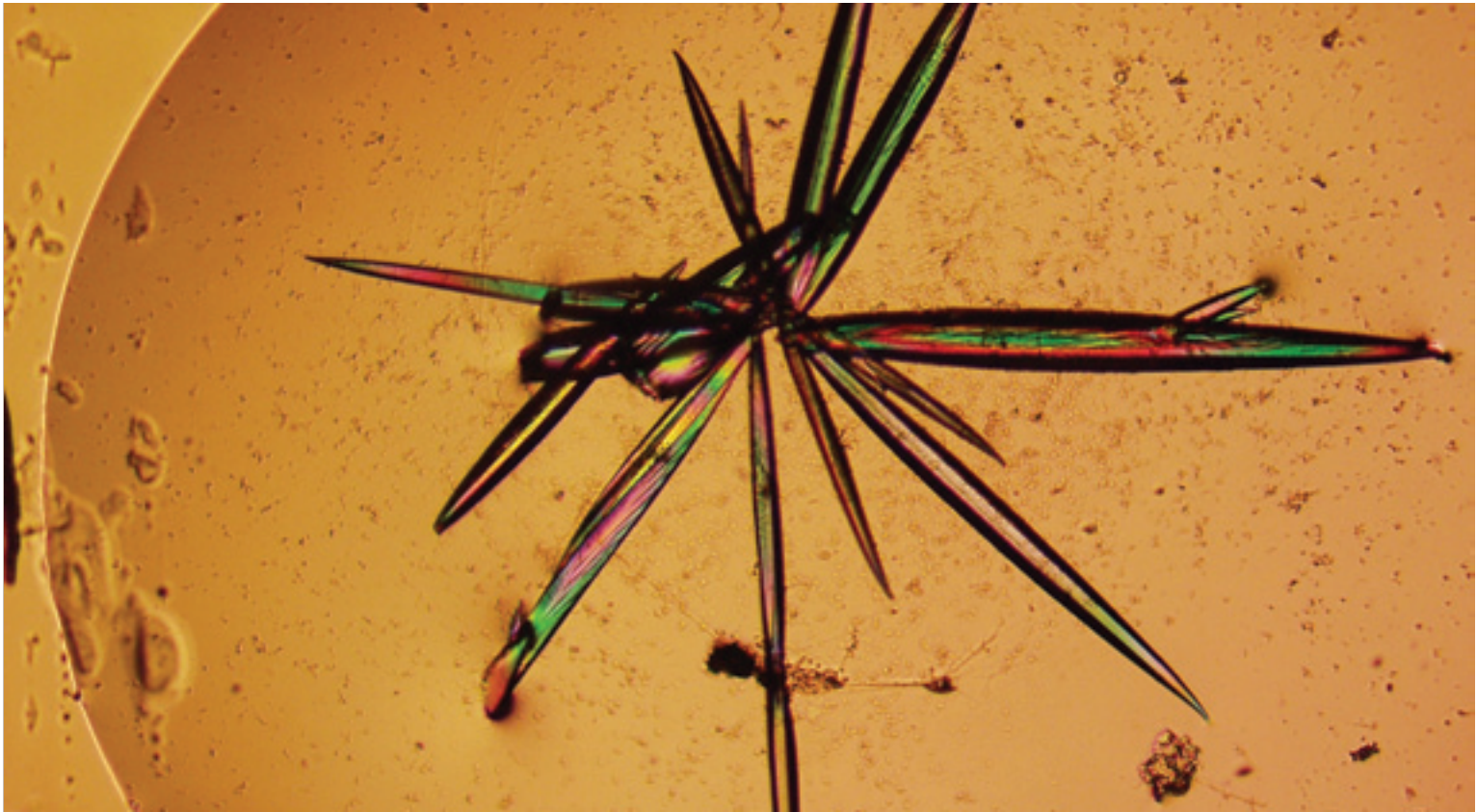




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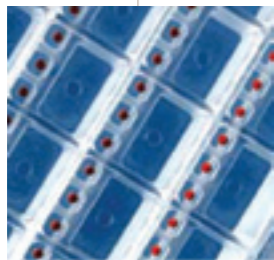
news from the protein crystallography lab

innovative laboratory solutions

■ storage ■ pipetting ■ screening ■ automation

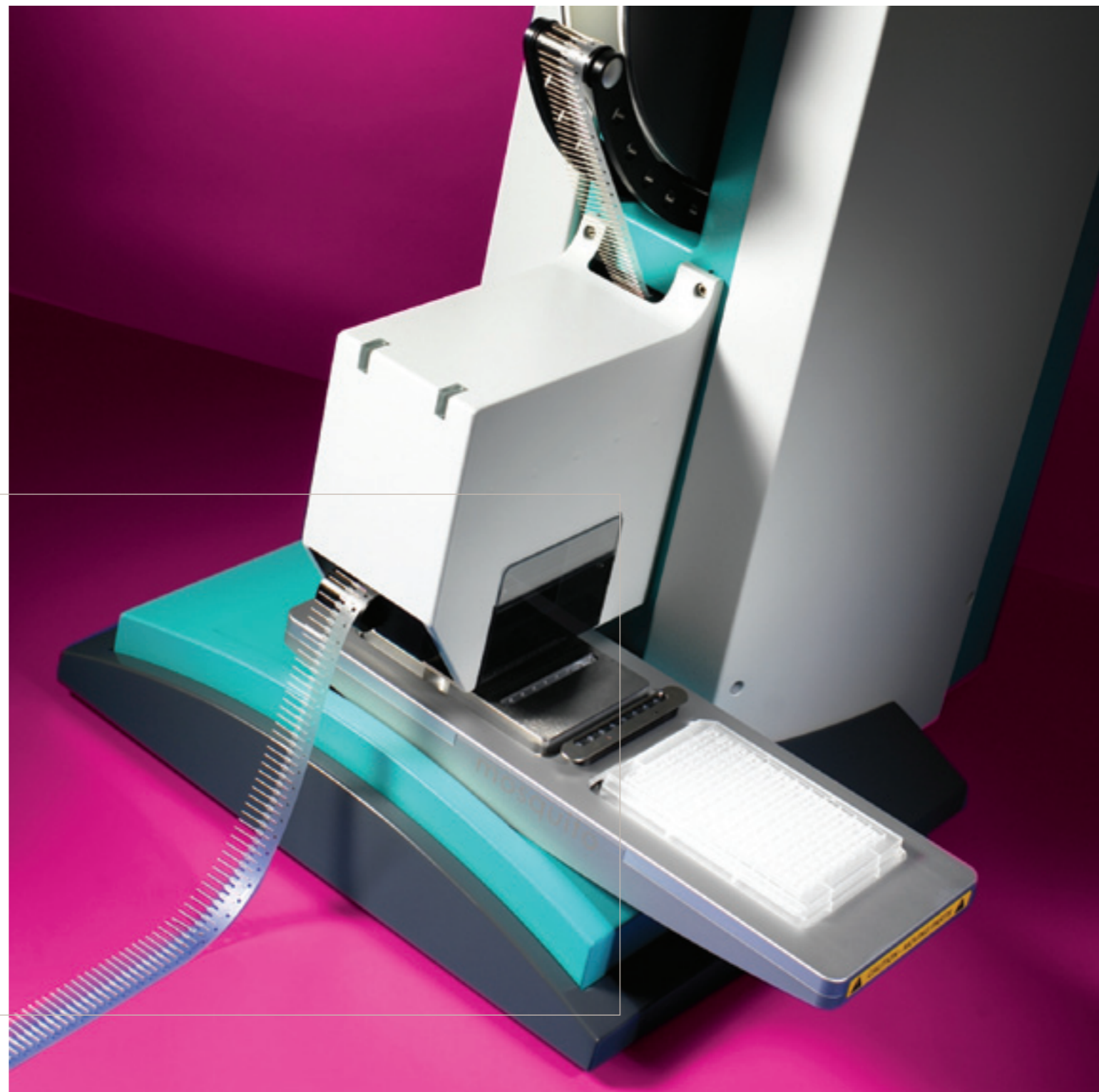
mosquito®

mosquito is the industry standard instrument for protein crystallographers throughout the world. But what makes our instrument so special?



Flexibility

- mosquito's positive displacement pipettes enable accurate aspiration and dispensing of any liquid irrespective of viscosity
- With a choice of two instruments, mosquito offers the ultimate in flexibility for protein crystallographers
 - mosquito Crystal is the crystallographer's choice for automated protein set-ups
 - The NEW mosquito LCP is a dedicated instrument that offers a fully automated solution to lipidic cubic phase screening
- mosquito is the only instrument capable of automating microbatch, seeding or additive screening set-ups in addition to both high-density 96-well hanging drop and sitting drop plate preparation without any set-up changes to the machine
- mosquito is compatible with all standard crystallography plates



Precision

The precise positioning of the pipettes permits:

- The accurate and reliable placement of drops in the centre of plate subwells without them distorting or coinciding
- The accurate placement of drops immediately on top of each other
- Reliable drop placement which enables easy automated imaging of membrane crystals

Repeatability

- The accuracy and reproducibility permit users to create multi-component drops per well, which allows simultaneous assessment of different protein concentrations and volume ratios

Quite simply,

mosquito makes crystallography faster, more cost effective and easier than ever before. ■

Automation Eases LCP Screening

TTP LabTech has extended its range of mosquito automated liquid handlers and now offers mosquito LCP, a dedicated instrument that offers a fully automated solution to lipidic cubic phase (LCP) screening.



This instrument was developed in collaboration with Professor Gebhard Schertler, now at the Paul Scherrer Institute in Switzerland, and Pat Edwards of the Structural Studies group at the MRC s Laboratory of Molecular Biology, Cambridge, UK, and has been designed to facilitate the crystallisation of membrane proteins, such as G-protein coupled receptors.

GPCRs remain one of the most important drug targets in drug discovery although research into them has been somewhat hampered by the problems associated with obtaining accurate structural data. Membrane proteins are known to be much more difficult to purify and crystallise than soluble proteins due to their native environment within the lipid bilayer of the cell membrane. As such, aqueous solutions are unsuitable for their reconstitution as they require lipids or detergents for them to be stable and retain their true structure.

Over 15 years ago Landau and Rosenbusch at the Biocenter in Basel developed a novel method for the crystallisation of membrane proteins that used lipidic cubic phases as the growth environment. Appropriate proportions of a lipidic substance (such as monoolein), water and protein can be used to form a matrix that enables membrane proteins to retain their structural integrity and activity.

The problem of mixing the protein and lipids was solved by Dr Martin Caffrey

¹ Cherezov & Caffrey: A simple and inexpensive nanoliter-volume dispenser for highly viscous materials used in membrane protein crystallization. J Appl. Cryst. (2005) 38: 398-400



This so-called LCP or *in meso* method has revolutionised the process of crystallising membrane proteins. However, there are a number of technical difficulties associated with the lipidic cubic phase method, which make the process difficult to perform and challenging to automate. One problem is the viscous nature of the lipids, which can be almost solid at room temperature. As a result the addition of protein to the lipid and the subsequent reconstitution can be hard to achieve. In addition, the accurate dispensing of the LCP, which is required for miniaturisation, and the precise positioning of drops, which is required for efficient imaging of the membrane crystals presents two other difficulties.

The problem of mixing protein and lipids was solved by Dr Martin Caffrey¹ who devised the two syringe method. This involves two coupled syringes, one containing lipid and the other containing protein. Moving the material backwards and forwards between the syringes results in the protein and lipid being mixed together to form lipidic cubic mesophases. There have been several crystallographers who have sought to develop instruments to automate this process, one of whom is Professor Gebhard Schertler. With the help of the MRC's technical and electronics workshops, Schertler devised an instrument that can scan plates for crystals and view them with a UV filter to distinguish protein crystals from salt. Using this apparatus he integrated a second arm with a micro syringe dispenser that could accurately dispense 50 or 100nL of the cubic phase across a specially developed UV transparent plastic plate. Then, along with his colleague Pat Edwards, Schertler approached TTP LabTech to develop a commercial automated instrument for the LCP drop set-up and buffer addition steps of this process. The result was mosquito LCP, an instrument that offers the full functionality of our renowned mosquito but incorporates technical innovations specific for LCP techniques.

Benefits of the mosquito LCP:

- uses a positive displacement syringe for accurate and repeatable dispensing of the LCP down to 50 nL
- automatically measures and calibrates the position of the syringe needle tip to within +/- 20 µm to facilitate precise drop placement and subsequent automated imaging of membrane protein drops and crystals
- has disposable, positive displacement mosquito tips that guarantee zero cross contamination of solutions and eliminate the need for time consuming wash steps
- minimises evaporation loss of the dispensed LCP
- has high throughput of at least 12 plates an hour
- enables both LCP and traditional crystallisation experiments to be set up in any commercially available plate

In summary, mosquito LCP provides significant benefits over manual processes due to the use of its unique disposable tip technology. For the precipitant additions step, this not only guarantees zero cross-contamination, but eliminates the need for time-consuming tip washing. Subsequently, high throughput rates of more than twelve 96-well plates per hour are easily achieved and due to the column by column dispensing pattern, evaporation of the dispensed LCP is minimised. Our instrument offers high throughput, high precision and unrivalled reproducibility. The additional programmable syringe module can also be used for zero loss dispensing of protein solutions or other expensive/highly viscous additives. The mosquito-LCP is a compact bench-top instrument that will fit anywhere and will be a valuable, versatile addition to any membrane protein crystallisation laboratory.

An Automated Microseeding Protocol for mosquito

Protein X-ray crystallography has undergone major advances during the last couple of decades; however, obtaining crystals of a suitable size and quality for the subsequent data collection can still be a time consuming process of trial and error.

One of the problems encountered is the instability of the proteins over the prolonged time periods that are required for the crystals to grow to an appropriate size for mounting and data collection. This can lead to degradation and ultimately incomplete protein structures.

Seeding is a well established method that is used to improve the quality and reproducibility of crystals. In particular, the microseeding protocol is a good method to induce rapid crystal formation. One of its main limitations however, is the time consuming nature of this technique as it is necessary to optimise conditions where seeding is likely to work. We describe here a protocol for mosquito that results in successful automated microseeding set-ups, thus leads to decreased time and reagent expenditure.

For hanging drop microseeding set-ups, the mosquito deck is loaded with an inverted hanging drop plate seal, a 96-well plate of screen buffers, and reservoirs of protein samples and seed stock. Initially, mosquito aspirates protein from the protein reservoir and

multi-dispenses 100 nl drops in columns across the plate seal (Step 1). Next, mosquito is used to aspirate 10 nL of seed stock from the reservoir and then moves to the first column of the screen plate and multi-aspirates 90 nL of screen solution (Step 2). This combined drop is dispensed accurately on top of the first column of protein drops on the plate seal (Step 3). Steps 1 and 2 are repeated to create a mirror image of the screen plate on the plate seal. Finally, the plate seal is inverted over the screen plate (using a simple alignment jig) so that each droplet of protein, seed stock and screen hangs over the corresponding screen well. The entire procedure takes under 3 mins per 96-well plate and eliminates both protein and seed stock wastage.

In a similar fashion, mosquito can be used to automate sitting-drop microseeding protocols. In essence the procedure is the same as that used for hanging drops, except that the protein drop is placed in the well and the combined seed stock and screen solution are dispensed on top of this drop. Using this method, varying amounts of seed stock can be added to the sitting drop and the resultant crystal growth is monitored.



NEW iQ plate for sitting drop set-ups

To facilitate the sitting drop technique, TTP LabTech has recently developed a new triple drop 96-well plate. The iQ plate has been created specifically for high throughput, low cost, low volume sitting drop protein crystallisation set-ups and is ideal for microseeding protocols. The new plate has a reservoir range of 30 - 80 μ L and, crucially, offers three identical sitting drop locations to facilitate high-density combinatorial experiments. The plate has been constructed from optically clear, low birefringence plastic for UV imaging and has large area flat bottom wells for optimal drop formation and crystal viewing. With SBS standard dimensions it has been designed to fit all common holders in addition to the mosquito plate deck, which makes it ideal for automated crystal screening. ■

Protein Crystallisation at the NKI: Aiming for Low Input and High Output

The Division of Biochemistry at the Netherlands Cancer Institute (NKI) hosts two protein crystallography groups lead by Titia Sixma and Anastassis Perrakis. The main goal of their research is to unravel the crystal structure of cancer-related proteins to gain an insight into the biochemical and biophysical properties of these proteins and provide a template for potential drug discovery research.

In 2004, the first steps into automation of crystallisation at NKI were taken by the acquisition of a mosquito, the second machine of its type that was sold in Europe. Beside the excellent performance of this instrument, the primary reasoning behind the purchase of this machine was the ease of use and low maintenance, two properties that makes mosquito ideal for a multi-user environment. At NKI mosquito was integrated with a Hydra II 96-well dispensing robot to fill up the reservoirs, and a Crystal Farm imaging system to create a complete crystallisation platform.

Patrick Celie, who is the research scientist and operator of the NKI protein facility, explains how the facility and users adapted to mosquito and the benefits that have been observed.

After a short transitional period the members of both crystallography groups had adapted to mosquito and switched from the traditional handmade microlitre drops to the automated nanolitre screening set-ups of mosquito.

The initial protocol we used with the mosquito preparation of 100 + 100 nanolitre droplets in one type of plate was adopted by everybody and is still used by a lot of our crystallographers. In addition many researchers have now designed their own programs including preparation of big droplets, multi droplets in different plates, crystal-seeding protocols and additive screening protocols. Furthermore, aside from the speed with which lab members adapted to the mosquito, we also gained empirical evidence that mosquito was the top ranking dispensing robot in our hands after we performed accuracy and reproducibility tests on several robots during the High-Throughput Macromolecular Crystallisation workshop organised at the NKI in December 2005.

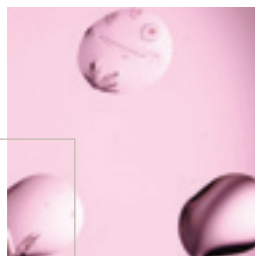
As is often the case in crystallography labs a number of the proteins that we work with at our facility are difficult to express and can only be purified in small amounts. This causes problems with the manual set-ups as too much protein is

required to optimise the crystallisation conditions. However, the nanolitre capabilities of mosquito meant that this ceased to be an issue as the protocol that we now follow only requires 16 μ L of protein per 96-well plate, so we can go ahead and work with proteins that were previously impossible. The success of growing crystals in nanolitre drops, and the fact that synchrotrons no longer require large crystals, has meant that these small crystals can now also be used for data collection. Due to these developments the mosquito has become successful as a crystal producing system, in addition to its screening application.

'Five years after purchasing our first mosquito, we bought one of the next generation instruments; the new improved design means that there is now better X- and Y-mobility of the plate deck, in addition to some more advanced features and technical improvements. With the anticipated delivery of a new crystal imaging system, that will be operational at 4 C, we may now add to our collection with a third mosquito that is specific for cold room work. For the future we expect an even higher throughput for the mosquitos. Due to the improvements in high-throughput protein expression technologies, we aim to increase the number of soluble proteins that can be subjected to crystallisation. In addition, the recently established NKI Protein Facility at the Division of Biochemistry will also offer protein crystallisation as part of its services and will strongly depend on the capabilities of the mosquito and the crystallisation platform. ■

mosquito Pays Dividends at Low Yields

Dr Vander Kooi is an Assistant Professor at the University of Kentucky, where he heads one of three crystallography groups.



“In my lab I work with proteins that are not only difficult to express but also produce very low yields”

DR VANDER KOOI

Dr Vander Kooi has been interested in structural biology since his graduate days when he was working with Dr Walter Chazin in the NMR lab at Vanderbilt University. He subsequently undertook a post-doc with Dr Daniel Leahy at John Hopkins University where he began studying the crystallographic structure of the neuropilin receptor. It was here that he also first used a first generation mosquito.

As a structural biologist, Dr Vander Kooi uses a range of biological tools and methodologies in his research but his preferred technique is protein crystallography. In his lab they primarily focus on the extracellular domains of cell surface receptors, oligomerisation of receptors, and the interactions of receptors with their ligands and co-receptors. The main receptor that Dr Vander Kooi and his group are interested in is the neuropilin receptor, which is involved in angiogenesis. During blood vessel formation, neuropilin binds VEGF and functions as a co-receptor for the VEGF-R receptor tyrosine kinases. Vander Kooi and his group are concerned with understanding neuropilin ligand binding specificity and receptor activation; the ultimate goal is to define and improve mechanisms of inhibiting the neuropilin signalling pathway.

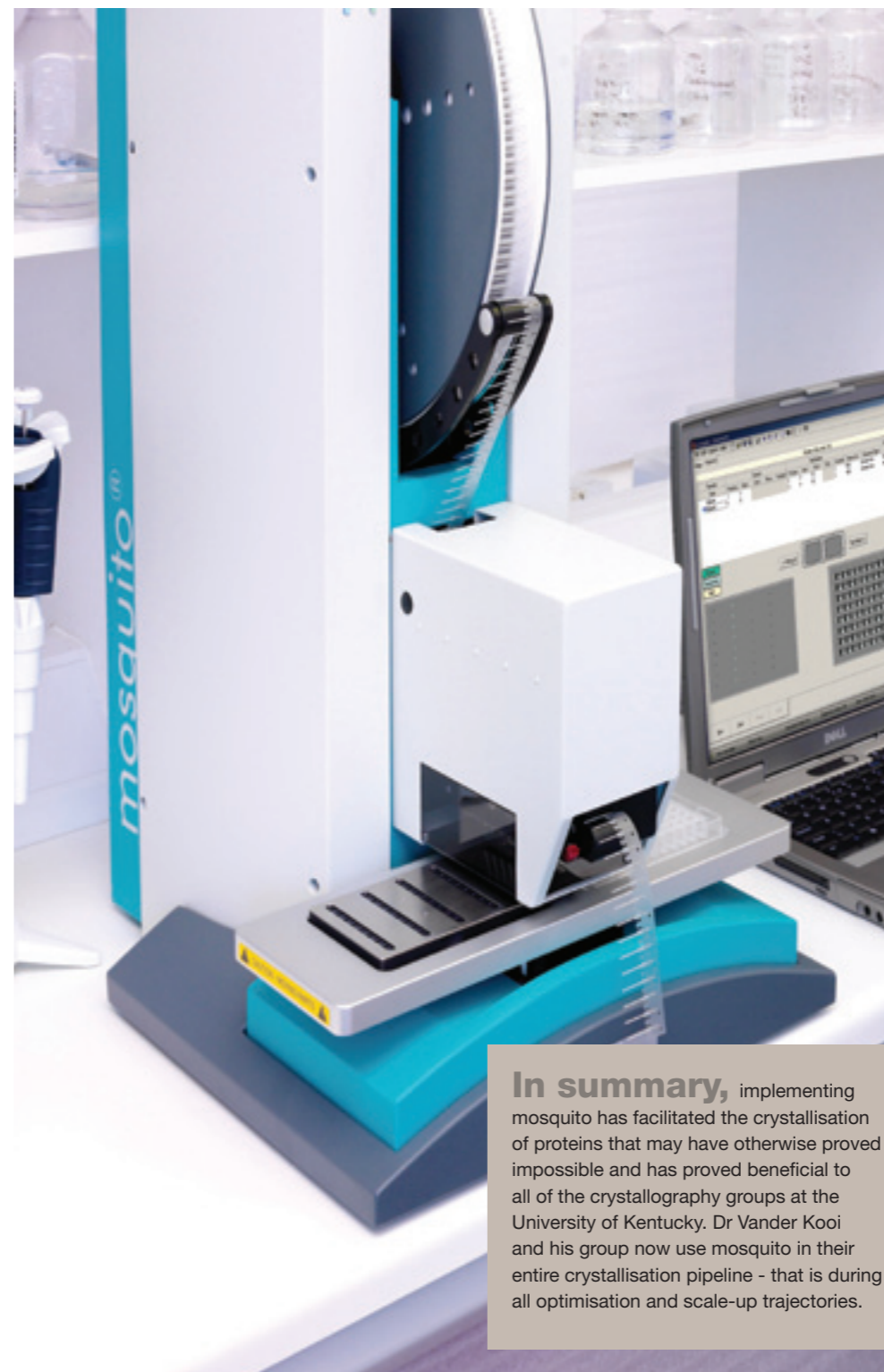
Currently Vander Kooi's group are investigating how the receptor interacts with its ligand and a range of inhibitory peptides. Dr Vander Kooi explains, "We are working with a large number of inhibitory peptides and using *in vitro* assays we have demonstrated that these can functionally bind to neuropilin receptors.

We now want to get structural information on the co-crystallisation of the neuropilin receptor with these inhibitors. Then we intend to correlate the potency of the inhibitory peptides with the structures that are formed. This will allow us to determine the binding pocket of the receptor and identify which residues on the peptides lead to receptor activation.

The neuropilin receptors have large extracellular domains which are cysteine-rich glycoproteins. As a result they are difficult to express in traditional prokaryotic expression systems and it is necessary to use eukaryotic cells instead. While such systems are ideal in that all the necessary post-translational modifications occur, which results in correctly folded proteins, the major limitation, is that the yields are often very low. Vander Kooi and his group have found that they typically only get 0.1 - 1.0 mg of protein from one litre of solution when using eukaryotic expression systems and this can cause logistical problems.

In my lab I work with proteins that are not only difficult to express but also produce very low yields. This makes crystallography using traditional methodologies very difficult and even impossible in some instances. However, the nanolitre capabilities of mosquito have helped us overcome this problem and enabled us to study all of our desired proteins, even those with exceptionally low yields' Vander Kooi commented.

We generally use a hanging drop protocol in our lab. Traditionally we used a one drop, one condition per well protocol, which meant that in a 96 well plate you could only test 96 conditions. If we needed to test different ratios of protein to mother liquor it was necessary to set up more



than one plate and of course this meant that more protein was required. However, since we have installed mosquito we use the three drop, three ratio method for all of our optimisation set ups. In this method, we produce three drops each of 200 nL total volume with a 3:1, 1:1 and 1:3 ratio of protein to mother liquor. This protocol is ideal for our initial screens as it only uses a total of 42 µL of protein solution for the 288 drops in one 96 well plate whilst giving unique hits at different ratios; this allows for much quicker lead to follow-up process. Furthermore, scaling up crystallisation drops is very rapid using the mosquito and, most importantly, highly reproducible. We now routinely transition from the initial 200 nL drops to 1.2 µL drops which is sufficient for growing large single crystals suitable for diffraction analysis.

The mosquito has been essential for me as a young investigator setting up a new structural biology laboratory', Vander Kooi explained. It allows dramatic savings in both resources and personnel. Without the nanolitre capabilities of mosquito we would not have been able to undertake a significant number of projects that we have so this instrument has enabled us to advance our research. We have made incredible savings in our lab due to the reduction of the total amount of reagents required to set up our initial optimisation screens. Furthermore, this instrument has proven to be a huge time saver; experiments and protocols that used to take 2 hours to set up can now be completed in about 2 minutes.

In summary, implementing mosquito has facilitated the crystallisation of proteins that may have otherwise proved impossible and has proved beneficial to all of the crystallography groups at the University of Kentucky. Dr Vander Kooi and his group now use mosquito in their entire crystallisation pipeline - that is during all optimisation and scale-up trajectories.

Moreover, all three crystallography groups at the university use mosquito and the three drop ratio protocol during their initial screening method and six other non-structural biology groups are now performing their own crystallisation trials using the mosquito demonstrating just how valuable and suitable mosquito is to crystallisation departments and multi-user environments.

Sanofi-aventis, Paris eases crystallography bottlenecks

Dr Alexey Rak leads the protein crystallisation and biophysics groups at Sanofi-Aventis, Paris, where they work on a number of protein targets in oncology and the central nervous system. His protein crystallisation lab is primarily concerned with high throughput crystallisation techniques and ways to optimise crystallisation conditions.

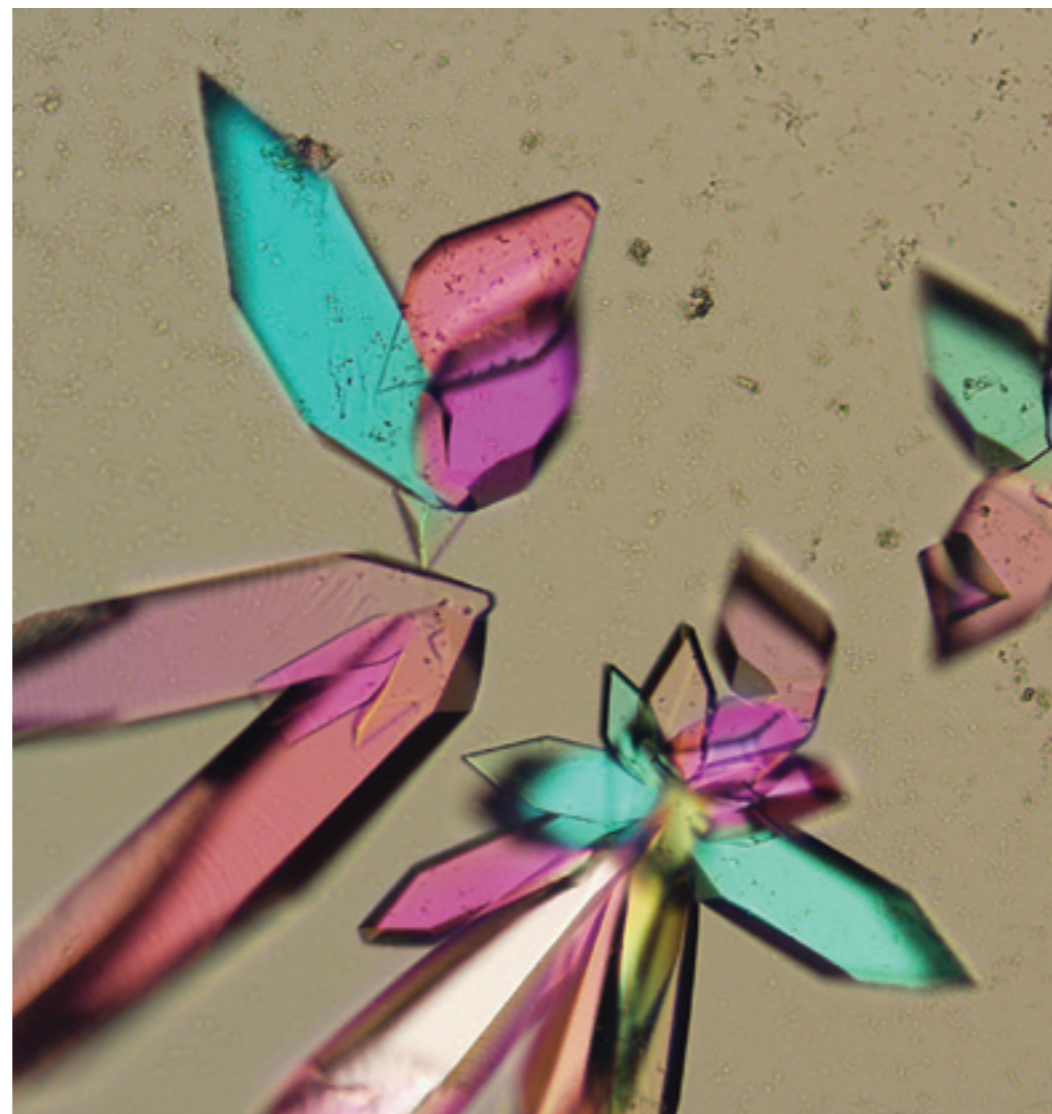


Dr Rak first used mosquito during his crystallography research whilst working at the Max Plank Institute of Molecular Physiology in Dortmund. As one of the first people in Europe to purchase a mosquito he has had over 4 years of experience in working with the instrument. When Dr Rak first joined Sanofi-Aventis the protein crystallography group were using non-contact dispensing robots. However, following his positive experience with mosquito at the Max Plank Institute Dr Rak initiated the purchasing of a mosquito automated liquid handler for his new lab.

In my lab we are focusing on high throughput crystallisation techniques for a number of protein targets. In particular we are interested in finding new crystallisation conditions and solving the structures of soluble and semi-integral membrane proteins, in addition to increasing the overall efficiency of these processes. We found that mosquito was superior in terms of set-up time, flexibility and at eliminating cross contamination compared to the other robots

“ In fact, mosquito has significantly improved the process of crystallisation ”

DR RAK



that we had previously been using commented Dr Rak. ‘In fact, mosquito has significantly improved the process of crystallisation within the group and we are now using the instrument in the majority of our crystallisation protocols.

One of the favoured protocols in Dr Rak’s lab is microseeding, a process where a small aliquot of the seeding stock, a supersaturated protein solution, is transferred to an undersaturated solution in order to form nucleation points. Crucial to the success of this protocol is the requirement for the seed stock

volume to be a minor fraction of the total drop volume. Traditionally, nucleation has been achieved using either a horsehair wand or an acupuncture needle to introduce the small aliquot of the seed stock to the crystallisation droplet, but this is a process that can be very time consuming. One approach to increase the speed and efficiency is through the automation of the seeding process, although this is not a simple task because of the problems crystallisation robots have with dispensing low volumes. The multi-aspirate function of mosquito can overcome this problem.

The relative volume of the seed solution to overall total volume has to be as small as possible to ensure the success of the microseeding technique. While automated liquid handlers struggle with dispensing very low volumes there is rarely a problem with the aspiration of small aliquots’ explains Dr Rak. ‘The protocol that we use here at Sanofi-Aventis typically uses 85 nL protein, 15 nL seed stock and 100 nL reservoir, which yields a droplet where the seed stock is only 7.5% of the total drop volume. Although the volume of our seed stock is very small we have found that mosquito is particularly well-suited to automating our microseeding protocol due to its multi-aspirate mode. This function enables one to perform serial aspirations and then dispense the cumulative volume, thereby overcoming the problem of low volume dispensing.

This has allowed us to perform our microseeding protocol in the following way: first we dispense the protein component of the droplet; we then aspirate the seeds and the reservoir; finally, the resultant volume is dispensed on top of the existing protein drop. This method ensures that the seeds are well preserved by the high concentration of precipitant in the reservoir solution before they come into contact with the protein. Another beauty of this protocol is the virtual elimination of cross contamination - the only potential contamination is of the reservoir with the seed stock but this is negligible and affects only that particular crystallisation plate and none of the stock solutions. Using this protocol has increased the efficiency of our seeding protocols by approximately three fold and we can now set up a 96 well plate in under two minutes.

Alexey Rak and his group have also used the mosquito to find new crystallisation conditions and generate new crystal forms. Furthermore, the group have found that using mosquito in their set-ups has resulted in improving crystal quality in some instances. To gain maximal structural information crystals need to be able to diffract X-rays to a resolution of 3 Å or better. During the screening for new crystallisation conditions it was found that using their mosquito microseeding protocol to reduce the nucleation points, Dr Rak’s group was also able to produce crystals of much better quality. This resulted in enhanced diffraction properties and the generation of more structural information.

“ Dr Rak summed up the benefits of having installed mosquito in the lab, ‘In all aspects of our research, mosquito has proven to be superior to the other crystallisation robots that we were previously using. The most useful attribute of mosquito to our research has been its multi-aspirate mode, which has enabled us to speed up our crystallisation screening set-ups. We have also found that mosquito has helped us optimise screening conditions and actually improve crystal structure.

mosquito is very easy to use and is an extremely reliable machine; it has enabled us to try out a number of different approaches to protein crystallisation and this has resulted in an improvement in both the quality and speed of our work. All in all, mosquito has increased both our efficiency and throughput, which has enabled us to become more cost effective and as a lab we are very happy’ ”



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