

# Sample Preparation and Data Collection for High-Speed Fixed-Target Serial Femtosecond Crystallography

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## Method Article

**Keywords:** fixed-target serial femtosecond crystallography, protein crystallography, virus crystallography

**Posted Date:** June 29th, 2017

**DOI:** <https://doi.org/10.1038/protex.2017.059>

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# Abstract

We have developed a protocol for high-speed fixed-target serial femtosecond crystallography (SFX) at X-ray free electron lasers (XFELs). Using the Roadrunner goniometer for fast scanning of microcrystals loaded on a micro-patterned silicon chip, our method allows for usage of the full 120 Hz repetition rate of the Linear Coherent Light Source (LCLS). By synchronizing the sample exchange to the arrival of the LCLS X-ray pulses our approach results in most efficient use of sample material and beamtime. The presented protocol describes the loading of microcrystals on micro-patterned silicon chips and subsequent data collection using the Roadrunner goniometer installed at an XFEL beamline. Data collection can be performed either at cryogenic or room temperature. We further describe the Roadrunner control and data acquisition software and data processing using cpxfel. Due to the small sample amounts required and the low background scattering levels achievable, the method is ideally suited for data collection of precious microcrystals from viruses or membrane proteins, where often only limited amounts are available. This protocol accompanies Roedig P et al, Nature Methods (10.1038/nmeth.4335, published online June 19, 2017).

## Introduction

**Experimental design** Serial femtosecond crystallography (SFX) at X-ray free electron lasers (XFELs) has evolved to a successful method for biological structure determination in recent years<sup>1–3</sup>. In the following, we present a protocol for highly efficient fixed-target SFX. Compared to other sample delivery methods, e.g. with jets, fixed target approaches require significant less sample and allow achieving higher hit rates, thereby making more efficient use of the limited beamtime at XFELs. Our method is based on sample delivery with micro-patterned silicon chips, which act as sample holders for more than 20,000 microcrystals. In combination with the Roadrunner, which provides the capability of fast scanning of the chips synchronized to the arrival of the X-ray pulses, diffraction data can be collected at the maximum LCLS repetition rate data of currently 120 Hz. The goniometer consists of a piezo-motor driven x,y scanning stage mounted on a rotation stage, which allows for data collection at different orientations of the chips. It is further equipped with an inline sample-viewing microscope, for alignment of the chip with respect to the X-ray beam and definition of the scan-grid. By minimizing the free path of the direct X-ray in air, the background scattering from air is significantly reduced. The method allows for data collection at cryogenic and also at room temperature. For cryogenic data collection cryogenically cooled chips already preloaded with sample are mounted on the goniometer. Cryogenic temperatures during loading and data collection are maintained by an open flow cryostat. For room temperature data collection, microcrystals are loaded on the chips directly on the goniometer. A gas stream at a controlled humidity level prevents the crystals from drying out. With respect to other room temperature fixed-target approaches<sup>4,5</sup>, no sealing of the chip with Mylar or Kapton foil is required in order to prevent the crystals from drying out. Before data collection chips are pre-aligned using the inline sample-viewing microscope and the scanning grid is defined using the graphical user interface of the Roadrunner goniometer. For data collection the chip is scanned through the XFEL beam in a meander-like fashion. The translation of the chips is

synchronized to the arrival of the XFEL pulses in such way, that the X-ray pulses always hit through the micro-pores of the chip. This guarantees an efficient sampling of the crystalline material on the chip at much faster sample exchange rates than realized by other setups<sup>6–8</sup>. With our method of sample preparation, obtaining a full dataset requires only micrograms of sample, which is significantly less than what is required other SFX experiments sample delivery methods, e.g. using liquid jets. This makes our approach particularly well suited for samples such as viruses or membrane proteins, where only small amounts of sample are available. The method has been initially developed for X-ray structure determinations from micro-crystals at synchrotron sources. The method has been successfully demonstrated at both cryogenic<sup>9</sup> and ambient<sup>10</sup> temperatures. Loading of the chip with CPV and lysozyme microcrystals is described in more detail in Ref. 9.

## Equipment

Reagents and Equipment • Micro-patterned silicon chips with magnetic mount carrying the microcrystals. Mounted and unmounted chips can be purchased from "Suna Precision GmbH, Germany":<http://www.suna-precision.com> • Roadrunner goniometer, which allows for fast (120 Hz) data collection consisting of an inline sample-viewing microscope, a high-precision fast scanning unit and an adjustable post-sample beam tube unit. • Motion controller DMC-4080 from "Galil":<http://www.galilmc.com> • Micropipette (1 µl – 10 µl) • Suspension containing microcrystals (ideally 1000 – 2000 crystals/µl) • Dewpoint generator (e.g. DG-4 dewpoint generator from Sable Systems International) for humidification of the gas stream (either helium or air) • Nozzle for releasing the humidified gas stream • Nozzle for helium purging of the direct beam path • Conventional filter paper • Equipment for data collection at cryogenic temperatures: - liquid nitrogen for cryo-freezing (CAUTION: Wear protective gloves and glasses to protect against cryogenic burn.) - open-flow cryostat - common sample handling tools used in cryo-crystallography - foam dewar for sample handling in liquid nitrogen - storage dewar for sample preparation prior to beamtime

## Procedure

**\*\*Chip inspection\*\*** The micro-patterned chips are manufactured from single-crystalline silicon and consist of an outer frame structure and an inner membrane part (Fig. 1). [See figure in Figures section.](#) The inner membrane has a thickness of less than 10 µm and is equipped with a regular array of micropores. Before mounting a chip on the goniometer, the integrity of the silicon membrane should be checked and that the chip is properly attached to its holder. Broken silicon debris or other not correctly oriented silicon material can cause strong silicon Bragg reflections if hit by the X-ray beam and thereby can potentially damage the detector. If it is required to rotate the chip during the measurements (to avoid incomplete data due to preferred orientation of the crystals on the chip) it is further recommended to carefully check for correct orientation of the chips on the goniometer. This is ideally done by slowly rotating a chip during a few line scans while exposing it to highly attenuated (transmission < 10<sup>-6</sup>) X-ray pulses and collecting diffraction images. **\*\*Chip loading\*\*** For sample loading onto the chips a drop of

typically 1 – 3  $\mu$ l microcrystal suspension is pipetted onto the top-side of the membrane area. Subsequently, the mother liquor is soaked through the pores by touching the bottom side of the chip with a wedge of filter paper (Fig. 2). [See figure in Figures section](#). In this way the mother liquor is wicked away efficiently and all crystals larger than the pore size are retained on the upper side of the chip membrane and arrange themselves according to the pore pattern. To prevent dehydration, the crystals are constantly maintained in a stream of humidified gas<sup>11</sup>. For room-temperature data collection sample loading is performed with the chip already mounted on the goniometer (Procedure A). For cryogenic data collection sample loading is performed in a similar way but can be performed offline in a laboratory (Procedure B). After sample loading the chips are immediately flash-frozen by plunging them into liquid nitrogen. For data collection chips are mounted onto the goniometer while maintaining cryogenic temperatures. For cryogenic chip handling standard tools for cryo-crystallography can be used.

**\*\*Procedure A: Chip loading for room-temperature data collection\*\***

**\*\*1\*\*** Start the Roadrunner software on the control computer. **\*\*2\*\*** Press the button “Set mount position”. This moves the goniometer stages to a pre-configured mounting position where the sample can be mounted without damaging the goniometer. **\*\*3\*\*** Mount the chip on the Roadrunner goniometer. **\*\*4\*\*** Press the button “Set sample in position”. The sample is then moved into the field of view of the inline sample viewing microscope. **\*\*5\*\*** Make sure that the humidified gas stream is turned on and well centered on the chip. Adjust the relative humidity of the gas stream using the dewpoint generator to the appropriate value. It should be matched to the concentration of precipitant and cryoprotectant of the mother liquor (see Ref. 12 for details). The equilibrium relative humidity can be determined in advance by monitoring a drop of mother liquor within the humidity stream. When equilibrium relative humidity is reached, the size of the drop remains constant. **\*\*6\*\*** Churn the suspension of crystals in order to obtain a uniform concentration of crystals within your mother liquor. Then apply a drop of 2 – 3  $\mu$ l of crystal suspension onto the top of the membrane part of the chip (Figure 2A). Avoid touching the chip with the tip of the micropipette, as the membrane is very fragile. The drop should be contained within the well on the top side of the chip. **\*\*7\*\*** Attach a wedge of filter paper to the bottom side of the chip so that the filter paper is oriented parallel to the chip (Figure 2B). Once the filter paper is in contact with the micropores, the mother liquor is soaked through the pores (Figure 2C) and crystals larger than the pore size are retained on the upper side of the chip (Figure 2D). Also see Ref. 9 for details. **\*\*8\*\*** If possible inspect the distribution of crystals on the top side of the chip with an optical microscope. A typical distribution of crystals is shown in Figure 3. [See figure in Figures section](#). If the concentration of crystals is too low, repeat steps 4 and 5 with the very same chip. If the concentration of crystals is too high, dilute the suspension with an extra amount of mother liquor and proceed with a new chip. **\*\*9\*\*** Rotate the chip by 90 degrees so that the flat side of the chip faces the X-ray beam and the crystals are on the other side in direction of the detector.

**\*\*Procedure B: Chip loading for cryogenic data collection\*\***

**\*\*1\*\*** Perform offline sample loading in the lab similar to the procedure as described in steps 5 – 9 in the previous procedure (Procedure A ‘Chip loading for room temperature data collection’). **\*\*2\*\*** After removal of the mother liquor (step 7) and, ideally, optical inspection with a microscope (step 8) directly dip the chip loaded with the crystals into liquid nitrogen for flash-freezing. Make sure to act fast in order to prevent the crystals from drying out. **\*\*3\*\*** Enclose the chip carefully with a vial while it is kept in liquid nitrogen. Transport the vial to the beamline, keeping it in liquid nitrogen all

the time. **\*\*4\*\*** Make sure that the open-flow cryostat is installed and aligned properly. **\*\*5\*\*** Wet mount the chip on the goniometer: Put the magnetic mount of the chip onto the goniometer and quickly remove the vial parallel to the chip. Take care that the chip is not damaged while the vial is removed. **\*\*6\*\*** Rotate the chip so that the flat side faces the X-ray beam. **\*\*Using the Roadrunner software for data collection\*\***

The main elements of the Roadrunner goniometer are high-precision piezo-scanning stages, which are operated in closed loop and allow for fast translation of the chip with a speed of up to 2.5 mm/s. The piezo motors are controlled by a DMC-4080 motion controller from Galil, which is capable of synchronized motion with respect to the LCLS pulse train at 120 Hz. For data collection the membrane of the chip is scanned line by line in a 2D meander scan. For chip alignment, the Roadrunner goniometer provides a rotational stage with a vertical rotation axis and a translation stage for movement in beam direction. The rotational stage is used during pre-alignment to ensure that no silicon Bragg reflections, which could possibly harm the detector, are excited. Additionally, through rotation of the chip during data collection it is possible to avoid preferred orientations of the crystals on the chip and therefore to cover a larger part of reciprocal space. The linear translation stage is used to move the chip in the center of rotation. **\*\*1\*\*** By pressing the button “Autofocus gonio Z” the chip surface is automatically moved into the focal plane of the inline sample viewing microscope and a sharp image of the chip surface should appear on the screen. **\*\*2\*\*** Now click on the “Clear raster” button to prepare for a new alignment grid. **\*\*3\*\*** Use the arrow buttons located on the top right part of the GUI to move the chip so that the upper left corner of the membrane of the chip appears on the microscope image. **\*\*4\*\*** Click on the “Set top left corner” button. Move the mouse pointer to the upper left micro-pore of the membrane area of the chip in the microscope image. A click with the left mouse button now defines the upper left corner of the scanning grid. **\*\*5\*\*** Use the arrow buttons again to move to the upper right corner. Now click the button named “Set top right corner” and click on the most right pore in the same row as the upper left micro-pore. Now the first upper line of the scan grid is defined. Now two guide lines are indicated on the microscope image. One straight line (in more or less) horizontal direction represents the first upper scan line which is overlaid to first row of micro-pores on the chip. The second line is oriented perpendicular to the first line and is meant to assist in defining the lower left micro-pore of the chip. **\*\*6\*\*** Use the arrow buttons to move to the lower left corner of the chip. Now click the button named “Set bottom left corner” and click on the position where the lowest left micropore of the chip intersects with the guide line. After this the scanning grid is defined and indicated as a green mesh on the microscope image. If the grid does not exactly coincide with pore positions it can be manually adjusted by using the right mouse button to drag and drop the grid in the microscope image. If necessary, the whole procedure can be repeated by starting again with point 2. **\*\*7\*\*** Enter the sample name in the field “Scan name”. **\*\*8\*\*** In order to achieve better coverage of reciprocal space during a scan, the chip can be automatically rotated in small increments during the scan. To do so, the starting and stopping angles of the goniometer have to be entered in the corresponding fields. **\*\*9\*\*** In case that only part of the chip is covered with microcrystals, it is possible to enter the number of the first and the last row to be scanned. Otherwise, the entire scanning grid will be scanned. **\*\*10\*\*** Enter the current X-ray pulse arrival frequency in the “Scan frequency” field. This frequency depends on the operation mode of the XFEL and/or potential settings of the pulse picker. **\*\*11\*\*** Define the number of acceleration pulses which are used to achieve a constant speed of the chip

and to synchronize the position of the micropores to the arrival of the X-ray pulses. A typically used value is 30. **\*\*12\*\*** Now the scan can be started by pressing the “Start Scan” button. The progress of the scan is indicated with a progress bar located on the bottom part of the GUI. **\*\*13\*\*** After the scan is finished, press the button “Set mount position” to drive the chip into a save position where it can be removed from the goniometer. **\*\*14\*\*** Remove the chip from the goniometer. **\*\*Data processing\*\*** LCLS detector images are stored in a sequential data format called XTC, which is beneficial in terms of fast data storage and reliability. For data analysis and processing, XTC data streams are converted into more convenient formats such as pickle or HDF5. The converted images can then be loaded into common programs for SFX data processing such as `_CrystFEL_`<sup>13</sup> and `_cppxfel_`<sup>14</sup>. In the following, conversion of XTC streams into individual pickle files is described by using the `_cctbx.xfel_` software suite, resulting in one pickle file per image. The hitfinder in the `_cctbx.xfel_` routine allows for filtering of the recorded detector images according to the number of possible diffraction peaks present on the image. In this way, blank images are sorted out and only possible crystal hits are used for further data processing. Diffraction images considered as hits are then passed into the processing pipeline of `_cppxfel_`, which includes indexing, integration, initial orientation matrix refinement and post refinement. The workflow and the underlying principles of `cppxfel` are discussed in Ref. 14. **\*\*1\*\*** Use a unix shell to log onto the SLAC computing environment, using your SLAC unix account username and password: `_ssh -X @pslogin.slac.stanford.edu_` **\*\*2\*\*** Log onto the psana machine used for XTC conversion: `_ssh -X psana_` **\*\*3\*\*** Setup your psana python environment by using one of the following commands. For c-shell: `_source /reg/g/psdm/etc/ana_env.csh_` For bash shell: `_source /reg/g/psdm/etc/ana_env.sh_` For further explanation see "here":<https://confluence.slac.stanford.edu/display/PSDM/psana+python+Setup>. Additional information can also be found "here":[http://viper.lbl.gov/cctbx.xfel/index.php/Set\\_up\\_PSDM\\_software](http://viper.lbl.gov/cctbx.xfel/index.php/Set_up_PSDM_software). **\*\*4\*\*** Setup `cctbx.xfel` as described "here":<http://viper.lbl.gov/cctbx.xfel/index.php/Setup>. **\*\*5\*\*** Follow the preparatory steps for image conversion as described "here":[http://viper.lbl.gov/cctbx.xfel/index.php/Preparatory\\_steps#Creating\\_a\\_mask\\_image](http://viper.lbl.gov/cctbx.xfel/index.php/Preparatory_steps#Creating_a_mask_image). These steps involve dark subtraction in order to account for temperature distortions in the detector and the creation of a mask image in order to mask of any bad pixels. Averaged light images might also be useful to flag up obvious errors in detector metrology. Misaligned quadrants of the detector will be recognized as non-continuous rings in the virtual powder patterns. In case the panel coordinates need to be redefined, see "here":[http://viper.lbl.gov/cctbx.xfel/index.php/Metrology\\_refinement](http://viper.lbl.gov/cctbx.xfel/index.php/Metrology_refinement). For further details regarding metrology refinement or contact your local support. **\*\*6\*\*** Submit a processing job to the cluster which will convert your image data to individual pickle files as described "here":[http://viper.lbl.gov/cctbx.xfel/index.php/Indexing\\_and\\_integration](http://viper.lbl.gov/cctbx.xfel/index.php/Indexing_and_integration). You need to edit a configuration file `\(*.cfg)` file which will contain the necessary image paths and input settings for dark run subtraction, bad pixel masking and hit-finding. Do not use the indexing and integration option of `_cctbx.xfel_` as these steps will be later performed using `cppxfel`. **\*\*7\*\*** Transfer the images to your local machine. For installation of `_cppxfel_` on your local machine follow the instructions as described "here":[http://viper.lbl.gov/cctbx.xfel/index.php/Cppxfel\\_Installation](http://viper.lbl.gov/cctbx.xfel/index.php/Cppxfel_Installation). The installation relies on three components: the DIALLS distribution, which will provide certain `_cctbx_` libraries for L-BFGS refinement, the



\_cppxfel\_ distribution and boost libraries, which allow for multicore threading. **\*\*8\*\*** Follow the instructions for indexing using the \_TakeTwo\_ algorithm of \_cppxfel\_ as described ["here":http://viper.lbl.gov/cctbx.xfel/index.php/Cppxfel\\_Indexing](http://viper.lbl.gov/cctbx.xfel/index.php/Cppxfel_Indexing). Prior to starting the indexing algorithm the DIALS<sup>16</sup> spotfinder is used for localization of strong spots within your diffraction images. Strong spots coordinates of each image are then output to individual text files used by \_cppxfel\_ for finding one or multiple indexing solutions. In case your diffraction data contains diffraction patterns from multiple crystals on one image ensure that the parameter "SOLUTION\_ATTEMPTS" in the parameter input file is followed by a number larger than one. An exemplary parameter input file used for indexing of SFX data is given in List 1. **\*\*9\*\*** Start initial orientation matrix refinement in order to produce a set of orientation matrices for each individual found indexing solutions \ (see ["here":http://viper.lbl.gov/cctbx.xfel/index.php/Cppxfel\\_Initial\\_Orientation\\_Matrix\\_Refinement](http://viper.lbl.gov/cctbx.xfel/index.php/Cppxfel_Initial_Orientation_Matrix_Refinement)). An exemplary parameter input file used for initial orientation matrix refinement is given in List 2. **\*\*10\*\*** Perform post-refinement of the integrated intensities, ideally against an external reference data set if available from a previous run, or from scratch if the first time \ (see ["here":http://viper.lbl.gov/cctbx.xfel/index.php/Cppxfel\\_Post-refinement](http://viper.lbl.gov/cctbx.xfel/index.php/Cppxfel_Post-refinement)). The algorithm will also break possible indexing ambiguities as discussed by Brehm & Diederichs<sup>15</sup>. Note that at the moment only two-fold indexing ambiguities can be broken without providing an external reference MTZ file. An exemplary parameter input file used for post refinement is given in List 3. **\*\*11\*\*** A final reflection MTZ file will be produced automatically during post-refinement. To assess common crystallographic statistical parameters such as  $CC_{1/2}$ ,  $R_{split}$ ,  $R_{merge}$ ,  $R_{meas}$  or  $R_{pim}$ , see ["here":http://viper.lbl.gov/cctbx.xfel/index.php/Cppxfel\\_Statistics](http://viper.lbl.gov/cctbx.xfel/index.php/Cppxfel_Statistics). **\*\*Phase extension\*\*** Density modification, cyclic averaging and phase extension were performed as described by Gouet et al.<sup>17</sup> **\*\*1\*\*** Fourier transform the reflection list with starting amplitude/phase information to a given resolution to produce a starting map. **\*\*2\*\*** Density modification preparation: Calculate envelope using GAP \ (version for download published in Ref. 18) for appropriate virus, space group and unit cell dimensions based on starting map. Repeat calculation every 10th cycle. **\*\*3\*\*** Density modification and cyclic averaging: Use envelope and NCS parameters to cyclically average the current electron density map using GAP in space group P1. **\*\*4\*\*** Use \_ccp4\_ program sfall to convert map file to reflection list. **\*\*5\*\*** Use \_ccp4\_ program sortmtz to sort reflection list. **\*\*6\*\*** Use \_ccp4\_ program CAD to remove systematic absences and restore correct space group. **\*\*7\*\*** Scale amplitudes to original values for each resolution shell, using Shellscale. **\*\*8\*\*** Phase extension: Add an incremental amount of amplitude information and return to step 1.

## Timing

Sample loading of the chip and removal of the mother liquor typically takes less than a minute. Additional time may be required for finding the optimum concentration of crystals in the applied suspension. For data collection at room temperature, these steps can be directly performed while the chip is mounted on the goniometer. For data collection at cryogenic temperatures, sample preparation including freezing of the sample can be performed in advance, prior to the beamtime, and takes a few minutes per sample. Wet mounting of the chip on the goniometer takes less than 1 minute. After the chip

has been mounted and the experimental hutch has been searched, alignment of the chip and defining a scanning grid using the control software takes another one to three minutes. Scanning of a whole chip takes less than 10 minutes.

## Troubleshooting

Procedure A, steps 6-8: If the concentration of microcrystals in the applied suspension is too high, soaking of the mother liquor may become impaired and may not result in the desired arrangement of the applied microcrystals. Similar problems may occur for mother liquors with very high viscosity, e.g. with high concentrations of common cryoprotectants such as polyethylenglycol used for data collection at cryogenic temperatures. To ensure that the applied microcrystals arrange according to pore pattern in this case, dilute the suspension of crystals by adding additional amounts of mother liquor. If the concentration of microcrystals in the applied drop of suspension is too low, steps 6-8 can be repeated several times until the desired coverage of the chip is achieved.

## Anticipated Results

The method allows X-ray structure determination from microcrystals at XFELs at either room or cryogenic temperatures in short time. In contrast to other approaches the method requires very small amount of sample and is well applicable also to large unit cell systems such as virus crystals.

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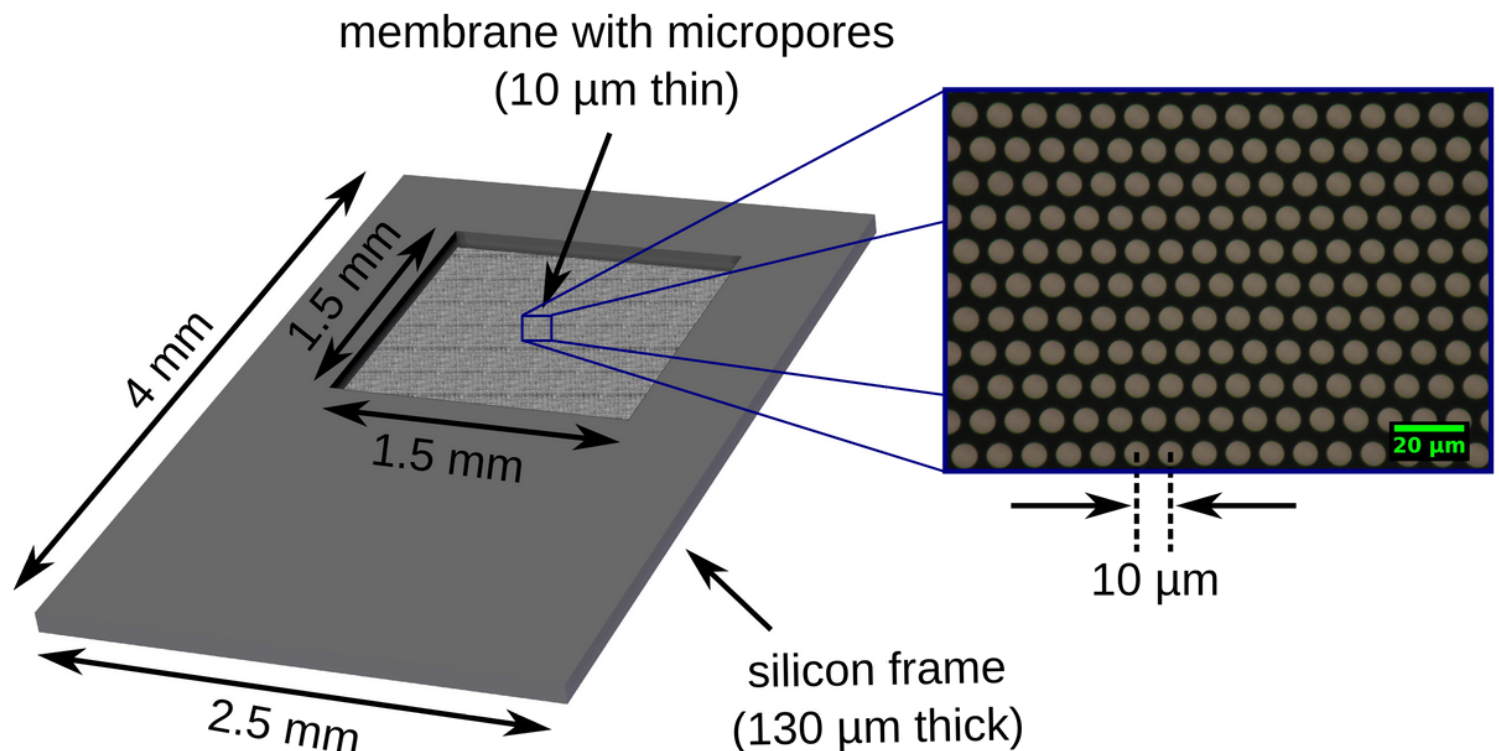
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## Acknowledgements

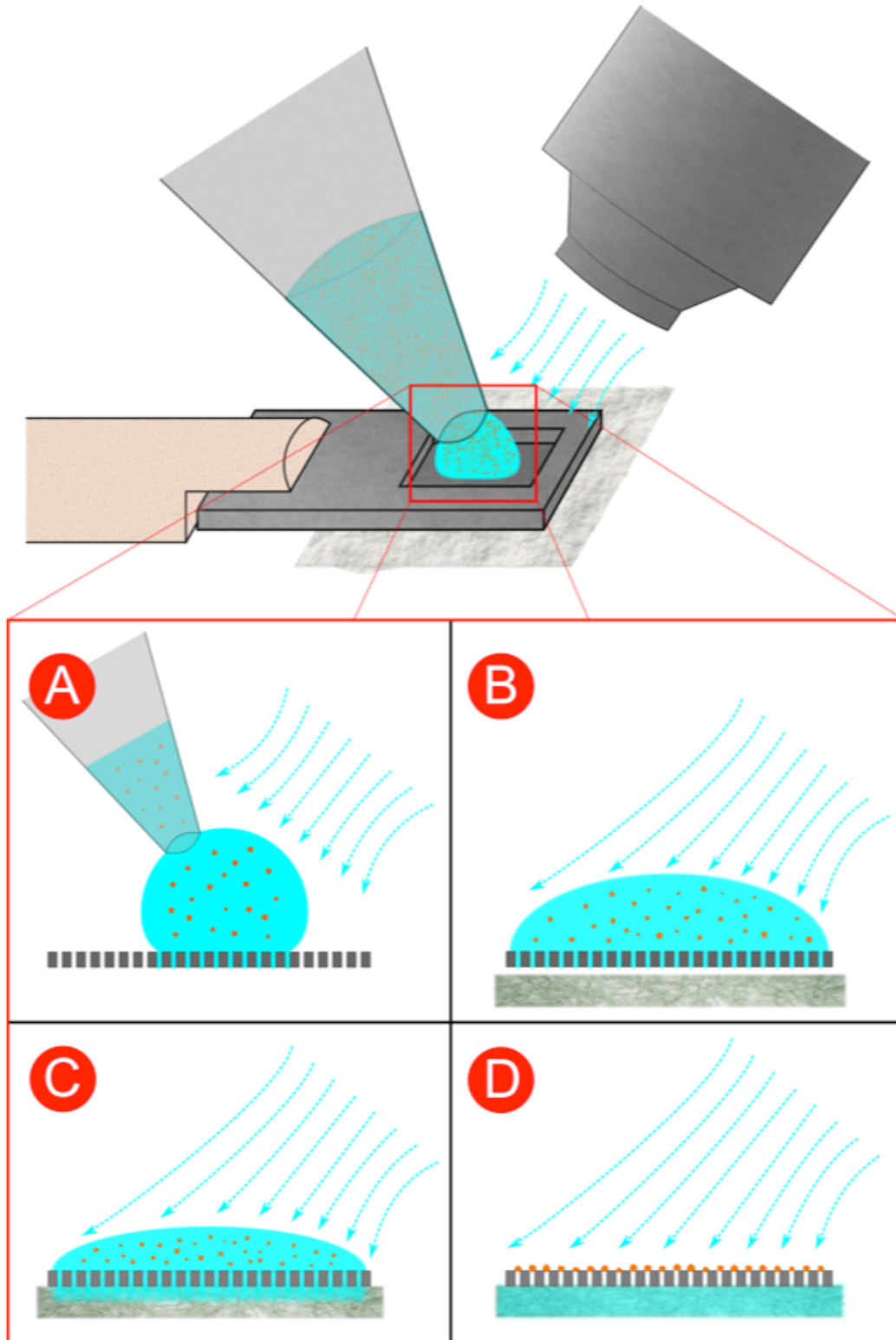
We thank LCLS staff for their support during experiment LH90. Use of the Linac Coherent Light Source (LCLS), SLAC National Accelerator Laboratory, is supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract No. DE-AC02-76SF00515. This work was further supported by the European Cluster of Advanced Laser Light Sources (EUCALL) and the Virtual Institute VH-VI-403 of the Helmholtz Association. HMG was supported by the Wellcome Trust (studentship 075491/04). DIS was supported by the Medical Research Council, grant MR/N00065X/1 and previously G1000099. NKS was supported by US National Institutes of Health grants GM102520 and GM117126 for data processing methods.

## Figures



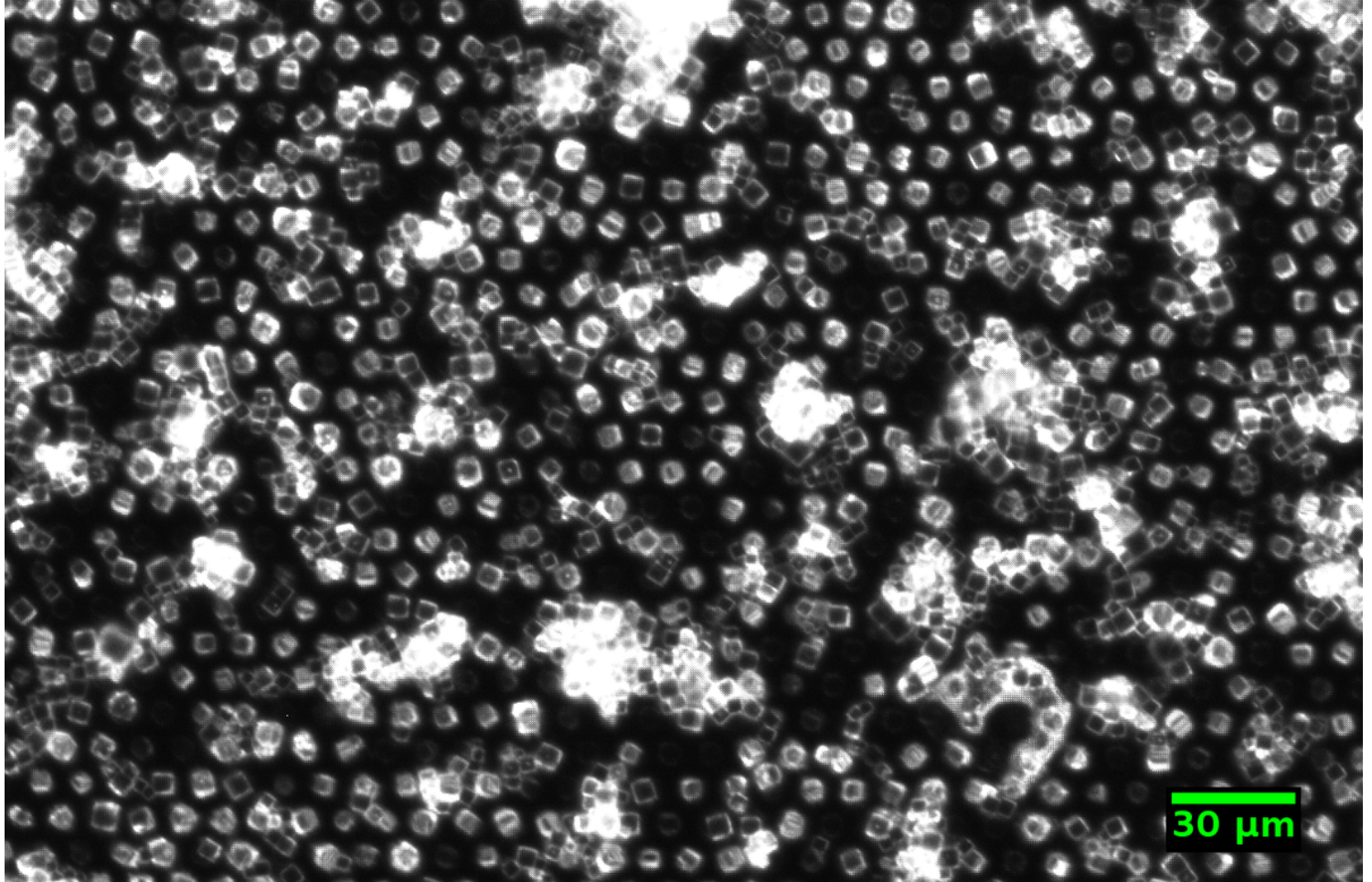
**Figure 1**

Dimensions and design of the silicon chip holder The 1.5 x 1.5 mm<sup>2</sup> sized membrane consists of more than 20,000 cylindrically shaped micro-pores, arranged in a hexagonal close-packing. Chips with micro-pore diameters between 1 µm and 30 µm are available, suited to variable crystal sizes. Image adapted from Ref. 9, licensed under CC BY 4.0.



**Figure 2**

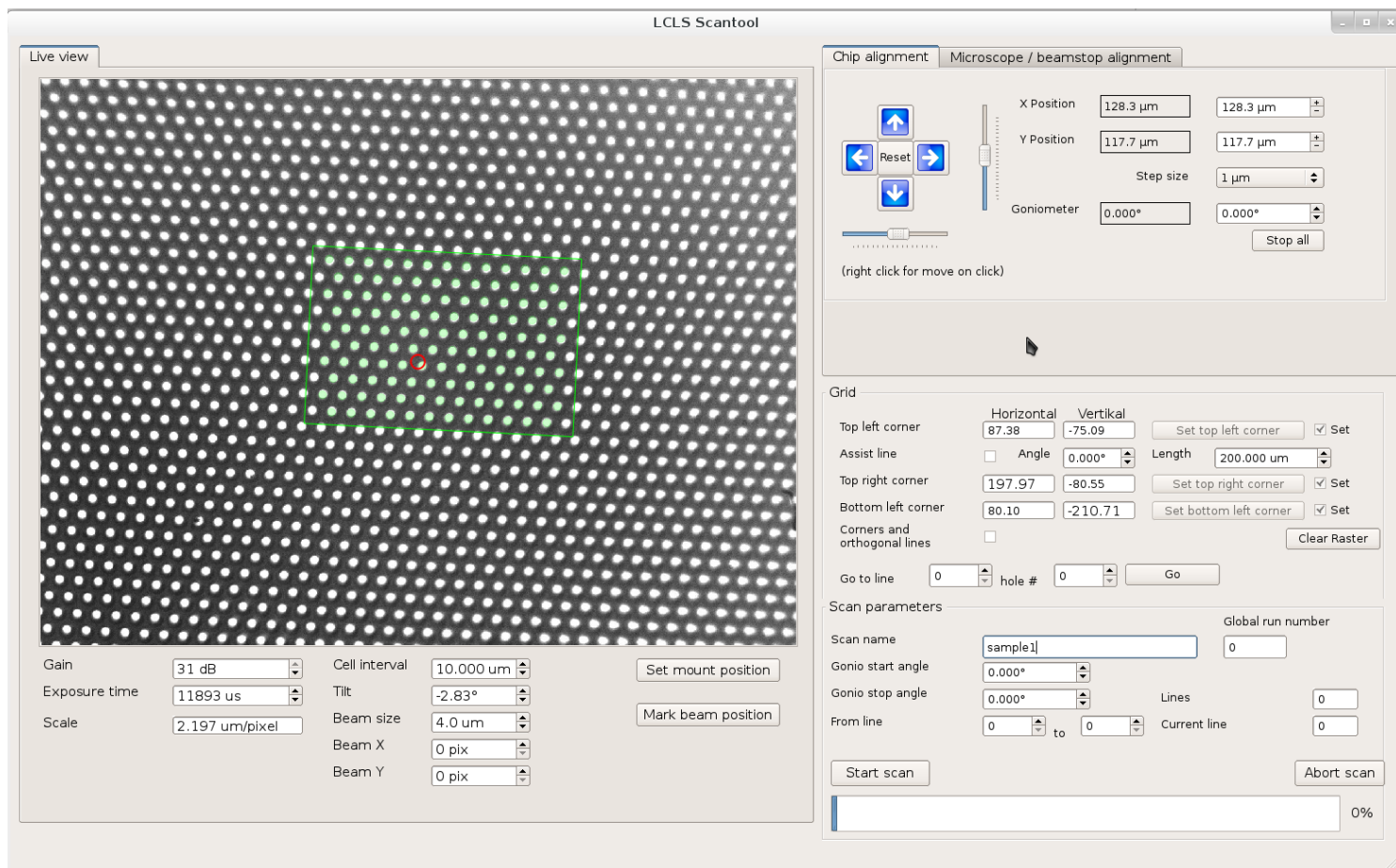
Illustration of chip loading A drop of 2 – 3  $\mu\text{l}$  of crystal suspension is applied onto the upper side of the membrane part (A). The mother liquor fills the microchannels and forms a meniscus on the lower side (B). By approaching with a wedge of filter paper from the bottom side, the mother liquor is soaked through the pores (C). Crystals larger than the pore size are retained on the upper side of the chip and arrange themselves according to the pore pattern (D). Dehydration of the crystals is prevented by a humidified gas stream. Image adapted from Ref. 9, licensed under CC BY 4.0.



**Figure 3**

Microscope image of cubic-shaped microcrystals aligned on the chip after sample loading For most part, the crystals arrange themselves according to the micropore pattern.





**Figure 4**

Control software for fixed-target serial femtosecond crystallography The tool allows full control of the Roadrunner goniometer for chip alignment and scanning. The camera image of the inline-viewing microscope shows the backside of a mounted chip with its hexagonal micro-pore pattern clearly visible. The user can define a rectangular scanning grid of arbitrary size and orientation for data collection. Scanning grid points are indicated by green circles and should superimpose on the micro-pore pattern. The motion controller of the high-precision piezo scanning stages is synchronized to the repetition rate of the XFEL to ensure that the X-ray pulses coincide with the scanning grid points.

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