Editorial comments:

1) All of your previous revisions have been incorporated into the most recent version of the manuscript. Please download this version of the Microsoft word document from the "file inventory" to use for any subsequent changes.

All changes made to the manuscript based on the editorial and reviewer comments, have been made to a new version of the document (R4).

2) In step 1.3.4 you use a sample that is not prepared in the protocol. This is a main point of the article and so its preparation should be included. Please keep protocol content and formatting guidelines in mind as you address this revision.

The isolation and preparation of an extra-cellular vesicle (EV) sample is a detailed process with many different steps. Adding this information to this manuscript will, in our opinion, distract the reader from tunable resistive pulse sensing for EV characterization. However, this process has been described and visualized in a JoVE publication before. We have added a short note right at the beginning of section 1.3, referring to this JoVE publication.

3) Please disregard the comment below if all of your figures are original.

If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

All of the figures are original.

4) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

The manuscript has been thoroughly been proofread by us.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript described the detailed protocol to quantify and profile the size distribution of EVs. Alternative method was also described to measure EVs directly in biological fluids. On the other hand, the limitation of the method is well described also. All these provide sufficient information for user to carry out the experiment.

Major Concerns:

The theory/working principle of the technique could be explained in more detail. i.e. the magnitude of current blockade is only affected by the particles diameter? How about other physical properties of the particles?

We have added text regarding the assessment of the zeta-potential of particles based on the blockade duration.

Minor Concerns:

1. In line 62-63 please provide reference for the mentioned processes.

A total of 5 references have been added to the manuscript to further clarify the mentioned processes.

2. Figure 2 is not displayed correctly.

Due to a hidden layer in the Illustrator file, figure 2 (as also mentioned by reviewer 2 and 3) can be confusing if opened in certain .pdf viewing software. When using Adobe Acrobat Reader the figure should display the correct information. We have made new versions of the Illustrator files with the hidden layers removed.

Additional Comments to Authors:

N/A

Reviewer #2:

Manuscript Summary:

The authors described the workflow for determining the concentration and size of Extracellular Vesicles (EV) using Tunable Resistive Pulse Sensing (tRPS). The standard operating protocol in part one is largely concordant with the training materials from Izon Science, so there is nothing much to be criticized about. In part two, the authors introduced an alternative protocol, which simplifies the calibration step for some samples by spiking the samples with calibration beads. The novel alternative protocol, though not yet fully supported by the factory software, is of general interest to researchers working in this field. However, some practical issues have to be addressed and they are outlined below.

Major Concerns:

As discussed by the authors in the introduction at line 56, the size of EVs can range from 50-1000nm. However, the authors described that 335nm polysterene beads can be used for spike-in standard in the alternative protocol. Since the author claimed that the alternative protocol is suitable for biological samples without filtering out the larger EVs, how did the authors avoid cross-talk between the spike-in standard and the EV sample?

We have added a note at the end of section 2.3 which explains this problem and offers a method to correct for the overlap of EVs and spiked polystyrene beads.

At line 264, the authors stated that "Measuring each individual sample once before recording replicates in order to distribute fluctuation in nanopore conditions equally over the different sample.". Should the replicates be performed right after the earlier attempts, such that the pore condition can be kept as close to each other as possible? Particularly for the alternative protocol, where replicates will be used for calibrating each other, maintaining constant pore conditions between replicates is vital for the accurate calibration of samples.

We fully agree with reviewer 2 that nanopore conditions should be kept as constant as possible. The text has been changes slightly to emphasize this more.

The representation of Figure 2 is confusing. Two overlapping X-axis were drawn and are obscuring each other. Besides, could the author explain why Time and Diameter could be represented by the same axis? It is not intuitive to think that only large particles can be recorded as the time of measurement increases.

As outlined above, this is due to a hidden layer in the Illustrator file that is displayed in some .pdf viewing software. The intended (Illustrator/pdf) file does not display the double axis.

In Figure 3B, only blockade height (nA) was shown, which is different from Figure 2B, where particle diameter (nm) was shown. Blockade height is not relevant to biologists unless that is calibrated into particle diameter. Since the alternative protocol has described the calibration of particle size using spike-in standards, particle diameters should be shown instead.

We have added subfigure 3C where one of the samples is displayed in nm rather than nA. We believe the subfigure 3B should remain in the manuscript since we use the blockade height plot, rather than particle sizing in nm, for the distinction of the two populations.

Minor Concerns:

Decimal points in Table 1 was written as comma, should be written as dot to be consistent with the rest of the article.

The commas in Table 1 have been changed to dots.

At line 99, one cannot "eliminate" electro-kinetic forces. I think the word "counter" better fits in here.

The word “eliminate” has been changed to “reduce the effect of” which better fits the desired effect of applying more pressure.

Additional Comments to Authors:

N/A

Reviewer #3:

Manuscript Summary:

The manuscript describes one of the methods for characterizing extracellular vesicles in terms of their size distribution and concentration. It is one of the three most used methods alongside nanoparticle tracking analysis and dynamic light scattering. The workflow is well described, language flows well and and the method is discussed in terms of its advantages and limitations. It gives an impression of a detailed yet balanced overview of the method. It deserves a video review well as it's not a very widely spread method. Having a detailed video overview of the method would help researchers to make a decision whether it suits their particular needs and, furthermore, if they already use it, is there anything they could do in order to optimise their workflow (e.g. using the "real-time" calibration using spike-in sample).

Major Concerns:

There are some points nevertheless that in my opinion would improve the manuscript from technical point of view.

1. The method description requires some clarification. While the authors explain well the principle of the system in Figure 1, it remains somewhat undefined what the word "tunable" in tRPS means. It may remain unclear for the reader whether this refers to the term "stretching" later in the manuscript. Also, it should be explained what that "stretching" is. It is a parameter that can be modified during measurement but it is not described in Figure 1 (i.e. what is its part in overall working principle of the device).

We have added information regarding the tunable aspect of the instrument in the figure legend of Figure 1 (therefore figure 1 has been slightly changed: added the arrow and “arm” text).   
Secondly we have added additional information in step 1.1.8.

2. The authors provide a recommendation which pore size to use for extracellular vesicles. If there is a rule-of-thumb which pore size should be chosen based on expected particle size then it could be mentioned (e.g. when perhaps researchers want to study properties of large apoptotic vesicles compared to EVs, etc.). If for new sample type several pore sizes should be tested to get an estimate which size to use in all future experiments, it should be more clearly mentioned.

We have updated step 1.1.2 to include all nanopores and their target size ranges for EV quantification and characterization.

3. At row 432, please clarify, does this mean that each sample should be measured using several different pore sizes for making sure that smaller particles are not being missed during measurement?

Additional text has been added to the manuscript explaining the trade-off between particle detection and nanopore clogging. This will explain in more detail the difficulties encountered when employing the “spiking method”, especially concerning the missing of a population of small EVs not reaching the detection threshold.

4. In discussion, the manuscript could benefit of a short but concise comparison between the described method and other used techniques such as nanoparticle tracking analysis and dynamic light scattering (e.g. performance, ease of use, potential artifacts, advantages, limitations, cost, ease of use, data quality).

We believe that describing the different alternative techniques with enough detail and accuracy is beyond the scope of this manuscript. In the introduction, we refer to a recently published review (vd Pol et. al 2013) where these techniques and others are described into great detail.

Minor Concerns:

Please check figures carefully again. In Figure 2, legends seem to overlap, also the graphs themselves seem to have been pasted on top of each other so that the underlying graph is partially visible.

In Figure 2A and text, it should be explained more explicitly how to read the particle rate graph. Particle rate is not any of the axis, as I understand it's rather the slope of the acquired points that makes up the "particle rate" (i.e. the average rate of detection of particles).

The text in the figure legend has been changed to “particle-count plot” which, as suggested, better describes the displayed data.

Figure 3B is said to represent particle size distribution, however the x-axis is in nA and not in nm (like in Figure 2B). Please correct the axis or terminology, please.

The blockade-size in nA is a measure for particle volume and thus particle size. However, as also suggested by reviewer 2 we have added subfigure 3C which illustrates the translation of figure 3b (in nA) to sizing in nm.

Additional Comments to Authors:

N/A