)/10,'valid');


%%
% take maxima for each pixel
atam=max(ata,[],3);
imshow(atam./max(atam(:))); title('Maxima for each frame of MA-averaged video');


%%
% recover background
```

```
bgs=imopen(imresize(atam,.1),strel('square',3));

bg=imresize(imfilter(bgs,fspecial('disk',5),'replicate'),size(atam));

imshow(bg,[]); title('Contrast-stretched background');


%%

% compensate for background and rescale

atam=atam-bg;

atam(atam<0)=0;

atam=atam./max(atam(:));

imshow(atam,[]); title({'Maxima for each frame of MA-averaged video';'(background-
compensated and rescaled)'});


%%

% binarize and label

L = bwlabel(atam>0.1,8);

imshow(atam>0.1); title('Binarized spots');


%%

% compute a mask representing areas covered solely by the

% background, which will used for illuminant strenght estimation later.


background_mask = atam<0.1;

% leave a very large 15 pixel margin around spots

background_mask = imerode(background_mask,strel('disk',15));

imshow(background_mask);  title('Mask of the background-only pixels');
```

```
%% Filter spots, and only keep isolated spots
mindistance = 7;
L2 = uint16(zeros(size(L)));
for i=1:max(L(:))
   Ld = imdilate(L==i,strel('disk',mindistance));
   if ~any(Ld & L~=0 & L~=i)
      L2(L==i)=i;
   end
end
RP = regionprops(L2,'Centroid','Area');
RP([RP.Area]==0)=[];

% draw
imshow(double(L>0)+double(L2>0),[]);
for i=1:length(RP)
      text(RP(i).Centroid(1),RP(i).Centroid(2),num2str(i),'Color','blue');
end
title('detected isolated spots (white), vs discarded spots (gray)');

%% Copy isolated spots in matrix of spots
% first prepare lookup table to copy frame pixels to appropriate spot ...
ssize=11;   % insert an odd number here
spotsLUT=zeros(ssize,ssize,length(RP));
for i=1:length(RP)
```

```
    r=round(RP(i).Centroid(2))-(ssize-1)/2:round(RP(i).Centroid(2))+(ssize-1)/2;
    c=round(RP(i).Centroid(1))-(ssize-1)/2:round(RP(i).Centroid(1))+(ssize-1)/2;
    r(r<1)=1; r(r>size(a,1))=size(a,1);
    c(c<1)=1; c(c>size(a,2))=size(a,2);
    [C,R]=meshgrid(c,r);
    spotsLUT(:,:,i)=sub2ind(size(a),R,C);
end


%%
% ... recover background from original movie ...
am=mean(a,3);
bgs=imopen(imresize(am,.1),strel('square',3));
bg=imresize(imfilter(bgs,fspecial('disk',5),'replicate'),size(am));
imshow(bg,[]); title('Contrast-stretched background');


%%
% ... then prepare spots 4D matrix using LUT from every frame
% Provides three variables:
% spots_orig -> original data of spots
% spots_bcorr -> background-corrected data of spots
% spots_bicorr -> background-corrected and multiplicative illuminant-corrected data of
spots


for i=1:size(a,3)   % for each frame in video
    thisframe=double(a(:,:,i));
```

```
    % compute spots_orig
    f_orig=thisframe;
    spots_orig(:,:,:,i)=f_orig(spotsLUT);

    % compute spots_bcorr
    f_bcorr=thisframe-bg;
    spots_bcorr(:,:,:,i)=f_bcorr(spotsLUT);

    % compute spots_bicorr:
    % ... first compute illuminant strength in this frame
    illuminant(i) = mean(thisframe(background_mask))/mean(bg(background_mask));
    f_bicorr=double(a(:,:,i))/illuminant(i)-bg;
    spots_bicorr(:,:,:,i)=f_bicorr(spotsLUT);
end

%%
% Plot graph of computed illuminant strength
plot(illuminant);
title('illuminant strength vs time');
ylabel('illuminant strength (1=average)');
xlabel('time');

%%
% spots matrix indices:
```

```
% spots( row, column, spotID, frame)


%%

% optionally, play movie consisting of isolated spots only


% movie:

%for i=1:size(spots,4)

%    imshow(reshape(spots(:,:,:,i),ssize,[],1),[]);

%    pause(0.05);

%end


%%

% Select which data to use for further processing (uncomment one line)


% spots = spots_orig;   % original spot data

% spots = spots_bcorr;  % bg corrected spot data

% spots = spots_bicorr; % bg and illuminant corrected spot data


spots = spots_bicorr;


%%

% show summary image of spot intensity evolution (rows are spots, columns

% are frames)

summary=[];

for i=1:5:size(spots,4)
```

```
    summary=[summary; reshape(spots(:,:,:,i),ssize,[],1)];
end
imshow(summary',[]);
ylabel('spot ID');
xlabel('time');
h=gca;
set(h,'Visible','on');
set(h,'XAxisLocation','top');
set(h,'XTick',3.5:ssize:ssize*size(summary,2));
set(h,'YTick',3.5:ssize:ssize*size(summary,1));
set(h,'XTickLabel',[]);
set(h,'YTickLabel',[]);



%% Process spot data
% compute average intensity for each spot (rows are spots, columns are frames)
data=squeeze(sum(sum(spots,1),2));
imshow(data,[]); title('spot intensities');


%%
% apply 5-frame MA filter
datam=convn(data,ones(1,5),'valid');
imshow(datam,[]); title('MA-filtered spot intensities');
```

```
%%
% normalize each spot w.r.t. its maximum intensity
datamn=datam./repmat(max(datam,[],2),1,size(datam,2));


%%
% plot the smoothed normalized intensity evolution of some of the spots
h=figure;
for i=1:14
   subplot(7,2,i)
   plot(1:length(datamn(i+28,:)),datamn(i+28,:)');
   axis([0 size(datamn,2) 0 1])
end
set(h,'Position',[1 1 700 1500]);
```

Table 6: Written code in Matlab for averaging all frames

```
%% Both Fluoro

a=aviread('C:\Documents and Settings\rajulapati\Desktop\41_n2_smallthermobox_mclamp1hr
before_sp43_2fl_1-5.avi');

a=cat(3,a.cdata);

%imshow(a(:,:,1)); title('Original video: first frame');

%%
```

```
aa=sum(a,3)/1;

%imshow(aa,[0 255])

imwrite(aa,gray(256),'C:\Documents and Settings\rajulapati\Desktop\2.bmp','bmp');

%% 1st Fluoro

a=aviread('C:\Documents and Settings\rajulapati\Desktop\41_n2_smallthermobox_mclamp1hr
before_sp43_1fl_52.avi');

a=cat(3,a.cdata);

%imshow(a(:,:,1)); title('Original video: first frame');

%%

aa=sum(a,3)/1;

%imshow(aa,[0 255])

imwrite(aa,gray(256),'C:\Documents and Settings\rajulapati\Desktop\1.bmp','bmp');

%% Background
```

APPENDIX-E

Table 7: Values obtained for trial 1 & trial 2 for zero, first, and second order fits to photobleaching of rhodamine under N_2 (trial 1, 2) and room air of 21% O_2 (trial 1, 2)

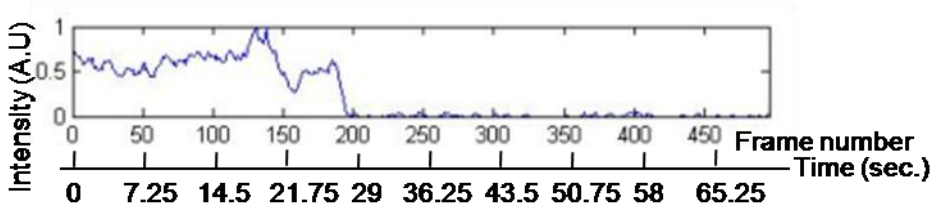
N_2						O_2					
Trail 1						Trail 1					
Zero order		First order		Second order		Zero order		First order		Second order	
Time	Number of Particles	Time	Number of Particles	Time	Number of Particles	Time	Number of Particles	Time	Number of Particles	Time	Number of Particles
1	401	1	5.993961	1	0.002494	1	354	1	5.869297	1	0.002825
4.406	380	4.406	5.940171	4.406	0.002632	4.406	298	4.406	5.697093	4.406	0.003356
7.875	363	7.875	5.894403	7.875	0.002755	7.875	251	7.875	5.525453	7.875	0.003984
11.343	344	11.343	5.840642	11.343	0.002907	11.343	191	11.343	5.252273	11.343	0.005236
14.776	333	14.776	5.808142	14.776	0.003003	14.776	139	14.776	4.934474	14.776	0.007194
18.265	312	18.265	5.743003	18.265	0.003205	18.265	111	18.265	4.70953	18.265	0.009009
21.734	288	21.734	5.66296	21.734	0.003472	21.734	95	21.734	4.553877	21.734	0.010526
25.203	283	25.203	5.645447	25.203	0.003534	25.203	87	25.203	4.465908	25.203	0.011494
28.671	277	28.671	5.624018	28.671	0.00361	28.671	72	28.671	4.276666	28.671	0.013889
32.14	275	32.14	5.616771	32.14	0.003636	32.14	65	32.14	4.174387	32.14	0.015385
35.609	255	35.609	5.541264	35.609	0.003922	35.609	51	35.609	3.931826	35.609	0.019608
39.218	245	39.218	5.501258	39.218	0.004082	39.218	47	39.218	3.850148	39.218	0.021277
42.687	236	42.687	5.463832	42.687	0.004237	42.687	38	42.687	3.637586	42.687	0.026316
46.14	232	46.14	5.446737	46.14	0.00431	46.14	36	46.14	3.583519	46.14	0.027778
49.609	221	49.609	5.398163	49.609	0.004525	49.609	30	49.609	3.401197	49.609	0.033333

N_2						O_2					
Trail 2						Trail 2					
Zero order		First order		Second order		Zero order		First order		Second order	
Time	Number of Particles	Time	Number of Particles	Time	Number of Particles	Time	Number of Particles	Time	Number of Particles	Time	Number of Particles
1.578	150	1.578	5.010635	1.578	0.006667	1.578	99	1.578	4.59512	1.578	0.010101
4.359	146	4.359	4.983607	4.359	0.006849	4.359	96	4.359	4.564348	4.359	0.010417
7.125	148	7.125	4.997212	7.125	0.006757	7.125	85	7.125	4.442651	7.125	0.011765
9.906	143	9.906	4.962845	9.906	0.006993	9.906	73	9.906	4.290459	9.906	0.013699
12.671	140	12.671	4.941642	12.671	0.007143	12.671	65	12.671	4.174387	12.671	0.015385
15.453	134	15.453	4.89784	15.453	0.007463	15.453	58	15.453	4.060443	15.453	0.017241
18.218	133	18.218	4.890349	18.218	0.007519	18.218	55	18.218	4.007333	18.218	0.018182
21	132	21	4.882802	21	0.007576	21	47	21	3.850148	21	0.021277
23.765	129	23.765	4.859812	23.765	0.007752	23.765	52	23.765	3.951244	23.765	0.019231
26.546	126	26.546	4.836282	26.546	0.007937	26.546	49	26.546	3.89182	26.546	0.020408
29.312	120	29.312	4.787492	29.312	0.008333	29.312	39	29.312	3.663562	29.312	0.025641
32.093	114	32.093	4.736198	32.093	0.008772	32.093	38	32.093	3.637586	32.093	0.026316
34.859	115	34.859	4.744932	34.859	0.008696	34.859	34	34.859	3.526361	34.859	0.029412
37.64	118	37.64	4.770685	37.64	0.008475	37.64	34	37.64	3.526361	37.64	0.029412
40.406	112	40.406	4.718499	40.406	0.008929	40.406	35	40.406	3.555348	40.406	0.028571
43.187	115	43.187	4.744932	43.187	0.008696	43.187	36	43.187	3.583519	43.187	0.027778
45.953	110	45.953	4.70048	45.953	0.009091	45.953	34	45.953	3.526361	45.953	0.029412
48.734	108	48.734	4.682131	48.734	0.009259	48.734	35	48.734	3.555348	48.734	0.028571
51.5	110	51.5	4.70048	51.5	0.009091	51.5	30	51.5	3.401197	51.5	0.033333

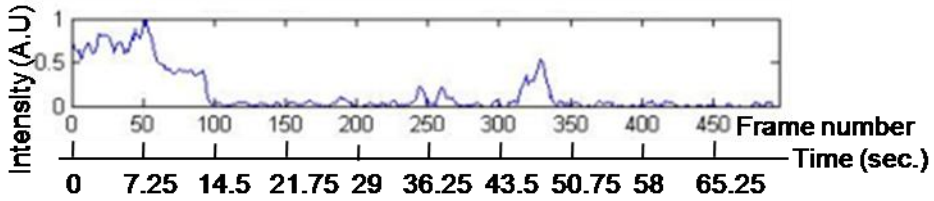
54.281	109	54.281	4.691348	54.281	0.009174	54.281	30	54.281	3.401197	54.281	0.033333
57.062	105	57.062	4.65396	57.062	0.009524	57.062	27	57.062	3.295837	57.062	0.037037
59.687	103	59.687	4.634729	59.687	0.009709	59.687	26	59.687	3.258097	59.687	0.038462
62.468	105	62.468	4.65396	62.468	0.009524	62.468	26	62.468	3.258097	62.468	0.038462
65.234	100	65.234	4.60517	65.234	0.01	65.234	22	65.234	3.091042	65.234	0.045455
68.015	97	68.015	4.574711	68.015	0.010309	68.015	22	68.015	3.091042	68.015	0.045455
70.781	97	70.781	4.574711	70.781	0.010309	70.781	23	70.781	3.135494	70.781	0.043478
73.562	95	73.562	4.553877	73.562	0.010526	73.562	21	73.562	3.044522	73.562	0.047619
76.328	95	76.328	4.553877	76.328	0.010526	76.328	17	76.328	2.833213	76.328	0.058824
79.109	93	79.109	4.532599	79.109	0.010753	79.109	17	79.109	2.833213	79.109	0.058824
81.878	92	81.878	4.521789	81.878	0.01087	81.878	16	81.878	2.772589	81.878	0.0625
84.656	91	84.656	4.51086	84.656	0.010989	84.656	14	84.656	2.639057	84.656	0.071429

APPENDIX-F

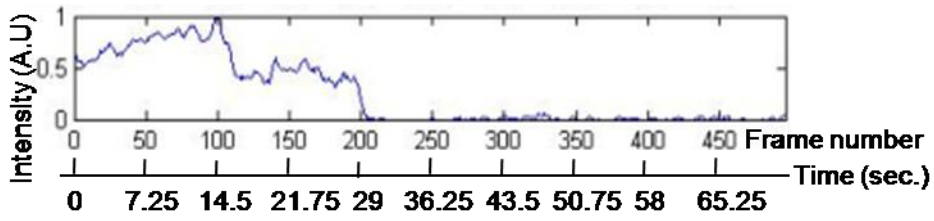
Figure 48: A plot of Intensity versus frame number for two closely spaced rhodamine molecules in rectangular Origami, showing a two-step photobleaching behavior (A – O).



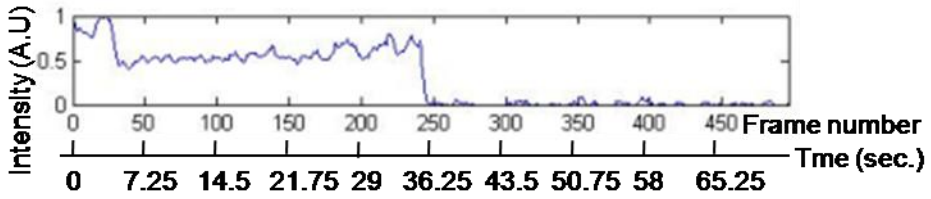
A



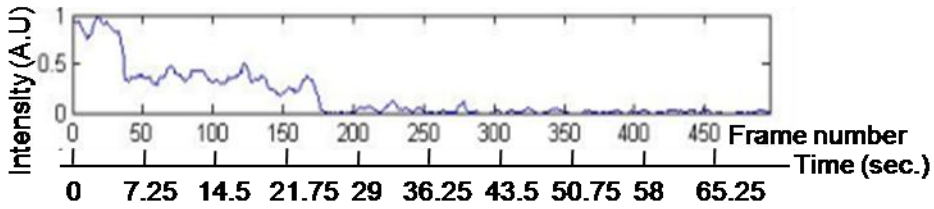
B



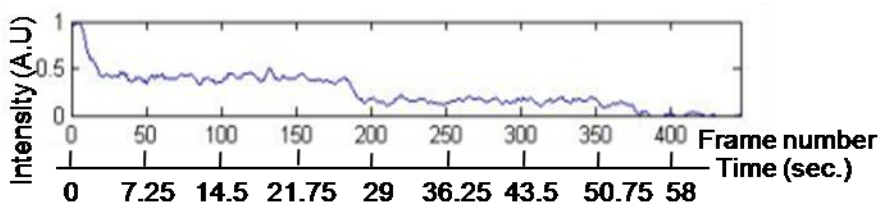
C



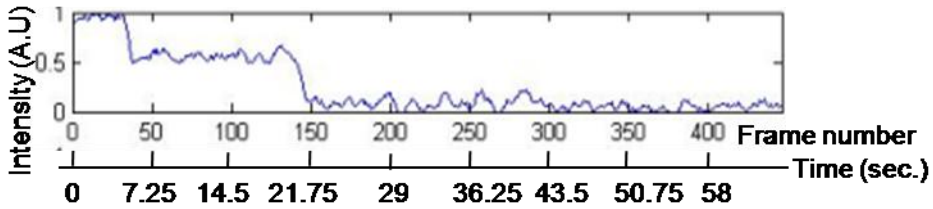
D



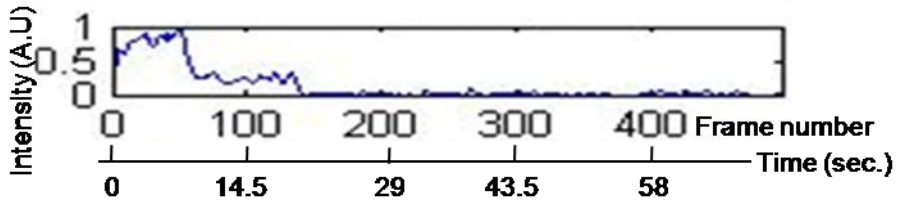
E


Intensity (A.U)
1
0.5
0
0 50 100 150 200 250 300 350 400 Frame number
Time (sec.)
0 7.25 14.5 21.75 29 36.25 43.5 50.75 58

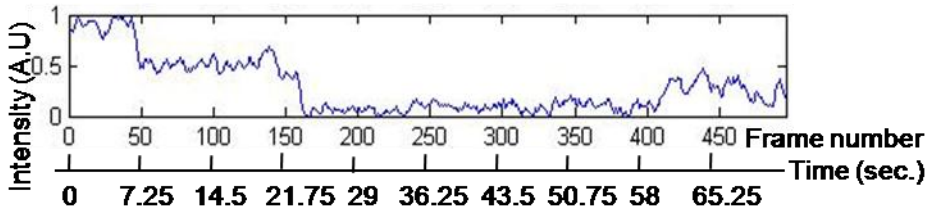
F


Intensity (A.U)
1
0.5
0
0 50 100 150 200 250 300 350 400 Frame number
Time (sec.)
0 7.25 14.5 21.75 29 36.25 43.5 50.75 58

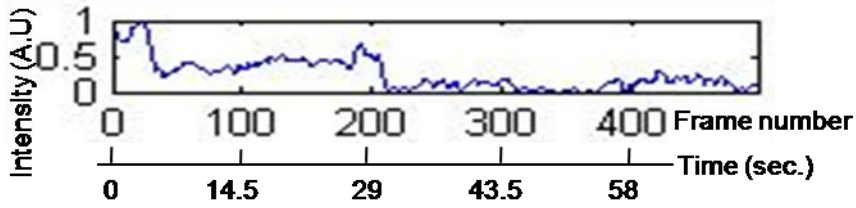
G


Intensity (A.U)
1
0.5
0
0 100 200 300 400 Frame number
Time (sec.)
0 14.5 29 43.5 58

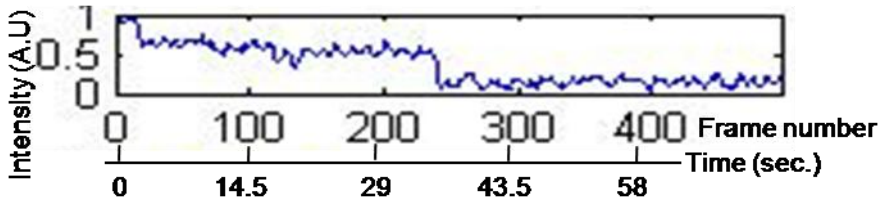
H


Intensity (A.U)
1
0.5
0
0 50 100 150 200 250 300 350 400 450 Frame number
Time (sec.)
0 7.25 14.5 21.75 29 36.25 43.5 50.75 58 65.25

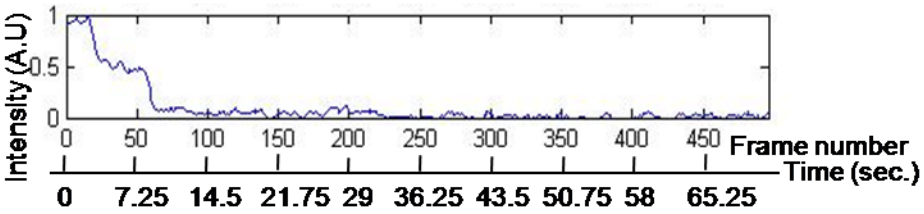
I


Intensity (A.U)
1
0.5
0
0 100 200 300 400 Frame number
Time (sec.)
0 14.5 29 43.5 58

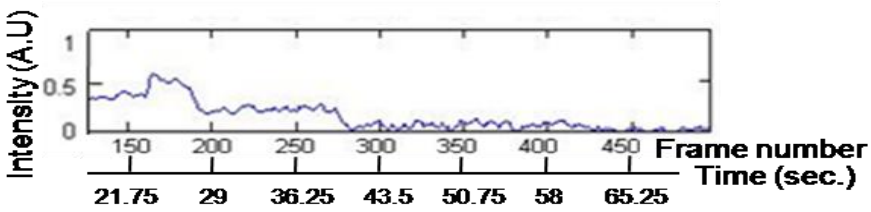
J


Intensity (A.U)
1
0.5
0
0 100 200 300 400 Frame number
Time (sec.)
0 14.5 29 43.5 58

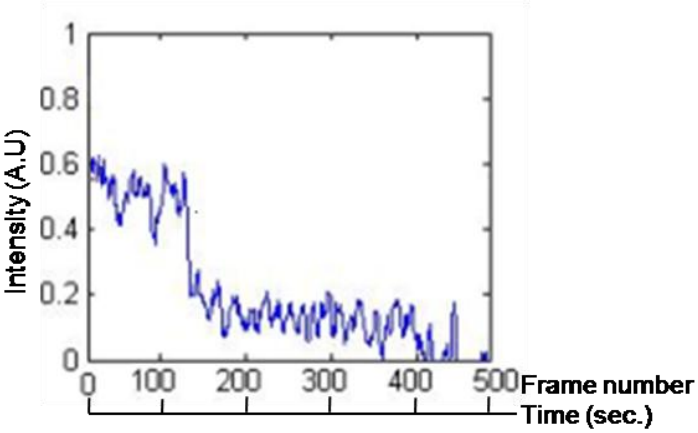
K


Intensity (A.U)
1
0.5
0
0 50 100 150 200 250 300 350 400 450
Frame number
Time (sec.)
0 7.25 14.5 21.75 29 36.25 43.5 50.75 58 65.25

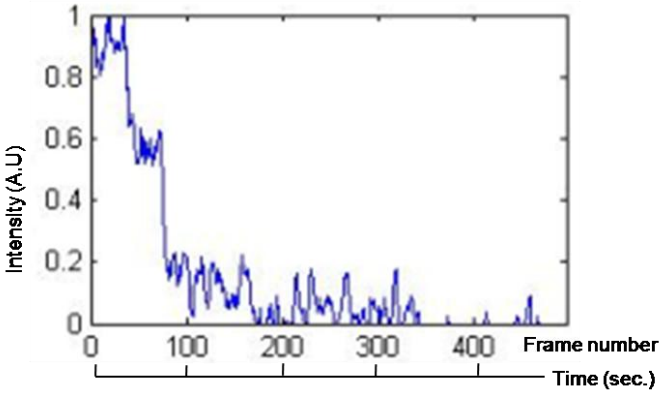
L


Intensity (A.U)
1
0.5
0
150 200 250 300 350 400 450
Frame number
Time (sec.)
21.75 29 36.25 43.5 50.75 58 65.25

M


Intensity (A.U)
1
0.8
0.6
0.4
0.2
0
0 100 200 300 400 500
Frame number
Time (sec.)

N


Intensity (A.U)
1
0.8
0.6
0.4
0.2
0
0 100 200 300 400
Frame number
Time (sec.)

O