July 3rd, 2018

Dear Editor,

I have the pleasure to submit the revised version of the method manuscript entitled: “High-Throughput Measurement of the Plasma Membrane Resealing Efficiency in Mammalian Cells”. We are thankful to the reviewers and editorial team for their very insightful comments. Based upon these comments, we have thoroughly re-written the entire manuscript to increase its clarity. We also shortened and clarified the abstract, which was providing too many complicated details. The title was modified to remove the word “kinetic”, as this term was misleading. Indeed, one reviewer thought that our goal was to measure the kinetic of plasma membrane resealing, but this is not the case. We use a kinetic assay that assesses the cell resealing efficiency over time, but does not measure how long it takes a given cell to reseal a given toxin pore. We also removed Table 1, as this table was providing unnecessary redundant information. We re-evaluated the analytical methods of this manuscript with the precious help of Dr. Chi Song from the Division of Biostatistics (OSU). We included calculation of the resealing efficiency, which characterizes the ability of cells to undergo plasma membrane resealing in any given experimental condition. We also calculated Z-factor and SSMD, and included intra-experiment statistical tools. Additional experiments were performed, as for example providing proof of concept, as requested by a reviewer. Below is a point-by-point answer to all comments we received.

Stephanie Seveau, PhD

**Editorial comments:**

*-Figure 1: Please include a space between number and its corresponding unit (e.g., 37 °C, 4 °C). Please also change “µl” to “µL”.*

We performed these changes.

*-Figure 2: Please make the scale bar line thicker/darker in panel A so it is easier to read.*

A larger scale bar was made in Figure 2A matching the thickness of the scale bars in Figure 2B.

*-Please provide an email address for each author.*

An email address was provided for each author. **Lines 16, 17, 20.**

*-Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.*

We performed these changes.

*-Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.*

This was corrected.

*-Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).*

This was corrected.

*-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 and Reagents. For example: ATCC®, EMD Millipore, Corning, SpectraMax, Axygen Scientific, SoftMax Pro7, etc.*

All commercial language was removed

*-Please revise the protocol to contain only action items that direct the reader to do something. The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”*

This was corrected.

*1.1.1: Please specify cell type, cell culture medium, and the volume of Trypsin-EDTA 0.25% used.*

This was corrected. **Lines 136-140.**

*1.1.2: Please provide the composition of growth medium used.*

The composition of growth medium was specified. **Lines 145-146.**

*-Please include single-line spaces between all paragraphs, headings, steps, etc.*

Single line spaces were placed between all paragraphs and steps.

*-Please revise to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.*This was accordingly corrected.

*-References: Please do not abbreviate journal titles.*

All journal titles are written in full. **Lines 706-835.**

**Reviewers' comments:**  
  
**Reviewer #1:**  
*1. Although the appropriate controls are outlined, there is a lack of statistical test to determine if this assay is robust enough for a high-throughput screen. Can the authors perform a statistical test such as the Z-factor (Z') (or other commonly used test) to assess a good discrimination between negative and positive controls?  
Birmingham, Amanda; et al. (August 2009). "Statistical Methods for Analysis of High-Throughput RNA Interference Screens". Nat Methods. 6 (8): 569-575. doi:10.1038/nmeth.1351*

We established a collaboration with Dr. Chi Song from the Division of Biostatistics (OSU) to re-evaluate the analytical methods. Z-factor and SSMD (strictly standardized mean difference) were determined to assess the robustness of the membrane repair assay. We also introduced a mixed effect model to provide statistical power within a single high-throughput screen involving four technical replicates. **Figure 7, lines 471-498, 594-602, 682-689**  
  
*2. As a proof-of-principle, can the authors show a defect in membrane repair using an siRNA or drug target known to be involved in plasma membrane resealing (e.g. Annexins, ESCRT, etc.)?*HeLa H2B-GFP cells were treated with the chemical inhibitor desipramine, which was previously shown to block membrane repair presumably via inhibition of the acid sphingomyelinase (ASM). Data presented in Figure 8 and corresponding analyses confirm that this drug inhibits membrane resealing, as evidenced by a decrease in the resealing efficiency measured in the presence of the drug. **Lines 500-522, Figure 8, and 604-617.**

*3. This method article is based on a previously published method article, which should be cited.  
Pathak-Sharma, S., Zhang, X., Lam, J. G. T., Weisleder, N., & Seveau, S. M. (2017). High-Throughput Microplate-Based Assay to Monitor Plasma Membrane Wounding and Repair. Frontiers in Cellular and Infection Microbiology, 7, 305. http://doi.org/10.3389/fcimb.2017.00305*Pathak Sharma et al. was referenced in the introduction. **Lines 80 and 137.**

*4. Figure 3 is similar to Figure 4A/B and therefore can be remove.*Figures have been remodeled to avoid redundancies, as advised.

*5. Line 296 (Results Figure 6), the authors say: "Indeed, HeLa H2B-GFP cells exposed to 0.5 nM LLO in M1 containing 1 μM TO-PRO-3 exhibit a 2-3-fold increase in fluorescence relative to the non-damaged controls without significantly affecting GFP intensity (Figure 6)." However, Figure 2A shows a 2-fold increase in M1 and a 3-fold increase in M2.*

The comment from the reviewer made us realize that the corresponding text lacked clarity. To avoid any confusion, we rephrased this section. **Lines 461-465.**

*6. Figure 6E is not described in the results section.*Figure 6E, now figure 7, is described in the results section. It shows the effect of LLO concentration on plasma membrane resealing efficiency. Indeed, efficiency decreases as the concentration of toxin increases. This reflects that high toxin concentrations cause damages that cells cannot repair efficiently. This also shows the LLO concentration range that is best suitable to the assay. **Lines 461-469 and 487-497, Figure 7.**

**Reviewer #2:**  
*7. This assay technically is examining cell wounding instead of membrane resealing. As dynamics of dye incorporation showed that intracellular dye concentration kept increasing until saturated.*

The assay directly measures cell wounding, as it measures the amount of dye that can penetrate the wounded plasma membrane. This assay also indirectly assesses the efficiency of cell resealing by comparing cell wounding in the presence (M1) and in the absence (M2) of extracellular Ca2+ (the latter condition completely prevents repair). To make this point clear, we rephrased the corresponding sections and included an equation for measurement of the resealing efficiency. **Lines 367-384.**

*8. I am a little concerned about incubating cells with PI/TO-PRO-3 for 30 min. It is not uncommon for cells to uptake these dyes either through passive transportation or endocytosis.*

Some water-soluble dyes can be taken up by pinocytosis. In our experimental conditions, uptake of the dye was negligible. Indeed, controls consisting of cells incubated with PI or TO-PRO-3 (without toxin) showed that there was no detectable increase in fluorescence intensity of the corresponding dyes over the 30 min duration of the kinetic assay **(Figures 3, 5 and 7).**

*9. How come cell count is not affected by LLO addition in Fig. 5b? If a cell is wounded and not repaired, GFP would diminish.*

HeLa cells do not express a soluble form of GFP; they express the protein chimera Histone 2B-GFP, a nuclear localized protein found in the octameric histone complex, which is wrapped by nuclear DNA and is thus physically immobilized within the nucleus. Despite plasma membrane damage, such nuclear components mostly remain in the nucleus and GFP fluorescence is not lost in damaged cells. In addition, the cell count is not affected by variation in GFP fluorescence intensity as presented in **Figures 4 and 6.**

*10. Please describe in the figure legend if cells in Fig. 4C/Fig. 6C is treated with LLO or not.*

Treatment conditions were specified in all figure legends.

*11. It is recommended to include a Tm-independent positive control for maximal wounding such as cell permeabilizing agent's saponin or triton.*We performed such controls, but they did not bring useful additional information so they were not included.

**Reviewer #3:**

*12. While of higher throughput than many of the mechanical injury approaches in the literature, the toxin used here injures the membrane by extracting cholesterol from the membrane. Due to wide recognition of the role of cholesterol in the membrane signaling and in membrane repair process this limitation of the approach needs to be clearly highlighted in the abstract and elsewhere in the protocol so as to prevent audience from using this approach for membrane injury applications that may be sensitive to cholesterol.*

CDC toxins bind to cholesterol, but do not extract cholesterol from the host cell plasma membrane **(lines 85-88).** Due to the high cholesterol content of the plasma membrane and the extremely low active concentration of the toxin, it is unlikely that the toxin affects signaling by affecting cell cholesterol levels. CDCs activate host cell signaling, as we and others have previously published, and this may affect the process of membrane repair as pointed out by the reviewer. This point is now discussed **lines 99-104, 649-653.**

*13. As membrane repair is undoubtedly sensitive to physiological temperature, a paragraph dedicated to discussing the limitation of rapid and repeated temperature switching of cells between 4 to 37 degrees on the plasma membrane as well as on the membrane repair process must to be included.*

We cannot exclude that temperature switches may affect membrane repair. As suggested, we included a comment relative to temperature switch during the experimental procedure. **Lines 658-661.**  
  
*14. The process of membrane repair occurs in seconds to minutes, while the readouts reported here show dye entry that continues for up to half hour, raising questions regarding the relevance of this approach to physiological repair process. As this slow response and readout limits the utility of the approach presented here for studying cellular responses at the seconds time scale that are required for efficient repair, this limitation needs to be discussed.*

This assay is not meant to measure the speed of repair in single cells. Its goal is to identify experimental conditions that affect the membrane repair efficiency. We do not need to develop an assay with a milli-sec time resolution to identify a defect in resealing. The previous title may have been misleading as it included the term “kinetic”. The assay is a kinetic assay, but we do not claim measuring the kinetic of repair. This point is now included in the discussion **Lines 542-545.**

*15. Previously published protocols in jove (PMID: 29364240, 24686523, 21750489) that avoid some of the above limitations. Discussing these protocols here will benefit the readers and are thus should be included here.*

We further explained in the discussion that the previous methods are not high-throughput, which is the goal of the present method. **Lines 636-643.**  
  
*16. The table needs cell borders as in its present form it is hard to align the various cells between the different columns.*

Table 1: Test conditions was removed.

*17. As there are alternate vendors for the microplate reader and imaging cytometer used here, it would be worthwhile to include discussion on if the equipment used here are unique and hence irreplaceable for this protocol or other equipment can also be used.*  
This type of equipment is offered by different vendors. We learned that Jove policy excludes adding any vendor name, so the specific company’s name was removed and we made clear that any plate reader that combines imaging and spectrofluorometry can be used. The described protocol is applicable to any multi-mode plate readers.

**Reviewer #4:**

*18. The protocol in this assay uses PI in the medium during the assay to measure the kinetics of PI influx into the cell. But adding PI at the end of the 30 min repair would also help assess the amount of PI influx that happens by pore formation during the kinetics and the PI influx due to remaining pores (not fully resealed cells). The authors could comment on this variation of the assay to compare PI influxes during pore formation or after repair has failed. Indeed from the kinetics, it is not necessarily clear whether higher doses of LLO cannot be repaired or whether the pores just continue to form over time, allowing PI to enter the cells before the pore is removed from the plasma membrane. Addition of a control condition where PI is added at the end of the repair time frame would address this question.*

We agree. We have used such alternative experimental strategy to assess the kinetics of repair of cells damaged by LLO in a distinct research article. The goal of the present approach is to screen for drugs/experimental conditions that affect the repair efficiency, not to measure the kinetic of repair. We removed the term “kinetic” from the title of this manuscript, which was misleading.

*19. The authors refer to the kinetics in M1 conditions as reflecting the resealing efficiency, but as such the fluorescence levels recorded reflect the presence of open LLO pores on the plasma membrane and the incorporation of PI into the cells. Therefore, resealing efficiency is better described as the difference between the M2 (repair restrictive where all the formed pores allow for PI entry) and the M1 (repair permissive where some non repaired pores also allow for PI entry) conditions. A change in the text of the abstract and introduction could more specifically address this.*

This is correct and we thought that this point was explained in the manuscript. Obviously, we were not sufficiently clear. Therefore, we carefully rephrased the manuscript. We included a method to calculate the resealing efficiency. **Lines 112-116, 367-384, 436-446, 465-469 and Figure 7.**

*20. LLO pore formation is not exactly synchronized. The start of pore formation is synchronized when switching to 37 °C, but secondary LLO pores continue to form slowly over time as is illustrated by the progressive increase in PI fluorescence in cells treated with LLO and in the presence of calcium (even at low LLO concentration), a condition where LLO pores should be removed from the plasma membrane and PI stopped from entering further into the cell. If all pores were formed immediately when cells were switched to 37 °c (synchronized), then fluorescence levels would first increase when pores are all formed and then plateau once pores are removed from the plasma membrane. The text should reflect this distinction to explain the progressive increase in PI visible in repair permissible conditions.*

We agree, and have clarified this point: only the initiation of pore formation is synchronized. We also better described the formation of pores and the inactivation of the soluble (unbound) form of the toxin. **Lines 93-95, 654-656.**

*21. Line 140: This seems to be the first use of the abbreviation PI for propidium iodide and should be indicated here.*

The abbreviation for propidium iodide was included **Line 189**  
  
*22. Line 150-151: In general, the authors should indicate how the fluorescence reading is organized during the assay (well by well, row by row or the whole plate at once ?). This would help clarify the actual kinetics of the assay when comparing samples in different wells. Can the authors explain how much time the fluorescence reading takes for the whole plate and whether there is a delay between reading the first wells and the last wells of the plate ? Indicating the shortest interval possible between readings would also help readers evaluate the assay's relevance for their own work.*

We agree that it is very important information to include. It takes about 30 sec to read the entire 96 well plate during the kinetic measurements, this time frame is short enough to not interfere with the test via introducing delay between wells located at different places. The microscopy image acquisition takes longer, but these images are taken only to enumerate cells before and after the kinetic measurement. Their longer time scale do not interfere with the kinetic assay that measures the repair efficiencies. **Lines 218-219**

*23. Line 162-163: Similarly, the authors should also indicate how long it takes to image each well for the whole plate and if there is a significant delay between the first and last well that could influence the interpretation of repair levels between samples in the first and last wells on the plate. This would be important if a kinetics were to use a shorter time frame where differences might be amplified between samples.*

The image acquisition read times were stated in the protocol. See response above and **Lines 244-248.**  
  
*24. Line 222-223: Please specify what homogenous cell count means. Same number of cells in each well? Same method/settings used for all the of the wells in the plate to get consistent normalization of the fluorescence kinetics?*

We re-phrased the sentence. We meant “same number of cells per well”. **Line 392-393, 669-671.**  
  
*25. Line 230: Please specify whether the fluorescence levels are normalized or not to the number of cells in each* well to plot the data of the kinetics. If not, why?

Fluorescence was not normalized to the number of cells. As explained in the discussion, normalization is not possible dues to the fact that resealing efficiency varies with toxin concentration relative to cell numbers. **Lines 669-673.**  
  
26. Line 294-295: Please Explain the importance of the difference in extinction coefficient between PI and TO-PRO-3 for this assay.

The importance of the extinction coefficient was explained. **Lines 456-458.**  
  
27. Line 334: Figure 4C legend: Please specify which condition (M1 or M2) the images are representative of.

We specified that figure 4C, now 4A, is under M1 conditions. **Line 560-561.**