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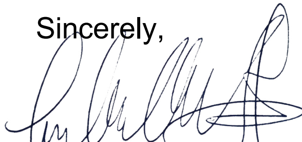
Journal of Visualized Experiments
1 Alewife Center Suite 200
Cambridge, MA 02140

Dear Vineeta Bajaj,

We thank the reviewers for their thoughtful comments and are encouraged that they are positive about our manuscript "**Cryogenic sample loading into a magic angle spinning NMR spectrometer that preserves cellular viability"**. The reviewers provided several clarifying suggestions. We have incorporated changes to address the reviewer's comments. Changes in the text are indicated with red text in the manuscript. A line by line response to the editorial and reviewer's suggestion is appended to this letter. All in all, these revisions improve the manuscript to the point, I believe, that it is ready for acceptance and publication. I hope that you and any of the reviewers will agree. I look forward to your decision.

Sincerely,



Kendra Frederick, Ph.D.

Point by point responses below. Critique in black, response indented.

<u>Editorial Comments:</u>

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• **Protocol Detail:**
1) 3.4.6: mention spin rate examples.

The spin rate of 12 kHz is mentioned in the text.

• **Protocol Highlight:** Please ensure that the highlighting is <2.75 pages.
1) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.

2) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

Highlighted steps now begin with section 2.2, rather than 1.2 to comply with length restrictions.

• **Discussion:** JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

We have slighted expanded the discussion to include an additional future application that was highlighted by reviewer 3.

• **References:**
1) Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For less than six authors, list all authors. For more than 6 authors, list only the first author then *et al.*): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage, (YEAR).]
2) Please spell out journal names.

Referencing conforms to journal style.

• **Commercial Language:** JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Tryp-L, cryovial, countess, etc.
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

All instances of cryovial have been changed to cryogenic vial. Tryp-L has been changed to trypsin and Countess has been changed to automated cell counter.

• **Table of Materials:** Please sort in alphabetical order.

The table is in alphabetical order.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Not applicable. Unpublished data.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The authors re-describe a protocol which is already described well in the literature. There is nothing new to report here, and the paper should not be published.

Major Concerns:
You can basically find the same protocol on any commercial site:

https://www.thermofisher.com/ch/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/freezing-cells.html

So this is already published and I would guide the authors to extend their literature searches beyond the NMR community to understand how this kind of work fits into the WIDER scientific community, ie: It's already been done.

Another section of this protocol is simply just detailing how to drop a sample into liquid nitrogen. This is what I would expect to see in an SOP and not in a publication. Is it reasonable to publish SOPs? I don't think so. Again, just because you're freezing cells in a new container (rotor compared to a cryovial) does not make it new or publication worthy.

In addition, the transfer of a frozen sample into an NMR spectrometer, is this really new compared to say, performing the same task on an EPR spectrometer? No new tools are described. The most important point is probably the marking of the rotor, but again, the cryoEM field would be well endowed with this knowledge and they have special pens for doing such a thing, again, there is a lack of engagement with the existing scientific literature and protocols already published to deal with such challenges. Again just because it's not published in an NMR journal does not mean it doesn't exist or is therefore novel.

As highlighted by the editor, novelty is not a requirement for publication in JoVE.

They also describe this is for DNP but show no DNP or how the slow freezinge (1oC/min) which is indeed how cells are cryo preserved normally) impacts DNP enhancements in cells.

As described in the introduction, performing DNP experiments on live cells was a significant motivator in our development of this protocol. Indeed, it is one (of several) exciting applications and this article is narrowly focused on cryogenic sample loading into a magic angle spinning NMR spectrometer which is a critical step towards this goal. We present data on cellular viability pre and post-NMR experiment to support the claim of cryogenic transfer. While we understand the enthusiasm, addition of DNP data (or even NMR data) would not provide any evidence of successful cryogenic sample transfer and is therefore outside the scope of this manuscript.

We also appreciate the reviewer’s curiosity about the impact of freezing rate on DNP enhancement. Indeed, we have a manuscript in preparation on this topic. However, that work is also beyond the scope of this manuscript and, quite honestly, would make for dull cinematography.

Reviewer #2:
Manuscript Summary:
In principle, DNP-NMR spectroscopy can enable structural characterization of molecules at an endogenous concentration by providing orders of magnitude signal enhancement. Practical applications of DNP-NMR are demonstrated only at cryogenic temperatures so far, therefore discussions on sample handling methods including cooling history during the sample preparation are desired. Here authors demonstrate the step-by-step sample handling procedure for mammalian cells at cryogenic temperature. The slow-cooling for mammalian cells as a cell-culture technique or pre-cooled DNP rotor handling for DNP-NMR spectroscopists can be seen somewhat typical, however, considering the difficulty of handling cryogenic samples, visualization of these procedures will be useful to those researchers who consider to join the field and try to adopt a state-of-the-art technique for their researches. I believe that this work presents an appropriate sample-handling procedure and make an effective record for visualization of research details.

Minor Concerns:
(1) As references for freeze-quenching reactions and protein folding, I believe that it is appropriate to include also a recent publication, https://doi.org/10.1073/pnas.1908006116, since it shows the current advancement for rapid freeze-quenching and MAS-DNP NMR spectroscopy, and makes a good example for the importance of handling pre-cooled rotors for Bruker DNP-NMR system.

We thank the reviewer for bringing this work to our attention and have included it in the introduction (new reference 18)

(2) Line 388-389 mentioned that uncontrolled freezing for purified proteins (overexpressed protein) does not compromise the sample quality, however, I would like to introduce a recent publication to authors: https://doi.org/10.1002/cphc.202000312, and emphasize that methods of cooling proteins need to be carefully chosen by considering types of proteins.

We have expanded the discussion to include this interesting study: "Uncontrolled freezing of samples containing purified proteins or cellular lysates does not typically compromise sample quality[7,27], although there are some indications that freezing rate may be an important variable even in purified systems[28]." (new reference 28)

(3) The figure showed that RT & MAS-NMR sample shows a negative effect on cell viability. While I believe that this is due to the detrimental effect of rapid spinning the cell sample, I would recommend including the spinning rate that was used in the experiment.

We haven't determined if the rate of temperature change (from room temperature to 100 K in a few minutes) or the high g-forces inside the rotor (12 kHz MAS) is more deadly to cells, but the combination is certainly terrible. We added the text "that had been pre cooled to 100 K" and included the MAS rate to clarify the details of the experiment in the figure legend for figure 2.

Reviewer #3:
Manuscript Summary:
Ghosh and coworkers describe a protocol for transferring previously frozen solid-state NMR samples into a pre-cooled MAS-NMR probehead for subsequent data acquisition. For whole-cell

NMR studies, this is a very necessary yet very difficult procedure. As the authors correctly point out, successfully being able to transfer a gently frozen and cryoprotected cell pellet into an NMR spectrometer, while maintaining cryogenic temperatures, enables the study of proteins and other biomolecules in their native environment and at their native concentration (albeit with the added variable of cryogenic temperature). Care is taken in the manuscript to denote seemingly trivial but quite critical steps, such as the rotor marking and orientation. As a result, the protocol will be easy to follow even for novice users. Shortcomings of the current protocol are also described, along with potential strategies for improvement. The protocol is well-documented, contains adequate images, and will be of interest to a wide range of NMR spectroscopists and structural biologists working to develop the field of whole-cell NMR.

Major Concerns:
None

Minor Concerns:
A few suggestions:
Step 3.3.2: the orientation of the rotor is quite important; the term "end cap" may be confusing to some. Perhaps "drive tip" or "drive cap" should be used instead.

We thank the reviewer for suggestions. “End cap” has been changed to “drive tip” throughout the text.

Step 3.4.6: the sample is already frozen and therefore mass redistribution should not be a problem; it then makes sense to spin up relatively rapidly to avoid warming up the cryogenic gas lines with low flows. Can the authors suggest some initial conditions (e.g. gas flow parameters)? Or has slow spin-up been found to perform better?

As the reviewer intuits, we spin the sample up relatively rapidly. Anecdotally, there is no correlation between experimental time (a half an hour versus several days) and post-experiment viability. Because cells experience more spinning force in longer experiments, this indicates that spinning is not an important variable. We have included guidelines for gas flow parameters in Step 3.4.6: “Immediately increase the bearing gas to ~200 mBar and the drive gas to 10 mBar. Once the sample spins, increase the bearing gas to 1000 mBar and drive gas to 200 mBar. As spinning stabilizes, increase the bearing to 2400 mBar and then increase drive gas from 200 mBar to 1700 mBar over several minutes. VT cooling gas is constant at ~1070 L/h.”

Step 5.1.2 (Note): "slow warming up decrease viability due to water recrystallization" -> I gather the authors refer to freeze-thaw processes from accidental heat transfer here, but the sentence needs to be clarified and perhaps expanded upon.

The sentence has been edited to clarify as follows: “*NOTE: Sapphire is an excellent heat conductor. Avoid touching the rotor with your fingers because heat transfer can cause local freeze-thaw events that compromise cellular viability.”*

Discussion:
(line 370): the word "while" is unnecessary in this context.
Removed.

(line 374): …other cellular systems; however, …
Corrected.

(line 376): …"the same the results" --> "the same results"
Corrected.