**Editorial Comments:**

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Complete.

2. Figures: Please include a space between the numbers and their corresponding units (i.e., 50 nm). Please order the figures based on their appearance in the text.

Complete.

3. Figure 2: Please add the panel labels (A-H) in the figure.

Complete.

4. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.

Complete.

5. Please provide an email address for each author.

Complete.

6. Please revise the Introduction to include all of the following:  
a) A clear statement of the overall goal of this method

Lines 34-35  
b) The rationale behind the development and/or use of this technique

Lines 37-40  
c) The advantages over alternative techniques with applicable references to previous studies

Lines 50-53  
d) A description of the context of the technique in the wider body of literature

Lines 60-62  
e) Information to help readers to determine whether the method is appropriate for their application

Line 62

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

Complete.

8. 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.”

Complete.

9. 3.1, 3.2, 3.3, 3.4, 3.6, 3.7, 3.8, 3.9: Please consider providing software screenshots as supplementary files to match each step.

Added supplemental file S2.

10. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Complete.

11. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

Complete.

12. Please note that Standard Access is checked in the uploaded ALA, while in the Questionnaire Responses Open Access is selected. Please be consistent.

Corrected.

**Reviewer #1:**

1) In principle, image processing algorithms should not be sensitive to rotation. This is essential in order to decrease the error coming from the manual intervention of the user and to facilitate the task of image analysis. Thus, I praise the authors for having evaluated the effect of rotating the analyzed images, because ideally, the computed perinexal space width should remain the same.  
As correctly observed by the authors, the strong dependence on rotation is an artefact of the dilation procedure due to the small size (3x3 pixels) of the used structural elements (dilation kernel shapes). The authors proposed a formula (line 248) to compensate for this effect. However, a motivation and a rigorous validation of this formula are missing. What is the rationale for this formula? Is it empirical or does it have some mathematical foundation? It would be desirable (e.g., in Fig. 5) to validate this formula (and its accuracy) by drawing parallel lines with a known separating distance and analyzing the image upon different rotations, rather than by processing an electron microscope image of an irregularly shaped perinexus as done in Fig. 5.

**Authors:** We appreciate the reviewer’s recognition of our efforts and constructive feedback on the rotation artifact correction. In the process of preparing the requested figures, we noticed an error in our formula, which, though empirically derived and validated, only functioned properly when applied to images of a certain spatial resolution. A full explanation of the new formula has been provided on lines 260-284 and Figure 8 has been updated as requested.

**R1:** 2) Morphological operations are very sensitive to contrast, blurred edges and to the manner images are prepossessed or recorded. This implies that if the prepossessing of the microscopy images is not standardized, false detection (in this case the manual outlining of the two membranes) may occur. The choice of the structure element (dilation kernel shape) might also depend on the contrast of the image. This should be assessed by artificially blurring the images or altering their contrast. This aspect is important because the membrane must be accurately outlined by the user.

**Authors:** The reviewer astutely identifies a key challenge with our method in that the original outline is user-generated. We have included clarification of gap junction and perinexus identification in the Protocol Section 2. We recognize that a limitation of the program is the reliance on the observer accurately identifying the relevant structure and a desirable and natural next step is to remove the human input and solve challenges including edge sharpness, contrast and signal-to-noise ratio as the reviewer has noted. In its current state, the described method is robust enough to identify relative differences between groups, even if absolute values are observer-dependent, as seen previously (reference 6).

**R1:** 3) A similar comment applies to the spatial resolution of the images. Would the algorithm give the same result if the images are taken at a higher or at a lower resolution? This should be ascertained by analyzing resampled images (up- and down-sampled), or, better, by analyzing microscopy images acquired at different magnifications.

**Authors:** The reviewer raises an intriguing question and we have included an additional low-resolution supplemental image (S6) and a brief addition to the discussion section, lines 440-442, to address differences in spatial resolution. The phantoms included in Figure 8 should also provide confidence that the results are valid for any spatial resolution, as the figures and supplement together span at least two orders of magnitude of potential spatial resolutions.

**R1:** 4) The algorithm presupposes that the section plane is perpendicular to the two membranes. How was this condition guaranteed? Was the section angle measured? The measured width will be overestimated if the section and membrane planes deviate from being perpendicular. If alpha denotes the angle of the deviation from perpendicularity, the measured width will be overestimated by a factor 1/cos(alpha). For alpha = 30 degrees, the width will be overestimated by 15% which is already substantial; for a very oblique section with alpha = 60 degrees, the width will be severely overestimated by a factor 2.

**Authors:** We appreciate the reviewer’s concern, as this is one of our greatest challenges with acquiring electron micrographs. We used the measurement of connexin gap junctions (GJW on page 7) to confirm that our sections are generally perpendicular to the structures we measure. Had they been substantially out of plane, we would have significantly overestimated their widths, as the reviewer noted. Still, it is difficult to say definitively that complex, and wider perinexal structures will stay in-plane even over the course of 200nm, and it is therefore critical that sufficiently large sample sizes are collected and that results are interpreted with a degree of caution. We would like to emphasize that perinexal values should not be interpreted to reflect in vivo spaces, but that our approach measures mean differences in perinexal width relative to some intervention. These points have been articulated in the discussion on lines 460-467.

**R1:** 5) If I understand correctly, the "centerline" is the locus of points for which the (ideally Euclidean) distance from the two membranes is equal. Assume P a point on the centerline and call A and B the closest points located on the respective membranes. Then, the distances PA and PB are equal, and the algorithm will return PA+PB as a result. However, if the two membranes are (locally) not exactly parallel, the points A, P and B will not be aligned, and the distance between A and B will be smaller than PA+PB because of the triangle inequality. This may lead to a further overestimation of the width of the intercellular space.

**Authors:** We appreciate the reviewer’s thorough analysis of our methodology. Of course one of the limitations of this algorithm is that as the membranes become less-parallel, it may over- or –underestimate the distance between them. However, due to the high spatial sampling frequency, small local variations have not been shown to affect the overall measurement and any differences resulting from such occurrences are smaller than our threshold for detecting differences between experimental groups. As we demonstrated in our published study using the manual technique (Reference 6), individual observers may measure different absolute values, but relative differences will still be detected.

**Reviewer #2:**

It is a technical report, but it is way too long and very hard to follow. It can be simplified much further.

**Authors:** We thank the reviewer for noting that the verbal description of the algorithm is long, complex, and at time tedious. In accordance with the JOVE vision, the written document should serve as a highly detailed source to explain concepts that were not clearly conveyed in the video, which for many users will be the first interaction with the material.

**Reviewer #3:**

One of few minor issues is that this Matlab-based pixel dilation method has been previously used for defining and calculating area/distance/border in biological images to achieve high precision and efficiency in image quantification in several publications (i.e. Sild et al. Neural Plast. 2013:853727; Yan et al. PLoS One. 2014, 8;9(8):e104357, and Papari et al. IEEE Trans Image Process. 2008 Oct; 17(10): 1950-62). This reviewer highly encourages the authors to cite these previous publications.

**Authors:** We appreciate the reviewer bringing these intriguing studies to our attention. However, we still have not found any methodology that utilizes serial image dilations to count the pixels between opposing edges. We do use a secondary dilation similar to Sild *et al*. to close gaps in our final image, and that citation has been included in the discussion. The other citations have been included in the introduction (Line 45) as they utilize image dilations for contour/edge detection but not in this particular application.

**R3:** Also, please provide some details about how to use morphological landmarks to identify perinexus and gap junction.

**Authors:** We appreciate the reviewer’s suggestion and have clarified the identification of the perinexus and gap junction in the Protocol Section 2.

**R3:** In addition, geometrically-diverse perinexi do appear in TEM images, such as the one in Fig 9C. Please provide an example to demonstrate the pixel dilation step-by-step for those perinexi with complicated morphology.

**Authors:** We agree the inclusion of dilation steps for more irregularly-shaped perinexi could be useful for the reader. For the sake of conciseness we have included a second example in the supplement S3 but defer to the reviewer if they would prefer additional examples.