A microfluidic platform to investigate the role of mechanical constraints on tissue reorganization

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MS TITLE: A microfluidic platform to investigate the role of mechanical constraints on tissue reorganization

AUTHORS: Sham Leilah Tlili, Francois Graner, and Hélène Delanoë-Ayari

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this study, Tlili and coauthors describe a new microfluidic platform to study how cell rearrangements take place in response to mechanical constraints. The platform allows large

deformations of aggregates over time scales of tens of minutes as they flow through a constriction in the device. This can be coupled to two-photon imaging to observe changes in cell morphologies – potentially linking cell scale changes to aggregate mechanical response. The time-scales and deformations are relevant to a number of developmental processes. Analysis of the deformations considering the aggregate as a viscoelastic liquid allows to separate the contributions of rearrangements and cell deformation. Overall, this is a paper with a lot of potential and I can see that the device might be useful but it is written for a Physics audience rather than a developmental biology audience and at times it stays quite superficial. As a consequence, it needs some major rewriting.

Comments for the author

Major issues:
-the paper is currently written for a Physics audience and there is very little introduction of the analysis methods used. I am a biophysicist and am somewhat familiar with the methods used by the authors but the description of the analysis methods in 2.2, 3.1 and 3.2 was so brief that I needed to go and read a few of the references to understand what they were doing. For example, in 3.2, the framework developed to look at flow of monolayers past obstacles is essential to understand the analysis. This needs to be explained clearly and a few diagrams would probably be helpful. The authors need to describe their methods in a more didactic way to reach a broad audience of developmental biologists and biophysicists. Otherwise, their work will not be read or cited as broadly as it deserves to.
- The authors state in 3.2 that they vary tau_relax and obtain a reasonable estimate of the deformations for tau_relax=20 min. If I look at the red curve on Fig 3c and compare to the medium grey one, the resemblance is not striking. What are the authors trying to match? Is it the order of magnitude of deformation? Or the temporal evolution? The temporal evolution is not very convincing because it overestimates the peaks in the deformation and overestimates the relaxation. This suggests that the rheology is not very well captured by the model. This may mean that there are phenomena not taken into account by the authors that are occurring in the tissue. An alternate reason might be that the authors include the effect of rearrangements as a dashpot – so this relaxation takes place over the whole aggregate whereas they have shown that rearrangements only happen in certain locations under certain conditions.
-In Fig 3c, why are there peaks in strain rate? Is it because the aggregate moves in stick-slip way? Are the peaks in strain rate linked to a sudden rearrangement of cell junctions? A more careful analysis may be very interesting.
-The analysis described in 3.2 identifies the region where the most rearrangements should take place. Do the authors observe rearrangements only in these regions? Are there enough observed rearrangements compared to predicted rearrangements? This would help justify the rheological assumptions and convince the readers of the power of the analysis. It would be useful to show on a real image where the rearrangements are predicted and show where rearrangements are observed.
-In fig 7, the authors consider linear rheology for the tissue but many publications highlight the presence of power law rheology in living tissues. The authors should discuss why, in their experimental conditions, it is OK to neglect power law rheology. How will this method allow to link cell rearrangements to aggregate rheology? Would it be better to use an FE approach with an element formulation that can undergo rearrangement under some specific conditions? This may allow to circumvent some of the issues I highlighted earlier.
-The observation of cell rearrangements in an aggregate in response to mechanical constraints is a strong point of the paper but, at times, it feels underutilised. For example, the authors state that they often see asymmetry in V+ and V-. Can they be a bit more quantitative? For example, they could compute a ratio or plot one vs the other? The authors highlight events with high V+ for epsilon_cell>0.3. Could they show a few of these? Is there any specific location where these occur? Is there loss of tissue integrity? Imaging cells expressing a GFP-tagged E-cadherin would be particularly interesting in this context.
-The authors show that the lifetime of vertices can be very long and hypothesise that this may be due to the turnover time of junctions. Could the authors block E-cadherin turnover with dynasore and determine if this impedes rearrangements?
-The authors mention that there are rapid and slow passages through the constriction. I presume Fig 3 is a slow passage. Could the authors show a rapid one too? During the relaxation afer a slow passage, a deformation field is created in the centre of the aggregate perpendicular to the

direction of passage. Does this surface tension induced strain field dissipate eventually and give rise to rearrangements at longer time-scales than tau_relax?
-The discussion is not very focused and I'm not sure what the main message is. The authors should briefly summarise the results and what the method has enabled and then focus on one or two interesting questions that it can be applied to.

Minor issues:
-The introduction needs to highlight the issues with the current experimental techniques for looking at aggregates that are solved by the current method. The main one, in my view, is that rearrangements are important in developmental biology but these are rarely observed in experiments on aggregates. So it is difficult to study them quantitatively and understand their biophysics.
-The constriction is 100 um high, signifying there are several cell layers throughout the aggregate. Do the authors observe different behaviours in the bottom or top layers compared to the middle layers?
-Fig 2a is difficult to visualise. I'm not sure what I am seeing even if I zoom in. Maybe the authors should reduce the density of arrows or find another presentation.
-Fig 3c is a bit messy. Can you put the names as inset or on the side. 3a needs a scale bar on the image varying from 1 (isotropic) to 3.
-Fig 4c: the red and green circles are not described anywhere and are not useful in my view.
-Fig 6: a green curve is mentioned in the legend for e. This seems to be in black in fact.

Reviewer 2

Advance summary and potential significance to field

In this manuscript, Tlili et al. describe a microfluidic platform that positions cell aggregates in a pseudo-2D configuration, and then passes them through a microfluidic channel while performing live imaging. Subsequent tracking of the deformations enables them to infer and model the pseudo-viscoelastic behavior of cell aggregates.

The paper is well written, and provides high quality figures that enable to visualize the kinetics of deformations at the cellular level during various situations of aggregate flow. It is interesting and seems sound. The experimental setup is well designed, and the quality of the resulting data quite impressive.

The aggregates measured are from a mouse embryonic carcinoma cell line, so the mechanical models developed probably have some relevance to inform the modelling of cell flow during embryogenesis. Nevertheless, given the stated scope of the journal, I have to note that the paper does not directly address any embryo related question any further.

Comments for the author

I have a few small suggestions/remarks (the most important ones concern code availability and Fig. 7):
"Biphoton microscopy" is very rarely used, "two-photon microscopy" has more than a thousand times more results on google scholars. To facilitate finding the paper in online searches, and to avoid any confusion, I'd recommend using the widely used appelation.
"these mesoscopic properties arise from the microscopic interplay between cells viscoelasticity and cell-cell interactions such as adhesion"
-> In the context of mechanics "mesoscopic" usually refers to the minimal volume over which there is enough averaging to define local quantities such as pressure/strain/stress. As such, if the mesoscopic level here is cell aggregates (and this seems to be correct according to the data that follows), viscoelasticity wouldn't be defined at the single cell/miscroscopic level. The definitions around this might benefit from being clarified a bit further.
"properties relevant for getting a better understanding of in vivo issues"
-> Is it a typo for "in vivo tissues"?
"Imaging in 3D cell rearrangements and cell shapes is still challenging. To avoid technical difficulties of 3D analysis, we design a quasi-2D flow experiment (Fig. 1) where the tissue is

physically constrained in the vertical dimension corresponding to the optical axis which forces it to flow in the plane of observation. We pre-confine the aggregate and use a capillary with straight sides (rather than a cylindrical pipette), thus creating a bidimensional flow (no dependency in z) that does not require 3D tissue reconstruction."
-> For the problem to really be 2D, one also has to assume that the aggregate is homogeneous. If there are core/shell differences, which is very common and a core reason for 3D cell cultures, or other more complex structures as found during embryo/embryoid body development, the 3rd dimension cannot be ignored.
This limits a lot the scope of application of the method to gastruloids (despite of what authors suggest) and embryos. The article would gain from stating this limitation and possibly suggesting how it might be overcome in future studies.
"For the introduction of the aggregate we use a 200 µm cone"
-> The picture seems to show a 200 µl (rather than µm) pipette tip, could there be a typo?
"We measure the two-dimensional velocity field $\vec{v}$(x, y, t) using a custom-made Matlab optic flow code based on the KanadeLucas-Tomasi (KLT) algorithm (24) with a level 2 pyramid."
-> I do not see a statement of code availability or link to github or similar.
To ensure the the method is reusable, it's very important that the code is released, with a minimal reproducible analysis on a test-dataset, together with the paper.
Fig. 7 legend "Schematic rheological diagram proposed for the while aggregate"
-> Typo for whole aggregate?
"How cell membrane trafficking, adhesion proteins and molecular motors dynamics affect rearrangements dynamics and are relocalized during junction remodeling in 3D tissues is still not well understood. Combining our method with cell lines endowed with fluorescent reporters of such proteins would enable to quantify their dynamics using fluorescence recovery after photobleaching technics for example."
-> Even more exciting opportunities that the authors don't mention might arise from blocking the action of some proteins, rather than just imaging them. Using KO lines or chemical inhibition of various pathways and effector proteins before measuring detailed mechanical parameters could lead to a lot of insights into the relative contributions of various components of the cellular machinery to tissue mechanics.
"For these kind of applications, it could be useful to develop parallel and/or sequential aspiration was done for single cells experiments."
-> Some words must be missing from the conclusion sentence for it to make sense.
Figure 7: since all the measurements are done on a single physical system, one would expect a unique model to fit all the experimental data. Is the model on the bottom right such a unifying model? Could we get curve fits of the various experiments based on a unique final model in this figure? In addition, I find it much nicer when symbol representation of models are depicted next to the data-fitting for which they were used, and the parameters are visually highlighted on the curve fits when applicable. I wonder if it would be possible to reorganize a bit the figures and text one way or another to put the theoretical models next to each experiment with corresponding curve-fits? And with the correspondance between parameters in the model and experimental measurements better highlighted? All parameters except G_ag are given in the legend, it would be helpful to also put the value of G_ag here for completeness - and these values might also be better presented in a table within the figure than scattered in the legend. The legend refers to a, b, c, d, subpanels which are not present within the figure. Last, in addition to the diagram, I wonder if it might also be useful to add the final stress vs strain model in equation-form on the side since this is the key final contribution from this paper that might be reused in future embryology studies.
Finally, no error intervals are given for the physical quantities reported. The authors should provide standard deviations or standard error of the mean across experiments at least for the most important parameters, i.e. those currently in the legend of Fig. 7.

First revision

Author response to reviewers' comments

Answer to Referee 1

- *In this study, Tlili and coauthors describe a new microfluidic platform to study how cell rear- rangements take place in response to mechanical constraints. The platform allows large deformations of aggregates over time scales of tens of minutes as they flow through a constriction in the device. This can be coupled to two-photon imaging to observe changes in cell morphologies - potentially linking cell scale changes to aggregate mechanical response. The time-scales and deformations are relevant to a number of developmental processes. Analysis of the deformations considering the aggregate as a viscoelastic liquid allows to separate the contributions of rearrangements and cell deformation. Overall, this is a paper with a lot of potential and I can see that the device might be useful but it is written for a Physics audience rather than a developmental biology audience and at times it stays quite superficial. As a consequence, it needs some major rewriting.*

We thank the Referee for this positive opinion and for very constructive comments. We have revised and rewritten the manuscript according to the Referees' suggestions.

- *The paper is currently written for a Physics audience and there is very little introduction of the analysis methods used. I am a biophysicist and am somewhat familiar with the methods used by the authors but the description of the analysis methods in 2.2, 3.1 and 3.2 was so brief that I needed to go and read a few of the references to understand what they were doing. For example, in 3.2, the framework developed to look at flow of monolayers past obstacles is essential to understand the analysis. This needs to be explained clearly and a few diagrams would probably be helpful. The authors need to describe their methods in a more didactic way to reach a broad audience of developmental biologists and biophysicists. Otherwise, their work will not be read or cited as broadly as it deserves to.*

We have added several explanations and Figs. 3, S2, S3. We hope this is now more suitable for biologists. If further clarifications are required, we will be happy to expand them in the Supplementary Material.

- *The authors state in 3.2 that they vary τ_{relax} and obtain a reasonable estimate of the deformations for τ_{relax}=20 min. If I look at the red curve on Fig 3c and compare to the medium grey one, the resemblance is not striking. What are the authors trying to match? Is it the order of magnitude of deformation? Or the temporal evolution? The temporal evolution is not very convincing because it overestimates the peaks in the deformation and overestimates the relaxation. This suggests that the rheology is not very well captured by the model. This may mean that there are phenomena not taken into account by the authors that are occurring in the tissue. An alternate reason might be that the authors include the effect of rearrangements as a dashpot - so this relaxation takes place over the whole aggregate whereas they have shown that rearrangements only happen in certain locations under certain conditions.*

In the revised version, we have improved the legibility of the figure (former Fig. 3c, current Fig. 4c), clarified the text, and indicated the limitations of the method. The text now writes :

In Fig. 4c, we compare the simulated variation with time of the deformation $\langle \varepsilon_{cell} \rangle_{box}$ with the experimental measurement $\langle \varepsilon_{cell} \rangle_{box}$ (red curve). We vary τ_r between 10 minutes (light grey) and infinity (black), the latter being a purely elastic limit. The simulated deformation *amplitude* agrees reasonably with the experiment when we use $\tau_r \approx 20$ min. The Fourier determination of deformation is sufficiently discriminant and reproducible to determine correctly the amplitude order of magnitude and we can reasonably exclude values of τ_r outside of the interval [15 min, 25 min] (Fig. S4).

• *In Fig 3c, why are there peaks in strain rate? Is it because the aggregate moves in stick-slip way? Are the peaks in strain rate linked to a sudden rearrangement of cell junctions? A more careful analysis may be very interesting.*

In the revised version, we address this question of the peaks. The text now writes :

Note that the determination of τ_r in Fig. 4c is not sensitive to the *shape* of the deformation evolution. The peaks in deformation are not systematically observed and their origin is unknown; they are likely due to a stick-slip friction on the glass coverslip (rather than to cascades of rearrangements, which we do not detect). Determining accurately the shape of the deformation evolution is beyond the scope of the present paper. It would require to improve simultaneously the time resolution and signal-to-noise ratio beyond the possibilities of the current Fourier method with our current image quality. This would be possible in principle using cell contour segmentation complemented with detailed tensorial analysis.

• *The analysis described in 3.2 identifies the region where the most rearrangements should take place. Do the authors observe rearrangements only in these regions? Are there enough observed rearrangements compared to predicted rearrangements? This would help justify the rheological assumptions and convince the readers of the power of the analysis. It would be useful to show on a real image where the rearrange- ments are predicted and show where rearrangements are observed.*

We have added a map of rearrangement positions (Fig. S5a), integrated along the duration of the whole movie, plotted as bars to indicate the direction. The number of rearrangements is sufficient to check qualitatively that there are more rearrangements in the region where the velocity gradient is highest (Fig. S5b), namely near the constriction entrance.

Our point here is to determine the timescale of cell shape relaxation. As cell divisions are rare on these timescale, we indirectly conclude that cell deformation relaxation is due to cell rearrangements (technically speaking, we follow each cell group using a Lagrangian approach). We do not actually predict the position or time of rearrangements. We agree with the Referee that the comparison between prediction and experiment would be an important validation, but it is out of the scope of the present work.

• *In fig 7, the authors consider linear rheology for the tissue but many publications highlight the presence of power law rheology in living tissues. The authors should discuss why, in their experimental conditions, it is OK to neglect power law rheology. How will this method allow to link cell rearrangements to aggregate rheology? Would it be better to use an FE approach with an element formulation that can undergo rearrangement under some specific conditions? This may allow to circumvent some of the issues I highlighted earlier.*

We now mention power-law rheologies, as follows:

For the sake of simplicity, since we focus here only on a characteristic time, we consider here only linear rheologies; future works might investigate more realistic power-law rheologies.

• *The observation of cell rearrangements in an aggregate in response to mechanical constraints is a strong point of the paper but, at times, it feels underutilised. For example, the authors state that they often see asymmetry in V_+ and V_-. Can they be a bit more quantitative? For example, they could compute a ratio or plot one vs the other?*

In the revised version we now plot these data, in a new Fig. 5c.

• *The authors highlight events with high V_+ for $\varepsilon_{cell} > 0.3$. Could they show a few of these? Is there any specific location where these occur? Is there loss of tissue integrity?*

In Supp. Movie 5, we have color-coded rearrangements, with rapid relaxation rearrangements ($V^+ > 1$ μm/min) represented in red while slow ones ($V^+ < 1$ μm/min) are in black.

In addition, we have expanded the text, as follows:

Examples of such highly deformed rearranging cells are visible in Figs. 4d and 5a; they occur mainly near the constriction entrance. They are usually compatible with the tissue confluence and integrity. High aspiration pressures and velocity can result in aggregate fractures (Supp. Movie 6): we do not analyse these experiments.

- *Imaging cells expressing a GFP-tagged E-cadherin would be particularly interesting in this context.*

We agree that it would be very interesting to be able to follow the cadherin expression level. We plan to do it in the future and we now mention it in the "Perspectives" section. But we do not have yet GFP cadherin F9 cell lines and this is beyond the scope of the present paper.

- *The authors show that the lifetime of vertices can be very long and hypothesise that this may be due to the turnover time of junctions. Could the authors block E-cadherin turnover with dynasore and determine if this impedes rearrangements?*

We did not block explicitly E-cadherin turnover, and we agree that it would be an interesting exper- iment to perform in the future. In the same spirit, we used a *a*-catenin null cell line to modify cell-cell junctions. The text now writes:

Or, cell-cell stress propagation can be modified for instance via an *a*-catenin null cell line. Since *a*-catenin links the cytoskeleton with cadherins, cell-cell junctions are modified. Aggregates form with cadherin-cadherin junctions, but cells have more irregular shapes and shorter range correlation in both shape and velocity, while rearrangements are dramatically impeded (Supp. Movie 13).

- *The authors mention that there are rapid and slow passages through the constriction. I presume Fig 3 is a slow passage. Could the authors show a rapid one too? During the relaxation after a slow passage, a deformation field is created in the centre of the aggregate perpendicular to the direction of passage. Does this surface tension induced strain field dissipate eventually and give rise to rearrangements at longer time-scales than τ_{relax}?*

The reviewer is right, former Fig. 3 (current Fig. 4) is a slow passage, as it is possible to measure the rearrangements occurring at the entrance of the canal. Supp. Movie 7 (further analyzed in Fig. 6) represents the case of a fast aspirated aggregate, where rearrangements did not have time to occur.

After the slow passage and the elasto-capillary deformation, cells should indeed relax the elastic deformation (as it starts to relax in Fig. 7, at the end of Supp. Movie 8), but we did not measure relaxation over longer timescales here. Moreover, we would also have to take into account divisions which would also contribute to this last relaxation. We now explain this better in the text, as follows:

After the fast cell shape relaxation, the aggregate does not entirely come back yet to its initial round shape. The aggregate then rounds up due to capillarity, while cells re-deform, within a few minutes (Supp. Movie 10, 11). Myosin is essential in this shape relaxation process since when blebbistatin is added, the relaxation is partial: the first fast relaxation is conserved, but not the second slow one (Fig. S7, Supp. Movie 12).

In turn, this new elastic deformation can eventually fully relax. This occurs at low shear rate, and long timescales, where cell divisions may also play a role.

- *The discussion is not very focused and I'm not sure what the main message is. The authors should briefly summarise the results and what the method has enabled and then*

focus on one or two interesting questions that it can be applied to.

We have strongly restructured the end of the article, briefly summarising the results, then emphasizing (and distinguishing) perspectives for biophysics and for developmental biology.

- *The introduction needs to highlight the issues with the current experimental techniques for looking at aggregates that are solved by the current method. The main one, in my view, is that rearrangements are important in developmental biology but these are rarely observed in experiments on aggregates. So it is difficult to study them quantitatively and understand their biophysics.*

In the revised version we have modified the introduction:

Cell-cell rearrangements are of high importance in developmental biology and are rarely observed in 3D experiments. There is a need to design a system where they could be properly quantified so as to understand which biophysical principles govern their dynamics.

The constriction is 100 um high, signifying there are several cell layers throughout the aggregate. Do the authors observe different behaviours in the bottom or top layers compared to the middle layers?

In the revised version we now mention this issue, as follows:

In principle, the flow could have a 3D structure, namely depend on the direction z perpendicular to the device plane. This would be the case for instance for an aggregate with a core-shell structure, or any heterogeneous tissue. In that case our set-up would be suitable to perform a 3D image analysis, provided the flow was slow enough. In the following, we present experiments performed on homogeneous 3D aggregates of cells which do not have core-shell structures (Fig. S1, Supp. Movie 2), and we check here that we can neglect the flow variation along z (Supp. Movie 3).

- *Fig 2a is difficult to visualise. I'm not sure what I am seeing even if I zoom in. Maybe the authors should reduce the density of arrows or find another presentation.*

We have improved its legibility.

- *Fig 3c is a bit messy. Can you put the names as inset or on the side. 3a needs a scale bar on the image varying from 1 (isotropic) to 3.*

We have improved its legibility and implemented the Referee's suggestions.

- *Fig 4c: the red and green circles are not described anywhere and are not useful in my view.*

We have suppressed them and clarified the figure.

- *Fig 6: a green curve is mentioned in the legend for e. This seems to be in black in fact.*

Corrected.

Answer to Referee 2

- *In this manuscript, Tlili et al. describe a microfluidic platform that positions cell aggregates in a pseudo-2D configuration, and then passes them through a microfluidic channel while performing live imaging. Subsequent tracking of the deformations enables them to infer and model the pseudo-viscoelastic behavior of cell aggregates.*
The paper is well written, and provides high quality figures that enable to visualize the

kinetics of de- formations at the cellular level during various situations of aggregate flow. It is interesting and seems sound. The experimental setup is well designed, and the quality of the resulting data quite impressive.

We thank the Referee for this positive opinion and for very constructive comments. We have revised and rewritten the manuscript according to the Referees' suggestions.

- *The aggregates measured are from a mouse embryonic carcinoma cell line, so the mechanical models developed probably have some relevance to inform the modelling of cell flow during embryogenesis. Nevertheless, given the stated scope of the journal, I have to note that the paper does not directly address any embryo related question any further.*

We indeed only present here results on F9 cell lines. But one of us (S. Tlili) has now started using this technique on mouse embryonic organoids (gastruloids). One member of H. Delano¨e-Ayari's lab is also using this technique to study development in Hydra. And we believe that this technique has a high potential for such study in the field. In the revised version we now write:

This setup will be very useful for understanding the mechanisms at stake in the response of 3D tissues to mechanical stresses and in particular to get new insights in the biophysics of cell-cell rearrangements which plays a central role in development. We envision that it could be used in many different systems such as Hydra (from which aggregates could easily be formed), mouse embryos, organoids, 3D bulk tissues of Xenopus, and could yield new information on key developmental processes.

- *"Biphoton microscopy" is very rarely used, "two-photon microscopy" has more than a thousand times more results on google scholars. To facilitate finding the paper in online searches, and to avoid any confusion, I'd recommend using the widely used appelation.*

In the initial version we had already used "two-photon" at most occurrences. In the revised version we only use "two-photon".

- *"these mesoscopic properties arise from the microscopic interplay between cells viscoelasticity and cell-cell interactions such as adhesion"*
In the context of mechanics "mesoscopic" usually refers to the minimal volume over which there is enough averaging to define local quantities such as pressure/strain/stress. As such, if the mesoscopic level here is cell aggregates (and this seems to be correct according to the data that follows), viscoelasticity wouldn't be defined at the single cell/miscroscopic level. The definitions around this might benefit from being clarified a bit further.

We have replaced everywhere the confusing words "microscopic", "mesoscopic" and "macroscopic" with "cell scale", "cell group scale" and "aggregate scale", respectively, and added Khalilgharibi 2016 in the bibliography (currently Ref. 2).

- *"properties relevant for getting a better understanding of in vivo issues". Is it a typo for "in vivo tissues"?*

Yes, corrected.

- *"Imaging in 3D cell rearrangements and cell shapes is still challenging. To avoid technical difficulties of 3D analysis, we design a quasi-2D flow experiment (Fig. 1) where the tissue is physically constrained in the vertical dimension corresponding to the optical axis which forces it to flow in the plane of observation. We pre- confine the aggregate and use a capillary with straight sides (rather than a cylindrical pipette), thus creating a bidimensional flow (no dependency in z) that does not require 3D tissue reconstruction." For the problem to really be 2D, one also has to assume that the aggregate is homogeneous. If there are core/shell differences, which is very common and a core reason for 3D cell cultures, or other more complex structures as found during embryo/embryoid body development, the 3rd*

dimension cannot be ignored. This limits a lot the scope of application of the method to gastruloids (despite of what authors suggest) and embryos. The article would gain from stating this limitation and possibly suggesting how it might be overcome in future studies.

In the case of F9 cell aggregate, there is no apparent shell pattern. We provide an equatorial section of an unperturbed F9 aggregate, where cell shape seems clearly homogeneous (Fig. S1). We agree with the Referee that in principle a severe radial gradient in physical properties could be observed. In this case, it would be very interesting and normally feasible to study the actual 3D flow structure in a quasi-static aspiration, as cell rearrangements are anyway slow. We now mention this point in the main text, as follows:

In principle, the flow could have a 3D structure, namely depend on the direction z perpendicular to the device plane. This would be the case for instance for an aggregate with a core-shell structure, or any heterogeneous tissue. In that case our set-up would be suitable to perform a 3D image analysis, provided the flow was slow enough. In the following, we present experiments performed on homogeneous 3D aggregates of cells which do not have core-shell structures (Fig. S1, Supp. Movie 2), and we check here that we can neglect the flow variation along z (Supp. Movie 3).

- *"For the introduction of the aggregate we use a 200 µm cone". The picture seems to show a 200 µl (rather than µm) pipette tip, could there be a typo?*

Yes, corrected.

- *"We measure the two-dimensional velocity field v(x, y, t) using a custom-made Matlab optic flow code based on the Kanade Lucas Tomasi (KLT) algorithm (24) with a level 2 pyramid." I do not see a statement of code availability or link to github or similar. To ensure the the method is reusable, it's very important that the code is released, with a minimal reproducible analysis on a test-dataset, together with the paper.*

Done. The text now writes:

All home-made codes and datasets are available upon reasonable request to the corresponding author.

- *Fig. 7 legend "Schematic rheological diagram proposed for the while aggregate". Typo for whole aggregate?*

Yes, corrected.

- *"How cell membrane trafficking, adhesion proteins and molecular motors dynamics affect rearrange- ments dynamics and are relocalized during junction remodeling in 3D tissues is still not well understood. Combining our method with cell lines endowed with fluorescent reporters of such proteins would enable to quantify their dynamics using fluorescence recovery after photobleaching technics for example."*
Even more exciting opportunities that the authors don't mention might arise from blocking the action of some proteins, rather than just imaging them. Using KO lines or chemical inhibition of various pathways and effector proteins before measuring detailed mechanical parameters could lead to a lot of insights into the relative contributions of various components of the cellular machinery to tissue mechanics.

We have added this remark in the perspectives section, as follows:

Other exciting insights could come from blocking the action of some proteins (using knock-out cell lines or chemical inhibition of various pathways and effector proteins), so as to quantify the relative contributions of various components of the cellular machinery to tissue mechanics. Studying the effects of drugs such as blebbistatin, which inhibits Myosin II could also brings new interesting pieces of

information, as shown by our proof of concepts experiments, see Fig. S7b, Supp. Movie 12. In the same way, cell-cell stress propagation can also be modified for instance using *a*-catenin null cell line. Since *a*-catenin links the cytoskeleton with cadherins, cell-cell junctions are then modified. Aggregates still form cadherin- cadherin junctions, but cells shape are more irregular and there is a shorter correlation range in both shape and velocity, while rearrangements are dramatically impeded (Supp. Movie 13).

- *"For these kind of applications, it could be useful to develop parallel and/or sequential aspiration was done for single cells experiments." Some words must be missing from the conclusion sentence for it to make sense.*

Corrected.

- *Figure 7: since all the measurements are done on a single physical system, one would expect a unique model to fit all the experimental data. Is the model on the bottom right such a unifying model? Could we get curve fits of the various experiments based on a unique final model in this figure?*

We now explain this better in the text, as follows:

To recapitulate the different behaviors observed in the previous sections, we propose a multi-scale rheological diagram of the aggregate (Fig. 8 and Table 1). It combines the contributions of intra-cellular rheology (cell deformations), inter-cellular rheology (cell rearrangements) and aggregate surface tension (elasto-capillarity). This complete rheological model could in principle apply to all experiments presented here (Figs. 4c, 6e, 7e). Ideally, all of them could be fitted with a single set of parameters, at least if elastic and viscous moduli are supposed constant. We however emphasize that only the aspiration part of the experiment is here submitted to a quantitative test. The relaxation part could be tested more quantitatively through a finite element simulation taking into account the geometry details.

- *In addition, I find it much nicer when symbol representation of models are depicted next to the data- fitting for which they were used, and the parameters are visually highlighted on the curve fits when applicable. I wonder if it would be possible to reorganize a bit the figures and text one way or another to put the theoretical models next to each experiment with corresponding curve-fits? And with the correspondance between parameters in the model and experimental measurements better highlighted?*

In the revised version, we have improved the legibility of these figures.

- *All parameters except G_{ag} are given in the legend, it would be helpful to also put the value of G_{ag} here for completeness - and these values might also be better presented in a table within the figure than scattered in the legend.*

We have clarified and homogeneized the notations throughout the whole text. A new Table (Table 1) now lists the quantities and their values (along with their method of determination). The notations in Fig. 8 and in its caption have been modified accordingly.

- *The legend refers to a, b, c, d, subpanels which are not present within the figure.*

Corrected.

- *Last, in addition to the diagram, I wonder if it might also be useful to add the final stress vs strain model in equation-form on the side, since this is the key final contribution from this paper that might be reused in future embryology studies.*

Although the Maxwell model uses stress, it is expressed here without requiring

explicitly the stress, and we do not measure it. Our approach here is purely kinematical.

- *Finally, no error intervals are given for the physical quantities reported. The authors should provide standard deviations or standard error of the mean across experiments at least for the most important parameters, i.e. those currently in the legend of Fig. 7.*

These parameters are now listed in Table 1. We mention in the caption of Table 1 that the values we indicate are only approximate orders of magnitude. In addition, we have added a new figure (Fig. S4) to show visually the precision and reproducibility of the method.

Second decision letter

MS ID#: DEVELOP/2022/200774

MS TITLE: A microfluidic platform to investigate the role of mechanical constraints on tissue reorganization

AUTHORS: Sham Leilah Tlili, Francois Graner, and Hélène Delanoë-Ayari
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This is a nice platform that will enable the study of the relationship between tissue- and cell-scale mechanics.

Comments for the author

The authors have constructively answered all of my points and the article is now ready for acceptance in my view. Well done!

Reviewer 2

Advance summary and potential significance to field

In this study, Tlili and coauthors describe a new microfluidic platform to study how cell rearrangements take place in response to mechanical constraints. The platform allows large deformations of aggregates over time scales of tens of minutes as they flow through a constriction in the device. This can be coupled to two-photon imaging to observe changes in cell morphologies - potentially linking cell scale changes to aggregate mechanical response. The time-scales and deformations are relevant to a number of developmental processes. Analysis of the deformations considering the aggregate as a viscoelastic liquid allows to separate the contributions of rearrangements and cell deformation. Overall, this is a paper with a lot of potential and I can see that the device might be useful in mechanical studies of developmental processes.

Comments for the author

The authors have improved the text and figures to clarify the points that were not clear, and the paper seems ready for publication to me.

Just a small note: access to the code is absolutely essential for other researchers to reproduce these results, and I think "available upon reasonable request" is not enough, it should be at least simply "available upon request" in its entirety, without any judgement of whether the request is reasonable or not.
And even much more appropriate would be to put the code in a repository, freely accessible online, such as github or a university website, or in the supplementary material, to be sure access to code is guaranteed without barrier and on a longer term.