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TITLE:
Cryogenic sample loading into a magic angle spinning NMR spectrometer that preserves cellular viability

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SUMMARY:
A protocol for cryogenic transfer of frozen samples into the DNP MAS NMR probe. The protocol includes directions for rotor storage prior to the experiment and directions for viability measurements before and after the experiment.

ABSTRACT:
Dynamic nuclear polarization (DNP) can dramatically increase the sensitivity of magic angle spinning (MAS) nuclear magnetic resonance (NMR) spectroscopy. These sensitivity gains increase as temperatures decrease and are large enough to enable the study of molecules at very low concentrations at the operating temperatures (~100 K) of most commercial DNP-equipped NMR spectrometers. This leads to the possibility of in-cell structural biology on cryopreserved cells for macromolecules at their endogenous levels in their native environments. However, the freezing rates required for cellular cryopreservation are exceeded during typical sample handling for DNP MAS NMR and this results in loss of cellular integrity and viability. This article describes a detailed protocol for the preparation and cryogenic transfer of a frozen sample of mammalian cells into a MAS NMR spectrometer.

INTRODUCTION:
The introduction of dynamic nuclear polarization for magic angle spinning nuclear magnetic resonance spectroscopy can increase the sensitivity of MAS NMR by several orders of magnitude. This has enabled detection of biomolecules at or near their physiological concentrations. DNP can and does provide the sensitivity required to detect an isotopically

labeled protein at endogenous (~1 μM) concentrations in complex biological environment[1]. Because there are well-established protocols to introduce isotopically-labeled molecules into unlabeled mammalian cells without affecting their viability, this opens up the possibility of studying isotopically-enriched biomolecules at their endogenous levels in their native environment. Moreover, because DNP enhancements are more efficient at lower temperatures[2-4], the experimental temperatures for DNP MAS NMR align neatly with those required for long-term storage of viable mammalian cells[5]. However, the conventional method of transferring a sample into a DNP MAS NMR spectrometer subjects it to temperature fluctuation rates that rupture mammalian cells.

MAS NMR experiments require that the sample be rotated about the magic angle at frequencies equal to or greater than the magnitude of the anisotropic interaction to be averaged to zero, typically at least 4 kHz and often much higher[6-9]. Samples are therefore packed into rotors that have a finned tip that is used to drive the rotation of the rotor by a stream of gas and have a mark at the other end so the rotation frequency can be monitored by a tachometer. Sample transfer for most MAS NMR instruments is accomplished by injecting the rotor from the exterior of the instrument into the stator at the end of the NMR probe with a stream of dry air or nitrogen gas. After the rotor reaches the stator, which holds the rotor at the magic angle, sample rotation is propelled by an air turbine mechanism. Separate streams of gas support, propel and control the temperature of the rotor. Inserting a rotor into the NMR spectrometer and achieving stable MAS spinning requires finely machined drive tips and tight control of the temperature and pressure of the separate streams of gas. Despite these technical demands, insertion and achieving stable MAS are largely automated for commercial MAS NMR probes for room-temperature applications.

However, the situation is more complicated for low temperature applications. Samples for low temperature applications are typically inserted into the spectrometer at room temperature and frozen in the stator. In the first minute, the sample temperature decreases quickly (> –100 °C/min) and the system temperature requires several minutes to equilibrate. Because of the interplay of temperature and pressure, insertion and approaching the desired MAS are often handled manually for low-temperature applications. Despite the requirement for manual intervention, freezing the rotor inside of the instrument is beneficial because it minimizes the introduction of water and condensation into the probe, which is critical for successful spinning. Not only can condensation and ice build-up from ambient moisture block gas lines, condensation or frost on the rotor itself can mechanically prevent MAS. Thus, samples for low temperature MAS NMR are typically frozen inside of the instrument at rates that exceed -100 °C/min.

Mammalian cells can retain their integrity through a freeze-thaw cycle if the cooling is slow[5,10-12], at a rate equal or slower than 1 °C/min. Alternatively cells also retain their integrity if the cooling rate is ultra-fast[13-15], at a rate faster than 10^4 °C/min. Rates intermediate to these two extremes rupture and kill mammalian cells due to ice crystal formation both inside and outside the cells, even in the presence of cryoprotective agents[16]. The sample cooling rates for a room temperature rotor inside a pre-cooled probe fall between these two extremes, thus to study cryogenically preserved intact viable mammalian cells, samples must be frozen before transfer into the instrument and transferred into the instrument without temperature fluctuations that could damage the sample or accumulation of frost on the rotor that could prevent the rotor from spinning. The protocol describes a method for frost-free, pre-cooled rotor insertion into a cryogenic MAS NMR system for study of cryogenically preserved intact viable mammalian cell samples. The cryogenic sample transfer described here was developed for NMR characterization of viable intact cells. However, it is applicable to any system where temperature fluctuations may compromise sample integrity. This includes any variety of complex systems, such as freeze quenched reactions for chemical and structural characterization of trapped reaction intermediates[17,18], enzymology[19,20] or protein folding[21,22].

PROTOCOL:

1. Culture and cryoprotection of mammalian cells

1.1. *Culture and harvesting mammalian cells*

1.1.1 Thaw an aliquot of frozen human embryonic kidney cells (HEK 293).

1.1.2 Culture HEK 293 cells in growth media (e.g. DMEM with 10% fetal bovine serum and 1% Pen-Strep) at 37 °C with 5% CO_2 in 100 mm plates for two to three passages (7-10 days).

1.1.3 Split the cells and culture in a 150 mm plate until cells attain 90-95% confluency.

NOTE: A 150 mm plate at > 90% confluency will be sufficient to fill two sapphire rotors with a diameter of 3.2 mm.

1.1.4 Harvest cells using 4 mL of trypsin (see table of materials) and 10 mL media. Transfer suspension to a sterile 15 mL conical and centrifuge at 673 x *g* (1000 rpm) for 5 min at room temperature. Remove supernatant.

1.1.5 Wash the cell pellet with phosphate buffered saline (PBS) (pH 7.4, $-CaCl_2$, $-MgCl_2$).

1.2. *Cryoprotection of cells*

1.2.1 Collect a 50 µL cell pellet in a microcentrifuge tube. Prepare a mixture of 50 µL PBS and 18 µL glycerol in a separate tube.

1.2.2 Gently mix the 50 µL cell pellet with 68 µL of glycerol-PBS mixture by adding the glycerol-PBS mixture to the top of the pellet and resuspending the pellet by gently tapping the side of the tube until no clumps remain.

NOTE: Gentle pipetting can also be used to resuspend the cell, however, make sure that cellular integrity is not compromised.

2. Cryopreservation of mammalian cells in an NMR rotor

2.1. *Transfer of cells into a 3.2 mm sapphire rotor.*

2.1.1. To make a funnel, cut a 200 µL pipette tip and insert the narrow end of the cut pipette tip into the 3.2 mm rotor.

2.1.2. Transfer the cells into the funnel sitting on the rotor. Place the rotor together with the funnel in a microcentrifuge tube and pellet the cells into the bottom of the rotor by centrifugation at 673 x *g* for 2-3 minutes at room temperature.

2.1.3. Remove the supernatant and any excess sample from the rotor. Repeat these two steps until the rotor is fully packed with the cells.

NOTE: *(Optional) Determine cellular viability of the sample before freezing. Resuspend 10 µL of the excess cell pellet in 100 µL of FBS-free DMEM. Mix 10 µL of the suspension with 0.4% trypan blue solution and immediately assess viability using an automated cell counter. Use only FBS free media to dilute cells as serum interferes with trypan blue staining.*

2.1.4. Seal the rotor with a silicon plug using commercially available packing tool.

2.1.5. Close the rotor with a ceramic drive tip by pressing it vertically downward. Avoid touching the delicate fins on the side of the drive tip.

2.1.6. Mark half of the bottom edge of the sapphire rotor with a silver permanent marker and the other half of the bottom edge of the rotor with a black permanent marker to allow accurate monitoring of the spinning of the rotor inside of the spectrometer.

NOTE: Imperfections in the marking of the rotor will prevent accurate counting of the spinning frequency, resulting in failure to achieve stable spinning. Because markers will not write on a frozen rotor and rotor warming compromises sample integrity, marking the rotor before freezing is a critical step.

2.2. *Cryopreservation of cells inside a 3.2 mm sapphire rotor.*

2.2.1. Place a cushion made by a piece of tissue or paper towel under the lid and at the bottom of the cryogenic vial (see table of materials).
NOTE: The tissue protects the rotor marking from damage incurred by bumping against the sides of cryogenic vials (see table of materials).

2.2.2. Place the 3.2 mm sapphire rotor into the cryogenic vial padded with tissue with marked end facing the bottom of the cryogenic vial.

2.2.3. Slow freeze the rotor by placing the cryogenic vial into the controlled rate (-1 °C/min) cooling container and place the container in -80 °C freezer for a minimum of 3 hours.

2.2.4. Transfer the cryogenic vial containing the frozen rotor to liquid nitrogen storage.

3. Cryogenic transfer of a frozen sample into the NMR spectrometer

3.1 Transport the frozen sample to the NMR facility.

3.1.1 Transfer the cryogenic vial containing the frozen rotor to a small dewar filled with liquid nitrogen for transport to the NMR facility.

3.2 Transfer of frozen rotor to the liquid nitrogen bath.

3.2.1 Fill a dry, thermally insulated wide mouth foam dewar with 500 mL – 1 L of liquid nitrogen.

3.2.2 Transfer the rotor from the cryogenic vial into the wide mouth foam dewar filled with liquid nitrogen.

3.2.2.1 Take the cryogenic vial from the transfer dewar in your hand and hold it just above the surface of the liquid nitrogen to protect it from the atmosphere.

3.2.2.2 While holding the cryogenic vial with the mouth pointing slightly downwards, unscrew the cap and let the rotor slide into the liquid nitrogen bath.

NOTE: Once the cap is unscrewed, the rotor has to fall from the cryogenic vial into the liquid nitrogen bath quickly (under 1 second) to prevent condensation from collecting on the rotor. The evaporation of liquid nitrogen forms a "nitrogen cloud" in the wide mouth foam dewar and prevents condensation on the rotor before it is submerged into the liquid nitrogen. Longer exposure of the rotor to air can lead to condensation of moisture on rotor walls which will re-condense into ice.

3.3 Cryogenic transfer of rotor to NMR sample catcher.

3.3.1 Prechill a 1.5 mL microcentrifuge tube by submerging it in the liquid nitrogen bath. Do not close the tube.

NOTE: *Inspect the rotor before transfer into the microcentrifuge tube. Using tweezers, hold the rotor just below the surface of the liquid nitrogen and check that the rotor markings are intact, that no ice deposits have formed on its walls and that the drive tip is intact. Ice crystal deposits on the rotor walls appear as white powder. Be careful to always hold the rotor by its body and not by the drive tip. Do not scratch the marking off the rotor with the tweezer-tips.*

3.3.2 Under the surface of the liquid nitrogen bath, use tweezers to transfer the rotor into the microcentrifuge tube with the drive tip facing the bottom of the microcentrifuge tube and the markings facing the opening of the tube.

NOTE: Always dry tweezers before submerging in liquid nitrogen or touching the rotor.

3.3.3 Using tweezers, hold the tube containing the rotor under liquid nitrogen by its neck.

3.3.4 With a second pair of tweezers, submerge the NMR sample catcher in the liquid nitrogen bath and hold it so that it is inclined at an acute angle with respect to the microcentrifuge tube.

NOTE: Minimize the time that the sample catcher is in contact with the liquid nitrogen to avoid freezing the O-ring. If the O-ring freezes, it will be very difficult to insert the catcher into the spectrometer.

3.4 Cryogenic rotor transfer from the NMR sample catcher to NMR spectrometer

NOTE: This step requires two people, one to operate the cryocabinet and one to transfer the sample from the liquid nitrogen into the probe.

3.4.1 Place the cryocabinet in ejection mode by pressing 'EJECT' on the cabinet.

NOTE: The ejection mode purges the dry and cold nitrogen gas flow at high pressure from the probe to the atmosphere in order to prevent the entrance of atmospheric moisture.

3.4.2 Transfer the rotor into the NMR sample catcher.

3.4.2.1 Insert the open end of the NMR sample catcher into the microcentrifuge tube while still under the surface of the liquid nitrogen.

3.4.2.2 Lift up both the microcentrifuge tube and NMR sample catcher to allow the rotor to fall into the sample catcher. Shake the NMR sample catcher and microcentrifuge tube in case the rotor is stuck on the rim of the sample catcher.

3.4.2.3 Leave the empty microcentrifuge tube on top of the NMR sample catcher to shield the rotor from air.

3.4.3 Remove the other empty NMR sample catcher from the probe and lay it on the floor.

3.4.4 Transfer the NMR sample catcher containing the rotor to your free hand, remove the microcentrifuge tube and insert it immediately into the probe.

NOTE: If the O-ring freezes, it will be difficult to tighten the sample catcher. Keep applying force until it slides into place.

3.4.5 Signal the person operating the cryocabinet to 'STOP EJECT' and 'INSERT'.

NOTE: The insertion mode guides the rotor from the sample catcher into the probe.

3.4.6 Spin up the sample to the desired spinning rate (e.g. 12 kHz) by adjusting the bearing and driving flow pressure controlled by the cryocabinet. (e.g. 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.)

NOTE: When lifting the microcentrifuge tube and NMR sample catcher out of the liquid nitrogen bath, make sure that microcentrifuge tube has enough liquid nitrogen inside it to surround the rotor. Minimize the time between transferring the rotor into the NMR sample catcher and inserting the NMR sample catcher into the spectrometer. All the steps in 3.4 should be completed within 30 seconds.

4 Cryogenic removal of the sample from the NMR spectrometer

4.1 Preparation of the liquid nitrogen bath and the cryogenic vial

4.1.1 Pour 500 mL - 1 L of liquid nitrogen into the wide mouth foam dewar and place the bath under the spectrometer.

4.1.2 Pre-cool the cryogenic vial. Submerge the empty cryogenic vial containing a piece of tissue paper in the liquid nitrogen bath.

4.2 Cryogenic transfer of rotor from probe to cryogenic vial

4.2.1 Reduce spinning rate to 0 kHz by ramping down the driving and bearing gas flow and eject the rotor by switching to the ejection mode.

4.2.2 Keep the ejection mode on, remove the sample catcher from the probe, drop rotor directly into the wide mouth foam dewar containing liquid nitrogen.

4.2.3 Using pre-chilled tweezers, transfer the rotor into pre-chilled cryogenic vial under the surface of the liquid nitrogen.

4.2.4 Cap the cryogenic vial. Prechill the cryogenic vial cap by dipping it into liquid nitrogen. Remove the cryogenic vial containing the rotor and liquid nitrogen from the bath and cap the tube with prechilled cap. Do not tighten the cap so that vaporizing nitrogen can be safely released.

4.2.5 Re-submerge the cryogenic vial in liquid nitrogen. The sample can be transferred to longer term liquid nitrogen storage or unpacked immediately for further analysis.

5 Unpacking rotor and viability measurements

5.1 Unpacking rotor and measuring viability

5.1.1 Pre-warm serum free media (DMEM) or PBS to 37 °C.

5.1.2 Remove rotor from liquid nitrogen. Remove drive tip and silicon plug.

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.

5.2 Measuring viability

5.2.1 Add 20 µL of warm media to the frozen cell pellet in the rotor and resuspend cells. Remove suspension with a pipette and mix the suspension with 100 µL of media.

5.2.2 Remove 10 µL of cell suspension and mix with equal volume of 0.4% trypan blue solution (v/v). Incubate at room temperature for 30 s to 1 min.

5.2.3 Measure viability using automated cell counter.

REPRESENTATIVE RESULTS:

Cryogenic insertion of pre-frozen samples of mammalian cells into the NMR spectrometer supports viability throughout the NMR experiment. Cellular viability and intactness can be assessed using a variety of methods. Here we used a standard dye-based measure of membrane integrity, which aligns well with other methods[23]. Intact cells are impermeable to trypan blue while cells with compromised membrane integrity are permeable. The number of trypan blue permeable and impermeable cells can be rapidly assessed using an automated cell counter. Using the protocol described here, the trypan blue permeability of mammalian cells after MAS NMR (i.e. at point 5.2.3) is similar to the trypan blue permeability of mammalian cells before any temperature change (i.e. point 2.1.3). However, if cells are slow frozen, then warmed to room temperature before insertion (i.e. following the protocol to point 3 before warming the rotor to room temperature before inserting into the chilled probe), cellular viability as assessed by trypan blue decreases to less than 10% of cells (Figure 2). Thus, freezing cells inside the spectrometer results in a loss of cellular membrane integrity while cryogenic insertion of frozen samples of mammalian cells supports cell viability throughout the NMR experiment.

FIGURE LEGENDS:

Figure 1. Schematic of cryogenic transfer of a frozen sample into a pre-cooled NMR probe. (A) Approach the surface of the liquid nitrogen and unscrew the top of the cryogenic vial . (B) Slide the rotor into the liquid nitrogen bath. (C) Submerge a microcentrifuge tube using tweezers and hold it in place until it cools completely. (D) Insert the rotor in the microcentrifuge with the drive tip facing the bottom of the tube. Push, rather than grab, with the tweezers. (E) Hold the

microcentrifuge tube just below the surface of the liquid nitrogen bath and visually inspect the rotor to ensure that it is frost free and well-marked. (F) Hold the sample catcher at an angle above the surface. (G) Lift the sample catcher and tube out of the liquid nitrogen as soon as the sample catcher is inside the microcentrifuge tube. (H) Shake the sample catcher if the rotor is caught on the rim. (I) Remove the empty sample catcher from the probe and lay it on the ground. (J) Remove the microcentrifuge tube from the sample catcher with the rotor inside, insert, tighten the sample catcher in the probe and press "INSERT" on the control console.

Figure 2. Cryogenic insertion of a pre-frozen sample of mammalian cells results in measurements of cellular intactness that are similar to samples of mammalian cells that have never been frozen. The percentage of trypan blue impermeable cells for samples that have never been frozen (e.g. step 2.1.3) is similar to that of cells for samples after MAS NMR (e.g. step 5.2.3 with 12 kHz MAS). Slow frozen cells (e.g. step 3) that were warmed to room temperature before insertion into the NMR instrument that had been pre-cooled to 100 K had much lower percentages of intact cells after MAS NMR with 12 kHz MAS.

TABLE OF MATERIALS:

(JoVE_Materials.xls)

DISCUSSION:

The cryogenic transfer of frozen samples into an NMR spectrometer is successful in preserving the viability of frozen mammalian cells through the NMR data acquisition. The success of this methodology is demonstrated in pre and post MAS NMR viability measurements. This approach is successful and generalizable to any system where temperature fluctuations may compromise sample integrity. The currently presented protocol is performed with the HEK 293 cell line. Because cryopreservation conditions for many mammalian cell lines are very similar, it is likely that the conditions reported here are translatable to other cellular systems; however, they may require further optimization of cryoprotectants, sample volumes, and freezing rates to attain the same results.

This methodology can be improved upon by unpacking the rotor faster post NMR experiment. This step is currently sub-optimal and its execution affects the viability of the cells. Before the cells can be resuspended in media, the drive tip and silicon plug must be removed from the rotor. The sample thaws unevenly when the rotor is held during removal of the drive tip and the silicon plug so shorter rotor handling times result in higher viabilities. The development of rotor holders or other tools to facilitate uniform thawing and quick removal of the drive tip and the silicon plug would aid in making the post-NMR assessment of viability more accurate.

With the application of DNP to MAS NMR, it is now possible to detect proteins and other biomolecules at endogenous physiological concentrations[24-26]. This opens up the possibility of studying biomolecules within their native environments. Maintenance of cellular integrity and viability throughout the experiment is likely to be critical in connecting the experimental outcomes of the spectroscopy to biological phenomena. 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]. However, samples of mammalian cells need to be frozen at controlled rate if preserving cellular intactness and viability is important for the interpretation. Here we present a protocol for freezing and transferring frozen samples of mammalian cells into a pre-cooled DNP MAS NMR instrument that avoids potentially damaging temperature fluctuations and supports measurement on viable cells.

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DISCLOSURES:

The authors have nothing to disclose.

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Approach the surface of the liquid nitrogen and unscrew the top of the cryovial.

Slide the rotor into the liquid nitrogen bath.

Submerge a microcentrifuge tube using tweezers and hold it in place until it cools down completely.

Insert the rotor into the microcentrifuge tube cap forward. We recommend pushing the rotor in rather than grabbing it with the tweezers.

Hold the microcentrifuge tube just below the liquid nitrogen surface and make sure that the rotor has no frost on it and that the marker did not come off.

Hold the sample catcher at an angle above the surface and be ready to quickly recover the rotor from the liquid nitrogen.

Lift the sample catcher and the tube as soon as the sample catcher is in the microcentrifuge tube.

Sometimes the rotor gets caught on the rim of the sample catcher. Shake the sample catcher to slip the rotor in.

Approach the probe. Remove the other sample catcher that blocks the insert/eject line. Lay it on the ground to free your hand.

Remove the microcentrifuge tube with the sample catcher as close as possible to the insert/eject line. Insert and tighten the sample catcher.

Figure

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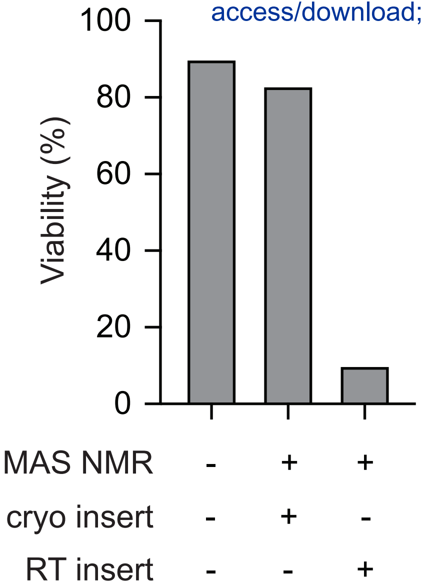

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.4% Trypan blue stain	Invitrogen	T10282	
100 mm cell culture dish	Thomas Scientific	430167	
150 mm cell culture dish	Nunc	157150	
3.2 mm sapphire rotor	Bruker		
45 ° angled forceps	Hampton Research	HR4-859	
AMUPol	Cortecnet	C010P005	
Black and Silver marker	Sharpie		
Cap removing tool	Bruker ?		
Cell culture grade water	HyClone	SH30529.03	
Ceramic cap	Bruker		
CoolCell	Corning/ Biocision	UX-04392-00 (Corning) / BCS-405 (Bioscion)	
Countess Cell Counting Chamber Slides	Invitrogen	C10288	
Countess II automated cell counter	Invitrogen	AMQAF1000	
Cryogen tubes	Nalgene	03-337-7Y	
d_8-glycerol	Aldrich	447498	
Deuterium oxide, 99.8 % atom D	Aldrich	756822-1	
DMEM	Gibco	10569-010	
DNP NMR system with 1.7 T cryogen free gyrotron	Bruker		
Foam dewar	Spearlab	FD-800	
Kimwipes	Kimwipes		
Packaging tool	Bruker ?		
Pen-Strep	Gibco	15140-122	

Powdered PBS	VWR	VWRRV0780
Protonated PBS	Gibco	10010-023
Silicon plug	Bruker	
Standard Vessel forceps		
Tryp-L	Gibco	12605010

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June 21, 2020

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.