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A Single Molecule Nanoactuator: Toward a Device for Drug Discovery at the Limits of Sensitivity

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Abstract

This work was initiated by single molecule studies of the molecular motor activity of the Type I Restriction – Modification enzyme *Eco*R124I at a time when the consensus viewpoint was that such motors could not manipulate microscale objects. Type I Restriction-Modification enzymes process DNA, prior to cleavage, by means of translocation and this work was described using such single molecule studies involving the use of a Magnetic Tweezer setup (which also demonstrated that a micron-sized magnetic bead could be pulled over several microns distance by the 20 nm motor).

Recently, this initial work has led to the development of an electronic version of the Magnetic Tweezer setup, which can also manipulate the DNA-attached bead, allowing its use as a biosensor and tool for drug discovery. The system can be used for a wide range of DNA-manipulating enzymes, many of which are potential drug targets.

Introduction

This work was inspired by studies with a novel molecular motor that is a Type I Restriction-Modification enzyme and it is from this early work that the questions about the capability of such motors first arose. In particular, our studies with *Eco*R124I [1] made it possible to show that biological molecular motors could be used to manipulate materials on the microscale and be used within artificially manufactured devices. However, as will become clear during this paper, our studies have moved away from the R-M enzyme toward other DNA manipulating enzymes as a way to seek commercial development of the device we have produced.

Type I Restriction-Modification Systems

Restriction and Modification (R-M) systems are bacterial protective systems that reduce horizontal transfer of DNA by recognising incoming DNA as ‘‘foreign’’, while ‘‘marking’’ the host DNA as ‘‘self’’ [2]. The most obvious example is illustrated by bacteriophage infection where the bacteriophage DNA is the ‘‘foreign’’, incoming, DNA, which is destroyed by the restriction enzyme (Figure 1). Modification provides the mechanism by which the host DNA is protected from restriction activity. Therefore, restriction enzymes are endonucleases, which recognise and bind at specific DNA sequences and then cleave the DNA unless the protective mechanism of modification prevents this [3]. Modification adds a methyl group to this specific sequence, which prevents cleavage by the associated restriction enzyme, affording the required protection to the host chromosome (Figure 1).

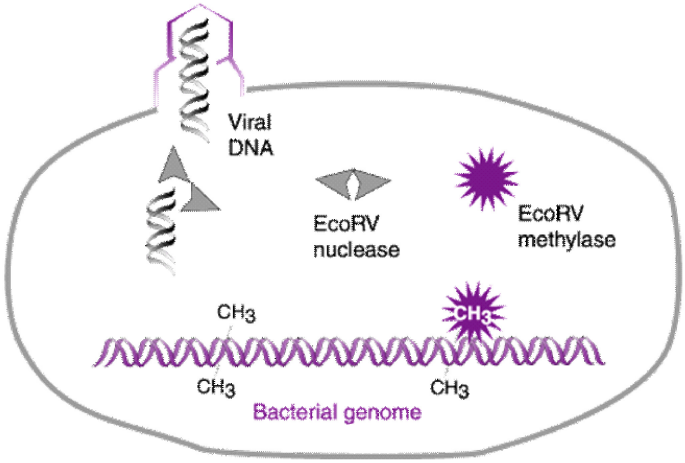

Figure 1. The processes of restriction and modification
The two processes associated with restriction and modification systems involve DNA cleavage by an endonuclease (R.*Eco*RV in this case) and DNA methylation by a DNA methyltransferase (M.*Eco*RV in this case). Methylation occurs within the same DNA binding sequence recognized by the endonuclease and prevents subsequent cleavage at that site.

Type I R-M systems were the first such enzymes identified and unlike the more common Type II enzymes used in Genetic Engineering, they combine the restriction and modification activities into a single, multifunctional, multi-subunit complex [4]. This presents an interesting regulation problem for the enzyme, which must be able to switch between the two activities in a controlled manner [5, 6]. The most obvious control occurs upon DNA-binding and involves the determination of the modification status of the DNA target sequence. If the target is methylated on both strands then the enzyme simply dissociates (this DNA is normally the host chromosomal DNA), while if the DNA is hemi-methylated (methylated on only one strand) then the R-M enzyme acts as a DNA methyltransferase and methylates the unmethylated strand of the target DNA (this would also, normally, be host chromosome DNA, but is hemi-methylated following DNA replication). However, if neither strand of the target DNA is methylated the R-M enzyme undergoes an ATP-dependent conformational change that switches the enzyme to an endonuclease and the DNA is cleaved in an ATP-dependent manner [7]. Cleavage by Type I R-M enzymes is a random process that results in fragments that are generally of random length [8] following a process that involves DNA translocation (Figure 2) [9, 10]. Cleavage appears to occur on a two-site DNA molecule following collision of two translocating enzymes [11], however, on single site circular DNA cleavage is independent of such collisions and appears to follow translocation of "all" of the available DNA [8, 12] suggesting that anything that prevents translocation induces the cleavage reaction [13].

Type I R-M enzymes consist of three different subunits encoded by the following genes [14]:

hsdS: DNA **S**pecificity – this gene encodes the subunit responsible for DNA sequence recognition and binding.

hsdM: DNA **M**odification – this gene encodes the subunit responsible for DNA methylation using the cofactor *S*-adenosyl methionine (AdoMet or SAM).

hsdR: DNA **R**estriction – DNA translocation and cleavage.

The subunits produced from these three genes are arranged with a stoichiometry of $HsdR_2$:$HsdM_2$:$HsdS_1$ (or $R_2M_2S_1$) in the fully functional holoenzyme (R-M enzyme), however, the subunit assembly pathways are complex and enzyme-dependent with a number of possible sub-assemblies [15, 16]. These sub-assemblies have, in some cases, a role in the control of the restriction and modification activities of the enzyme [5].

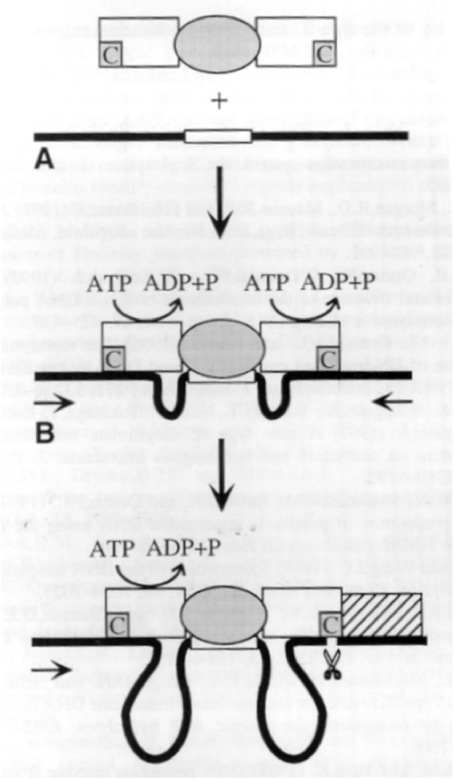

Figure 2. DNA cleavage by a Type I Restriction-Modification enzyme
DNA cleavage by a Type I R-M enzyme involves DNA translocation followed by cleavage at a random site. The core MTase ($HsdM_2$:$HsdS_1$) is illustrated by the grey oval and this complex will bind two HsdR subunits (illustrated by the clear squares) to produce the fully functional R-M enzyme. Binding of this enzyme to an unmethylated recognition sequence (clear rectangle on the DNA, which is represented by a thick black line), in the presence of the cofactor ATP results in DNA translocation (pulling of the DNA) through the bound complex. This continues until a blockage is met (represented by the hatched square) when translocation is stopped and cleavage occurs – this results in a random position of the cleavage site, relative to the binding site, on each molecule as initiation of translocation is asynchronous.

Type I R-M system are divided into four families based on complementation assays, cross-antigenic interactions, biochemical properties, or gene order [3, 17 – 19] – Type IA [20] (typified by the *Eco*KI and *Eco*BI systems), Type IB [21] (typified by *Eco*AI and *Cfr*A), Type IC [22] (typified by *Eco*R124I and *Eco*DXXI) and Type ID [23] (*Kpn*AI). Type IA, IB and ID are all located on the chromosome, but the first members of the Type IC systems were isolated from conjugative plasmids [24]. However, more recent members of this family have been isolated from the chromosome [25, 26]. The location of the *Eco*R124I and *Eco*R124II R-M systems on the conjugative plasmid R124 present a particular problem for control of restriction versus modification activities as this control must be temporal

and modification of the new host chromosome, following conjugal transfer, must occur before any restriction activity [27, 28] and, as mentioned above, for *Eco*R124I this control appears to involve control of subunit assembly with the final assembly step with the second HsdR subunit being the key stage [5]. The $HsdR_1$:$HsdM_2$:$HsdS_1$ sub-assembly complex retains methylation activity and translocation activity [29], but lacks endonuclease activity and the weak P_{RES} promoter limits production of the HsdR subunit, enabling this temporal control.

Magnetic Tweezer Technology

The simplest device for measuring DNA translocation, such as that produced by Type I R-M enzymes, is a Magnetic Tweezer device. Magnetic Tweezers allow real time monitoring of protein-DNA interactions without surface interference and with femtonewton sensitivity. In addition, these systems can measure DNA displacements as low as 10 nm as well as being able to produce negative, or positive, supercoils into the DNA, one turn at a time, through manipulation (spinning) of the magnetic bead [30, 31].

In the study of DNA associated proteins and enzymes the Magnetic Tweezer technique has been found to be a powerful tool [32], it comprises a single DNA molecule anchored at one end to a surface and, at the other end to a micron-scale magnetic bead [33 – 38]. Magnets are used to pull and rotate the microbead, thus stretching and twisting the DNA molecule. The vertical magnetic force causes the DNA to extend and provides a restoring force to restrict the bead's transverse Brownian fluctuations. DNA translocation, winding or twisting induces vertical movement of the bead that can be measured by the Magnetic Tweezer setup.

Comparison of helicases and translocators

The *Eco*R124I motor was used to demonstrate motor activity at the single molecule level, but more recently our interest has moved to DNA helicases. However, there is a close relationship between Type I R-M enzymes and DNA helicases [39]. Although the docked HsdR subunit of *Eco*R124I has primarily been described as a translocase in the literature [40], primary sequence analysis suggests that this motor subunit shares a great deal of sequence identity to the Superfamily 2 (SF2) helicases and would be classified under the scheme proposed by Singleton *et al.* [41] as a SF2β helicase (the β denoting the requirement for a double stranded substrate).

The difference between the helicases and translocases are indeed primarily based upon the biochemical characteristics of the system, with both helicases and translocases moving directionally along DNA strands (either single or double stranded) utilising ATP as an energy source, with the main difference being that helicases unwind the substrate and

translocases simply track it. The motor domain responsible for ATP hydrolysis is also highly conserved between the two types of motor, with both types utilising two RecA-like core domains for ATP hydrolysis and movement.

Both translocases and helicases often have secondary functions, the *Eco*R124I translocating complex being a Type IC restriction endonuclease has coupled endonuclease activity, whereas the non-structural protein 3 (NS3) of the hepatitis C virus, possibly the most well researched SF2 helicase couples its' helicase domain with an N-terminal protease domain that is required for processing of viral polyprotein [42].

These similarities shared between the translocases and helicase have allowed us to broaden the reach of our research and therefore the device to allow the study of SF2 helicases from *Plasmodium falciparum* as potential anti-malaria targets.

Results and Discussion

This paper represents a review and short history of two EC-funded projects (MOL SWITCH) and BIONANO-SWITCH), which were born out of a question set by KF in the late 1990's – "*can we detect the movement produced by a biological molecular motor through its ability to manipulate microscale objects*". Of course, this concept was driven by the elegant work on myosin [43, 44], where single molecule experiments were used to characterise the mode of action of a motor that can influence movement on the macroscale.

The first step along the path, which we will describe in this paper, was a bid for funding to detect motor activity through induced motion of DNA-attached nanoscale gold particles. However, this first step was quickly halted by a referee's comments that "*Stoke's Law indicated that this was not possible as the motor could not generate sufficient force to manipulate such objects*". However, despite this major set-back, a chance meeting between KF, Prof. Cees Dekker and Prof. David Bensimon, at a Bionanotechnology Conference in Berkley, California, led to the idea of measuring motor activity for a Type I R-M enzyme using a Magnetic Tweezer setup and this work built upon the knowledge generated by bulk-solution, biochemical studies.

Measurement of DNA Translocation

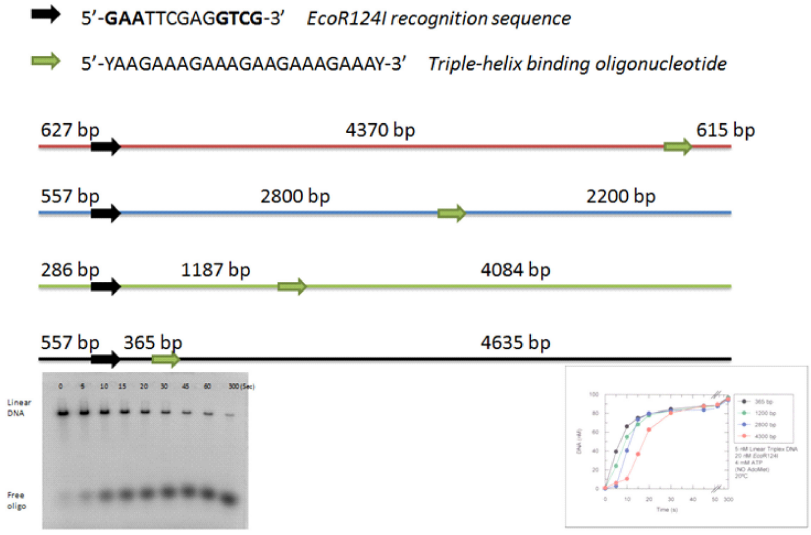

Figure 3. The concept of the triple-helix displacement assay
Four plasmids were prepared with binding sites for the *Eco*R124I enzyme toward one end (black arrows) and triple-helix binding sites at variable distances from the *Eco*R124I binding site (green arrows). Translocation by the *Eco*R124I motor resulted in displacement of a radiolabelled triple-forming oligonucleotide (agarose gel inset at left) and the concentration of bound triplex-forming oligonucleotide against time produced the inset graph showing lag periods at the beginning of displacement.

The first measurements of DNA translocation by the Type I R-M enzyme *Eco*R124I were a collaborative effort with Dr. Mark Szczelkun at the University of Bristol. The work was based on an assay using a triple helix displacement assay developed by Szczelkun [45] in which the distance between the DNA-binding sequence of the *Eco*R124I enzyme and a triple helix binding site was varied (Figure 3). Displacement of the triple-helix-binding oligonucleotide was measured with a radiolabelled sample and the results analysed using agarose gel electrophoresis (Figure 3 inset). Analysis of the data, using a multistep model for translocation and measuring the lag periods for displacement, determined that the speed of translocation was ~400 bp s^{-1}. This provided much of the required background knowledge that was required for single-molecule analysis of translocation and allowed a comparison of the kinetics measured using single molecules as against bulk-solution measurements.

The first step toward single molecule studies with *Eco*R124I was the use of an Atomic Force Microscope (AFM) to analyse the motor bound to DNA and the quality of some of the data obtained from Dekker's laboratory is illustrated (Figure 4), which encouraged us to measure loop-sizes for the translocating molecules and determine the rate of translocation [46]. However, the results obtained were some 10-fold lower than expected due to errors in time measurement with this process. Despite this drawback, the AFM analysis also allowed us to

closely examine the initiation complex for translocation (obtained by using the non-hydrolysable analogue of ATP – ATPγS) and identify an ssDNA ''bubble'' associated with initiation of translocation [46].

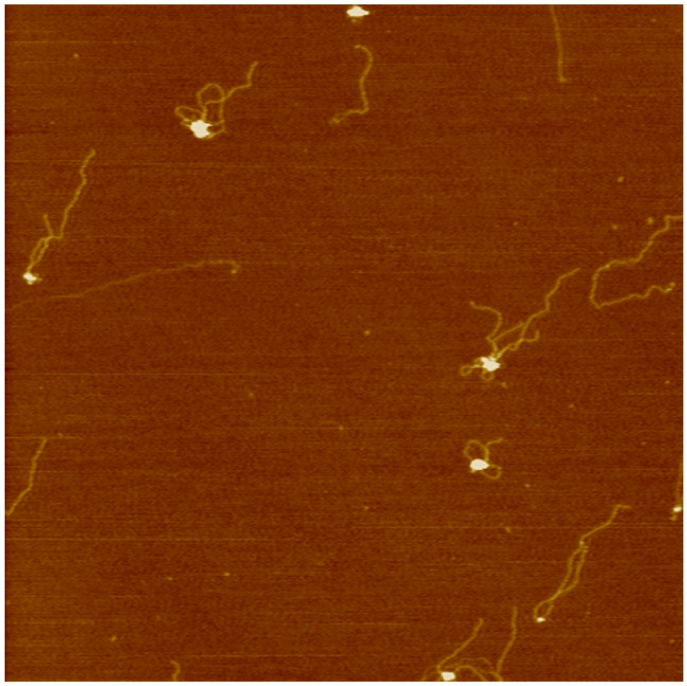

Figure 4. AFM analysis of *Eco*R124I bound to DNA
Images of dsDNA with bound *Eco*R124I enzyme showing loops that are indicative of DNA translocation. There are also some mini-aggregates where several *Eco*R124I enzymes are bound together and a few examples of only the bound MTase.

The AFM work showed that the protein was very clean, bound DNA as expected and seemed to translocate DNA as expected (we believed that the slow translocation rate was an artefact of the timing mechanism following fixing of the complexes onto a mica surface). Therefore, we initiated work with the Magnetic Tweezer setup that could measure the speed of translocation and the forces generated [47]. A typical trace from the Magnetic Tweezer experiments is shown in Figure 5 and shows a motor that has high processivity, moves rapidly from stationary to full speed and resets after a random time period [47]. Analysis of the slope of these traces gave a speed for a single-motor subunit (HsdR) of 450 bp s^{-1}, which is very close to the figure obtained in the bulk experiments (where many of the motors will be idle), which was a very re-assuring situation. Force measurement provided unexpected results as increasing the force on the DNA (pulling opposite to the motor activity) did not stall the motor, but rather decreased processivity until at about 8 pN applied force there was little or no translocation observed. This force is very similar to that generated by motors such as myosin [48] and kinesin [49]. Further, a more detailed analysis using the Magnetic

Tweezer setup allowed us to demonstrate how the stalling of the motor led to dissociation of the motor unit and that the two motor subunits, present in the *Eco*R124I holoenzyme [16], were independently functional motors [29].

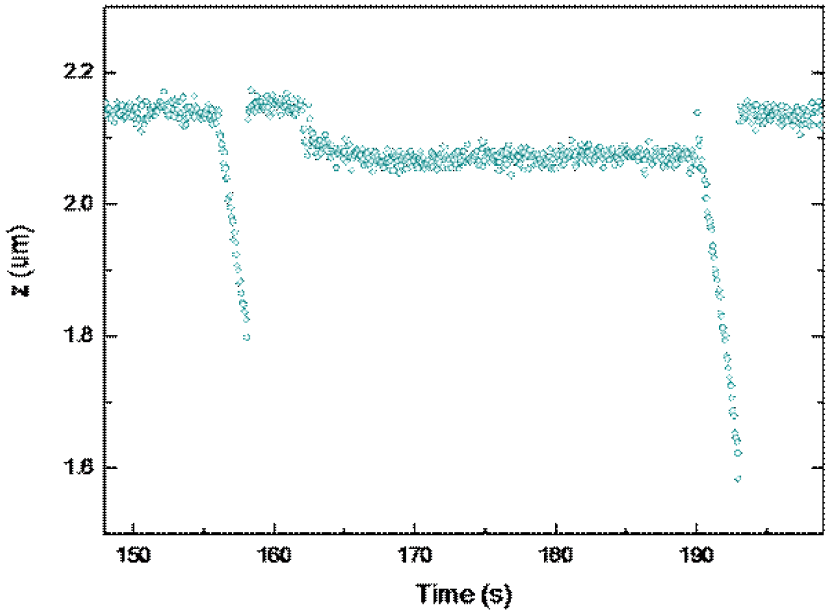

Figure 5. Output from a Magnetic Tweezer analysis of DNA translocation.
A typical trace from a Magnetic Tweezer setup where a 4 kbp linear DNA molecule was attached to the surface of the flow cell through a PCR product, containing 10% Digoxygenin-dUTP, ligated to the plasmid and immobilised on a monolayer of anti-DIG antibodies. At the opposite end of the plasmid is another PCR product, incorporating biotinylated nucleotides, also ligated to the plasmid and the attached to a streptavidin-coated Dynal^{TM} 3 micron bead.

In parallel with this work, David Bensimon's group were also investigating the motor activity of another enzyme that could translocate DNA – FtsK – also using a Magnetic Tweezer setup and they were able to show that FtsK is the Ferrari of the two motors with a speed that is 10-fold that of *Eco*R124I (approaching 2 μm s^{-1}). In addition, FtsK does not require a specific binding site, but attaches to almost any DNA and then pulls the DNA through the bound complex in a highly processive manner [50].

This work (funded through the EC grant MOL SWITCH – http://www.nanonet.org.uk/molswitch) had now answered the question set by KF – the two DNA translocating enzymes were motors that could indeed manipulate micron-scale objects (the beads used in the Magnetic Tweezer setup varied between 1 and 3 μm diameter and, perhaps even more interesting, the longest "run" observed moved these beads > 3 μm distance – governed by

the height of the flowcell, or the total length of the DNA substrate). Therefore, the scene was set for the next question: *Could a biological molecular motor be used as a nanoactuator within a useful device?*

The use of *Eco*R124I as one potential motor within a nanoactuator presented an interesting problem – DNA cleavage – the normal activity of *Eco*R124I, to cleave unmethylated DNA was a potential problem within a magnetic Tweezer setup as this cleavage would result in loss of beads, but our initial experiments were performed with the previously described subunit-assembly intermediate complex of *Eco*R124I, which has only one HsdR subunit per MTase – an R_1-complex ($R_1M_2S_1$) that does not cleave DNA. Interestingly, although stalling of the enzyme, even as the complete holoenzyme (R_2-complex), was frequently seen in the Magnetic Tweezer, DNA cleavage was a rare event. However, to overcome any such problems, as part of the MOL SWITCH Project, cleavage deficient mutants of HsdR were prepared by one of the Partner Groups [51]. Therefore, we now had two motors that were able to act as nanoactuators within any proposed device and had shown that we could detect movement of a bead using an optical Magnetic Tweezer Setup.

An electronic Magnetic Tweezer

The first step toward using biological molecular motors within a biosensing device was to simplify the optical Magnetic Tweezer setup, which was primarily designed as a tool for research laboratories (see http://www.picotwist.com) and the concept (Figure 6A) we imagined was a biological molecular dynamo, where a biological motor would move a magnetic bead within range of a sensor that could detect this movement in the vertical plane. It was clear that two types of sensor were possible – a magnetoresistive device [52] or a Hall Effect device [53] and two new partners, working with such sensors, were added to the consortium involved in the MOL SWITCH Project to develop a new proposal.

At about the same time there was an EC call for projects in the area of Synthetic Biology and it was clear that developing a generic biosensor that uses a biological molecular motor as the transducer of the biosensor, outputting an electronic signal from detection of a biological event, would be Synthetic Biology. Such a device would require construction of novel biomaterials that would allow surface attachment of biomolecules and would be a clear example of integration of silicon-based devices with single biomolecules. From this concept the EC-funded project BIONANO-SWITCH was born.

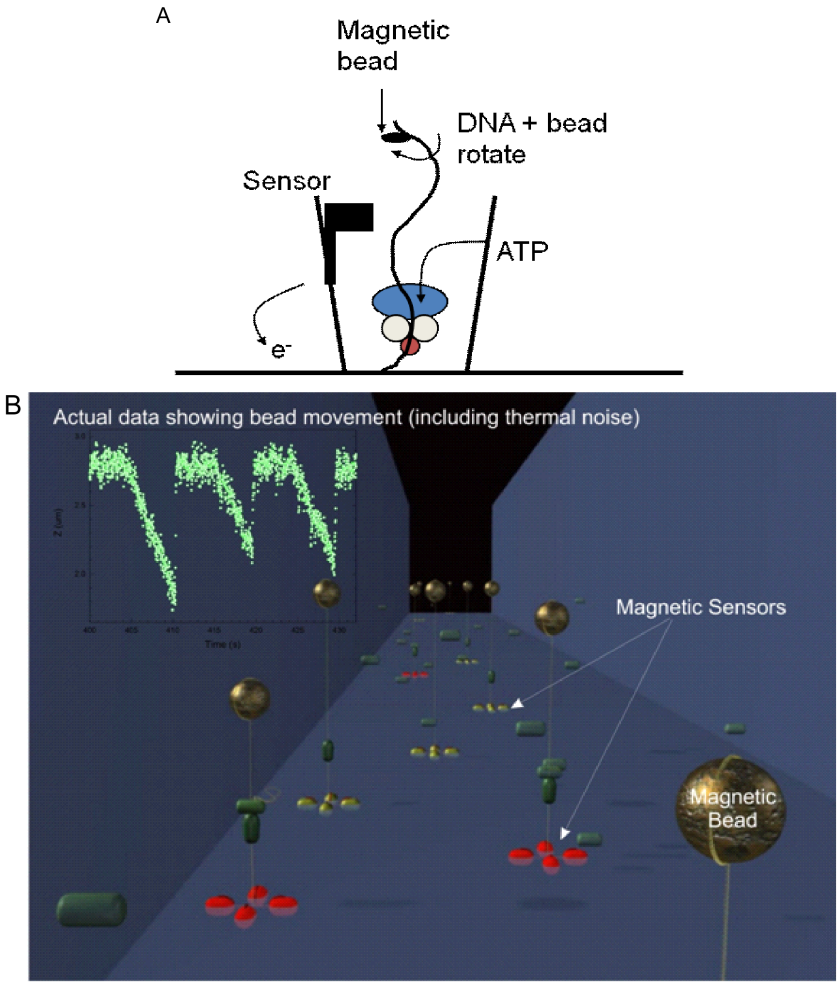

Figure 6. The "MOL SWITCH" Device

(A) A simple cartoon that was used to suggest the concept of the original MOL SWITCH Project. In the proposed device a DNA molecule is attached to a surface, at one end, and to a magnetic bead at the opposite end in a manner analogous to that used in a Magnetic Tweezer setup. The motor can be introduced, will bind and translocate the DNA, pulling the magnetic bead past an electronic sensor, which outputs an electrical signal, acting as a molecular dynamo. **(B)** This image is taken from an animation available at www.bionano-switch.info and shows a view inside of a flowcell of a Lab-on-a-Chip device that incorporates the proposed Electronic Magnetic Tweezer device. Sensors are located on the floor of the flowcell and attached above these sensors is a single DNA molecule attached to a Magnetic bead. The DNA is stretched using an external magnetic field and movement of the bead, by a molecular motor, is detected by the sensor (indicated as a red sensor).

A Synthetic Biology approach to a biosensor design

In order to construct a generic biosensor, which would employ some of the concepts of Synthetic Biology during development, we proposed a modular construction that ''bolted'' a single-molecule nanoactuator as the transducer (output) onto an existing detection technology – the Sandwich ELISA – using a high throughput system [54]. The proposed device employed a ''Molecular Amplifier'' to both improve the signal-noise ratio of the ELISA and to enable release of a biological motor, from an immobilised form, following antigen detection within the ELISA setup. In addition, we imagined the use of DNA aptamers [55] as replacement for the antibodies within the ELISA system which would make the biosensor totally generic (in theory aptamers could be raised to almost any ligand).

The bulk of the Genetic Engineering aspects of the BIONANO-SWITCH Project involved construction of a ''cassette like'' or ''Lego'' system, to mimic systems previously described in this area [56], to link the proteins of interest to other moieties (e. g. biotin, Glutathione-*S*-Transferase and proteases). Eventually all of the biomaterials were created, although with a few problems of protein insolubility for specific constructions – an important aspect of the ''Lego'' approach for Synthetic Biology is that biomaterials cannot be ''engineered'' in a way that is more feasible with chemicals and certain protein-fusion constructs can behave in unpredictable ways – trials of the Generic Biosensor/Molecular Amplifier concepts demonstrated that while the system worked, there was no amplification of the signal. The driving force behind this work was to demonstrate the application of Synthetic Biology techniques within a commercially viable device, but the complexity of the system and the unexpected low turnover of one of the fusion proteins make commercial exploitation of this seem unlikely. This is also an important message for the future of devices based around Synthetic Biology – the unpredictable behaviour of novel bio-constructs suggests that an important aspect of the iGem [56] ''Lego-like'' approach to the production of novel proteins requires a background database of observations with such constructs, which must include unexpected results including reduced solubility and reduced function of protein fusions. Without such an information store many devices will be labour intensive during the development stages, may repeat existing data or knowledge and result in failure.

The proposed nanoactuator device

Despite the above comments, one aspect of the BIONANO-SWITCH Project was very successful, which involved integration of biomaterials, including the biological molecular motors, within a silicon-based sensor. Perhaps this is one aspect of Synthetic Biology that will be commercially viable and this reflects both robustness and reliability of biological molecular machines.

The nanoactuator device will be described in detail elsewhere, but the concept of the device can be understood from Figure 6B. The transducer element is a single biomolecular motor that manipulates a single magnetic bead attached to a single DNA molecule. In effect the sensor is a single molecule switch, which provides an on-off signal of motor activity. Once again our interest in such a device was driven by the concept of commercial application of the device and market research carried out in Portsmouth indicated the most lucrative market might be drug discovery. Many biological molecular motors (topoisomerases, helicases, polymerases etc.) are drug targets and the advantages that can be gained by using single molecule studies have already been clearly demonstrated with topoisomerases [57, 58]. In the proposed device we can readily imagine a multiple channel system (illustrated with a two channel setup in Figure 7A) where one channel can detect normal motor activity, while the other channels can be used to detect drug-induced loss of activity (Figure 7B). Our own work has focussed on the use of helicases from *Plasmodium falciparum* that have been proposed as potential antimalarial drug targets [59], but this work is currently at a very early stage.

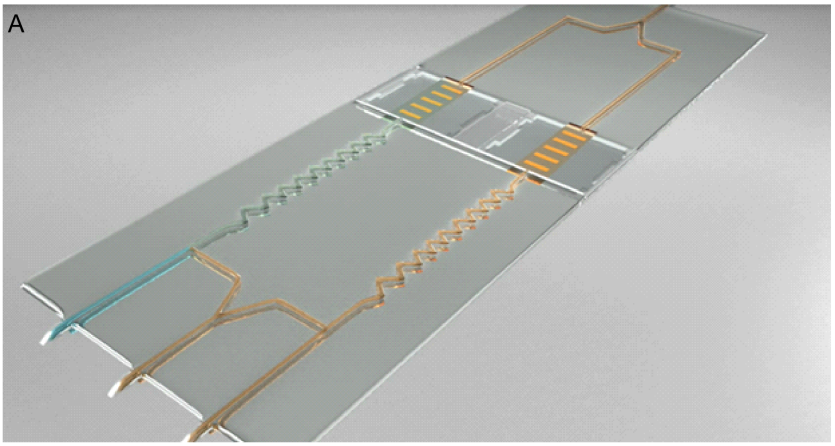

Figure 7. The proposed nanoactuator device.
(A) A two-channel flowcell of the proposed commercial version of the Electronic Magnetic Tweezer in which one can see the attachment sites for the DNA molecules (gold pads) that are fed buffer, motor and drugs through the microfluidics.

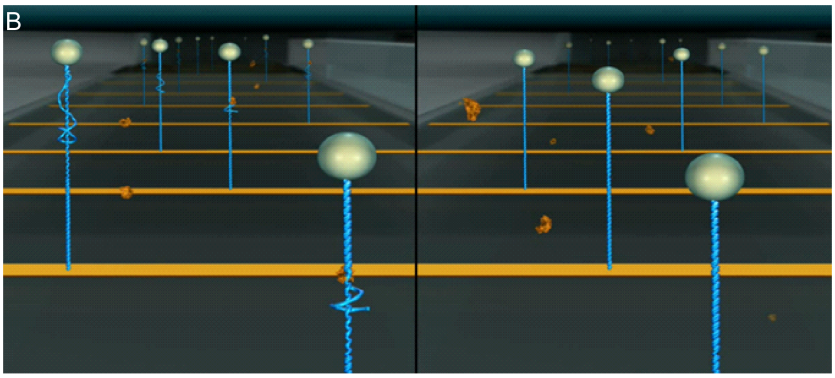

Figure 7. The proposed nanoactuator device.
(B) A view of the above flowcell, side by side, showing DNA+beads attached to the gold pads and functioning helicase molecules in the left channel unwinding DNA from a gapped substrate, but helicase+drug molecules in the right channel that show loss of function. Electronic signals from the device measure loss of activity from the drug-target interaction at the single molecule level and, therefore, at a sensitivity based on the equilibrium constant of the drug-target interaction.

Conclusion

Biological molecular motors are surprisingly robust and very powerful. The (early) concept that such motors could not manipulate objects on the microscale was both wrong and surprising as a viewpoint, but several groups have now been able to disprove this assumption and this suggests a potential for such motors in useful devices. This idea is one of the more promising aspects of Synthetic Biology, where integration of biomaterial within silicon devices has been shown to be surprisingly successful and we have also been able to demonstrate efficient surface attachment of biomolecules, at precise location (< 100 nm error), at the single molecule level. Our attempts to apply the "cassette" approach for the construction of novel protein fusions was very mixed and protein insolubility, or loss of function, was a major issue, but was also unpredictable – we suggest that the eventual application of such techniques within Synthetic Biology requires a detailed database of results to avoid repetitive failures for specific constructs. Finally, we believe we have developed a single molecule biosensor, which can be used in the area of drug discovery, that detects drug-target interactions at the single molecule level where the sensitivity of the system is determined by the chemical equilibrium constant of the interaction (actually by k_{off}). We believe this system offers an opportunity to screen both existing and new drugs at a level not previously possible against DNA manipulating targets. Adaptation of the device to novel targets (e. g. protein-protein interactions and chromatin remodelling) provides a huge potential for future application of the technology.

Acknowledgements

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