Parts exchange:
why molecular machines are like used cars

Proteins, so small that one billion would fit on a full stop, carry out most of the vital activities in living cells; they drive chemical reactions, transport cargoes, communicate with the outside world and even segregate chromosomes. A novel approach now allows us to monitor single proteins in complicated molecular machines, and it seems that biological components wear out and get replaced just as they do in man-made machines.

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In his 1959 lecture on nanotechnology (though before the actual phrase was invented) entitled *There's plenty of room at the bottom*, the celebrated physicist Richard Feynman noted how biological systems do "all kinds of marvellous things – all on a very small scale". He could not have been more right, and there are now literally hundreds of examples of natural biological machines known to exist at the 'nanometre' scale, one hundred times smaller than the wavelength of light (Figure 1). An important subset of these types of machine is the so-called 'molecular motor'. These motors contain some of the most fundamental machines of living organisms.

Life has existed on Earth for, at the best current estimate, about four billion years. By using genetic analysis on existing organisms we can now estimate that molecular machines have probably existed in some form for the majority of this period, and so have to be considered as one of the most important characteristic features of life itself.

Ingenious strategies have evolved over many millions of years to perform very diverse tasks: some motors shunt material around inside living cells or use ion gradients to make cellular fuel; they drive DNA synthesis and ultimately cell division; they cause individual cells to swim or crawl; and still others cause movement of large populations of cells in the tissues and organs of complex multi-cellular organisms. In many ways these tiny machines are analogous to those of our larger scale man-made equivalents: they typically have separate functioning parts whose collective properties ultimately transform energy from a chemical form into mechanical work or motion, much like a car using several different components that work together to cause it to move.

However, unlike its macroscopic companions, these 'nanoscopic' machines function in an environment in which the size of the motions and the dimensions of the machine itself are comparable to the random thermal fluctuations of surrounding molecules, typically of water in which all living components are ultimately bathed. This has led to the concept of molecular motors being immersed in a 'thermal bath'. It is this crucial difference which allows molecular motors to perform with, sometimes, exceptional efficiency compared to the macroscopic man-made equivalents. It is remarkable how such machines have evolved to carry out complicated and varied functions while being composed of relatively few single protein molecule components.

Biological motors do, at least superficially, have much in common with those of the macroscopic world (Figure 2). For example, instead of using electricity as a fuel to bring about some mechanical motion, many molecular motors will use the flux of charged ions such as protons, which is in essence the same as electricity though without the wires! But even though the total power output is much higher in a macro-motor than in a single biological one, the efficiency of conversion of the fuel to useful mechanical energy can sometimes be very much higher in the latter (under certain circumstances almost 100%!)

Nature's primordial wheel

In our research, we have focused upon on a type of molecular machine which causes

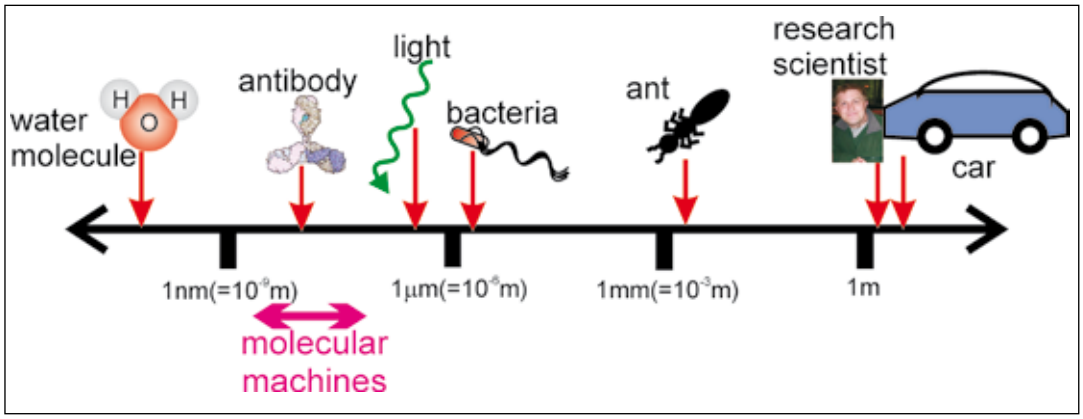

Figure 1. A ruler linking the nanoscopic and macroscopic worlds. This so-called 'mesoscopic scale' is the length range over which we can describe the properties of constituent matter without resorting to describing properties of the constituent atoms themselves. Here we have illustrated mean typical effective length dimensions of various common objects, spanning a length range equivalent to ~10 orders of magnitude from something as small as a single water molecule (~0.2 nanometres), through to the typical wavelengths of visible light (~500 nanometres), up to the macroscopic world including things such as people and cars (a few metres and beyond).

whole bacterial cells to swim, called the bacterial flagellar motor. Bacteria use these rotary motors in their membranes to rotate semi-rigid helical extra-cellular flagella. The cell body counter-rotates and the bacterium swims through its environment, one which is all viscosity and no inertia at that size (for us it would be like trying to walk through treacle instead of air). Despite this, bacteria reach some of the fastest known relative swimming speeds, up to 150 microns/sec, or 100 body length/sec.

Bacteria use this swimming to reach or stay in their optimum environment for growth, making it an essential process in many positive and negative bacterial activities, from colonising roots as a means of bringing about nitrogen fixation to populating the gut to cause food poisoning. This rotary motor, which in many ways can be seen as a primordial wheel of the natural biological world, contains all the components you might expect from a rotary motor, principally a ring of 'stator' units which generate force to rotate an inner 'rotor' ring. The rotor ring connects to a central shaft running through the cell membranes, and this shaft in turn is connected to the outside world via various protein molecule adaptors to the helical filament (Figure 3a and b).

The fundamental generation of force by the stator components is energised by a constant flux of ions across the cell membranes into the cell from the outside, down both a charge and concentration gradient. These ions are either protons or sodium ions, depending on the particular species of bacterium, and the force is known as the protonmotive and sodium-motive force respectively. The precise details of the molecular mechanisms by which this machine effects its rotary function are still far from determined.

To help us understand how such machines work, we looked more closely at the force-generating stators of the bacterial

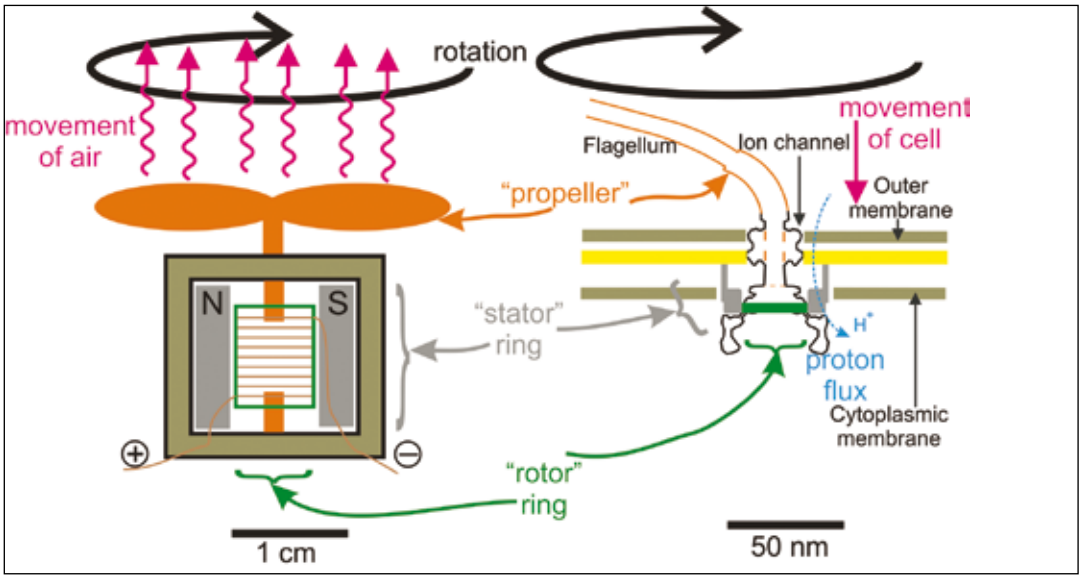

Figure 2. Electric fan and biological motors compared. The biological motor that rotates the flagellum is essentially the same as an electrical motor with a fan attached but made up of many different proteins. Stator units in the bio-motor are equivalent to magnets in the macro-motor, the proton (H^+) flux across the outer and cytoplasmic membranes to electricity, the flagellum to the propeller and the movement of the cell itself to the induced motion of air. The bio-motor is also about 20 million times smaller than the electric motor!

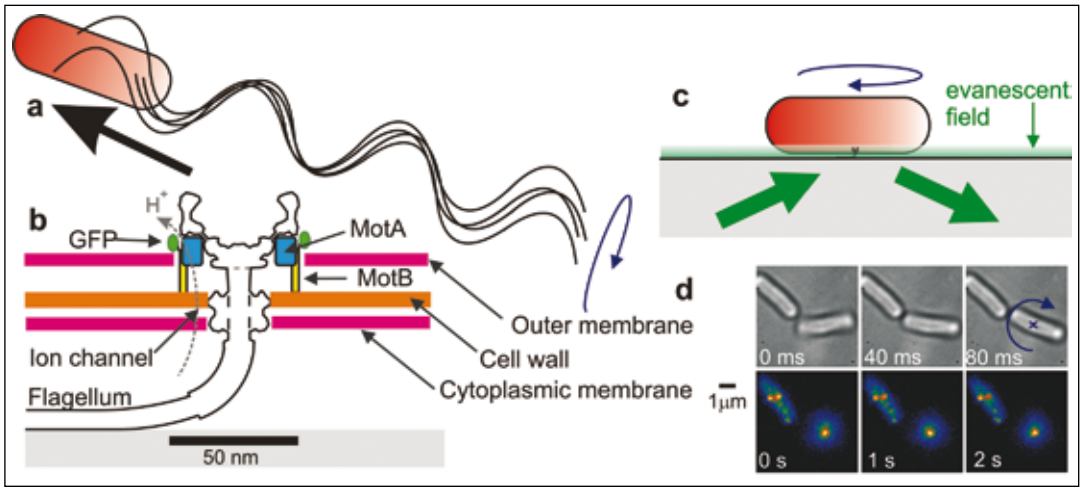

Figure 3. Visualising components of functioning molecular machines. a) schematic of an *E. coli* bacterium swimming in the direction of the black arrow by means of concerted rotation of several stiff, helical filaments bundled together as one super-filament. At the base of each filament is a molecular machine embedded in the cell membrane called the bacterial flagellar motor. b) a filament tethered to a glass microscope slide (grey), with a cross-section through the motor showing the inner and outer membranes (magenta), the cell wall (orange), the proton flux that drives motor rotation (grey arrow) and the proteins MotA (blue) and MotB (yellow). MotA and MotB make up the 'stator' unit, and here MotB has been tagged with GFP (green). c) the antibody-tethered bacterial cell rotation assay, showing the typical extent of the evanescent excitation field with TIRF laser illumination (green). d) consecutive non-fluorescent (top) and TIRF (bottom) image frames. Rotation of a freely-tethered cell is marked in blue, adapted from Leake *et al,* 2006.

flagellar motor, and here we had help from the genus *Coelenterata*. A species of deep-sea jellyfish, *Aequorea victoria*, produces a naturally fluorescent molecule called green fluorescent protein (GFP) first discovered in 1962 (Shimomura *et al*, 1962) and now known to be used by many different marine organisms which all live at such ocean depths that hardly any light can penetrate from the surface. These animals all use GFP to glow in the dark using the tiny quantities of ultra-violet light that reach these depths to excite the molecule; telling other animals where they are, rather than lighting their way. A crucial breakthrough in the use of GFP came when the gene was cloned in 1992 by Prasher *et al*. Subsequent researchers were then quick to express it into several other organisms including bacteria and nematodes or roundworms thereby demonstrating that no enzymes specific to the jellyfish were required to generate this bio-luminescence.

Using genetic engineering the gene encoding GFP has been modified to make it brighter and to glow with different colours. The gene for GFP can then be fused to a gene encoding a completely different protein from a completely different organism and when the genetic code is read the protein encoded by this gene will be fused to a GFP molecule, resulting in every single one of these expressed protein molecules possessing a fluorescent tag. Since 1999, several homologues to GFP have been discovered in many other classes of organism including sea anemones, corals and even crustaceans, indicating a wide evolutionary diversity for this category of proteins. In the past 10 years the use of the GFP family as 'molecular reporters' for the location of tagged proteins in living organisms has increased enormously. They are widely used as non-invasive probes to study different biological systems, from the level of whole organism tissue-patterning down to single individual cells, including monitoring of protein–protein interactions and measurement of a cell's internal environment including pH as well as ion-sensing.

Using advanced microscopy techniques we can cause the GFP tag to glow, telling us very precisely where the fusion protein is in the cell at any given time. We used this trick to follow the localisation of the stator protein of the bacterial flagellar motor of the model bacterium *Escherichia coli* by fusing the GFP tag onto MotB which is known to be one of the required components of each stator unit (Figure 3b).

Imaging molecular machine components

The GFP molecule was introduced into the bacterial flagellar motor using genetic encoding (as opposed to retrospective binding of a dye tag) to our molecule of interest. This involved deleting the gene responsible for expressing the MotB protein and then re-inserting a fusion gene made in the lab consisting of the MotB DNA sequence with that for GFP added on to the N-terminus at exactly the same position in the original chromosome (Leake *et al*, 2006), hence with a labelling efficiency for MotB of, in effect, 100%.

Since the new fusion gene is under the control of the same native gene 'promoter' (the sequence of DNA which denotes the start-point of a gene) the levels of expression of the GFP-MotB fusion protein are roughly the same as that for the MotB protein in the unmodified 'wild-type' cell strain. Hence, a highly physiologically-relevant result. However, visualising a single GFP molecule in a single living cell is no mean feat; the signal is exceptionally weak and the background noise, either from other GFP molecules or from natural so-called 'autofluorescent' molecules, can be very high making the imaging contrast extremely poor.

To improve this situation we employed an advanced microscopy technique called total-internal-reflection fluorescence or TIRF (Axelrod *et al*, 1984). Here, excitation light, typically from a laser, is directed onto a microscope slide from a very oblique angle such that instead of being refracted and transmitted through the biological specimen the beam is actually reflected at the boundary between the microscope slide and the water-based solution in which the specimen is bathed. The reflection of the laser light however generates a so-called 'evanescent' excitation field on the water side of the boundary. This excitation field decays steeply with distance from the slide; after ~100 nanometres the field intensity is ~1/3 of the value at the slide surface, whereas at ~1000 nanometres from the slide, the typical width of a bacterial cell, the corresponding intensity has dropped to only a few thousandths of a percent (Figure 3c). The significance of this is that only fluorescent molecules very close to the slide are excited, whereas those in the rest of the specimen, or any present in the surrounding solution, are not. This localised excitation can increase the imaging contrast to such an extent that detection of single molecules in living samples becomes possible.

To see the GFP-MotB molecules at the bacterial flagellar motor we use a very simple but clever technique to tether each cell to the microscope slide. The bacterial flagellum is made of thousands of copies of a single protein, flagellin. Antibodies can be easily raised to this protein and these antibodies used to coat a microscope slide. Bacteria then attach to the glass surface by their flagella (Silverman and Simon, 1974). This means that the flagellum can no longer rotate. However, the motor is still being driven by the ion gradient, therefore the cell body is forced to rotate about its point of tether attachment on the slide, rather like holding a car by one of its wheels and watching the car rotate.

These cells are easily visualised in the microscope, rotating very close to the glass surface because the flagellum is attached to the glass surface, bringing the flagellar motor within the evanescent field of the TIRF microscope. When we look at these rotating cells using TIRF we see bright spots which correspond to the centre of cell rotation (Figure 3d), indicating a high density of GFP-MotB protein molecules at the bacterial flagellar motors. When we measure the actual intensity values of these bright spots frame-by-frame under continuous TIRF illumination we see them decay with time in what appears to be a roughly exponential fashion.

More detailed analysis, especially towards the end of each trace, shows the decay curves are composed of many small steps, either of roughly similar size or approximately integer multiples of this (Figure 4a and b). This effect is due to a phenomenon called 'photobleaching'; many fluorescent molecules such as GFP will emit a roughly constant brightness of light but then, after an average period of time characteristic of that molecule, will stop emitting light as a result of some chemical damage. This means that the size of the small steps we observe is a measure of the brightness of a single GFP molecule or, less frequently, two or perhaps three GFP molecules which photobleach during the time window of each image frame. By measuring this very accurately we can extrapolate back to the brightness at the beginning of each trace.

This gives a precise estimate of how many photoactive GFP molecules were present in each spot, and therefore how many MotB molecules are present in the bacterial flagellar motor, an example of a Fluorescent Unitary Counting or FUC technique (Leake *et al*, 2006). This leads to an estimate, with this particular molecular machine, of ~22 molecules (Figure 4c). Additional data suggests that there are two of these MotB molecules per stator unit, therefore indicating that there is a ring of ~11 stators in the functional motor.

Molecular components on the move

This value is, however, only a snapshot of the average number of MotB molecules present in the motor at any one time; there is a danger in thinking that because this number does not appear to change from snapshot to snapshot then everything must be static. It turns out

that these molecules are far from stationary. In *E. coli* there are typically between four and eight bacterial flagellar motors per cell, each containing an average of 22 MotB molecules – however there is a pool of ~200 MotB molecules not present in the motors but rather free to diffuse in the cell membrane.

The presence of these additional membrane-localised fluorescently-tagged MotB proteins makes tracking any individual GFP-MotB a difficult task because they result in the membrane appearing almost uniformly bright. We can remedy this by generating a highly intense laser focus to photobleach all GFP molecules in a highly localised region of the cell membrane which then appears as a bleached circle of diameter ~1000 nanometres on the TIRF image. Having thus eradicated the bulk of background fluorescence in this region we can then watch with clarity as individual GFP-MotB components diffuse into the bleached zone. The brightness of such diffusing components suggests that they are composed of two GFP-MotB molecules, consistent with a single stator unit, and on average these molecules take about two minutes to diffuse from one pole of the 3 micron rod-shaped *E. coli* to the other.

We can then perform the same experiment but direct the laser focus at a bacterial flagellar motor itself. What we see is an initial sudden decrease in spot brightness to close to zero, due to photobleaching of the GFP-MotB stator ring bound at the motor. However, we subsequently observe roughly half of the original brightness coming back over a period of five minutes or so, an example of fluorescence recovery after photobleaching (FRAP). Similarly, if we direct the laser focus to be ~1 micron distance from a motor, then watch the motor at similar subsequent times we see the spot brightness *decrease* with time (Figure 5), in effect fluorescence loss in photobleaching (FLIP). Using the information available from the speed of diffusing stator units in the cell membrane these data suggest that each individual stator unit will spend on average only ~30 seconds in each motor. In other words, the force-generating components of a functional motor are constantly on the move; a truly remarkable feat if one considers attempting to change one of the wheels of a car whilst travelling down a motorway at high speed.

An ingenious evolutionary mechanism

The car analogy does not stop there. Cars are more than a collection of wheels: there is the engine, the fuel and its tank, a steering-wheel, gears, a clutch, shock-absorbers, brakes, the bodywork. The list goes on, and with each item it is not difficult to think of a biological molecular machine equivalent in the single living cell. Our results with the bacterial flagellar motor may be just the tip of the iceberg: continuous changing of molecular components may be a universal feature of natural nanoscopic machines.

The techniques we have developed have the potential to shed light upon these processes in the future, but we should take a back-seat for a moment in our analogous car to ask why this could be such an important feature of biological cells. Why should components constantly turn over? It may simply be a consequence of making more than are needed and the proteins in the pool simply exchange with the proteins in the motor when diffusion brings them into contact. On the other hand some components may go wrong. Parts of a molecular machine that perform distinct functions are rarely single molecules *per se* but rather small collections of molecules bound in well-defined conformations to each other. But the bonds between such molecules are far from permanent, so in essence after a given time components may develop 'cracks'. Constantly kicking-out old components and exchanging

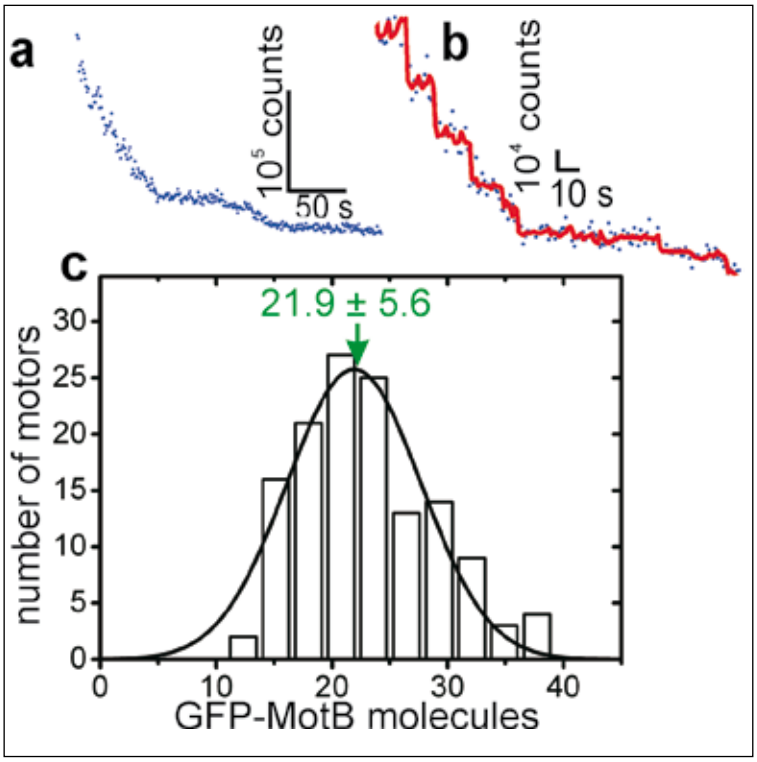

Figure 4. Counting molecular components in machines. a) raw intensity vs time plot for a typical continuous TIRF illumination of a flagellar motor. b) expansion of (a) above (blue) with overlaid filter showing distinct steps (red). c) histogram for the distribution of many such motors for the prediction of the number of GFP-MotB molecules present at the motor with mean and standard deviation error indicated.

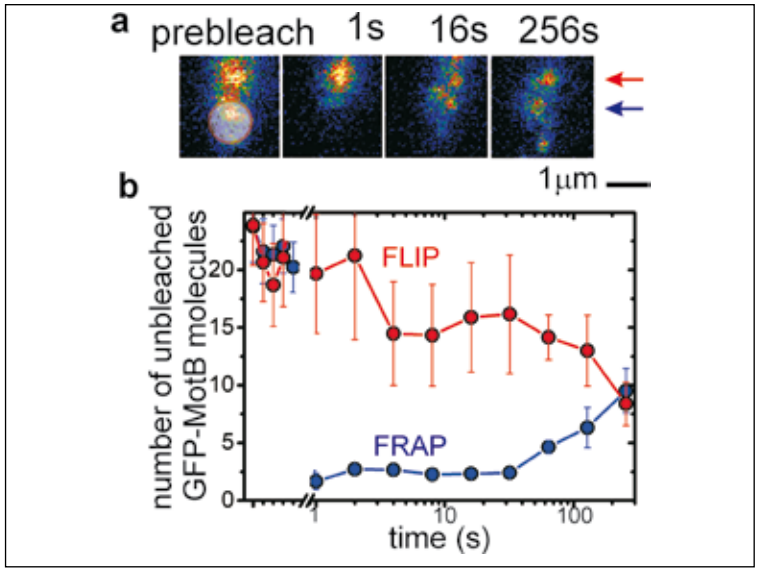

Figure 5. Seeing molecular components turnover. a) time-lapse images for fluorescence recovery (FRAP) and loss (FLIP) experiments following photobleaching with a focused laser (marked with a white circle on the 'prebleach' image) on a single cell. One of the motors showed a recovery of fluorescence (blue arrow), whilst another distant from the original laser focus shows a loss (red arrow). b) measured mean variation in number of photoactive GFP-MotB molecules at the motor, measured for several motors for FRAP (blue) and FLIP (red) experiments (errorbars = 1 standard deviation).

those for new ones may be an evolutionary mechanism to ensure that worn-out or broken components never dominate a molecular machine, thereby securing a continuous functional output.

Cells do indeed contain a machinery for identifying damaged proteins and either breaking them down into their constituent amino acids or refolding them, depending on their level of damage. Refolding of proteins made non-functional by denaturation and/or misfolding due to factors such as pH, mechanical stress and heat is assisted by molecular machines called 'chaperones' (Hemmingsen *et al*, 1988), and the prevalence of heat as a damaging factor may account for their alternative name of heat-shock proteins. The best example for the complete degradation of damaged proteins is the so-called 'proteasome', a comparatively large barrel-shaped complex which is a feature of both highly complex eukaryotic cells (Voges *et al*, 1999) as well as some comparatively simpler bacteria (Festa *et al*, 2006). Here, proteins are first targeted by specific enzymes which introduce tags of a protein called ubiquitin (so called because it is ubiquitous across all eukaryotic cells). This ubiquitin tag-motif is recognised by the proteasome complex which then acts as a site for several proteolytic reactions which require energy in the form of ATP, in which the protein is then broken down into smaller peptide components which can then be recycled for use in other biological processes.

It has been known for over 20 years that similar machines extend into the plant kingdom as well (Ohad *et al*, 1984), suggesting that the ability to detect and repair/replace proteins is a highly conserved evolutionary feature of life in general. However, at present at least, there is no direct evidence that a dedicated molecular machine can directly detect which individual components are faulty or not in a working motor so it may be that, to allow for some damage, the motor loses some perfectly good components along with the faulty ones, randomly exchanging motor-bound stators with the membrane pool. For this approach to work in the cell there must ultimately be a continuous source of new, fully functional components as well as a sink for degraded faulty ones, which ensures that the average number of functional molecules generally exceeds the faulty ones. For any given rate of damage this is a simpler mechanism than detecting damaged proteins in all individual molecular machines.

A consequence of this is that components that are re-incorporated into a molecular machine may have been used by several machines before and some, hopefully not too many, may even themselves be faulty (but hopefully most will be recognised by the cell's repair/destroy system). In other words, they are in effect second-hand parts with several previous owners whose service-history is far from exemplary.

There is an intriguing endpoint to this argument, which addresses a very heated issue of so-called 'intelligent' design. Richard Dawkins made the comment in his book *The Blind Watchmaker* that "biology is the study of complicated things that give the appearance of having been designed for a purpose". This particular biological machine of the bacterial flagellar motor has often been cited by some groups of creationist theologians as a clear example of a natural biological feature that is so intricate and perfectly suited to its function that some grand Intelligent Designer (*sic*) must have been ultimately responsible. The experimental facts actually now suggest that the bacterial flagellar motor is far from perfectly designed. Not only is there a good evolutionary basis for the design (Berg, 2003; Pallen and Matke, 2006), is it also probably composed of second-hand parts. In addition, although the efficiency of the machine when measured by tethering a cell to a microscope slide (Figure 3c) is sometimes almost 100%, when the cell is freely swimming the efficiency actually drops to almost 5%, not the efficiency you would want from a new

car! The reason is that, for a bacterial cell to swim, the rotation of the motor has to be coupled to a flagellar filament to bring about actual propulsion. However, the filament is in essence a helical-shaped rod which, when it rotates, causes not only the cell to move forwards but also lots of the surrounding water to moving backwards and sideways (Purcell, 1997) – plus there are frictional losses between the cell and the surrounding water. Thus, lots of energy which might have been utilised in forward motion of the cell is in effect dissipated elsewhere, thus manifest in a relatively low efficiency for swimming. And so the automobile analogy motors on, albeit not with a slick, new car, sparkling and freshly waxed, but rather with a second-hand model, perhaps a little worn and weathered and not as fast and efficient as might be desired, but ultimately capable of getting from A to B.

Obtaining the blueprints for the architecture of biological nano-machines is essential for understanding the workings of the cell. Here we have counted the number of proteins in such a machine, a tiny rotating motor, and for the first time have seen that individual components are rapidly replaced. This may indicate that biological components wear out and need replacing just as they do in man-made machines. The future of this research lies not only in exploring other bio-machines with similar techniques, but also in extending the work into the multi-colour regime by tagging several different proteins with different coloured tags in the same living cell. In doing so we may be able to observe many different components of the same machine at the same time, or to monitor individual components of different co-operating machines simultaneously. In this way we may be able to get an insight into the very heart of systems-level biological complexity itself.

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Relevant websites

www.physics.ox.ac.uk/users/leake Additional details on techniques and bio-systems described here.

www.aip.org/pt/jan00/berg.htm An excellent introduction to bacterial motility.

http://people.cryst.bbk.ac.uk/~ubcg16z/hsplec.html Details of molecular chaperone repair systems.

www.sysbio.ox.ac.uk Description of systems-level bio-research at Oxford University.

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