Mapping the electrostatic profile of cellular membranes

Sharon Eisenberg, Ehud Haimov, Glenn Walpole, Jonathan Plumb, Michael Kozlov, and Sergio Grinstein

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|--------------------|------------|
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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)
1st Editorial Decision

RE: E19-08-0436
TITLE: Mapping the electrostatic profile of the cellular membranes

Dear Dr. Grinstein

Thank you for submitting your article entitled “Mapping the electrostatic profile of the cellular membranes” to MBoC. It has now been viewed by two experts in membrane biology. As you will see from the verbatim comments of the reviewers, each provided a thoughtful review with a set of somewhat overlapping concerns. Both reviewers thought that an appropriately revised manuscript would be a significant contribution to membrane cell biology. Most of the reviewer comments require explicit clarifications or revisions, and some perhaps additional experimental work. It was also noted that the data in Table 1 do not indicate errors. I am sure you will be able to address these comments by revising the manuscript, and I have therefore placed the manuscript in the “revise and re-review” category. Please provide a listing of your responses and revisions when you submit the revised version. I look forward to reading the revised manuscript.

Best regards
Tom Martin

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Dear Dr. Grinstein:

The review of your manuscript, referenced above, is now complete. The Monitoring Editor has decided that your manuscript is not acceptable for publication at this time, but may be deemed acceptable after specific revisions are made. Any specific areas to be addressed are outlined in the reviewer comments included below.

A reminder: Please do not contact the Monitoring Editor directly regarding your manuscript. If you have any questions regarding the review process or the decision, please contact the MBoC Editorial Office (mboc@ascb.org).

When submitting your revision online please use the link below, and include a cover letter that details, point-by-point, how the Monitoring Editor's and reviewers comments have been addressed. When entering the author names online, enter them exactly as they appear on the manuscript title page. Please send only the latest revised manuscript. DO NOT resend any previous versions. Authors are allowed 180 days to submit a revision. If this time period is inadequate, please contact us immediately at mboc@ascb.org.

Revised manuscripts are assigned to the original Monitoring Editor whenever possible. However, special circumstances may preclude this. Also, revised manuscripts are often sent out for re-review, usually to the original reviewers, when possible. The Monitoring Editor may solicit additional reviews if it is deemed necessary to render a completely informed decision.

To prepare for the possible acceptance of your revised manuscript, submit final, publication-quality figures with your revision.

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Line Images. Prepare line drawings at one-column width (less than 8.47 cm) or less if the graph or histogram is relatively simple. Symbols should be at least 1 mm high and large enough to be distinguishable from the lines connecting them.

To submit the cover letter, revised version, and figures, please use this link (please enable cookies, or cut and paste URL): Link Not Available

Please contact us with any questions at mboc@ascb.org.

Thank you for submitting your manuscript to Molecular Biology of the Cell. We look forward to receiving your revised paper.

Sincerely,

Eric Baker
Journal Production Manager
MBoc Editorial Office
mbc@ascb.org

Reviewer #1 (Remarks to the Author):

In this manuscript, Eisenberg et al, use the distribution of a series of previously built positively charged Prenylated or charged helix- based constructs to assess the membrane potential of the inner membrane of the PM/Golgi/ER/Mitochondria. They first use the rate of dissociation of the probes from the PM to assess the membrane potential of the PM by comparing the same with a GUV construct of varying PS ratios (surface charge) and experimentally determined zeta potential. They then use the thermodynamic principle that at equilibrium the chemical potential of each probe in individual compartments must be the same. Therefore for all the probes at equilibrium, regardless of their charge, \( \Delta \psi = \psi_{PM} + \Delta \psi_{org} \). This allows the authors to estimate the membrane potential of internal organelle with respect to the PM \( \Delta \psi = \psi_{PM} - \psi_{org} \) resulting in their final equation, \( \Delta \psi = \frac{1}{2} \ln \left( \frac{C_{PM}}{C_{org}} \right) + \frac{1}{2} \ln \left( \frac{C_{PM}}{C_{org}} \right) \) where they extract the value of \( \ln \left( \frac{C_{PM}}{C_{org}} \right) \) from literature. Using the measurements of \( \ln \left( \frac{C_{PM}}{C_{org}} \right) \) (Appendix Table 2) the authors find that while the PM is -35 mV, the inner membranes are much lower and have values as tabulated in Table 1.

While these are interesting results and represent perhaps a first attempt at providing a quantitative estimate of the membrane potential at the surface of internal organelles, there are a number of concerns that need to be addressed before these results are convincing to this reviewer.

1) First, the authors have used three different cell systems to make measurements that they present in a unified manner. The measurements of the colocalized partitioning are from RAW264.7 macrophages, whereas the probe dissociation data are collected from HeLa cells. The data regarding intra organelar partitioning is obtained from the RAW cells while data regarding the kinetics is obtained from HeLa cells (see Appendix Table 2 and Fig 3F, respectively). It is not clear how comparable in terms of their surface charge properties these cells are, and if this may result in an erroneous averaging of data.

2) A key measurement in this analysis is data regarding the partitioning of the probes with respect to the PM, ie the measurement of \( \ln \left( \frac{C_{PM}}{C_{org}} \right) \). The authors state 'To this end, we derived equations that enabled us to calculate the differences between the surface potentials of intracellular organelles, based on the measured amounts of the probes bound by each organelle, normalized per unit surface area.' After reading the methods and figure legends it is impossible for this reviewer to understand how the authors have carried out their analysis. Detailed procedures including work flows and controls would be necessary to understand how the authors arrive at \( \ln \left( \frac{C_{PM}}{C_{org}} \right) \); they must have normalized a number of data sets including the
relationship of concentration of dye, fluorescence intensity and area occupied by one dye versus the other, methods they have completely glossed over, to arrive at the data presented in Appendix table 2.

3) In measuring the kinetics of the different helix-charged peptides, the authors set up to measure the recruitment of the +8 charge peptide and compare the off rates with the experimentally measured off rates of a similar peptide on GUVs of different charge lipid composition. Here it would be useful if the authors could also use some of the other charged peptides to see if the kinetics they determine from the in vitro measurements provide some correlations with the variety of assumptions that they make- if that differently charged peptides have similar non-specific hydrophobic association/dissociation rates, or that the rates they measure for the dissociation from the Golgi-membrane localized (or any other organellar membrane except the mitochondrion) are in the range of what they are able to capture in vitro.

4) The authors glibly state that to obtain the ratio of $\text{L/L}\$ they require a measurement of $\text{A/A}\$, which they obtain from the literature (Appendix Table 1). There is no detail or reference given about how they extract this data from the literature.

5) In general, where possible it would be worth providing original data as well as statistics and propagate errors for example in Table 1 so that the reader is able to determine for themselves how these measurements and their interpretation may be trusted.

6) An explanation that the authors give for the huge variation in the measurement of $\text{Q}$ for the internal organelles is the possibility that $(\text{Q}_\text{int}} - \text{Q}_\text{cyt})/ \text{Q}_\text{cyt}$ may be subject to inter organelle differences and not cancel out to zero. It would be worth testing whether the different probes do indeed have differences in interactions with the same membrane after screening of the surface charge. This manuscript has more than its acceptable share of explicit (and unexplored), and more concerning, implicit assumptions that abound in the determination of a quantitative property of the cells membranes.

Reviewer #2 (Remarks to the Author):

This work is the logical developments of previous beautiful studies by the Grinstein group of the electrostatic features of the cytosolic leaflet of cellular organelles (See notably Yeung et al 2006 Science, Yeung et al 2008 Science). Whereas these previous works were mostly qualitative, the aim of the current study is to provide a more quantitative view of the electrostatic properties of the plasma membrane vs other compartments. Compared to the aforementioned studies, the current analysis benefit from two technical improvements.

First, the authors consider the surface area of the various organelles of interest using the membrane probe FM4-64. Because this probe is impermeable, the authors devise a clever protocol by which the cell membrane is briefly permeabilized to allow the probe to enter the cell, before a resealing and washing step.

Second, they devise an imaginative method to measure the koff of the electrostatic probes. Specifically, they modify the probe to allow its displacement from the membrane of interest to the mitochondria where the probe is eventually bound thanks to rapamycin-induced pairing. The off rate is then compared to that observed with giant liposomes of defined zeta potential. The calibration curve (koff vs zeta potential) enables estimation of the zeta potential.

Overall, I found the manuscript interesting. However, I noticed several flaws that might require clarification.

#1. The first figure sounds to me very close to what was published before by the same group, notably Figure 3 of Yeung, T. et al. Membrane phosphatidylserine regulates surface charge and protein localization. Science 319, 210-213 (2008). I was quite surprised that this paper was not mentioned in the reference list.

#2. I understand the importance of determining the actual surface area of organelles to normalize the fluorescence signal obtained from the charge probes. Technically, however, the corresponding method section is not very clear and I don't understand how the authors perform the experiments (Fig 2F). Is it a three-color measurement; one for the organelle, one for the surface, and one for the charge probe?

#3. The GUVs used to calibrate the zeta potential (Fig 4) are probably symmetric in lipid composition in contrast to the plasma membrane. Please discuss for the lay reader the effect of lipid asymmetry on the zeta potential. For example, should the theoretical zeta potential of a membrane containing 15 mol % PS that is symmetrically distributed equal that of a membrane containing 7.5 mol % or 15 mol % of PS present only on the cytosolic leaflet? In other words, does the asymmetric distribution of anionic lipids influence the potential seen by a soluble protein on the slipping surface?

#4. The authors assume that the hydrophobic residues present on the charge probes should not interfere with their organelle-binding properties. What is the evidence for this assumption? Lipid packing and therefore hydrophobic insertion might depend on cholesterol concentration and lipid saturation ratio, which are known to differ between organelles.

#5. One striking observation of this work but also of the previous studies of Yeung and Grinstein is that the ER is not permissive for the binding of negatively charged proteins as compared to other organelles, mostly PM and endosomes. Yet, a recent study by
Tsuji T suggests predominant localization of phosphatidylserine at the cytoplasmic leaflet of the ER (Proc Natl Acad Sci U S A. 2019 Jul 2;116(27):13368-13373). How can this observation be reconciled with the present work?
Dr. Tom Martin  
Monitoring Editor  
Molecular Biology of the Cell  

Re: E19-08-0436

Dear Tom,

My apologies for the inordinately long time we required to submit the revised version of our manuscript “Mapping the electrostatic profile of cellular membranes”. The closure of our laboratories for several months because of the pandemic and an inevitable change in laboratory personnel caused the delay. I do hope that you can consider this submission as a revision, despite the fact that we missed the original deadline for resubmission.

On the positive side, I believe that we have been able to address every one of the reviewers’ comments and suggestions, and made the appropriate additions and changes to the manuscript. An itemized list of the changes made follows, preceded by the reviewers’ original comments (in blue), for reference.

Reviewer #1 (Remarks to the Author):

In this manuscript, Eisenberg et al, use the distribution of a series of previously built positively charged Prenylated or charged helix- based constructs to assess the membrane potential of the inner membrane of the PM/Golgi/ER/Mitochondria. They first use the rate of dissociation of the probes from the PM to assess the membrane potential of the PM by comparing the same with a GUV construct of varying PS ratios (surface charge) and experimentally determined zeta potential. They then use the thermodynamic principle that at equilibrium the chemical potential of each probe in individual compartments must be the same. Therefore for all the probes at equilibrium, regardless of their charge, \[ \mu_{PM} = \mu_{GC} = \mu_{ER} = \mu_M \]. This allows the authors to estimate the membrane potential of internal organelles with respect to the PM (\( \Delta \phi_i = \phi_i - \phi_{PM} \)) resulting in their final equation,  
\[ \Delta \phi_i = (\mu_{PMZ0} - \mu_{iZ0})/Z_e + kBT/Z_e \ln (NPMZ/NiZ)(NLi/NLPM), \]

by making a number of assumptions, they reduce this to  
\[ \Delta \phi_i = kBT/Z_e \ln (NPMZ/NiZ)(NLi/NLPM) \]

where they extract the value of \( NLi/NLPM \) from literature. Using the measurements of \( NPMZ/NiZ \) (Appendix Table 2) the authors find that while the PM is -35 mV, the inner membranes are much lower and have values as tabulated in Table 1.

While these are interesting results and represent perhaps a first attempt at providing a quantitative estimate of the membrane potential at the surface of internal organelles, there are a number of concerns that need to be addressed before these results are convincing to this reviewer.

1) First, the authors have used three different cell systems to make measurements that they present in a unified manner. The measurements of the colocatalized partitioning are from RAW264.7 macrophages, whereas the probe dissociation data are collected from HeLa cells. The data regarding intra organellar partitioning is obtained from the RAW cells while data
regarding the kinetics is obtained from Hela cells (see Appendix Table 2 and Fig 3F, respectively). It is not clear how comparable in terms of their surface charge properties these cells are, and if this may result in an erroneous averaging of data.

-We performed experiments to document that the hierarchical distribution of the charged probes applies to the two cell types studied. Moreover, we tested both the prenylated and the amphiphilic helical probes in both cell types. The results of these experiments are now presented in the new Figures 1-4 that replace and extend the original Figure 1. In a nutshell: the prenylated probes behaved identically in RAW264.7 and in HeLa cells, as did the helical probes. The only noticeable difference between the two types of probes was the observation that a considerable fraction of the +2-helix probe is soluble, likely because the hydrophobic component is insufficient to retain it when the electrostatic component is reduced.

2) A key measurement in this analysis is data regarding the partitioning of the probes with respect to the PM, ie the measurement of \( NPMZ/NIz \). The authors state "To this end, we derived equations that enabled us to calculate the differences between the surface potentials of intracellular organelles, based on the measured amounts of the probes bound by each organelle, normalized per unit surface area." After reading the methods and figure legends it is impossible for this reviewer to understand how the authors have carried out their analysis. Detailed procedures including work flows and controls would be necessary to understand how the authors arrive at \( NPMZ/NIz \); they must have normalized a number of data sets including the relationship of concentration of dye, fluorescence intensity and area occupied by one dye versus the other, methods they have completely glossed over, to arrive at the data presented in Appendix table 2.

- The data in Appendix Table 2 were obtained by dividing the percentage of the total cellular probe found in the plasma membrane by that in the indicated organelle. For clarity, we have now included an additional Table (Appendix Table 3) that lists the individual percentages measured for each probe in every one of the organelles, which were used to calculate the numbers presented in Appendix Table 2. The means, standard errors and number of determinations for each organelle and probe are specified in Appendix Table 3. These were obtained using cells that were triple-labeled with the indicated charge probe, organelar marker and intracellular FM4-64, as described in detail in the legend and text to Figure 5. As described in the revise manuscript, FM4-64 was used to quantify the total amount of cytosolically-exposed endomembranes that was used for normalization.

3) In measuring the kinetics of the different helix-charged peptides, the authors set up to measure the recruitment of the +8 charge peptide and compare the off rates with the experimentally measured off rates of a similar peptide on GUVs of different charge lipid composition. Here it would be useful if the authors could also use some of the other charged peptides to see if the kinetics they determine from the in vitro measurements provide some correlations with the variety of assumptions that they make- if that differently charged peptides have similar non-specific hydrophobic association/dissociation rates, or that the rates they measure for the dissociation from the Golgi-membrane localized ( or any other organelar membrane except the mitochondrion) are in the range of what they are able to capture in vitro.

- As requested by the reviewer we synthesized a helical peptide with a reduced charge, NH\(_2\)-SALAALFARLRKWFKKG-C-COOH (FITC+4), and measured its dissociation from giant liposomes of varying lipid composition and hence different charge. The results of these new experiments are presented in the revised Figure 7F and described on page 10 of the revised paper. Briefly, the dissociation time was considerably shorter for the +4probe, compared to the
probe with +8 charges and the dependence on the concentration of PtdSer was also steeper for the +8 probe, as anticipated.

4) The authors glibly state that to obtain the ratio of \( \frac{N_L P_M}{N_L i} \) they require a measurement of \( \frac{A_P M}{A_i} \), which they obtain from the literature (Appendix Table 1). There is no detail or reference given about how they extract this data from the literature.

-We have now added a legend to Appendix Table 1 that indicates that the values used are a consensus derived from comparison of the area ratios reported for various cell types in the references below:
Loud, AV (1968) J Cell Biol 37, 27-46;
Weibel, ER, et al. (1969) J Cell Biol 42, 68-91;
Mori, H and Christensen, AK (1980) J Cell Biol 84, 340-354;
Sokol, RJ, et al. (1987) J Anat 151, 27-35;
Kerr, JB (1988) Anat Embryol 79, 191-203;
Brunner, M, et al. (1992) Cell Tissue Res 268, 283-286;
Keller, B, et al (1991) Acta Anat 141, 324-328;
Hirano, D et al. (2001) Med Electron Microsc 34, 240-248.

5) In general, where possible it would be worth providing original data as well as statistics and propagate errors for example in Table 1 so that the reader is able to determine for themselves how these measurements and their interpretation may be trusted.

-As requested, we now include standard errors alongside the mean values presented in Table 1. The errors \( \delta(\Delta \varphi_i) \) of the voltages appearing in the Table were calculated according to the propagation of uncertainty theorem using the following formula:

\[
\delta(\Delta \varphi_i) = \frac{k_B T \delta}{Ze} \left( \frac{N_{PMZ}}{N_{IZ}} \right)
\]

The procedure used to calculate the errors is now described in the Appendix (page 24 of the revised manuscript).

6) An explanation that the authors give for the huge variation in the measurement of \( \varphi_i \) for the internal organelles is the possibility that \( \frac{\mu_{PMZ0} - \mu_{iZ0}}{Ze} \) may be subject to inter organelle differences and not cancel out to zero. It would be worth testing whether the different probes do indeed have differences in interactions with the same membrane after screening of the surface charge. This manuscript has more than its acceptable share of explicit (and unexplored), and more concerning, implicit assumptions that abound in the determination of a quantitative property of the cells membranes.

-Screening the surface charge does indeed cause relocation of the probes. This can be demonstrated by allowing entry of calcium into the cells using a divalent cation ionophore. An example is shown in the movie available using the following link:

https://drive.google.com/drive/folders/1i96mVtsH7rpDSjX_UTPZD7JcczCvl8l6

The movie shows frames acquired over 5 min at 30 sec intervals. In this experiment cells transfected with the +8-helix probe are treated with ionomycin after the first frame, resulting in
an acute release of the probe from the plasma membrane. We have elected not to include such results in the present manuscript, because we published similar experiments earlier using both the prenylated and amphiphilic probes (Yeung T, et al. (2006) Receptor activation alters inner surface potential during phagocytosis. Science. 313, 347-351).

Reviewer #2 (Remarks to the Author):
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First, the authors consider the surface area of the various organelles of interest using the membrane probe FM4-64. Because this probe is impermeable, the authors devise a clever protocol by which the cell membrane is briefly permeabilized to allow the probe to enter the cell, before a resealing and washing step.
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Overall, I found the manuscript interesting. However, I noticed several flaws that might require clarification.

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-We have corrected this omission and now mention the 2008 reference on pages 4 and 6 of the revised version.

#2. I understand the importance of determining the actual surface area of organelles to normalize the fluorescence signal obtained from the charge probes. Technically, however, the corresponding method section is not very clear and I don't understand how the authors perform the experiments (Fig 2F). Is it a three-color measurement; one for the organelle, one for the surface, and one for the charge probe?

-We apologize for the lack of clarity and detail in the original version. These were indeed three-colour determinations that took advantage of the broad spectral properties of FM4-64, which was visualized with excitation at 514 nm and emission at 640 nm to enable simultaneous visualization of green and red fluorescent proteins. This is now explicitly stated in the Methods section (page 15) and in the revised legend to Figure 5 (that was Figure 2 in the original version but has been renumbered because of the inclusion of the new Figures 1-4).

#3. The GUVs used to calibrate the zeta potential (Fig 4) are probably symmetric in lipid composition in contrast to the plasma membrane. Please discuss for the lay reader the effect of lipid asymmetry on the zeta potential. For example, should the theoretical zeta potential of a membrane containing 15 mol % PS that is symmetrically distributed equal that of a membrane containing 7.5 mol % or 15 mol % of PS present only on the cytosolic leaflet? In other words,
does the asymmetric distribution of anionic lipids influence the potential seen by a soluble protein on the slipping surface?

-We thank the reviewer for this suggestion and have included the following statement to this effect on page 9 of the revised version: Note that the lipid composition of the two monolayers that constitute the membrane of the giant liposomes is identical, unlike that of biological membranes. However, in both instances the surface potential is dictated exclusively by the exofacial monolayer.

#4. The authors assume that the hydrophobic residues present on the charge probes should not interfere with their organelle-binding properties. What is the evidence for this assumption? Lipid packing and therefore hydrophobic insertion might depend on cholesterol concentration and lipid saturation ratio, which are known to differ between organelles.

-Indeed, unique features of individual organellar membranes such as lipid packing or acyl chain composition could affect the partition of the probes. This caveat has been added to pages 5-6 of the manuscript: Preferential partition of the prenyl group or of the hydrophobic chains of the helix probes into membranes of varying acyl chain composition or lipid packing may have affected their distribution, but the overall similarity of the results obtained with both types of probes lends confidence to the above conclusions, which are also consistent with earlier findings (Yeung et al., 2006; Yeung et al., 2008).

#5. One striking observation of this work but also of the previous studies of Yeung and Grinstein is that the ER is not permissive for the binding of negatively charged proteins as compared to other organelles, mostly PM and endosomes. Yet, a recent study by Tsuji T suggests predominant localization of phosphatidylserine at the cytoplasmic leaflet of the ER (Proc Natl Acad Sci U S A. 2019 Jul 2;116(27):13368-13373). How can this observation be reconciled with the present work?

-The results of Tsuji et al. are indeed unexpected and different from those reported by others in mammalian cells and in yeast, the same model used by Tsuji and colleagues. Detailed perusal of the novel method used by Tsuji et al may account for the detection of PtdSer on the cytosolic surface: the authors used detergents at two different stages of the preparation of their samples (2.5% SDS at an early stage and then 0.1% Triton X-100), possibly causing scrambling of luminal lipids to the cytosolic surface. We feel that discussion of the existing discrepancies in the distribution of PtdSer in the ER would be outside the scope of our manuscript and therefore have chosen not to mention the contradictory findings.

Please thank the reviewers on our behalf for all their efforts and valuable comments, which contributed greatly to the evolution of this manuscript. We would like to thank you also for handling the paper.

We look forward to hearing from you soon.

Sincerely,
Sergio Grinstein
Senior Scientist
Program in Cell Biology
The Hospital for Sick Children and
Professor of Biochemistry
University of Toronto
Dear Dr. Grinstein

Thank you for resubmitting the revised version of your manuscript Mapping the electrostatic profile of cellular membranes. The revisions meticulously address all of the substantial and detailed comments of reviewers. With apologies for some delay in handling this, I am happy to indicate that the manuscript will be recommended for publication at MBoC.

Best regards
Tom Martin

Monitoring Editor
Molecular Biology of the Cell

Dear Dr. Grinstein:

Congratulations on the acceptance of your manuscript.

A PDF of your manuscript will be published on MBoC in Press, an early release version of the journal, within 10 days. The date your manuscript appears at www.molbiolcell.org/toc/mboc/0/0 is the official publication date. Your manuscript will also be scheduled for publication in the next available issue of MBoC.

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Sincerely,

Eric Baker
Journal Production Manager
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