Nano World Journal
https://doi.org/10.17756/nwj.2018-060
Research Article
Open Access

Langmuir-Blodgett Protein Multilayer Nanofilms by XFEL

Eugenia Pechkova[1, 2*] and Claudio Nicolini[2-6]

[1]*Laboratories of Biophysics and Nanotechnology, Department of Experimental Medicine, University of Genova Medical School, Italy*
[2]*Fondazione EL.B.A. Nicolini, Pradalunga, Bergamo, Italy*
[3]*School of Molecular Sciences, Arizona State University, Tempe, AZ, USA*
[4]*NanoWorld High Tech LLC, Tempe, AZ, USA*
[5]*Foreign Member, Russian Academy of Sciences, Russian Federation*
[6]*HC Professor, Moscow State University, Russian Federation*

***Correspondence to:**
Prof. Eugenia Pechkova, PhD
Laboratories of Biophysics and Nanotechnology
Department of Experimental Medicine
University of Genova Medical School
Genova, Italy
E-mail: eugenia.pechkova@gmail.com

Received: December 17, 2018
Accepted: December 27, 2018
Published: December 31, 2018

Citation: Pechkova E, Nicolini C. 2018. Langmuir-Blodgett Protein Multilayer Nanofilms by XFEL. *NanoWorld J* 4(4): 48-53.

Published by United Scientific Group

Abstract

Serial femtosecond crystallography (SFX) at X-ray free electron lasers (XFELs) has created many new opportunities for protein crystallography, including radiation damage mitigation and the study of dynamics at room temperature. This field is rapidly evolving, requiring new methods of macromolecule organization into diffracting arrays, since current methods of sample preparation and delivery are often the bottleneck which limits productivity. We propose here the Langmuir-Blodgett (LB) protein nanofilm technology as a novel approach for direct "on chip" protein molecules organization into the 3D ordered diffracting arrays. The combination of this advanced technology with the XFEL for fixed target SFX has the potential to become an important tool for the structure determination of proteins that are difficult to crystallize, such as membrane proteins of life science interest and with pharmaceutical industry impact.

Keywords

Serial femtosecond crystallography, X-ray free electron lasers, Photolithography masks

Introduction

SFX by XFELs has now become established as an important tool for the structure determination of proteins, as well as for time-resolved structural studies in which radiation damage must be avoided [1-3]. A number of sample delivery approaches have been developed for SFX such as liquid jets [4], whose disadvantages include the clogging of the nozzle by larger crystals, which have to be filtered prior to experiment, large sample consumption, and low hit rates (on the order of 1-10%). Better results are obtained with 'tooth paste'-like lipidic cubic phase (LCP) jets [5, 6]. However, delicate crystals may be damaged from the pressures and shear forces of the delivery process itself, and neither method is suitable for few-layer crystals. Despite these disadvantages, the liquid and LCP injectors are currently indispensable for studies on proteins in liquid environments, in particular for time-resolved studies down to the femtosecond scale.

Another method for fast sample delivery at XFELs involves the use of fixed substrates, which are mounted on mechanical translation stages and raster-scanned through the beam. Solid substrates are more appropriate to serial crystallography of static samples for high throughput data collection. The micro-patterned

structure of the chip facilitates automatic measurements by raster scanning of the individual wells of the chip, where the crystals are arranged in a periodic array. The technology uses photolithography masks and includes a number of approaches, among them: (1) silicon dice with thin silicon nitride (Si_3N_4) membrane windows (also commercially available from Silson, Inc. or similar), recently used also for 2D crystal analysis [7-9]. XFEL data collection in transmission geometry through ultrathin Si_3N_4 membranes allows an extremely low scattering background which is ideal for nanocrystals scattering experiments; (2) silicon or Si_3N_4 mesh sealed with a polyimide film in order to prevent the crystals from dehydration, designed for room temperature measurements in a defined atmosphere. Depending on the dimensions the chip can harvest several hundreds of crystals [10, 11]; (3) microchip from single crystalline silicon with a periodic structure of micropores designed to work at cryogenic temperatures [12], but also used at room temperature [13, 14] and serves as sample holder for several thousand microcrystals. This fixed target system, specifically designed for high throughput data collection at LCLS and allows very low background data collection of X-ray data at 120 Hz. Moreover, fixed target chip can be used with goniometer-based experiments [15]. Alternatively, several methods were developed to grow the protein crystal *in situ* using array-like plates, including microfluidic devices [16].

The main drawback of fixed target approaches is an increase in background scattering that arises from the relatively thick (>50 nm) device material layer that can interfere with weak diffraction patterns. Indeed, Si_3N_4 or graphene has an ultralow scattering backgrounds. Single crystalline Si-substrates of about 10 micron thickness used in microchips with etched holes have in principle also a low scattering background but scattering from the holes and Bragg peaks appearing during oscillation experiments could obstacle the data collection. Another problem is that the crystals have to be removed from their native-growth condition. Moreover, alignment and centering procedures become challenging for microcrystals, where optical refraction effects in the material surrounding the crystals can shift the apparent position of the crystal and further decrease signal-to-noise ratio.

The general disadvantage of both liquid injectors and fix target approaches is the bottleneck of protein crystallization, still unsolved. Novel methods of the protein molecule ab initio organization into the 3D ordered diffracting array have to come to the forefront. LB protein nanotechnology can offer the elegant solution to this problem.

The aim of this research is to overcome the drawbacks of existing methods of the sample preparation and delivery. We suggest the LB protein multilayer nanofilms on the Si_3N_4 chip surface as a fixed target for femtosecond XFEL study. The specific advantages of the LB protein multilayer films with respect to other solid substrates are following: (1) utilization of highly ordered protein LB multilayers for diffraction data collection could bypass the bottleneck of protein crystallization which is leaving still unsolved large part of important proteins, like the membrane ones; (2) possibility to study of much smaller crystallites; (3) due to high stability of LB films, multilayers long-range order can be increase by heating and cooling technique; (4) the experiments can be done in dry atmosphere without humidification or in vacuum using single sample for whole data collection.

For this proof of principle, we have used phycocyanin protein, a potential drug for cancer treatment [17]. The optimal technology for phycocyanin LB film deposition and XFEL data collection in transmission will therefore be defined.

Materials and Methods

Phycocyanin protein

Phycocyanin protein was the courtesy of Dr. Raimund Fromme, ASU. The native protein was isolated from cyanobacteria *Thermosynechococcus elongatus* as described in [18].

Thermosynechococcus elongatus cells were preprocessed with a microfluidizer to break the cell walls. This was followed by a series of centrifugation cycles to isolate the thylakoid membrane. Phycobiliproteins such as phycocyanin and allophycocyanin were isolated by ultracentrifugation of the supernatant obtained after microfluidizer treatment. Cell debris and larger particles were spun down at 50,000 g for 1 h. The supernatant was concentrated using Centricon spin filters with a molecular-weight cutoff of 100 kDa to obtain concentrated protein at concentration of 50 mg ml^{-1} [18].

Langmuir-Blodgett (LB) protein nanofilm technology

Developed over the last decade appears to be a break-through technology for the structural proteomics [19, 20]. The method consists in the bringing the protein molecules to the air-water interface of the LB trough, compression of the created monolayer by means of Teflon barriers up to a surface pressure corresponding to the highly packed and ordered system, deposition of the resulting monolayer using Langmuir-Blodgett (vertical lift) or Langmuir-Schaefer, LS (horizontal lift) method to the solid substrate. A number of proteins, not crystallizable by classical methods, were successfully crystallized by the LB homologous protein LB nanotemplate method with a reduced number of trials and under standard supersaturation condition. The submicron crystals obtained by this method appear to be exceptionally stable to highly intense synchrotron radiation and their structure is highly ordered, analogous to crystals grown in earth orbit, as confirmed by clustering analysis and molecular dynamics. An LB film with its exceptional properties (long range order, thermal and storage stability) allows one to create the same pattern in three dimensional crystals, as confirmed by Atomic Force Microscopy (AFM) [21] and Grazing Incidence Small Angle Scattering (microGISAX) [22]. LB multilayers can be created by the same method, by multiple consequent deposition of the protein monolayers.

Highly ordered LB phycocyanin multilayers (20 layers) were deposited onto the Si chip with the thin (200 nm) Si_3N_4 windows directly for data collection. LB multilayers

of phycocyanin (20 monolayers) were deposited on the Si_3N_4 chips of 10 x 10 mm^2 with the membrane window of dimension of 5 x 5 mm^2 and thickness of 200 nm (supplied by Silson Ltd, Warwickshire, England). LB nanofilms were prepared by Nima 611 LB trough with a Teflon bath (Figure 1). Optimal conditions of the ordered film formation were identified (sub-phase composition, barriers' speed, surface pressure of deposition). The protein solution was spread onto the subphase surface by the Hamilton syringe. The Silson slide was firmly kept by the vacuum gadget (Hampton research, California, USA, HR8-098). Every deposited monolayer was dried by the nitrogen flux prior to next deposition.

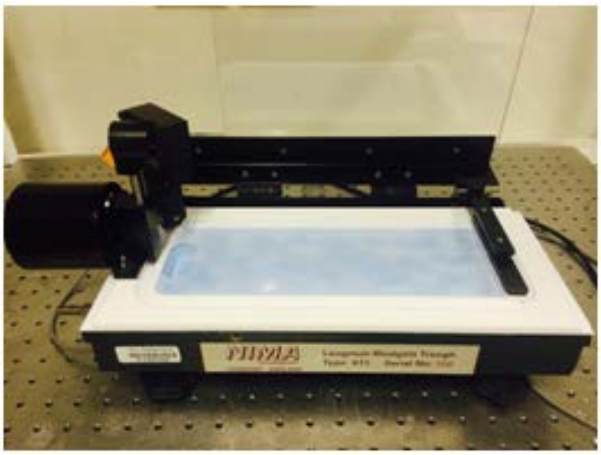

Figure 1: Langmuir-Blodgett Nima 611 Trough with the phycocyanin protein deposited on the water surface of the teflon bath.

Long-range ordered LB protein multilayers after heating and cooling

Previous synchrotron radiation and scanning tunneling microscopy studies confirm the exceptional long-range order and thermal stability of the protein LB multilayers. The synchrotron μGISAX and diffraction studies of protein LB multilayers film show that heating of the protein LB multilayered film, deposited onto the 5 x 5 mm Si chip, up to 150 °C and subsequently cooling it to the room temperature leads to a quite more ordered arrangement, as demonstrated in [23]. Micro-GISAX measurements resulted in new insights previously unforeseeable on the type of reorganization taking place within the LB multilayers, suggesting a possible model for the reorganization of the structures in the film, in terms of higher packing and long-range order. As previously confirmed, the in-plane correlation length indeed decreases with heat, compatibly with the merging of layers. Decrease of in-plane correlation length is likely due to the loss of water caused by LB formation and the heating procedure. The dramatic increase of long-range order in the LB multi-layered enzyme films after heating and cooling, made previously apparent by μGISAX was studied by powder X-ray diffraction beamline (ID11 beamline at ESRF) result in clear appearance in 2D and 3D space respectively of the diffraction peaks typical of the corresponding protein crystal structure (2.6 Å -10 Å resolution in d-spacing) [24].

For this study, the multilayered LB films (20 layers) were divided in two sample groups: the first group was incubated at 20 °C, while the second group was heated up to 150 °C for 10 minutes (in the pre-heated oven), and then cooled down to the room temperature in order to increase the long-range order. The AFM analysis of heated and unheated samples will be presented in details elsewhere [25].

XFEL data collection

To warrant fixed target chip mounting the Si chips with frame dimensions of 10 x 10 mm^2 and Si_3N_4 membrane window dimensions of 5 x 5 mm^2 and thickness 200 nm, compatible with the Roadrunner II goniometer at Macromolecular Femtosecond X-ray Crystallography (MFX), were glued onto an aluminum frame that can be placed on the goniometer using a magnetic mount (Figure 2). Before mounting, the chips were already covered with multilayered LB film. After the chip is mounted onto the goniometer, it was rotated edge-on in the in-line camera view, and moved into the X-ray interaction region (and center of rotation of the microdiffractometer). As dehydration is not the issue for the LB dry films, no stream of humidified helium or similar procedure is needed. However, the humidity sensors were used to measure the humidity during the experiment.

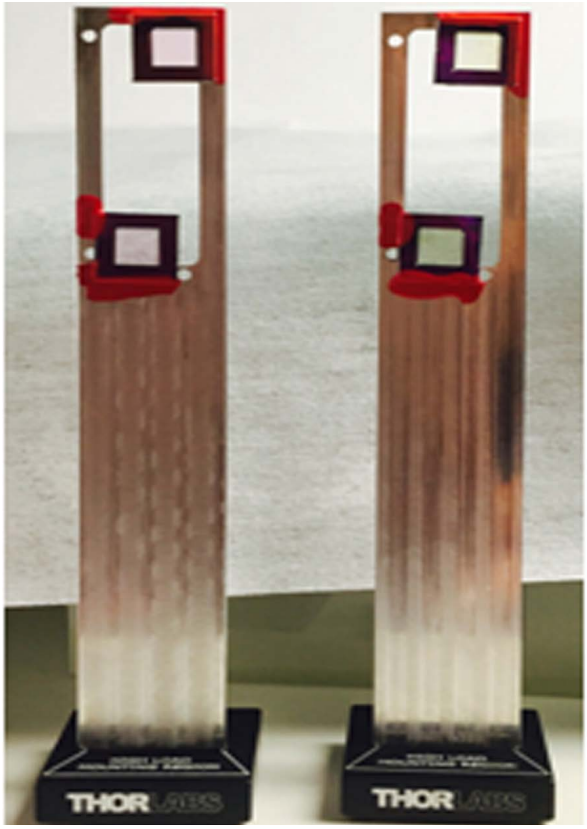

Figure 2: Silson Si chips with Si_3N_4 membrane windows covered with LB protein (phycocyanin, left and photosystem I, right, for comparison) multilayers, glued onto an aluminum frame to be placed on the goniometer using a magnetic mount. The blue phycocyanin and green PSI color can be clearly through Si_3N_4 membrane windows. On the same frame, heated and unheated protein nanofilms samples were mounted.

For data collection, Roadrunner II goniometer at the MFX instrument with CSPAD frame rate of 120 Hz was

used. The detector distance was 313 mm. We used 6 - 9 keV, ~ 20 fs X-ray pulses, and 1 x 2 μm^2 beam size for LB multilayers. Data collection modes for SFX will include raster scanning (automated translation of the sample between exposures).

All data collection was performed at the room temperature.

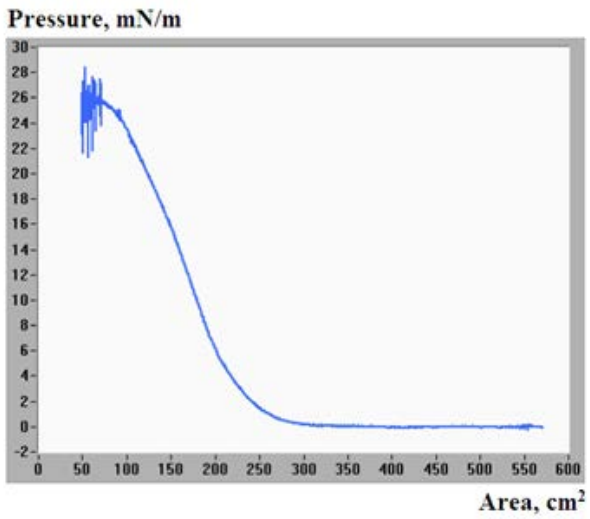

Figure 3: LB isotherm of phycocyanin protein. Several monolayers depositions (five) were performed at the surface pressure of 26 mN/m.

Results and Discussion

Langmuir Blodgett (Figure 1) isotherms of phycocyanin were studied in order to find optimal conditions for multilayers deposition. The optimal subphase composition was determined as following buffer: 0.05 M MES, 20 mM $MgCl_2$, pH 6.5. The barrier speed has to be rather high in order to obtain protein monolayer on the surface (100 cm^2 /min).

We found that surface pressure of 26 mN/m corresponds to the highest point of the LB isotherms before the monolayer breakage, therefore this surface pressure was used for highly packed phycocyanin multilayers (20 layers) deposition on the Si_3N_4 chips (Figure 3).

The preliminary XFEL diffraction results were obtained by MFX instrument, LCLS at SLAC, entirely confirmed the feasibility of the proposed pioneer experiments.

Two types of samples were analyzed: LB film of 20 monolayers kept at room temperature (Phyco 20 rt) and LB film of 20 monolayes heated up to 150 °C for 10 min and cooled down to the room temperature (Phyco 20 150).

At the beginning, the empty Si_3N_4 chip was scanned by the Roadrunner system with 2 microns-sized beam and the step of 90 microns in order to study the stability of the Si_3N_4 membrane to the XFEL beam. Even with 100% X-ray transmission, the Si_3N_4 membrane remained stable and not destroyed by XFEL beam (Figure 4A). This result is important, since taking into account the low number of protein multilayers, the X-ray transmission should be very high for this type of experiments.

After this, Si_3N_4 chips covered with the phycocyanin LB multilayered films were analyzed, as summarized in the Table 1. We were able to collect 22920 images from room temperature LB film sample and 22080 images from heated up to 150 °C and cooled LB film sample, which remained stable after this experiment (Figure 4B). The cracks on Si_3N_4, visible at the figure 4, had appeared during the scanning experiment (toward the end), and they are apparently due to the XFEL pulses. It is worth to notice that at the beginning of the experiment no cracks were observed. At the end of the experiment all slide was covered with these rather regular cracks, which however do not significantly affect the data collection (Table 1).

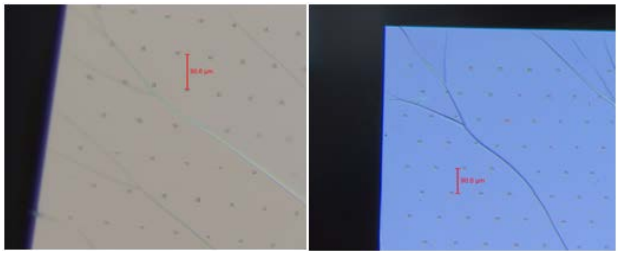

Figure 4: (A) XFEL scanning of the Si_3N_4 membrane of 200 nm thickness. **(B)** XFEL scanning of the phycocyanin LB multilayered film (20 monolayers), heated up to 150 °C and cooled down to the room temperature. The distance between scanning points is about 90.6 micron.

It is worth to notice, that the LB films of the phycocianin, heated to the 150 °C maintain the same blue color as those kept at the room temperature (similarly, PSI LB multilayers remain green after heating and cooling).

At MFX, novel auto-detect and shoot mode permits automatically identify crystal positions by image recognition software and then this position is exposed to a single X-ray pulse. In order to obtain full data sets, the sample holder can be tilted (at least ± 45°) to collect oscillation series, using an attenuated beam. Single-shot diffraction patterns from the protein crystals (Figure 5) will be analyzed using CrystFEL [26]. Oscillation series can be analyzed with conventional crystallographic software.

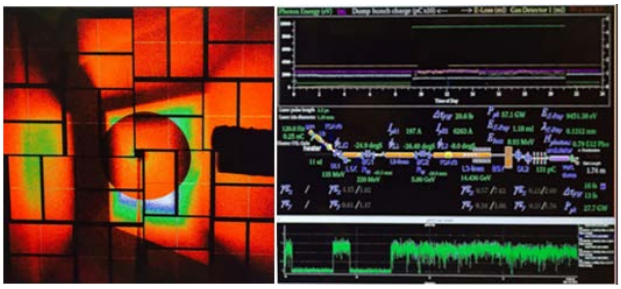

Figure 5: XFEL data collection at LCLS from protein LB multilayers. **(A)** XFEL diffraction pattern of phycocyanin multilayers and **(B)** XFEL data collection control panel at MFX, LCLS, SLAC.

Conclusions

Diffraction data from the LB protein multilayered samples can be collected with 100% X-ray transmission, without any damage of the surrounding sample area. In

Table 1: Data collection from the LB phycocyanin multilayered films.

S*	Duration (sec)	X ray transmission %	T °C	Humidification (%)				Energy (eV)	Freq (Hz)	Puls E (mJ)	Puls Duration (fs)
				Sens 1	Sens 2	Sens 3	Sens 4				
1	2:55	0.5-100	24.2	24.9	25.52	24.61	25.67	9456	120	1.6	20
2	3:11	5-100	24.2	24.9	25.52	24.61	25.67	9456	120	1.6	20
3	2:27	5-40	24.2	24.9	25.52	24.61	25.67	9456	120	1.6	20
4	37	62	24.2	24.9	25.52	24.61	25.67	9456	120	1.6	20

*Sample number

(1) Si_3N_4 window 200 nm thickness

(2) 20 layers of phycocyanin, room temperature

(3) 20 layers of phycocyanin, heated to 150 °C and cooled to room temperature

(4) 20 layers of phycocyanin, heated to 150 °C and cooled to room temperature

principle, the proposed nanotechnology allows highly ordered LB film formation for any type of protein. Thus, diffracting 3D protein arrays of any given protein could be ab initio produced, avoiding the bottleneck of protein crystallization. The same experiments can be also done by CXI (Coherent X-Ray Imaging) instrument at LCLS with the advantage of the vacuum atmosphere and consequently, without air scattering [27]. Further combination of LB nanotechnology with ad hoc designed and constructed fixed target chips, including those of advanced nanomaterials (e.g. graphene) could result in generalized procedure of protein sample preparation and delivery at XFEL, significantly reducing time and material consumption for optimal results in routinely successful protein structure resolution by SFX. The comprehensive interpretation of obtained crystallographic data can be done by Monte Carlo integration of the high-quality diffraction intensities, from which experimental phases can be determined [28], resulting in an experimental electron density map good enough for automated building of novel protein structure, can open a new avenue in structural proteomics.

Acknowledgements

This work was performed during Eugenia Pechkova's sabbatical leave at Arizona State University. The authors appreciate the help of group of Prof. Petra Fromme at Biodesign Center for Applied Structural Discovery, Arizona State University and of Prof. John Spence, Department of Physics, Arizona State University for the XFEL experiment and LB instrumentation, correspondingly. We also would like to thank Dr. Raimund Fromme for his courtesy of supplying of phycocianin protein.

References

1. Fromme P, Spence JC. 2011. Femtosecond nanocrystallography using X-Ray lasers for membrane protein structure determination. *Curr Op Struct Biol* 21(4): 509-516. https://doi.org/10.1016/j.sbi.2011.06.001
2. Chapman HN, Fromme P, Barty A, White TA, Kirian RA, et al. 2011. Femtosecond X-ray protein nanocrystallography. *Nature* 470(7332): 73-77. https://doi.org/10.1038/nature09750
3. Kupitz C, Basu S, Grotjohann I, Fromme R, Zatsepin NA, et al. 2014. Serial time-resolved crystallography of photosystem II using a femtosecond X-ray laser. *Nature* 513(7517): 261-265. https://doi.org/10.1038/nature13453
4. Weierstall U, Doak RB, Spence JCH, Starodub D, Shapiro D, et al. 2008. Droplet streams for serial crystallography of proteins. *Exp Fluids* 44(5): 675-689. https://doi.org/10.1007/s00348-007-0426-8
5. Liu W, Wacker D, Gati C, Han GW, James D, et al. 2013. Serial femtosecond crystallography of G protein-coupled receptors. *Science* 342(6165): 1521-1524. https://doi.org/10.1126/science.1244142
6. Weierstall U, James D, Wang C, White TA, Wang D, et al. 2014. Lipidic cubic phase injector facilitates membrane protein serial femtosecond crystallography. *Nat Commun* 5: 3309. https://doi.org/10.1038/ncomms4309
7. Frank M, Carlson DB, Hunter MS, Williams GJ, Messerschmidt M, et al. 2014. Femtosecond X-ray diffraction from two-dimensional protein crystals. *IUCrJ* 1(Pt 2): 95-100. https://doi.org/10.1107/S2052252514001444
8. Casadei CM, Tsai CJ, Barty A, Hunter MS, Zatsepin NA, et al. 2018. Resolution extension by image summing in SFX of two-dimensional membrane-protein crystals. *IUCrJ* 5(1): 103-117. https://doi.org/10.1107/S2052252517017043
9. Hunter MS, Segelke B, Messerschmidt M, Williams GJ, Zatsepin NA, et al. 2014. Fixed-target protein serial micro crystallography with an x-ray free electron laser. *Sci Rep* 4: 6026. https://doi.org/10.1038/srep06026
10. Zarrine-Afsar A, Barends TR, Müller C, Fuchs MR, Lomb L, et al. 2012. Crystallography on a chip. *Acta Cryst D* 68(Pt 3): 321-323. https://doi.org/10.1107/S0907444911055296
11. Murray TD, Lyubimov AY, Ogata CM, Vo H, Uervirojnangkoorn M, et al. 2015. A high-transparency, micro-patternable chip for X-ray diffraction analysis of microcrystals under native growth conditions. *Acta Crystallogr D Biol Crystallogr* 71(Pt 10): 1987-1997. https://doi.org/10.1107/S1399004715015011
12. Roedig P, Vartiainen I, Duman R, Panneerselvam S, Stübe N, et al. 2015. A micro-patterned silicon chip as sample holder for macromolecular crystallography experiments with minimal background scattering. *Sci Rep* 5: 10451. https://doi.org/10.1038/srep10451
13. Roedig P, Ginn HM, Pakendorf T, Sutton G, Harlos K, et al. 2017. High-speed fixed-target serial virus crystallography. *Nat Methods* 14(8): 805-810. https://doi.org/10.1038/nmeth.4335
14. Roedig P, Duman R, Sanchez-Weatherby J, Vartiainen I, Burkhardt A, et al. 2016. Room-temperature macromolecular crystallography using a micro-patterned silicon chip with minimal background scattering. *J Appl Crystallogr* 49(Pt 3): 968-975. https://doi.org/10.1107/S1600576716006348

15. Cohen A, Soltis SM, González A, Aguila L, Alonso-Mori R, et al. 2014. Goniometer-based femtosecond crystallography with X-ray free electron lasers. *Proc Natl Acad Sci U S A* 111(48): 17122-17127. https://doi.org/10.1073/pnas.1418733111

16. Heymann M, Opthalage A, Wierman JL, Akella S, Szebenyi DM, et al. 2014. Room-temperature serial crystallography using kinetically optimized microfluidic device for protein crystallization and on-chip X-ray diffraction. *IUCrJ* 1(pt 5): 349-360. https://doi.org/10.1107/S2052252514016960

17. Jiang L, Wang Y, Yin Q, Liu G, Liu H, et al. 2017. Phycocyanin: a potential drug for cancer treatment. *J Cancer* 8(17): 3416-3429. https://doi.org/10.7150/jca.21058

18. Fromme R, Ishchenko A, Metz M, Chowdhury SR, Basu S, et al. 2015. Serial femtosecond crystallography of soluble proteins in lipidic cubic phase. *IUCrJ* 2(Pt 5): 545-51. https://doi.org/10.1107/S2052252515013160

19. Pechkova E, Nicolini C. 2004. Protein nanocrystallography: a new approach to structural proteomics. *Trends Biotechnol* 22(3): 117-122. https://doi.org/10.1016/j.tibtech.2004.01.011

20. Pechkova E, Nicolini C. 2017. Langmuir-Blodgett nanotemplates for protein crystallography. *Nat Protoc* 12(12): 2570-2589. https://doi.org/10.1038/nprot.2017

21. Pechkova E, Sartore M, Giacomelli L, Nicolini C. 2007. Atomic force microscopy of protein films and crystals. *Rev Sci Instrum* 78(9): 093704. https://doi.org/10.1063/1.2785032

22. Pechkova E, Gebhardt R, Riekel C, Nicolini C. et al. 2010. *In situ* μGISAXS: I. Experimental setup for submicron study of protein nucleation and growth. *Biophys J* 99(4): 1256-1261. https://doi.org/10.1016/j.bpj.2010.03.069

23. Pechkova E, Tripathi S, Nicolini C. et al. 2009. MicroGISAXS of Langmuir-Blodgett protein films: effect of temperature on long-range order. *J Synchrotron Radiat* 16(pt 3): 330-335. https://doi.org/10.1107/S0909049509002763

24. Nicolini C, John W, Pechkova E. 2015. Synchrotron Diffraction of multilayered LS PGA films after heating and cooling. *NanoWorld J* 1(1): 4-8. https://doi.org/10.17756/nwj.2015-001

25. Pechkova E, Nicolini C. 2018. Langmuir-Blodgett protein multilayer nanofilms by AFM and Cryo-EM. *Langmuir* (Submitted).

26. White TA, Kirian R, Martin AV, Aquila A, Nass K, et al. 2012. CrystFEL: a software suite for snapshot serial crystallography. *J Appl Crystallogr* 45(2): 335-341. https://doi.org/10.1107/S0021889812002312

27. Pande K, Hutchison CD, Groenhof G, Aquila A, Robinson JS, et al. 2016. Femtosecond structural dynamics drives the trans/cis isomerization in photoactive yellow protein. *Science* 352(6286): 725-729. https://doi.org/10.1126/science.aad5081

28. Kirian RA, White TA, Holton JM, Chapman HN, Fromme P, et al. 2011. Structure-factor analysis of femtosecond microdiffraction patterns from protein nanocrystals. *Acta Crystallogr A* 67(Pt 2):131-140. https://doi.org/10.1107/S0108767310050981