

MOL SWITCH

Newsletter

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2nd Mol Switch Workshop

*Dr Keith Firman -
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It was proposed that the Mol Switch Consortium would meet annually to discuss progress, problems and results. Therefore, the 2nd meeting was planned for around month 12 of the project. Following a brief discussion with John Gallop, it was decided that we would combine the 2nd Workshop with a NanoNet Workshop and, at the same time, include a similar Workshop for another 5th Framework Consortium, to be co-ordinated by John Gallop. John will host these events at the National Physical Laboratory, Teddington, UK. The NanoNet Workshop will be titled "Force Microscopy for Biological Systems". The event will be held over a two-day period, which will enable us, once again, to hold a number of focussed group meetings for Mol Switch.

The proposed date for the 'Workshop' is now 17/18 December 2003. To be confirmed...

Reports From the Various Groups

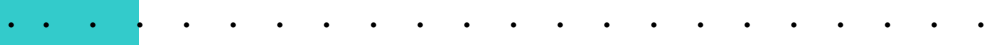
University of Portsmouth - UK appointed Dr Terry Robinson, a Postdoctoral Worker, for the Project. Dr Robinson is well qualified as a Molecular Biologist and his wide experience in communication (ex-army, mature student), will be very beneficial with regard to communication across



the Network. Protein production (for assembly of the molecular motor) is fully established, with a number of improvements to the purification protocol leading to better yields. An improved method for production of DNA substrates, for use with the

magnetic tweezer set-up, has been proposed (involving homopolymer tailing of PCR products and subsequent ligation) and initial studies of the success-rate have been initiated. The potential date for the final availability to the Consortium Members who require this material, will, in all probability, be in month 6. Measurement of the ATPase activity of the motor and its sub-assemblies is well underway and a number of interesting observations have already been made.

ATPase assay techniques have now be extended to smaller volumes, gradually moving to a situation where the surface/volume ratio is very high and



including silicon surfaces (WP3). Following initial discussions with Prof Favilla during the 1st Workshop, together with subsequent discussions at Portsmouth with all parties involved, it was decided that a fluorescence displacement assay would be the best first-step toward measuring DNA translocation. Investigation of the most reliable technique is underway.

National Physical Laboratory - UK appointed Dr. Olga Kazakova (Senior Research Scientist, permanent post). Existing staff, who are working on the MOL SWITCH project, are Mrs. Lizzie Brown, Dr. Ling Hao and Dr. John Gallop. Mrs Lizzie Brown is also studying for an external PhD, in an AFM-based project, at Imperial College, London. The NPL group, to facilitate work on MOL SWITCH, has acquired a second AFM/MFM. It is a Veeco (Nanoscope) Multimode instrument, which is more versatile than the existing AFM and has improved liquid scanning capability.

During the first four months of the MOL SWITCH project, NPL work has concentrated on the following areas: immobilisation of DNA on Mica Substrates, action of methylase enzyme on DNA and subsequent AFM imaging, preliminary experiments on commercial streptavidin-coated magnetic beads and design considerations for magnetic readout, using either MFM or sub-micron Hall bars.

Imaging of DNA in non-contact mode on mica has proved very successful and reproducible images of uncut and cut (using XmnI enzyme) double stranded DNA has been observed. In addition, under some conditions when the methylase enzyme is added, (e.g. varying incubation time) some DNA strands are observed to have enzyme still attached and supercoiled loops have been formed. Some statistical analysis of the loop size versus the incubation time, similar to work already carried out by the Delft group, will be attempted shortly.

Streptavidin-coated magnetic beads have been obtained from Dynabead. These are 1µm and 2.7µm in diameter. Preliminary analysis of the beads has been carried out, including

measurements of their magnetisation curves using a SQUID magnetometer. Dispersion of the beads on mica has been observed with the AFM and magnetic force imaging of the beads has commenced. We have shown that beads may be attached and released from magnetised MFM tips. The next stage is to attach beads to biotinylated DNA, followed by attachment to a suitably prepared substrate using a DIG-reaction. This will be coupled to the work (described above) at the University of Portsmouth.

A literature survey, of sub-micron Hall bar measurements, has been undertaken. A mathematical model is under construction, to determine the sensitivity of Hall bars, for detection of sub-micron sized magnetic beads. In addition, contact has been established with a group at Imperial College, London, who possess a scanning sub-micron Hall magnetometer and we hope to carry out some preliminary measurements using this system, shortly.

TU Delft – THE NETHERLANDS appointed Dr John van Noort on the Project, from 01-01-2003. Studies with the type I restriction-modification enzyme EcoR124I have shown DNA bending by the MTase. An initial endonuclease (ENase) complex in the presence of non-hydrolysable ATP_γS, results in an unusual extrusion of DNA shown to be required for initiation of translocation. A paper describing these results is under preparation for submission to EMBO Journal. Measurement of the rate of translocation, during the 'early' stages of the process, suggests a different rate for translocation compared to that observed from bulk solution (which measures the 'end-rate'). Some 'aggregation' or dimer/trimer formation, during surface analysis of translocating complexes, was observed. Relaxation of expanding loops, perhaps through DNA slippage, was detected using the magnetic tweezer set-up. A 'block' against the initial translocation event, at about 400-bp, suggests a two stage process. Initial measurement of movement, using magnetic tweezers, has been carried out.

ENS/CNRS Paris – FRANCE appointed Dr Omar Saleh, an American PostDoc, who joined them in December 2002. He receives his salary from both a foreign affair French Ministry Grant and MOL SWITCH. His project concerns the RNA-polymerase enzyme, a molecular motor that translocates along the DNA duplex. Work is progressing on a DNA construct for the study of RNA polymerase translocation. Work is in progress on chromatin remodelling translocases and type I restriction enzymes. One publication on helicases, for submission to Science, is in preparation.

We are looking for start-up funds, to establish a company commercializing the magnetic tweezers set-up. Contacts have been established with a sub-contractor.

We are mainly studying molecular motors working on DNA, including RNA polymerase (an enzyme that translocates along DNA in order to make RNA), DNA remodelling factors, and DNA helicases. We have been preparing the DNA molecule to study RNA polymerase. The idea is to simultaneously attach a single DNA molecule both to a substrate and a bead by utilizing, respectively, bonds between digoxigenin/anti-digoxigenin and fluorescein/anti-fluorescein.

A biotin molecule incorporated upstream of the RNA polymerase promoter sequence will provide the attachment point for the polymerase enzyme. We have successfully tested both the digoxigenin and the fluorescein attachments, and are working on incorporating the promoter and the biotin in the middle of the molecule.

We have been actively characterising DNA remodelling factors such as Brg1 and BrM, using our magnetic tweezers set-up. By preparing a positively, or negatively supercoiled molecule, we have evidence that these enzymes create positive supercoiled bursts, which extend (contract) negatively (positively) supercoiled DNA. In the single molecule regime, these bursts typically last 10 seconds and have an amplitude of four superturns. This study has been conducted on naked DNA. We are in the process of repeating this experiment with chromatin fibres. We are also

Initiation of DNA Translocation:

Initiation of DNA Translocation is Associated with an Unusual DNA Structure

Dr Keith Firman and Prof Cees Dekker

We were fortunate; in that the Mol Switch funding was preceded by a small travel grant awarded to Dr Keith Firman (an EMBO Short Term Fellowship), which allowed us to 'kick-start' single molecule studies with the EcoR124I molecular motor in collaboration with the group at TUDelft. This work has been quite productive and led to a 'Paper' describing an unusual DNA Structure, during the initiation of translocation. This work was carried out by John van Noort, together with Thijn van der Heijden and was a major part of Thijn's 'Masters Degree Thesis' (which we are pleased to report, was a success).

investigating the translocase activity of a type I restriction enzyme. Finally, we are continuing the study of DNA helicases, which also present a translocase activity.

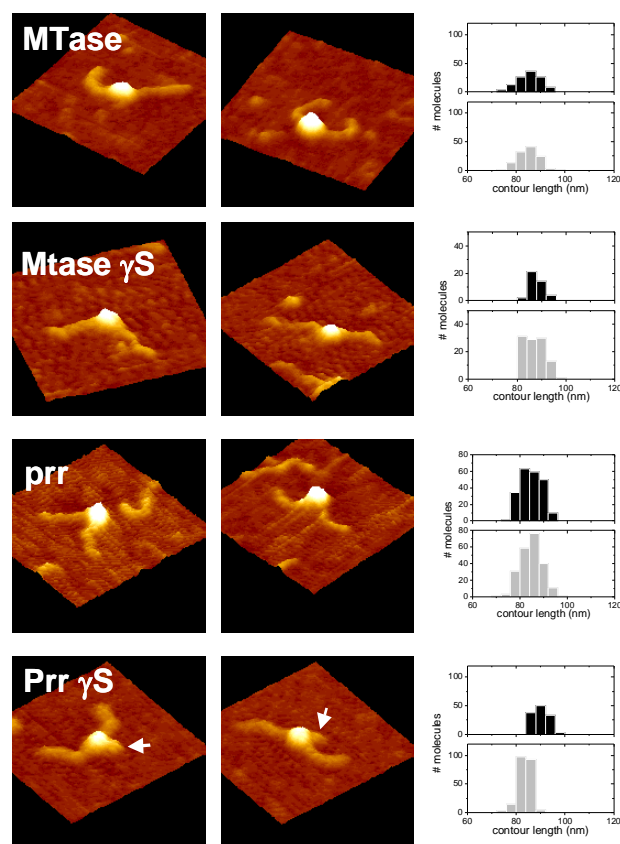
Institute of Microbiology, Czech Academy of Sciences – CZECH REPUBLIC after recalculating personnel costs, according to our new Wage Schedule, the sum allocated will cover one and a half persons equal to 2,520 hours on the Project. Dr. I. Holubová, 1,680 hours on the Project, Ing. Stěpánka Vejsadová, 840 hours on the Project.

Testing of an applicable system, for selection of restriction deficient mutants, has been completed. For mutagenesis of motif X in both *hsdR*(R124I) and *hsdR*(prr1), plasmids pACYCR124 and pCPD127 has been chosen, respectively, for their reliable complementation of restriction phenotype *in vivo*. Appropriate complementary mutant oligonucleotides and sequences primers have been designed and those for D151A substitution already purchased. Experiments will begin, immediately, on receipt of the Mutagenesis Kit. Using the Quick-Change XL Site-Directed Mutagenesis Kit we have performed the control experiments with oligos and plasmid provided in the Kit. The efficiency of mutagenesis was 96%. Accordingly, we set the reaction conditions with our oligos (for D151A substitution within the *hsdR* gene) and plasmid pACR124. First 50 plasmid DNA were purified and transformed into both JM109(DE3)/R124 r^+m^+ and JM109(DE3)/R124-25 r^-m^+ . We are currently testing the restriction phenotype looking for plasmid which does not complement restriction in JM109(DE3)/R124-25 and reduces (even abolishes) restriction in JM109(DE3)/R124. This data should be available over the next few weeks.

Latest update – Portsmouth have now received six individual mutants that have been confirmed as such, using DNA sequence analysis.

University of Parma – ITALY has financed a graduate student with one short-term (3 months) fellowship, in order to start the Project work. In May, they will finance a second 6-month postdoctoral fellowship to begin the work with the confocal microscope, which has just arrived. This second fellowship should then be renewed for the whole project period. Both will be financed with MOL SWITCH funds. We have initiated measuring ATPase activity of EcoR124I complex, using an enzyme coupled fluorescence assay. We will write an initial report on the ATPase activity measurements shortly, in collaboration with the University of Portsmouth. Fluorescence studies of the translocation process will start shortly using a displacement assay, following discussions, of the most appropriate technique, with the University of Portsmouth.

Figure 1



The basis of the work was to investigate contour length of DNA molecules in the presence and absence of EcoR124I MTase, or, a hybrid R1-complex (motor) with and without ATP γ S. The hypothesis leading to this work was the possibility that the motor might wrap the DNA around itself prior to translocation (to overcome topological problems associated with this first step in translocation) and that using the non-hydrolysable ATP analogue would trap such a complex. Therefore, we assumed such a complex would result in a reduction of contour length when measured by AFM. Much to our surprise, the reduction in contour length was only 8-9 nm and a short 'bulge' in the DNA (Figure 1) was accompanied by this event.

The full details of this work will be submitted to EMBO Journal.

Nanotech may spark fierce ethical row

From the BBC Web pages

The following article is taken from the BBC's web Pages in an 'un-doctored' form. It is the type of article that is supposed to stimulate the ethical debates that involve BioNanotechnology. Any responses and comments would be interesting and useful...

By Alex Kirby

BBC News Online environment correspondent
A confrontation over nanotechnology could be as bitter as the current debate over biotechnology, researchers fear. They say the emerging knowledge has the power to revolutionise society. But its power to exploit the potential of extremely small-scale systems is outrunning our capacity to digest its implications.

The researchers say the only hope is rapidly to close the gap between the science and ethics of nanotechnology.



A mini-sub cruises an artery (Image: Science Photo Library)

The warning comes in a study by the Joint Centre for Bioethics (JCB) at the University of Toronto, Canada.

The study, Mind the Gap: Science and Ethics in Nanotechnology, is published in the UK journal Nanotechnology.

The cause of the researchers' concern is the process of building working devices, systems and materials molecule by molecule, by controlling matter measured in billionths of a metre.

Small is effective

Perhaps more significantly, nanotechnology is about exploiting the unique and powerful electrical, physical and chemical properties found at that scale.

The new science has developed from advances in microscopy, materials science, molecular-level manipulation, and the relationship between classical and quantum physics.

It has already seen single-molecule transistors, an enzyme-powered bio-molecular motor with nickel propellers, and a minute carrier able to cross from the blood to the brain to deliver chemicals to fight tumours.

Hypothetical advances suggested include cheap, light materials strong enough to make space transport economical, and the ability to remove greenhouse gases from the atmosphere.

Some enthusiasts even claim it may

be possible to revive people now in suspended animation, though they have little support.

The JCB researchers say nanotechnology raises unique questions that may require specific regulations.

Problem areas include:

- Equity: who will benefit - just the rich, or the poor as well?
- Privacy and security: invisible microphones, cameras and tracking devices could improve security, and help catch terrorists. What are the military applications?

- Environment: what will the new nano-materials do when they are released?
- The study says although research is still in its infancy, with most applications perhaps years away, "the backlash against the new technology is already gathering momentum".

Awful warning

Research and development spending on nanotechnology is growing fast - in the US up from \$432 million in 1997 to \$604m by 2002, in Japan from \$120m to \$750m over the same period.

“Go into a British pub and say 'nanotechnology', and nobody knows what you're talking about”

Dr Peter Singer, JCB

The researchers say: "There is a danger of derailing

nanotechnology if serious study of its ethical, environmental, economic, legal and social implications does not reach the speed of progress in the science."

They say they fear "a showdown of the type we saw with genetically-modified crops".

One of the authors, Dr Peter Singer, said: "Nanotechnology is going to cause a major revolution that will have a profound impact on society."

Hope for the poor

"Technology promising such massive changes in our lives will be viewed with suspicion and perhaps outright fear."

Dr Singer told BBC News Online: "There's a lot of hype around nanotechnology, but there's also great potential."

"The science is barreling forward, but the ethics aren't, and there's very little public engagement."

"Go into a British pub and say 'nanotechnology', and nobody knows what you're talking about."

"The first step is a fully-informed public - that's the gap we have to close, so we can optimise the benefits and minimise the risks," he said.

"The key equity issue is how we can use nanotechnology to help development, to narrow the gap between the rich and the poor worlds."

"I don't want the science to slow down. I want the ethics to catch up," said Dr Singer.

this technology (see Figure 2 and 3).

Surface Attachment of Motor

A key aspect of the work involving the EcoR124I molecular motor, which was discussed at the 1st Mol Switch Workshop, was a need to easily attach the motor to surfaces, nanoparticles, fluorophors etc. It was agreed at the 1st workshop that the Portsmouth Group would investigate this possibility. We mentioned a possible cysteine in the motor subunit (HsdR) that might be available for such work.

We are now pleased to confirm this work! The HsdR(prrI) hybrid motor subunit (N-terminus from HsdR(prrI) and C-terminus from HsdR(R124I) with

Self-assembling DNA Substrates

Dr Keith Firman

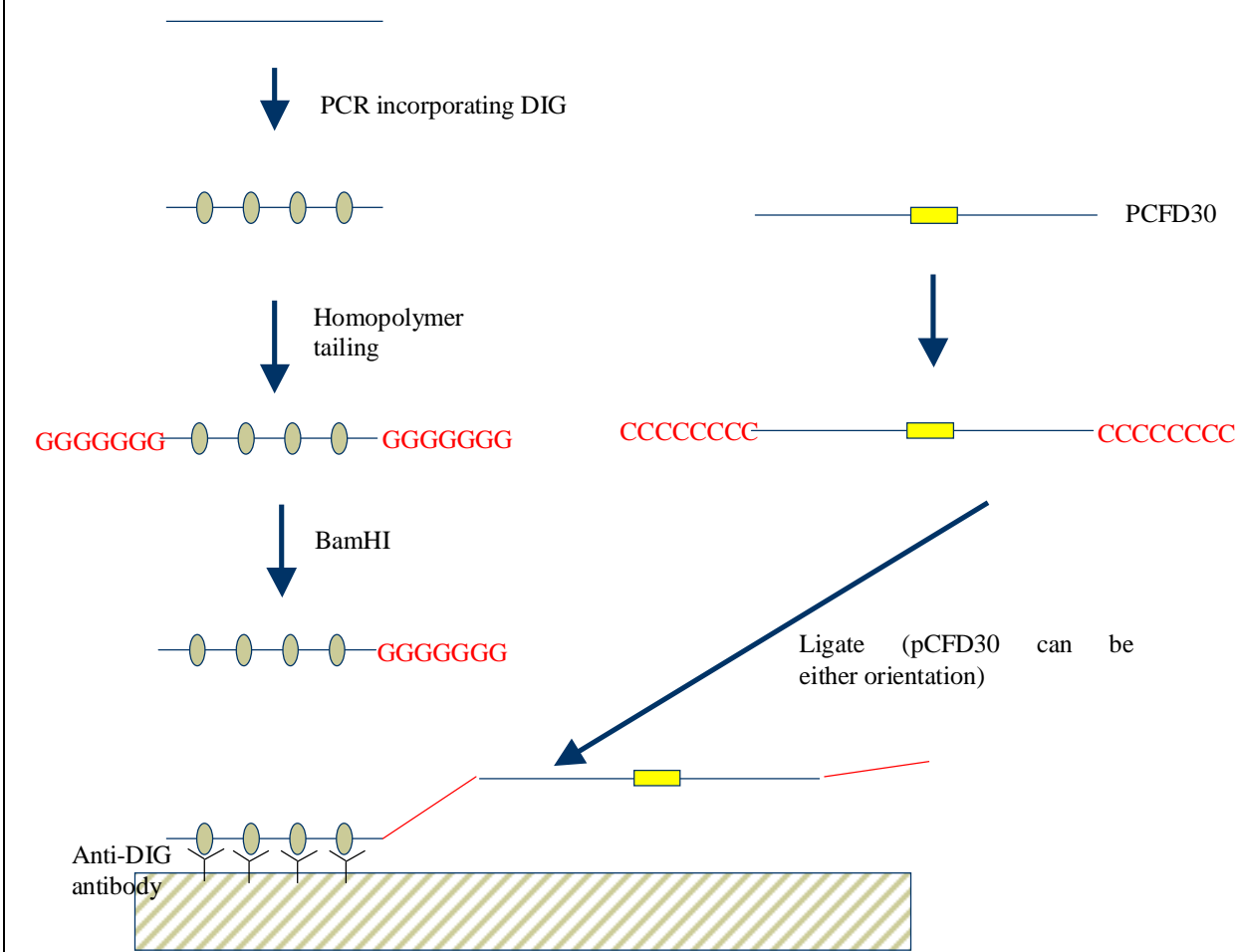
The magnetic tweezer hardware, used by TUDelft and ENS/CNRS/Paris6/7, requires a DNA substrate that can be anchored (to a derivatised glass surface) at one end, using a DIG/antiDIG system, and that can also be attached to a magnetic bead at the other end of the DNA (through a biotin/streptavidin linkage). The current technology uses a three-stage method based around two PCR products (one incorporating DIG and the other biotin), which are ligated to a linear DNA substrate with suitable binding sites for the system under study. However, TUDelft report problems with

the efficiency of ligation and the subsequent purification of un-nicked DNA. Therefore, at Portsmouth, we have begun an investigation of possible alternative techniques for substrate production.

The current (best) working (alternative) method makes use of homopolymer tailing produced by the enzyme terminal transferase (a template-independent DNA polymerase). This enzyme can add single base4s to the 3'-ends of dsDNA in a controlled manner. We have determined the best rates for addition of dGTP and dCTP tails to both pCFD30 (a recombinant plasmid carrying a single recognition site for EcoR124I) and a PCR product with random incorporation of digoxigenin (DIG) moieties. We can now proceed to a self-assembly system based around

Figure 2

Stage 1



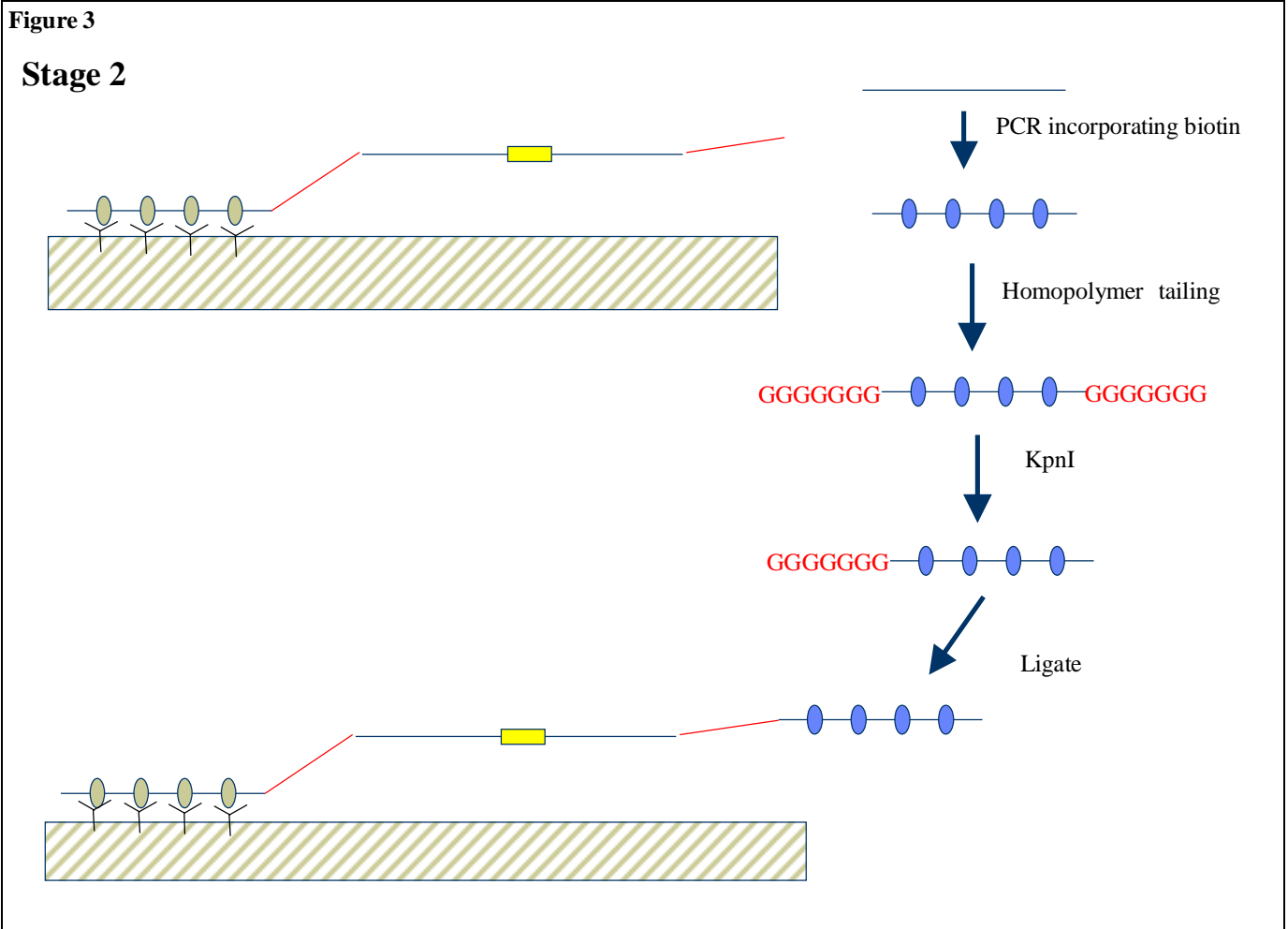
the junction at the unique PstI restriction site in *hsdR* is predicted to have three cysteine residues from the published DNA sequence. This suggests that one of these cysteines will be 'free', while the others are likely to form a disulphide bridge. Terry Robinson has assayed available cysteines in this protein, in both folded and un-folded states, and has discovered five molar equivalent cysteines! This indicates an error somewhere in the published DNA sequence and that two disulphide bridges are likely. In

addition, he has shown that one cysteine is 'freely' available in the folded protein. This cysteine is predicated at position 3 from the DNA sequence and is likely to be free for binding HsdR top surfaces etc. We will now begin work to determine the true availability of the cysteine for attachment of ligands and surfaces. This cysteine now makes it considerably more feasible for us to attach a fluorophore to the motor. In addition, we will also be able to attach a fluor to the DNA and measure FRET

during/following translocation (as originally described in the Mol Switch project). We have also shown that protein fusion to HsdR, at the N-terminus, does NOT inhibit motor activity (as measured by DNA cleavage and ATPase activity). Therefore, it seems highly probable that attachment of the motor to ligands and surfaces, through this cysteine, will NOT inhibit motor activity.

Figure 3

Stage 2



E-mail correspondence:

David N. Guedj, Ph.D.
European Commission -
Information Society Directorate
General - INFOS

Wrote:

Dear Dr. Firman,

Thank you for the 1st Management Report of Mol Switch you sent me.

I would be very happy to attend the 2nd project workshop but still have to check my agenda as we have a significant number of FP5 projects coming to an end in December.

Best Regards,
 David Guedj

Terry Robinson

Wrote:

Ciao Roberto,

With regard to Keith's recent e-mail on the subject of cysteine fluorescent probes (Cy3 and Cy5). I believe we should also consider the Alexa Fluor Thiol-reactive probes available from Molecular probes.

It appears that these particular probes give a substantial 'confidence' benefit, with regard to the microarray monitoring of variable plate assay data. These Fluors also possess a greater resistance to photo-bleaching than their cy3 and cy5 counterparts. Whilst wanting your initial thoughts on the above, could you also remind me of the optimal wavelength of your fluorometer.

Terry

Roberto Favilla

Wrote:

Dear Keith and Terry,

Following your last emails, I think that Alexa-633 is probably more appropriate both in terms of photostability and of maximum absorbance since my pulsed diode

laser emits at 637 nm. With the labelled protein, we could start doing single molecule studies (e.g. photostability and flu corr. spectr., e.g. FCS could possibly say something new, or at least confirm knowledge, about protein subunit association as a function of subunit ratio, with or without (unlabelled) DNA. As far as FRET measurements are concerned, these will start later, once we have seen something on the labelled protein only. In any case I think that Alexa-647 (or the similar but apparently less photostable analogue Cy5) should be appropriate to label DNA because it absorbs mostly where Alexa633 emits mostly (i.e. near 650 nm). On the other hand emission of Alexa647 is also very close to that of Alexa-633, so that there might be problems to separate them well for FRET measurements. Other Alexa red-derivatives, such as Alexa-660, might also be appropriate or even better acceptors, because, though their absorbance is less overlapped with Alexa-633 fluorescence, their fluorescence is more separated from that of Alexa633, so that there should be less cross-talk between the two parallel SPAD detectors (one for Alexa633 flu, the other for Alexa650 flu). However, how easy it will be to separate them is difficult to predict at the moment (this depends much on the filter set one uses). Before ordering the "right" filter set I want to be sure which is the best one for our purposes (unfortunately these filters are very expensive), and this of course also depends on the fluorophore couple(s) we decide to use.

Best regards
 Roberto

Marc Bailey

Wrote:

Dear Dr Firman,

I'm a member of the biotechnology team at NPL with a strong background in molecular biology. As

part of the single molecule sequencing project, John and Olga have asked me for advice on attaching DNA to a surface and magnetic beads as a preliminary step to using the AFM to measure translocation of the DNA by the methylase. I am very happy to provide input but would like an opportunity to discuss the system with you and check with you whether you have already designed and undertaken the DNA labelling experiments.

At this stage I'm guessing that the best way to approach the problem would be to amplify the region of interest of the plasmid by PCR using modified oligos, one biotinylated at the 5', the other modified at the 5' with dioxigenin (DIG). The DIG modified terminus of the DNA can then be surface anchored using standard reagents from Invitrogen while the other end can be attached to an avidin-coated paramagnetic bead.

However there are other ways of achieving the end-labelling and this strategy requires the sequence of the plasmid template to identify the best oligonucleotide binding sites.

Best wishes
 Marc Bailey

Marc Bailey

Wrote:

Dear Keith,

I'm not sure I understand why both strands of the template need to be attached to the surface. Why is free rotation a problem? I'm guessing that as the methylase progresses it introduces supercoils into the template ahead of it in an analogous manner to polymerase progression. Constraining both strands at both ends of the template should prevent these supercoils from being released but will put a turning force on the bead (and the surface).

If one strand is free to rotate relative to the other then there should be less turning force applied by the DNA template to the bead.

What effects do you expect these differences to have on the behaviour of the enzyme? If there is no introduction of supercoils by the enzyme then I'm still not sure why both strands need to be tethered to the bead and solid surfaces?

The second question is that Olga is keen to start work on the beads where the DNA has been attached. Do you have any biotinylated template that Olga can have? We can biotinylate DNA here but the only thing I know about the template is that it is linearised by XmnI. Do you have a plasmid map? Is there any benefit to working with template that only has one end tethered? What kind of surface would you want to use for tethering via DIG? Do you have a protocol for coating surfaces with anti-DIG antibody?

Best wishes
Marc

The reply is based around the self-assembling DNA section (Page 5).

Keith Firman

Wrote:

Dear Roberto

Do you have the capability to carryout this type of assay for translocation by EcoR124?

Xu, H.Q., Zhang, A.H., Auclair, C., and Xi, X.G. (2003) Simultaneously Monitoring DNA binding and helicase-catalyzed DNA unwinding by fluorescence polarization. Nucl. Acids. Res. 31: e70-.

Regards,

Keith

Roberto Favilla

Wrote:

Dear Keith,

I have given a quick look at the very interesting article on translocation measures by anisotropy. What I can say at the moment is that our spectrofluotometer (LS-50 Perkin Elmer) allows one to perform anisotropy measurements, but I'm afraid instrumental sensitivity is not enough to get reliable anisotropy values at such a dilution as nanomolar range. Actually they use an anisotropy dedicated instrument (Beacon 2000), which also allows for very small volumes (150 uL), whereas in our case we need a cuvette filled with at least 500 uL. Another little disadvantage is that acquisition can only be done manually by us, not automatically every 8 seconds as done by them. In any case I can try with a nanomolar fluorescein solution and see if I can read something. In principle my stopped flow allows the acquisition of anisotropy values automatically as a function of time, but I have never used it in such a mode. I may try and then let you know how it works.

Provided that sensitivity is high enough, it would be OK because we could use small volumes as well, also mixing (e.g. addition of ATP) would be very rapid.

Turning to FRET, I have seen that Mol Probes sells a compound called QSY21 (cat. no. Q-20132), which acts just as a quencher with fluorophors such as Alexa-633 but is not fluorescent itself. Therefore in case of FRET no fluorescence should be seen. This could be a simpler alternative to measure FRET.

The last thing I wanted to mention is that the use of alternative FRET acceptors of the Alexa family, such as Alexa-647, 660, 680 or even 700 could be useful, because they have an absorbance spectrum progressively shifted towards the red, and therefore an increasingly smaller overlap with the fluorescence spectrum of the donor, say Alexa-633. Since the critical Forster distance R_0 is directly proportional to this overlap, it decreases more and more as the acceptor absorbance is shifted more and more into the red region. In other words, depending on the distance of closest approach between donor and acceptor, the use of one or another Alexa acceptor may be more or less useful. I have had a problem with one of the two single photon detectors of the confocal microscope. I have to send it back to Berlin for proper check. I hope it is not burnt, otherwise I have lost 12,000 Euro.....

Best regards

Roberto