Revision letter to editor: Title: **LabVIEW-operated novel nanoliter osmometer for ice binding protein investigations. Manuscript number 4189**

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Dear Editor,

We thank the reviewers for their effort in reviewing our manuscript and for their helpful remarks. We have improved the manuscript by addressing these remarks and comments. Please find below explanations for the changes and answers to the reviewers' remarks, as well as quotes from the relevant changes in the text. For clarity, we numbered the reviewers' comments and have marked them in *italic*. Citations from the manuscript are in smaller font.

Reviewer 1: *Summary*
1.1 The MS deals with measurement of TH created by IBP using nanolitre osmometer. The technique provided in this MS is generally very useful and significant to understand the interaction of proteins and ice.
To automatize the control of the ramping rate of temperature and video recording of the data, the authors developed LabVIEW-operated nanoliter osmometer. Overall this system is convenient and advanced for studying the kinetics of the ice-protein interaction as well as TH measurement.

We thank the reviewer for his/her supportive comments. Below we address the minor comments.

Major Concerns
None

1.2
Minor Concerns
Page 2, line 1: the word "cold-environment" is, I think, not a good fit in this case. Please use other words.

First sentence changed in the manuscript to " Ice-binding proteins (IBPs), including antifreeze proteins, ice structuring proteins, thermal hysteresis proteins, and ice recrystallization inhibition proteins, are found in cold-adapted organisms and protect them from freeze injuries by interacting with ice crystals."

1.3)
Page 2, line 3: I would like to ask the author to rewrite the sentence to make readers understood clearly. This sentence needs some polishing.

We changed the sentence to include a more detailed description of the TH phenomenon. We hope it is more clear now.- " IBPs adsorb to the surfaces of ice crystals and prevent water molecules from joining the ice lattice at the IBP adsorption location. Ice that grows on the crystal surface between the adsorbed IBPs develops a high curvature that lowers the temperature at which the ice crystals grow, a phenomenon referred to as the Gibbs–Thomson effect. This depression creates a gap (thermal hysteresis, TH) between the melting point and the nonequilibrium freezing point, within which ice growth is arrested (8-10), see Fig 1."

1.4)
Page 4, 3.4) Form a single ice crystal~ needs changing. It should sound like this. "Increase the temperature slowly until you find a single ice crystal (or a single ice crystal is formed), and then stop increasing the temperature. This point can be noted as its melting point."

Changed in the manuscript to:
" Melt the ice until a single crystal remains by adjusting the temperature. The final size of the crystal should be around 10 μm. Switching to a 50X objective at this point is recommended. This adjustment is interactive, and the final steps are typically performed using small temperature steps of 0.002°C. The highest temperature at which melting has ceased is determined to be the melting point.."

1.5)
Page 4, 3.5) Please change "Adjust~" to "Adjust the ramping rate as desired (or as you wish)"
Changed in the manuscript to: "Adjust the ramping rate as desired"

1.6)
Page 5, 3.7) Please change "Observe~" to "Observe the crystal shape as temperature decreases. At some point you may detect a sudden burst of ice crystal which is a crystal burst temperature."

Changed in the manuscript to: "..Observe the crystal shape as temperature decreases. At some point you may detect a sudden burst of ice crystal. The temperature at which this happened is noted as the crystal burst temperature."

1.7)
Page 5, 4.3) Please delete "exactly"

Done.

1.8)
Page 5, 4.4) Please make two sentences. The second one may be "Calculate the TH activity as a function of time to evaluate the time-dependence of TH activity.

Changed in the manuscript to: "Document the temperature at which the crystal burst occurs. Calculate the exposure time (the time between crystal formation and the crystal burst).

4.5) Repeat the experiment for various delay times and plot the TH activity as a function of exposure time to evaluate the time-dependence of TH activity."

1.9)
Additional Notes to the Author
As described in the MS, it would be good if the authors make this system available for the IBP community.

We agree and indeed we wrote in the manuscript that the software is available upon requ

est. Page 6: "The LabVIEW software and the designated adapting electric circuit design for the Clifton

stage are available upon request"

Reviewer 2:

Summary

The article definitely provides a sound description of a very delicate measurement procedure. It took me about a month to set-up the technique myself. Having a well-described protocol would have helped me a lot. I am looking forward to seeing the video. The development and description of a new nanoliter-osmometer is a significant contribution to the field, as Clifton Nanoliter Osmometers (the standard instrument for this kind of measurements) are no longer built. Nevertheless, the description of the experimental set-up needs to be improved.

2.1)

Rational and background are mostly well explained, although the authors omitted to mention that the technique is also applicable for the measurement of the osmolality of very small samples - although the name of the instrument of course implies that - ..

Indeed this system is designed to measure osmolarity. We add a section describing the Clifton instrument usage and its limitation: " Nanoliter osmometers, such as the Clifton Instrument (Clifton Technical Physics, Hartford, NY, no longer available) were design to measure the osmolarity of a solution by measuring the melting point depression of droplets with nanoliter volumes. These devices were used to measure the osmolarities of biological samples, such as tears (11), and were found to be useful in IBP research. Manual control over the Clifton nanoliter osmometer limited the experimental possibilities. Temperature rate changes could not be controlled reliably, the temperature range was limited to 4000 mOsmol (about –7.5 C), and it was not possible to record the temperature as a function of time. "

2.2)

..and for the measurement of recrystallization inhibition (Kiko 2010). For the measurement of recrystallization inhibition also another method exists (Knight et al 1988), which should be cited.

Indeed recrystallization inhibition is important function of IBP and can be monitored with nanoliter osmometer. Here is the text we added regarding recrystallization:

".. The nanoliter osmometer additionally allowed us to test the recrystallization inhibition of IBPs (5, 13). In general, recrystallization is a phenomenon in which large crystals grow larger at the expense of small crystals. IBPs efficiently inhibit recrystallization even at low concentrations (14), (15). We used our LabVIEW-controlled osmometer to quantitatively follow the recrystallization of ice and to enforce a constant ice fraction using simultaneous real-time video analysis of the images and temperature feedback from the sample chamber (13). The real-time calculations offer additional control options during an experimental procedure."

2.3)

Furthermore the use of the term ice binding protein is a bit misleading. Most authors

call proteins that cause thermal hysteresis "Antifreeze proteins", also the term "Thermal hysteresis proteins" was sometimes used. The term "ice-binding protein" was introduced for protein solutions that cause recrystallization inhibition, but not thermal hysteresis. Further work showed that there is actually no mechanistic difference between ice-binding and antifreeze proteins. Also ice-binding proteins are able to cause thermal hysteresis if present at high enough concentrations. Please see Kiko (2010) for a further discussion on that topic. I would propose that the authors use the term antifreeze proteins and note that these are also sometimes called ice-binding proteins or thermal hysteresis proteins. Including all three terms in the article will increase the chance that researchers searching for techniques to characterize this class of proteins will actually find it. All three terms should also be included in the keywords.

We thank the reviewer for pointing to the naming of the proteins. Indeed several naming of the proteins are used, including thermal hysteresis proteins, antifreeze proteins, and ice structuring proteins. In addition there are recrystallization inhibition proteins. Further still there are the ice nucleating proteins that interact with ice as well. Recently the name ice binding proteins was put forward by Raymond (Ice-binding proteins from sea ice diatoms (Bacillariophyceae). J. Phycol. 42, 410-416 2006), and Davis and Braslavsky (J Mol Biol. 2012 Mar 9;416(5):713-24) as a name to describe both antifreeze proteins and recrystallization inhibition proteins that while share their activity, are used differently in nature in freeze avoidance and freeze tolerance creatures. This name is actually used to reduce the misleading naming of antifreeze proteins that can work as recrystallization inhibition in solutions that contains ice. Indeed other naming were introduce to reduce this confusion such as "ice structuring proteins" (by Unilever). Still this new name did not catch on. The name ice binding proteins is currently gain momentum; for example, the international conference that was take place recently has the name, ice binding proteins conference (http://pldserver1.biochem.queensu.ca/IBP_meeting_2011/). Thus we would like to keep the name Ice binding proteins, and added the other names in the text and in the keyword as suggested. We add to the abstract the following sentence:
" Ice-binding proteins (IBPs), including antifreeze proteins, ice structuring proteins, thermal hysteresis proteins, and ice recrystallization inhibition proteins, are found in cold-adapted organisms and protect them from freeze injuries by interacting with ice crystals."

2.4)
Major Concerns
A good description of the cooling stage, including the copper disc is needed or the supplier needs to be mentioned. Also the whole measurement set-up needs to be described better to justify the title of the article.

We added a paragraph with the description of the cooling stage as well as a figure with stage details.

Page 3: "**The cold stage system**
The cold stage assembly (Fig. 5) consists of a set of thermoelectric coolers that cool a copper plate. Heat is removed from the stage by flowing cold water through a closed compartment under the thermoelectric co olers. A 4 mm diameter hole in the middle of the copper plate serves as a viewing window. A 1 mm diamet er in-plane hole was drilled to fit the thermistor. A custom-made copper disc (7 mm in diameter) with seve

ral holes (500 µm in diameter) was placed on the copper plate and aligned with the viewing window. Air was pumped at a flow rate of 35 mL/sec and dried using Drierite (W.A. Hammond). The dry air was used t o ensure a dry environment at the cooling stage. The stage was connected via a 9 pin connection outlet to a temperature controller (Model 3040 or 3150, Newport Corporation, Irvine, California, US). The temperat ure controller was connected via a cable to a computer GPIB-PCI card (National instruments, Austin, Texa s, USA). “

2.5)
Minor Concerns
The short abstract should mention the different terms for antifreeze proteins and in one or two more sentences explain the measurement principle and introduce the term "thermal hysteresis".

We add the name antifreeze proteins to the short abstract. The word limit (50) of the short abstract does not allow for adding further sentences. We describe the thermal hysteresis in the long abstract.

2.6)
Long abstract:
Include the terms "Antifreeze proteins" and "Thermal hysteresis proteins" in the abstract.

Done.

2.7) Mention that the Nanoliter-Osmometer can also be used to measure recrystallization inhibition and osmolality of very small samples.

Done, see above section 2.1 and 2.2.

2.8) Antifreeze proteins were also found in crustaceans (Kiko 2010).
Second sentence: Sort the list according to taxonomic knowledge. It could also include a reference to algae (Raymond et al 1994) although these are covered under plants.

We added the recommended references to the manuscript. We organized the naming in a better way we hope, even thought we mix kingdoms (plants, fungi and bacteria) with phylum (Arthropods that include insects and crustaceans) as well as fish which are under the phylum of chordate, which is not easily recognized by the non biological audience. On the other hand the fish are well known in the field of antifreeze proteins. This kind of list are common in the AFP literature.

Thus the sentence changed to: " IBPs are found in a variety of organism, including fish (1), plants (2, 3), arthropods (4, 5), fungi (6), and bacteria (7)."

2.9) First sentence of second abstract: point missing.
… dependence of the TH activity. We showed …

The point has been added.

2.10) Protocol text:
Sample preparation: I believe the particle size should be 200 µm not mm. Please provide a sketch of the cover slip with the particle or improve the text. The text is a bit confusing. One could get the impression that the Drierite particle is surrounded entirely by glue, but if I understand it correctly it is enclosed in between the two coverslips, which are glued together at the edges.

We improved the text describing the coverslipe. Further details of the assembly will be shown in the video.

1. " **Double-layer coverglass assembly.** A coverglass assembly was prepared to allow for sample observation without condensing moisture on the cover glass surface. This was achieved by placing a Drierite (W.A. Hammond Drierite, Xenia, Ohio, USA) particle (2 mm in diameter) between two coverslips that were then glued with a hot glue gun. This configuration prevented condensation that could block the view when the sample was cooled to low temperatures and removed the need to blow dry air onto the observation window."

2.11) I think that the cooling stage set up needs to happen before loading the samples and therefore is a preliminary procedure. At least I always did it like that. Please check that the order is correct.

We change the order as suggested.

2.12) Please provide the supplier and model of the cooling stage or, if it is a custom made stage provide a detailed description of the stage, including some technical drawings. Also a scheme of the set-up (connection to the computer, tubings etc.) would improve the manuscript. The title of the manuscript implies that a nanoliter-osmometer is described. Currently, this is not the case, it is rather the measurement principle that is described. The description of the osmometer is not complete. Please make sure that this is the case.

" The cold stage, described previously (9, 10), contains a metal block through which water circulates, ther eby functioning as a heat sink. Attached to this block are thermoelectric coolers that may be driven using a commercial temperature controller that can be controlled via LabVIEW modules. Further details are provi ded below. The major advantage of this system is its sensitive temperature control (0.002°C). Automated t emperature control permits the coordination of a fixed temperature ramp with a video microscopy output c ontaining additional experimental details. "

And

"**The cold stage system**
The cold stage assembly (Fig. 5) consists of a set of thermoelectric coolers that cool a copper plate. Heat is removed from the stage by flowing cold water through a closed compartment under the thermoelectric co olers. A 4 mm diameter hole in the middle of the copper plate serves as a viewing window. A 1 mm diamet er in-plane hole was drilled to fit the thermistor. A custom-made copper disc (7 mm in diameter) with seve ral holes (500 μm in diameter) was placed on the copper plate and aligned with the viewing window. Air was pumped at a flow rate of 35 mL/sec and dried using Drierite (W.A. Hammond). The dry air was used t o ensure a dry environment at the cooling stage. The stage was connected via a 9 pin connection outlet to a temperature controller (Model 3040 or 3150, Newport Corporation, Irvine, California, US). The temperat ure controller was connected via a cable to a computer GPIB-PCI card (National instruments, Austin, Texa s, USA). "

2.13) Step 1.3: please provide the rubber tube diameter and manufacturer, as well as the syringe type, volume and manufacturer.

Done.

(2.14) Step 1.5: please provide more information on the protein solution used. What is MpIBP actually? How was it prepared. Is it a recombinant protein? Which concentration was used?

Added to the protocol text (2.5) - " (2.4 μM *Mp*IBP-GFP in 20 mM $CaCl_2$ and 25 mM Tris-HCl at pH 8, see reference (10) for the preparation details)"

(2.15) Step 2.2: what is the floe rate of the dried air? Is it cooled to avoid a change in temperature in the cooling stage?

The air was dried by column of drierite (details in table) with a flow rate of 35 ml/sec. The air was not pre-cooled. The temperature is dynamically controlled by the thermo-coolers which compensate for the heating of the air. We added the information in the manuscript. Page 3"…Air was pumped at a flow rate of 35 mL/sec and dried using Drierite (W.A. Hammond). The dry air was used to ensure a dry environment at the cooling stage."

(2.16) Step 3.4 Better write: Let the temperature slowly increase until a single ice crystal (10 μm size) is present … Mentioning of the 50X objective here could be omitted.

We change the text to ".. 3.3) Initially, the solution droplet will be clear. At low temperatures, typicall y –35°C, the droplet changes color, indicating that the solution has frozen. Immediately after the sample h as frozen, increase the temperature slowly until the bulk ice begins to melt.
3.4) Melt the ice until a single crystal remains by adjusting the temperature. The final size of the crystal should be around 10 μm. …"

If one do not switch to higher magnification at this point, it is difficult to accurately control the crystal size. Thus we would like to keep the recommendation. "..Switching to a 50X objective at this point is recommended.."

(2.17) If the ice crystal is still present, then this is not the melting temperature! For a

more precise measurement, melting temperature and thermal hysteresis should be measured in two separate runs.

Please note that the melting temperature is the equilibrium temperature in which ice is not growing or melting and not the temperature of the disappearance of the ice. It is important to have only small fraction of ice in the droplet to limit the osmolats (salts mostly) concentration change due to the present of the ice. A 10 micron wide crystal, is less than 0.001 of the volume of a 100 micrometer diameter droplet and thus does not change significantly the concentration. We measured the melting temperature by measuring the temperature in which the crystal is marginally melting.

(2.18) Fiure 1: *Celik et al 2010 is not cited correctly. It should probably be a (7)*

Corrected.

(2.19) Figure 3: *How many measurements were done per time point?*

Added to the figure legend – "Each time point is the average of 3–6 experiments"

(2.20) Discussion:
GFP-tagged IBPs: define abbreviation GFP; if it is the GFP-IBP from paper (6), give a reference to it here.

We defined GFP and added a relevant reference (10).

(2.21) A discussion of the actual data could be beneficial here. E.g. the work presented makes clear that kinetics impact on the actual TH readout and that the initial small (10 µm) crystal should be kept just below the melting point for ten minutes to obtain proper read outs. Crystal-size also impacts on the TH readout. Provision of a very standardized protocol for this kind of measurement is an actual strength of the article and could be further emphasized.

We further emphasized this point as suggested: "… The time dependence described indicates th at the cooling rate may influence the TH readings. Thus, we suggest including a report of the time during which the crystal was exposed to the solution prior to cooling, as well as the cooling rate. We typically wai ted 10 min prior to ramping down the temperature at 0.01°C steps each 4 sec…"

(2.22) Additional Notes to Authors
(((For the process of aspiration and dispension of nanoliter amounts I actually used a rubber tube that is filled with immersion oil and closed at one end with a pulled capillary (not used for sample aspiration). I then attached a pulled capillary filled with immersion oil to the other end of the rubber tube (tube length about 7 cm; one needs to leave the capillary tube with the blunt end in a small pool of immersion oil for some time, so that it fills by itself. Same with the tubing.) The capillary is then hold with one hand and the tubing can be pressed with the other hand to push out some of the immersion oil. Thereafter, the capillary is introduced into the sample (or tissue) and the pressure on the tubing released, which leads to the aspiration of liquid. After insertion into the sample holder, the sample is dispersed trough squeezing the tubing again. The authors might try this technique, which works well also if very small sample amounts

need to be handled. I found it difficult to do so with a microliter-pump)))

We thank the reviewer for the suggestion. We will try it. Still, in order to control the small amounts that are handled with air pressure we used a very fine opening of the capillary. We estimate it on the order of one micron size. We ensure the proper size of the opening with fine bubbling of air, as was discussed in the capillary preparation. Under these conditions the handling becomes simple and not very sensitive to the pressure. It will be shown in the video.

Reviewer 3:

(3.1) Summary

The idea behind this is very good - both Clifton nanolitre osmometers and Otago osmometers are somewhat difficult to use and need some practice. I am somewhat skeptical about having 0.002 C control - but it is possible I suppose.

We add a graph with temperature measurement of the holder as measured with our instrument (fig 4). This shows fluctuations of 0.002 ^{o}C. Some gradients within the droplets are present, on the order of 0.01 ^{o}C/ 100 microns. Still, there is a good control on the temperature of a crystal that is located at a constant position.

(3.2) Major Concerns

-In sample preparation part where do the copper discs come from and what do they look like?

We included a figure with description of the disc (fig 5) and described it in the text. Page 3: "..A custom-made copper disc (7 mm in diameter) with several holes (500 μm in diameter) was placed on the copper plate and aligned with the viewing window. "

(3.3) -Equally the double layer cover glass is a novel idea - but would not dry air blown over the cover glass do the same job?

Blown dry air over the upper cover glass probably will be fine, but the static solution works well and very simple to use. Once the double glass is formed it can be used for several months.

(3.4) -Also its states a 200 mm drierite particle - a typo I am sure.

Changed to - 2 mm Drierite particle.

(3.5) Minor Concerns

How does someone with a Clifton stage access the "designated adapting electric circuit"?

We added: "..The LabVIEW software and the designated adapting electric circuit design for the Clifton stage are available upon request.."

(3.6) Additional Notes to the Author

The idea is good, but please expand on the details.

We expand on the details in this revision.
We thank the reviewers for the constructive review.