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# Lipidico Injection Protocol for Serial Crystallography Measurements at the Australian Synchrotron

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| **Corresponding Author:** | Connie Darmanin<br>La Trobe University<br>Melbourne, VIC AUSTRALIA |
| **Corresponding Author's Institution:** | La Trobe University |
| **Corresponding Author E-Mail:** | C.Darmanin@latrobe.edu.au |
| **Order of Authors:** | Peter Berntsen |
| | Rama Sharma |
| | Mick Kusel |
| | Brian Abbey |
| | Connie Darmanin |
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**TITLE:**

# Lipidico Injection Protocol for Serial Crystallography Measurements at the Australian Synchrotron

**AUTHORS AND AFFILIATIONS:**

Peter Berntsen[1], Rama Sharma[1], Mick Kusel[2], Brian Abbey[1], Connie Darmanin[1*]

[1]Australian Research Council Centre of Excellence in Advanced Molecular Imaging, Department of Chemistry and Physics, La Trobe Institute for Molecular Science, La Trobe University, Melbourne, 3086, Australia

[2]Kusel Design, Melbourne, Victoria, Australia

Email addresses of co-authors:

Peter Berntsen (p.berntsen@latrobe.edu.au)
Mick Kusel (mick@kuseldesign.com.au)
Brian Abbey (b.abbey@latrobe.edu.au)
Rama Sharma (rsharma1@swinburne.edu.au)

Corresponding author:

Connie Darmanin (c.darmanin@latrobe.edu.au)

**KEYWORDS:**

serial crystallography, high viscous injector, lipidic cubic phase, small crystals, X-ray crystallography, synchrotron

**SUMMARY:**

The goal of this protocol is to demonstrate how to prepare serial crystallography samples for data collection on a high viscous injector, Lipidico, recently commissioned at the Australian synchrotron.

**ABSTRACT:**

A facility for performing serial crystallography measurements has been developed at the Australian synchrotron. This facility incorporates a purpose built high viscous injector, Lipidico, as part of the macromolecular crystallography (MX2) beamline to measure large numbers of small crystals at room temperature. The goal of this technique is to enable crystals to be grown/transferred on glass syringes to be used directly in the injector for serial crystallography data collection. The advantages of this injector include a very rapid response to changes in the flow rate without interruption of the stream. Several limitations for this high viscosity injector (HVI) exist. There is a restriction on the allowed sample viscosities to >10 Pa.s. Stream stability can also potentially be an issue depending on the specific properties of the sample. A detailed protocol for how to set up samples and operate the injector for serial crystallography measurements at the Australian synchrotron is presented here. The method demonstrates preparation of the sample, including the transfer of lysozyme crystals into a high viscous media (silicone grease), and the operation of the injector for data collection at MX2.

**INTRODUCTION:**

Serial crystallography (SX) is a technique that was developed initially in the context of X-ray Free Electron Lasers (XFELs)[1-4]. Though fixed target approaches can be used for SX[5-7], typically, injector systems are employed to deliver crystals in a continuous stream to the X-ray beam. Because it combines data from a large number of crystals, SX avoids the need for any crystal alignment during the experiment, and enables data to be collected at room temperature[8,9]. With the aid of a suitable injector, the crystals are streamed one-by-one into the X-ray interaction area and the resulting diffraction data is collected on an area detector[9,10]. To date SX has been successful in solving a number of protein structures[1,11-13] including crystals too small to measure using conventional crystallography. It has also provided new insights into time-resolved molecular dynamics by exploiting the femtosecond pulse duration of the XFEL. By initiating pump-probe reactions with optical laser sources, in-depth studies have been carried out on photosystem II[14,15], photoactive yellow protein[16,17], cytochrome C oxidase[18], as well as bacteriorhodopsin[19-21]. These studies have probed the electron transfer dynamics that occur following light activation demonstrating the significant potential of serial crystallography for understanding time-resolved biological processes.

Development of serial crystallography is also becoming increasingly prevalent at synchrotron sources[9,12,20,22-24]. Synchrotron based SX allows for large numbers of individual crystals to be measured efficiently at room temperature using an appropriate injector system. This approach is suitable for smaller crystals hence, in addition to requiring a fast frame-rate detector to collect the data, a micro-focused beam is also required. Compared to conventional crystallography, SX does not involve the mounting and alignment of individual crystals in the X-ray beam. Because data from large number of individual crystals is merged, the radiation dose received by each crystal can be substantially reduced compared to conventional crystallography. Synchrotron SX can also be applied to the study of time-resolved reactions, even down to the millisecond regime, provided a detector with a sufficiently high frame rate is available (e.g., 100 Hz or more). Several serial crystallography experiments have been carried out at the synchrotron using injectors that were initially developed at XFEL sources[20,22,23]. The two most common types of injector are the Gas Dynamic Virtual Nozzle (GDVN)[25] and High Viscous Injector (HVI)[9,24,26-28]. The GDVN is ideal for injecting low viscosity, liquid samples, but requires high flow rates to achieve stable streams, which in turn leads to high sample consumption rates. By contrast, HVI's are suitable for high viscosity samples which allows generation of a stable stream at much lower flow rates, leading to much lower sample consumption. The HVI injector, therefore, favors delivery of samples where a viscous carrier is preferable (e.g., lipid-based for membrane proteins) and/or large quantities of sample are not available. SX injectors are generally challenging to use and require extensive training to operate. They also involve lengthy sample transfer protocols, as the sample needs to be loaded into a specialized reservoir, this generally has a high risk associated with it of sample being lost either in the 'dead volume' or via leakages in the connections. Therefore, it is desirable to optimize the injector design to mitigate any losses prior to the sample reaching the X-ray beam.

Recently, the first SX results were published using Lipidico[23], with a lysozyme target, and using an Eiger 16M detector. This injector design limits sample wastage by minimizing the number of

steps involved in going from initial crystallization to the transfer of crystals into the injector followed by the delivery of sample to the X-ray beam. This manuscript describes and demonstrates the sample transfer procedure starting from sample preparation, moving on to the injection process, and finally data collection, using the same crystallization vessel. The operation of the injector is also described.

**PROTOCOL:**

**1. Preparation of crystals in a high viscous media using glass syringes**

1.1. Centrifuge the crystal solution gently (~1,000 *x g*, ~10 min at 22 °C) to form a soft crystal pellet and remove the excess buffer. This will result in a high concentration of crystals in the pellet which can be used for data collection.

NOTE: To prevent dilution of the viscous media increase the crystal concentration at this step. Optimize the ratio of viscous media to crystal volume for each sample to obtain a high concentration of crystals whilst maintaining a high viscosity for the media. Centrifuge the crystal solution to form a pellet and remove excess buffer to increase the crystal concentration in the solution, as described in Darmanin et. al.[29].

1.2. Set up two 100 µL syringes with a coupler.

1.3. Fasten the coupler to the end of the first syringe and add 28 µL of crystal solution to the top of the syringe. Slowly, insert the plunger into the top of the syringe and gently push the solution down to the end of the coupler tip, removing any air bubbles that form. Do this by following one of the two methods discussed below.

NOTE: The volume of crystals to be added to the high viscous media can be varied if it does not significantly change the viscosity of the overall sample.

1.3.1. First method: Use rapid pressure changes to burst the air bubbles.

1.3.1.1. Carefully place one gloved finger on the top of the blunted coupler needle point and (very gently) apply pressure to create a seal. Do not apply too much pressure as this may result in a needle stick injury.

1.3.1.2. Now pull the plunger back to draw the solution away from the needle point, this will generate a buildup of pressure in the syringe.

1.3.1.3. Quickly release the pressure at the top of the syringe by removing the gloved finger from the blunted needle tip. Be careful, if the sample is too close to the tip when the finger is removed it may spray out resulting in loss of sample. The rapid change in pressure within the syringe will burst the air bubbles. Repeat until all bubbles have been removed.

1.3.2. Second method: Using the 'human centrifuge' to remove air bubbles.

1.3.2.1. Place the syringe in one hand with the needle facing upwards and the plunger wedged between two fingers, so that it cannot move.

1.3.2.2. Quickly rotate the arm holding the syringe in one direction 2–3 times, the resulting centrifugal force on the sample will force any air bubbles out of the syringe. Be careful, if done too slowly sample can be lost.

1.3.2.3. Inspect the syringe for air bubbles, if bubbles remain, repeat steps 1.3.2.1 – 1.3.2.2 until all the air bubbles are removed.

1.4. Using a fine spatula, add ~42 µL of high vacuum silicone grease directly to the top of the second syringe. Push the plunger all the way down to the end, removing all the air bubbles and ensuring that there is no air gap in the end of the syringe.

NOTE: The precise composition of the silicone grease is not critical as it is acting as an inert carrier. A viscosity value of >10 Pa.s or ratio of approximately 60:40 silicone grease to crystal solution is optimal for injection, however, it is possible this may vary depending on the exact characteristics of the sample. Adjust the volume of crystal solution added to the silicone grease to optimize the crystal concentration in the mixture, as outlined in the discussion. High-viscous media other than silicone grease can be used as long as they are compatible with the sample[30-33].

1.5. Ensure there are no air bubbles in either of the two syringes and attach the syringes together using the coupler. Hold the syringes vertically by griping just the end of the syringe as warming up the syringe due to the heat generated from fingers can affect the sample.

1.6. Mix the sample together by gently depressing the plunger on the crystal solution side so that it mixes into the silicone grease and then push the plunger on the silicone grease side, so that it pushes the sample back towards the crystal solution side. Gently, repeat this process 50 to 100 times so that the sample is mixed thoroughly and looks homogeneous.

NOTE: If the crystals are grown directly in highly viscous media (e.g., lipidic cubic phase, LCP), steps 1.1–1.6 are not required. Protocols for growing crystals within the syringes can be found in Liu et.al.[34, 35]. Follow this protocol up to sample consolidation, step 10, where all the sample is now contained in one syringe and the crystallization buffer is removed. The addition of 7.9MAG is not required for this protocol. Instead, remove all excess liquid from the syringe by gently pushing the plunger down in the sample until no more liquid remains in the syringe.

1.7. Visualize the crystals under an optical microscope either through the syringe or, for best results, extract a small amount (~1 µL) on to a glass slide and place a cover slip on the top.

1.8. Check crystal concentration.

1.8.1. Determine the crystal concentration in the silicone grease by counting the number of crystals in a specific area using the optical microscope images. For best results, a high density of crystals uniformly dispensed throughout the media is ideal (>$10^6$ crystals/mL) in order to obtain a high crystal-hit rate.

1.8.2. Adjust the crystal concentration in the syringe by changing the ratio of crystals to viscous media in steps 1.3 and 1.4.

1.8.2.1. To decrease the crystal concentration, dilute the crystals in the syringe by increasing the amount of silicone grease/HV media at step 1.4 or by diluting the crystal pellet in solution with the crystallization buffer prior to setting up the syringes in step 1.1.

1.8.2.2. To increase the crystal concentration, decrease the amount of silicone grease in step 1.4 but be mindful not to reduce the viscosity below 10 Pa.s.

NOTE: The crystal concentration reported here was optimized for this specific sample and crystal size. However, the ideal crystal concentration will depend on the crystal size, beam size, the needle Internal Diameter (I.D.), and the incident X-ray flux. This can be determined from the hit rate with an optimal hit rate of ~30% considered as 'good'. In practice, the crystal concentration must be optimized for different samples during beam time to obtain the desired hit rate. Start with a high concentration of crystals, $10^9$ crystals/mL. The procedure for adjusting the crystal concentration is given in Liu et.al.[35].

1.8.3. The protocol can be paused here until the injector is ready for data collection.

1.8.3.1. If the crystals are grown in LCP, then seal sealed them and let them remain in syringes for up to a few days at room temperature.

1.8.3.2. If the crystals have been transferred to a different high viscous media (e.g., silicone grease), then perform a stability test of the crystals over time should prior to the measurement. This will determine how long the crystals are stable in the inert media (ideally > 8 h) and the time limit for data collection. Here, lysozyme crystals were stable for days in silicone grease and did not show signs of dissolving when inspected using an optical microscope.

1.9. Move all the sample into one syringe prior to disconnecting the coupler. Disconnect the coupler from the syringe and attach the injection needle. Screw the needle firmly into the base of the syringe and ensure that it is fastened tightly to prevent any leakages. A 108 µm I.D. needle (needle length 13 mm, point style 3) was used for this experiment. Depending on the crystal size and the beam size attach either a 51 µm or 108 µm I.D. needle.

NOTE: Pre-testing of the sample stream prior to beam time is required to be able to select the correct injector needle size. Depending on the sample characteristics (i.e., viscosity, charging, buffer composition) the samples will flow differently with different needle I.D. Therefore, it is recommended to test a variety of needle sizes to produce the most stable stream with the

smallest possible I.D. needle to maximize the signal-to-noise ratio during data collection.

1.10. If the injector is already mounted and aligned on the beam line move to section 3 to mount the sample syringe directly onto the injector.

NOTE: The protocol can be paused here.

**2. Injector mounting and control**

2.1. Mount the injector onto the beamline. Remove the cryogenic nozzle on the MX2 beamline and replaced with the injector. Loosen the clamping screw that holds the cryogenic nozzle and attach it to the bracket located next to the motorized stage.

2.2. Lift the injector up from its trolley by holding the black handle and place it on the motorized stage.

2.2.1. To fasten the injector to the motorized stage; hand tighten the black clamping screw knob.

2.3. Mount an empty syringe onto the injector

2.3.1. In the injector computer control interface (e.g., EPOS position control software), select **Homing Mode** under **Tools** in the software menu. Then select **Negative Limit Switch,** and **Start Homing**, let the software run until the screw is retracted sufficiently for the syringe to fit under the screw. If left to run unsupervised the limit switch stops the screw automatically.

2.3.2. Mount an empty ('dummy') syringe onto the injector by inserting the needle through the slit in the syringe holder. Line up the syringe against the bracket.

2.3.3. Fasten the syringe with two O-rings.

2.3.3.1. Wrap the first O-ring across the mid-section of the syringe attaching it to the hooks on either side of the syringe.

2.3.3.2. Loop the second O-ring around the hooks on the upper section of the syringe and then put one part of the O-ring on the top of the glass syringe as shown in the demonstration (**Figure 1B**).

2.4. Align the injector to X-ray beam

2.4.1. Move the motorized stage with the injector towards the X-ray interaction point via the beamline control software. This region of intersection can be visualized using an inline camera. The size and position of the beam is denoted by a red cross on the screen and the motorized stage can be moved to align the needle with the X-ray interaction region.

2.4.2. Adjust the x and y position of the stage to align the needle with the red cross.

2.4.3. Adjust the z position until the needle tip comes into focus.

2.4.4. Verify alignment of the needle tip and X-ray beam visually by checking that the syringe tip meets the crosshairs which are visible in the optical microscope image generated by the beamline camera.

2.4.5. Once the needle tip is aligned move the tip above the cross hairs ~100 µm. This prevents x-ray scatter from the needle tip.

NOTE: Mounting and aligning the injector on the MX2 beamline at the synchrotron must be carried out by the beamline scientists and can be completed in less than 30 min.

**3. Mounting the sample syringe**

3.1. Replace the empty syringe with the sample syringe by following step 2.3.

3.2. Place the injector cap on the sample syringe plunger head.

3.3. Move the drive screw to the top of the cap.

3.4. In the injector control software select **Velocity Mode**.

3.5. Input 3000 rpm (~115 nL/s) as the **Setting Value** and press **Apply Setting Value**.

3.6. When the drive screw touches the cap on the syringe plunger head, stop the motor.

3.6.1. Set the rpm value to zero in the injector control software by changing the **Setting Value** to '0' as the screw approaches the cap.

3.6.2. Once contact is made, activate the preset value by pressing **Apply Setting Value** to stop the screw instantly.

3.7. Gently wiggle the cap to make sure that it is firmly held in place.

NOTE: Whenever a new set value is entered in the **Setting Value** box in the control software it is only activated after pressing **Apply Setting Value**.

**4. Running the injector**

4.1. After the cap drive screw has made firm contact with the top of the plunger change the rpm **Setting Value** to 100. This is equivalent to ~ 4 nL/s.

4.2. Visually inspect the needle tip when the sample first comes out or look at the beamline camera image of the needle to observe when the sample starts to extrude from the needle tip.

4.3. Perform a search of the hutch, close the hutch door, and turn on the X-ray beam. From this point the injector must be operated remotely from outside of the hutch.

4.4. Tune the rpm until a stable stream is generated.

4.4.1. Decrease the rpm **Setting Value** to 90.

4.4.2. Repeat step 4.4.1, decreasing the rpm **Setting Value** in increments of 10, to slow down the stream but still maintain a stable stream. For silicone grease a value of 30 rpm was used.

NOTE: The typical rpm range varies between 30 – 100 rpm (1 – 4 nL/s) depending on the sample characteristics and X-ray exposure time. In general, the rpm should be tuned to produce the slowest flow rate (to minimize sample consumption) whilst maintaining a stable stream.

4.5. Managing a sample stream that does not flow well.

4.5.1. Keep increasing the rpm **Setting Value** in increments of 10 until the stream gets straighter. If the stream does not stabilize, even after increasing the rpm **Setting Value** to the maximum value of 100 rpm try to improve the stability by implementing one of the two methods described below.

4.5.1.1. First method: Insert a polystyrene sample catcher underneath the sample stream to help guide the sample. This method works well for highly charged sample streams.

4.5.1.2. Second method: Insert a venturi suction funnel to the sample catcher. To supply air to the funnel, connect it to the air outlet tube located in the hutch. This method can aid in directing and stabilizing the sample flow independent of the charge of the stream.

4.6. Once a constant and steady stream is achieved, start data collection, and optimize the detector distance as per a normal X-ray crystallography experiment.

NOTE: The rpm is converted to flow rate using the supplied conversion table in the Supplementary Information, 'Lipidico Device Calculator'. Enter the rpm value, needle diameters, and syringe volumes to calculate the flow rate using this calculator.

**REPRESENTATIVE RESULTS:**

Lipidico is an HVI built as an alternative delivery system for use on MX2 (**Figure 1**). It is ideally suited for SX where crystals are either grown in lipidic cubic phase or transferred to a high viscous inert media.

To demonstrate the injector application silicone grease mixed with lysozyme crystals was used

to collect SX data at the MX2 beamline at the Australian synchrotron. To mount the injector on the MX2 beamline the cryogenic nozzle is removed and replaced by the injector as shown in **Figure 1**. The syringe samples are mounted directly on the injector and fastened using O-rings (**Figure 1B**). Data collection can be initiated within minutes after a sample change. The injector is designed for the rapid sample control with a simple setup that is easy to operate for users who are unfamiliar with SX.

[place **Figure 1** here]

The injector is compatible with various types of highly viscous carriers. The optimal sample running speed for the injector is crucial for the stream stability, too slow will cause a curling effect/stream expansion and too fast will result in the stream breakup. The optimal speed will vary depending on the carrier system used. **Figure 2** and **Figure 3** show silicone grease tested at different injector speeds and demonstrates how the stream behavior can vary. A slow flow rate (**Figure 2A**, 2.3 nL/s) results in expansion of the silicone grease extruding from the injector while a faster flow rate (**Figure 2C**, 6.6 nL/s) produces a thinner stream. Curling of the viscous media stream has regularly been observed (**Figure 3A**). To overcome this issue two innovative solutions were tested: a polystyrene catcher and a venturi suction funnel below the needle. The polystyrene catcher introduces a weak electrostatic force and worked best on highly charged samples, as in the case of silicone grease (**Figure 3B**), while the venturi suction funnel aids in guiding the stream vertically downwards to the catcher, independent of sample charging. Depending on the characteristics of the sample either option can be used successfully.

[place **Figure 2 and 3** here]

A glass syringe containing 26 µL of lysozyme crystals (crystal size 10 µm x 10 µm x 20 µm) suspended in silicone grease was used (**Figure 4A**). Lipidico generated a constant sample stream, using the 108 µm needle I.D., with an average flow rate of 1.14 nL/s (with the motor set to 30 rpm). A peak finding algorithm[36] was used to assess the hit rate and a sufficient amount of data (total of 224,200 images) was collected within 38 min of data collection time, enabling structure retrieval (PDB code 6MQV). **Table 1** provides a summary of the data collection statistics and **Figure 4B** shows a representative image of the electron density map surrounding one of the di-sulfide bonds in the lysozyme structure[20].

[Place **Figure 4** here]

[Place **Table 1** here]

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Images of Lipidico, a viscous injector used at the Australian synchrotron for SX experiments.** (**A**) Shows a photo of Lipidico incorporated into MX2. The red arrow indicates the direction of the X-ray beam. (**B**) A closer view of the sample holder region on Lipidico. The syringe is held in place by two O-rings and the sample is collected in the catcher. (**D**) A close-up view of

the sample stream showing the injector needle and the sample waste catcher which can be altered to incorporate a polystyrene/venturi suction catcher seen under the needle in (**A**). The figure has been adapted from Berntsen et. al. 2019[23] with the permission of AIP Publishing.

**Figure 2: The effect of a high viscous carrier stream using different injector speeds**. 100% silicone grease was tested at different sample velocities to assess its flow characteristics. (**A**)**.** 2.3 nL/s (30 rpm), (**B**) 3.5 nl/s (90 rpm) and (**C**) 6.6 n/s (170 rpm).

**Figure 3: Stream behavior of 100% silicone grease demonstrating curling of the viscous media.** (**A**) Curling effect at 2.3 nl/s (30 rpm) (**B**) The effect of adding the polystyrene catcher to the sample waste holder with the injector speed operating at 2.3 nL/s (30 rpm).

**Figure 4: Lysozyme images.** (**A**) Optical images of the lysozyme crystals in silicone grease. A cross polarized (40x magnification) image of lysozyme crystals mixed with a high viscous media, silicone grease, imaged using a visible light microscope. The size of the crystals range between 15 and 20 µm and (**B**) A representative image of the electron density map (2Fo-Fc, 1σ) surrounding the disulfide bonds of lysozyme. The figure has been adapted from Berntsen et. al. 2019[23] with the permission of AIP Publishing.

**Table 1: Summary table showing SX data statistics from Lipidico**. Lysozyme crystals were mixed with a high viscous media, silicone grease, and streamed through the X-ray interaction region of the MX2 beamline. This is a summary of the complete table of results published in Berntsen et. al. [23] . The table is adapted here with the permission of AIP Publishing.

**DISCUSSION:**

An alternative HVI has been developed, ideal for carrying out SX experiments at synchrotron sources. It has two key advantages over existing HVIs. First, it is easy to install on the beamline allowing rapid switching between conventional crystallography and SX, just ~30 minutes is required for instalment and alignment on MX2. Second, the sample syringes used to grow crystals can be directly used as the reservoirs for injection, limiting wastage during sample transfer. The protocol for changing samples has been described and demonstrated. The design eliminates the need for a complicated gas stream to control the jet compared to other HVIs. A simple process for changing the flow rate of the injector was demonstrated which can be adjusted without a delayed response.

The most crucial steps for successful SX data collection are optimizing the crystal concentration, obtaining a homogeneous mixture of high viscous media, and producing a stable stream for data collection. The optimal crystal concentration and homogeneity can be achieved by centrifuging the crystals down to a pellet and adding a higher concentration of crystals to the carrier media, ensuring that the sample is thoroughly mixed in the coupler system. Once the crystal concentration is optimized, the injector flow rate can easily be adjusted during beamtime to obtain a stable stream. Curling of the viscous stream is commonly observed with high viscous samples. Two solutions are presented: use of a polystyrene sample catcher for charged samples or the addition of a venturi suction funnel acting as a catcher. This has been successful in

controlling the stream in the initial tests, but under different sample conditions curling of the stream may result in sticking of the sample to the needle point. It is possible that this could be overcome by changing the surface charge properties of the needle by adding special coatings. Needles coated with different chemicals (i.e. silicone) have previously been successfully adapted for use with liquid handling robots and can be custom ordered from manufactures to suit the syringe[37].

The injector is not limited to silicone grease and can be used with other highly viscous liquids. Several different alternatives have been demonstrated[23] which can be successfully used for crystal injection and are described in a number of other publications[22,30,33]. The upper limit on sample viscosity using this HVI is still under investigation, however sample viscosities of up to 25 Pa.s (using 100 % silicone grease) have resulted in a stable stream. However, when the sample viscosity was reduced to <10 Pa.s (using 70 % silicone grease sample) a reliable stream could not be produced. Hence, 10 Pa.s represents the current lower limit on the sample viscosity for injection using this HVI. The needle I.D. is also an important consideration. Although 51 µm I.D. was successfully tested whilst injecting various carriers without any crystals, the introduction of crystals disrupts the sample flow. Hence, with crystals introduced into the matrix, there is a much higher likelihood of blockage when using the 51 µm I.D. needle compared to the larger needle size. When a blockage occurs a critical buildup of pressure can occur, in other injectors this could result in syringe breakage. However, the design of this HVI includes a safety mechanism where the drive mechanics will pull away from the syringe plunger if the pressure becomes too high until a deactivation switch is enabled. This results in the drive being stopped and prevents the glass syringe from rupturing.

Several limitations for this injector exist. The injector is specifically designed for highly viscous samples, therefore, liquid like samples cannot be used directly for sample delivery. To overcome this limitation, crystals grown in buffer systems can be mixed with a suitable inert media as described in step 1, however, there is a possibility the crystal may become unstable. Therefore, screening of a variety of inert media[26-29] should be investigated first to ensure sample stability is maintained. Secondly, the size of the crystal and quality of crystal packing will affect the diffraction quality. The MX2 beamline operates at an optimal beam size of 22 x 12 µm (H x W) and flux ~$10^{12}$ photon/s. The optimal crystal size to carry out SX at MX2 is ~10 µm and has been shown to yield a high signal-to-noise ratio. However, it is possible to collect data on smaller sized crystals if they are well ordered and diffract to high-resolution. There is an option to slit down the beam size at this beam line to ~7.5 µm, however, this comes at a cost since it reduces the incident flux which needs to be considered during the experiment.

The development of this injector makes SX easily accessible to mainstream crystallographers. The successful operation of Lipidico, opens the door to a fast and easy method for SX data collection at a wide variety of synchrotron sources. It enables room temperature data collection on crystals which are 10 µm or less at MX2, limiting radiation damage effects for individual crystals. It also provides a new opportunity in Australia to carry out millisecond time resolved SX which is the current state-of-the-art for crystallographers. Future applications of this injector system extend to X-ray characterization of highly viscous materials using Small Angle X-ray Scattering (SAXS)

allowing the injector to be readily adapted to other beamlines at the Australian Synchrotron.

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**DISCLOSURES:**

MK works for Kusel designs. Kusel Design, a custom laboratory device developer, was engaged by Dr Peter Bentsen of La Trobe University and Dr Tom Caradoc-Davies of ANSTO and to develop a low-cost device to enable high viscous studies in the MX2 beam line at the Australian Synchrotron. The device was developed in close consultation with Dr. Caradoc-Davies. The authors have no competing financial interests.

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Figure 1

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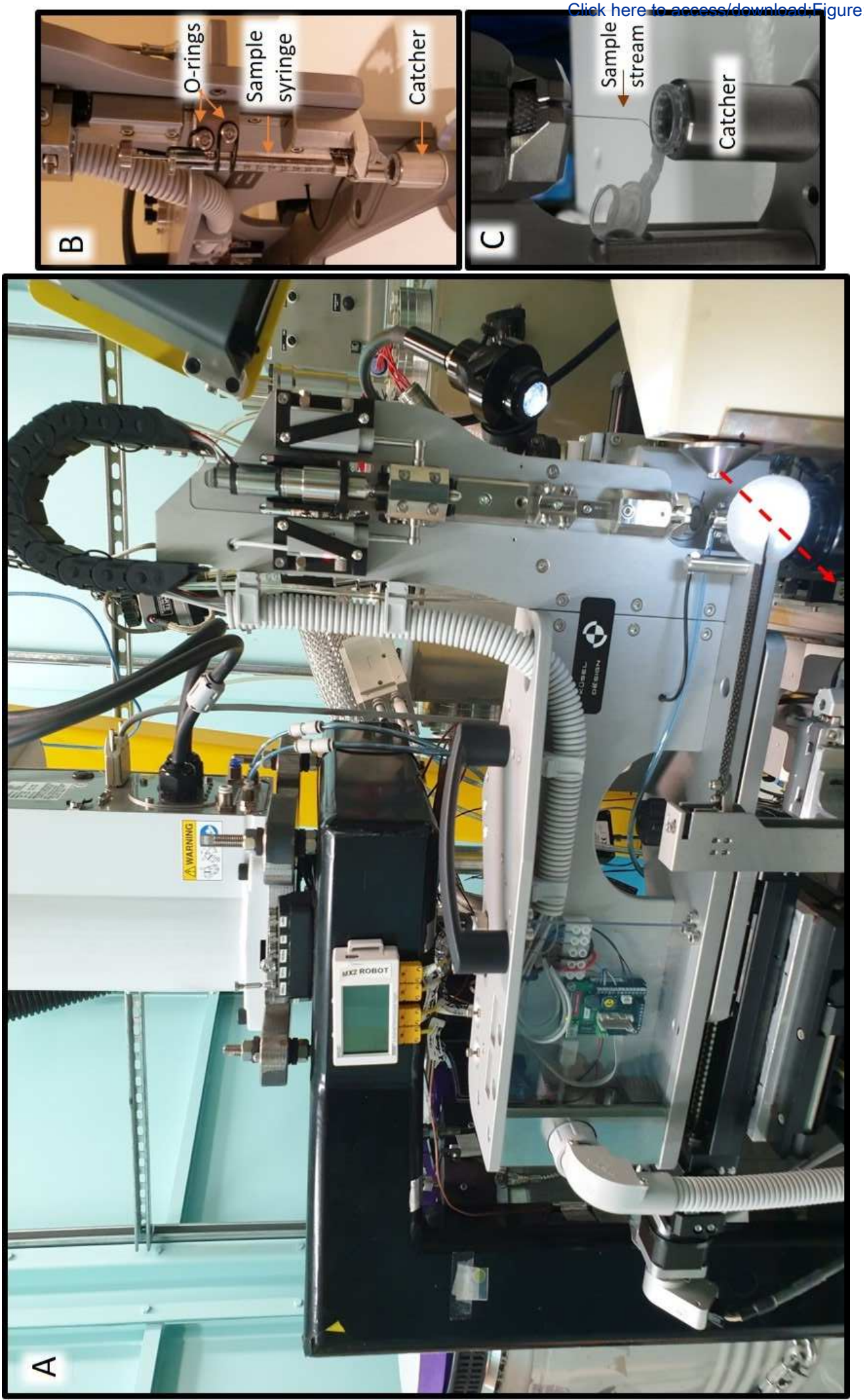

Figure 2



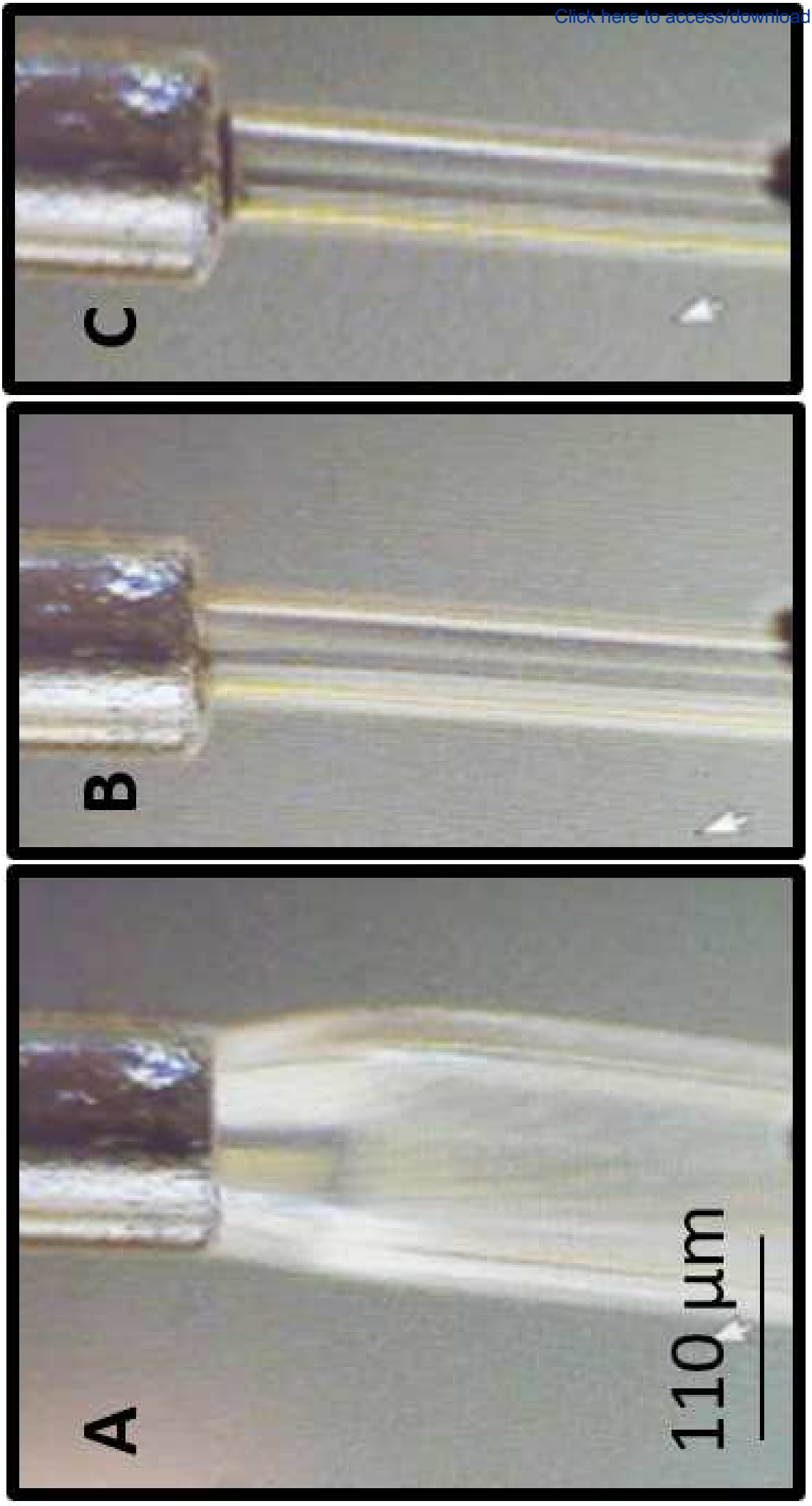

Figure 3

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Figure 4



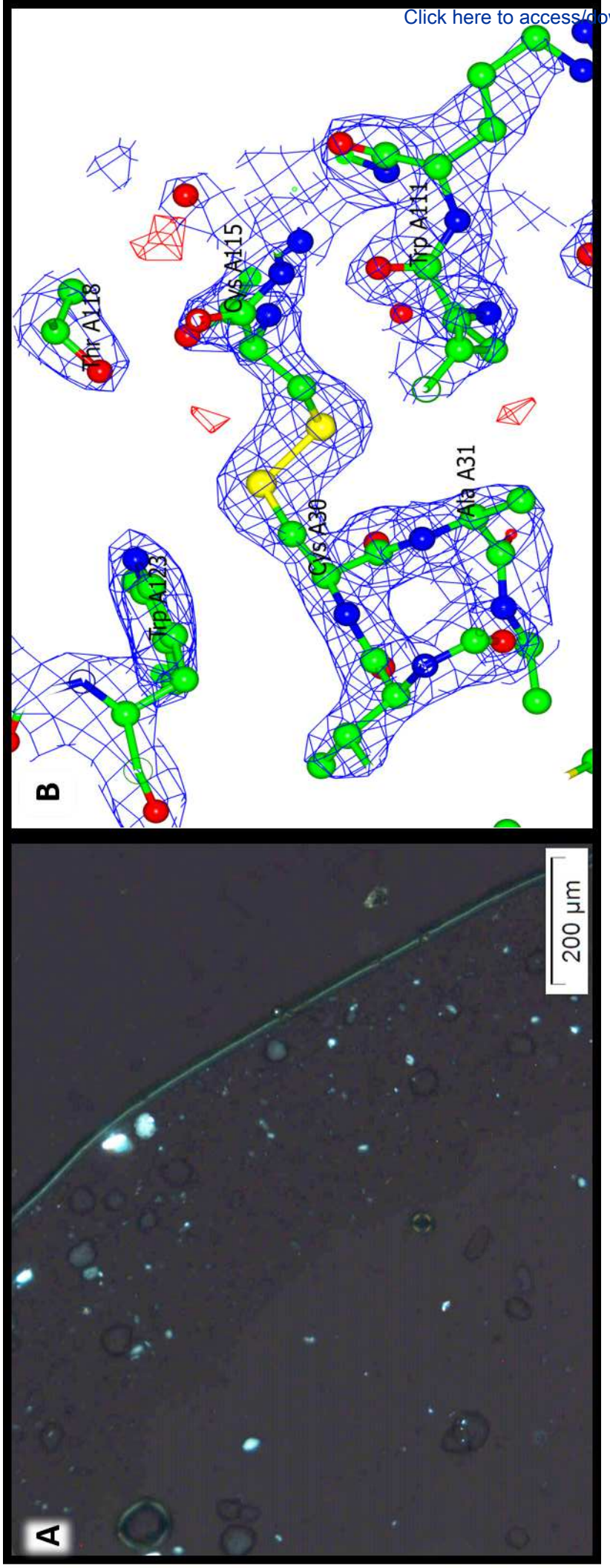


Table 1



| Data statistics | Lipidico, MX2 beamline |
|---|---|
| **Detector, frame rate** | Dectris EIGER X 16M, 100Hz |
| **Sample-detector distance (mm)** | 300 |
| **X-ray energy (KeV)** | 13 |
| **Beam size (WxH) (μm)** | 12x22 |
| **No. of frames** | 224200 |
| **Hit rate** | 2.95 |
| **No. of indexed frames** | 4852 |
| **Resolution (Å)** | 17.74-1.83 |
| **Completeness** | 99.44 |
| **Space group** | $P4_32_12$ |
| **Unit cell** | |
| **a, b, c (Å)** | 78.68, 78.68, 34.48 |
| **α, β, γ (°)** | 90, 90, 90 |
| **I/σ(I)** | 5.08 |
| **Redundancy** | 73.97 |
| **$CC_{1/2}$** | 0.96 |
| **$R_{work}/R_{free}$ (%)** | 18.7/26.3 |

| Name of Material/Equipment | Company | Catalog Number |
|---|---|---|
| Hen eggwhite lysozyme | Sigma-Aldrich | L6876 |
| | | |
| High vacuum silicon grease | Dow Corning | Z273554-1EA |
| Injector needle (108 µm ID) | Hamilton | part No: 7803-05 |
| Glass gas-tight syringes, 100 µl | Hamilton | part no: 7656-01 |
| | | |
| LCP syringe coupler | Formulatrix | 209526 |
| Lipidico injector | La Trobe Univerity/ANSTO | |

**Comments/Description**

Used to grow crystals for testing the injector and the crystals are transferred into silicon grease. https://www.sigmaaldrich.com/

Used for testing of injector. https://www.sigmaaldrich.com/ www.hamiltoncompany.com

Syringes used for sample injection. www.hamiltoncompany.com

Syringe coupler to mix the samples

This is a specific piece of equipment that can be accessed through La Trobe University / ANSTO Australian Synchrotron Facility

Dear Editor,

Thank you for taking the time to view our manuscript. We have addressed all your comments below and have included the changes in the word document. Track changes can be used to see the edits.

Yours Sincerely,

Connie Darmanin

Editorial comments:

*Comment 1: Even if this is not a commercial, I have reduced the usage of the term. This is because our policy states that the video narrative is objective and not biased towards a particular product featured in the video. Please check.*

Response: We have checked the manuscript and have no objections.

*Comment 2: line 73 Please expand HVI*

Response: This has been incorporated.

*Comment 3: The protocol needs to be action steps in imperative tense. Please ensure all steps are written in imperative tense as if describing someone how to perform the action with all specific details. Please remove the redundancy and make the steps crisp. Please ensure that each step contains no more than 2-3 actions. We cannot have paragraphs of text in the protocol section.*
*In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.*

Response: We have corrected this through out all steps and have tracked out changes. We have also revised the notes so they are more concise.

*Comment 4: line 107 We cannot have two notes following one step. Only one note can follow one step. So moved here.*

Response: We have corrected this and moved the note to the correct step it should be linked to.

*Comment 5: line 145 For how long at what temp?*

Response: This line has been incorrectly edited and we have clarified this in step 1.1.

*Comment 6: What is human centrifuge? Please bring out clarity.*

Response: We have clarified this in step 1.3.2 line 143.

Comment 7: We cannot have two notes following one step. Only one note can follow one step. So moved here.

Response: We have fixed this so that it now make sense and the only one concise note exists.

*Comment 8: How small?*

Response: This is an incorrect addition by the editor. We have fixed this and added sufficient details to explain the steps preceding this.

*Comment 9: line 192 Please check the new step number.*

Response: We have checked all the steps and edited accordingly.

*Comment 10. Line 224, We cannot have two notes present under one step. Also we cannot have paragraph of texts in the protocol section. Protocol step should mostly be numbered action step in order with all specific details associated with it in imperative tense.*
*Some of the details can be converted to protocol steps and other details can be moved to the discussion.*

Response: We have fixed this so only one note is present and included it in the protocol steps.

*Comment 11: line 239 We cannot have two notes present under one step. Also we cannot have paragraph of texts in the protocol section. Protocol step should mostly be numbered action step in order with all specific details associated with it in imperative tense.*
*Some of the details can be converted to protocol steps and other details can be moved to the discussion.*

Response: We have fixed this and converted the information to steps.

*Comment 12: line 298, We cannot have two notes present under one step. Also we cannot*

*have paragraph of texts in the protocol section. Protocol step should mostly be numbered action step in order with all specific details associated with it in imperative tense.*

*Some of the details can be converted to protocol steps and other details can be moved to the discussion.*

Response: We have fixed this and converted the information to steps.

*Comment 13: line 271 Internal diameter? Please expand all abbreviations during the first time use. What was used in your lysozyme experiment?*

Response: This information has been added.

*Comment 14: line 292 This is not a step, please convert int a note.*
*Please include how mounting and aligning is performed. Please include all the button clicks, knob turns, command lines, etc. if any. e.g., Click on open to* ***open*** *the software, then click* ***calibrate****. Check all the boxes which appear red.*

Response: We have deleted this expanded this section to include the injector mounting at the beam line.

*Comment 15: line 343, How do you visually check this?*

Response: This is visually done using the online cameras. We have incorporated this into the manuscript in step 2.3.

Comment 16 : line 366, How? Manually?

Response: We have expanded step 3 to include these details.

*Comment 17: line 428, Please include how each step is performed.*

Response: All steps have been included in step 4.

*Comment 18: line 464, Please use imperative tense.*

Response: We have updated the manuscript throughout with imperative tense.

*Comment 19: line 506, Please include the lysozyme results as well to show how this was used.*

Response: We have included an image of the lysozyme structure showing the electron density in Figure 4B which was solved using this injector. This information was also updated in the text line 542 and in the Figure legend, line 570.

*Comment 20: line 531, Which panel?*

Response: We updated this to Figure 3B

*Comment 21: line 578, Please do not embed the table here, please upload as a separate file in .xlsx format.*

Response: This table was removed and uploaded separately in .xlsx format.

*Comment 22: line 584, As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:*

*a) Critical steps within the protocol*
*b) Any modifications and troubleshooting of the technique*
*c) Any limitations of the technique*
*d) The significance with respect to existing methods*
*e) Any future applications of the technique*

Response: We have updated the discussion to include an extra paragraph to address the limitations (line 628) and the last paragraph (line 643).

*Comment 23: line 659, Added here. Please disclose his position. Also please include if there were anything provided by Kusel designs for the experimental purpose.*

Response: We have added more information here to disclose Mick Kusel involvement.

*LIPIDICO DEVICE CALCULATOR: lipid speed, volume flow rate, motor speed*

| | | |
|---|---|---|
| Hamilton syringe bore size | 1.46 | mm |
| device/syringe dead volume | 4.5 | µL |
| needle bore diameter | 110 | µm |
| needle outside diameter | 210 | µm |
| lipid volume in syringe | 26 | µL |
| lipid stream diameter | 110 | µm |

reference figures

critical input figures

resultant figures

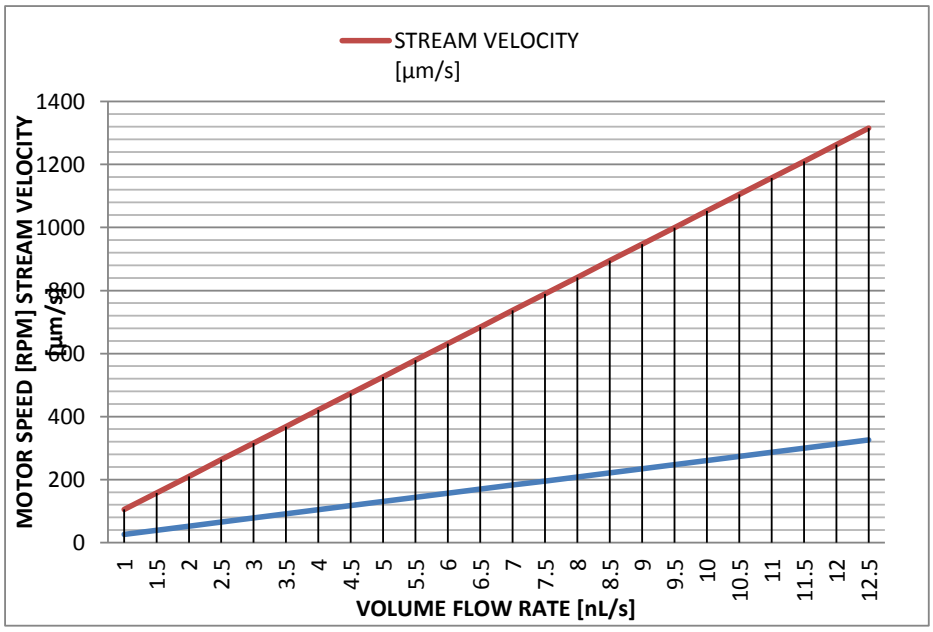
| | | |
|---|---|---|
| volume flow rate target | 8.0 | nL/s |
| required motor speed | 209 | rpm |
| lipid stream velocity | 842 | µm/s |
| lipid extrusion duration | 0 | hours |
| | 45 | minutes |

| | | |
|---|---|---|
| lipid stream velocity target | 5000 | µm/s |
| required motor speed | 1239 | rpm |
| lipid volume flow rate | 47.5 | nL/s |
| lipid extrusion duration | 0 | hours |
| | 8 | minutes |

| | | |
|---|---|---|
| motor speed input | 1000 | rpm |
| lipid volume flow rate | 38.4 | nL/s |
| lipid stream velocity | 4036 | µm/s |
| lipid extrusion duration | 0 | hours |
| | 9 | minutes |

**Custom Needles**

| Gauge | O.D. X I.D. (mm) | Point Style | Small RN |
|---|---|---|---|
| 34 | 0.16 x 0.05 | 3 or 4 | 207434 |
| 33 | 0.21 x 0.11 | 2, 3 or 4 | 7803-05 |
| 32 | 0.24 x 0.11 | 2, 3 or 4 | 7803-04 |
| 31 | 0.26 x 0.13 | 2, 3 or 4 | 7803-03 |
| 30 | 0.31 x 0.16 | 2, 3 or 4 | 7803-07 |
| 29 | 0.34 x 0.18 | 2, 3 or 4 | 7803-06 |
| 28 | 0.36 x 0.18 | 2, 3 or 4 | 7803-02 |
| 27 | 0.41 x 0.21 | 2, 3 or 4 | 7803-01 |




Graph showinig motor speed v's flow rate

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