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TITLE:

An all-in-one sample holder for macromolecular X-ray crystallography with minimal background scattering

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KEYWORDS:

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SUMMARY:

A novel sample holder for macromolecular X-ray crystallography along with a suitable handling protocol is presented. The system allows crystal growth, crystal soaking and in situ diffraction data collection at both, ambient and cryogenic temperature without the need of any crystal manipulation or mounting.

ABSTRACT:

Macromolecular X-ray crystallography (MX) is the most prominent method to obtain high-resolution three-dimensional knowledge of biological macromolecules. A prerequisite for the method is that highly ordered crystalline specimen need to be grown from the macromolecule to be studied, which then need to be prepared for the diffraction experiment. This preparation procedure typically involves removal of the crystal from the solution, in which it was grown, soaking of the crystal in ligand solution or cryo-protectant solution and then immobilizing the crystal on a mount suitable for the experiment. A serious problem for this procedure is that macromolecular crystals are often mechanically unstable and rather fragile. Consequently, the handling of such fragile crystals can easily become a bottleneck in a structure determination attempt. Any mechanical force applied to such delicate crystals may disturb the regular packing of the molecules and may lead to a loss of diffraction power of the crystals. Here, we present a novel all-in-one sample holder, which has been developed in order to minimize the handling steps of crystals and hence to maximize the success rate of the structure determination experiment. The sample holder supports the setup of crystal drops by replacing the commonly used microscope cover slips. Further, it allows in-place crystal manipulation such as ligand soaking,

cryo-protection and complex formation without any opening of the crystallization cavity and without crystal handling. Finally, the sample holder has been designed in order to enable the collection of in situ X-ray diffraction data at both, ambient and cryogenic temperature. By using this sample holder, the chances to damage the crystal on its way from crystallization to diffraction data collection are considerably reduced since direct crystal handling is no longer required.

INTRODUCTION:

The knowledge of the three-dimensional structure of biological macromolecules constitutes an important cornerstone in all basic biological, biochemical and biomedical research. This even extends to certain translational aspects of such research, such as for instance drug discovery. Among all methods for obtaining such three-dimensional information at atomic resolution X-ray crystallography is the most powerful and the most prominent one as is evidenced by the fact that 90% of all available structural information is contributed by X-ray crystallography[1]. The major prerequisite of X-ray crystallography, which is at the same time its major limitation, is that diffraction-quality crystals have to be produced and prepared for the diffraction experiment. This step still constitutes one of the major bottlenecks of the method.

Historically, diffraction data from protein crystals were collected at ambient temperature. Individual crystals were carefully transferred into glass or quartz capillaries prior to data collection, mother liquor was added to the capillaries so that the crystals would not dry out and the capillaries were sealed[2–4]. Since the 1980s, it became more and more apparent that due to the ionizing properties of X-radiation and the imminent radiation sensitivity of macromolecular crystals, data collection at ambient temperature poses severe limitations on the method. Consequently, approaches were developed to mitigate radiation damage effects by cooling macromolecular crystals down to 100 K and to collect diffraction data at such low temperature[5,6]. For working at low temperatures, the mounting of the samples in capillaries became impractical due to the low rate of heat transfer. In spite of this, there are ongoing efforts to also use capillaries, in particular from counter-diffusion crystallization experiments, for low-temperature diffraction work[7,8], but, irrespective of that, it became the standard approach in macromolecular crystallography to mount macromolecular crystals held by a thin film of mother liquor inside a thin wired loop[9,10]. Even though a number of improvements (e.g., the introduction of lithographic loops and similar structures[11]) have been made over time to this loop-based mounting, the basic principles that were developed in the early 1990s are still in use today. It may be safely stated that most diffraction data collections on macromolecular crystals nowadays still rely on this approach[5].

Over time, there were some interesting new developments and modifications of the loop-based mounting method, but these approaches have so far not been widely adopted in the community. One is the so-called loop-less mounting of crystals, which was developed to achieve lower background scattering[12–14]. Another one is the use of graphene sheaths to wrap the crystalline samples and to protect them from drying out. Graphene is a well-suited material in that respect because of its very low X-ray scattering background[15].

More recently, developments in the field of sample mounts were mainly focused on

standardizing the mounts with the aim of increasing sample throughput[16] or on designing mounts, which can hold more than one sample[17], such as for instance patterned membranes on a silicon frame, which are capable of holding hundreds of small crystals mostly in the field of serial crystallography[18–22].

All of the sample mounting methods discussed so far still require some degree of manual intervention, which means that there is an inherent danger of causing mechanical damage to the sample. Therefore, novel approaches are being sought by engineering the sample environment such that diffraction data of crystals can be collected within their growth environment. One such method is termed in situ or plate-screening[23,24] and it is already implemented at a number of macromolecular crystallography beamlines at various synchrotron sources worldwide[25]. However, the use of this method is limited by the geometrical parameters of the crystal plate and the space available around the sample point of the instrument.

Yet another approach is realized in the so-called CrystalDirect system[26]. Here, entire crystallization drops are harvested automatically. The foils on which the crystals have been grown are custom-cut using a laser and directly used as the sample holder[27].

In the work described here, the aim was to develop a sample holder, which would allow a user to move the crystalline sample from its growth chamber to the data collection device without touching it and which would enable the user to manipulate the sample easily. Since many researchers in the field of macromolecular crystallography are still using the 24-well crystallization format for optimizing crystal growth by modifying conditions identified in large screening campaigns, the new sample holder was designed to be compatible with this format. In the following, the design of the new sample holder will be described and the handling and the performance of the sample holder for in situ data collection and ligand soaking will be demonstrated. Finally, the suitability of this new sample holder as well as its limitations for the various work steps will be discussed.

PROTOCOL:

CAUTION: For all subsequent work, it is very important that the yellow-colored polyimide foil must not be touched with unprotected fingers, because of possible contaminations to the sample holder. Also, the usage of protected forceps is highly recommended.

1. The sample holder

1.1. Use one of the three types of sample holder.

NOTE: Three different versions of the new developed sample holder are shown in **Figure 1**. All of them contain a black plastic support structure, an airtight COC foil on the outside and a microporous structured polyimide foil on the inside. Type 1 (**Figure 1A**) contains a fixed outer plastic ring, whereas for types 2 and 3 (**Figure 1B,1C**) the outer ring can be broken off mechanically at the designated respective break points for use in automated sample transfer

systems (see red arrows in **Figure 1B**). The design of the sample holders allows the setup of multiple crystallization drops on the yellow polyimide foil. It does not compromise the monitoring of crystallization experiment, as the material is highly transparent for visible light. The 21 µm thick polyimide foil also features 5 µm pores, which allows simple crystal manipulation by soaking later on. Since the transmission of X-rays is close to 1.0 at all commonly used diffraction data collection energies in macromolecular crystallography, the contribution of the foil to the background scattering in a diffraction experiment is negligible[28].

2. Setting up crystallization drops

2.1. Create a clean and dust-free surface using a damp lint-free cloth. Take one sample holder from its box and gently place it, yellow foil facing up, on the cleaned surface to avoid damage or unwanted puncture of the backside COC foil.

2.2. Set up the crystallization drops with a maximum recommended volume of 2 µL on the yellow foil as it would be done on commonly used cover slides. Place the drops gently to avoid any rupture or piercing of the foil using a pipette. On a sample holder of type 1 (**Figure 2A**) up to three drops can be placed, whereas on sample holders of type 2 and 3 two drops are the recommended maximum (**Figure 2C**).

2.3. Flip the sample holder over and place it onto a pre-greased cavity of a 24-well Linbro style plate. Use the positioning aids (see red arrows in **Figure 1A**) of the sample holder to guide it to its optimal position.

2.4. Ensure the correct position of the sample holder in order to avoid unwanted evaporation (**Figure 2A**).

3. Observing crystal growth

3.1. By placing the crystallization plate under a transmission light microscope, with or without polarizers, monitor crystal growth without any disturbance of the experiment (**Figure 4**).

3.2. When using the smaller 18-mm sample holders of type 3 (**Figure 1C**), which were designed for use on SBS footprint plates, use an imaging robot capable of handling SBS-footprint plates to monitor the crystal growth in a more automated way.

4. Crystal manipulation

NOTE: It is recommended to perform all subsequent steps under a transmission light microscope.

4.1. Cryo-protection

4.1.1. Gently pierce the outer COC foil using a fine cannula. Make sure the inner yellow foil remains untouched. The puncture should be right next to the drop that is to be manipulated

(**Figure 3A,3C**).

4.1.2. Use a fine paper wick and insert it in the poked hole. Carefully push the wick forward until it touches the yellow polyimide foil. Keep the wick in contact with the perforated foil. The wick will suck away all excess solution. The time required for complete liquid removal depends on the viscosity of the solutions and the mother liquor composition **(Figure 3B**).

4.1.3. After all liquid is sucked away, gently retract the paper wick. Remember the position of the drop, since it may not be visible after removal of the mother liquor.

4.1.4. Take a standard pipette to apply a small volume of cryo-protectant solution, max. 3 µL, using an extruded tip (e.g., a gel loading tip) through the same hole. Once the liquid is dispensed, retract the tip. The porosity of the yellow foil allows for diffusion across the foil. The time to attain cryo-protection of your crystals highly depends on the employed solution and its components.

4.1.5. To reseal the self-healing COC foil, gently place a protected finger on the hole for about 1 s and slide it across the puncture. The slight pressure in combination with the elevated temperature will promote the resealing of punctures, which are not too large.

4.2. Ligand soaking

NOTE: Excess mother liquor may be removed before ligand soaking. To do so, follow the steps described in 4.1.1 to 4.1.3.

4.2.1. Dissolve the ligand in mother liquor in the desired concentration in a reaction tube.

4.2.2. Spin the solution for 10 minutes at 12,000 x *g* in order to remove insoluble particles. Use a temperature-controlled centrifuge if needed.

4.2.3. Gently place a volume of max. 3 µL of ligand containing solution in the gap between the COC foil and the polyimide film using a long-extruded pipet tip. Retract the tip.

4.2.4. To reseal the self-healing COC foil, gently place a protected finger on the hole for about 1 s and slide it across the puncture (see also 4.1.5).

4.2.5. Incubate the experiment for some time in order to allow for diffusion across the membrane. The soaking time highly depends on the viscosity of the diffusing solution and its components[29].

4.2.6. Repeat steps 4.2.1 to 4.2.5 multiple times to subsequently soak different ligands.

5. In situ diffraction data collection at ambient temperature

NOTE: In order to minimize solvent scattering, remove excess solution before data collection.

5.1.1. Ensure a stable humidity controlled beamline environment with pre-established conditions[30].

5.1.2. Gently lift the transparent COC foil at the designated point using forceps and peel it off like one would remove the lid from a yogurt cup (**Figure 6B**).

5.1.3. Gently lift the sample holder from its cavity and insert it immediately into a pre-prepared magnetic sample holder base. No glue is necessary for this step (**Figure 6B**).

5.1.4. Apply gentle pressure to ensure the correct positioning of the sample holder within the base.

5.1.5. Mount the sample holder on a beamline goniometer and ensure correct positioning of the holder. Depending on goniometer geometry the sample holder can be rotated by up to 160° without causing any shadowing during the diffraction experiment.

5.1.6. Use a paper wick and gently touch the yellow polyimide foil from the backside to remove excess mother liquor. Please note, that at that stage ligand soaking or cryo-protection may be performed just as well. The sample is now ready for centering and diffraction data collection.

5.1.7. When using a sample holder with removable outer ring, apply gentle pressure by holding on to the outer ring and break it off at the designated break points (**Figure 6C**). The sample is now ready for centering and diffraction data collection.

6. In situ diffraction data collection at cryogenic temperature

NOTE: It is recommended to remove residual mother liquor from the sample by performing the steps 4.1.1. to 4.1.3. before continuing with the next steps to minimize solvent scattering. Most samples may be transferred to liquid nitrogen without prior cryo-protection[31]. If cryo-protection is needed, see steps 4.1.1. to 4.1.5.

6.1.1. Gently lift the COC foil at the designated point using a forceps and peel it off (see step 5.1.2) (**Figure 6A**).

6.1.2. Take the sample holder off the cavity and mount it on a magnetic sample holder base. Gentle pressure may be applied in order to ensure correct and tight fitting (see step 5.1.5, **Figure 6B**).

NOTE: The symmetrically arranged designated break points allow for simple removal of the outer ring of the sample holder by applying gentle pressure (see step 5.1.8., **Figure 6C**). Now, the sample holder is ready and can be plunged into liquid nitrogen. The geometry of the sample holder types 2 and 3 (**Figure 1B,1C**) allows their transfer into standard SPINE sample vials, which

can be used for robot assisted sample mounting (**Figure 6D**).

REPRESENTATIVE RESULTS:

The sample holder type 1 has been designed so that it fits onto a well of a 24-well Linbro style plate. Each individual sample holder contains positioning aids on either side of the outer rim in order to ensure optimal positioning on the rim of the well (**Figure 1A, 2A**). Up to three individual crystallization drops of maximum volume 2 µL each can be placed onto the yellow polyimide foil (**Figure 2B**). For sample holders of types 2 and 3, it is recommended to set a maximum of two drops of maximum volume 2 µL each. 24 sample holders can be fitted onto one 24-well Linbro plate (**Figure 3D**).

A crystallization experiment on a 24-well Linbro plate using sample holder type 1 was set up. 1 µL of hen egg-white lysozyme solution (15 mg/mL) was mixed with 1 µL of mother-liquor comprising 50 mM NaAc pH 4.7, 500 mM NaCl and 25% (w/v) PEG-6000 on the yellow polyimide foil on the sample holder (**Table 1**). The drop was equilibrated at 293 K against 500 µL of mother-liquor and crystals of the size 40-50 µm were observed after 5 hours (**Figure 4**). Crystal growth can be observed using a transmission light microscope (**Figure 4**) with or without a polarizer. High transparency films ensure best observation and monitoring of crystal growing conditions using both, a conventional light microscope or an automated crystal imaging system. Crystal growth observation using UV-light was not tested.

After removing the mother liquor from around the crystals, a sample holder with hen egg-white lysozyme crystals was taken from the crystallization plate and placed in a humidity-controlled airstream on HZB-MX beamline 14.3 [32]. Diffraction data were collected at ambient temperature in 1°-increments using a 150 µm beam at 13.8 keV energy with 4 x 10^{10} photons/s and an exposure time of 5 s per image. A typical diffraction image is shown in **Figure 5**. No elevated background scattering on the diffraction image can be detected. Further experimental details as well as associated data processing statistics are listed in **Table 2**.

FIGURE AND TABLE LEGENDS

Figure 1: Schematic view of the new sample holders. The sample holders consist of a black plastic support, which is covered on the outer side with an amorphous cyclic olefin copolymer (COC) foil. This foil (colored in blue) is highly transparent and self-healing. It also ensures gas tightness of the experiment. The inner foil (colored in yellow) is made of bio-inert polyimide, which is highly transparent for X-rays. On this foil, the crystallization drops can be placed. The outer rim of the sample holder contains two positioning aids indicated by the red arrow (panel **A**), which allows accurate placement of the sample holder on the individual pre-greased cavity of the crystallization plate. (**A**) Sample holder (type 1) with 22 mm diameter with a fixed external support ring. (**B**) Sample holder (type 2) with 22 mm diameter with removable external support ring. (**C**) Sample holder (type 3) with 18 mm diameter with removable external support ring. The latter two have been developed for using them in a high-throughput fashion with automated sample mounting robots using SPINE standard. The designated break points are highlighted by the red arrows in panel **B**. The black arrow in panel **C** indicates the positioning marker. The

protruding pins at the outer perimeter of the yellow foil are necessary to align the polyimide foil during the production process.

Figure 2: **The sample holder may be used on a 24-well Linbro plate in the same way as the commonly used microscope cover slips.** It seals the cavity airtight. Positioning aids ensure the correct positioning of the sample holder on the cavity (red arrows in panel **A**). Up to three individual drops may be placed onto a type 1 sample holder (panel **B**), whereas the recommended maximum number of drops placed on a type 2 or 3 sample holder is two. The maximum recommended volume for each drop is 2 µL.

Figure 3: 24 type 1 sample holders fit on a 24-well plate. The sample holders can be placed in two orientations on the 24-well plate as indicated (panel **D**). A cannula is used to pierce the back COC foil in order to remove excess liquor from a crystallization drop (panels **A** and **C**) by using a paper wick gently inserted in the same hole (panel **B**).

Figure 4: Image of hen egg-white lysozyme crystals observed through a transmission microscope equipped with a polarizer. Individual crystals are easily discriminated from precipitated protein solution. The crystals in this image are of an average size of 40 µm x 50 µm.

Figure 5: A typical X-ray diffraction image of a lysozyme crystal grown on the sample holder. Prior to exposure to X-rays all excess mother liquor was removed from around the crystal. Diffraction data were collected at ambient temperature on BL14.3 at the electron storage ring BESSY II[32] using a humidity controlled sample environment with 97.5% relative humidity. No elevated background due to the sample holders can be observed. The dashed lines in the image indicate the resolution rings.

Figure 6: The sample holder is prepared for diffraction data collection. First, the COC film is lifted gently by using a forceps and then peeled off (panel **A**). Subsequently, the sample holder is removed from the cavity and inserted into the central hole of a magnetic base until indicated by the marker (panel **B**). By holding on to the central part, gentle pressure is applied to the outer ring to free the central part using the symmetrically arranged designated break points (panel **C**). After the removal, the sample holder can be plunged into liquid nitrogen and transferred into standard SPINE vials. Placed, for instance, in pucks they can be transported to synchrotron sites where automated sample-mounting robots recognize them as regular samples (panel **D**).

Table 1: Experimental details of the described crystallization experiment.

Table 2: Diffraction data collection and processing statistics.

DISCUSSION:

Suitability for crystallization experiments. The new sample holders can be used for standard hanging drop crystallization experiments using either 24-well Linbro type plates (types 1 and 2), or 24-well SBS footprint plates in which each well has a diameter of 18 mm (type 3). They can be used instead of the standard microscope cover slips. The amorphous COC foil ensures the

airtightness of the system. The monitoring of the crystallization experiment is possible using a transmission light microscope, because of the use of high clarity foils. To the best of our knowledge, no other sample holders exist for 24-well crystallization plates, which would allow crystal manipulation or diffraction experiments, without mechanically removing the crystal from the drop, in which it is grown. This is of particular importance, since many researchers in the field still rely on such plates for crystal optimization, due to the fact that larger drop volumes can be used compared to 96-well sitting-drop plates. With these larger drop volumes, larger crystals may be obtained.

Suitability for crystal manipulation. Due to the self-healing properties of the outer COC foil and the microporous structure of the inner yellow polyimide foil, the crystal environment is accessible and the crystals can be manipulated without mechanically transferring them to other containers. This makes the sample holders very convenient. The only other system we know of, which allows this indirect and gentle access to the crystal is the CrystalDirect system[26]. However, CrystalDirect is less flexible since special 96-well crystallization plates have to be used. The foil, on which the crystals are growing, is the same that seals the crystallization experiment and it is not self-healing. This means that an aperture that has been pierced into the foil by laser ablation for ligand or cryo-protectant delivery to the crystals will remain open, increasing the chance for liquid evaporation. In contrast to our design, crystals will not be directly exposed to the environment even if the COC foil gets pierced a number of times.

Suitability for in situ diffraction experiments at ambient temperature. The sample holder can be removed from the crystallization plate in a straight-forward manner, stuck onto a magnetic base and put on a beamline goniometer. For a diffraction experiment at room temperature, it is advisable to put the sample into an air stream of defined humidity[33]. The mother liquor around the crystal may be removed prior to putting the sample holder on the goniometer in order to reduce the scattering background. Such a set-up is stable for hours.

Suitability of the used material for operation and storage at 100 K. Neither the material used for the production of the sample holder nor the polyimide film are adversely affected by cooling them down to low temperatures[34]. Hence, working with the sample holder at low temperature (e.g., 100 K) does not pose a serious problem.

Suitability for in situ diffraction experiments at 100 K. For data collection at 100 K in a nitrogen stream, the sample holder needs to be removed from the crystallization plate as in the previous paragraph, stuck onto a magnetic base and put into a gaseous nitrogen stream at 100 K on a beamline goniometer. If desired, the sample may also be cryo-protected, although it is likely that for naked samples this may not be necessary in most cases[31]. For experiments at 100 K, the sample holders type 2 and 3 are better suited because the outer plastic ring can be removed. Hence, they are of smaller size and should therefore be less prone to icing. However, even a sample holder of type 1 may be used. Given a not too high humidity in the experimental hutch and a properly aligned cryo-system icing up of the holder is not really a problem.

Limitations. The sample holder's geometry permits unobstructed diffraction data collection by

the rotation method over a total rotation range of 160°. This is sufficient so that complete diffraction data sets can be obtained for most crystal systems. In cases where this is not possible, data from more than crystal need to be merged together. When crystals are grown together, it may be possible to adjust the size of the incident X-ray beam so that only parts of individual crystals are exposed. In extreme cases, one may need to resort to a data collection strategy similar to the MeshAndCollect approach[35]. In summary, while there are certain limitations associated with the sample holders, these can be overcome in most cases. Of course, it is always possible that situations are encountered, in which none of this is possible. In such cases, one may need to resort to other crystal mounting methods.

We have described a novel type of sample holder for macromolecular crystallography and we have demonstrated the suitability of the sample holders for various applications. Taking into account the simple and reproducible handling of protein crystals, as well as the unique properties of the sample holders, we believe that these sample holders will prove to be a valuable addition to the arsenal of sample holders for macromolecular crystallography.

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DISCLOSURES:
Patent applications regarding the reported sample holder have been filed by Helmholtz-Zentrum Berlin with the following registration numbers and registration dates with the German Patent and Trademark Office: DE 10 2018 129 125.6, registration date November 20th, 2018; DE 10 2018 125 129.7, registration date October 11th, 2018; DE 10 2017 129 761.8, registration date December 13th, 2017. A subsequent international patent application via the PCT route, using the priority of DE 10 2017 129 761.8 has been filed, PCT/DE2018/101007. A registration of a utility model with the number DE 20 2018 106 955.1 was filed on December 6th, 2018. The sample holder has been made commercially available under the trade names XtalTool and XtalTool/HT by Jena Bioscience, Jena, Germany. https://www.jenabioscience.com/crystallography-cryo-em/data-collection/xtaltool. The authors have nothing further to disclose.

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Figure 1

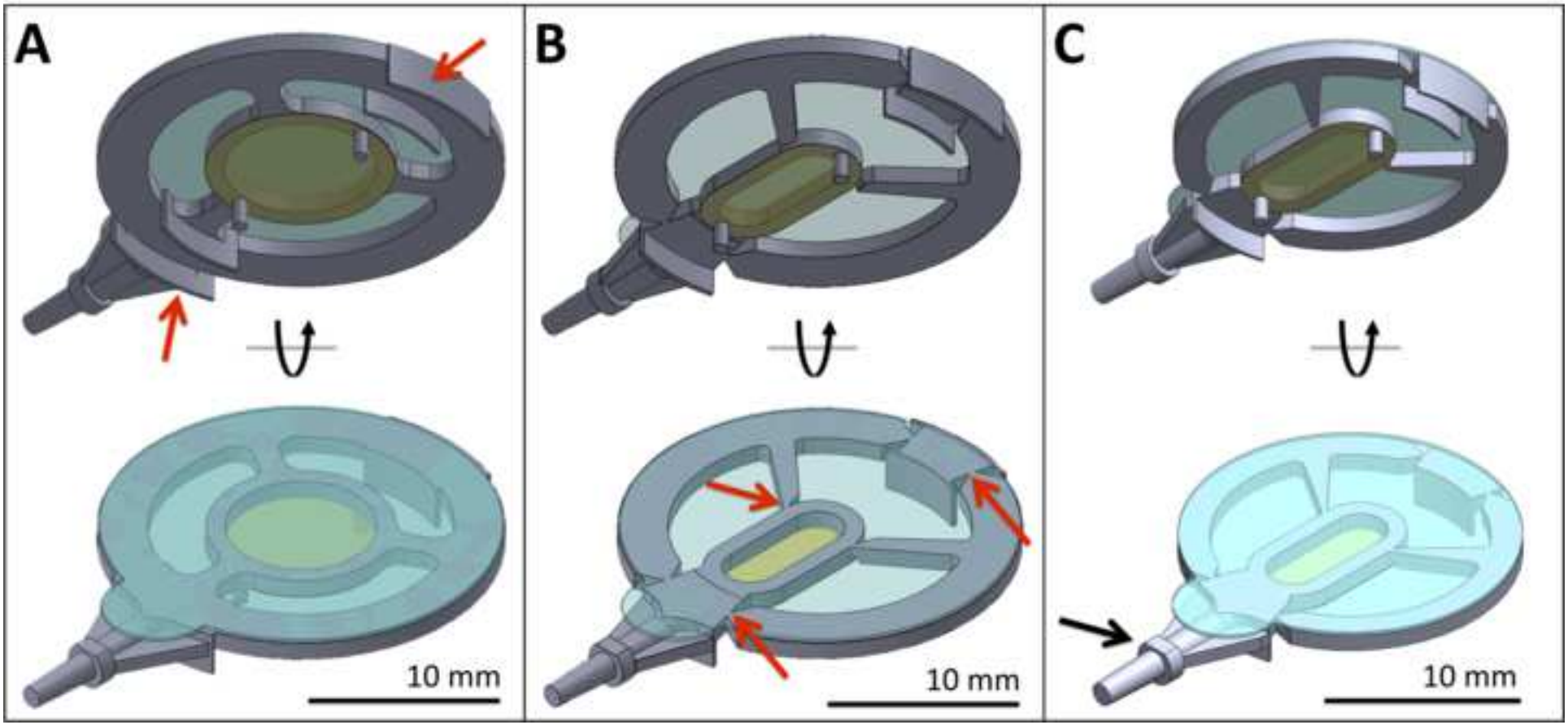

Figure 2

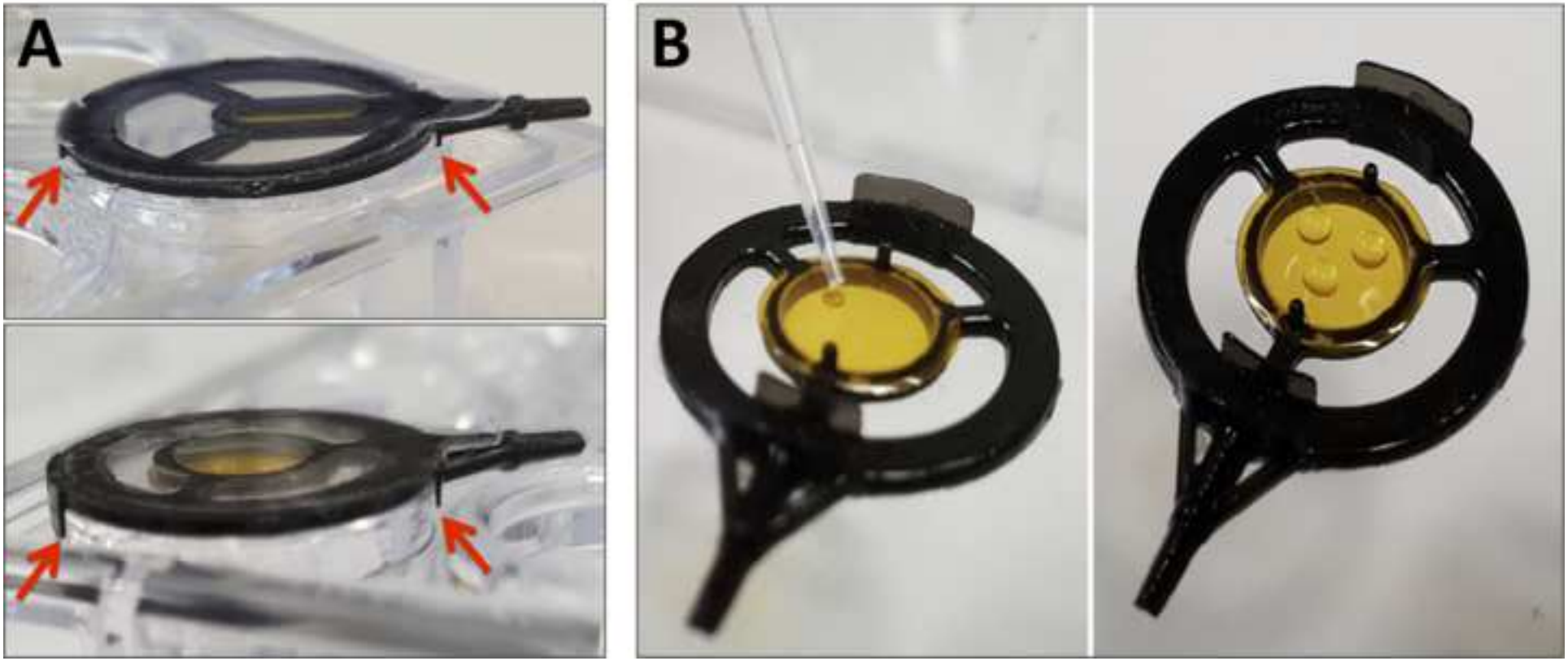

Figure 3

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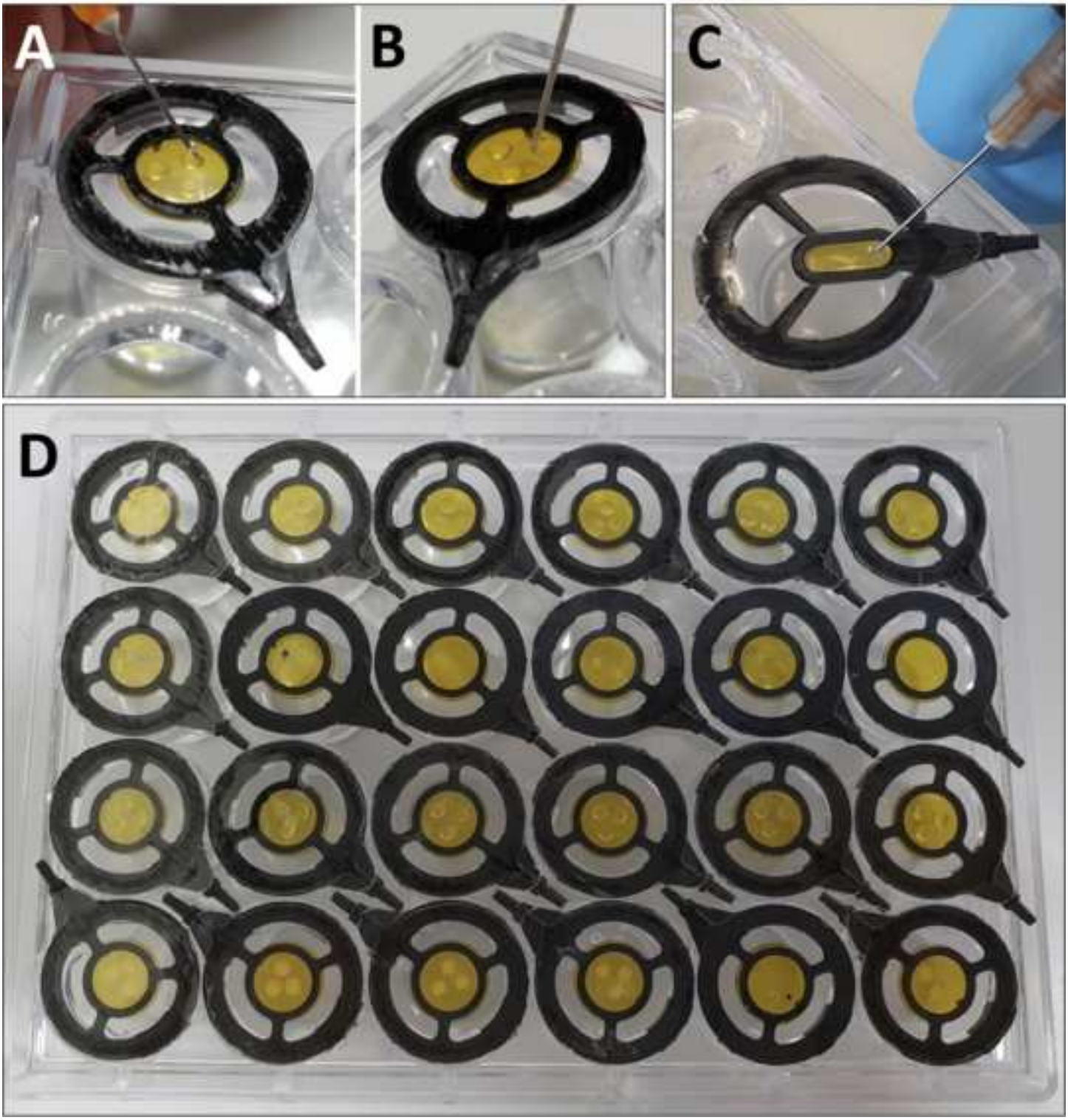

Figure 4

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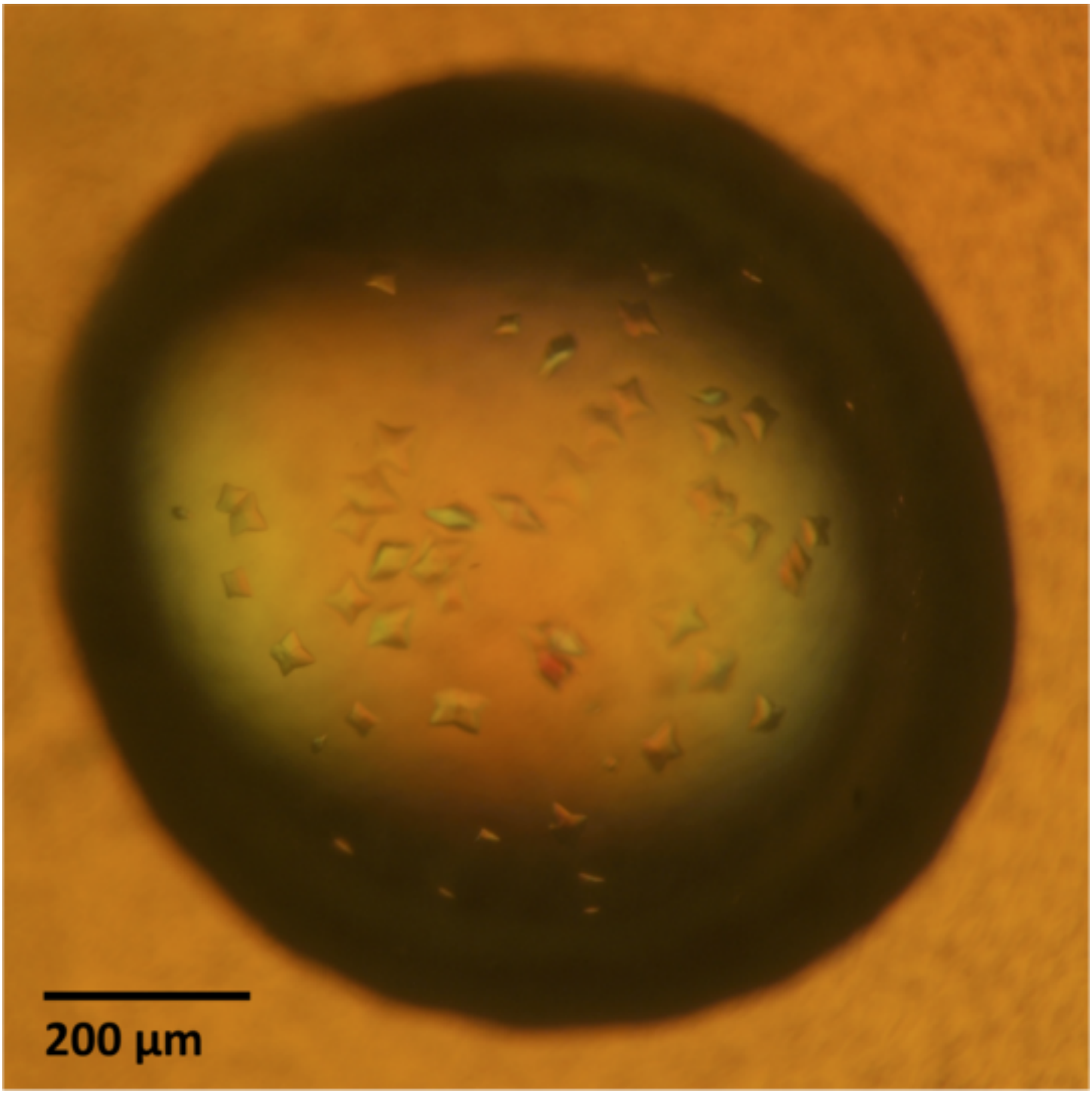

Figure 5

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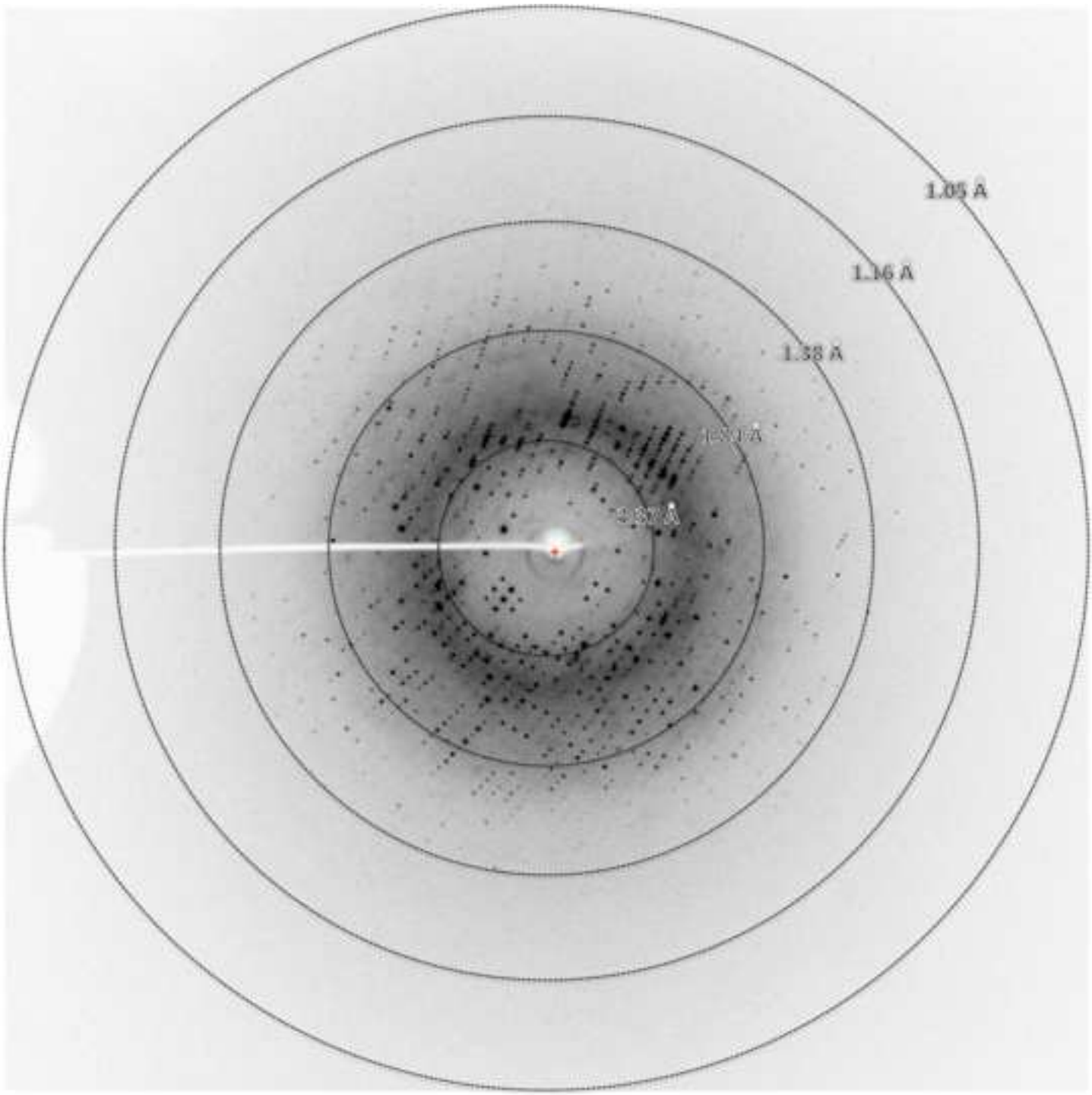

Figure 6

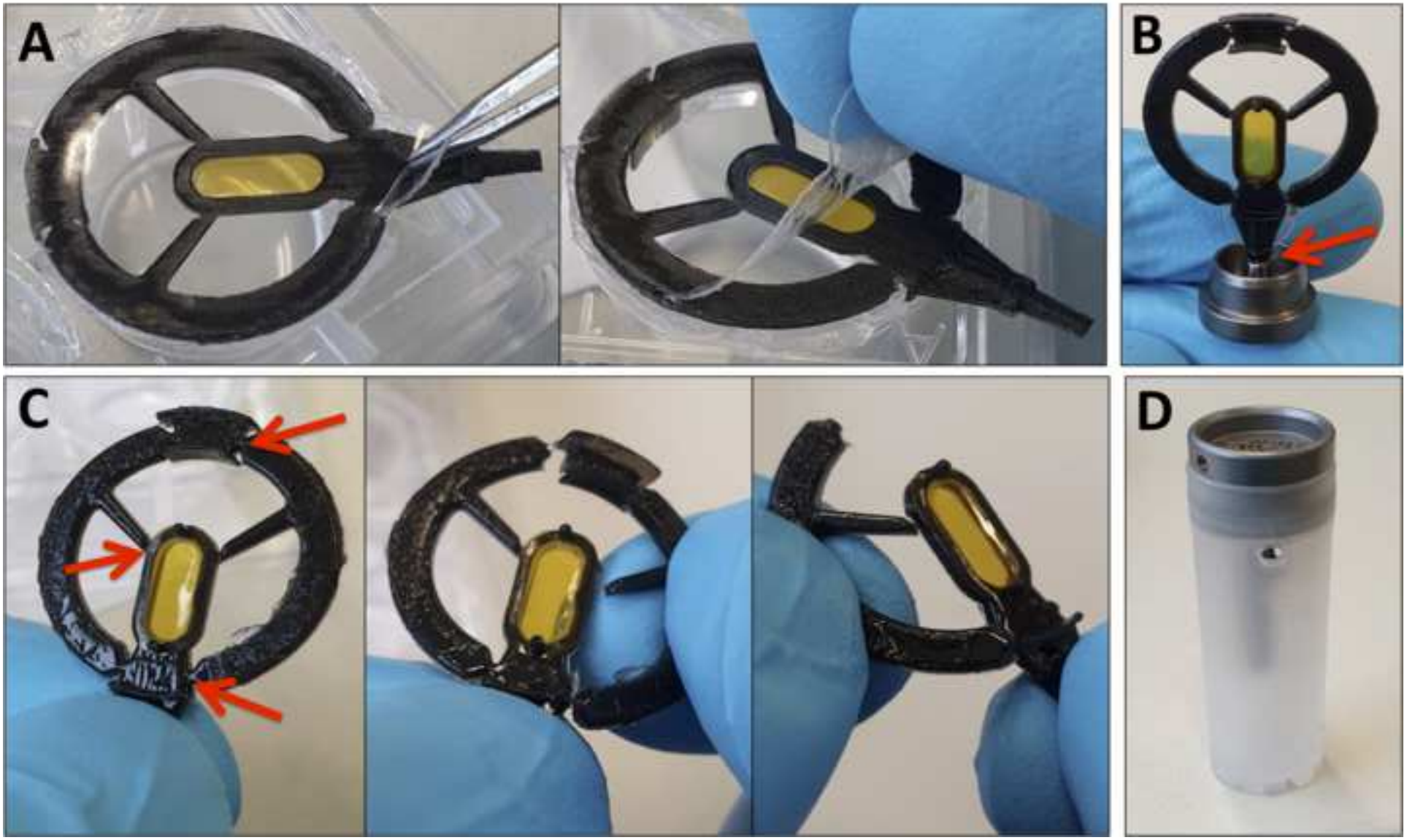

Table 1

Crystallization details

Method	Hanging drop, vapor diffusion method
Plate type	SuperClear Plates
Temperature (K)	293
Protein concentration (mg mL^{-1})	15
Composition of reservoir solution	50 mM NaAc pH 4.7, 500 mM NaCl, 25 % (w/v) PEG-6000
Volume and ratio of drop	2 µL total, 1:1 ratio (protein : mother liquor
Volume of reservoir	500 µL
Incubation time	12 hours

Table 2

Data collection and processing	
Wavelength (Å)	0.89429
Temperature (K)	293
Detector	Rayonix MX225 CCD
Crystal-detector distance (mm)	120
Rotation range per image (°)	0.5
Total rotation range (°)	120
Exposure time per image (s)	5
Space group	$P\,4_3 2_1 2$
Unit-cell parameters (Å)	a = 79.01, b = 79.01, c = 37.95
Mosacity (°)	0.07
Resolution range (Å)	39.50 - 1.35 (1.37 - 1.35)
Total number of reflections	191940 (8932)
Number of unique reflections	27020 (1292)
Completeness (%)	99.88 (99.20)
Multiplicity	7.1 (6.9)
Mean $I/\sigma(I)$	15.0 (1.9)
R_{meas}[35] (%)	6.3 (107.0)
R_{pim}[36] (%)	2.4 (40.4)
$CC_{1/2}$[37]	99.9 (68.5)
ISa[38]	16.1
Wilson B-factor (Å^2)	17.0

Table of Materials

Click here to access/download;Table of Materials;Feiler-et-al_Table_of_Materials-revised.xlsx

Name of Material/ Equipment	Company	Catalog Number
AF Satetiss	RS Components	101-5738
Cannula	Dispomed Neoject	25 G 5/8" 0.5 x 16, Ref:10026
COC foil	HJ-Bioanalytik GmbH	900360
ComboPlate	Greiner Bio-one / Jena Bioscience	662050 / CPL-131
Cryo Vials	Jena Bioscience	CV-100
Eppendorf Research Plus	Eppendorf	3123000012
Eppendorf Tubes	Eppendorf	30125150
Forceps Usbeck	FisherScientific	10750313
GELoader Eppendorf Quality	Eppendorf	30001222
Magnetic CryoVials	Molecular Dimension	MD7-402
Microfuge Thermo	ThermoFisher Scientific	R21
Paper wicks	dental2000	64460
Rotiprotect Nitril-eco	Carl Roth	TC14.1
SuperClear Plates	Jena Bioscience	CPL-132
UHU super glue	UHU GmbH & Co KG	45545
VeroBlackPlus	Alphacam	OBJ-40963
XtalTool	Jena Bioscience	X-XT-101
XtalTool HT	Jena Bioscience	X-XT-103 / X-XT-104
XtalToolBases	Jena Bioscience	X-XT-105

Comments/Description
lint-free paper, multiple retailer
multiple retailer
pre-greased plate, multiple retailer
0.1 - 2.5 µL volume
1.5 mL g-Safe Eppendorf Quality, manufacturer reference number
extruded tips (0.2 - 20 µL), manufacturer reference number
Set of paper wicks, multiple retailer
powder free, multiple retailer
pre-greased plate
manufacturer reference number, multiple retailer
manufacturer reference number
sample holder set
SPINE compatible sample holder set
Magnetic sample holder bases set

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Author(s): Christian Feiler, Dirk Wallacher & Manfred Weiss

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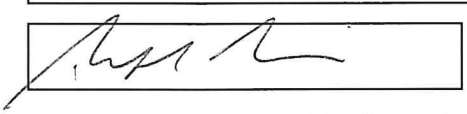
Signature:	
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Berlin, Feb 22, 2019

Ref.: Revised Manuscript Submission

Dear Editor(s) of JoVE,

Thank you very much for your comments on our manuscript on a new sample holder for macromolecular crystallography. In the following we would like to answer all points raised by the referees and by you. We hope that you find our comments and the concomitant changes we applied to our text and the figures satisfactory and that our manuscript is now suitable for publication in JoVE. Please note that the line numbers may be different depending on whether you view the clean manuscript or the manuscript with tracked changes. In this letter, we refer to the line numbers of the clean manuscript.

Comments from the Editor:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have tried our very best to thoroughly proofread our manuscript for spelling and grammar errors.

2. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an .xls/.xlsx file. Please sort the Materials Table alphabetically by the name of the material.

The revised Materials Table now includes all essential supplies, reagents and used equipment and is sorted alphabetically by the name of the material. When using trade names of individual materials, the manufacturer reference number is also given. If a given material is available from more than one vendor, a comment was added to the respective line.

3. Please provide more dimensions of the sample holder. Can the STL file be provided? If someone were to repeat the protocol, how would they make the holder or where can they obtain it?

In order to provide more information on the dimensions of the sample holder, we have redone Figure 1 with scale bars added. I am very much afraid to say that we will not be able to provide the associated STL files due to the pending patent applications. The sample holder has already been

made commercially available under the trade names XtalTool and XtalTool HT by the company Jena Bioscience (Jena, Germany), respectively. Sample holders can be either purchased directly from Jena Bioscience or via one of their distributors. Please refer to the Jena Bioscience website: *https://www.jenabioscience.com/crystallography-cryo-em/data-collection*. Note: If the provision of the STL file is a mandatory requirement for publication on your side, we will have to withdraw the manuscript. Please advise!

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We have edited the manuscript and removed all commercial language including all trademark symbols, registered symbols, etc. All relevant information was added to the Materials Table including references. We would like to point out, however, that in your example the term CrystalDirect was already used for the system before it became commercially available. If we use other terms for it, this sound very artificial and it will be utterly confusing to the readers.

5. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

We have revised our protocol and adapted it to answer the “how” questions in all necessary steps. References have been added to steps for further information (lines 198, 206 and 230). We have also redone some of our figures. For instance, the setting up of crystallization drops is now illustrated with a renewed Figure 2 to allow a better visual illustration how this is step needs to be performed.

6. 2.1: How is the surface cleaned and made dust free?

Detailed information is now included within the protocol. From line 138 onwards, the text reads now as follows: “Create a clean and dust-free surface using a damp lint-free cloth. Take one sample holder from its box and gently place it yellow foil facing up on the cleaned surface to avoid damage or unwanted puncture of the backside COC foil. Introduced scratches may disturb later on the crystal observation.” Information about the recommended material was also added to the Materials Table.

7. 2.2: What is the desired volume? It is best to provide a specific example here instead of generalized protocols. As the representative results used is lysozyme, please provide these details throughout the protocol.

We have added more detailed information throughout the protocol. To elucidate the handling, we included the new panel B of Figure 2 and added a new table summarizing the experimental details for crystallization (Table 1) and data collection (Table 2).

8. What is the reservoir? Concentrations and volumes?

Detailed information about the used reservoir composition has now been given in the respective paragraph starting from line 253. In addition, the new Table 1 provides summarized details.

9. What are the incubation conditions?

The protocol was adapted to provide detailed information regarding the recommended volumes. The paragraph in the representative results section describing the experimental procedure in detail was significantly revised and many more details regarding the incubation conditions are now provided (line 253 and following).

10. Please provide all volumes and concentrations used throughout.

In order to not be too repetitive throughout the text, we have added a paragraph in the representative results section, which gives the requested information. Experimental details used in the reported case are provided in this section. We have also included Table 1 in order to provide precise information on the experimental setup.

11. Please be more neutral and discuss some limitations of the sample holder in the discussion.

We have added a new paragraph on the limitations of the sample holder at the end of the discussion section. This new paragraph reads as follows: **Limitations.** The sample holder's geometry permits unobstructed diffraction data collection by the rotation method over a total rotation range of 160°. This is sufficient so that complete diffraction data sets can be obtained for most crystal systems. In cases where this is not possible, data from more than crystal need to be merged together. When crystals are gown together, it may be possible to adjust the size of the incident X-ray beam so that only parts of individual crystals are exposed. In extreme cases, one may need to resort to a data collection strategy similar to the MeshAndCollect approach (Zander *et al*., 2015). In summary, while there are some limitations associated with the sample holders, these can easily be overcome in most cases. Of course, it is always possible that situations are encountered, in which none of this is possible. In such cases, one may need to resort to other crystal mounting methods.

Comments from referee 1:

Minor Concerns: Use of commercial words/language like "easy", "not really a problem".

We have corrected the protocol accordingly.

Comments from referee 2:

Manuscript Summary:

In this article, the author devised a simple device that allowed data collection screening in situ without the need for direct manipulation of the fragile protein crystals. Also, as background signal from buffer diffraction is a problem, this new invention could drastically decrease buffer content of the crystal for data collection. The manuscript is well-written and explains the idea and method clearly. The beauty of this design is that the device, or in other words, a substitute of the widely used glass cover slide, is compatible with the widely used 24-well Linbro style plate. The use of two layers of membranes, namely a self-healable COC membrane and a liquid permeable polyimide membrane allowed sealing for crystal growth and buffer exchange or drain with ease. The design itself is smart and should increase the efficiency of the screening process.

Major Concerns: However, the downside of the design is that, even though this gadget seems suitable for screening crystals without direct manipulations of the crystals, it does not allow for single crystal being isolated and harvested for data collection if there're a bunch of crystals appeared in a single drop.

This is indeed true. We have also stated this in the manuscript on page 11, lines 431-440. It is clear that there is no one solution to all possible problems. Avoiding manipulations thus means leaving the crystals alone. However, if optimized crystal growth will not lead to individual or well-separated crystals, there is still the chance that by tuning the size of the incident X-ray beam only one single crystal will be exposed at a time. We have also stated this explicitly in a new paragraph on limitations in the Discussion section.

What's more, currently, we routinely used robot to set up 96-well sitting drop plate for screening crystal growth conditions and most of the time we only use 24 well plate for optimization of crystal growth conditions, and if single crystal do occur, then this device should for sure avoid direct manipulation of the crystal. But most of the time, multiple crystals are seen in a single drop. And if a crystal is seen that diffract x-ray well and further data collection is desired, then there's a problem that other crystals around will hamper data collection at an angle parallel to the membrane. For crystals like those formed by lysozyme, even if there're multiple crystals in a single drop, it should not be a problem because each crystal diffracts quite well and one can harvest a full data set by combining the data collected from multiple crystals. However, in circumstances like some particular crystal diffract well while others diffract poorly, it is always desired to isolate and harvest the single crystal for diffraction screening and for data collection.

This comment is a continuation of the previous comment. The here described sample holders are designed for optimization experiments of crystal growth conditions, which were identified from large screening campaigns. This means that initial crystallization conditions are already known from experiments such as the one described by the referee. By varying the ratio between mother liquor and protein solution, the number of expected nucleation sites may be tuned. Other than in the screening plate, the sample holder does not only allow to test but also collect diffraction data from each crystal individually. Diffraction data collection might be hampered due to the parallel orientation of crystalline material on the membrane. However, the sample holder allows the usage of a kappa goniometer in order to minimize the chance of illuminating multiple crystals at a given time. We further like to refer to Zander *et al.* MeshAndCollect: an automated multi-crystal data-collection workflow for synchrotron macromolecular crystallography beamlines. *Acta Cryst.* **D71**, 2328–2343. This reference is listed in the manuscript as reference 35.

What's more, for crystal grown in a cluster or overlay 3 dimensionally, it is still a problem even with current design, limiting its possible applications.

Again, this comment is a continuation of the two previous comments. In the case of crystals grown in clusters, the addition of additional mother liquor from the back of the crystal growth support might help to relocate some clusters and allow data collection on individual crystals (or parts of them). As already mentioned the design reported here is not magic. There will always be cases, when one has to resort to other approaches.

Comments from referee 3:

Manuscript Summary:

The manuscript submitted by Feiler *et al*. describes a new sample holder for macromolecular crystallography. In this approach, crystals are grown on a polyimide foil attached to a 3D-printed plastic holder using conventional vapor-diffusion method. The holder is then affixed to a magnetic base for subsequent X-ray analysis at either room-temperature or under a nitrogen stream. The system can be conveniently used for crystal treatments such as ligand soaking, solvent removal and cryo-protection. The principles of the method are very similar to the Crystaldirect™ method by Cipriani *et al*. but applied to a popular crystallization plate type used for manual crystal optimization. Like Crystaldirect™, crystal manipulation (typically using a "fishing" loop) is avoided but unlike Crystaldirect™, the presence of the plastic holder frames limits the range of oscillation for crystallographic data collection, an inconvenience that is not clearly mentioned in the manuscript. The method is of interest for crystallographers and has the potential to expand serial crystallography approaches. The manuscript is worth publishing but would need to address the following comments prior to publication.

Minor Concerns:

l. 98. Refer to Table 1 of Aller *et al*. 2015 Methods in Molecular Biology and l. 100. Add Crystaldirect™ reference here (citation 27).

We have added the two references at this point.

l. 103. The authors present the disadvantages of the Crystaldirect™ approach, namely the need for opening the crystallization chamber and the potential difficulty of isolating single crystals. Both aspects are however still present in the method described in the present manuscript. The authors may want to tune this sentence differently.

In response to this comment, we have deleted the sentence the respective sentence from the text.

l. 106. Replace "our aim was to develop" with "we aimed at developing" and l. 111. Replace "we would like to describe … and to demonstrate" with "we describe…and demonstrate"

The text has been changed in order to avoid the use of the first person.

l. 121. Figure 1. Add a scale bar.

The new and redone Figure 1 contains now a scale bar in all panels.

Figure 1. Describe the purpose of the 2 little pins protruding out. Does it puncture the foil to hold it in place?

The protruding pins are used during the manufacturing process to align the polyimide foil. An explanatory sentence was added to the first section the description of the sample holder, page 3, lines 139-140.

Figure 1. Highlight the breaking point for 1B and 1C.

The designated break points are now highlighted by red arrows, which have been added to Figure 1, panel B. We have further induced the new Figure 6 in order to illustrate the breaking of the sample holder for data collection.

l. 123. Add the thickness of the foil and comment on the absorption at common energies used in macromolecular crystallography.

The thickness of the foil and comments on absorption have been added on page 3, line 131.

l. 126. Does the yellow color affect the polarized light or UV light?

Polarized light is not affected by the yellow color. Figure 4 was taken on a transmission microscope using a polarizer in place. UV light we have not tested, since we have no such facilities available in our laboratory. We have stated this on page 7, lines 258-259 and page 8, lines 309-312 of the manuscript.

l. 146. It would be good to have a dedicated figure illustrating this point.

Unfortunately, we are unable to address this point since we do not have access to an imaging robot or an imaging hotel in our laboratory.

l. 168. The use of a finger to cover the hole sounds a bit rudimental. Please explain in more details.

We agree with this comment of the referee, but sometimes it is the simplest solutions, which are the best. We have added a few more details on this to the protocol.

l. 173. Replace "4.1.5" with "4.1.3"

Thank you for pointing this out. Done.

l. 192. Description used on point 6.1.1 (l. 213) reads better.

We have followed the suggestion and introduced the respective changes.

l. 198. What does "correct positioning" means (a figure may illustrate this better)? What about icing? Please describe the limitation in oscillation range.

In order to illustrate the term “correct positioning” we have included indicating arrows in Figure 2. We would also like to refer to the protocol text to the figure panel 2A for better understanding. We have collected data after the sample holder was left for 1 hour on the goniometer and no significant icing was observed. This is now stated in the text on page 10, lines 389-393. A paragraph regarding limits on the rotation angle has been added on page 10, lines 394-397.

l. 205. Refer to Fig 1 and indicate the breaking points.

We have revised Figure 1 and included that point.

l. 215. Is the holder attached with glue? Describe what a "pre-prepared" magnetic base is.

Since the sample holder is a device for single use, we have designed the attachment to the base so that it works without glue. The sample holder is clamped onto the magnetic base by inserting it all the way to the indicated marker. We have revised Figure 1C by highlighting the marker, up to which the sample holders need to be inserted into the base, to explain this in detail. Further, the new Figure 6 illustrates the preparation procedure.

l. 220. How stable are both the sample holder and the film in LN2?

Both are stable in liquid nitrogen. The stability of the sensitive polyimide film has been tested at 77 K previously by Yano and Yamaoka (1995). Cryogenic properties of polymers. *Progress in Polymer*

Science. **20**, 585–613. The appropriate reference has been added to our list of references as reference no. 34.

l. 221. The transfer into vials only works for case described on Fig 1B and C (not A). Please amend.

This has been clarified on page 6, line 241-243.

l. 229. What is the recommend volume for the drops?

We have added recommended drop volumes and drop numbers for all types of sample holders in the manuscript, page 7, line 249-252.

l. 279. The use of the polarizer could be illustrated in a figure as well.

Figure 4 was taken using a polarizer. We have now also included the possible usage of a polarizer in the figure description.

l. 242. Add beam size, flux and dose.

The requested information has been added to the text on page 7, line 265.

l. 243. It would be good in this paragraph to add the merging statistics if enough data were collected.

We have now included a new Table 1 that provides the merging statistics for a data set collected using the sample holder.

L. 253. Add thickness of the foil.

The thickness of the foil was added to the general description.

l. 277. Replace “Observation of crystal growth” with “Picture of hen egg-white crystals”

The requested change has been made.

Figure 5. Add resolution rings. Indicate where the background from the foil is.

Figure 5 was revised and includes now distinct resolution rings.

Comments from referee 4:

Manuscript Summary:

This paper describes an interesting approach using a newly designed device to handle crystals grown in drops, highly decreasing their manipulation before X-ray diffraction data acquisition. The device was conveniently prepared to be used with Linbro plates, which are still highly used for protein crystallization experiments, despite many other plates have been made commercially available. The device has also been made commercially available by Jena, showing its putative commercial potential. There are some issues which could be improved/changed for a better understanding of the reader.

Major Concerns:

Whereas I agree that the use of capillaries became impractical for cryoprotection many years ago, at present, protein crystals grown in capillaries by the counterdiffusion technique can be readily

prepared in the lab, sent to a synchrotron source in a dewar, and robotically mounted for data acquisition under a nitrogen steam, totally avoiding crystal manipulation; furthermore, ligand soaking and cryoprotection can also be achieved by counterdiffusion once the crystals have been prepared just by placing a cut capillary inside a mother liquor containing the compound of interest (Gavira et al. (2002). Acta Cryst D58:1147-54; Martinez-Rodriguez et al. (2008). Acta Cryst F64, 50-53). This information could be added in the introduction section, where the authors describe the different methodologies describing the problems of protein crystal handling.

We have added the method and the associated references to the introduction section of the manuscript (page 2, lines 75-77).

One of the major drawbacks of the device might be a high number of crystals in the drop (e.g., Fig 4). Whereas this issue could be minimized by setting up different drops reducing nucleation, I think this issue should be mentioned somewhere in the paper (maybe a footnote), just in case crystal improvement is not feasible; this issue would difficult data acquisition if proper centring is not allowed due to the limitations of positioning the goniometer, even when we know that the corresponding datasets could be treated afterwards to discriminate between different crystals. Thus, a short explanation for putative users of the holders might help to understand how to overcome some minor issues on the handling at the X-Ray source.

We would like to thank the reviewer for this remark and we have included a paragraph explaining the limitations of the device and how they can be overcome. The sample holder is designed for optimization of crystallization conditions. It allows multiple drops to be set. Tuning the ratio of protein to mother liquor may optimize the number of nuclei to grow fewer, but larger crystals. If this is not applicable to the specific case, using kappa angles on the goniometer can increase chances of proper centering and data collection in challenging cases. This is now all mentioned and discussed on page 10, lines 394-399.

I also suggest the authors to consider the gelling of the crystallizing drops as they will fix the crystal for further measurements.

While we agree that gelling might be an option for immobilization of crystals; we do believe that it is not really necessary, since the crystals will hold on to the sample holder by mere surface tension. Since gelling might also interfere with ligand soaking or cryo protection, we would rather refrain from adding this as a recommendation.

I miss some statistics of crystals collected using the classical "fishing out" method and crystals measured using the holder presented in this work would reinforce the holder benefits.

We have now included some data processing and merging statistics of a collected data set using the sample holder (see Table 2).

However, the main concern is not about the science but about the protocol itself. I think that the protocol needs a large revision to be understandable and useful for readers, mostly students. For instance, I have missed some images or extra information showing the kind of cannula used to pierce the foil, the fine paper wick, or how to reseal the foil after manipulation.

We have included a new Figure 2 to illustrate how to use a cannula and the fine paper wick and we have tried to be as informative as possible in the protocol, in particular for novice users.

I am sure it is quite straightforward, but since I could not see the holders to make an idea, it would help readers to know what kind of materials to be used.

The full list of materials used in now in the revised Materials Table.

A short sentence (a movie?) describing how the holders with crystals can be easily sent/transported to a synchrotron source might be added.

Thanks a lot to the reviewer for mentioning this. This is exactly the reason why we are trying to publish this work in JoVE.

In section 4 needs a picture or illustration on how and where to perforate the COC film.

A new panel for illustration on how to prepare the sample holder for ligand soaking and removal of mother liquor was added to Figure 2.

Section 4.2 needs a fully rewritten. If the solution is located between the polyimide and the COC films, will the ligand migrate through the polyimide film to the crystal sample? Please explain it clearly.

Yes, the reviewer is exactly right in this. We have thoroughly reviewed section 4.2 and clarified it in various places. Further, the new panels in Figure 2 should help to understand the procedure further.

In 4.2.4., do you mean that just by touching the COC film it will auto-seal it? Please explain it better.

Yes, this is indeed the case. We are now provided a more extensive explanation in the protocol.

Section 5 & 6: how is the holder fix to the magnetic cap? How does it looks like?

Figure 6 was added to elucidate, how the holder is fixed on the magnetic cap. The procedure was also added to the text, page 5, line 209 and is illustrated in the new figure 6.

Is there any problem with the humidity control system and the polyimide foil? A picture exemplifying the set-up could be of great help.

The employed polyimide does not cause any problems with the humidity controlled sample environment. The material itself is rather inert to humidity and is therefore not prone to swelling are absorb a significant amount of humidity. Taptimthong et al. have tested this previously with slightly thicker material of 25 µm. The result was that the total humidity absorption was negligible (Taptimthong *et al*. (2014). Flexible Magnetic Writing / Reading System: Polyimide Film as Flexible Substrate. *Procedia Technology* **15**, 230–237.). Within the humidity controlled sample environment, its porous structure allows to keep an equal humidity level on either side. We now have included new Figure 6 to exemplify the workflow.

With best regards,

Manfred Weiss

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