Chapter 20

***In Meso In Situ* Serial X-ray Crystallography (IMISX). A Protocol for Membrane Protein Structure Determination at the Swiss Light Source**

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Running Head: *In Meso In Situ* Serial X-ray Crystallography (IMISX)

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Abstract

The lipid cubic phases (LCP) has enabled the determination of many important high-resolution structures of membrane proteins such as G protein-coupled receptors, photosensitive proteins, enzymes, channels and transporters. However, harvesting the crystals from the glass or plastic plates in which crystals grow is challenging. The *in meso in situ* serial X-ray crystallography (IMISX) method uses thin plastic windowed plates that minimize LCP crystal manipulation. The method, which is compatible with high-throughput *in situ* measurements, allows systematic diffraction screening and rapid data collection from hundreds of microcrystals in *in meso* crystallization wells without direct crystal harvesting. In this chapter, we describe a IMISX protocol for *in situ* serial X-ray data collection of LCP-grown crystals at both cryogenic and room temperatures which includes the crystallization setup, sample delivery, automated serial diffraction data collection and experimental phasing. We also detail how the IMISX method was applied successfully for the structure determination of two novel targets - the undecaprenyl-pyrophosphate phosphatase BacA and the chemokine G protein-coupled receptor CCR2A.

Key Words: *In Situ*, IMISX, Lipid Cubic Phase, Mesophase, Membrane Proteins, Microcrystals, Serial Crystallography, Synchrotron, X-ray, X-ray Free Electron Lasers

1. Introduction

Membrane proteins (MPs) play an important role in signal and energy transduction, metabolism, transport, as well as contributing to the structural integrity of cells. They account for approximately one third of cellular proteins and represent close to 50% of drug targets in humans (1). The atomic resolution structure of a MP serves as the basis for understanding a protein's mode of action and is valuable for structure-based drug design (2). However, MPs are challenging to express, to purify and to crystallize, in part, reflecting the complex membrane environment from which they derive. Traditionally, detergent micelles have been

used to extract and solubilize MPs by mimicking, to some degree, the lipid bilayer of the native membrane. Alas, micelles are imperfect mimics. The lipid bilayer of the lipid cubic phase (LCP) or *in meso* crystallization provides a closer analogue to the native membrane and has been used successfully to support MPs crystallization (3,4). The LCP enables MPs to stack in sheet-like assemblies giving rise to crystals with type I packing, which tend to be small, well ordered and can give rise to high-resolution diffraction (5). The LCP method has had a major impact in the field of MPs structural and functional biology, and has contributed many high-impact structures including G protein-coupled receptors, rhodopsins, transporters, ion channels, enzymes and an assortment of complexes (6,7). It has also strongly contributed to the recent success of injector-based serial X-ray crystallography at both free-electron lasers (XFELs) (8-12) and synchrotrons (13-17).

The traditional glass plate designed for the LCP method provides stability during crystallization and the plate can be stored for long periods without water loss (18). However, the process of harvesting crystals from the mesophase in the glass plate is technically demanding (19) and can lead to damage and/or crystals loss. We developed an improved method called *in meso in situ* serial X-ray crystallography (IMISX) which avoids direct crystal harvesting (20). It employs a double sandwich plate and its utility has been demonstrated with the structure determination of several MPs, including a GPCR (21). The IMISX method has been further refined for data collection under cryogenic conditions (IMISXcryo) (21) and for heavy atom soaking directly in IMISX wells (22). Independently, Broecker *et al.* have developed a similar method with lower X-ray scattering and absorption by using thinner plastic windows and for ease of use with 3D-printed holders (23,24).

A microfocus beam with a high flux density facilitates the detection of tiny crystals which are difficult to do otherwise with large synchrotron beams at conventional macromolecular X-ray crystallography (MX) experimental stations (25,26). Some of the more notable LCP case

studies involving use of a microfocus beam are those in the field of GPCRs (27-30). The Nobel Prize in Chemistry awarded to Lefkowitz and Kobilka in 2012 recognized the important contributions made by the LCP method. While the method has been successful, it is still a challenge to obtain high quality data from single LCP crystals of $< 20\ \mu\text{m}$ in size. Accordingly, partial data sets from many crystals must be assembled to generate a complete data set, thus the term of serial X-ray crystallography (31). The serial method was actually standard in 1980s for the measurement of virus crystals at room-temperature, but its modern variants utilize hundreds to thousands crystals in high-throughput manners by taking advantage of the advances in sample delivery methods (8,13,17,32-35), detector technology (36,37), together with automation in data collection and processing (38-40).

Here, we review the LCP *in situ* serial crystallography method as implemented at the SLS and describe step-by-step procedures for the crystallographic structure determination of MPs, from IMISX plate assembly, well harvesting, heavy atom (HA) and ligand soaking, to *in situ* serial data collection and experimental phasing (see **Note 1**).

2. Materials

2.1 IMISX plate

In situ serial crystallography enables X-ray data collection directly on crystals where and as they grow. It significantly reduces the effort and time devoted to sample preparation and structure determination. It started with crystals in thin glass-walled capillaries (10 μm thickness) which were used for *in situ* data collection with counter-diffusion crystallization (41,42). The crystals in optically clear glass are easy to observe and the watertight property of the glass provides a hermetic seal and a reproducible environment for crystal growth. However, the viscous nature of the LCP makes it difficult to perform *in meso* crystallization and screening in such thin microcapillaries. Although the traditional LCP sandwich glass plate enables high throughput screening of crystallization conditions, the glass sandwich plate cannot be used

directly for X-ray diffraction screening due to poor X-ray transparency and strong scattering. Therefore, other materials have been explored to lower X-ray background scattering and increase transmission (43). Thin plastic polymers have been tested including polydimethylsiloxane (PDMS, 14-45 μm), polyvinyl chloride (PVC, 50 μm), polyethylene terephthalate (PET, 50 μm) and cyclic olefin copolymer (COC, 50-100 μm), and applications have been reported in microfluidic devices for on-chip characterization (44-46) and for the data collection on MPs (47,48). Of these materials, COC proved to be relatively water-tight and to have good X-ray transparency and scattering properties which make it a useful material for *in situ* experiments. Importantly, easy to handle 50-100 μm thick COC film became commercially available. To date, COC has been used with 96-well SBS *in situ* plates including CrystalQuick™ X (49), CrystalDirect™ (50), and *In Situ*-1 Plates™ (MiTeGen) for *in situ* diffraction experiment at room temperature. The IMISX plate, which uses 25 μm thick COC film, was successfully used for structure determination of weakly diffracting LCP crystals (20-22,51). Subsequently, 13 μm thick cyclic olefin polymer (COP) with similar properties as COC (52) has been used for the structure determination of a GPCR (53). Thinner materials like 3.5 μm biaxially-oriented polyethylene terephthalate (Mylar) (23), 250-500 nm graphene (54-56) and 1-20 μm silicon nitride (57-60) have been investigated as well for X-ray crystallography. The thin film window material is normally combined with 96-well perforated double-stick tape for high-throughput LCP crystallization using a robot (61). The detailed protocols for IMISX plate assembly (Fig. 1), sample harvesting (Fig. 2), as well as serial data collection implemented at the SLS are described below. All parts and materials can be purchased individually from commercial sources and assembled in house (see Note 2).

2.1.1 Thin plastic films

Two 112 mm $\times$ 77 mm pieces of 25 μm thick COC or of 13 μm thick COP are required. The COC film is available from MiTeGen (Ithaca, NY), Molecular Dimensions (Suffolk, UK),

Zeonex (ZenorFilm, Düsseldorf, Germany) and SWISSCI (Neuheim, Switzerland). The COP film can be provided through Zeonex (ZenorFilm, Düsseldorf, Germany).

2.1.2 Perforated double-stick spacer tape

Double-stick spacer tape measuring 112 mm × 77 mm and 50, 64 or 140 µm thick with 5, 6 or 7 mm diameter perforations spaced 9 mm apart center-to-center and double-stick gasket 2 mm-wide and 140 µm thick with outer dimensions of 118 mm × 83 mm and inner dimensions of 114 × 79 mm are required. The materials can be sourced from SAUNDERS (Chicago, IL, US).

2.1.3. Glass plates

124 mm × 84 mm No. 1.5 glass 0.15 mm thick thin glass plate and 127.8 mm × 85.5 mm standard 1 mm thick glass plate are required. These can be purchased from MARIENFELD (Lauda-Königshofen, Germany).

2.1.4 Other materials and tools needed for the IMISX plate assembly

Milli-Q water, silanizing agent (RainX), tissue paper, adhesive tape, 10 or 20 µL pipette, and micro tip.

2.2 Sample preparation prior to the data collection

2.2.1 Sample holder

To support *in situ* serial and high-throughput screening and data collection, many MX beamlines are equipped with a sample holder for SBS *in situ* 96 well plate (52,62,63) where protein crystals can be screened directly *in situ* and diffraction data can be recorded at room temperature. However, radiation damage of the sample at room temperature is a limiting factor and increases sample consumption (64-66). The problem is often exacerbated when working with small LCP crystals. Later, thick polycarbonate plastic with 75 holes on a standard goniometer-based allowed LCP crystallization and data collection directly at room and cryogenic temperatures was developed (34). Other supports for holding a single *in situ* well were made and modified for cryogenic measurements at a synchrotron. They include the

DiffraX™ sample holder (52), 3D-printed holders (23,24) and Y-shaped IMISX sticker-supports (21,22).

The IMISX technology was designed around the MX facilities at the SLS. The original prototype used a square support made of 25 μm thick COC which imposed a very slight curvature (**Fig. 3a**) on the *in situ* sample once glued in place (21). Although this curvature increased the rigidity of the sample in the cryostream, it turned out to be inconvenient for large area grid scans because crystals distributed throughout the bolus were at different focus levels and on different rotation axes. Later, a Y-shaped sticker support (**Fig. 3b**) was designed to create a flat IMISX well and to facilitate large area grid scans (22). The Y-shaped support is made conveniently and in medium-throughput fashion using a bespoke puncher which works directly with 25 μm COC on 141 μm double-stick tape (**Fig. 4**). Recently, the mounting of the sample on the support was simplified using a new 3D-printed support which can hold most LCP sample preparation types (24). We adapted the latter 3D design (unpublished results) to enable HA/ligand-soaking capability directly in the IMISX well for IMISX-experimental phasing (IMISX-EP) (**Fig. 3c-d**). The details method will be described in section 3.2.

2.2.2 Heavy atoms

Currently, 40% of the unique MP structures on record in the PDB have been solved by the *in meso* crystallization method and most of them have had to be solved by experimental phasing with either selenomethionine (SeMet) labeling or HA soaking (22,67). The IMISX method is compatible with soaking in a manner that does not involve crystal handling (22). Using dedicated sample holders, it is possible to add the HA soak solution to the LCP bolus and to remove it post-incubation, through the cut end of the IMISX well.

The optimal HA concentration and soak time can be screened using a gel shift assay (68) or analyzed with the HATODAS II server (69) by uploading the protein sequence. The HA can be sourced from Hampton Research (California, USA).

2.2.3 LCP crystallization

Monoolein (9.9 MAG), 7.8 MAG, and cholesterol (70). Gryphon LCP (Art Robbins) and mosquito[®] LCP (TTP Labtech), heat block, 50 or 100 μ L Hamilton glass syringes and coupler.

2.2.4 Other materials and tools needed for sample preparation and harvesting

Glass-cutting tool, tweezers, scissors, scalpel or blade, brayer or hand-held roller, 3D printed rectangular or round support, 25 μ m thick COC film, 141 μ m double-stick tape, Y-shaped puncher, magnetic goniometer base, glove, magnetic wand, cotton bud, adhesive tape, microscope, 10 or 20 μ L pipette, micro tip, incubator, timer, liquid nitrogen (LN_2), container for LN_2 , sample storing puck, and Dewar.

2.3 MX beamlines

2.3.1 X-ray beam characteristics

The IMISX method was developed at the two undulator beamlines X06SA-PXI and X10SA-PXII at the SLS, Paul Scherrer Institut (PSI). Both beamlines provide fast and stable energy (wavelength) selection in the range from 5.5 to 17.5 keV for X06SA-PXI and from 6 to 20 keV for X10SA-PXII. Beamline X06SA-PXI is optimized for micro-focusing applications and features a beam down to $2 \times 1 \mu\text{m}^2$. Beam size can be varied to match the dimensions of the crystal thereby maximizing the diffraction signal. The X10SA-PXII beamline utilizes three apertures to resize the beam to a limiting dimension of $18 \times 10 \mu\text{m}^2$. A recent micro-focusing upgrade with kinoform diffractive lenses delivers a $5 \times 2 \mu\text{m}^2$ beam (71). Both PX beamlines are equipped with a high-precision D3 diffractometer, which is essential for fast and continuous grid scanning (72).

2.3.2 Detector

For serial crystallography, a fast detector is required to expeditiously scan a large area, to locate microcrystals, and to collect hundreds of partial data sets at high speed. Both the EIGER 16M (37) at X06SA-PXI and the EIGER2 16M (Dectris Ltd.) at X10SA-PXII are state-of-the-art

single-photon-counting, pixel-array detectors with low-noise, fast frame-rate and negligible dead-time, which make them ideal for serial synchrotron crystallography.

2.3.3 Software

The software suite developed at the SLS for IMISX applications includes: 1) a fast scan program in DA+ GUI (73), 2) an automated serial data collection CY+ GUI (22), 3) an automated data processing (*adp*) program (74) and 4) an automated data merging (*adm*) pipeline (40). The suite includes real-time data quality monitoring and feedback to inform concurrent data collection.

3. Method

The optimized IMISX crystallization and serial data collection protocols described here are available at the SLS. All of the procedures are carried out at 20°C unless indicated otherwise. Instructional, open-access videos describing implementation and effective use of the IMISX method are available on-line (21).

3.1 Performing an IMISX crystallization trial

3.1.1 Assembling the IMISX plate

1. COC films come from the manufacturer sandwiched between two protective covers. Remove one protective cover from each of two COC films with the help of a small piece of adhesive tape (**Fig. 1a**). Place the two films on a clean working surface with the exposed COC side face up. Apply 5-10 μ L silanizing agent to the exposed surfaces and spread it evenly on the films with a tissue to create a hydrophobic surface (**Fig. 1b**). One film (Film1) will serve as the base film for the wells in which crystals grow. The other (Film2) functions as the cover for the well.
2. Rinse the silanizing agent off the treated films using 5-10 μ L MilliQ water (**Fig. 1b**).
3. Remove the protective white paper from a 96-well double-stick spacer (6 mm diameter perforations) (**Fig. 1c**) and apply it - with its sticky surface down - to Film1 from the

previous step (**Fig. 1d**). Use the roller or brayer to expel air from between the tape and Film1 and to ensure a tight bond between the two. This composite piece will be referred to hereafter as Film1*. (see **Note 3**).

4. Remove the protective white paper from the double-stick tape gasket and apply it, sticky surface down, to a 1 mm thick base glass plate. The two should be arranged so that their upper left hand corners are aligned. Use the roller to make sure the tape and glass stick firmly together (**Fig. 1e**).
5. At the middle and at the four corners of the 1 mm base glass plate from 3.1.1 step 4 and to a No. 1.5 cover glass plate apply 5-10 μ L MilliQ water (**Fig. 1e**).
6. Use small pieces of adhesive tape to remove the protective cover from Film1* (with double-stick tape attached) and the protective brown paper from the 96-well double-stick spacer (**Fig. 1d**). Place Film1* with its COC film facing down on the 1 mm base glass plate carefully positioned and centered within the double-stick gasket (**Fig. 1e**).
7. Use adhesive tape to remove the second protective cover from Film2 (**Fig. 1d**). With Film2 oriented with its silanized surface up, place it on the No. 1.5 thin cover glass plate with at least 2 mm from the sides, as shown (**Fig. 1e**). MilliQ water on the plate will help secure Film2 uniformly flat on the cover glass plate. A paper tissue can be used to squeeze/absorb excess water from between the cover glass plate and Film2 and to ensure the two are held firmly together by capillary forces.

3.1.2 High throughput crystallization trial setup using the LCP robot

The Gryphon LCP and mosquito[®] LCP are popular robots for setting LCP crystallization trials. Both robots are equipped with a mesophase dispensing arm for dispensing the viscous protein-laden LCP. A protocol for using these robots that includes instructional on-line videos has been published (75). Here, we describe a modification to this protocol specifically for setting up IMISX plates in high-throughput fashion.

1. Melt the hosting lipid (usually monoolein 9.9 MAG with or without an additive lipid such as cholesterol) on a heat block at 42°C and warm up a 100 μ L Hamilton syringe on the same heat block. Prepare the protein solution at a concentration typically at or above 10 mg/mL.
2. Fill a 100 μ L Hamilton syringe with 20 μ L protein solution and fill the warm 100 μ L syringe with 30 μ L molten lipid. Connect the two syringes together with the narrow-bore coupler. Be careful not to over- or to under-tighten the coupled syringe mixing device. Mix the contents of the two syringes immediately after connecting them. Our preference is to move the protein solution into the lipid as the first step in the mixing process. Continue to mix the contents until it becomes viscous and optical clear. The volume ratio of lipid to the protein solution is 3 to 2 based on the 9.9 MAG phase diagram and 1 to 1 for the short chain lipid, 7.8 MAG (4).
3. Turn on the humidifier that provides a humid atmosphere over the deck of the robot for at least 10 minutes before use and make sure the humidity level has reached 80% before dispensing mesophase onto plates.
4. When using the mosquito[®] LCP for the crystallization trial, first follow the LCP syringe wizard that is part of the mosquito[®] LCP protocol to assemble the Hamilton syringe complete with LCP from section 3.1.2 step 2 and needle on the robot's LCP dispensing arm. The wizard will automatically calibrate the X and Y horizontal coordinates of the needle tip. The needle height can be adjusted manually to ~ 0.1 mm above the bottom of the well in the receiving plate to avoid damaging or making a tool mark on the IMISX film if the parameters of the standard glass plate type is used in the protocol of plate type. Alternatively, a new plate type can be setup and added using the Options| Plates menu of mosquito[®] LCP software without changing the needle height. Measure the plate height with a plastic film on the standard glass base plate by using Vernier caliper. If the Gryphon LCP

is being used, first prepare a Gryphon LCP syringe with a calibrated needle. The calibration of the Gryphon syringe can be done by using Configuration| Configure Global Settings, Syringe menu in the Gryphon LCP software. The LCP sample then can be transferred from a Hamilton syringe to the Gryphon LCP syringe. Follow the instruction of the Exchange Solo Syringe of the Gryphon LCP protocol to assemble the Gryphon syringe on the LCP dispensing arm. Needle height can be adjusted manually to ~ 0.1 mm above the bottom of the well through the Dispense menu of the Gryphon software if the parameters of the standard glass plate type is used in the protocol of plate type. Alternatively, the new plate type will need to be setup for the IMISX plate through the Configuration| Open Labware Editor| Create New Labware menu of Gryphon LCP software without changing the needle height. Use the Vernier caliper to measure the plate height with a plastic film on the glass base plate.

5. Activate the robot to dispense typically a 50 nL LCP bolus plus 800 nL precipitant solution sequentially into each well. When all 96 wells have been filled, cover the plate with No. 1.5 thin cover glass plate with Film2 attached (COC face down toward the sample) that was prepared in section 3.1.1 step 7 (**Fig. 1f**). Place a 1 mm thick glass plate on the top of the assembled IMISX plate and use a roller or brayer to effect a uniform and a hermetic seal. Note that the LCP bolus can deform by brayering action without a 1 mm thick glass plate on the top of assembled IMISX plate.
6. The IMISX plate described here has the same footprint as a SBS plate and can be directly stored in an incubator/imager (Rock Imager[®] RI1000, Formulatrix, Waltham, Massachusetts, USA) without a base plate frame support. The Rock Imager[®], equipped with a top-of-plate barcode scanner, can be used for the IMISX plate incubation, storage and crystallization progress monitoring. Add new plate type identifier as IMISX plate to the Plate Type list in the Rock Maker[®] software using the Plate Type Editor. Print a barcode

with the IMISX plate type, apply it to the assembled plate from section 3.1.2. step 5 and incubate them in the Rock Imager® typically at 4°C and/or 20°C for quantitative tracking of crystallization progress.

3.2 Preparing the IMISX well for data collection

3.2.1 Harvesting using sticker support

1. Screen plates for crystals and mark the wells which contain crystals.
2. Use a glasscutter to cut and to free a square of cover glass on the selected well (**Fig. 2a**), remove the freed cover glass and clean away glass shards using a brush or with a piece of adhesive tape (**Fig. 2b**).
3. Follow the edge of the square from section 3.2.1. step 2. to cut the inner plastic IMISX well using a blade or a scalpel (**Fig. 2c**) and carefully remove the freed plastic well with its crystal-laden bolus using a tweezers (**Fig. 2d**).
4. Prepare a piece of 25 µm COC film and a 141 µm double-stick tape. Remove both protective covers from the COC film and remove the protective white paper from one side of the double-stick tape. Place the sticky side of double-stick tape down on the COC film and then use the roller or brayer to expel air from between the tape and film and to ensure a tight bond between the two (**Fig. 4a**).
5. Use the Y-shape puncher (**Fig. 4b**) on the film prepared in the previous step to generate a Y-shaped mount and then make a hole on the middle of the neck of the Y-shape support (**Fig. 5a**).
6. Remove the protective cover from one face of the Y-support (**Fig. 5b**). Pass the pin through the centering hole in the Y-support sticky face up (**Fig. 5c**).
7. Position the well on the exposed sticky surface of the Y-support (**Fig. 5d**).
8. Fold the Y-support back on itself and the two parts pressed together to secure the well firmly to the pin (**Fig. 5e-g**). Adjust the pin position to be just below the LCP bolus (**Fig.**

5h). Trim the edges of the IMISX well to fit it to the storage puck (**Fig. 6a**). The IMISX well securely attached to the pin on a base is now ready for precipitant removal, and HA or ligand soaking (**Fig. 6b-c**).

9. For snap cooling in liquid nitrogen, waft away by hand the stagnant warmer layer of gas above the liquid nitrogen that fills to the brim a small Dewar and rapidly snap-cool the mounted IMISX sample by plunging it edge down into the liquid nitrogen for long-term storage.

3.2.2 Mounting the harvested IMISX well on a 3D printed support

1. Prepare the IMISX sample as described in section 3.2.1 steps 1-3.
2. Insert a 3D printed support into a standard goniometer base and mount the IMISX well on the support (**Fig. 3c-d**). Position the well so that the LCP bolus is approximately at the center of the support window (see **Note 4**). The round-shaped support is well suited for boluses in the sponge phase (**Fig. 3c**). The rectangular-shaped support should be used for boluses that are in the cubic phase (**Fig. 3d**). (see **Note 5**).
3. Trim the edges of the IMISX well to fit the rectangular- or round-shaped support (**Fig. 6a**). Use a cotton bud or tissue paper to remove precipitant solution from the cut well (**Fig. 6b**). Follow the section 3.2.3 for HA or ligand soaking, or section 3.2.1 step 9 for sample storage.

3.2.3 IMISX-soaking with HA or ligand

The HA or ligand-containing solution is applied directly to the IMISX wells without touching the crystals and in a closed environment (minimal air exposure). The method (22) is described below.

1. Prepare an IMISX sample as described in section 3.2.1 or 3.2.2.
2. Snip off one corner of the well with a scissor (**Fig. 6a**) and wick away the precipitant solution from around the mesophase bolus using a cotton bud or tissue paper (**Fig. 6b**).

3. Pipet the heavy atom reagent or ligand solution, dissolved in the same precipitant solution, into the well via the open corner and manipulate so as to make contact with and to fully bathe the crystal-laden mesophase bolus (**Fig. 6c**).
4. Store the samples in a box with wet tissues at 20°C and then check the crystals at different time intervals under the microscope. The recommended time course is from 1 min, and every 10 min up to 1 h. If soaking is allowed to proceed for too long and/or at too high a concentration, it can lead to the crystal dissolving or fracturing. This should be avoided.
5. Snap-cool the IMISX well immediately in liquid nitrogen to terminate the derivatization process.

3.3 Serial data collection

3.3.1 Beamline setup and serial data collection:

In what follows, we describe how serial data collection is performed at PXI-X06SA and PXII-X10SA at the SLS.

1. The SLS uses the data acquisition software DA+ GUI (74) and the automated serial data collection protocol CY+ GUI (40) for serial crystallography data collection. At the start of a measurement, adjust the beam size to the crystal dimensions to optimize the signal-to-noise ratio ($10 \times 10 \mu\text{m}^2$ or $20 \times 20 \mu\text{m}^2$ typically). Place all sample pucks into the Dewar of the sample changer.
2. Set the data acquisition software (DA+) to sample changer mode and mount the strengthened COC sandwich on the magnetic head of the goniometer (**Fig. 7**).
3. Orient the IMISX well normal to the beam with the well/bolus positioned in the cross-hairs of the high-resolution on-axis sample-viewing microscope which corresponds to the position of the beam (see **Note 6**) (For reference, we define the X, Y and Z positioning of the crystal as follows. X corresponds to the crystal position along the rotation axis of the goniometer which is aligned to intersect orthogonally with the X-ray beam axis. Z

corresponds to the position along the beam axis and Y to the position along the axis perpendicular to both X and Z.). This step should be performed as quickly as possible to shroud the sample in a uniform stream of cryogenic nitrogen gas and to limit the possible ice formation. The same setup with the cryo-jet switched off can be used for *in situ* serial data collection at room temperature.

4. With the on-axis sample microscope, zoom in until a 1-2 mm diameter bolus can be seen, refocus on the bolus itself (this is done by moving the sample along the beam or Z-axis). Make a 1-2 mm grid with the single grid cell size $10 \times 10 \mu\text{m}^2$ or $20 \times 20 \mu\text{m}^2$ to fit to the size of crystals and to cover the entire bolus (**Fig. 8a**). For the initial screen, a raster scan at a typical speed of 50 Hz (20 ms exposure time per cell with a flux of 2×10^{12} photons s^{-1}) should provide enough diffracting spots for a successful spot finding operation in the 5 to 50 Å-resolution range using DISTL (Diffraction Image Screening Tool and Library) (76). If the mesophase is of the more fluid sponge type, the crystal containing bolus may be spread over a bigger area ($> 2 \times 2 \text{ mm}^2$).
5. The resulting finely sampled grid map should accurately locate all diffracting crystals in the bolus (**Fig. 8a**) and provide a ranking of diffraction spots in heat map form (**Fig. 8b**).
6. Launch the automated data collection protocol CY+ after completing the grid scan for serial data collection on crystals above a defined diffraction quality threshold over a specified rotation range (typically 10-20°) and a defined beam attenuation (see **Note 7**). Note that such a data collection scheme does not align individual crystals to the goniometer rotation axis. In practice, this is not an issue because the rotation range is small (10-20°).
7. In case of problems with preferred orientation (20), repeat data collection with the IMISX chip through a range of angular sweeps (typically $\pm 30^\circ$, $\pm 45^\circ$ or $\pm 60^\circ$ with respect to the X-ray beam). Monitor processing and merging results for completeness (**Fig. 9**).

3.3.2 Strategy for phasing the structure and structure determination

1. Follow section 3.3.1 steps 1-4 for centering the LCP bolus. Raster scan the LCP bolus to establish crystal coordinates in the bolus. If HAs are present, scan the absorption edge of the HA employed (note that the fluorescence signal may not come from the crystal but from the surrounding mesophase). Adjust the energy to the peak of the absorption edge to maximize f'' (77).
2. Check the quality of diffraction from the raster scan. Follow section 3.3.1 steps 5-7 for data collection (see **Note 8**).
3. Where relevant, monitor the anomalous signal using *adp* (74) and *adm* (40) and continue to record diffraction until there is sufficient multiplicity in the anomalous data.
4. Determine the sub-structure using SHELXC/D (78).
5. Once the sub-structure is known, launch phasing programs such as SHELXE (78), CRANK2 (79), autoSHARP (80) or PHENIX AutoSol (81), which combine density modification and automatic model building.

4. Successful cases and applications

The IMISX method was introduced in 2015 and has been continually optimized for serial data collection and phasing of novel structures. In what follows, we present results obtained with two non-reference target MPs to demonstrate its usefulness with novel targets. The first successful use of the IMISX method was to solve the structure of undecaprenyl-pyrophosphate phosphatase (BacA) (PDB code 5OON) from *Bacillus subtilis* (51), a MP with a unique interdigitated inverted topology involved in the peptidoglycan synthesis (**Fig. 10a**). Crystals of 20 μm in maximum dimension were initially obtained using the LCP crystallization method in standard glass sandwich plates. A few of these diffracted to 2.8-Å resolution after conventional loop harvesting but they did not yield a complete data set. The crystallization of BacA was readily reproduced using the IMISX plate with the same screen as for the glass sandwich plate. Experimental phasing was performed by HgCl_2 co-crystallization using the IMISX-EP method

(22). Without directly crystal handling and harvesting, two wells containing a total of 66 crystals with average dimension of $2 \times 15 \times 20 \mu\text{m}^3$ were collected (**Fig. 10b**). Of the 66 data sets collected, 36 came from well 1 recorded over a 30° wedge per crystal and 30 were from well 2 recorded over a 15° wedge per crystal. After merging 54 partial data sets, a complete data set at 2.6-Å resolution provided enough Hg anomalous signal for phasing the structure by Hg-SAD. Later, the native BacA crystals were derivatized by directly soaking the HgCl_2 in IMISX well to demonstrate the *in situ* soaking protocol (section 3.2.3 IMISX-soaking). The merging of 360 partial data sets yielded an interpretable experimental SAD map using IMISX-soaking with Hg. Parenthetically, the structure was also solved by the SIRAS method by merging 94 partial data sets from native crystals and 271 partial data sets from Hg-derivatized crystals.

The second case is from GPCR family - the prime target of LCP crystallization method and structure-based drug screening in industry. In a collaboration with Boehringer Ingelheim Pharma GmbH and leadXpro AG, we recently reported a CC Chemokine Receptor 2A (CCR2A) structure in complex with MK-0812 (PDB code 6GPX) at 2.7-Å resolution using the IMISX method (**Fig. 10c**) (53). In this study, the IMISX method was used with a thin COP film of 13 μm . Partial data sets of $10\text{-}15^\circ$ were collected from crystals of $80 \times 20 \times 10 \mu\text{m}^3$ in size on average (**Fig. 10d**). Finally, 77 small wedges of data were merged to yield a complete data set and the structure was solved by molecular replacement (MR). The new CCR2A ligand (MK-0812) was clearly identified with well-defined hydrogen-bonds to protein residues. The structure provided key information on the residue E291^{7,39} for the antagonist binding and provides new insights for the drug design on the high selective CCR2 antagonists.

In summary, IMISX has emerged as a valuable alternative for the structure determination of microcrystals grown *in meso*. As shown here, the method reduces crystals handling and makes the screening of the LCP crystals highly effective. It is generally applicable. In addition, the

IMISX well is a suitable medium for serial femtosecond crystallography (SFX) applications at XFELs, providing an alternative to the well-established LCP injector-based methods. As a fixed-target method with small footprint, the IMISX setup can be used directly at both cryogenic and room temperatures as demonstrated in recent experiments at the Swiss X-ray free-electron lasers (SwissFEL) (unpublished results).

5. Notes

1. Some of the text included in this report is taken verbatim from (20-22).
2. IMISX kit that includes COC film, base glass plate with double-stick gasket, cover plate and silanizing reagent is available from MiTeGen.
3. Large quantities of base and cover films (Film1* and Film2) can be prepared up to step 3 in section 3.1.1 (**Fig. 1d**) prior to the crystallization trial. The hydrophobic surface so created is stable for several months in storage under ambient conditions. For convenience, Film1* and Film2 can be placed on the base glass and cover glass plates (**Fig. 1e**), respectively, as outlined in section 3.1.1 without removing the protective paper from double-stick gasket and base film. The base glass plate with Film1* and cover glass with Film2 are stable in storage for several months. Be careful to keep the plate and film in a dust-free environment.
4. It takes approximately 2 min per well to complete steps 1 to 2 in section 3.2.2. Mounted wells can be stored for a short time (~ 1.5 h) on moist tissue without dehydrating. In this way, IMISX wells can be conveniently prepared together in groups of 10-15.
5. Some precipitant solutions can cause the LCP to convert to the more fluid sponge phase. Sponge phase samples can move and spread in the well during harvesting. Therefore, the round-head or Y-shaped sticker support (**Fig. 3b-c**), which has a bigger area in which to perform the grid scan, is recommended for this kind of sample.

6. The mesophase bolus should be at or close to the center of the support. If the sticker support has been used, first look for the pin extending from the goniometer base. The bolus should be at the other end of the pin. The mesophase bolus appears brown in color when viewed with the in-line microscope and crystals should be visible if the precipitant solution has been removed properly.
7. Set up the parameters for serial data collection. First, set up the threshold and neighborhood number of the grid output to adjust the number of crystals to be shot. If known, one can provide the cell parameters, space group and resolution range for data collection. For a new structure, the space group can be set to 0 for automatic space group identification. The program will provide an estimate of the time required to complete the data collection operation.
8. Unlike for native data collection, the dose per crystal should ideally not exceed ~5 MGy for phasing experiments (22,77). It can be estimated with knowledge of the incident flux, exposure time, wedge, crystal chemical composition and size.

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Figure legends

Fig.1 Steps involved in assembling an IMISX plate. **(a)** Prepare two COC films. With the aid of a piece of adhesive tape, remove one of the protective covers from both COC films. **(b)** Treat the exposed surface with silanizing agent, rinse with water and blot dry with a tissue. **(c)** Remove the protective white paper from one side of the perforated spacer tape. **(d)** Apply the spacer tape, sticky side down, to the silanized surface of the COC film from step **(b)** and remove the second protective covers from both COC films. Also remove the second protective paper from the upper surface of the double-stick tape from COC base plate. **(e)** Remove the protective paper from the upper surface of the double-stick gasket from the glass base plate. Place few drops of MilliQ water on the upper surface of the glass base plate and glass cover plate. Place the COC base plate and cover plate prepared in step **(d)** and place it bottom side down firmly onto the glass plate to be held in place by capillarity. **(f)** Use the LCP robot to dispense mesophase and precipitant on base glass plate with COC base plate, and seal using the cover glass plate with the COC film facing the sample. Place a standard glass plate on top of the cover glass plate and brayer to provide tight seals all round.

Fig.2 Steps for removing a well from an IMISX plate. **(a)** Use a glasscutter to cut and to free a square of cover glass on the selected well. **(b)** Remove the freed cover glass. **(c)** Follow the edge of the square from the previous step to cut the inner plastic IMISX well using a blade or a scalpel. The arrows on the side view of the well indicate the cutting position. **(d)** Remove the freed plastic well using a tweezers. Reproduced under a Creative Commons Attribution (CC-BY) License (<https://creativecommons.org/licenses/by/2.0>) from (20,21) Copyright: © 2015, 2016 Huang et al.

Fig.3 Evolution of the IMISX support (a) Square support. Adapted under a Creative Commons Attribution (CC-BY) License (<https://creativecommons.org/licenses/by/2.0>) from (21). Copyright: © 2016 Huang et al. (b) Y-shaped support (c) Round-shaped support (d) Rectangular-shaped support.

Fig.4 The materials needed to make a sticker support. (a) Double-stick tape with the COC film and Y-shaped support (b) Y-shaped puncher.

Fig.5 Steps involved in securing an IMISX well to a Y-shaped support and subsequently to a mounting pin. (a) Parts: mounting pin in base, IMISX well, Y-shaped support with a centering hole and made from double-stick tape. (b) Protective cover is removed from one face of the Y-shaped support. (c) The pin is passed through the centering hole in the Y-shaped support sticky face up. (d) The well is positioned on the exposed sticky surface of the Y-shaped support. The precipitant that surrounds the crystal-laden mesophase is seen clearly in the IMISX well. (e) (f) and (g) The Y-shaped support is folded back on itself and the two parts pressed together to secure the well firmly to the pin. (h) The IMISX well securely attached to the pin on a base ready for precipitant removal, heavy atom or ligand soaking and/or snap cooling in liquid nitrogen. Reproduced under a Creative Commons Attribution 4.0 International License (<https://creativecommons.org/licenses/by/4.0>) from (22). Copyright: © 2018 Huang et al.

Fig.6 Trimming an IMISX well and soaking with HA or ligand. (a) Snip off the top and both sides of the IMISX well. (b) Wick away precipitant solution from around the mesophase bolus using a cotton bud or tissue paper. (c) Pipet heavy atom reagent or ligand solution into the well via the open corner.

Fig.7 Experimental setup of an IMISX well on a goniometer with the crystal-laden mesophase bolus positioned in the X-ray beam and in the cryostream at 100 K for SX data collection on beamline PXI-X06SA at the SLS. Reproduced under a Creative Commons Attribution (CC-BY) License (<https://creativecommons.org/licenses/by/2.0>) from (21). Copyright: © 2016 Huang et al.

Fig. 8 Screenshots of the fast grid scan GUI (DA+) and automated serial data collection GUI (CY+). **(a)** DA+ GUI: A grid scan covers the complete LCP bolus (outlined with a dashed line) with the diffraction hits represented as a heatmap. The grid scan parameters are configured in the lower-left panel of the GUI. **(b)** CY+ GUI: The selected crystal hits, identified by DA+ in **(a)**, are labelled automatically with white dots and overlaid on the DA+ grid scan heatmap. The outline of the LCP bolus is shown so that panels **(a)** and **(b)** can be compared. The crystal hits selection criteria and parameters for serial data collection are configured in the left panel of the GUI. Reproduced under a Creative Commons Attribution 4.0 International License (<https://creativecommons.org/licenses/by/4.0>) from (22). Copyright: © 2018 Huang et al.

Fig. 9 The *adm* merging results and statistics of 100 PepT_{St} LCP crystals in the *adp*-tracker with ‘SX-View’ mode. Reproduced under a Creative Commons Attribution 4.0 International License (<https://creativecommons.org/licenses/by/2.0>) from (40). Copyright: © 2019 Basu et al.

Fig. 10 Overall structures of the MPs solved by IMISX method and screenshots of the crystals in a well from an IMISX plate **(a)** Overall structure of BacA. Reproduced under a Creative Commons Attribution 4.0 International License (<https://creativecommons.org/licenses/by/4.0>) from (51). **(b)** Crystals of BacA. The size of the beam on the sample and scale bar are shown.

(c) Overall structure of CCR2A. (d) Crystals of CCR2A. (c) and (d) are reproduced and adapted from (53) Copyright (2019), with permission from Elsevier.

Figures

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