Answers to reviewers' and editors' comments

We would like to thank the reviewers and the editorial board for their very constructive comments. All of them were taken into account to revise the manuscript and the video. Changes in the revised text are highlighted in red, as well as our answers below.

Editorial and production comments

Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Done
2. Please revise the title for conciseness. Please reflect the revised title in the video as well. The title has been shorten
3. How can one obtain/get the ChipX? A sentence has been inserted in Step 1. to indicate that ChipX can be obtained from the authors
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. Done (all product names are referenced in the Table of Materials)
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. Done (see below)
6. Please provide an example for the protocol instead of a generalized protocol. We need specific values throughout. The protocol has been updated accordingly
7. What enzyme solution is used and at what concentration? Done
8. 1.1.5: What are the dimensions of the piece of tape? Done
9. 1.2.5: Incubate at what temperature? Done
10. 2.15: As described where? Done
11. Please provide the diffraction data collection parameters. Done (see representative results and table 1)
12. Please discuss some limitations of the protocol in the discussion. Done

Changes to be made by the Author(s) regarding the video:
1. Video Editing Notes
• 01:15 Directly after the chapter title card leaves the screen, there is a black flash frame before the animation of the X3 chip. Consider using a fade down to and from white and eliminate the black flash frame. Done
• 04:10 Similar issue here as 01:15. Avoid flashing black or white frames. Consider fading down to white and fading back up to the chapter title card. Done
• 04:47 It looks like the outgoing image of the channel with the crystal in it is being matched to the incoming closeup of the channel. A neat idea, but perhaps increase the duration of the dissolve (fading across two shots/images), as right now it seems "glitchy" instead of smooth, because the incoming image appears too quickly. Done

• 05:02 This transition could use a little work as well, similarly to the other title card transitions. The fading out of the graphics should complete before the fading in of the chapter title card. Done
• 09:55 Black flash frame right before chapter title card Done
• 10:01 The background behind Raphael here shifts or jerks a bit at the beginning. Consider starting playback a little later to trim out the glitch Done
• 10:30 The dubbing here is a little obvious. Try applying some frequency equalization to his voice to knock out the low-ends / bass to try better match the original audio. Some reverb might help too (the dubbing sounds too "clean" compared to the live audio recorded by Claude Done

Please upload a revised high-resolution file here:
https://www.dropbox.com/request/zE6EOlNMqYYH4PC3Panj?oref=e

Reviewer #1:

Manuscript Summary:
This paper describes a method of using the ChipX counter-diffusion microfluidic device for the crystallization, potential ligand soaking, and then in situ room temperature X-ray diffraction data collection of protein crystallography data.

Major Concerns:
1. Line 118: In Step 10 of the labeling protocol, why is the flow-through from the spin column recovered? Perhaps there is a difference in terminology here, but to me the "flow-through" would be the filtrate that passes through the filter (thus lacking protein), rather than the retentate (containing protein) that sits above the filter.

Related to this point, the authors could specify a "protein containing" solution where relevant for emphasis.

The procotol is correct and corresponds to the original one published by Pusey and coll. (ref 7). The column is a desalting device used to exchange the buffer and the protein is recovered in the flow-through. We rephrased this part to better indicate in which buffer the protein is.

2. The authors provided useful guidelines for adapting crystallization conditions from vapor diffusion to counter diffusion. Can they also comment on the potential importance (or lack thereof) of inspecting samples over time? Depending on the crystallization conditions the counter diffusion concentration gradient could take hours or days to develop and ultimately stabilize. How important is this transient period in terms of successful crystallization results as compared to vapor diffusion?
Related to this point, would it be useful/informative to describe how the location in the channel where crystals form can be correlated to a specific concentration. For instance, where in the channel did the crystals of the CCA-adding enzyme form? Done (representative results and discussion).

3. In the procedural discussion related to ligand soaking and Figure 4, it might be useful to specify the total volume of the reservoir. Based on the schematic, I assume that 5 µL of crystallization would not be enough to fill up the well. Perhaps 10 µL would not fill the reservoir either. At what point should the user be concerned about overflow? Done (see 1.2.1)
4. How is the device secured to the chip holder for X-ray analysis? Are there set screws? Please clarify this part of the procedure and any relevant considerations. Done (see 5.1)
5. For users who are not familiar with room temperature data collection, are there any considerations and/or indications of data quality lost that would be useful to communicate? Done (representative results and discussion)
6. The authors state that "the counter-diffusion method implemented in ChipX is very efficient at screening the supersaturation landscape and at finding nucleation and growth conditions." While I absolutely agree, this statement is given without explanation. I do not think that a detailed discussion of this point belongs in this type of protocol paper, but it might be useful to refer the reader to papers where they can read more about this aspect of the method. Done (see discussion)
7. Similarly, the statement that crystal quality is preserved by avoiding physical handling could be supported by references. Done
8. How is the volume needed for an individual counter-diffusion experiment in ChipX only 300 nL if 5-6 µL are needed to fill the chip? Done (explained in 1.1)
9. Can the authors comment about any general trends in the usability of their device with regards to successful data collection on small crystals? There is a certain amount of background scattering and signal attenuation associated with the device. Are they able to collect data on crystals that are equal in size to the thickness of the device? Half of that? What is known? Done (see discussion)

Minor Concerns:
1. In the Introduction, please clarify that the acryonym 'CCA' in CCA-adding enzyme refers to a nucleotide sequence. I did not realize this until I got to the more detailed description in the representative results section. A sentence was added in the introduction
2. Line 87: When talking about "recovering the extra solution in the crystallant reservoir," would the authors please comment on the method used for this? Are standard pipets sufficient? Is there any particular technique involved? Done
3. Line 134: the use of the term "binocular" in this context seems odd. I think of binoculars as being used to magnify things at a distance. Perhaps the description "stereomicroscope" could be used in place of binoculars, and differentiated from the already mentioned "microscope" as an inverted/upright microscope? Done
4. Lines 175-176: The discussion of chip orientation with respect to the X-ray beam could be improved via reference to a figure that more clearly highlights the device architecture. Done
5. Line 232: The phrase "The whole is sealed" was unclear. Are the authors referring to the entire device or holes in the device? Done
6. In the caption for Figure 1 I might suggest specifying right/left in the image for the single inlet vs. the crystallization solutions, rather than "one side" and "the other side." Done

7. In Figure 1 we can easily discern the ABCD labels for each of the channels. However, it is unclear what the smaller labels along the length of each channel are. Would the authors please include an inset image that highlights this aspect of their device? (One might also consider showing a photograph, rather than just the schematic). Done. This point is nicely illustrated in the movie (see 4:15)
8. It might be useful for the authors to label some key residues in their electron density map in Figure 7 to give readers who are not intimately familiar with this protein a point of reference. Done

Reviewer #2:
Manuscript Summary:
The purpose of this video is to grow protein crystals in microfluidic channels using the diffusion technique. Protein crystals can be measured directly in X-ray beam without any extra steps for crystal handling.

Major Concerns:
I do not have any major concerns and I like this ChipX techniques.

Minor Concerns:
All steps in the video are very clear to follow up and all expiations from the beginning to the end are easily understood. In the section II and III (2:26 to 4:10), it would be much better if the view could be zoomed in more to see the loading the process.

Zoomed views have been inserted in the video as suggested at 2:30 and 3:27.