We thank the referees for their careful review of the manuscript and their helpful comments. We have revised the manuscript to address the comments, and we provide a detailed response to each comment below.

**Editorial comments:**

1. **Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.**
   We have checked the whole manuscript for spelling and grammar issues.
2. **Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).**
   We have checked the protocol text and removed all personal pronouns.
3. **2.4: How to set up the cycles?**
   All settings including chamber and reactor pressure, length of the cycles and purges are automated to achieve a defined rate of deposition. These parameters may vary among the various models of equipment. The pre-configured recipes are often prepared by the vendor or tool responsible in the clean room, and communicated to the user as deposited film thickness per unit time. It is not possible for us to determine the deposition parameters to the extent a specialist would be able to. This will in nearly all instances be the situation which the readers find in their own shared clean room facilities. We added this information as a note to the protocol following 2.4.
4. **Lines 319-323: Please write all text in the imperative tense.**
   The tense in protocol section 4 (lines 319-323 before revision) has been corrected.
5. **Reference 15: Please add journal title and page number.**
   The format of the reference 15 before revision, now 17, has been corrected.

Reviewers' comments:

Reviewer #1:

Minor Concerns:

**The authors have included many notes in the protocol to help those who want to reproduce the results in this protocol. However, for my personal point of view, it is important to have a "trouble-shooting" section in the protocol to list all the possible problems during the experiments. If things go well, it is straightforward. But in many cases, things could go wrong due to some minor changes. "Trouble-shooting" section can serve as a guide to solve the problems.**

We appreciate your concern and agree with the importance of trouble-shooting for experiments. We followed the guidelines of JoVe about providing information for sub-optimal experiments to demonstrate the range of possible outcomes in discussion. We included a detailed description of the results to expect if critical steps were not followed, and instructions on how to avoid them. This part, also represented by Figure 2, constitutes the trouble-shooting. We also added notes throughout the protocol for every critical step and associated potential complications.

Reviewer #2:

Minor Concerns:

1. **While piranha treatment of the Al2O3-coated microscope slides ought to be avoided, the authors may want to comment on other less aggressive methods of treating surfaces for use with this method. In particular, RF/plasma and UV/ozone treatments which may allow less-rigorous storage of substrates, and provide a way of activating surfaces just prior to use for (potentially) better reproducibility.**

   The samples should ideally be used immediately after deposition. The optimal storage requires positioning the surfaces inside polypropylene wafer carriers, followed by enveloping the carriers in clean room-compatible plastic bags, which are to be nitrogen flushed before vacuum sealing. The purpose is to avoid exposing the surface to air-borne contaminants. If necessary, the surfaces can be kept in air tight containers at RT at maximum for 5 days. Longer storage is not recommended. For users who do not have easy access to a clean room nearby, and purchase or obtain the surfaces from abroad, re-oxidizing the substrates by means of oxygen plasma or ozone treatment may be an alternative solution[1]. We added this information as a note to the protocol following 2.7.

2. **It's not entirely clear why $Al_2O_3$ is required and not simply suitably cleaned borosilicate surfaces. While not necessary for reproducing the authors results, it may be helpful to share briefly what other substrates the authors have tried, the results, and reference their prior work documenting work with other substrates.**

   $Al_2O_3$ surfaces provide optimal adhesion strength, where lipid films can attach strongly enough to spread as a double lipid bilayer membrane, but also can de-attach and form tubular networks upon removal of $Ca^{2+}$ ions. We previously tested the same experiment on $SiO_2$, where the multilamellar vesicles spread as a double lipid bilayer membrane, but no tubular network formation was observed upon addition of chelators[2]. De-attachment and tube formation are observed only on $Al_2O_3$ or plasma etched Al[3]. Our investigations revealed that the major contributing parameter leading to such phenomenon was the zeta potential of the surfaces which for Al and $Al_2O_3$ were close to zero (mV), and for $SiO_2$ significantly negative. The zeta potential of borosilicate is similar to $SiO_2$[4], therefore adhesion of lipid films on borosilicate is equally strong and irreversible. In fact, multilamellar lipid reservoir contact with borosilicate surfaces typically leads to immediate rupture and formation of single lipid bilayers[5]. We included a paragraph on this in the discussion (highlighted in green).

3. **It is asserted by the authors that lipid preparations are sensitive to rotation velocity, rapid pressure changes, and final pressure value. Please explain with supporting information, even if only anecdotal, or add a citation for this assertion.**

   The formation of a homogenous lipid film of uniform thickness in the preparation vessel is the most important requirement for the rotavap procedure. Too fast rotation leads to turbulences, and too slow rotation leads, due to gravitation, to the accumulation of a thick layer of fluid at the bottom of the flask. During the subsequent overnight swelling process, a very inhomogenous lipid mass is produced, that does not respond well to the final

sonication step, and the resulting fractions are of different composition. To our knowledge, this has never been systematically studied, as the procedure was originally optimized to obtain unilamellar vesicles with attached multilamellar vesicles. The size of multilamellar vesicles produced by the method under the conditions specified is most suitable for the depositions described in this article. Thicker lipid deposits often led to excessively large multilamellar aggregates, that do not break down to the appropriate size during sonication.

Similarly, pressure and time within the range specified in the method ensures slow desolvation. With chloroform as solvent, too rapid of a drop in pressure cools down the mixture, resulting in increased viscosity, aggregate formation and uneven film formation. It is noted that the long process time of 6 hours is recommended in order to remove solvent to the greatest extent possible, as the organic solvent partitions into the lipid material upon rehydration.

We added this information as a note to the protocol following 1.2.

4. **What is the purpose of the glycerol? Are there other similar agents that could fulfill the same purpose?**

   The glycerol is used to prevent the complete dehydration of the lipid film and enables bilayer separation[6]. We have not tested alternative agents for the same purpose but we added a note below section 1.3 stating the reason for using glycerol.

   Note to the reviewer: The effectiveness of antidessicants has been systematically studied by Shapiro et al. in 1985[7]. For example, Base oil, folicote, SAG 470 (at 1–10%); foam fighter F, and klearol (at 2.5–10%); 2251 oil (at 5–10%); and several other oils (at 10%) were listed besides glycerol as effective antievaporants.

Reviewer #3:

Major Concerns:

**My major concern is that the authors presented this artificial lipid nanotube networks as a suitable model as a bottom-up-generated structural model for the cellular ER. This claim has not been substantiated by data, was only proposed in their original research article (reference 4, Bilal and Gozen, Biomater. Sci. 2017), and thus should not be presented as a fact. The generation of a dynamic artificial lipid nanotube network itself is very interesting. Given that JOVE does not require novelty for publication, I strongly suggest toning down the claim of ER-relevance in the text (particularly in the title and the introduction), so as not to mislead readers in their research.**

We show in this protocol, as we showed in the original publication, the similarity of the morphology and dynamic tubular rearrangements of our lipid-based model to cellular ER (Figure 1). We originally suggested that it may serve as a 'bottom-up" model for the ER, based on morphological features and similarities in the self-organization of the structures. This is a degree of similarity that has been found to be noteworthy. We originally suggested further, and maintain that here, that the complexity of this early model can potentially be gradually increased. Exactly here lies the purpose of the current manuscript, enabling the interested

readership to build this model at their convenience, and explore further directions. This appears to us as a reasonable approach for obtaining new facts on behalf of the validity of this model, which in the future may be verified or falsified. It can, in any case, enable additional, more focused research. We assume that we have been invited to prepare our method paper with exactly this future potential in mind. We do appreciate greatly that the referee emphasizes the other possible angles may exist for the reader. Therefore we added the following sentences to the introduction (highlighted in green): In addition to serving as a bottom-up model for the still incompletely understood cellular ER, the lipid route to nanotube networks described in this protocol can be interesting for researchers studying self-assembly, nanofluidics, single molecule and colloid transport phenomena, Marangoni flow and others.
Reviewer 2 also suggested that the structures we obtain may enable interesting research in a wider context, which we thankfully adopt and incorporate.
Minor Concerns:

1. **Please clarify the model and settings of the ultrasonic water bath used in Protocol 1.5.**
   We specified the model of the ultrasonic bath in the Table of materials in line 22. We operate at 35 kHz at RT as now specified in 1.5.
2. **Please clarify if it is necessary to flash freeze the lipid suspension in LN2 in Protocol 1.7.**
   Flash freezing the lipid suspensions in LN is not necessary at any stage of the protocol. Direct storage at -20 °C is sufficient. The reviewer makes an interesting connection to cryopreservation, where flash-freezing is indeed a way to preserve the structure of membranes. However, to our knowledge the subsequent re-warming to RT is also known to cause substantial harm to the membranes. This might be one of the reasons why flash-freezing has not found widespread application in the preparation of giant vesicles. We added an informative sentence as a note to the protocol following 1.7.
3. **Please clarify Protocol 3.3. Is the 1 ml HEPES buffer deposited directed on the glass slide with the dried film of lipids (after desiccation in Protocol 3.2)? A volume of 1 ml is rather large to be deposited on a microscope slide/coverglass.**
   Indeed, 1 ml HEPES buffer is deposited directly on the glass slide for rehydration of the dry lipid film. There are examples employing similar protocols showing deposition of such volumes on cover slips with photographs[6]. We note that that large amount cannot be deposited on strongly hydrophilic surfaces (e.g. $SiO_2$), where due to the interfacial tension the droplet immediately spreads out. On more hydrophilic surfaces, such as SU-8 polymer-coated slides, even 1.5 ml can apparently be deposited, as is evident from the images in the above mentioned article. Clean borosilicate slides tend to support droplets of several hundred microliters up to 1.5 ml without problems. Since the coverslip does not need to be moved, we do not consider this a technical problem. The volume of rehydration buffer effects the density of vesicle suspension (the number of vesicles per unit volume) which is subsequently transferred to the observation chamber. Depending on the volume of the observation chamber and the desired vesicle density, the rehydration volume can be tuned to 0.5-1 ml. We included this information in the protocol as a note following 3.3.

**I cannot parse the description "... exposure of the dehydrated lipid film more than 20 min at room temperature leads to evaporation of the buffer and partial dehydration of the previously rehydrated vesicles". How does the exposure of the dehydrated lipid film lead to evaporation of the buffer and partial dehydration? Does the author mean "exposure of rehydrated lipid"?**

We apologize for the confusion, this was a mistake in wording that we overlooked. By mistake we used the word dehydrated instead of rehydrated. We very much appreciate that the reviewer noticed what all authors have apparently overlooked. It is now corrected.

**References**


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3 Gözen, I. *et al.* Thermal migration of molecular lipid films as a contactless fabrication strategy for lipid nanotube networks. *Lab on a Chip.* **13** (19), 3822-3826, (2013).

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7 Shapiro, M., McLane, W. & Belli, R. Laboratory Evaluation of Selected Chemicals as Antidesiccants for the Protection of the Entomogenous Nematode, Steinernema feltiae (Rhabditidae: Steinernematidae), Against Lymantria dispar (Lepidoptera: Lymantriidae). *Journal of Economic Entomology.* **78** (6), 1437-1441, (1985).