*Circulation Research* Peer Review Report for DOI: 10.1161/CIRCRESAHA.120.317266

**Title: Beat-By-Beat Cardiomyocyte T-Tubule Deformation Drives Tubular Content Exchange**

In an effort to promote greater transparency in peer review, the authors and reviewers of this *Circulation Research* article have opted to post the original decision letter with reviewer comments to the authors and the authors' response to reviewers for each significant revision.

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May 30, 2020

Dr. Eva A Rog-Zielinska
University of Freiburg
Institute for Experimental Cardiovascular Medicine
Elsaessestrasse 2q
Freiburg 79102
Germany

RE: CIRCRES/2020/317266: Beat-by-beat changes in cardiomyocyte T-tubular nanostructure as a driver of T-tubular content exchange

Dear Dr. Rog-Zielinska:

Your manuscript has been carefully evaluated by three external reviewers and the editors as a Regular Article. Although of potential interest, the paper is not acceptable for publication in Circulation Research in its present form.

As you will gather from the reviews, the referees identified a number of substantive conceptual and methodological problems. The editors concur. Major issues include concern regarding the statistics and concerns about the interpretation.

Given the extensive new data that would be required for a responsive revision, we would understand if you were to decide to submit the paper elsewhere. Nevertheless, the editors see this manuscript as potentially important and would be willing to evaluate a revised version if you feel that you can effectively address the reviewers' concerns and are willing to perform the extensive new experiments required. The paper would be reviewed again, with no assurance of acceptance.

As detailed in the reviewers' critiques, a responsive revision would require a substantial amount of new data. In particular, the editors feel that additional data would be necessary to address the issue of how the 7 hearts were divided between the 3 experimental conditions. In a revised manuscript it would also be necessary to address the issue raised by reviewer 2; whether the amount of "observed" beat-to-beat fractional volume changes is incompatible with biophysical constraints. It would also be necessary to address issues regarding the change in cell volume with altering NCX. Finally, issues regarding the "eccentricity" index also need to be addressed

NEW REQUIREMENTS:

Upon revision, authors of manuscripts that contain cropped gels/blots will be required to submit a separate PDF file that contains the entire unedited gel for all representative cropped gels in the manuscript. Authors should label each gel as "Full unedited gel for Figure _" and highlight which lanes of the unedited gel correspond to those shown in the cropped images within the manuscript. For more information, please go to https://www.ahajournals.org/res/manuscript-preparation.

All research materials listed in the Methods should be included in the Major Resources Table file, which will be posted online as PDF with the article Supplemental Materials if the manuscript is accepted. A template Major Resources Table file (.docx) is available for download here: AHAJournals_MajorResourcesTable_2019.docx. Authors should reference the PDF in their Methods as follows: "Please see the Major Resources Table in the Supplemental Materials."

To read the comments to authors from the reviewers, please see below.

Please note that revised and resubmitted manuscripts are not assured of publication, and that fewer than 15% of all papers submitted to Circulation Research are eventually published.

Our current guidelines allow authors 90 days to complete the revision. If the manuscript is resubmitted within 90 days, one or more of the original reviewers will be re-consulted. If you need more than 90 days to submit a revised paper, please notify the editorial office.

PLEASE READ: During this unprecedented and challenging time, the health and safety of you, your family, and your community is of utmost importance. We appreciate that one aspect of this current situation is the inability to continue research work and that some measures will lead to a significant hindering of research progress. Please know that we are flexible regarding turnaround times for revisions and other tasks during this stressful time and deadlines will be extended as needed. If you are able to, please contact us if you need any extensions or if you experience any challenges around manuscript preparation; we will work with you.

If you choose to revise, please include a detailed response to each of the referees' and editors' comments, providing each comment verbatim in bold followed by your response and giving the exact page number(s), paragraph(s), and line number(s) where each revision was made. If you make substantive changes to the manuscript, please provide a clear description of what you did and where. If you insert important sentences, paragraphs, or sections in response to the comments, please also include them in your response. Please indicate clearly any deletions. Additionally, a marked up version of the revision with the changes highlighted or tracked should be uploaded as a supplemental file. Each page of the revised manuscript should be numbered in the top right corner, using your manuscript number followed by /R1 to denote a first revision.

Please ascertain that your resubmitted manuscript adheres to the Instructions to Authors as they appear online at https://www.ahajournals.org/res/author-instructions. Revisions that do not conform to the current limits on numbers of words (8000 total) and display items (maximum of 8 tables and/or figures) may be returned to the authors for abbreviation. If you cannot reduce the overall word count, the editors may deem an extended print version appropriate; the authors should provide written assurance that they will cover the costs of the pages that are in excess of these limits. Note that paying for excess display items is not an option. Please refer to the Instructions to Authors for further details regarding our policy on page limits, articles with extended print versions, and related costs. No such limits apply to the online supplementary information, which can include supporting data and/or expanded text to offset the limits on the print version. Such online supplementary information can be cited in the print version as appropriate.

As of January 1, 2020, all corresponding authors of articles accepted to AHA Journals are required to link an ORCID iD to their profile in the AHA Journal submission system. To avoid potential processing delays in future, we recommend that you link an ORCID iD to your profile when you submit your revision. To register with ORCID or link your profile, please go to "Modify Profile/Password" on the submission site homepage (insert journal homepage link), and click the link in the "ORCID" section.

We wish to thank you for having submitted this manuscript to Circulation Research.

Sincerely,

Jane E. Freedman, MD
Editor-in-Chief
Circulation Research
An American Heart Association Journal

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**REVIEWER COMMENTS TO AUTHORS:**

**Reviewer #1:**

In this manuscript, Rog-Zielinska et al provide evidence that the shape of the transverse tubular system changes during contraction with their cross section being most circular at intermediate sarcomere length and more elliptical at shorter and longer lengths. Correlated with this, diffusion of dextrans along the transverse tubule is accelerated at intermediate sarcomere lengths. Although interesting, the work does not provide direct evidence of consequences of alterations in t-tubular diffusion for cardiac function.

There are no page numbers in the manuscript. This makes it harder to refer to specific places.

Fig. 1. The reviewer is concerned about the method used to produce contracture which involves Na+ removal and addition of 10 mM caffeine. How do the authors exclude the possibility of changes of intracellular volume, rather than sarcomere length per se having effects? For example, it has been shown that altering NCX can affect cell volume (Takeuchi et al, J. gen. Physiol. 2006, 128, 495-507).

Fig 1. I am unclear about the statistical approach used. Apparently 7 hearts, 29 tissues samples , 125 cells and 539 TT were used. This raises the following questions. (1) How were the 7 hearts distributed between the three experimental conditions? (2) How did the authors deal with the issue of pseudoreplication given that, no matter how many tissue samples, cells or TT were studied, the data come from 2 or 3 hearts per condition? Specifically, how is statistical significance in Fig 1C estimated with such small numbers of hearts without problems of pseudoreplication?

Fig 1 (and elsewhere). Why is the "eccentricity" index used? I am concerned by the following. (1) "eccentricity" is a very non-linear function of the minor/major axis ratio. By my calculations, a decrease of eccentricity from 1.0 to 0.9 corresponds to a change of axis ratio from 0 to 0.43 whereas an identical sized change of ratio (from 0.7 to 0.6) corresponds to the axis ratio only increasing from 0.71 to 0.8. In other words the slope of the relationship between eccentricity and axis ratio changes fourfold. Why not plot and analyse the measured ratio? (2) In Fig 1D, it appears that there are some points with values of eccentricity around 1.0. This corresponds to a minor axis of around zero size. How are such measured?

Fig 1. The examples of Fig 1A show that in the contracture case the long axis of the TT is parallel to the z line. In contrast, in the stretch case the long axis is perpendicular to the z line. Is this a general finding?

Fig 2. There may also be a statistical issue here. It is unclear whether separate TT from a given cell have been treated as independent. This would be an error.

As acknowledged by the authors, the measurements of diffusion are based on 10 kDa dextrans. If we assume that diffusion is proportional to the square root of MW then the diffusion of a potassium ion will be considerably faster thereby questioning the importance of the effects.

Minor points
Page 3, third full para. You state, "Between 65% and 80% of the L-type current that triggers intracellular Ca2+ release is thought to flow across TT membranes. In contrast, the Na+ -Ca2+ exchanger (i.e. the main pathway for removal of 'trigger-Ca2+') appears to be more evenly distributed between TT and surface sarcolemma." As written, this is vague, the fact that 65-80% of the L-type current may flow across t-tubules does not mean that 65-80% of the channels are located on the tubules. It could be that the movement of Ca2+ per channel is different in the two locations. I would also suggest that the authors reread some of the papers they are citing to see how convinced they are of the statistical significances of all the reported differences.
It is odd that no mention is made of the well known changes of ion concentrations in the t-tubules in skeletal muscle (Almers, Fink & Palade, J. Physiol 1981, 312, 177-207; Barry and Adrian, J. memb. Biol. 1973 14,243-92).

Page3, line 3. There is still some question as to the role of t-tubules in atrial myocytes.

**Reviewer #2:**
In the MS "Beat-by-beat changes in cardiomyocyte T-tubular nanostructure as a driver of T-tubular content exchange" Dog-Zielinska et al present a very interesting set of experimental observations on dynamic changes of the t-system in cardiac myocytes.

The data presented constitutes the most convincing demonstration that this reviewer has seen to date that dynamic beat-to-beat deformation of the t-system could play a role in facilitating t-system luminal volume exchange.

The methodology is quite convincing and uses state of the art imaging approaches. I have got a few questions regarding the observed changes and their interpretation.

1) The observed fractional volume change by about ~20% (section on "Volume:Surface Ratio of TT at different SL") over a beat would have to enter and leave the t-tubule mouths in each beat. Alternatively, some of the water volume would have to cross the t-tubular membranes each beat. Is this biophysically reasonable?

To make the issue mentioned above more explicit, the argument above is similar to that used to explain the fast iso-volumetric contraction of ventricular myocytes on the basis that there is simply not enough time to move significant volume of water across the membrane on a beat-to-beat basis.

I.e. is it compatible with our biophysical understanding that there are ~20% total volume changes in the t-system every beat? This topic should be carefully discussed and would ideally be able to exclude the possibility that the amount of "observed" beat-to-beat fractional volume changes is incompatible with biophysical constraints.

2) In connection with the previous point, the careful analysis of the intensity signal from t-tubules during beating should reveal changes in the local t-system luminal volume, similar as described in reference 5 that the authors cite. Briefly, local t-system volume is generally smaller than the PSF and volume changes should be reflected in tubule intensity changes when the dextran signal is analysed over a beat. The reviewer appreciates the challenge arising from motion but the authors should make an attempt at analysing this. In fact, the summed intensity in a chosen region should be proportional to total t-system volume in that volume.

3) The degree of decrease in FRAP recovery time constant during dynamic length changes was statistically significantly different from the rest case. What is less clear is if the observed difference (~16%) would be expected to have actual physiological impact on phenomena such as accumulation/depletion etc. Such a quantitative consideration should be part of a careful discussion.

4) There is previous literature about the presence and effect of the glycocalyx in t-tubules (e.g. "Parfenov, A. S., Salnikov, V., Lederer, W. J. & Lukyánenko, V. Aqueous Diffusion Pathways as a Part of the Ventricular Cell Ultrastructure. Biophysical Journal 90, 1107-1119 (2006)" and references therein) that should be considered in terms of particle size of dextran and interpretation of the results in this study.

Minor: wouldn't the surface to volume ratio values given in the text have a unit associated with them? I could not see any.

**Reviewer #3:**
This is a nice well-focused study providing clear quantitative data about dynamic deformation of the T-tubules (TT) that appear to occur during each heartbeat and which may be functionally relevant in facilitating the mixing of TT content with the extracellular space. Importantly, this mixing may limit local ion depletions or accumulations that may occur in the TT. I have minor suggestions for improvement.

Introduction ¶4: It is a misstatement to imply that Na-Ca exchanger are evenly distributed between TT and surface sarcolemma. The most direct quantitative measurements on this point (ref 25) measured 3.6 times higher density of NCX current in TT vs surface (and similar 3-fold relative concentration for Na/K-ATPase pump current). There is likely to still be imperfect balance of fluxes into and out of TT, especially kinetically because Ca current is very rapid and Ca extrusion (and Na movement by Na/K-ATPase) are slower and influenced by the intracellular SR Ca release and transient amplitude.

Further to this point (and relevant later), Bers' group measured dynamic and transient extracellular [Ca] depletions during individual beats in rabbit ventricular tissue (PMID: 6829789, 3681259, 2705515) of 2-3%. Those measurements of interstitial space [Ca] were attributed to Ca influx via Ca current mainly in the TT. Since the TT is likely to be only 3-5% of interstitial space, that imply a 40-90% depletion of Ca in TT at each beat. That was also examined by diffusional modeling in TT and interstitial space (PMID: 1646660). Your FRAP studies suggest roughly a 3-fold acceleration of mixing due to contraction, such that the foregoing may be a good argument for the functional importance of your novel observations.

The data are clear and well presented for these transverse 250 nm long "pieces" of TT. Are these all near the surface, or was there a difference with respect to depth into the myocyte? Did you also analyze any longitudinal TT network components in these images? The longitudinal components would be expected to experience very different mechanical stresses...

The manuscript is well written, but the Introduction and Discussion could be a bit more concise (and still convey the main concepts).

Typo (¶5 of Discussion): "weans" should be "wanes"

**Manuscript # CIRCRES/2020/317266 "Beat-by-beat changes in cardiomyocyte T-tubular nanostructure as a driver of T-tubular content exchange"**

## Response to reviewers' comments

### Reviewer #1:

*In this manuscript, Rog-Zielinska et al provide evidence that the shape of the transverse tubular system changes during contraction with their cross section being most circular at intermediate sarcomere length and more elliptical at shorter and longer lengths. Correlated with this, diffusion of dextrans along the transverse tubule is accelerated at intermediate sarcomere lengths. Although interesting, the work does not provide direct evidence of consequences of alterations in t-tubular diffusion for cardiac function.*

**R1-1** We agree that our paper does not focus on consequences of the above mentioned static alterations in t-tubular (TT) diffusion on organ function.

The novelty of the paper is related to the fact (i) that TT surface-volume ration changes with sarcomere length (SL) on a beat-by-beat basis; (ii) that this affects TT diffusion during static deformation in the way the reviewer summarised above; and (iii) that – even though this should be expected to slow down TT diffusion (as cells spend most of their mechanical cycle at lengths different to 'intermediate') – we see a speed-up of TT diffusion in dynamically contracting cells.

The functional relevance of the paper is therefore the message that the mechanical activity of cardiac myocytes accelerates the exchange of TT lumen and 'bulk' extracellular space by adding an advective component to TT content exchange by diffusion. This is conceptually new, and will form the subject of follow-on research, including cardiac cells with healthy and pathologically remodelled TT. While this future research is outside the scope of the current communication, we have added a more in-depth discussion of the existing literature [lines: 105-114] and our results [lines: 312-319]. Please see also comment **R3-3** below.

*There are no page numbers in the manuscript. This makes it harder to refer to specific places.*

**R1-2** We apologise for this omission. We have added page and line numbers for easier reference.

*Fig. 1. The reviewer is concerned about the method used to produce contracture which involves Na+ removal and addition of 10 mM caffeine. How do the authors exclude the possibility of changes of intracellular volume, rather than sarcomere length per se having effects? For example, it has been shown that altering NCX can affect cell volume (Takeuchi et al, J. gen. Physiol. 2006, 128, 495-507).*

**R1-3** We thank the reviewer for raising this point. In the publication mentioned by the reviewer, the authors observed that NCX block results in cell swelling, but only in the presence of ouabain.[1] We did not use ouabain. Cell swelling following NCX block was

observed after ~40 min. In our experiments, the delay between induction of contracture by $Li^+$/caffeine and tissue fixation was 2-3 minutes [lines: 380-384] – a period during which no cell swelling has been reported in the literature. Prompted by the reviewer's concern, we conducted a careful re-examination of our data and found no signs of swelling in any samples, including contractured and stretched tissue. Below, we provide a quantification of mitochondrial volume (which generally serves as a sensitive indicator of onco/osmotic stress)[2] which confirms that no differences were seen between resting and contractured states (Fig. R1). This is now stated [lines: 271-273] and the figure is now included in the manuscript's supplement (Fig. S7).

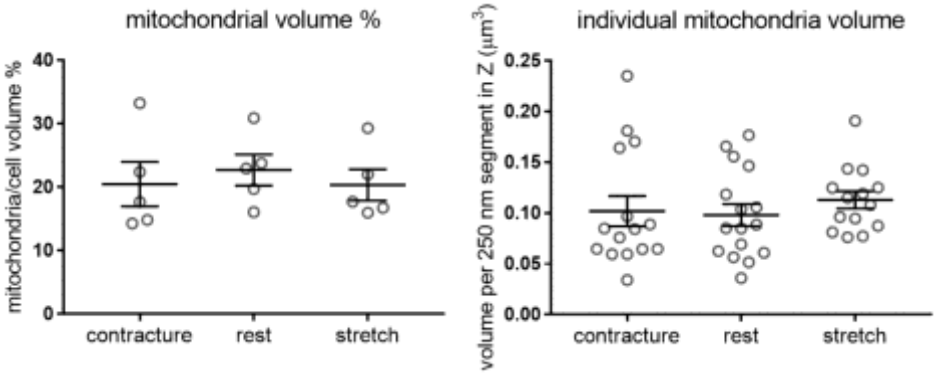


***Figure R1. Lack of changes in mitochondrial volume between contracture, rest, and stretch (tissue data).*** *Left: Partial mitochondrial volume per total cell volume in n=5 representative electron tomographic volumes per mechanical state (each stack containing on average 8 mitochondria). Right: absolute volume of individual mitochondrial segments contained within these stacks (250 nm depth). Stacks representative of N=2 or 3 hearts for contracture/stretch or rest, respectively. Statistical significance assessed using one-way AVONA; p=0.811 (left), p=0.659 (right).*

*Fig 1. I am unclear about the statistical approach used. Apparently 7 hearts, 29 tissues samples, 125 cells and 539 TT were used. This raises the following questions. (1) How were the 7 hearts distributed between the three experimental conditions? (2) How did the authors deal with the issue of pseudoreplication given that, no matter how many tissue samples, cells or TT were studied, the data come from 2 or 3 hearts per condition? Specifically, how is statistical significance in Fig 1C estimated with such small numbers of hearts without problems of pseudoreplication?*

**R1-4** We are grateful to the reviewer for pointing out the lack of clarity in our data presentation. We now provide a detailed distribution of all data (see Table R1; this is now included as Table S1 in the manuscript's supplement).

To address the issue of possible pseudoreplication we have consulted the University of Freiburg Institute of Medical Biometry and Statistics, and subsequently modified our analyses to use hierarchical statistics methods [lines: 484-495]. We fit linear mixed effects models with random effects associated with individual heart, tissue fragment, and cell, for the tissue data (Fig. 1, S1) and heart sample and cell for the cell data (Fig. 2, S2) and FRAP data (Figures 3,4). This new analytical approach reconfirmed previously reported results, and demonstrated that there were no significant effects of heart, sample, or cell, and that observed effects are attributable solely to changes in SL.

***Table R1. Data distribution across individual hearts.*** Stated are the number of tissue fragments, cells, and individual TT analysed, as well as the prescribed mechanical state: contracture, rest, and stretch.

| | Heart 1 | Heart 2 | Heart 3 | Heart 4 | Heart 5 | Heart 6 | Heart 7 |
|---|---|---|---|---|---|---|---|
| Mechanical state prescribed | Contracture | Contracture | Rest | Rest | Rest | Stretch | Stretch |
| Tissue fragments | 4 | 5 | 3 | 2 | 4 | 5 | 8 |
| Cells | 15 | 15 | 8 | 14 | 10 | 26 | 37 |
| TT | 51 | 73 | 53 | 62 | 28 | 84 | 188 |
| Summary | 9 tissue fragments<br>30 cells<br>124 TT | | 9 tissue fragments<br>32 cells<br>143 TT | | | 11 tissue fragments<br>63 cells<br>272 TT | |

In addition, we provide a breakdown of data points (here, for demonstration purposes, focussing on eccentricity data) in relation to sample source. Figure R2A demonstrates that even within single hearts, data points are distributed across a wide range of SL, illustrating the inherent SL heterogeneity present within the muscle – a phenomenon previously described in skeletal muscle.[3] This may be linked to the fact that any mechanical perturbation, applied to the whole heart, will not affect each cell and sarcomere equally, owing to the complex cardiac structure and non-linear organisation of cell layers and connective tissue. In our experiments, the globally prescribed mechanical states shifted the distribution of the 'point cloud' towards shorter or longer SL, while maintaining a range of individual SL, so not pseudo-replicating same numbers. This is why we believe that expressing our measured parameters for each TT in relation to the nearest SL is the most appropriate approach.

To further demonstrate this point, we present our data as average eccentricity/cell (colour-coded, Fig. R2B), and as representative point distributions within single tissue fragments and cells (Fig. R2C). This data is now included in the manuscript's supplement as Fig. S8.

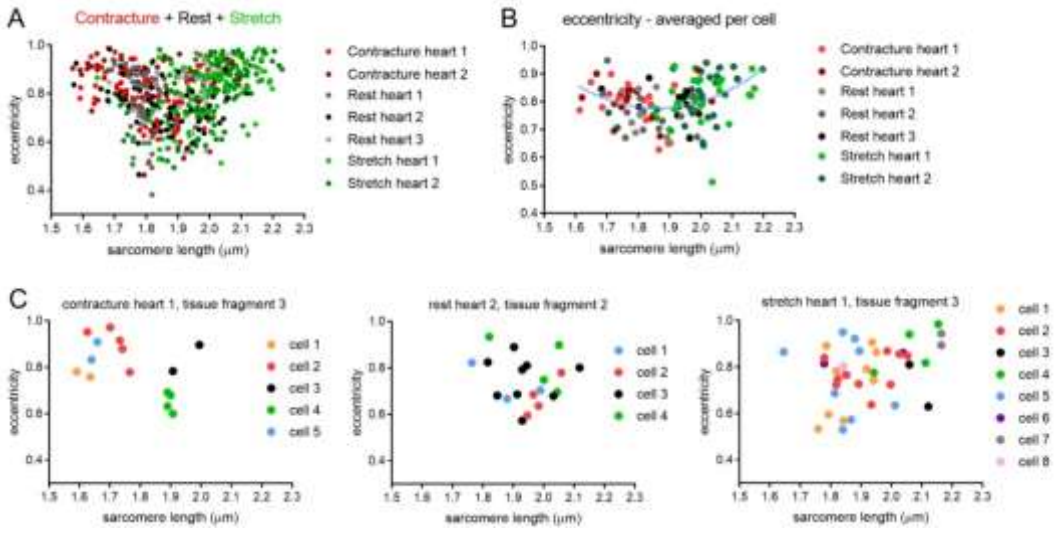


***Figure R2. Representative per-sample data distribution (here shown TT eccentricity in tissue) demonstrating the high degree of SL heterogeneity within individual samples, and the close relation of read-outs to SL length.*** *A: Distribution of individual data points across all 7 hearts studied (colour-coded, see also Table S1). B: Data averaged per cell, statistical analysis was performed by comparing a mixed effects model to a constant model; quadratic fit, p<0.0001. C: Representative distribution of data points in individual tissue fragments obtained from hearts preserved in contracture (left), rest (middle), and stretch (right). Note the presence of heterogeneity of SL even within individual cells (colour-coded).*

*Fig 1 (and elsewhere). Why is the "eccentricity" index used? I am concerned by the following. (1) "eccentricity" is a very non-linear function of the minor/major axis ratio. By my calculations, a decrease of eccentricity from 1.0 to 0.9 corresponds to a change of axis ratio from 0 to 0.43 whereas an identical sized change of ratio (from 0.7 to 0.6) corresponds to the axis ratio only increasing from 0.71 to 0.8. In other words the slope of the relationship between eccentricity and axis ratio changes fourfold. Why not plot and analyse the measured ratio?*

**R1-5** The minor:major radius ratio can indeed be used to describe elliptic structures – though, to be fair, it shows non-linear behaviour as well (see Fig. R3B and D).

As the surface-to-volume ratio of a deforming tubular structure, *i.e*. the biological phenomenon we wish to describe, is highly non-linear as well, so we see no inherent problem with non-linearity. That said, we now provide graphical representations of eccentricity *and* minor:major radius data (Fig. R3; Supplemental figures S1A,C and S2C,D; referenced in the manuscript text [lines: 155-156,171]); for interrelation between minor:major radius and eccentricity data, see Fig. R3E.

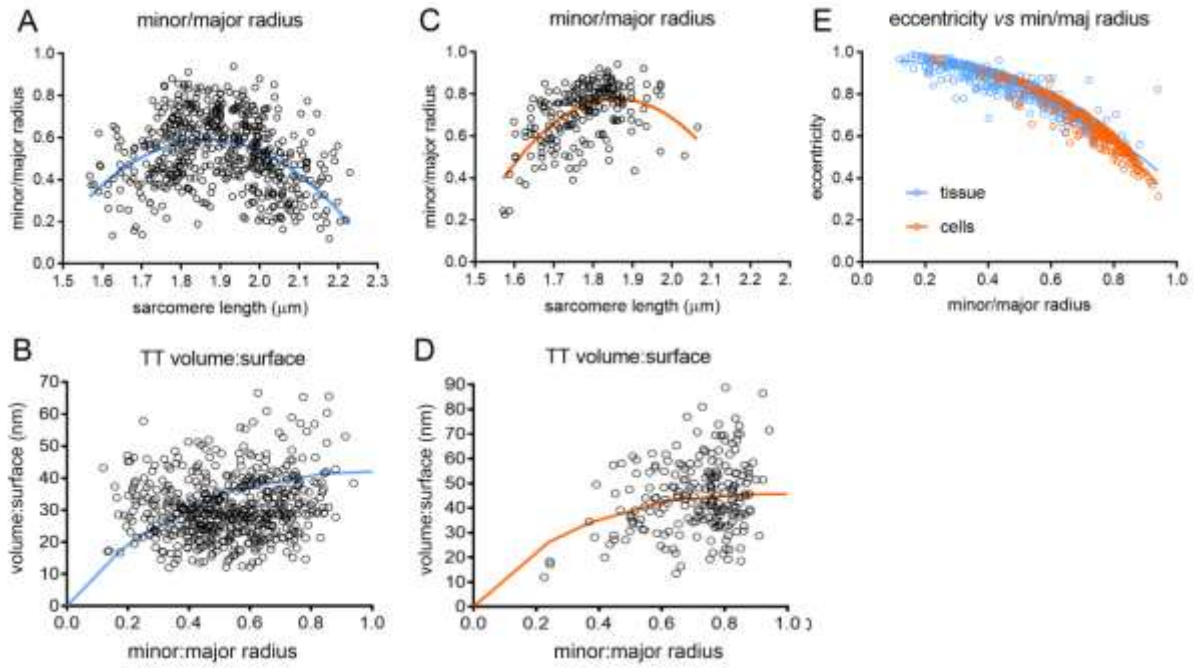


***Figure R3. Data presented based on assessment of minor:major radius of TT. A-D:*** *TT minor:major radius as a function of SL in tissue (A) and cells (C); TT volume:surface as a function of minor:major radius in tissue (B) and cells (D). Statistical significance was assessed comparing a mixed effects model to a constant model; p<0.0001 (A-C), p<0.001 (D).* ***E:*** *Relationship between eccentricity vs minor:major TT radius data in tissue (blue) and cells (orange).*

*(2) In Fig 1D, it appears that there are some points with values of eccentricity around 1.0. This corresponds to a minor axis of around zero size. How are such measured?*

We used IMOD software ('imodinfo -e' function) to quantify the eccentricity of fitted ellipses. The highest recorded value was 0.9872, which still contains a visible lumen (in this case, the minor and major axes lengths were 91.78 nm and 583.43 nm, respectively; top panel in Fig. R4B).

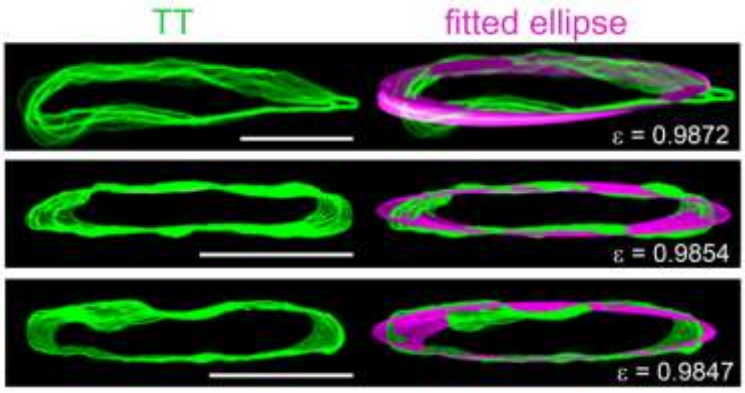


***Figure R4. High eccentricity TT examples**. Shown are 3D outlines reconstructed from electron tomography (green) and fitted ellipses (magenta). Corresponding eccentricity values are stated. Scale bar = 200 nm.*

*Fig 1. The examples of Fig 1A show that in the contracture case the long axis of the TT is parallel to the z line. In contrast, in the stretch case the long axis is perpendicular to the z line. Is this a general finding?*

**R1-6** Indeed: this is a key part of the message of our communication. Please see Fig. 1C and 2C (Rose plots), Fig. S1B and S2B (individual points), and text [lines: 157-160, 254-258].

*Fig 2. There may also be a statistical issue here. It is unclear whether separate TT from a given cell have been treated as independent. This would be an error.*

**R1-7** In response to your feedback, we have performed a more rigorous statistical assessment of our data, using a linear mixed effects model, and find that conclusions are not affected by measuring multiple TT from one cell / multiple cells from one heart (see **R1-4**, above). For the purpose of demonstration of data heterogeneity in single cell preparations, we include here the distribution of points across two separate cell isolations (Fig. R5A), the averaged data per cell (Fig. R5B), and for cells within one experimental condition (Fig. R5C).

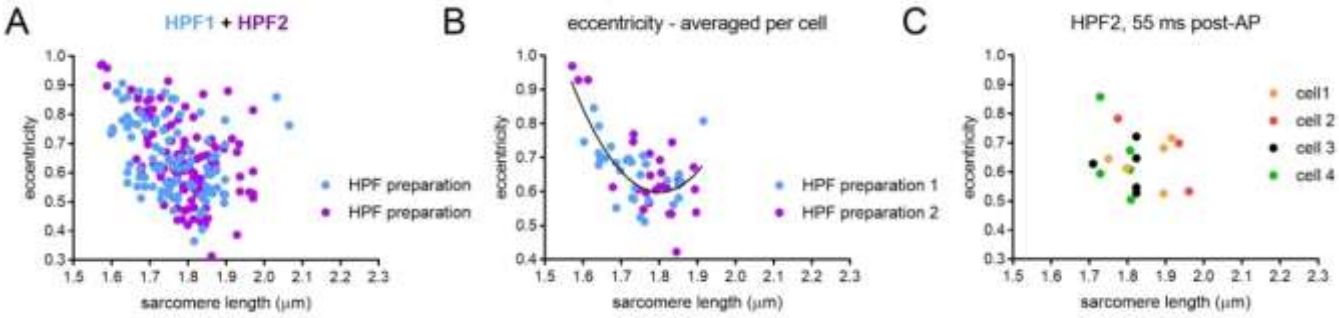


***Figure R5. Representative per-sample data distribution (here shown TT eccentricity in cells) demonstrating the high degree of SL heterogeneity within individual samples, and the close relation of read-outs to SL length. A:** Distribution of individual data points across 2 high-pressure frozen (HPF) preparations. **B:** Data averaged per cell, statistical analysis was performed by comparing a mixed effects model to a constant model; quadratic fit, p<0.0001. **C:** Representative data distribution within one experimental group. Note the presence of heterogeneity of SL even within individual cells (colour-coded).*

*As acknowledged by the authors, the measurements of diffusion are based on 10 kDa dextrans. If we assume that diffusion is proportional to the square root of MW then the diffusion of a potassium ion will be considerably faster thereby questioning the importance of the effects.*

**R1-8** Our reason for choosing dextran particles was to explore TT luminal content movement. This required a membrane-impermeable fluorescent reporter, and the 10 kDa offered the best compromise between 'small size' and 'assured homogeneity' in dextran diameter. Based on previous studies (in mouse), only particles < 11 nm are thought to be able to penetrate TT (see also **R2-5**).[4] The exact choice of dextran was guided by our preliminary studies and the supplier's advice. Anything larger than 10 kDa would be expected to behave a highly branched sugar, potentially introducing artefacts. The other alternative - 3 kDa (1.4 nm Stokes radius) - was not recommended as it exhibits a rather large heterogeneity of sizes, with particles often below 1.5 kDa, which have previously been shown to enter cells (e.g. *via* connexins). This could have compromised our analysis, which relied on selective 'labelling' of TT content. Last, but not least, the dextran particles we used bind to on average double the amount of dye molecules (1.5 for 10 kDa *vs* 0.7 for 3 kDa), providing a brighter signal needed to visualise near-/sub-diffraction spatial domains.

As the reviewer points out, this limitation of using 10 kDa dextrans is acknowledged in the discussion [lines: 332-335]; the approach allowed us to successfully monitor TT content exchange dynamics.

In future experiments we hope to pursue the question of what the effect of the here uncovered advection-assisted diffusion is on individual ion concentrations, focussing initially on calcium (for which an imbalance between in- and out-flow pathways in TT and surface membranes has been suggested). This would ideally involve the use of ion indicators, genetically targeted to the TT lumen, but this is outside the scope of the present report.

**Minor points**

*Page 3, third full para. You state, "Between 65% and 80% of the L-type current that triggers intracellular Ca2+ release is thought to flow across TT membranes. In contrast, the Na+ - Ca2+ exchanger (i.e. the main pathway for removal of 'trigger-Ca2+') appears to be more evenly distributed between TT and surface sarcolemma." As written, this is vague, the fact that 65-80% of the L-type current may flow across t-tubules does not mean that 65-80% of the channels are located on the tubules. It could be that the movement of Ca2+ per channel is different in the two locations. I would also suggest that the authors reread some of the papers they are citing to see how convinced they are of the statistical significances of all the reported differences.*

**R1-9** We thank the reviewer for highlighting this. We provide now a more detiled review of the literature; see Introduction [lines: 92-101] and also **R3-2**, below.

*It is odd that no mention is made of the well known changes of ion concentrations in the t-tubules in skeletal muscle (Almers, Fink & Palade, J. Physiol 1981, 312, 177-207; Barry and Adrian, J. memb. Biol. 1973 14,243-92).*

**R1-10** The suggested publications are now discussed in the manuscript [lines: 112-114].

*Page3, line 3. There is still some question as to the role of t-tubules in atrial myocytes.*

**R1-11** We agree. In recent years, a number of reports (incl. contributions by the authors) have highlighted the previously underestimated relevance of atrial transverse and axial

tubules for cell function, specifically calcium cycling, in health and disease.[5-7] We are not sure whether there any specific questions that the reviewer would like us to respond to, beyond the text in [lines: 325-328].

**Reviewer #2:**

*In the MS "Beat-by-beat changes in cardiomyocyte T-tubular nanostructure as a driver of T-tubular content exchange" Rog-Zielinska et al present a very interesting set of experimental observations on dynamic changes of the t-system in cardiac myocytes.*

*The data presented constitutes the most convincing demonstration that this reviewer has seen to date that dynamic beat-to-beat deformation of the t-system could play a role in facilitating t-system luminal volume exchange.*

**R2-1** We thank the reviewer for this kind assessment of our work.

*The methodology is quite convincing and uses state of the art imaging approaches. I have got a few questions regarding the observed changes and their interpretation.*

*1) The observed fractional volume change by about ~20% (section on "Volume:Surface Ratio of TT at different SL") over a beat would have to enter and leave the t-tubule mouths in each beat. Alternatively, some of the water volume would have to cross the t-tubular membranes each beat. Is this biophysically reasonable?*

*To make the issue mentioned above more explicit, the argument above is similar to that used to explain the fast iso-volumetric contraction of ventricular myocytes on the basis that there is simply not enough time to move significant volume of water across the membrane on a beat-to-beat basis.*

*I.e. is it compatible with our biophysical understanding that there are ~20% total volume changes in the t-system every beat? This topic should be carefully discussed and would ideally be able to exclude the possibility that the amount of "observed" beat-to-beat fractional volume changes is incompatible with biophysical constraints.*

**R2-2** We are grateful to the reviewer for raising this point. We have removed the linear fit (from which the 20% value was derived), as it did not take into account *a-priori* knowledge about TT shape, and show only the model fit that takes into account the realistic geometry of TT (Fig. 1E, 2E). Based on this non-linear fit, the surface-volume mismatch would in fact appear to be 36%. This TT volume change is considerable, especially when taking into account the time constraint of a single beat, and the fact that such change would have to occur twice per beat. But – that is the data.

Where does this volume go? We see three possibilities: (i) it might enter the cell; (ii) it might re-distribute in TT and/or 'modulate' TT surface area, or (iii) it could be exchanged with bulk extracellular space. We hope to act in the spirit of the Reviewer's suggestion in proposing a thought experiment, regarding (i), trans-sarcolemmal water flux of such amplitude.

If 36% of TT volume entered the cell during each of the peak TT deformations (*i.e*. during end-diastole and end-systole), this would transiently dilute a near-TT volume of cytosol that one might geometrically describe as a 'hollow cylinder', whose inner radius *r* is equivalent to TT radius. If, for argument sake, one assumed that the 'thickness' of that hollow cylinder is

equal to TT radius, then its volume would be 3x larger than that of TT.[1] If one further assumes that 'free water content' in cytosol is a maximum of 8% of hollow cylinder volume *V*,[8] and if this is 'diluted' by 36% of TT volume *v*, then the new water concentration in V would be (0.08 x 3 *v* + 0.36 *v*) / 3*v* = 0.2. In other words, water content in the proximity of TT would increase by a factor of 2.5, twice per heartbeat. Consequently, local 'free ion concentrations' would drop by the same factor, both in end-diastole and in end-systole. If that was the case – one would expect that we should long have discovered these fluctuations. Alas, there are no indications in the published literature that indicate that such swings to occur.

In addition, a volume equivalent to the 36% TT volume (that would be supposed to enter the cell as a consequence of TT surface-volume-mismatch) would have to leave the cell into TT, twice on every cycle, as well. While one, perhaps, might be able to construe a TT hydrostatic pressure driving force for trans-sarcolemmal water flux from TT into the cell, it would seem more difficult to imply a driving force for water back into the 'relaxing' TT.

So, overall, the notion of a 36% TT volume flux *via* the sarcolemma seems implausible. That doesn't exclude the possibility of small levels of trans-sarcolemmal water flux (even though we see no indication of osmotic changes even in sustained contracture; see Fig. R1 [Fig. S7]); this is a question for further research.

Re possibility (ii), we present evidence for cyclic stretch- and contraction-induced caveolar membrane integration in TT. This would alleviate the 36% TT volume mismatch by about a third, according to our data. Whether there is additional re-distribution within the TT, perhaps involving axial segments, is a questions for further research.

Re possibility (iii), we present direct evidence for advective exchange between TT lumen and bulk extracellular space, as the apparent speed of TT luminal diffusion is increased in contracting cells.

So, from all we can see in published data and our own findings, there is little to suggest that there is cyclic trans-sarcolemmal water exchange. If it did exist, it would be relatively small, as otherwise we would expect to see intracellular ion concentration changes. Instead, we observe caveolar membrane integration and contraction effects on TT diffusion, which combined offer the more straightforward explanation for how the cell copes with TT deformation.

*2) In connection with the previous point, the careful analysis of the intensity signal from t-tubules during beating should reveal changes in the local t-system luminal volume, similar as described in reference 5 that the authors cite. Briefly, local t-system volume is generally smaller than the PSF and volume changes should be reflected in tubule intensity changes when the dextran signal is analysed over a beat. The reviewer appreciates the challenge arising from motion but the authors should make an attempt at analysing this. In fact, the*

[1] The 'hollow cylinder volume' $V = \pi \times h \times (R^2 - r^2)$,
where $R$ = outer radius of hollow cylinder and $r$ = inner radius of hollow cylinder
If $R - r = r$, then the volume ratio of hollow cylinder $V$ to that of its cylindrical core $v$ with a radius of $r$ is
$V/v = \pi \times h \times ([2r]^2 - r^2) / \pi \times h \times (r^2) = \mathbf{3}$

*summed intensity in a chosen region should be proportional to total t-system volume in that volume.*

**R2-3** We thank the reviewer for this suggestion. In the past, we did indeed use light microscopy in an attempt to probe volume and surface of TT in living cells, but this was done in static cells, and it required geometric assumptions regarding TT shape – which were provided by electron microscopy.[9]

Our data here has been obtained by imaging in confocal mode, and in case of contracting cells, FRAP traces are analysed during diastole only (to exclude the possibility of artefacts arising from TT movement relative to the focal plane). We have evaluated our data and conclude that for the proposed assessment, we would need to combine fast acquisition of z-stacks (to account for TT movement in the z-direction) with observation of a second label for TT membranes (to be able to obtain a ratio of volume and surface information), and apply custom motion tracking post-acquisition to obtain reliable information. This is not possible with our current techniques.

Instead, we used electron tomography (ET) to directly quantify true TT geometry, using AP-synchronised HPF to address the temporal delay previously encountered in other studies. This allowed us to go well beyond the diffraction limit of the visible light and to provide 'ground-truth' level information. We believe that based on this principal confirmation of the presence of advection-assisted TT diffusion, it now makes sense to develop techniques in a targeted manner that would allow one to implement the approach, suggested by the reviewer, in freely beating cells. We are up to that challenge, but it is outside the scope of the present communication.

*3) The degree of decrease in FRAP recovery time constant during dynamic length changes was statistically significantly different from the rest case. What is less clear is if the observed difference (~16%) would be expected to have actual physiological impact on phenomena such as accumulation/depletion etc. Such a quantitative consideration should be part of a careful discussion.*

**R2-4** We agree that the biological significance of observed changes had not been adequately discussed by us. We now include a more in-depth discussion of the potential relevance of our findings [lines: 312-317], and propose several aspects that can be pursued in future work (*e.g.* beating rate and contraction amplitude-dependence of diffusion speed [lines: 317-319], possible mechanical modulation of caveolar signalling hubs [lines: 282-284]). Please see also **R3-3**.

*4) There is previous literature about the presence and effect of the glycocalyx in t-tubules (e.g. "Parfenov, A. S., Salnikov, V., Lederer, W. J. & Lukyánenko, V. Aqueous Diffusion Pathways as a Part of the Ventricular Cell Ultrastructure. Biophysical Journal 90, 1107-1119 (2006)" and references therein) that should be considered in terms of particle size of dextran and interpretation of the results in this study.*

**R2-5** We thank the reviewer for pointing out this important paper. Inspired by this, we include more comprehensive considerations regarding the possible impact of glycocalyx on particle penetration and diffusion, and we have added reference to this work, as well as to work

showing that cardiac plasma membrane exhibits low-affinity calcium binding properties [lines: 294-298].[10]

We particularly want to thank the reviewer for drawing our attention to the study by Parfenov et al. The authors used particles of different sizes to probe the diffusion within the TT network. Aside from noting that glycocalyx is capable of slowing down the diffusion of particles, as well as of trapping particles within TT, the authors found that the presence of particles within TT was increased in contracting cells. The authors attributed this to the trapping of particles by the glycocalyx. We think these findings are compatible with the here substantiated advective TT content exchange during mechanical activity, leading to improved penetration of particles inside TT (now mentioned in the discussion [lines: 330-331]).

*Minor: wouldn't the surface to volume ratio values given in the text have a unit associated with them? I could not see any.*

**R2-6** Indeed – many thanks (nm; now stated)!

**Reviewer #3:**

*This is a nice well-focused study providing clear quantitative data about dynamic deformation of the T-tubules (TT) that appear to occur during each heartbeat and which may be functionally relevant in facilitating the mixing of TT content with the extracellular space. Importantly, this mixing may limit local ion depletions or accumulations that may occur in the TT. I have minor suggestions for improvement.*

**R3-1** We thank the reviewer for this kind summary.

*Introduction ¶4: It is a misstatement to imply that Na-Ca exchanger are evenly distributed between TT and surface sarcolemma. The most direct quantitative measurements on this point (ref 25) measured 3.6 times higher density of NCX current in TT vs surface (and similar 3-fold relative concentration for Na/K-ATPase pump current). There is likely to still be imperfect balance of fluxes into and out of TT, especially kinetically because Ca current is very rapid and Ca extrusion (and Na movement by Na/K-ATPase) are slower and influenced by the intracellular SR Ca release and transient amplitude.*

**R3-2** We thank the reviewer for pointing this out; we now offer what we hope to be a more thorough summary of existing literature [lines: 92-101].

*Further to this point (and relevant later), Bers' group measured dynamic and transient extracellular [Ca] depletions during individual beats in rabbit ventricular tissue (PMID: 6829789, 3681259, 2705515) of 2-3%. Those measurements of interstitial space [Ca] were attributed to Ca influx via Ca current mainly in the TT. Since the TT is likely to be only 3-5% of interstitial space, that imply a 40-90% depletion of Ca in TT at each beat. That was also examined by diffusional modeling in TT and interstitial space (PMID: 1646660). Your FRAP studies suggest roughly a 3-fold acceleration of mixing due to contraction, such that the foregoing may be a good argument for the functional importance of your novel observations.*

***R3-3*** *We are most grateful to the reviewer for this insightful comment; an expanded discussion of the literature [lines: 105-114] and our results [lines: 312-319] is now included in the manuscript.*

*The data are clear and well presented for these transverse 250 nm long "pieces" of TT. Are these all near the surface, or was there a difference with respect to depth into the myocyte? Did you also analyze any longitudinal TT network components in these images? The longitudinal components would be expected to experience very different mechanical stresses...*

**R3-4** The minimum distance between the surface sarcolemma and TT included in the analysis was 400 nm. In cases where the lateral sarcolemma was included within the imaging volume, TT were separated from the surface by a myofibril, *i.e*. usually by more than 1 μm (Fig. R6).

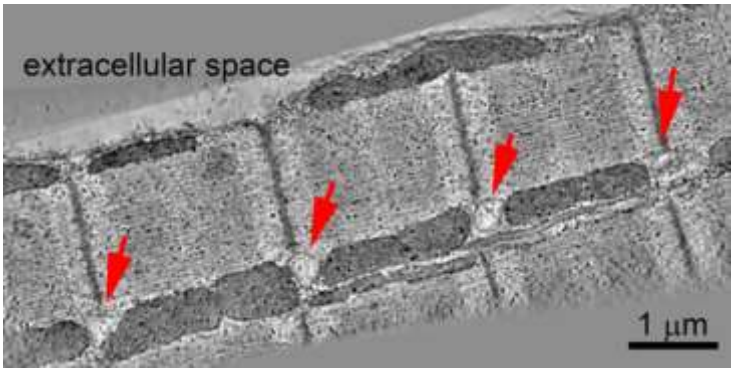


***Figure R6. Representative distribution of TT included in the analysis.*** *TT (red arrows) in relation to the lateral surface sarcolemma (if included in the imaging volume – most often this was not the case, as TT were imaged even more 'centrally'). All TT included in the analysis were imaged at least 400 nm 'below the surface plasma membrane'.*

We agree with the reviewer that in the future studies, focus should be placed on the possible heterogeneity of TT deformation upon stretch and contracture in relation to depth inside the cell. This applies equally to longitudinal elements of the TT. We suspect that cell-depth related phenomena may also be species-dependent, given the differences in TT mouth configuration that can affect TT luminal access.[11]

Our knowledge of the electro-mechanical relevance of axial elements is still limited. A handful of reports indicate they are active contributors to excitation-contraction coupling,[5,6,12] and their relative presence increases during pathological remodelling (as reviewed in[13]). In future studies we intend to explore the relevance of axial elements – whether mechanical (providing 'spare' membrane / accommodating extra volume during stretch?) or electrical (role in $Ca^{2+}$ fluxes, aiding $Ca^{2+}$ diffusion between neighbouring TT?).

*The manuscript is well written, but the Introduction and Discussion could be a bit more concise (and still convey the main concepts).*

**R3-5** We have revised the text with clear and concise communication in mind.

*Typo (¶5 of Discussion): "weans" should be "wanes"*

**R3-6** This has now been corrected – many thanks.

……..…

## References


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September 28, 2020

Dr. Eva A Rog-Zielinska
University of Freiburg
Institute for Experimental Cardiovascular Medicine
Elsaessestrasse 2q
Freiburg 79102
Germany

RE: CIRCRES/2020/317266R1: Beat-by-beat changes in cardiomyocyte T-tubular nanostructure as a driver of T-tubular content exchange

Dear Dr. Rog-Zielinska:

Your manuscript has been carefully evaluated by 5 external reviewers and the editors as a Regular Article. We regret to inform you that the paper is not acceptable for publication in its present form.

As you will gather from the reviews, the referees identified a number of statistical issues that need to be addressed. The editors concur.

Despite these concerns, the editors see this paper as potentially important and wish to encourage revision. If you would like to revise the manuscript in accordance with the suggestions of the reviewers and editors, we would be willing to evaluate a new version. The manuscript would be reviewed again, with no assurance of acceptance.

**********
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**********
Among the concerns cited by the reviewers, the editors feel that the most important issues that need to be addressed are the statistical and technical issues and addressing the remaining concerns of reviewer 3.

NEW REQUIREMENTS:
Upon revision, authors of manuscripts that contain cropped gels/blots will be required to submit a separate PDF file that contains the entire unedited gel for all representative cropped gels in the manuscript. Authors should label each gel as "Full unedited gel for Figure _" and highlight which lanes of the unedited gel correspond to those shown in the cropped images within the manuscript. For more information, please go to https://www.ahajournals.org/res/manuscript-preparation.

All research materials listed in the Methods should be included in the Major Resources Table file, which will be posted online as PDF with the article Supplemental Materials if the manuscript is accepted. A template Major Resources Table file (.docx) is available for download here: AHAJournals_MajorResourcesTable_2019.docx. Authors are required to upload the Table at the revision stage. Authors should reference the PDF in their Methods as follows: "Please see the Major Resources Table in the Supplemental Materials."

To read the comments to authors from the reviewers, please see below.

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Our current guidelines allow authors 90 days to complete the revision. If the manuscript is resubmitted within 90 days, one or more of the original reviewers will be re-consulted; the editors may also choose to obtain additional opinions from new reviewers. If you need more than 90 days to submit a revised paper, please notify the editorial office. In general, extensions over the revision time limit will not be granted except under special circumstances at the editors' discretion.

If you choose to revise, please include a detailed response to each of the referees' and editors' comments, providing each comment verbatim in bold followed by your response and giving the exact page number(s), paragraph(s), and line number(s) where each revision was made. If you make substantive changes to the manuscript, please provide a clear description of what you did and where. If you insert important sentences, paragraphs, or sections in response to the comments, please also include them in your response. Please indicate clearly any deletions. Additionally, a marked up version of the revision with the changes highlighted or tracked should be uploaded as a supplemental file. Number each page in the top right corner, using your manuscript number followed by /R2 to denote a second revision.

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As of January 1, 2020, all corresponding authors of articles accepted to AHA Journals are required to link an ORCID iD to their profile in the AHA Journal submission system. To avoid potential processing delays in future, we recommend that you link an ORCID iD to your profile when you submit your revision. To register with ORCID or link your profile, please go to "Modify Profile/Password" on the submission site homepage, and click the link in the "ORCID" section.

We wish to thank you for having submitted this manuscript to Circulation Research.

Sincerely,

Jane E. Freedman, MD
Editor-in-Chief
Circulation Research
An American Heart Association Journal

**********

**Reviewer comments to the Authors:**

**Reviewer #1:**
No further comments.

**Reviewer #2:**
The authors have responded largely constructively to issues raised and I am satisfied with the responses.

**Reviewer #3:**
The authors have been responsive to my comments, and the manuscript is improved. However, there are minor comments that I still have.
1. While the authors acknowledged my point (based on functional measurements) about the nearly 4-fold higher density of NCX in T-tubules,25 that point in the Introduction is left as "more evenly distributed" in the text. Since the majority of Na influx occurs vis NCX and >80% of Ca influx occurs in the T-tubules, depletions of Ca and even Na ions could occur in the T-tubules. The Ca depletion is more likely to have functional consequences. It was also surprising that the likely primary functional benefit of the advective T-tubule flux described (i.e. to stabilize luminal [Ca] against depletion) was only poorly articulated in the Discussion.

2. The longitudinal tubules should also shorten with SL shortening, but might increase their cross-sectional area, except that there would tend to be lateral compressive forces as well. While it is OK to put off a detailed analysis to a later study, since these elements should also be visible in their images, it might have been of interest to include comment about that -at least in the EM images.

**Statistical Reviewer:**
Please provide precise p-values with two significant digits (rather than P<0.0x). Scientific notation is strongly encouraged. These can be provided with other additional statistical details (eg normalization procedures, tests establishing normality, sample sizes, named statistical tests, named post hoc correction, raw/corrected pvalues) in a supplemental table if that is more convenient.

How were representative images/figures chosen? Please note the approach used to select representative images in the main text or figure legends.

Please give estimates of fit.

Please evaluate the use of statistical language throughout the paper "lack of effects" or "lack of changes" should likely be "lack of significant effects", "lack of significant changes", similarly, be mindful if claims of "no difference" should be revised to note "no statistically significant difference"

Figure 2 B is listed twice in the legend.

What steps were taken to ensure that curve fitting was not driven by outliers? Eg, Fig 2E appears to be strongly defined by three data points. Also, the authors don't specify the statistical test they used for model fitness, so it's difficult to interpret the meaning of these p-values.

In S4A it's not clear what each group was compared to to calculate each p-value.

Some tests (eg t-tests, ANOVA) used assume normality and independence of samples (when multiple measures

are made from a smaller number of cells/tissues/hearts, these assumptions are violated), however it is not clear how normality was established. Note that common tests of normality are not powered to detect departures from normality when n is small (eg n<6) and in these cases normality should be support by external information (eg from larger samples sizes in the literature) or non-parametric tests should be used.

Please show exact data values (eg fig S6)

The authors should also provide the exact equations they used for the mixed effect models

**Technical Reviewer:**
Comments to Authors on Rigor Checklists:
The current study was evaluated for inclusion of guideline items present in the Circulation Research checklists for rigor, transparency, and reproducibility. The reviewer has identified a number items that would constitute moderate deviations from Journal standards. Below is a detailed list of reviewer queries that will need to be addressed by the authors in revision:

In vitro checklist:
1) Authors must complete and submit an "In Vitro Checklist."
2) Although not mandatory, Circulation Research encourages authors present data in scatter/dot plots as opposed to bar graphs. Please communicate to the editor/reviewers why this was not consistently performed throughout (i.e., Figure S6).

In vivo checklist:
1) Authors must complete and submit a "Long In Vivo Checklist."
2) Where animals are described, please provide additional baseline characteristics, including age, chow, bedding, and source (from what laboratory or vender animals were procured).
3) Checklists indicate that randomization and allocation concealment were performed; however, this was not evidenced in the text.
4) In the manuscript, please provide a brief description of blinding procedures used.
5) In the manuscript's text, please indicate whether any animals were excluded from analyses, and if so, based on what criteria said exclusions were made. If no animals (or data points) were excluded, explicitly state this in the text.
6) Provide a brief description of a priori power calculations used in the determination of group sizes. If power calculations were not performed, authors should specify how group sizes were determined in their absence.
7) Only female animals were used in experiments. In the manuscript, please provide written justification for not considering sex as a biological variable in the study.
8) Statements regarding author conflicts of interests must be provided in the manuscript.

Other:
1) Per the Journal's recommendations, authors will need to submit a "Major Resources Table."

Comments to the Authors on Research Guidelines and Reporting:
No deficiencies.

**Manuscript # CIRCRES/2020/317266/R2**

**"Beat-by-beat changes in cardiomyocyte T-tubular nanostructure as a driver of T-tubular content exchange"**

## Response to reviewers' comments

**<u>Reviewers #1 and #2</u> were satisfied with the previous set of responses.**

**<u>Reviewer #3:</u>**

**The authors have been responsive to my comments, and the manuscript is improved.**

We thank the reviewer for the kind assessment.

**However, there are minor comments that I still have.**

**1. While the authors acknowledged my point (based on functional measurements) about the nearly 4-fold higher density of NCX in T-tubules,25 that point in the Introduction is left as "more evenly distributed" in the text. Since the majority of Na influx occurs vis NCX and >80% of Ca influx occurs in the T-tubules, depletions of Ca and even Na ions could occur in the T-tubules. The Ca depletion is more likely to have functional consequences. It was also surprising that the likely primary functional benefit of the advective T-tubule flux described (i.e. to stabilize luminal [Ca] against depletion) was only poorly articulated in the Discussion.**

Taking this very helpful advice into consideration, we have amended the text accordingly, incl. lines: 93-100, 229-230, and 305-306.

**2. The longitudinal tubules should also shorten with SL shortening, but might increase their cross-sectional area, except that there would tend to be lateral compressive forces as well. While it is OK to put off a detailed analysis to a later study, since these elements should also be visible in their images, it might have been of interest to include comment about that -at least in the EM images.**

Many thanks for this comment. Indeed, we expect the mechanical activity to have effects on longitudinal elements (axial tubules: AT) that may differ from those on TT. The nature of AT changes is hard to predict, as 'AT space' shortens with sarcomere length, while the interfibrillar gap decreases during contracture.[1]

Unfortunately, given (i) the relative sparsity of AT (compared to TT) in healthy rabbit myocytes,[2] (ii) the fact that our ET data sets contain finite subcellular volumes (at the resolution required, each tilt-series measured just ~2,500 nm × 2,500 nm × 250 nm), (iii) the need to focus ET data gathering on target-structures (here TT), and (iv) the high variability of individual tubular structures that calls for an analysis of large numbers (for this paper, a total of 753 TT has been reconstructed), our ET data do not at this point contain sufficient

information to allow a robust scientific analysis of the impact of the cell mechanical state on AT structure.

We re-evaluated our tissue data and – where possible – measured the minor:major radius of the cross-section of AT fragments. This preliminary analysis is in keeping with the notion that AT also deform during the cell mechanical cycle (Fig. R2-1). However, it would be premature to deduce any systematic change from the available n = 22 data points. To do this, one would have to change the study design, and model individual AT along at least a half-sarcomere each. Such data is not currently available to us.

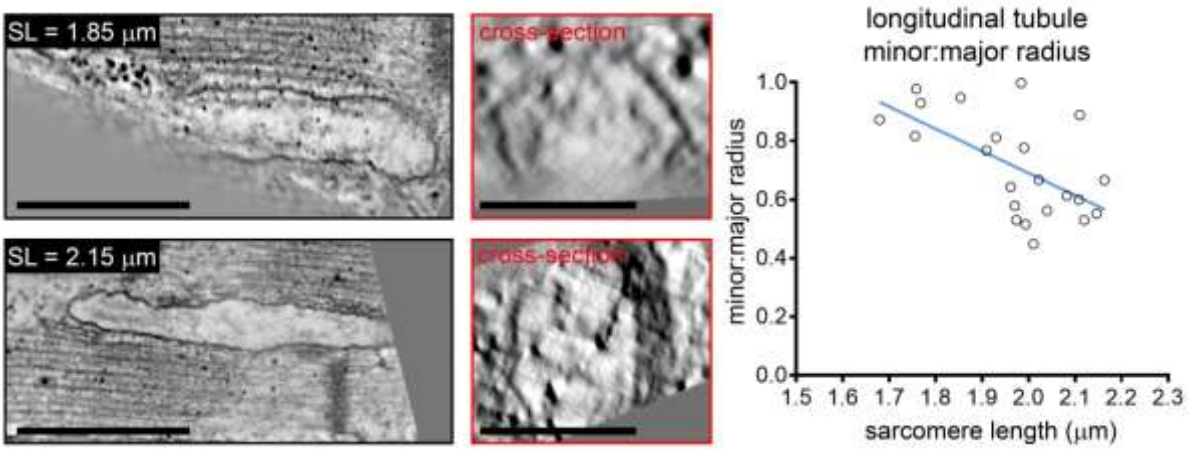


**Figure R2-1. Examples of AT shapes in cardiomyocytes with different sarcomere length.** AT morphology was examined in chemically fixed tissue, preserved at different SL (left panel – electron tomography slices, middle panel – virtual slices demonstrating cross-sectional shape). Right panel – minor:major radius of the AT cross-section as a function of SL; n = 22; blue line shows linear fit. Scale bar = 500 nm (left) and 200 nm (right).

**Statistical Reviewer:**

**Please provide precise p-values with two significant digits (rather than P<0.0x). Scientific notation is strongly encouraged. These can be provided with other additional statistical details (eg normalization procedures, tests establishing normality, sample sizes, named statistical tests, named post hoc correction, raw/corrected pvalues) in a supplemental table if that is more convenient.**

Exact p-values are now provided in Supplemental Table 3, [lines: 838-840].

**How were representative images/figures chosen? Please note the approach used to select representative images in the main text or figure legends.**

The representative images were chosen based on the conclusions of the quantitative analysis, to convey statistically confirmed results. This statement is now included in the text [lines: 506-508]. An exception is Figure 3A which contains images of dextran-labelled cell fragments – chosen to demonstrate the size of the analysis area.

**Please give estimates of fit.**

These are now provided in Supplemental Table 3, [lines: 838-840].

**Please evaluate the use of statistical language throughout the paper "lack of effects" or "lack of changes" should likely be "lack of significant effects", "lack of significant changes", similarly, be mindful if claims of "no difference" should be revised to note "no statistically significant difference"**

We agree that lack of positive confirmation of a difference is not the same as positive confirmation of the lack of a difference. We have re-checked the text according to the above suggestions and indeed identified two previously ambiguous statements in the Figure legends [lines: 744 and 802]. Many thanks for pointing this out.

**Figure 2 B is listed twice in the legend.**

Many thanks – this has now been fixed.

**What steps were taken to ensure that curve fitting was not driven by outliers? Eg, Fig 2E appears to be strongly defined by three data points.**

We have tested the datasets for the presence of outliers (using both two-sided Grubbs' test and robust regression and outlier removal [ROUT] test). No outliers were identified. Of note, the fit presented in Fig. 2E (as well as 1E) is a shape-based geometric approach, with the high-SL part of the curve mostly determined by the assumption that at ε=1 the volume:surface ratio is 0. This is described in the main test [lines: 182-185], we now also provide an additional statement in the relevant figure legends [lines: 701 and 719].

**Also, the authors don't specify the statistical test they used for model fitness, so it's difficult to interpret the meaning of these p-values.**

The coef test within Matlab was used. This is a test which returns the p-value for an F-test with the hypothesis that all fixed-effects coefficients, except for the intercept, are 0. This statement is now included in the Supplemental Table 3 [lines: 838-840].

**In S4A it's not clear what each group was compared to to calculate each p-value.**

This has now been clarified in the figure legend [lines: 788-789] and in Supplemental Table 3, [lines: 838-840].

**Some tests (eg t-tests, ANOVA) used assume normality and independence of samples (when multiple measures are made from a smaller number of cells/tissues/hearts, these assumptions are violated), however it is not clear how normality was established. Note that common tests of normality are not powered to detect**

**departures from normality when n is small (eg n<6) and in these cases normality should be support by external information (eg from larger samples sizes in the literature) or non-parametric tests should be used.**

Data presented in Fig. 4B, S4A, S6, and S7 have now been assessed for normality using D'Agostino-Pearson normality test. Due to small sample numbers in some of the groups included in Fig. S4A, S6, and S7 the data were re-analysed using non-parametric Kruskal-Wallis test. This did not change the conclusions of the study. Figure legends have been updated accordingly [lines: 788-789, 798, 807-808].

**Please show exact data values (eg fig S6)**

Data is now presented as individual points.

**The authors should also provide the exact equations they used for the mixed effect models.**

This has now been included in Supplemental Table 3, [lines: 838-840].

**Technical Reviewer:**

**1) Authors must complete and submit an "In Vitro Checklist."**

Done.

**2) Although not mandatory, Circulation Research encourages authors present data in scatter/dot plots as opposed to bar graphs. Please communicate to the editor/reviewers why this was not consistently performed throughout (i.e., Figure S6).**

This has now been made uniform throughout the manuscript (specifically Fig. S6).

**3) Authors must complete and submit a "Long In Vivo Checklist."**

Done.

**4) Where animals are described, please provide additional baseline characteristics, including age, chow, bedding, and source (from what laboratory or vender animals were procured).**

We now include the requested information in the main manuscript file [lines: 373-375, 394-395, 449-450].

**5) Checklists indicate that randomization and allocation concealment were performed; however, this was not evidenced in the text.**

This information has now been included in the main file [lines: 505-506].

**6) In the manuscript, please provide a brief description of blinding procedures used.**

All imaging files were assigned a name designed to not reflect the sample type/treatment (date and number for functional studies, grid box coordinates for structural observations). Analysis was performed after all numerical data was collated. A brief statement is now included [line: 506].

**7) In the manuscript's text, please indicate whether any animals were excluded from analyses, and if so, based on what criteria said exclusions were made. If no animals (or data points) were excluded, explicitly state this in the text.**

Now stated [lines: 369-370].

8**) Provide a brief description of a priori power calculations used in the determination of group sizes. If power calculations were not performed, authors should specify how group sizes were determined in their absence.**

*A priori* sample size was determined based on an exploratory pilot study using tissue fragments, and under the guidance of the most variable parameter – volume:surface ratio. Based on the high variability of the dependent predictor within the tissue data set, we decided to design an experiment with power of 0.95, and significance level of $\alpha = 0.01$. The optimal sample size was determined to be 521 (ultimately we performed 539 observations, this includes the pilot dataset). For our cell based studies, we employed a different strategy, whereas we decided to focus on 2 rather than 3 main mechanical states – additionally, observed variability was expected to be lower. We predicted a sample size of 141 (ultimately, 214 observations were included). Finally, functional experiments were designed with power of 0.9, and based on own previous and literature data of parameter variability,[3,4] and mathematical predictions based on structural data (applies to 'static' data, predicted sample size 75, performed = 89). "Dynamic" functional data sample size was calculated based on literature and own experimental data ('static", see Fig. S4B, predicted 27, performed 25). A short statement is now included in the main file [line: 504].

**9) Only female animals were used in experiments. In the manuscript, please provide written justification for not considering sex as a biological variable in the study.**

Our structural studies were conducted using female rabbits, whereas our functional studies have been performed using a mixed population. The structural read-out is a purely biophysical phenomenon, and we have no reason to expect sex-based differences in outcome. In contrast, our functional analyses were designed to investigate biological consequences of the structural observation – and as such were performed on a mixed population for post-hoc assessment of possible sex-specific differences; we did not detect any effects of sex.

**10) Statements regarding author conflicts of interests must be provided in the manuscript.**

Done [lines: 529-530].

**11) Per the Journal's recommendations, authors will need to submit a "Major Resources Table."**

Done.

## References


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October 25, 2020

Dr. Eva A Rog-Zielinska
University of Freiburg
Institute for Experimental Cardiovascular Medicine
Elsaessestrasse 2q
Freiburg 79102
Germany

RE: CIRCRES/2020/317266R2: Beat-by-beat changes in cardiomyocyte T-tubular nanostructure as a driver of T-tubular content exchange

Dear Dr. Rog-Zielinska:

Your revised manuscript has been carefully evaluated by a technical reviewer, a statistical reviewer and the editors as a Regular Article. While we are interested in your paper, further minor revision is required before we can accept the manuscript for publication in Circulation Research. Specifically, there are several formatting issues that need to be addressed. Please submit your revision at your earliest convenience.

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