The Need for Speed

Advances in High-Speed AFM for Biological Imaging

Almost since the time of its invention in 1986 by Binnig, Quate and Gerber, the Atomic Force Microscope (AFM) has been recognised as possessing great potential in solving some of the most elusive problems in the biosciences. Indeed in his Nobel prize-winning lecture Binnig identified biological imaging as being one of the main motivators for developing AFM.[1] The use of AFM has expanded in recent decades with thousands of papers now being published each year that report on AFM studies of biological systems.[2]

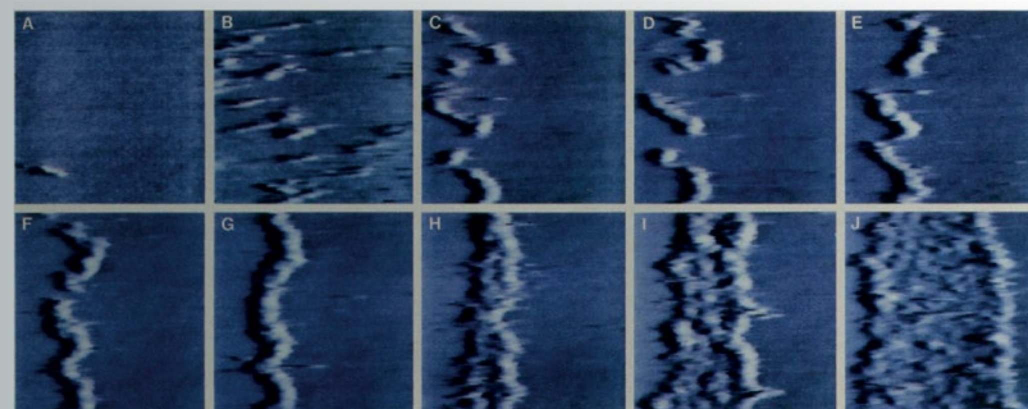


Fig 1: *Clotting of the human blood protein fibrinogen in real time; an early example of AFM imaging of dynamic biological processes before the advent of high-speed AFM. The ten images were taken before the addition of the clotting enzyme thrombin (A), and then at incubation times of between 9 and 33 minutes (B-J). The relatively slow frame rates available with AFM at the time of this study limited the technique's applicability to other biological processes. Each image is 450nm square. Images taken from reference [5].*

For those not familiar with AFM, the concepts surrounding it can seem alien. One way to think of AFM as a technique is that it 'feels' rather than 'sees' the surface. The basic concept of AFM remains the same as when it was first developed, although there have been endless technical improvements and refinements. A sharp silicon or silicon-nitride stylus (or 'tip') with a nanometre-scale sharpness attached to a cantilever is used to scan line-by-line over a sample immobilised on a flat substrate. The backside of the cantilever is reflective, allowing a laser beam to be reflected off the cantilever and into a photosensitive detector. Deviations in the reflected laser beam position are indicative of the intermolecular forces between the tip and sample, allowing a three-dimensional image of the sample to be built up over time.

In the biological sciences AFM has a number of advantages over other forms of microscopy.

Resolution is limited only by tip sharpness hence atomic scale imaging is possible; molecular imaging, for example protein structure, is increasingly commonplace and imaging on the length-scale of larger biological samples such as cells and macromolecular assemblies is now well-established. This places it at higher resolution than conventional optical microscopy and roughly equivalent to super-resolution light microscopy[3] with the further advantage of not having to rely on fluorescent markers. AFM also has multiple advantages over electron microscopy including the ability to image while fully immersed in fluid, allowing biological samples to be imaged in their native state. Finally, AFM is applicable to a wide variety of different samples with a general rule of thumb being; if it's solid and can be immobilised on to a surface, AFM will be able to image it.

AFM has seen many developments since its invention that have benefitted researchers in solving many fundamental biological questions. Refined imaging modes have given higher resolution and less invasive ways of visualising biological samples while force spectroscopy techniques have allowed the detection and manipulation of single molecules such as proteins and nucleic acids, giving an insight into tertiary structures and their relation to biological function. The technical development of AFM has gone hand-in-hand with the wider acceptance of AFM in the biosciences and as a result AFM manufacturers now typically market their devices to the non-expert as well as the more experienced user. The entry barriers to bio-AFM in terms of technical expertise are significantly lower than they were 20 years ago.

However, one of the main drawbacks of AFM remains its relatively slow image acquisition speed.

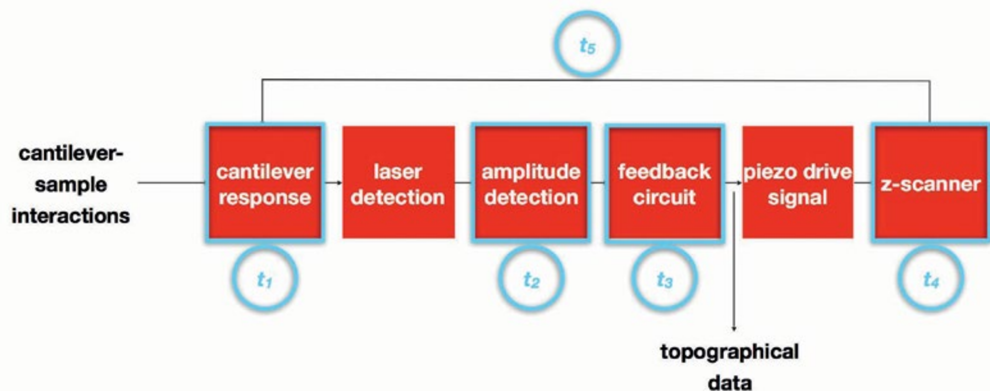


Fig 2: Flow diagram of tapping mode AFM operation with steps that involve significant time delays highlighted in blue. These time delays must be minimised for high-speed AFM to be realised. Interactions between sample and cantilever causes damping in the cantilever oscillation over time t1. This in turn causes a change in the amplitude of the reflected laser signal, which is measured over time t2. A proportional-integral-derivative (PID) feedback circuit computes the associated change in tip-sample distance, over time t3, which determines the topographical data readout. This data is fed back into the piezoelectric element and the cantilever height is adjusted allowing for the z-scanner response time t4. In some cases when the cantilever scans a steep incline the cantilever tip detaches from the surface and an additional delay t5 takes place as the tip returns to the surface.

Standard commercial AFMs allow high resolution images to be captured over a time scale of minutes. Quite apart from being low throughput compared to most optical techniques this drawback means that conventional AFM is unable to effectively address research questions that relate to active biological agents, where activity might occur over time scales of seconds or even fractions of a second. Such dynamics are fundamental to biological processes relating to cells, organelles, biopolymers and macromolecular assemblies. Imaging these dynamics using other forms of microscopy has limitations in terms of resolution, therefore there is an obvious advantage to adapting AFM to operate at faster speeds.

In this article we review the progress made in bio-AFM from its earliest conception up to present developments in high-speed AFM. We explain the various technical factors that have contributed to high-speed AFM and present a selection of the key findings in the literature in recent years. Finally we describe progress made here at Leeds in using AFM to understand phase separated biomembranes and appraise the future of high-speed AFM in the biosciences.

A Brief History...

The history of AFM of biological samples has been well covered by a number of review articles on the subject[2] and it would be impossible to list all of the large number of incremental changes that have led to the capabilities that AFM now possesses. Instead we highlight a number of technological milestones that have made substantial improvements to how AFM can be applied to biological samples.

The first of these is the development of AFM imaging in liquid samples in 1987, which was essential when studying hydrated biological samples. Over the following two years, developments in micro-fabrication of cantilevers gave improved resolution and optical beam deflection techniques improved sensitivity. The combined strength of high resolution and liquid imaging was demonstrated in 1994 with the first images of protein surfaces[4] however the delicate nature of many biological samples remained a limiting factor. A major breakthrough in bio-AFM came in 1994 with the invention of tapping mode in liquid,[5] where the cantilever is oscillated at its resonant frequency, the surface topography being determined by the damping of the oscillation from intermolecular forces between tip and sample. This minimised contact and lateral forces between tip and sample. Further refinements, particularly soft cantilevers and improved control over contact force have meant that contact mode also remains useful for biological applications. More recently a new mode, peak force tapping, has been developed in which the probe is oscillated at a non-resonant frequency and captures force vs. distance data on each oscillation. This allows the tip-sample interaction to be carefully minimised and is ideal for imaging delicate samples such as live cells.

A clear drawback of AFM for biological applications has historically been its slow capture time, which makes it difficult to visualise biological processes in real time. Furthermore no other imaging method is capable of directly visualising biological samples in aqueous environment beyond optical resolutions making this drawback one that is vital to overcome.

Early studies found "low hanging fruit" through biological processes that took place over minutes rather than seconds. Some examples from the early 1990s included the imaging of fibrinogen clotting at 1 minute intervals after the addition of thrombin, as shown in Figure 1 [6] or the binding of RNA polymerase to DNA strands[7] Even so AFM frame rates from these early studies remained limited. Truly high-speed AFM is in many ways the last piece of the puzzle.

What makes it High Speed?

Recent years have seen many technological advances that have ultimately led to faster capture AFM times. This advancement has been incremental and has relied on solving a number of instrumental limitations. AFM data collection can be seen as a multi-step process in a feedback loop, starting with

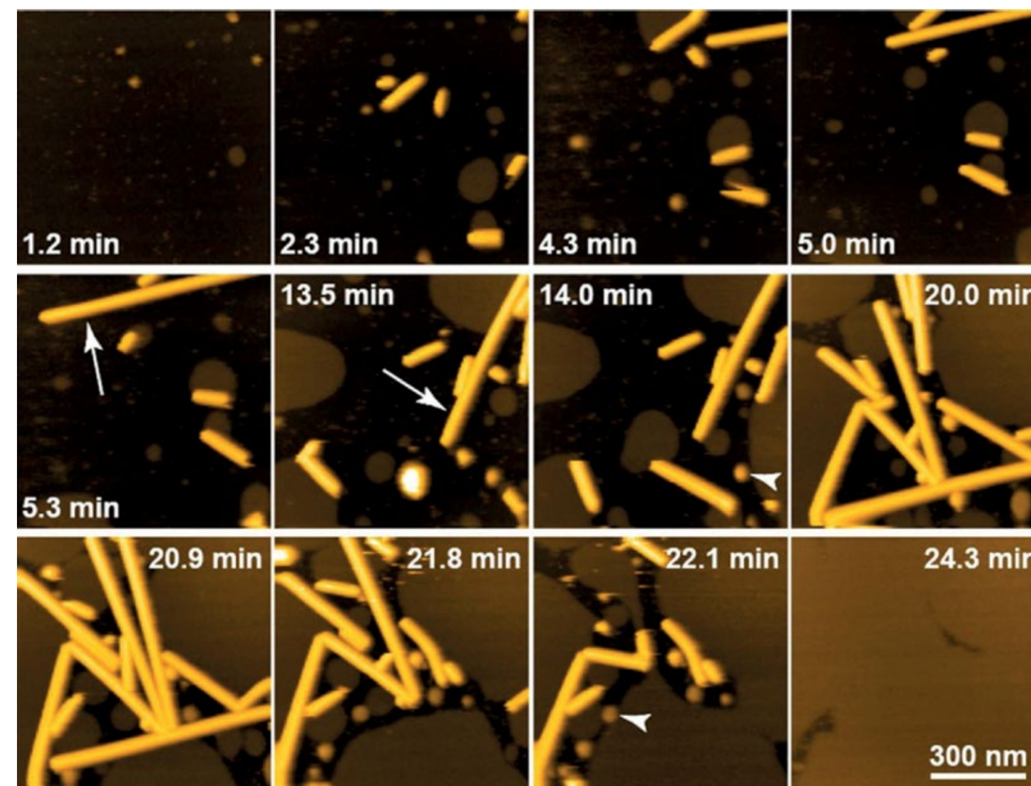


Fig 3: Flow diagram of tapping mode AFM operation with steps that involve significant A selection of high-speed AFM images of the formation of supported lipid bilayers. Membranes were made of a ternary mixture of lipids (DOPC, DOPS and biotin-cap- DPPE) and sonicated. It has generally been assumed that the bilayer forms by absorption of vesicles on to the mica surface and eventual rupture. However, this study has shown that the bilayer formation is also driven by intermediate tubular structures (indicated by arrows) of height 20nm as well as by unruptured vesicles. Image taken from reference [12].

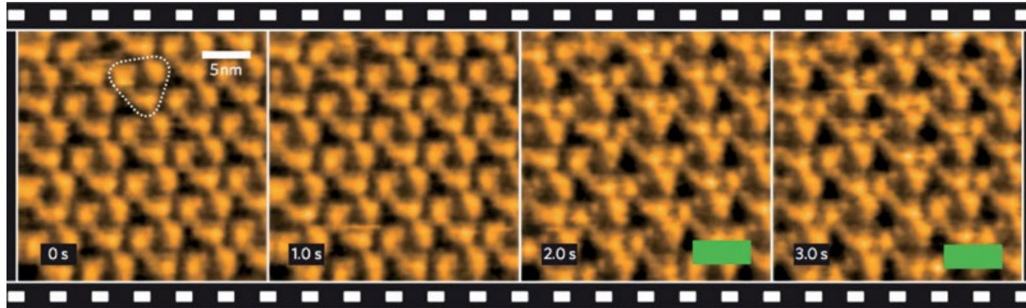


***Fig 4:** Successive high-speed AFM images, taken at 1 frame per second, of a trimeric array of the photosensitive protein Bacteriorhodopsin on a mica surface. A single trimer is highlighted by a white triangle. Upon illumination with green light (2.0s and 3.0s above) the array undergoes a clearly noticeable structural transition; each monomer exhibits a lateral protrusion and the entire trimer rotates by 7° counterclockwise. Image taken from reference [13].*

the interactions between the cantilever and sample and, after a number of intermediate steps, finishing with a read-out of topographical data followed by the movement of the cantilever to the next pixel in the image and the adjustment of the cantilever parameters for the next measurement. Owing to either mechanical or computational reasons there are finite time delays associated with a number of these intermediate steps that must be minimised if high-speed AFM is to be achieved. The oscillating cantilever has a finite response time, the change in cantilever amplitude takes time to detect, the feedback circuit takes time to integrate this signal and the z-scanner that adjusts the cantilever height also has a mechanical inertia. A further source of time delays is the phenomenon known as 'parachuting' where the cantilever tip can detach from the surface entirely when scanning a steep incline. This results in a delay in order to allow the tip to return to the surface. Figure 2 shows a flow diagram of each of the steps in the feedback loop, with steps that involve significant time delays marked in blue and their associated delay times labelled t1-t5. Various innovations in AFM have minimised each of these time delays.

Cantilevers have also been miniaturised [8] to allow higher resonant frequencies and higher sensitivities thereby improving response times but with the added complication that lasers need to be focussed onto a smaller area. Optical detectors have been designed that measure the oscillation amplitude of the reflected beam at every cycle of oscillation, dynamic feedback controllers have been devised that automatically change the feedback gain parameters[9] and new techniques including active damping and counteracting piezoactuators have been used to reduce noise in the z-scanner, thereby also increasing its response time.[10]

Thus, it would be wrong to identify any one of these advances as the 'silver bullet' that has led to high speed AFM. Rather their cumulative effect on improving imaging speed has led to the high-speed capabilities that AFM now possesses.

Some Highlights of Recent Work

In recent years, a number of key studies have demonstrated high-speed AFM to be a uniquely useful technique in understanding dynamic processes on the nano-scale.[11] One simple but elegant paper in this field has been the imaging of supported lipid bilayer formation in real time. Supported lipid bilayers are useful model systems that mimic the properties of native biological membranes. A typical method for forming a supported lipid bilayer is the 'vesicle' rupture method in which lipid vesicles are formed and absorbed to a surface, eventually rupturing and forming a continuous planar membrane once their surface area coverage reaches a threshold. In a 2010 study,[12] this process was imaged in real time and it found that, before the bilayer was fully formed, tubular membrane structures with a height of 20nm formed. A selection of images over longer time scales are shown in Figure3. These tubular structures merged with the unruptured vesicles to form the bilayer. This study was the first to elucidate precisely the formation mechanism of a lipid bilayer, a widely used sample for many biological experiments.

High-speed AFM has also been used to study the relationship between the structure and function of the light sensitive protein Bacteriorhodopsin. Bacteriorhodopsin is a protein complex that forms two-dimensional crystalline arrays, typically referred to as 'purple membrane', that occupy up to 50% of the surface area of archaeal cells. Its role is to harvest chemical energy by undergoing conformational changes when exposed to light, thereby creating proton gradients in the membrane that are converted into energy. Bacteriorhodopsin comprises 7 helical motifs that span the membrane and it has generally been accepted that the protein channel opens when two helices, the 'E' and 'F' helices, tilt away from the centre of the protein. However, this process has been difficult to visualise due to the short timescales involved. In a 2010 study Shibata et al[13] addressed this by using a mutant form of Bacteriorhodopsin (D96N) whose photocycle is of the order of 10's of seconds and forming a planar array from it. The protein array was imaged using high-speed AFM at 1 frame per second, before and after exposure to green light. Figure 4 is taken from this work and shows successive images of the protein structure. Before illumination the proteins are in array of trimers. After illumination, a noticeable change in structure can be seen. Image analysis showed that each protein monomer exhibited a lateral protrusion, associated with the movement of the helices, and the entire trimer rotated by approximately 7° counter clockwise, thereby clearly showing the structural changes associated with the Bacteriorhodopsin function.

As well as studies of sample formation and structural changes in response to external stimuli, high-speed AFM has also been used to study active molecular systems. The molecular motor protein myosin V moves along actin filaments inside the cell, transporting vesicle cargos. Myosin V has two heads which bind to the actin surface. Mechanistically it moves by stepping each of the two heads alternatively along the actin filament, with each head moving from behind to in front of the other with each step, much like walking. This 'walking' has been visualised for the first time with high-speed AFM.[14] Previously the only images of myosin had been stationary and immobilised in a non-native environment with electron microscopy.

With high-speed AFM, however, native conditions were better approximated by binding an actin filament to a lipid bilayer using a biotin-streptavidin linkage. The bilayer was given a slightly positive charge through addition of the lipid DPTAP, which prevented the myosin from standing perpendicular to the surface and allowed the side-on imaging of its structural dynamics through high-speed AFM. The Myosin V motors were found to take successive

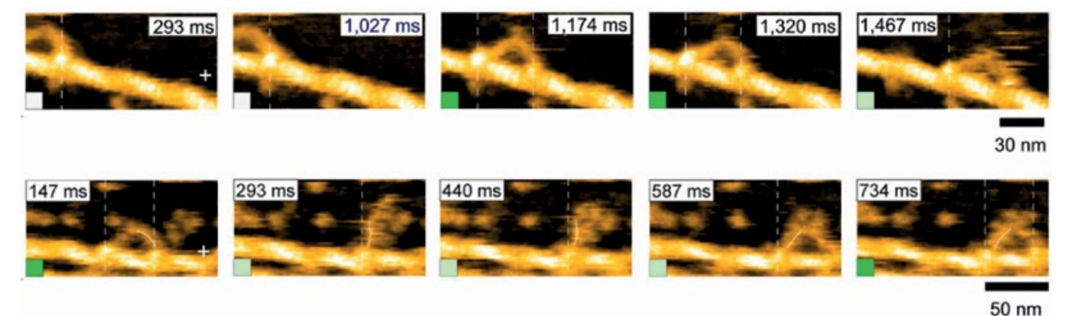


***Fig 6:** Successive high-speed AFM images of the molecular motor protein Myosin V interacting with actin filaments. (a) Images show the processive 'walking' movement of Myosin V along the actin filament. Each step taken is approximately 36nm in length, in line with data from the literature. (b) At higher speed the individual steps of the Myosin can clearly be resolved and the mechanism is shown to be in agreement with the 'lever arm swing' hypothesis, the swinging lever highlighted by a thin white line. Image taken from reference [14].*

36nm steps, a finding consistent with previously observed behaviour of myosin motors.

These clearly visualised steps are shown in Figure 5. The study also confirmed the hypothesis of a 'lever-arm swing' whereby subtle changes in the myosin head's structure were propagated to the attached lever arm of the myosin, resulting in large displacements that facilitate the myosin's 'walking'. Each of these studies demonstrates the capability of high-speed AFM to directly observe biological molecules at high resolution. High-speed AFM is both more widely accepted and more widely used and it is likely that as more groups use it, a wider range of biological samples and biological phenomena will be studied.

Our Research at Leeds

At the University of Leeds we seek to use AFM to understand the fundamental properties of supported lipid bilayers. Lipid bilayers make up the cell membrane, the chemical barrier that encloses the cell, and defines it's boundary. As cells need to communicate, obtain energy and excrete waste, a huge number of cellular processes are located in the cell membrane.

The membrane is also an important organelle when it comes to disease: currently over 60% of approved drug targets involve the cell membrane and its components.

Supported lipid bilayers are one of a number of model systems used to study the surface of the cell membrane for the purpose of understanding the behaviour of biological membranes.

For example, the current understanding of the cell membrane states that it can be considered as a two dimensional fluid with regions saturated in certain types of lipid, so-called 'lipid rafts'. While the existence of lipid rafts is now gaining more widespread acceptance there are still many open questions, especially relating to the length scales on which such rafts persist and their typical lifetimes. These questions are confounded by the fact that behaviour seen in model systems is not revealed in living cells.[15] Recent studies have suggested this discrepancy is due to a lack of complexity in model systems but questions remain about whether a planar system like a supported bilayer can ever truly be used to represent the curved surface of cell.[16]

In our laboratory we use a commercially available Bruker fast-scan AFM, capable of achieving frame rates of up to the order of 8 frames per second. Although this does not match the speeds of devices currently under development by various labs, as described above, this is still significantly faster than standard AFM imaging. We use AFM to study supported lipid bilayers where raft-like domains can be distinguished clearly by differences in surface height. [17] The resolution of AFM exceeds that of other techniques making it ideally suited to investigating sub-micron sized domains. The advent of high-speed AFM has extended this capability by allowing short-lived lipid domains to be effectively imaged. Figure 6 (a) shows a series of images captured at 0.2 frames per second of a bilayer composed of a mixture of lipids: 30% DOPC, 35% Sphingomyelin and 35% Cholesterol. A bilayer of this composition is known to phase separate with raft-like 'liquid ordered' domains standing slightly higher than the 'liquid disordered' background. In the final frame of Figure 6, one of the smallest domains shown by a white circle dissolves into the liquid disordered background phase. We postulate that the cause of this is the high energy cost of maintaining small lipid domains due to a large proportion of the lipid molecules being located on the boundary between two phases. Thus the constituent molecules of the smaller domains diffuse through to the larger domains, a thermodynamic process known as 'Ostwald Ripening'. Ostwald Ripening is a common occurrence in many diverse systems including crystals, emulsions and quantum dots but our data, shows the first directly observed instance of this process in lipid bilayers. Figure 6 (b) shows a similar process where the bilayer composition is 60% DOPC, 25% sphingomyelin and 15% cholesterol.

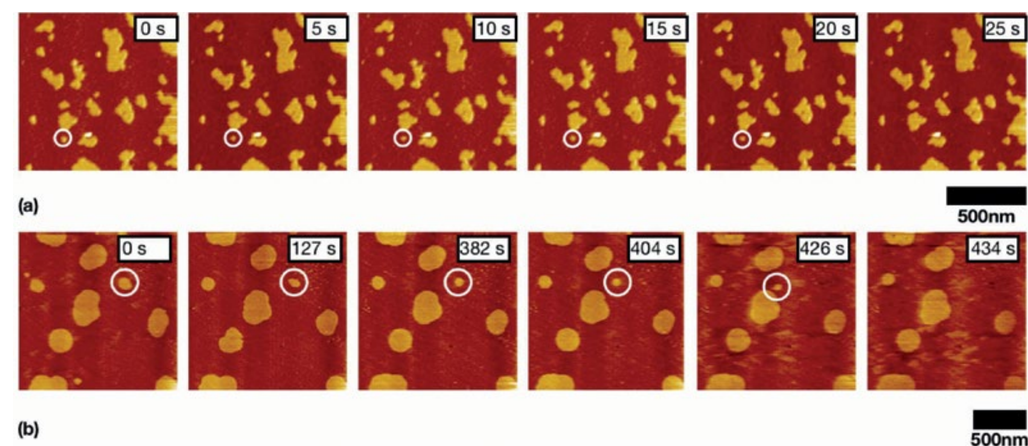


Fig 6: *Successive high-speed AFM images of lipid bilayer prepared from a ternary lipid mixture; 30% DOPC, 35% Sphingomyelin and 35% Cholesterol (a) and from 60% DOPC, 25% Sphingomyelin and 15% Cholesterol (b). In both bilayers the membranes are phase separated into a liquid disordered background phase and raft-like liquid ordered domains. In (a) small raft-like domain is shown to disappear in the final frame. This process is known as Ostwald ripening. A similarly process is shown in (b) where a single small domain is shown to diffuse from right to left, shrinking and merging with a larger domain in the process.*

Again, the bilayer is phase separated into a liquid disordered phase and raft-like liquid ordered domains. However in this set of images a smaller domain (highlighted by a white circle), rather than dissolving, diffuses towards and coalesces with a larger domain.

These findings are not just interesting from a fundamental perspective but rather they are also highly relevant for the wider use of lipid bilayers as model systems. A key criticism in the literature of lipid bilayers as a representative model system, has been that formation of the membrane on a solid support prevents the effective diffusion of lipid domains in the membrane in the way that would occur in a cell membrane. Counter arguments have been made that this concern is unfounded due to a 1nm hydration layer that sits between the bilayer and the solid support [18] but without a means of direct and fast high resolution imaging of domains it is difficult to resolve this question. The data in Figure 6 appears to support the hydration layer argument by showing for the first time that lipid diffusion is not prevented by having the bilayer on a solid support. These findings are preliminary and we have identified many opportunities for further study. For example, it would be insightful to image phase-separated bilayers at critical points, where the energy required to maintain domains is tiny and the bilayer fluctuates between different phases over small scales in time and distance.[19] It has been postulated that this critical behaviour may be characteristic of naturally occurring lipid rafts.[20]

High speed AFM could also be instructive in studying the interactions between bilayers and biological reagents. For example we have recently reported on the antimicrobial peptide Polybia-MP1, a host-defense peptide with known anticancer properties and we have shown through AFM and other techniques that the peptide causes pores to form in the membrane through an interaction with two lipids PS and PE.[21] We show that these two lipids synergistically combine to enhance membrane poration: both are required for pores to form. It is thought that the anticancer properties of MP1 stem from the fact that these lipids are expressed on the outer leaflet of the membrane of various cancerous cells. Studying pore formation in real time could give further insights into the mode of action of this and other membrane-acting anticancer agents.

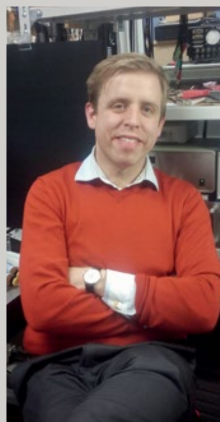
The Future

High speed AFM has opened up a wealth of new opportunities to directly observe and measure the fine structure, dynamics and function a range of

biological systems. This has given new insight into the function of biological systems, even those which have already been extensively studied through other techniques. The key take-home message is that high speed AFM allows a directness of measurement of dynamic biological systems at small length scales, a capability not shared by any other form of microscopy. Furthermore the technique has much unrealised potential as our laboratory's initial findings from model supported lipid bilayer systems shows.

At present the usage of high speed AFM is expanding. After years of development the first commercially available mass-market high speed AFMs are now available with frame rates of the order of a few images per second. At the same time, continuing technological development has led to even more impressive devices that comfortably achieve the order of tens of frames per second. Specialists in the field seem to concede that scan speeds cannot exceed 100Hz under the current modes of AFM configuration.[11] Instead future developments are more likely to revolve around functional augmentation such as sub-surface imaging, through scanning near-field ultrasound holography[22] or scanning acoustic microscopy[23]. One of the first reviews of bio-AFM contained just 9 references from the literature from a limited number of laboratories. A similarly comprehensive review today would have to reference thousands of studies from laboratories all over the world. Similarly, current comprehensive reviews of high speed AFM contain references to just tens of published studies from a small number of laboratories. It should be expected that years from now that high speed AFM will be available to a wider range of laboratories and therefore applied to a much wider range of systems. This will lead to new insights that, although they cannot now be envisaged, are likely to have a major impact on the bioscientific research in the future.

About the Author



Anders Aufderhorst-Roberts obtained his PhD in Biological and Soft Matter Physics from the University of Cambridge in 2013. Following his PhD he carried out postdoctoral work in the research group of Simon Connell at the University of Leeds, where he used a range of advanced atomic force microscopies to characterise phase separation in supported lipid membranes. From 2016 he is a postdoctoral researcher in the lab of Gijsje Koenderink at FOM Institute AMOLF in Amsterdam, the Netherlands where he studies the rheology and microstructure of composite cytoskeletal filament networks. His other research interests include nanomechanical mapping of soft materials, buffer mediated membrane patterning and lipid-specific antimicrobial peptide action for anti- cancer applications.

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