**Editorial comments:**

Changes to be made by the author(s) regarding the written manuscript:

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

We have proofread the manuscript for spelling and grammar issues.

2. Please revise lines 504-505 to avoid previously published text.

These lines have been revised.

3. Please provide an email address for each author.

The email address for each author is now listed on the title page.

4. 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. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Jackson Laboratory, Sigma, Pharmco-Aaper, Baxter, etc.

We have removed commercial names from the manuscript and updated the table of materials and reagents accordingly.

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

We have revised the manuscript to avoid the use of personal pronouns.

6. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). 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.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

We have revised the manuscript and used the imperative tense whenever possible.

7. 1.5: Please describe how to confirm GCaMP6f expression by genotyping or add a relevant reference.

We have added the detail of how GCaMP6f was confirmed with genotyping in the revised manuscript.

8. 2.1: Please specify the concentration of isoflurane.

The concentration of isoflurane used is now included in the revised manuscript.

9. 2.2: Please provide the composition of Krebs-Ringer bicarbonate solution. If it is purchased, please cite the Table of Materials.

The KRB solution composition is now inserted into the revised manuscript.

10. 2.5, 3.1, 3.18, 3.19, 4.1, etc.: 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. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

We have revised and simplified the protocol and ensured that the protocol steps are shorter throughout.

11. After you have made all the recommended changes to your protocol (listed above), 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.

The text deemed most appropriate has been highlighted.

12. 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. Please do not highlight any steps describing anesthetization and euthanasia.

The text deemed most appropriate has been highlighted.

13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

The text deemed most appropriate has been highlighted.

14. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

Figures have been uploaded as requested.

15. Figure 2H: The x-axis in the middle panel is cut off. Please fix it.

This has been corrected.

16. Discussion: Please also discuss any limitations of the technique.

Limitations of the technique are now discussed.

17. References: Please do not abbreviate journal titles.

Journal titles are not abbreviated.

18. Please revise the table of the essential supplies, reagents, and equipment to include the name, company, and catalog number of all relevant materials.

Now revised.  
  
**Reviewers' comments:**  
  
**Reviewer #1:**

Manuscript Summary:  
The authors have a rich publication history utilizing elegant calcium imaging preparations with a rigorous analysis focused on providing quantitative parameters to describe the observed phenomena. The manuscript titled "Applications of spatio-temporal mapping and particle analysis techniques to quantify intracellular Ca2+ signaling in situ" primarily provides a step by step protocol detailing their Ca2+ acquisition process and Ca2+ video analysis protocol that can be readily applied to other tissues in addition to the intestinal ICC imaging described here. The generation of ICC targeted GCaMP6f expression using the Cre-Lox system under control of a tamoxifen inducible cKit-Cre is described, and followed by the method of preparation and Ca2+ imaging of the intestinal ICCs. The authors then describe an improved ROI method for analyzing Ca2+ videos using spatio-temporal maps to allow for quantification of Ca2+ events beyond simple intensity-time plots including production of informative STM plots, F/F0 values, spatial spread and velocity of Ca2+ events, and rise/decay/total time of events. The authors then describe a step by step procedure using a custom software Volumetry to analyze an entire FOV of a complex Ca2+ recording in an unbiased and efficient manner. Some explanation of the power and limitations of the protocols described here are discussed and evidence given for their practical applications in other tissue beds.  
  
Minor Concerns:  
The protocols are written in a clear and succinct manner. The STM protocol outlined in section 3 was easily followed by this reviewer using ImageJ and the described results from Figure 2 were readily achieved using Ca2+ recordings from non-ICC cells. However, the bias inherent to the user generated ROI is still a potential downfall. The STM created from the rectangle ROI used in Volumetry appears to be an average value of (STMAvgRow) the intensity across either the y or x axis, and I am curious if signal amplitude could be inadvertently diluted if the Ca2+ is highly localized and substantially smaller than the width of the ROI.

The reviewer is correct in this observation. We have added a statement in the discussion regarding the potential dilution of the calcium signal amplitude if the width of the ROI is drawn too wide.

Tangentially, taking intensity measurements from a single line through the midpoint axis of a cell as described in section 3.12 is also subject to the proximity of the Ca2+ event to the user generated line for the STM. This may be easily disregarded in spindle like longitudinal cells such as the ICC-DMP as the authors point out in Section 4.1, but may be worth adding a few sentences in the discussion for awareness to these potential issues in the broader application of this method.

As the reviewer suggests, we have added additional information in the discussion highlighting these key areas of analysis consideration.

The PTCL analysis using Volumetry is especially powerful in its ability to analyze an entire field of view in an unbiased manner. Widespread use of the protocols listed will likely depend on the means and ease of availability and acquisition of the Volumetry program. For section 4.3 and 4.4 the reader is told to run the STK Filter Differentiate and a STK Filter-Gauss KRNL, and are told that a value of 2 and 5 work well respectively. It may be helpful to provide what the reasonable range for the values one should expect to use to prevent or the conditions that would lead to changing those values from what is suggested.

As the reviewer suggests, we have added additional information in the discussion describing more details about the range of differentiation values to be applied across different recordings and also the consequences on noise and signal to noise ratios of applying too low or too high a differentiation value. We added the following statement to further clarify the role of the differentiation function. We have added the following statement to the manuscript to further clarify the differentiation function in Volumetry. “*Note: Differentiation in this context will reduce the intensity of areas of recording (pixels) that show no dynamic activity over the number of frames specified. Thus, if a value of ‘2’ is inserted, each pixel in every frame of the recording is analysed and if within that frame there is no dynamic change in pixel fluorescence 1 frame before and 1 frame after, the intensity within those pixels will be subtracted from the recording. Thus non-dynamic background noise is removed and signal to noise is increased.”*

In the section utilizing Image J, I would suggest to mention or consider adding the plot profile command in section 3.7 and 3.17 to provide a visual image of the calcium intensity trace, whereupon the background or baseline intensity value taken from the histogram can be simply and quickly confirmed visually across time (information lost in the histogram) prior to subtraction and normalization.

We would not recommend using the plot profile command at this juncture of the protocol as the plot command profile does not give an accurate representation of intensity against time for stacked TIFF images in ImageJ. While the command for plot profile generates very accurate plots of fluorescence taken from single TIFF images such as the STMs described, in order to gain accurate plot profiles from the movie itself, further calibrations for space and time would need to be inserted into the raw movie, which would serve to lengthen and complicate the analysis protocol.

Line 282 Would using the median or mode, as opposed to the mean, be less sensitive to potential outlier signal events within the background ROI (or incidental inclusion of the targeted cell population). Introduction of outlier signal noise within this background ROI could inadvertently result in some signal loss.

In our experience, the use of the mean value for subtraction purposes yields the most consistent normalization for movie recordings. While as the reviewer suggests, the use of the mode or median can be beneficial in certain recordings, in order to consistently apply the same analysis to all recordings the mean is the most consistent representation of background noise (at least for our recordings).  
  
Minor Edits:  
Line 141/2: For consistency with the rest of manuscript stick with "seconds"

Edited.

Line 181: Assume Safflower "Oil"

Correct, now edited.

Line 404: "wave speed plugin" should be cited or in the very least provide the website address to access the plugin mentioned.

Upon searching for the appropriate plugin online to provide a link, it appears that this plugin is now longer available for public download and thus all reference to its implication has been removed from the manuscript.

Line 556 I assume should read Fig 4D-E.

Correct, now edited.

**Reviewer #2:**

Manuscript Summary:  
This article describes the uses of Cre-Lox recombination to genetically insert a calcium indicator into a specific cell type - known as the Interstitial Cell of Cajal (ICC). Then, the study describes how to analyze calcium transients within ICC. While cre-lox recombination is not new, nor is the use of genetically encoded calcium indicators, the analysis approaches are useful. The methods of analysis appear to have been used previously in the Baker S et al. J. Physiol. paper, but this JOVE article explains in more detail how to use Volumetry. The analytical methods in this paper could be useful to the field, not just ICC. In fact, as there are increasingly few laboratories imaging ICC (I don't know any other laboratories doing imaging of ICC anymore, my feeling is the analysis methodology in this paper would more likely be useful to others using imaging in other cell types e.g. central neurons or brain slices. There are some bold statements that need to be reworded and supported by references. There are also some additional new references that need to be inserted, before any further editorial consideration can be given. The figures are clear and references suitable - when modified.  
  
Major Concerns:  
Line 84: it is stated "While newer Ca2+ indicator dyes such as Cal520/590 have improved the photobleaching difficulties associated with older dyes somewhat, it still remains a concern for investigators." Do the authors have a reference to support this statement ? Reference 24 (Thomas et al. (2000) Cell Calcium) does not show that new calcium indicators have less photobleaching than older indicators as it was published in 2000, and the new indicators were not around then. Please explain what data this comments is based on.

We have now added new references that point out photo-bleaching effects of the Cal lines of calcium indicator dyes (Flagmeier et al., 2017; Tsutsumi et al., 2015; Rietdorf et al., 2014), highlighting that photobleaching still occurs with preparations loaded with Cal 520 and in a quantitative study was found to have similar photobleaching effects as Fluo 4.

Whilst genetically encoding calcium indicators have some clear benefits they do involve genetically modifying the animal. To provide a balanced view, the authors need to acknowledge that an important benefit of not using genetically-modified animals is the animals do not have to be genetically modified. In the introduction please insert:  
"Whilst genetically-encoded calcium indicators can offer some clear advantages, recent studies have revealed that calcium imaging can be successfully performed from large populations of different neurochemical classes of neurons simultaneously using conventional calcium indicators that are not genetically encoded into the animal (Spencer NJ et al. 2018; J. Neuroci). This approach used post-hoc immunohistochemistry to reveal multiple different classes of neurons firing at high frequency in synchronized bursts, and avoided the potential that genetic modifications to the animal may have interfered with the physiological behaviour the investigator seeks to understand (Spencer NJ et al. 2018; J. Neuroci; Hibberd T et al. AJP, 2018).

We have inserted the requested statement and the appropriate references.  
  
Line 83: It is stated "While newer Ca2+ indicator dyes such as Cal520/590 have improved the photobleaching difficulties associated with older dyes somewhat, it still remains a concern for investigators." Do the authors have a reference to support this statement ? That is, has anyone published that newer calcium indicators confer less photo-bleaching than older indicators, or is this a subjective view ? If the author's don't have a reference to support this statement, then please reword this to state that this comment reflects the authors personal view (if that is indeed what they believe).

As stated above, we have provided new references to appropriately account for our statement in this paragraph.

Line 111: Again, it is stated "Another advantage of GECIs over traditional Ca2+ indicators is that photobleaching is reduced, as compared to dye-loaded specimens, particularly at high magnification and high rates of image capture." Again, is there a study that has systematically quantified this and published it ? If so, please include the reference. If not, reword the statement to say this is purely the authors views (again, if they believe this to be true). Otherwise, without any reference, the statement is ill-founded.

We have added an appropriate reference to the manuscript to substantiate our statement here. We draw particular attention to the paragraph in the cited paper (Barnett et al., 2017)*“The remarkable redistribution of the GCaMP chromophore from neutral to anionic forms has two important consequences. First, with 470 nm excitation, the Ca2+ sensor is dark at low Ca2+ concentrations, and only fluorescent in active cells, producing an excellent signal-to-noise ratio in complex tissues like the brain. Second, since the neutral form of the chromophore is not absorbing the 470 nm light, the Ca2+ sensor is only susceptible to bleaching during the brief periods that the cell is active.”*

The methodology provided to run Volumetry appears to be written clearly and should be able to be followed by others.  
  
References that need to be inserted  
Hibberd T et al. (2018) AJP; https://www.ncbi.nlm.nih.gov/pubmed/28935683  
Spencer NJ et al. (2018) J. Neurosci: <https://www.ncbi.nlm.nih.gov/pubmed/29807910>

We have added the requested references.  
  
Minor Concerns:  
In the abstract: Change the words "the biological information" to "some biological information".

Edited as requested.  
  
**Reviewer #3:**   
Manuscript Summary:  
Overall, the method seems to be a worthwhile one and worthy of publication in JOVE. A useful description of the experimental method used to acquire the Ca signals is presented and I see no particular problems with that.  
The analysis workflow for the STM and particle analysis is presented in a very basic step by step fashion which would benefit in some places with a clearer explanation of the processing function taking place at that point.  
  
Major Concerns:  
316 Normally, the signal to noise of the created linescan will need to be improved. Do this by  
317 clicking 'Auto' on the B&C HUD.  
  
This procedure simply adjusts the image contrast to make the linescan image optimally visible to the viewer. It does not improve signal-noise and should not be referred to as doing so.

The reviewer is indeed correct here. We have edited this statement to say that the contrast and not signal to noise of the linescan needs to be improved.

322 Selection' function in the ImageJ interface, draw an ROI on an area of the STM that is in focus  
323 and displays the most uniform and least intense area of fluorescence (F0).  
  
I'm not sure what " an area of the STM that is in focus" means. If this is a confocal image there are no "out of focus" regions. The ROI should be placed on a uniform region with containing the lowest intensity.

The reviewer is correct, we have removed the emphasis on focus for this step of analysis.  
  
362 that when the calibration bar is inserted, ImageJ creates a new STM containing it, leaving the  
363 original version without the calibration bar intact and separate.  
  
It didn't do this when I tried it.

If when inserting the calibration bar, the ‘Overlay’ box is ticked, then a new STM will not be created which may have happened to the reviewer in this instance. We have clarified this detail in the revised manuscript.  
  
399 3.26 The velocity of a propagating Ca2+ event can be determined by drawing a line along the  
400 propagating front of the event and calculating the slope of the line. This may be performed  
401 manually in a similar manner described in step 3.24, by determining x,y values for where the  
402 line begins (x1,y1) and ends (x2, y2), these values will be displayed in the lower left side of the  
403 ImageJ interface when the mouse cursor is situated on the STM. Alternatively, plugins to  
404 calculate velocity such as the 'Wave Speed' ImageJ plugin are available to download from  
405 various sources.  
  
This seems a rather time consuming process, it might be more useful to describe the use of the Wave Speed plug-in. Or alternatively write an ImageJ script to do it.

As stated above in response to reviewer 1, upon searching for the appropriate plugin online to provide a link, it appears that this plugin is now no longer available for public download and thus all reference to its use has been removed from the manuscript.

In a more general point here concerning this method Since once of the main arguments for the use of STMs was that they were more objective than simple fixed ROIs, I was slightly concerned to see that the identification of the Ca event by the placement of the horizontal and vertical is entirely manual.  
I think something needs to be said here about how the user should go about selecting events consistently and objectively. Should every visible event be analysed for instance?

As per the reviewers suggestion we have included the following statement in our manuscript about consistently selecting Ca2+ signals for analysis. *“Note: Experimenters will need to design specific criterion for thresholding valid Ca2+ events in these recordings. In our experiments, we designated a Ca2+ event as being valid for analysis if its amplitude was >15% of the maximum amplitude event in the control section of recording. However, these thresholds will depend on the specific tissues and cells under study and are only arbitrary guidelines that require specific optimization for every type of tissue and cell.”*  
  
436 4.3 In order to accurately calculate Ca2+ signals from the entire FOV, the movie will firstly  
437 undergo differentiation and smoothing to remove background interference and increase the  
438 signal to noise ratio. In the Movie Window, right click to bring up a menu and using right clicks  
439 access 'STK Filter-Differentiate', right click again to input a value to differentiate, press ENTER  
  
This needs further explanation. It's not clear what "differentiation" means in this context or why it should improve the signal-noise ratio? Also what sort of "background interference"?  
I presume the smoothing is to increase the signal-noise ratio?

We have added the following statement to the manuscript to further clarify the differentiation function in Volumetry. “*Note: Differentiation in this context will reduce the intensity of areas of recording (pixels) that show no dynamic activity over the number of frames specified. Thus, if a value of ‘2’ is inserted, each pixel in every frame of the recording is analysed and if within that frame there is no dynamic change in pixel fluorescence 1 frame before and 1 frame after, the intensity within those pixels will be subtracted from the recording. Thus non-dynamic background noise is removed and signal to noise is increased.”*

The smoothing function does not affect signal to noise but helps in removing potential high frequency shot noise from the camera.

448 4.5 Start to create PTCLs by firstly selecting a quiescent period of the movie (20-40 frames)  
.....  
483 active Ca2+ transient PTCL at your determined threshold point. To save this as a coordinate  
  
I find this whole section 448-483 hard to understand given the lack of explanation of what the aim of the processing is here. Since this is a very crucial part of the analysis it would benefit from being made clearer.  
In the initial stage 4.5.-4.6, a threshold is determined semi-empirically from frames with no Ca activity isolating "particles" which correspond to the ICC-MY cells separated from background noise.  
This threshold is then used throughout the analysis of subsequent frames? So is the intent to set the threshold just above the cell resting Ca fluorescence?  
When Ca release events occur the number and area of a particles increase presumably because more of the ICC-MY cells area exceeds resting Ca conc.?  
  
Can you define the criteria for selecting Ca release sites more explicitly and explain why it was chosen? E.g. why particles overlapping after 70 ms? Is this the expected duration of events?  
I am also slightly puzzled about the criterion that there should be no overlap with another particle in the preceding frame.  
If the threshold used to initially locate the cells during a quiescent period was used to detect these particles then there would always be a particle present.  
It would just get bigger in area when an event occurred. I may be misunderstanding something here.

Yes - the threshold equates to greater than the background intensity level of ICC-MY in the non-active state, i.e. during the inter slow-wave period. We have amended the text in the revised manuscript to make this more clear.

Indeed - events that transiently exceeded the “background” intensity level after both differentiation and smoothing filters were applied were considered as Ca2+ transients.

The choice to filter out Ca2+ events < 70ms was made mostly to filter out occasional random noise aggregations that could appear in 1 frame.  These aggregations rarely lasted for more than 1 frame in the same spatial position and could be filtered temporally.  The duration of Ca2+ transients was much longer (>150ms) and the temporal filter did not affect them.

Initiation particles had no overlap with other particles in the preceding frame - Ca2+ transient particles were overlapped through their duration.

The threshold choice, size + temporal filtering eliminated most/all background associated with labeled-cells in a transiently quiescent state.  Therefore any particle was inherently a dynamic increase in Ca2+ intensity.

Minor Concerns:  
  
203 2.4 Following this equilibration period, in situ Ca2+ imaging of small intestinal ICC-MY and  
204 ICC-DMP can be performed using confocal microscopy. Due to the benefits of GECIs described  
205 above, high-resolution time-lapse images (>30 frames per second, FPS) combined with high  
206 power objectives (60-100x) can be used to acquire movies of dynamic Ca2+ signals in ICC.  
  
Is it spinning disk confocal that is being referred to here, rather than point scanning. If so, better to be specific since a point scanning confocal would probably be operated in line scan mode obviating the need for the type of analysis described.

A spinning disc was in use here and we have specified this in our revised manuscript.

298 'Brightness/Contrast' (B&C). This will bring up a new heads up display (HUD) where various  
299 aspects of brightness and contrast may be modified. Left click on the 'Auto' option once to  
300 reveal the increased quality in the movie. Leave this B&C HUD open for future use.  
  
Heads up display (HUD) is not a normal term for this. "pop-up dialog box", or just popup box would do.

We have amended our terminology from HUD to pop up box as the reviewer suggests.  
  
**Reviewer #4:**   
Manuscript Summary:  
In situ calcium imaging provides an opportunity to study cellular activity within its native cellular environment. Cell-specific expression of genetically encoded calcium sensors has made it possible to perform high resolution Ca2+ imaging with low background and reduced photobleaching. However, there are a lack of techniques, particularly in enteric neuroscience for unbiased analysis of complex Ca2+ signaling patterns.  
The authors outline a protocol to perform unbiased analysis of Ca2+ recordings from interstitial cells of Cajal (ICC) within the small intestine. They also quantify and describe the complex Ca2+ signaling patterns observed in ICCs located in different layers of the small intestine. The protocol describes two different approaches to quantify Ca2+ signals based on the pattern of cellular activity. They have used a spatiotemporal mapping based method (STM) for analysis of ICCs within the deep myenteric plexus, and a particle based technique for ICCs surrounding the myenteric plexus.  
This protocol can be applied for extensive characterisation of complex subcellular Ca2+ signaling patterns in a cellular network. An advantage of the technique is the unbiased identification of regions within the field of view using a consistent thresholding protocol, as this can be a major source of operator error when defining ROIs. Using PTCL analysis, subcellular calcium signaling domains can be identified and characterized. The analytical techniques described can extract more quantitative data from Ca2+ recordings than traditional ROI-based methods, thus providing extensive spatial and temporal information.  
  
Major Concerns:  
NA  
  
Minor Concerns:  
\*It is informative to see different analysis routines for varying cellular activity, but what about its applicability to other complex cell types or systems, such as enteric neurons and/or glia? Enteric glial cells also have slower calcium response profiles, in the order of seconds, depending on stimuli.

We have included a paragraph in the discussion describing how the PTCL based analysis outlined in this protocol has been applied to other complex tissues and suggest that it may also be applicable to neuronal networks also. *“PTCL analysis can be easily adapted to different intact preparations other than that described in this protocol. For example, a recent study used PTCL analysis to study novel rhythmic Ca2+ events occurring in the intact cellular networks of the lamina propria of the rat urinary bladder 55, 56 and thus could be easily applied to other complex, intact cellular systems such as neuronal systems.”*

\*It is a bit hard to follow the analysis routines in section 3 and 4. It will be helpful to include a flow chart giving an overview of the analytical steps.

We have simplified the analysis routines in section 3 and 4 and shortened the action steps. The protocol is now easier to follow and the design of figures 2-4 are provided in a step by step fashion to follow the text.

\*It may be informative to give some background about the particle based analysis technique (advantages and principle?).

We have included a paragraph in our discussion outlining the advantages of using PTCL based analysis approaches to in situ imaging “*PTCL analysis provides a streamlined technique to quantify complex, subcellular Ca2+ events occurring in the network. Moreover, it also allows all Ca2+ events in the network within a given FOV to be analyzed, rather than using arbitrary ROIs, which only provide information on frequency and intensity within the ROI. An advantage of the PTCL analysis described here is that by applying differential and Gaussian smoothing filters to recordings, a large amount of noise can be removed from movies that may contain contaminating light from cells not of interest or due to non-dynamic bright spots or inclusions.”*

\*It appears that more information can be extracted from this data. What about analyzing the directionality and coordinated activity of the network?

This is certainly a possibility and in fact has been done for analyzing wave propagation directionality in a crude manner in ICC-MY (Drumm et al., 2017; J Gen Physiol). However, due to the length of the protocols already described we do not have enough space to include any detailed information on this possibility in the current manuscript.

\*Authors should state the reason for using GCaMP6f, over GCaMP6 or GCaMP6s. It would be appropriate to mention this as the choice of GECI is dependent on the system being studied, and this in turn influences data analysis and interpretation.

We have included the following statement and reference in our revised manuscript to clarify our selection of GCaMP6f. *“Note: GCaMP6f was used due to its reported efficiency in reporting localized, brief intracellular Ca2+ signals in situ and in vivo. 54”*

\*Were any drugs added to reduce contractions (e.g. nicardipine)? If not, were there any movement artefacts (contractions/relaxations) during recording? Which motion stabilization algorithms or protocols were used (names)?

Nicardipine was applied during recordings to prevent tissue movement. The following statement has been inserted into the revised manuscript to clarify this. *“Note: To reduce tissue movement, nicardipine (0.1-1M) was applied during recordings as described previously.37–41”*

\*In the long abstract, the authors should state that the technique focuses on analyzing data from GECIs.

We have included this point in the long abstract as suggested.

\*This may fall outside the scope of the article, but the authors could add notes about using this same technique to analyze calcium dye loaded cells/tissue, i.e., whether the described technique can be used or not? If so, what are the considerations that must be taken into account? (Heppner et al., 2017)

We have added the following paragraph to the discussion of our revised manuscript to address this point “*While this paper focused on Ca2+ imaging in intact tissues with GECIs, these analysis techniques can also be run on isolated cells and tissues loaded with traditional Ca2+ indicator dyes. The STM based analysis has been used to successfully quantify localized Ca2+ signals and Ca2+ waves from spindle shaped interstitial cells and smooth muscle cells from a variety of preparations 11, 58–61. Furthermore, the PTCL analysis routines described here have also been applied to in situ network preparations visualized with Cal 520 56, 57. However, these studies also retain the disadvantages of such dye loading protocols such as ambiguous cell identification and problems with signal to noise*.”  
  
Software:  
\*Will the steps for software analysis in the video be as detailed as the protocol, i.e., will the video include step by step instructions for software use?

Certain steps will be included in the video as decided by the JOVE editorial team.

\*Including an ImageJ macro for steps outlined in section 3 will ensure accurate reproducibility of the protocol. It could also be a list of the commands from the ImageJ 'Macro Recorder window'.

As the selection of regions for background noise, F0 and lines to be drawn for linescans require drawing lines and ROIs on different regions of the movies / cell and these are different in every recording, we do not feel that a Macro for the detailed analysis described in section 3 would be beneficial for users at this time.

\*Can ImageJ be used for section 4?

Image J does have the capability for pixel based PTCL analysis routines. However, the design and implementation of such routines is not conducted in our laboratory and all PTCL based analysis is conducted using the Volumetry software as described.

\*Please include a link or reference to the 'Wave Speed' ImageJ plugin (section 3.26).

As stated above in response to reviewer 1, upon searching for the appropriate plugin online to provide a link, it appears that this plugin is now longer available for public download and thus all reference to its implication has been removed from the manuscript.