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To the
Editors of
JoVE

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Ref.: Revised Manuscript Submission

Dear Editors,

Thank you very much for your comments on our manuscript on the workflow for crystallographic fragment-screening at the HZB. 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.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We acknowledge the editor's comment and have thoroughly revised the entire manuscript in this respect.

2. Please revise your title to "Workflow and Tools for Crystallographic Fragment Screening".

We are a bit surprised by your request to change the title of our manuscript, but are willing to comply with it to some extent. we would propose a compromised title version such as: "**Workflow and Tools for Crystallographic Fragment Screening at the Helmholtz-Zentrum Berlin**". The addition of "at the Helmholtz-Zentrum Berlin" is absolutely crucial as this protocol (as any suchlike protocol) is in parts very specific to the experimental site in Berlin. This is because these kinds of experiments require the use of large-scale facilities, with adjusted protocols for each of them (hence see the contribution by the DIAMOND light source and the ESRF to this methods collection). We definitely want to avoid giving the impression that our manual "as is" is applicable to every other laboratory/facility, also out of respect for our colleagues at other CFS facilities. The protocol outlined here concerns the crystallographic fragment screening at the HZB only. Several steps of the protocol (by the nature of large-scale facilities) are bound to be carried out at the HZB. Nonetheless, the reader not intending to use our facility may still extract the transferrable parts of the protocol and use the know-how in his/her own laboratory. This is even an advantage of our protocol, e.g. steps 1 and 2 are indeed transferrable to any laboratory. However, the other steps are site-specific, which needs to be reflected in the title of the manuscript.

3. Please provide an email address for each author.

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4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Kapton loops (MiTeGen Dual Thickness 191 MicroLoops (LD)); Hampton Research; Falcon tube; Unipuck foam dewars; beamline 14.1 or SPINE pucks to beamline 14.2 dewar, DIAMOND light source etc

We understand this point very well and revised the manuscript accordingly. However, we need to state that some of these names are used in the scientific community as such. There is simply no other description for the device. The comprises Beamline 14.1, SPINE pucks, Unipucks, DIAMOND light source. There are no replacements for this, and we are sure that you do not want us to replace DIAMOND Light Source for instance by “the synchrotron located in the vicinity of Cambridge”. Also, not detailing equipment parts by using the terms commonly used in the field can create utter confusion. For example, if we would call the unipucks only “pucks” this would be too general as the different types of pucks are fundamentally different in their specifications.

5. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We acknowledge the editor’s comment and have thoroughly revised the manuscript in this aspect.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We recognize the editor's comment and changed the manuscript accordingly.

7. The Protocol should contain only action items that direct the reader to do something. Please move section 1 in the protocol to the introduction and sections 2 and 3 to the Discussion.

We acknowledge the removal of explanatory part from the protocol but found that the mentioned sections were all better suited in the introduction as these points need to be taken care of prior to execution of the protocol. We have adjusted the introduction accordingly.

8. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps.

We acknowledge the editor's comment and added more details where it seemed necessary for understanding.

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. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

9. Wherever possible, list materials, reagents, software, equipment in the Table of Materials and cite the Table of Materials in the text. Hence, remove all lists of materials to be used from the protocol.

We acknowledge the editor's comment and removed the list of materials from the protocol part. The Table of Materials has been updated to include all materials there and cited appropriately at the points of the protocol where workplaces are arranged (i.e. 1.2 and 2.2)

10. Please include a one line space between each protocol step and then highlight up to 3 pages of protocol text for inclusion in the protocol section of the video.

We thank the editor for the reminder. We included the one-line spaces now for all protocol steps where they were missing and have highlighted the protocol parts to be filmed in yellow.

11. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

We are thankful for the reminder to provide a scale bar for microscope images. We added the bar scale in figure 2.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes how to perform a Fragment screening campaign at Bessy (Helmholtz-Zentrum Berlin). Fragment screening is a powerful method to facilitate structure-based drug design and drug discovery. Here the authors clearly describe how to streamline the process. This significantly lowers the threshold for new users of this method and also helps and guides experienced users. The method is very clearly described and everything is presented with the clarity and rigor that this group is known for.

We are very happy about the overall enormously positive feedback by Reviewer #1.

Minor Concerns:
The authors should mention how the streamlined method also can be used to analyze prescreened fragment libraries. They could also mention advantaged and disadvantages of prescreening libraries using different, orthogonal methods.

We are grateful for the suggestion to include the topic of prescreening fragment libraries and orthogonality of different biophysical methods. We are aware that in the field of fragment screening several different methods can be applied. It has shown by us and others that screening the same fragment library with different methods could result in very low overlap of hits between the different methods applied. Nevertheless, we have included two more sentences about this in the introduction. There it reads now:

"Several biophysical methods may be applied for fragment screening, the most popular being nuclear magnetic resonance, X-ray crystallography, surface plasmon resonance and thermal shift assays. These methods are used either in an parallel or in a sequential way, with the aim to increase the confidence in the hits and reduce the numbers of false positives or false negatives, respectively. However, a recently conducted comparative study[6] suggested that sequential screening cascades are to be avoided due to the low overlap between the different methods."

It is clear that the topic of pre-screening is so large, that an entire article could be written on it. However, this would be widely out of scope for this manuscript, which is not a review article, but a practical protocol about **crystallographic** fragment screening as a first-step screening technique as performed at the HZB, with the Protocol/Video as the central part. And at the HZB, we have – for reasons detailed above – refrained from including pre-screening in our workflow.

What does the sporadic yellow highlighting of text mean?

We thank the reviewer for the question about the yellow highlighting. It is mandatory for a JoVE produced video manuscript to highlight protocol text in yellow that will be filmed later on, as described in the author guidelines/ the template. The video is actually the main and central part of the publication. We apologize that this was not communicated along with the manuscript.

Reviewer #2:
Manuscript Summary:
Very well-described and thorough protocol for fragment screening by X-ray crystallography.
Major Concerns:
The recommended soaking drop size of 0.4 microliters is too small. For the inexperienced hand it will lead to evaporation of the liquid before the crystal can be harvested, which may lead to crystal damage. Please address why such a small drop size has been selected. A usual protocol for such a screening would involve soaking in a drop at least 1 microliter in volume.

We recognize the concerns of the reviewer about the small drop size of 0.4 µl. However, in respect to screening campaigns in macromolecular crystallography, such small drop sizes are very common and also well established. In our publication concerning the validation campaigns of our library (Wollenhaupt et al., 2020, doi.org/10.1016/j.str.2020.04.019) and all conducted user campaigns, we always use 0.4 µl and it works very well, in our experience, even for users that are fairly new in the field of macromolecular crystallography. Additionally, at other synchrotron sites that perform such screening campaigns on a weekly basis similar drop sizes are used, for example at XChem (DIAMOND) 0.2 – 0.6 µl drops. Also, a recent collaboration headed by the group at DESY screening several thousand crystals of the SARS-CoV-2 protease using drop sizes of 0.5 µl (preprint: Günther et al., 2020, doi.org/10.1101/2020.11.12.378422). Many more examples could be given. Thus, it is very convincing that these drop sizes are well established in the crystallographic community and no changes or further elaboration on that is necessary in the manuscript.

Reviewer #3:

Manuscript Summary:

Wollenhaupt et al. report on the procedures of crystallographic fragment screening at the BESSY synchrotron. The article is generally very well written, sound and concise. The article is of high relevance for the crystallographic community and the JoVE journal is a very well suited medium for this information. I recommend publication after minor corrections, as detailed below.

General comments:

Some text is marked in yellow for reasons not obvious to the reviewer. I ignored this.

The journal seems to provide videos for the procedures. These were not accessible through the review system.

We thank the reviewer for noticing the yellow highlighting and lack of a video. JoVE is a video-based journal. The video is actually the main and central part of the publication. It will be based on the protocol part and will only be produced after the manuscript is peer-reviewed. It is mandatory for a JoVE produced video manuscript to highlight protocol text in yellow that will be filmed later on, as described in the author guidelines/ the template. We apologize that this was not communicated along with the manuscript.

Minor concerns:

Line 144: " The number of hits obtained in a CFS campaign is not only dependent on the druggability of the target protein and the suitability of the crystal lattice (see above) but it also on the choice of the library, its quality and its presentation."

Please specifiy in more detail, which factors are meant when referring to "choice", "quality" and "presentation" of the library.

We thank the reviewer for the suggestion to elaborate more on this particular topic. Nevertheless, we have to recognize that this manuscript is not an exhaustive review, but a practical guide to performing CFS experiments with the protocol (and the resulting, to be filmed video) as a central part. We have tried to incorporate the suggestion of the reviewer mildly in the introductory part of the manuscript.

The changed paragraphs read now:

“The number of hits obtained in a CFS campaign is not only dependent on the druggability of the target protein and the suitability of the crystal lattice (see above) but it is also dependent on the quality of the library. Library quality comprises two aspects: the selection of the compounds for the library and the confectioning of the compounds, i.e., in which physical form they are presented for the experiment. For compound selection different strategies can be employed. Most library designs include the maximization of the chemical diversity of the fragments. A strategic focus could be to include the chemical tractability of the fragments for follow-up design, which has been applied for instance in the DSI poised library [10]. Yet another strategic focus for library design could be to maximize the representation of commercially available chemical space of fragments by shape- and pharmacophore-based clustering, as has been exemplified by the F2X libraries developed at HZB [11].

[… and about the “representation” further below:]

“With respect to the physical presentation of a library, two approaches are most commonly adopted: the fragments are either used as DMSO stock solutions or the fragments are dried and immobilized on ready-to-use plates.”

Line 172: "We recommend preparing additional mock-soaks that will later...". Please define "mock-socks" at this point (e.g. "data sets without a soaked ligand"). It becomes clear later, but may be unclear at this point for many readers.

We appreciate the suggestion of the reviewer to define “mock-soaks” in more detail at an earlier stage. We therefore added the following paragraph as second-to-last paragraph in the introduction:

“Mock-soaks are defined as soaking experiments on protein crystals using the same soaking solution as the fragment soaks for the same incubation time, but no fragments present. If the soaking solution is equal to the crystallization condition, the crystals may be directly harvested from the crystallization plate.”

Line 216: " Add milli-Q or distilled water..." -> Add deionized water ... (milli-Q is a device from Millipore)

We are grateful for the reviewer pointing out the use of the word milli-Q. We replaced the term as suggested by the referee at every position where “milli-Q” was used in the former version of the manuscript.

Line 351:
" Nevertheless, samples should be prioritized:
a) First collect one diffraction dataset of each fragment soak, prioritizing the better of the two duplicates
b) Collect duplicate diffraction datasets in case of failed data collection, loss of diffraction or severe ice rings
c) Collect diffraction datasets of apo crystals.
d) Collect the remaining duplicates."
Even after repeated reading of this section, the procedure is not entirely clear to the reviewer. Is this the suggested procedure?:
a) First, collect a data set for one crystal of each fragment, based on the prioritization in step 6.6.
b) Collect a second diffraction dataset for those fragment data sets, that suffer from failed data collection, loss of diffraction or severe ice rings in the data collection of the first crystal.

c) Collect diffraction datasets of apo crystals.
d) Collect data sets of the remaining duplicates of each fragment.

We thank the reviewer for this very valuable comment, which helped us to improve the manuscript. We adopted the suggestions of the reviewer with slight modifications so that it reads now:

"Nevertheless, samples should be prioritized:

a) First, collect diffraction data sets for one sample per fragment condition, based on the prioritization in step 2.6. / 2.7, i.e. collect the data for the higher prioritized duplicate.

b) For those experiments in a) that suffered from failed data collection, loss of diffraction or severe ice rings, collect data for the second duplicate sample for the respective fragment condition.

c) Collect diffraction datasets of apo crystals (if prepared according to steps 1.24 and 2.12).

d) Collect diffraction datasets of the remaining duplicates of each fragment condition."

Line 375: Is this procedure of excluding fragment-bound data sets from the ground state models necessary if apo data sets have been collected for the purpose of determining the ground state for PanDDA analysis?

We are thankful for the question of the reviewer and would like to explain the procedure in more detail. The apo data sets **can** be used exclusively for the characterization of the ground state. In such a case, a second round of PanDDA excluding fragment-bound datasets would not lead to new information because the datasets used for the ground state remain the same. However, due to differences between crystals, even apo crystals, it is not recommended to solely depend on apo data sets for the ground state characterization. It is best to leave the decision of picking datasets used for the ground state characterization to the PanDDA algorithm, as it finds the datasets that are very similar. In this case rerunning PanDDA by excluding fragment-bound datasets results in an improved ground state model and can extract additional hits. As the reviewer pointed out with the question, it is not clearly stated in the manuscript how to use the apo data sets. Therefore, we reformulated the entire paragraph, explaining what a ground state model is and also make the suggested procedure more comprehensible. The paragraph reads now:

"A key element for hit identification is PanDDA. Details are explained in the respective publications [13, 23], in brief: PanDDA automatically calculates electron density maps of a set of data sets in a CFS campaign. These are then assumed as non-binding fragment conditions and averaged to generate the so-called ground state model. The ground state model is then used to derive local discrepancies between each electron density map and the ground state map, using voxel-associated Z-scores. Then, for areas of high Z-scores a so called PanDDA-map is created by fine-tuned subtraction of ground state density from the respective map. This largely enhances the visibility of fragment binding events.

To maximize the outcome of PanDDA, the authors recommend a two-step approach. Firstly, performing a PanDDA run (pandda.analyze) with standard settings. Even if mock-soaked crystals have been collected, their identity will not be included as a parameter (which is possible nonetheless) in order to enable an unbiased generation of the ground state model by PanDDA from all available data. After that the output data is evaluated by the user via a so-called PanDDA

inspection in Coot [24]. Here, hits with relatively high confidence should be noted, concluding the first step.

Secondly, the authors recommend re-running the pandda.analyse step excluding the preliminary hits (determined in the first step) from the ground state model via the

--exclude_from_charactersation="<list-of-bound-dataset-ids>"

command line option. Further details are described on the PanDDA help pages (https://pandda.bitbucket.io/). This way, datasets that are clear hits and thus would obscure the ground state model if included there, are disregarded, leading to an improved ground state model and thus to improved results overall. Finally, a thorough PanDDA inspection is performed to complete the hit identification."

Line 421: "The photographs were taken on the BioMAX beamline for AR samples collected there as part of the F2X Entry validation10." Include the information "BioMAX beamline (MAX IV synchrotron, Lund, Sweden)".

We thank the reviewer for pointing out the missing information and changed the manuscript as suggested.

Line 472: "It may be, that the weak binding of a fragment*,* does not provide enough energy to induce a protein motion." Suggestion, add: "...in particular if the crystallized conformation is stabilized by crystal packing forces." The protein conformation is also stabilized by crystal packing forces, which the fragment also needs to compensate. Omit the comma *,*.

We would like to thank the reviewer for the proposed addition to our statement. It is an important point to include the crystal packing forces and therefore we changed the text as proposed.

Line 478: "A rather theoretical limitation seems to be when a protein is not made to interact with anything and does not contain any surface..." I think, this is not so theoretical. It is very likely that the hit rate will depend on the type of interaction sites. Suggestion: "Different proteins may contain more or less druggable sites. Protein-protein interactions are usually mediated by extended flat surfaces, that are more difficult to target. The fragment binding hit rate will therefore likely depend on the stucture of the protein molecular surface.

We are grateful for the reviewer's suggestion to rephrase this part of the manuscript. We changed the respective paragraph in the discussion, largely adopting the proposed statement of the reviewer. It reads now:

"Different proteins may contain druggable sites to a greater or lesser extent. For example, protein-protein interactions are usually mediated by extended flat surfaces that are more difficult to target. The fragment binding hit rate will therefore likely depend on the structure of the protein's molecular surface. In an extreme case, a protein might not contain any suitable surface hot spots that serve as target sites for fragment binding. Thus, despite a meticulously performed experiment, no fragment hits will result from the screening. However, the authors have so far not encountered such a situation."

When specifying hit rates, the authors should also mention that a *large portion* of the hits can also occur at crystal contacts or sites that are unlikely to influence protein function (enzyme inhibition,

receptor modulation). Therefore the number of hits that are useful for drug design is significantly lower than the overall hit rate.

We appreciate the reviewer bringing up this topic. However, our experience with in-house and user campaigns does not suggest a general “large portion” of hits at crystal contacts or on site presumably “unlikely to influence the protein function”. However, we do acknowledge that there is a minor part of such hits. To this end we included the following paragraph in the discussion, after the paragraph specifying expected hit rates. The new paragraph reads:

“A general caveat of crystallographic fragment screening is the presence of crystallographic contact sites. These could either occlude a priori known active sites (to be checked before the screening, see above), or, these contact sites also often provide pockets and hot spots where fragments can bind. Such fragment hits will be artifacts of the crystallization lattice and will likely not bind to the protein in solution. However, this is for most projects usually the minor portion of the hits obtained. For example, in the F2X-Entry Screen validation campaign using endothiapepsin (EP) and the spliceosomal protein-protein complex of Prp8RnaseH and Aar2 (AR), most of the hits occurred in promising sites 11. For EP, 27 out of the 37 observed binding events were located in the active site, i.e. the peptide cleft of this protease. The 10 remote binding events comprise two solvent exposed binding events and eight crystal contact binding events (corresponding to five unique hits). Excluding those crystal contact hits would still reflect an overall rate of 24% unique hits for the EP campaign. It is also important to notice that binding events remote of a known active site (except crystal contact binders) could also potentially be interesting, e.g. revealing new hot spots or allosteric sites of the protein. For the AR campaign (in the same publication), of the 23 observed binding events, seven were located at crystal contacts, one was located at the direct interface of the two proteins, seven were located at known protein-protein interactions sites with other binding partners of the larger biological context (hence different assembly stages of the spliceosome), eight binding events revealed two hot spots on AR of yet unknown function and one being at a solvent exposed surface of Prp8RnaseH. Therefore, excluding the events at crystal contacts and the Prp8RnaseH singleton, the number of potentially useful binding events is 15 (corresponding to 14 unique hits) thus a hit rate of 15.6%. These hits can be starting points for design of protein-protein interaction modulators or for tool compounds aimed to explore the two discovered Aar2 hot spots. Taken together, also in line with conducted user campaigns, often only a minor portion of hits in crystallographic fragment screening has to be disregarded as artefacts. However, this will also be largely target dependent”

With best regards,

Manfred Weiss

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