**We thank both the Editor and the Reviewers for their thoughtful and insightful comments. We feel that the process of addressing their concerns has considerably strengthened the manuscript.**

**Editorial comments:**  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.  
  
We have made minor changes throughout the document.  
  
2. Please remove all commercial language: Sparkleen, catalog numbers, PowerPC, National instruments, LabVIEW VI, Kimwipe, etc.

We have removed commercial language. Section 8 of the original manuscript was specific to the commercial high content microscopy software that we used to analyze our images. To accommodate the prohibition of commercial language, we have rephrased this in more general terms and included a caution statement recommending the use of a commercial tool generated by professional software engineers for this step without specifying any of the several viable choices in the marketplace.

3. Manuscript, Figure 1 and 2: Please use SI units, e.g. use “μL” instead of “μl”. Please ensure that the L in these abbreviations are capitalized.

We have corrected liters to a capital L, and corrected imperial units.  
  
4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

We have made minor changes throughout the document, and rearranged some text to conform.

5. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.  
  
We have made minor changes throughout the document.  
  
6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

We have made changes throughout the document and moved some text to the discussion.  
  
6. Some additional details are needed in the protocol:  
1.6: Please use the Celsius grade.

Done

2.10: Cure plate where and at what temperature?

At room temperature. This has been stated specifically in the text.

3.2.3: What other parameters for sonication are used?

8 minutes in at 42,000 Hz in a 60W device in DI water. This has been stated specifically in the text.

3.3.2: Secure the plate how?

The plate is secured with clamps. Figure 1 has been added to illustrate the clamps on the device. References to securing the plate throughout the protocol have been updated to make clear that the plate is secured with clamps.

3.4.3: How many seconds exactly?

10. This has been added.

Remove what?

The foam pad and the components that weight it down. This step has been reworded for greater clarity.

4.2: Please provide an stl file.

An .stl file is being provided.

4.3: What are the plasma treatment parameters? Same as before?

Yes. This has been clarified in the text.

7. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

The text block at the start of 3.1.1 has been cut in light of this comment and a related comment from Reviewer #1 and moved to the end of the first paragraph of the Discussion.

8. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

An appropriate subset of the protocol has been highlighted.  
  
9. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

As above.  
   
**Reviewers' comments:**  
**Reviewer #1:**  
Manuscript Summary:  
The paper titled "Method for High Speed Stretch Injury of Human Induced Pluripotent Stem Cell-Derived Neurons in a 96 Well Format" aims to present a platform for studying brain injury in vitro with human-derived neuronal cultures. While stretch injury models have been used for many years to study mechanisms of trauma in neuronal cultures, this paper focuses on two specific challenges in this field which would make the technique attractive to researchers: (1) Increased throughput and (2) incorporation of Human cell lines. In general, the paper describes the necessary components required for this method to be reproduced successfully, and therefore I recommend it for publication, given that the subsequent issues are addressed.  
  
Major Concerns:  
1. There needs to be a general increase in the amount of quantification of mechanics associated with the model. Previous stretch devices were designed to generate highly reproducible strain and strain rates as these have been shown to be critical for neuronal injury. The major limitation of moving to high throughput is the risk of losing precision in the injury across samples. In its current form, I have no idea of how precise this instrument is and this would be my single hesitation in using it. There are many instances in which the authors describe potential pitfalls and subsequently offer suggestions as to how to avoid these, however, these are not sufficient. Expanding on this, the use of a voice coil actuator is a clever design, however, plate trajectories and induced substrate strain traces need to be included. I would want to see that the PID controller combined with the voice coil is sufficient to accurately produce specific strain and strain velocity profiles. Furthermore, to be complete I would also include traces with indenters partially and fully loaded in order to vary the load on the coil.  
  
Figure 2 has been added to quantify the kinematics of the device in detail. Panels B and C are reproduced from a prior publication1 under the terms of the Creative Commons license governing that publication. Panel A is a new figure of freshly generated data that allows the reader to compare the motion of the device when the plate is loaded with 52 posts to the motion when not loaded. This addresses the concern raised in the last sentence of the above paragraph. Also, numerical results have been added to the first paragraph of the Representative Results quantifying the variability in the mechanical strain across wells and plates.  
  
2. The figures included to support this technique are fairly weak. The multi-panel indicating the relationship between Laminin concentration and cell density is minimally helpful.

Cell density and laminin concentration are the two experimental parameters that most strongly influence the health of the cultures on silicone. These parameters cannot be optimized independently because one influences the other, as explained by the figures. Furthermore, optimizing uninjured cultures is not sufficient. Cell density and laminin concentration also influence the injury phenotype. This fact is also illustrated by the figures. These trends are vital to the design of successful experiments. We have included these figures to illustrate these trends to those who wish to use this technique in the hope that it will accelerate their efforts to optimize their cultures.

It is also over-saturated.

While calcein AM evenly labels the entire cytoplasm, the neurites are much fainter structures than the soma because their volume is much smaller. In these images, the dynamic range is intentionally adjusted to optimize visualization of the neurites. This adjustment inevitably leads to some saturation of the soma but this is worthwhile because the morphology of the neurites is more important in this context than the internal features of the soma. This practice is standard in the field2-4. The reviewer’s concern is understandable because we neglected to explain this in our previous draft. We have explained this choice in the legend for Figure 3 of the revised manuscript. This point is also addressed in step 7.4 in the revised manuscript.

The inclusion of time points or comparison to controls would be informative.

We have added data from the 24 hour post-injury time point so that it can be compared to the 4 hour post-injury time point that was originally presented.

For example, you might include an example of an optimal culture at several time points next to a control dish using the manufacturer’s protocol to illustrate minimal differences in culture quality metrics such as those provided in the following figure. Such a figure would provide an example for the appearance of a quality sample as well as provide evidence for the optimization of the culture protocol on the required flexible silicone substrate.

Figure 3B in the revised manuscript has been added to allow comparison of cultures on conventional rigid substrates with cultures on the required, flexible, silicone substrate.

The second figure is also fairly weak. Even if we are not reviewing for quality of data, my primary concern is that this figure doesn't emphasize the strength of the technique that is being described in the paper. I am assuming that this entire dataset is generated from 1 well, which should be emphasized if true in order to highlight the high throughput nature of the device.

This entire data set was not generated from a single well. It was also not generated from a single plate. All the wells in a plate must be injured with the same strain. Therefore, an experimental design that requires multiple strain levels must include multiple plates.

Proper statistics should also be included, again to highlight the shear volume of data that can be generated from a single plate.

We have added ANOVAs identifying which main and interaction effects in each data set are statistically significant. The results of these statistical tests are presented in the third paragraph of the Representative Results in the revised manuscript.

The injury levels should also be presented in proper units. Why indicate a 1 mm vs 2 mm indentation, which tells me nothing, when the actual numbers of strain have been measured.

The categorization of data by displacement has been replaced with categorization of data by strain in Figure 4 of the revised manuscript.

Also, it is unclear how neurite length was calculated. Clarifying this with an image would be helpful.

It is difficult to be highly specific about how neurite length was calculated because we used commercial software for this purpose and the Editor has asked us to eliminate all commercial language from the revised manuscript. We have rewritten section 7 to address this concern while also respecting the Editor’s wishes on this point.   
  
Minor Concerns:   
1. Implementation of this technique requires multiple custom fabricated parts. It is understood that these parts are difficult to describe in the text alone, but it will be essential to include drawings with necessary dimensions. For instance, section 3.1.1 describing the stretching plate is not sufficient to reproduce the device. From the text, it is also unclear if one will have to write the necessary supporting LabVIEW scripts or if they are provided.

The reviewer raises an important point about the challenges of describing a protocol that requires custom-built tools. The process of building the device is not a part of this protocol and the editor has specifically instructed us not to include material that is beyond the scope of the protocol. We have included a schematic of the device to better orient the reader as Figure 1 in the revised manuscript. We consider a complete set of technical documents necessary to reproduce the device (technical drawings with tolerances, bills of materials, LabVIEW code etc.) inappropriate for publication in the peer-reviewed literature so we will instead make them available to interested groups by informal communication. We have specified in the first paragraph in the Discussion of the revised manuscript that these documents are available upon request. When we share these documents, we will do so on an “as is” basis to assist those interested in reproducing our device. However, if we included these numerous, detailed documents as part of this publication, we would commit ourselves to guaranteeing that every single document is without error and might in theory have to retract the paper or publish an erratum over an inconsistency in the drawings or a bug in the code. Guaranteeing this level of accuracy across all these numerous documents would substantially delay publication of the paper. Therefore, we feel that informal communication is the more appropriate method for disseminating this information.  
  
2. Section 4 may be one of the most important for producing consistent injuries across laboratories, and the depth of the text is appreciated. However, I suggest including a table of measured values and expected variability.

Figure 2 in the revised manuscript provides results on measured strains and variability. These parameters are also quantified in the first paragraph of the Representative Results section in the revised manuscript.

Please also include a statement about the expected calibration requirements - how often, is the full procedure with high-speed camera necessary each time.

Our practice is to repeat the indenter-block alignment step (3.3) every time we wash the indenter (i.e. for every experiment) and to repeat characterization of membrane stretch (section 4) any time we alter the indenters or the plates. This practice is now described at the end of the first paragraph in the Discussion in the revised manuscript.

3. Given the importance presented within the paper on substrate preparation, it would be informative to provide control images of neurons cultured using the manufacturer’s protocol. It would be helpful to clarify in section 5 the differences in culture methodology for the iCell neurons on glass vs stretchable surfaces. In my experience, culturing neurons on stretchable surfaces is always more difficult compared to rigid surfaces, so this section may be best utilized to highlight these differences and simply direct the researcher to the iCell protocol for steps that adhere to the manufacturer’s protocol.

Figure 3B in the revised manuscript allows comparison of cells cultured on conventional rigid substrates according to the manufacturer’s protocol with cells cultured on silicone using our optimized protocol.

4. The discussion addresses the "…primary physical driver of variability in the system…" and there are suggestions for minimizing this error. However, there needs to be numbers included to inform the researcher how much variability to expect. The primary allure to this type of technique is the ability to injure many wells simultaneously. However, I don't have any idea what sort of variability to expect across wells within a single stretch as well as within a well across multiple stretches. These numbers will be useful both when assembling this type of device as well as for proper experimental design.

The newly added Figure 2 provides quantitative results describing measured strains and their variability. Numerical results quantifying the variability in strains across wells in a single plate and from plate to plate have been added to the first paragraph of the Representative Results section of the revised manuscript.

**Reviewer #2:**  
Manuscript Summary:  
This is a very nice piece of work, which will constitute an important contribution to the literature. The authors are commended for their efforts. A number of minor points need addressing before the paper can be fully accepted for publication.  
  
Major Concerns:  
None  
  
Minor Concerns:  
(1) Protocol 1.2. Was this washing only with H2O?

Membranes are washed with soapy water. Section 1.2 has been reworded to be more explicit on this point.

(2) Plate Fabrication steps 2.2 and 2.3. What is the actual time (text says "at the last minute")? What is the time between rinses?

APTES solution should be diluted no more than 60 seconds before the introduction of the plate. The text has been updated to clarify this point.

(3) Section 3. A picture or schematic of the apparatus would be helpful.

A schematic of the apparatus has been added to the revised manuscript as Figure 1.

(4) Section 3.4.1. Was the water de-ionised?

Yes, but it does not need to be. The tubes of water simply function as ballast in this step. This passage has been reworded for greater clarity on this point.

(5) Section 3.5.5. What is the full range? The typical range is interesting too.

The typical range of plate displacements is 1-4mm. The maximum is 5mm. These values have been added to the text.

(6) Section 4.2. 3D printing us stated. Is this necessary or could it be fabricated alternatively?

3D printing is not necessary although it is the approach we employed. The text has been adjusted to clarify this point.

(7) Section 4.17.1. Can a schematic of the wells C05-E08 be provided?

In reflecting on this comment, we concluded that the description of the process for choosing how many wells to image was too prescriptive. We have made the language in this section more general so the reader can make their own choices for their experimental design in characterizing the induced strain. The choice of how many wells to image at a time, and how many times to image them, will depend on the reader’s judgement and understanding of their application.

(8) Section 5.17. Better to say "0.033". Do the authors want to mix SI and Imperial units?

.33 has been replaced with 0.33. All imperial units have been converted to SI.

References

1 Sherman, S. A. *et al.* Stretch Injury of Human Induced Pluripotent Stem Cell Derived Neurons in a 96 Well Format. *Sci Rep.* **6** 34097, doi:10.1038/srep34097, (2016).

2 Morrison, G. *et al.* Evaluation of inter-batch differences in stem-cell derived neurons. *Stem Cell Res.* **16** (1), 140-148, doi:10.1016/j.scr.2015.12.025, (2016).

3 Wheeler, H. E., Wing, C., Delaney, S. M., Komatsu, M. & Dolan, M. E. Modeling chemotherapeutic neurotoxicity with human induced pluripotent stem cell-derived neuronal cells. *PLoS One.* **10** (2), e0118020, doi:10.1371/journal.pone.0118020, (2015).

4 Sirenko, O., Hesley, J., Rusyn, I. & Cromwell, E. F. High-content high-throughput assays for characterizing the viability and morphology of human iPSC-derived neuronal cultures. *Assay Drug Dev Technol.* **12** (9-10), 536-547, doi:10.1089/adt.2014.592, (2014).