nature portfolio

Peer Review File

Active foam dynamics of tissue spheroid fusion

Corresponding Author: Professor Bart Smeets

This manuscript has been previously reviewed at another journal. This document only contains information relating to versions considered at Nature Communications.

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript by Ongenae et al “Active foam dynamics of tissue fusion dynamics” combine experimental observations of the fusion of spheroids of human periosteum cells and numerical simulations to make a link between active mechanics of individual cells , which can be tuned by the action of drugs, and tissue material properties. These results have numerous applications in tissue engineering and regenerative medicine.
Do spheroids fuse like liquid droplets? This is the subject of this article focusing experimentally and theoretically on the fusion and arrested fusion of cell aggregates.

I have few naïve questions before the discussion of the paper.
The case of arrested fusion means that they are soft elastic materials. My first question is how these soft beads can minimize their surface energy to become spheroids? A drop of foam is never spherical. Do arrested coalescence require the formation of extracellular matrix during the fusion?
My second question is that the dynamics of fusion leads to the measurement of a characteristic velocity which is the ratio of a surface tension divided by a viscosity. The action of drugs is not easy to explain because a fluidification of the tissue will decrease the viscosity but how it’s action on the surface tension is related?

The paper is composed of two main parts:

I) Experimental results interpreted by an analytical model of viscoelastic fusion

- Equation 1 applies an earlier model developed by some of the authors in Ref. 13. The model is a modified version of the classical viscous sintering model of Frenkel and others, to which an elastic element is added to predict arrested coalescence. It raises several questions:

1)A common feature between the Frenkel-Eshelby viscous model and this more recent viscoelastic description is their common prediction of short-term dynamics that scale as a ~ t1/2, where a is the radius of the contact. This scaling arises from the assumption that the viscous or viscoelastic response occurs at the scale of the entire aggregate, a volume of order R03. Such an assumption contrasts with the Michel and Shanahan kinetic model based on JKR theory (Johnson, Kendall, Roberts” Surface Energy and the Contact of Elastic Solids”), where viscous dissipation occurs only in the region of significant elastic deformation, of order a3, resulting in a ~ t1/3. The Michel-Shanahan scaling (Michel F, Shanahan R CRAS Paris 1990)has been shown to correctly predict the spreading of cellular aggregates on a substrate, a phenomenon closely resembling aggregate fusion (Douezan et al , PNAS 2010). The JKR-MS Michel-Shanahan model of the kinetics of the JKR experiment (viscoelastic solid fusion model) leads to arrested coalescence as in eq. 1, but with different dynamical scaling laws. However, they lead to the same expression for the fusion times.

2) Moreover, the evidence for such arrested coalescence in the current data is weak. The authors state that arrested coalescence is observed under certain conditions shown in Fig. 1E. This statement needs to be better supported. How do the authors define an "equilibrium angle" in the experiments? How are they sure that equilibrium has been reached? Is the observation of an equilibrium angle less than 90° statistically significant?
3)How the measure of α deduced from granular shape index is related to the aggregate surface tension?

4) The Michel-Shanahan model has been extended to viscoelastic liquids (Viscoelastic Liquid Fusion Model; Beaune et al, Langmuir 2022) to describe the fusion of cellular aggregates. This model, rather than the widely used Frenkel-Eshelby model, correctly predicts the observed dynamics of aggregate fusion in the absence of arrested coalescence. Can you check if this model fit your experimental data?This article also describe the interpenetration of cells from the fusion of labeled and unlabeled aggregates. Can the authors compare the dynamics of interpenetration in the two cases?

II)Active foam model

The proposed numerical model, which the authors have perfected from previous papers, is a valuable contribution that provides insight, notably by describing the role of cell migration in aggregate dynamics.

The quantification of single cell trajectories in experiments is truly remarkable. The time-averaged mean square relative displacement show that cellular rearrangements occur frequently and are greater in the absence of ROCKi . Can you give an order of magnitude of the cell diffusion coefficient?

In Figure 1C, the analytical model of Eq. 1 is fitted to the experimental data. In Fig. 3D,E the analytical model of Eq. 1 is fitted to the numerical model. Can the authors provide the values of the fitting parameter of the analytical model and discuss their meaning regarding the conditions fitted? Specifically, in the fitting to the numerical model in Fig. 3, can the fitted parameter values of the analytical model be deduced, or made sense of, from the parameter values of the numerical model? They explore the role of cell-cell friction on tissue dynamics and jamming transitions. How the tissue viscosity η t is related to the cell viscosity η ? Do the authors can relate the viscosity of cellular aggregates to the level of expression of the cadherins?

It will be interesting to test from this numerical model the two analytical viscoelastic fusion models (Shanahan versus Frenkel) leading to different dynamical scaling laws.

The model's focus is on arrested coalescence and jamming, which is not clearly observed in the experiments. To better illustrate the model's capabilities, the authors could compare to experimental data from the literature where arrested coalescence would be more apparent. The comparison of the mobility data with model predictions is slim, again because experiments correspond to fluidized conditions, far from the jamming transition where model predictions are most interesting.

Conclusion

The experimental part is very rich and brought new features on spheroids shape and cell motility, in particular how cells of the two aggregates mix by diffusion at the interface.
They are interpreted by a viscoelastic model. An earlier model developed in the past by Shanahan in the spirit of JKR, leads to different dynamical scaling laws. It must be introduced and discussed.
To describe the interplay between single cell properties and tissue mechanics, the authors develop a 3D active foam model with deformable cells parameterized by relative cell-cell tension and motility. The model reproduces fusion dynamics while capturing cell shape and motility. The fusion of aggregates at a macroscopic scale is linked to fluidization at a microscopic scale.
Even if the experimental observations can “globally” be explained by existing, simpler viscoelastic fluid models (except for the surprising observation that fusion dynamics for less granular spheroids upon ROCKi removal decelerated despite increased cellular rearrangements, which cannot be explained by the proposed numerical model either, as mentioned in the article's discussion), the experimental part is useful to enter in the complexity of the simulations.
I recommend publication if more experimental data on arrested coalescence are added to the paper to allow a real confrontation between jamming and arrested coalescence and if similar earlier works are introduced and discussed.

Francoise Brochard Wyart

(Remarks on code availability)
The paper of Shanahan is in french and difficult to find!

Reviewer #2

(Remarks to the Author)
The subject of the article is timely and of fundamental relevance to tissue development. The manuscript is generally well-written and insightful. The specific points we list below concern mainly unclear writing/definitions, overly assertive statements, and lack of a comprehensive contextualization of the authors' work within the existing body of literature on cell-based modeling and tissue fusion.

The provided code consists mostly of Python wrapper scripts and looks clean. However, MPacts (https://mpacts.com) was used as a backend to do all the simulations. The software seems to be closed-source. We notice that one of the co-authors, Bart Smeets, is listed as one of the developers of Mpacts, and several academic papers are listed that used the software.

As such, we were not able to check the implementation of the model. We consider it absolutely necessary that the simulation code is made public when the paper is published.

Specific points:
It is unclear to me why the authors cite only Ref. 25 in the introduction to support the statement that "Recent expansions of these models also include inter-cellular pores". There are rather many such models, also in 2D. For an overview, see Table 1 in Vetter et al., Comput. Phys. Commun. 299, 109128 (2024).

A quick search for previous experimental work on tissue and spheroid fusion reveals a body of literature that the authors don't mention in their work. Some of them report contact angles and fusion times. Although some of these systems will be different from the author's spheroids, it would nevertheless appear appropriate to discuss how the results presented in this manuscript compare to these prior studies. A starting point could be the following publications (in no particular order):
Ray & Niswander, Development (2012) 139: 1701–1711
Olsen et al., Acta Biomaterialia (2015) 13: 188-198
Lindberg et al., Adv. Sci. (2021) 8: 2103320
Rago, Dean & Morgan, Biotechnology and Bioengineering (2009), 102: 977-1282
Livoti & Morgan, Tissue Engineering Part A (2010) 16, 2051-2061
Hajdu et al. Journal of Tissue Engineering and Regenerative Medicine (2010) 4: 659-66

Are \tau_\Gamma and \tau_\epsilon the two free fit parameters used in Fig. 1? This isn't clearly stated.

In the caption of Fig. 1, it is unclear to me how many repetitions were done. It is stated that "Data acquired from a single experiment, n = 7, 10, 8, 9", but what is n, if it is a single experiment?
In all figures that have them, it is unspecified what the error bars represent.

On page 3, the statement "there is no singular connection between cell-cell tension and fusion time" is potentially misleading. What do the authors mean by "singular"? Single? Simple? This sentence isn't clearly enough linked to the data.

On page 4, the authors write that "fusion proceeded notably slower in absence of ROCKi". This seems to be in contradiction to Fig. 1D, which shows that the Bleb/ROCKi+ condition lets them fuse even slower.
In Fig. 2E, how is "height" defined? Height of what, measured how?

In Fig. 2G, what is the uncertainty of the shown MSRD curves? How to the authors ensure statistical significance in the different results between the conditions?

How many repetitions were made for each condition in Figs. 3F,G, 4C,D, and Supplementary Fig. 4? Is there sufficient statistical power to support the claim that "Fusion time-scale first increases with pa at low motility, while it decreases with pa at higher levels of motility"? Fig. 3G seems rather noisy, such that the statement on the dependency on pa seems unsupported (assuming each pixel in the plot shows a single simulation).

Apparently based on the data shown in Fig. 3c, the authors state on page 6 that "the critical active pressure to fluidize the tissue material scales as pa,crit $\propto$ $\alpha^{-1/2}$". This strong statement is not sufficiently supported by the data. It looks like it may be approximately correct, given the coarse data, but this is not conclusive by any means. Verifying a power-law relationship as claimed here would require fine data over at least about two orders of magnitude, and a fit that yields an exponent that is statistically consistent with the claimed one. As I'm assuming the authors do not have such data, the statement should be weakened clearly to reflect the uncertainty.

The term "Normalized unique contacts" is not clearly defined. The caption of Fig. 4 writes that it is the total number of unique contacts during fusion divided by average number of contacts. Unique in what way? How is "during fusion" defined? Averaged over what? Time, or the cells? This makes it hard to interpret Figs. 4C,E,F,H.

The origin of the dimensionless activity parameter A is not explained. Where does this scaling come from? Is it just a phenomenological observation that the authors came up with by trial and error? Is there an intuitive derivation or interpretation for it? Are the exponents -1/2 and 1/4 exact, or just guessed? This needs to be clarified to allow the readership to judge its credibility.

On page 7, it is written that the activity parameter A scales differently from the effective temperature in an active vertex model, citing ref. 16. For clarity, it would be helpful to explain why the authors consider this comparison meaningful, even though the quantities (A vs. temperature) are different. Moreover, the cited reference 16 mentions the scaling Teff~v0^2 as a conjecture, not a conclusive result, but from the authors' text it appears as though this was a fact.

On page 7, the statement "the tissue viscoelastic timescale sharply increases with activity" is made. It is not clear to me how this is supported with data, as I can't find this relationship shown anywhere.

Fig. 4g is unclear. What is x? Are the curves from individual simulations, or averaged?

Fig. 5 introduces another time τm that doesn't appear elsewhere in the manuscript (only in the caption of Extended Data Fig. 2).

In Eq. 4, the symbol T is undefined, and the integral should probably be a definite integral.
In the section "3D active foam model" of the Methods section, the definition of the internal reaction traction Tc,i (which seems to be a vector) appears to be a scalar integral without direction.

In Eq. 4 of the Supplementary Note, the volume strain history term is unexplained. What is its purpose, and how did the authors ensure that their solutions are not history-dependent (or is such a dependence intended)?

The authors do not provide a link to a code repository. I would strongly encourage them to make their source code publicly available.

Writing errors:
Line below Eq. 1: "visco-elastic" -> "visco-elastic time"

The figures were not always correctly referenced, e.g. where it says figure 3c, figure 4c seems to be meant. Also in the supplementary table for the forth condition the units for ROCKi say micro instead of micromolar

Page 6: "Fig. 3k" -> "Fig. 3d"

Discussion: "tissue shapes an decreased" -> "tissue shapes and decreased"

Page 9: "This procedure performed" -> "This procedure was performed"

Below the definition of τf in the Methods, the spheroid radius before fusion, R0, is mistakenly called a0.

First line of page 12: "between between"

There are some formatting issues in the bibliography, such as missing volume numbers, wrong capitalization ("lscience" instead of "iScience") and so on.

Figure 2b: description says four conditions but there are only three displayed, TGF-b is missing.

Figure 2c: in the description C8 condition is forgotten.

Caption of Extended Data Fig. 2: "time affect fusion" -> "time affects fusion"

Caption of Extended Data Fig. 5: "shape index ¿ 5.41"

(Remarks on code availability)
The provided code consists mostly of Python wrapper scripts and looks clean. However, MPacts (https://mpacts.com) was used as a backend to do all the simulations. The software seems to be closed-source. We notice that one of the co-authors, Bart Smeets, is listed as one of the developers of Mpacts, and several academic papers are listed that used the software.

As such, we were not able to check the implementation of the model. We consider it absolutely necessary that the simulation code is made public when the paper is published.

Reviewer #3

(Remarks to the Author)
Ongenae et al. developed a 3D computational model based on the recently introduced "active foam" framework to capture the dynamics of spheroid fusion. Unlike earlier versions, this approach successfully replicates complex 3D cell dynamics and incorporates a novel parameter—cell-cell friction—which the authors highlight as a critical factor for accurately modeling

tissue fusion. Notably, the study reveals that jamming and arrested coalescence can arise even when cells exhibit an "unjammed" shape index. The use of high cell-cell friction to explain these phenomena is particularly compelling.

While the computational model is robust, the biological alignment with it remains somewhat unclear to me. However, I view this ambiguity as a strength, as it opens the door to extensive experimental exploration. For instance, what role does cellular granularity play? And why can fusion occur without substantial cell rearrangement? These are intriguing questions that could inspire follow-up studies.

Overall, I find the study to be meticulously executed. I have only one major concern: is ROCKi exerting only the expected effects, or could some of the "unexpected" outcomes be due to off-target effects? I suggest the authors explore alternative inhibitors targeting actin to clarify this. Additionally, it would be valuable to examine the effects of enhanced actomyosin contractility using drugs like calyculin or jasplakinolide. Testing these conditions could significantly strengthen the manuscript and won't take too long.

I strongly recommend this paper for publication in Nature Communications. However, I encourage the authors to make the text more accessible for readers less familiar with computational modeling and tissue mechanics, as this could broaden the work's impact.

Minor Comment:
The statement, "Elimination of either ROCKi or TGF-β1 resulted in deceleration of fusion," warrants reconsideration. Figure 1c shows a small difference between C8 and TGF-β1, but this is not reflected in Figure 1d. Based on this discrepancy, it may be inaccurate to claim that the elimination of TGF-β1 significantly decelerates fusion.

Alessandro Mongera

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The paper, "Active Foam Dynamics of Tissue Spheroid Fusion," is suitable for publication on Nature.com.
The authors carefully address my main questions.
They also checked their data against the Shanahan viscoelastic model.
They compared the results of interpenetration dynamics to those of aggregate fusion with hybrid particle-cells and found good agreement for the cell diffusion coefficient.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
I recommend accepting the manuscript for publication. The work is of high scientific quality. The authors have made considerable effort to improve the manuscript and have addressed almost all points raised satisfactorily.

There is one exception, namely that the backend solver ("MPacts") is available only in binary form in a docker container, not open source, and to our knowledge, was also not made accessible elsewhere before.

(Remarks on code availability)
The backend solver ("MPacts") is available only in binary form in a docker container, not open source, and to our knowledge, was also not made accessible elsewhere before.

Reviewer #3

(Remarks to the Author)
I'm happy with all the changes of this revised version.

(Remarks on code availability)

In-depth reply to the reviewers

"Active Foam Dynamics of Tissue Spheroid Fusion" – *Nature Communications*

General remarks

We thank the referees for their in-depth evaluation of our manuscript and our presented results, providing helpful suggestions and important concerns on both experimental and computational issues. We also apologize for the long duration of our revisions, as we performed substantial additional experimental measurements and data analyses. In this document, we provide an in-depth reply to all individual remarks. In general, we carried out several additional experiments and analyses to better support our results and strengthen the conclusions. Here is a short overview of the main new additions in the manuscript:

1. We performed additional repeats of the two-photon time-lapse imaging experiment to image single cell motion during spheroid fusion and measure relative cell diffusivities during this process. This facilitates statistical analysis comparing the effective relative diffusivity between 4 studied experimental conditions. These results are presented in the **revised Fig. 2-E-H.**
2. We performed additional experiments of fusion of larger spheroids (500 cells), presented in the **new Extended Data Fig. 1**. This further demonstrates visco-elastic fusion dynamics and equilibrium fusion angles below 90 degrees.
3. We analyzed our fusion experiments using an alternative visco-elastic model of spheroid fusion suggested by referee 1 and present these results in **new Supplementary Fig. 4-5.**
4. We numerically estimate the empirical scaling exponents relating tissue fluidity to active cell mechanical properties. These results are presented in the **new Supplementary Fig. 8**, and the methodology in the **new Methods section "Scaling exponents of tissue fluidity"**.
5. We provide a theoretical rationale for the observed scaling of tissue fluidity based on the balance of active protrusions and cortical retraction determining the probability of topological transitions. This theoretical analysis is presented in the **new Methods section "Theoretical scaling of tissue fluidity"**.
6. As suggested by referee 3, we also tested treatment with Jasplakinolide, which stabilizes actin and prevents filament disassembly. These findings are presented in the **new Supplementary Fig. 1**.
7. We added a **Data Availability** and a **Code Availability** statement, which link to a zenodo repository for experimental data, simulation scripts and software, and a GitLab page for source code and demo scripts of our simulations.

Changes to the manuscript can be inspected in the provided `'diff.pdf'` file highlighting all changes compared to the originally submitted version.

Reviewer #1 (Remarks to the Author)

The manuscript by Ongenae et al "Active foam dynamics of tissue fusion dynamics" combine experimental observations of the fusion of spheroids of human periosteum cells and numerical simulations to make a link between active mechanics of individual cells , which can be tuned by the action of drugs, and tissue material properties. These results have numerous applications in tissue engineering and regenerative medicine.
Do spheroids fuse like liquid droplets? This is the subject of this article focusing experimentally and theoretically on the fusion and arrested fusion of cell aggregates.

We agree with the reviewer that these results have numerous applications in Tissue Engineering and Regenerative Medicine. To this end, we would like to stress one of the strengths of this work is that we use spheroids of human periosteum-derived cells which are adult progenitors with direct downstream applications, as a clinically relevant cell source for bone and cartilage engineering.

I have few naïve questions before the discussion of the paper.
The case of arrested fusion means that they are soft elastic materials. My first question is how these soft beads can minimize their surface energy to become spheroids? A drop of foam is never spherical. Do arrested coalescence require the formation of extracellular matrix during the fusion?

This is a valid remark: Assuming all equal, mechanical conditions that give rise to arrested fusion also would have prevented a spherical aggregate in the first place. As the reviewer correctly noted, our experiments show mostly full or nearly full coalescence of cell aggregates. In the work of Oriola et al. (2022),

> Oriola, D., Marin-Riera, M., Anlaş, K., Gritti, N., Sanaki-Matsumiya, M., Aalderink, G., ... & Trivedi, V. (2022). Arrested coalescence of multicellular aggregates. Soft Matter, 18(19), 3771-3780,

arrested coalescence was observed. In their case, larger aggregates (which were still spherical) failed to fuse completely. However, it is reasonable to expect that the tissue properties might have changed from the spheroid formation phase to the fusion phase, including that the spheroid would have simply grown between formation and fusion (which will also promote arrest).

My second question is that the dynamics of fusion leads to the measurement of a characteristic velocity which is the ratio of a surface tension divided by a viscosity. The action of drugs is not easy to explain because a fluidification of the tissue will decrease the viscosity but how it's action on the surface tension is related?

The model is parameterized by two timescales, the visco-capillary time-scale $\frac{R_0\,\eta}{\Gamma}$, and the visco-elastic timescale $\frac{4\eta}{G}$. In our experiments, the increased fusion timescale at complete or near-complete coalescence is predominantly caused by an increased visco-capillary time (since G must be low to reach complete coalescence). An increase of fusion timescale could be caused by increased tissue viscosity, but also by decreased tissue surface tension. As discussed in the following section, we also observe an increase in relative cell-cell tension at the cell scale, which aligns with a reduction in aggregate surface tension. While our fusion experiments alone cannot differentiate between the contributions of increased viscosity versus reduced surface tension, our aggregate contour analysis indicates that at least part of the increased fusion timescale is likely due to a decrease in tissue surface tension.

The paper is composed of two main parts:

I) Experimental results interpreted by an analytical model of viscoelastic fusion

- Equation 1 applies an earlier model developed by some of the authors in Ref. 13. The model is a modified version of the classical viscous sintering model of Frenkel and others, to which an elastic element is added to predict arrested coalescence. It raises several questions:

1) A common feature between the Frenkel-Eshelby viscous model and this more recent viscoelastic description is their common prediction of short-term dynamics that scale as a ~ t1/2, where a is the radius of the contact. This scaling arises from the assumption that the viscous or viscoelastic response occurs at the scale of the entire aggregate, a volume of order R03. Such an assumption contrasts with the Michel and Shanahan kinetic model based on JKR theory (Johnson, Kendall, Roberts" Surface Energy and the Contact of Elastic Solids"), where viscous dissipation occurs only in the region of significant elastic deformation, of order a3, resulting in a ~ t1/3. The Michel-Shanahan scaling (Michel F, Shanahan R CRAS Paris 1990)has been shown to correctly predict the spreading of cellular aggregates on a substrate, a phenomenon closely resembling aggregate fusion (Douezan et al , PNAS 2010). The JKR-MS Michel-Shanahan model of the kinetics of the JKR experiment (viscoelastic solid fusion model) leads to arrested coalescence as in eq. 1, but with different dynamical scaling laws. However, they lead to the same expression for the fusion times.

The scaling of the contact radius with time indeed can give valuable information on the dominant type of material behavior that drives early fusion. As noted by the reviewer, our model assumes bulk liquid-like behavior, with an apparent long-time elasticity (which we later relate to jammed configurations through simulations of the computational model), arising from the inability of cells to undergo topological changes. As such, our model assumes that the cell aggregate is initially

liquid. The early time scaling is thus the same as the Frenkel-Eshelby viscous model. Alternatively, as explored in Douezan et al. PNAS 2010, one may assume that early fusion displays visco-elastic behavior. In that case, the JKR-MS Michel-Shanahan model can be used to model its kinetics.

The referee is also correct in that these models differ in their assumptions on where viscous dissipation and elastic deformation occurs: In the full aggregate (*Frenkel-Eshelby* and visco-elastic extensions) versus only at the contact region (essentially all extensions of Hertz / JKR such as JKR-MS). Our modeling choice was motivated by the following reasons:

1) We observe active aggregates, with frequent rearrangements, which are expected to spread viscous dissipation and elastic stress across the aggregate.
2) The aggregates are also very small, so small movements of single cells have repercussions on the aggregate scale.
3) Experiments are full fusion or near-full fusion (as the referee correctly points out in the following point, see below). In simulations, the full spectrum can be obtained, but the arrested fusion corresponds to cases of highly granular behavior and porous aggregates.
4) Our model fits our data well in all observed conditions, both in theory and experiments.

Yet, it is an important assumption that should have been more clearly stated. In the revised manuscript, we more clearly state the assumptions of the theoretical model in the method section “Visco-elastic model for tissue spheroid fusion”. We now state on p10:

> “Assuming that viscous dissipation and elastic deformation occur throughout the bulk of the fusing spheroids, fusion dynamics can be expressed in function of visco-elastic material properties by the governing equation …”

Further considerations on this general point are provided in the replies to the subsequent remarks of the referee.

2) Moreover, the evidence for such arrested coalescence in the current data is weak. The authors state that arrested coalescence is observed under certain conditions shown in Fig. 1E. This statement needs to be better supported. How do the authors define an "equilibrium angle" in the experiments? How are they sure that equilibrium has been reached? Is the observation of an equilibrium angle less than 90° statistically significant?

We agree with the reviewer that our data *does not conclusively show arrested* coalescence (at very long timescales). The existence of ‘arrested coalescence’ is not the main conclusion of our paper and we should have been indeed more careful in concluding this from the data in Fig. 1E. Rather, what the data, in combination with our model, show is that the viscous and elastic

timescales differ between studied conditions. We further address this remark in-depth below in further replies to the referee.

The equilibrium angle is defined based on model fits, and stems from the fitted coefficient beta discussed in the methods section "Visco-elastic model for tissue spheroid fusion". In the revised manuscript, we included an additional Extended Data Figure, the new Extended Data Figure 1, to illustrate the estimated equilibrium angle in more detail. Moreover, we performed new experiments containing larger spheroids of 500 cells to demonstrate the applicability of the visco-elastic model. We show model fits in all conditions for both small N=100 and larger N=500 spheroids, together with fitted values for fusion time and equilibrium fusion angle. Using a 1-sided t-test, we also test whether the equilibrium fusion angle is statistically significantly different from 90°. These results are also provided in the **new Extended Data Fig 1**. However, we do not consider this conclusive evidence of arrested coalescence because:

1) These equilibrium angles are still close to 90 degrees and thus reflect nearly spherical aggregates;
2) The value of this equilibrium fusion angle hinges on the validity of the proposed visco-elastic model, which is only an approximation of the real biological material.

In the revised manuscript of the experimental section, we now revised statement discussing the results in Fig 2E. We now state on p2:

> "Despite substantial variation between replicates, most conditions exhibit nearly complete coalescence, with equilibrium fusion angles close to 90° (Fig. 1E and Extended Data Fig. 1)."

3) How the measure of α deduced from granular shape index is related to the aggregate surface tension?

The measure of α as deduced from granular shape index is the relative cell-cell tension, which parameterizes the ratio between the tension at the cell-cell interface and the cell-medium interface. The aggregate surface tension is the apparent surface tension if the entire aggregate is modeled as a liquid droplet, and for which Young-Laplace law holds at the aggregate scale. The aggregate surface tension is a parameter of the visco-elastic fusion model presented in Eq. 1. In the general case, there is no direct, trivial relationship between the aggregate surface tension and α, which is a cell-scale parameter. Rather, this relationship would depend on the cell configuration and packing in the cell aggregate. However, previous work of the authors investigated this relation in numerical simulations (Ref 33, see Fig 4 therein):

Cuvelier, M., Pešek, J., Papantoniou, I., Ramon, H., & Smeets, B. (2021). Distribution and propagation of mechanical stress in simulated structurally heterogeneous tissue spheroids. Soft Matter, 17(27), 6603-6615.

In the limit of high adhesion (thus low α) and large cell number (thus reducing discrete packing effects), the hydrostatic pressure in the aggregate can be approximated as (Eq. 5 in Ref 33), with adhesive tension ω and equivalent aggregate radius R_{agg}:

$$\sigma_{th}^{h} = \frac{2\omega}{R_{agg}}.$$

This would imply that in this limit, based on Young-Laplace law, the aggregate surface tension is $\Gamma = \gamma(1-\alpha) = \omega$, i.e, decreased α implies increased aggregate surface tension, if γ is kept equal. In the case that $\alpha = 0$, i.e. no tension at the cell-cell interface, a true liquid droplet, this trivially reduces to $\Gamma = \gamma$, since there is only tension at the periphery, which is just the cell-medium tension. However, it should be noted that this is only an approximation and breaks down in the granular limit (high α) and in case intercellular pores are significant in the aggregate. On this topic, we further kindly refer the referee to Box 1 of the reference below and their cited references, demonstrating the same relation,

David, R., Luu, O., Damm, E. W., Wen, J. W., Nagel, M., & Winklbauer, R. (2014). Tissue cohesion and the mechanics of cell rearrangement. Development, 141(19), 3672-3682.

We also added this reference in the revised manuscript and cite it when we make the statement that “Cell-cell interfacial tension governs tissue surface tension at the spheroid scale”

4) The Michel-Shanahan model has been extended to viscoelastic liquids (Viscoelastic Liquid Fusion Model; Beaune et al, Langmuir 2022) to describe the fusion of cellular aggregates. This model, rather than the widely used Frenkel-Eshelby model, correctly predicts the observed dynamics of aggregate fusion in the absence of arrested coalescence. Can you check if this model fit your experimental data?

We thank the referee for this suggestion. We applied the model proposed in Beaune et al. Langmuir 2022 to our experimental data, namely, we fitted Eq. 15 from that paper:

$$\frac{a^3}{R^2} \approx L_e\left(1 - e^{-\frac{t}{\tau_1}}\right) + v_f t.$$

Here, a is the current contact radius, R is the aggregate radius, L_e is an elastic length-scale, τ_1 is the visco-elastic relaxation time of the Kelvin-Voigt spring-damper and v_f is the characteristic

fusion velocity at long times. This model is explained in the **new Supplementary Note on "Alternative visco-elastic model for spheroid fusion"**, and the results of these fits are shown in the **new Supplementary Fig. 4**. As can be seen in this figure, this model also fits the general trend of our data well in most studied conditions. From these fits, we also extracted the parameters L_e, τ_1, and v_f, as shown in the **new Supplementary Fig. 5**. This model assumes an initial visco-elastic relaxation, followed by a liquid viscous response at long timescales. One issue this interpretation has is that most cases (apart from Bleb/ROCKi$^+$) showcase full fusion (to a single sphere). In that case, the fitted value of L_e will trivially result in the final aggregate radius, with $v_f \approx 0$. As such, it cannot represent a true material property of an initial relaxation ($L_e \propto \frac{\gamma}{E}$): It can only provide a lower bound of the actual elastic length-scale of the cellular material.

Nonetheless, it should be remarked that particularly the fits in Bleb/ROCKi$^+$ conditions are well compatible with the Beaune et al. 2022 model, with some experiments showing a region with a $a \propto t^{1/3}$ scaling, as reported in their paper. These are relatively inactive and granular aggregates, which are not fully fused. In this regime, the model of local deformation/dissipation in the contact region could be more appropriate to model the experiment than the model proposed in our paper. The Beaune et al. 2022 model predicts decreased elastic length-scale for Bleb/ROCKi-, indicating increased stiffness (see Supplementary Fig. 5), since $L_e \propto \frac{\gamma}{E}$. This is compatible with our visco-elastic model, where we see a reduction in the equilibrium fusion angle (**new Extended Data Fig. 1**), or, correspondingly, a decrease in the elastic timescale τ_ϵ, indicating increased stiffness.

In the revised manuscript, we now explicitly discuss the assumptions made in our model. Furthermore we added a **new Supplementary Note on the "Alternative visco-elastic model for spheroid fusion".** The results of these analyses are presented in **the new Supplementary Fig. 4-5,** where we show the result and interpretation of applying the Beaune et al. (Langmuir) 2022 model to our data.

In the revised manuscript, we now state on p3:

> "We also examined an alternative model for viscoelastic fusion dynamics, proposed by Beaune et al. (2022) based on earlier work on tissue spreading [Douezan2011]. This approach assumes that both viscous dissipation and elastic deformation are confined to the contact region between fusing bodies (see Supplementary Notes for details). When we fitted this model to our data, the results were consistent with our primary findings: We observed a reduction in the characteristic elastic length scale following treatment with blebbistatin and ROCK inhibitor (Supplementary Fig. 4-5).

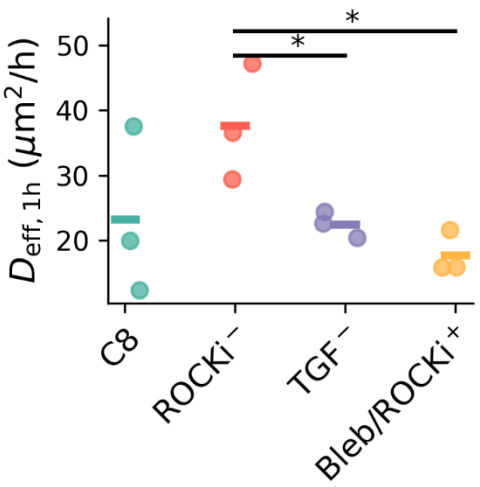
We thank the referee for reminding us of these works.

This article also describe the interpenetration of cells from the fusion of labeled and unlabeled aggregates. Can the authors compare the dynamics of interpenetration in the two cases?

We did not experimentally characterize the dynamics of interpenetration as was done in Beaune et al. *Langmuir* 2022. We did, however, measure the movements of individual cells relative to each other and observe that topological changes occur (see Fig 2G), i.e. cells are actively moving around. In Beaune et al., the effective cell diffusivity was estimated around 1.4×10^{-2} μm/s. To obtain statistics on the relative diffusion, we performed additional two-photon microscopy experiments of fusing spheroids in the 4 different conditions. These results are presented in the **new revised Figure 2G-H**. In all cases, we observe midly subdiffusive behavior at all timescales, likely due to elastic effects, caging and the geometric constraints of the spheroid. However, an effective diffusivity can be estimated, for example for a lag-time of 1h as:

$$D_{\mathrm{eff,1h}} = \frac{MSRD(1h)}{6 \times 1h}$$

These results are presented in the **new revised Figure 2E-H** (and shown below).



Here, we observe that aggregates in absence of Rho kinase inhibitor are more active, while aggregates in presence of blebbistatin and additional rho kinase inhibitor are less active. The diffusivity coefficients at these timescales are roughly between 10 and 50 $\mu\mathrm{m}^2/\mathrm{h}$, corresponding to values between 0.28-1.39 $\times\ 10^{-2}$ $\mu\mathrm{m}^2/\mathrm{s}$. These values are well compatible with the reported values of diffusivity in Beaune et al 2022. However, it should be stressed that these values are characterizing the *relative* diffusion, rather than the absolute diffusion, i.e. how much do cells move relative to each other when initially in contact in an aggregate.

As shown in Fig. 4G, simulations of the computational model predict that complete fusion can still occur in cases where microscopic mixing is very slow. In this case, cell deformations play a crucial role in the fusion response: A model of soft, spherical cells would always strongly overestimate the degree of mixing (or MSRD) required to achieve full fusion, as discussed e.g. in Ref. 13. This is one of the main strengths of our computational model, as it predicts a regime of liquid-like fusion behavior (full fusion) yet minimal mixing at the time-scale of fusion. This regime occurs at low α and low to intermediate levels of active cell motility, essentially a version of the density-independent jamming transition studied first in vertex models, Ref 24.

In the revised manuscript, we added a comparison of these estimates of the relative diffusivity based on the MSRD. Moreover, on p5 we discuss the compatibility with the estimates from Beaune et al. 2022 as:

> "Based on the MSRD at a 1-hour lag time (Fig.2h), we estimated effective relative diffusivities ranging from 7 to 52 $\mu m^2/h$, consistent with previous values for encapsulated microparticles [Beaune et al. 2022]"

II)Active foam model

The proposed numerical model, which the authors have perfected from previous papers, is a valuable contribution that provides insight, notably by describing the role of cell migration in aggregate dynamics.

We thank the reviewer for this assessment of our model.

The quantification of single cell trajectories in experiments is truly remarkable. The time-averaged mean square relative displacement show that cellular rearrangements occur frequently and are greater in the absence of ROCKi .

Again, we thank the reviewer for this assessment, and also for the appreciation for this very time-consuming and technical experiment. It took quite some expertise from microscopy experts and is very time-consuming, both in data acquisition and data processing.

Can you give an order of magnitude of the cell diffusion coefficient?

The effective relative diffusivity of the cells varies greatly in simulations, depending on relative cell-cell tension (Fig 4A), cell motility (Fig 4B) and other parameters (see Extended Data Figures 3-6).

In the revised manuscript, we now report adimensionalized simulation data, since the scale of time is always relative to an arbitrary timescale (such as the persistence time τ_p). However, a comparison with experimental data can be made by assuming a timescale that gives (order of magnitude) similar fusion times. For example, in **revised Fig. 4F**, we compare the experimental MSRD at 60 minutes with simulations assuming a persistence time τ_p = 100s. Here, simulations explore a very wide range of cell diffusivities. By assuming that r=8.88 µm like in the experiments, the effective relative diffusivity for a lag time of 1 h would range between 0.13-390 µm^2/h. In case of extremely active (motile) cells, simulated aggregates showcase strong active shape fluctuations (with decreasing apparent equilibrium fusion angles).

In Figure 1C, the analytical model of Eq. 1 is fitted to the experimental data. In Fig. 3D,E the analytical model of Eq. 1 is fitted to the numerical model. Can the authors provide the values of the fitting parameter of the analytical model and discuss their meaning regarding the conditions fitted? Specifically, in the fitting to the numerical model in Fig. 3, can the fitted parameter values of the analytical model be deduced, or made sense of, from the parameter values of the numerical model?

The values of the fitted parameters of the curves shown in Fig. 3D-E are shown in the heatmap Fig 3F-G and further in the **new supplementary Fig. 6**. In Fig 3F-G, the dashed lines represent the cross-section of the parameter space shown in Fig 3D (vertical) and 3E (horizontal). The equilibrium fusion angle decreases below 90 degrees upon decrease of cell activity and increase of relative cell-cell tension: Here the cellular material is jammed and the spheroid appears solid-like rather than liquid-like. Furthermore, the value of the fitted parameters are also plotted upon variation of persistence time and relative cell-cell tension (Extended Data Fig. 3C-D), cortical tension and cell motility (Extended Data Fig. 4C-D), spheroid size (Extended Data Fig. 5C-D) and cell-cell friction (Extended Data Fig. 6 C-D).

They explore the role of cell-cell friction on tissue dynamics and jamming transitions. How the tissue viscosity η t is related to the cell viscosity η ?

This is a very good question.

1. Cell viscosity itself is not a model parameter: The cell is approximated as an inviscid incompressible cytoplasm surrounded by a viscous cortical acto-myosin of viscosity η, which will determine the apparent cell viscosity.
2. Tissue viscosity is an 'emergent' property in our simulations. We did not measure viscosity *in silico* (although one could in principle perform a simulation of a spheroid micropipette aspiration, similar to Ref. 23). Yet, tissue viscosity can be reasonably expected to correlate with tissue fluidity (higher fluidity → lower apparent tissue viscosity). However, by

assuming this, we implicitly assume that we study the long timescale tissue viscosity, on a timescale where cell rearrangements can help fluidize the tissue. This might not correspond to the context of a mechanical measurement.

In our work we derive an explicit scaling parameter for tissue fluidity which increases with active motility and persistence time, and decreases with relative cell-cell tension, and visco-elastic relaxation time of the cell cortex, $\tau_\gamma = \eta / \gamma$, with cortex (and not cell) viscosity η. Hence, we expect that the critical active pressure to unjam the system scales as $\eta^{1/4}$. Cell-cell friction further complicates this picture as it also strongly reduces cell rearrangements, thus increasing the apparent tissue viscosity.

Do the authors can relate the viscosity of cellular aggregates to the level of expression of the cadherins?

We did not perform measurements of cadherins. However, based on results of our computational model and existing literature, the expression of cadherins is expected to affect tissue viscosity in a few interesting ways:

1. Differential interfacial tension or cell-cell adhesion: As discussed in Ref. 42, Maître et al. Science 2012, cadherins will locally downregulate myosin contractility and thereby increase the apparent adhesive tension. They can also contribute directly to adhesion energy through adhesive ligands.
   Both mechanisms are mechanically equivalent and result in a decrease in relative cell-cell tension α. In our model, decrease cell-cell tension increases fluidity of the tissue and would thus reduce apparent viscosity. However, this only holds for confluent tissue (see Ref. 25), as for porous tissue, increased adhesive tension reduces tissue fluidity. Please note also our recent work on this topic:
   https://www.biorxiv.org/content/10.1101/2025.04.17.649329v1
2. Cadherin turn-over: In our model, cell-cell friction is an effective property resulting from bond turnover at the cell-cell interface. More stable cadherin bonds (=lower turnover rate, or higher bond lifetime), will proportionally increase cell-cell friction and thereby strongly decrease tissue fluidity / increase tissue viscosity.

Hence, we propose that an increased expression of cadherin could either increase or decrease tissue fluidity, depending on whether the tissue is porous and whether also bond turnover rate is affected.

It will be interesting to test from this numerical model the two analytical viscoelastic fusion models (Shanahan versus Frenkel) leading to different dynamical scaling laws.

We fitted both models to the fusion curves shown in Fig 3E, where we simulated fusion at a fixed relative cell-cell tension for varying cell motility (color scale).

As can be appreciated in the figure below, both models fit these simulated fusion curves very well. Comparing the fitted parameters, there is a clear correspondence between the fitted elastic length-scale L_e from the Beaune et al. (2022) model and the equilibrium fusion angle from our model. For full fusion, L_e converges to the final aggregate radius ($R_f \approx \sqrt[3]{2}\, R_0$) whereas the equilibrium fusion angle converges to 90 degree.

We agree with the referee that it would be very interesting to use this simulation to investigate in more detail the rheological scaling of fusion under various continuum assumptions and the apparent visco-elastic properties of fusion. We hope the reviewer understands that we would prefer to reserve such a comprehensive comparison for future work.

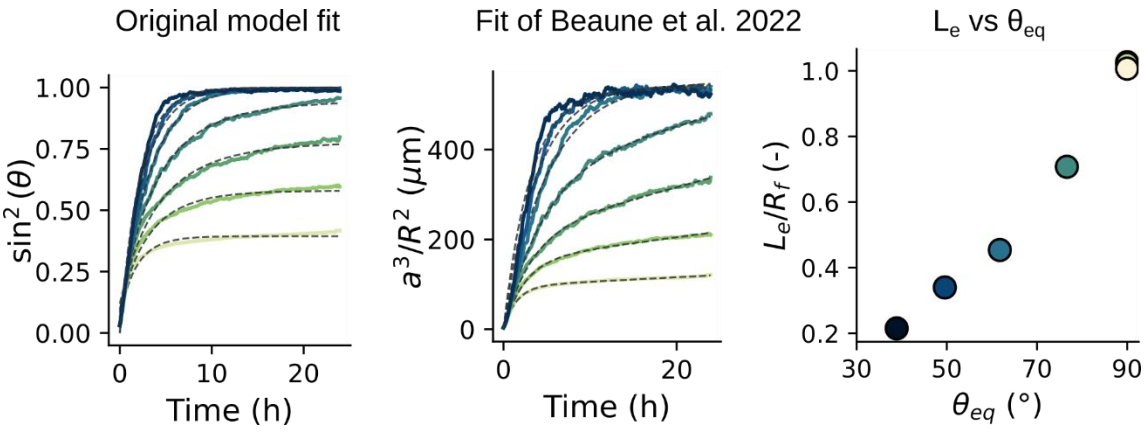


Figure: Comparison of fusion curves varying $\frac{p_a r}{2\gamma}$ with fits of the original fusion model presented in the manuscript, and the alternative model proposed in Beaune et al. (2022).

The model's focus is on arrested coalescence and jamming, which is not clearly observed in the experiments. To better illustrate the model's capabilities, the authors could compare to experimental data from the literature where arrested coalescence would be more apparent. The comparison of the mobility data with model predictions is slim, again because experiments correspond to fluidized conditions, far from the jamming transition where model predictions are most interesting.

We would like to clarify that the primary focus of our work is not arrested coalescence per se, but rather on understanding how cell-scale physical properties (e.g., surface tension, active fluctuations, adhesion) influence fusion behavior and tissue-scale rheology. While arrested coalescence is an important limit captured by our model, our main interest lies in exploring the

transition between fluid-like and arrested dynamics, and how this transition is governed by microscale parameters.

We agree that the range of behaviors observed in our experiments is narrower than in our simulations. Nevertheless, we believe that the experimental data meaningfully complement the simulations. In particular, they demonstrate similar microscale mechanical properties (e.g., relative cell-cell tension) and reproduce macroscopic viscoelastic fusion behavior in line with our model predictions. A detailed comparison suggests that under certain experimental conditions, especially for Blebbistatin-treated and ROCKi+ samples, the tissues exhibit fluid-like behavior but are close to the jamming threshold. This proximity is also reflected in the fusion angle decreasing below 90°, which is consistent with the model's prediction near the transition regime.

We also acknowledge the reviewer's suggestion to compare with more strongly arrested experimental systems. Indeed, Oriola et al. (Soft Matter, 2022), Ref. 11, have reported clear experimental evidence of arrested coalescence in larger multicellular aggregates. However, such large-scale aggregates are beyond the scope of our imaging and simulation capabilities, particularly in terms of tracking dynamic fusion events using two-photon microscopy. As such, while we can qualitatively compare our findings with theirs, a detailed quantitative comparison is not feasible. We do note, however, that our analytical framework is conceptually very similar to the one developed in that study.

Conclusion

The experimental part is very rich and brought new features on spheroids shape and cell motility, in particular how cells of the two aggregates mix by diffusion at the interface. They are interpreted by a viscoelastic model. An earlier model developed in the past by Shanahan in the spirit of JKR, leads to different dynamical scaling laws. It must be introduced and discussed. To describe the interplay between single cell properties and tissue mechanics, the authors develop a 3D active foam model with deformable cells parameterized by relative cell-cell tension and motility. The model reproduces fusion dynamics while capturing cell shape and motility. The fusion of aggregates at a macroscopic scale is linked to fluidization at a microscopic scale. Even if the experimental observations can "globally" be explained by existing, simpler viscoelastic fluid models (except for the surprising observation that fusion dynamics for less granular spheroids upon ROCKi removal decelerated despite increased cellular rearrangements, which cannot be explained by the proposed numerical model either, as mentioned in the article's discussion), the experimental part is useful to enter in the complexity of the simulations. I recommend publication if more experimental data on arrested coalescence are added to the paper to allow a real confrontation between jamming and arrested coalescence and if similar earlier works are introduced and discussed.

Again, we want to stress that our study is not about the phenomenon arrested coalescence alone. This is more clear in the revised manuscript. Nonetheless, we kindly point the referee to the **new Extended Data Fig. 1**, where we present additional fusion experiments, including experiments using larger aggregates of ±500 cells. This combined data demonstrate that - while fusion angles are still close to 90 degrees, there are still conditions where it is consistently below 90 degrees.

In the revised manuscript, we also discuss and refer earlier works suggested by the referee, including Douezan et al. 2011 and Beaune et al. 2022.

Reviewer #2 (Remarks to the Author)

The subject of the article is timely and of fundamental relevance to tissue development. The manuscript is generally well-written and insightful. The specific points we list below concern mainly unclear writing/definitions, overly assertive statements, and lack of a comprehensive contextualization of the authors' work within the existing body of literature on cell-based modeling and tissue fusion.

We thank the referee for this assessment, and hope the revised manuscript and this rebuttal letter adequately address the concerns raised by the referee.

The provided code consists mostly of Python wrapper scripts and looks clean. However, MPacts (https://mpacts.com) was used as a backend to do all the simulations. The software seems to be closed-source. We notice that one of the co-authors, Bart Smeets, is listed as one of the developers of Mpacts, and several academic papers are listed that used the software.

As such, we were not able to check the implementation of the model. We consider it absolutely necessary that the simulation code is made public when the paper is published.

We thank the reviewer for raising this important point regarding code accessibility and reproducibility. To ensure transparency and reproducibility, we now provide a link in the revised manuscript to a public GitLab repository that includes all relevant model code developed for this work. This includes:

1. All Python source code that defines the simulation setup and physics;
2. The relevant C++ subroutines that were used for performance-critical components;
3. The binary of the Mpacts particle-based simulation backend. This serves as an execution layer that translates Python-defined mathematical expressions into efficient low-level routines for particle-based simulations.

All code implementing the physical model described in this paper is fully open and available. Furthermore, since the mathematical expressions used in Mpacts are specified at the Python level and are directly inspectable, the reviewer and future readers can fully verify the implementation details that are scientifically relevant to this work.

We provide a general overview of the software and source code here

https://gitlab.kuleuven.be/mebios-particulate/publications/mpacts-active-foam-dynamics-of-tissue-spheroid-fusion

Moreover, we also added a fully annotated 'demo script' (containing the full model in a demo fusion simulation), which can be found here:

https://gitlab.kuleuven.be/mebios-particulate/publications/mpacts-active-foam-dynamics-of-tissue-spheroid-fusion/-/tree/main/scripts/fusion_demo

Here, you will find

1. Detailed instructions on how to run the demo simulations using the attached docker container, which is registered in the Gitlab repo.
2. A detailed overview of the components in the script, together with links to the relevant source code that implements them.

We believe that this demo script serves as the best entry point when exploring the underlying source code related to the active foam model.

Lastly, we would like to clarify that while the senior author was involved in the early development of Mpacts, they have no financial stake, ownership, or operational role in the company or its current development. We hope this clarification addresses the reviewer's concerns and satisfies the code availability requirements of the journal.

In the revised manuscript, we added the following Data Availability statement:

> "The datasets generated and analyzed during the current study are publicly available on Zenodo [zendo reference] This repository includes raw microscopy data, processed datasets, simulation software and scripts, analysis tools, and relevant metadata necessary to reproduce the results presented in this article."

and the following Code Availability statement:

"The source code related to the simulations presented in this study is publicly available on GitLab [Gitlab reference] To facilitate reproducibility, the complete software environment—including all dependencies—is provided as a Singularity image. This image, together with the simulation scripts used to generate the reported results, is archived and documented in the Zenodo repository [zenodo reference]."

Specific points:
It is unclear to me why the authors cite only Ref. 25 in the introduction to support the statement that "Recent expansions of these models also include inter-cellular pores". There are rather many such models, also in 2D. For an overview, see Table 1 in Vetter et al., Comput. Phys. Commun. 299, 109128 (2024).

We apologize for this omission, and we acknowledge that there are quite a lot of papers containing vertex-like cell representations with intercellular pores. In the revised manuscript we also cite the provided reference of Vetter et al., as well as Brown et al. Nat Comp Sci 1.11 (2021): 754-766.

A quick search for previous experimental work on tissue and spheroid fusion reveals a body of literature that the authors don't mention in their work. Some of them report contact angles and fusion times. Although some of these systems will be different from the author's spheroids, it would nevertheless appear appropriate to discuss how the results presented in this manuscript compare to these prior studies. A starting point could be the following publications (in no particular order):
Ray & Niswander, Development (2012) 139: 1701–1711
Olsen et al., Acta Biomaterialia (2015) 13: 188-198
Lindberg et al., Adv. Sci. (2021) 8: 2103320
Rago, Dean & Morgan, Biotechnology and Bioengineering (2009), 102: 977-1282
Livoti & Morgan, Tissue Engineering Part A (2010) 16, 2051-2061
Hajdu et al. Journal of Tissue Engineering and Regenerative Medicine (2010) 4: 659-66

We thank the referee for pointing out these publications. We did not aim to exclude important works if they are relevant for our results.

- *Ray & Niswander, Development (2012) 139: 1701–1711*
  This (interesting) review paper mainly deals with biological fusion in development. While described with the same term (fusion), some of these mechanisms, such as neural tube fusion, bear little resemblance to the fusion investigated in our work.
- *Olsen et al., Acta Biomaterialia (2015) 13: 188-198*
  This paper describes some nice example of cellular mixing and is thus relevant for

discussion of tissue fluidity. While qualitative, they show how low collagen content spheroids showcase both increased fusion and increased cell mixing compared to high collagen content spheroid. This observation is consistent with our findings and simulations.

Lindberg et al., Adv. Sci. (2021) 8: 2103320

Also this work, while rather qualitative on the fusion dynamics, showcases that there is a link between degree of fusion (which is approximated by the 'area change' they show in Fig3, as higher area change would correspond to a greater fusion angle) and the ability of cells to interpenetrate, which is shown in Fig4A,D.

In the revised manuscript, we cite the previous two works in discussion, p8

"Our results show that complete fusion, corresponding to the coalescence of liquid droplets at the macroscopic level, is closely linked to tissue fluidization at the microscopic level, as illustrated in Fig. 5. This finding is compatible with previous experimental results that show that increased fusion is associated with a higher degree of cell mixing [Olsen2015,Lindberg2021]."

- *Rago, Dean & Morgan, Biotechnology and Bioengineering (2009), 102: 977-1282*
  and
  Livoti & Morgan, Tissue Engineering Part A (2010) 16, 2051-2061
  These are excellent examples of using the properties and degree of spheroid fusion during biofabrication to obtain various different geometries. We now cite these work in the discussion.
- *Hajdu et al. Journal of Tissue Engineering and Regenerative Medicine (2010) 4: 659-66*
  While interesting, we believe that these results are too qualitative to help understand our results

Are \tau_\Gamma and \tau_\epsilon the two free fit parameters used in Fig. 1? This isn't clearly stated.

The actual fitted parameters are the fusion timescale τ_f, the constant β (see methods) and an initial (small) fusion angle θ_0, see last equation (now labeled Eq. 4 in the revised manuscript) in the methods section "Visco-elastic model for tissue spheroid fusion". We clarified this in this method section, where we now state on p11:

"We fitted Eq. (4) to experimental and simulated fusion curves (t, θ) with fit parameters β , τ_f and θ_0 using 'curve fit' from the Python package SciPy [55]."

In the caption of Fig. 1, it is unclear to me how many repetitions were done. It is stated that "Data acquired from a single experiment, n = 7, 10, 8, 9", but what is n, if it is a single experiment?

During one experiment, we monitor fusion of multiple spheroid pairs in parallel. There are two sources of variability in the experimental outcome: variability between individual cases (spheroids) within the same experiment, and additional variability between different experiments. In this case n refers to the amount of spheroid doublets on which this plot is based. We repeated the same experiment 5 times, as can be seen in Supplementary Fig. 2. In Fig 1. D-E, the pooled result of these experiments is presented. Here n represents the total combined number of repeats across these 5 experiments. We fitted a fusion curve through every single repeat (single fusion curve of xone spheroid doublet). Note that n varies between conditions because 1) there are empty wells or wells with more than two spheroids, 2) some contain pre-fused aggregates, which are discarded or 3) the spheroids never made contact, or no contact point could be determined.

In the revised caption of Fig. 1 we now clarify that Fig. 1D-E stems from 5 different experiments.

In all figures that have them, it is unspecified what the error bars represent.

We clarified what error bars represent in all figures containing box plots and error bars. In Fig. 1D-E, the horizontal bars represent the mean and extrema (min-max) of the data. We thank the reviewer for pointing this out

On page 3, the statement "there is no singular connection between cell-cell tension and fusion time" is potentially misleading. What do the authors mean by "singular"? Single? Simple? This sentence isn't clearly enough linked to the data.

We rephrase this on p3 to now state:

> "Thus, while cell-cell tension governs tissue surface tension, the observation of faster fusion times in C8 implies that fusion time cannot be directly inferred from cell-cell tension alone, as additional factors likely contribute."

We believe that this statement is directly supported by the data presented.

On page 4, the authors write that "fusion proceeded notably slower in absence of ROCKi". This seems to be in contradiction to Fig. 1D, which shows that the Bleb/ROCKi+ condition lets them fuse even slower.

Our phrasing was confusing here. We meant that fusion proceeded notably slower in absence of ROCKi compared to C8 (which contains ROCKi). In other words, we observe that fusion times first decrease (faster fusion) when adding ROCKi but then increase again when adding additional ROCKi and blebbistatin.

In the revised manuscript, we now clarified this on p5 as:

> "Interestingly, despite decreased relative cell-cell tension and increased cell motility, fusion proceeded notably slower in absence of ROCKi compared to C8, which contains ROCKi."

In Fig. 2E, how is "height" defined? Height of what, measured how?

In Fig. 2E, height refers to the position along the z-axis in a 3D image stack, relative to the bottom of the spheroid (which is arbitrary). This vertical spatial information is represented in a 2D image using a method called depth encoding, where each z-slice is assigned a specific color to indicate its depth, instead of using a monochromatic look-up table for the representation of pixel intensities, and subsequently reducing from a 3D stack to a 2D image by taking the maximum intensity projection.

We clarified this in the revised manuscript and now state in the figure caption on p4, Fig. 2:

> "E: Time-lapse of cell nuclei (DAPI staining) from two-photon microscopy imaging colored by z-position (relative to the bottom of the spheroid)."

We also changed the label 'Height' to 'z-position' in Fig. 2.

In Fig. 2G, what is the uncertainty of the shown MSRD curves? How to the authors ensure statistical significance in the different results between the conditions?

In the original manuscript, we could not establish statistical significance, which is a valid concern. To address this, we have performed additional experiments to obtain 3 independent repeats for each condition, allowing statistical comparison. Note that these experiments are extremely time-consuming reaching the limits of devices, both in experimental time as well as in data analysis.

In the revised manuscript, we now changed **Fig. 2-F,G,H** to present the results of these experiments. These experiments confirmed our initial conclusions, and from statistical analysis, we establish that ROCKi^{-} is significantly more active than both Bleb/ROCKi^{+} and TGF^{-} conditions.

How many repetitions were made for each condition in Figs. 3F,G, 4C,D, and Supplementary Fig. 4?

In all parameter studies presented in Fig 3-4, we performed 10 random repeats for each studied parameter combination, as follows:

1. For that parameter combination, we create a virtual library of 10 spheroids, indexed [1,2, … 10] as described in the methods section
2. We then simulate fusion between [1/2, 2/3, … 9/10, 10/1], where each spheroid is randomly rotated and two spheroids are positioned next to each other. This gives rise to 10 random repeats. Note that due to the combinatory effect, they are not precisely 'independent repeats' but they are for all practical purposes.

In the revised manuscript, we now clarified this in Method section "Simulation setup", p13,

> "To simulate fusion, two spheroids are initially positioned with minimal overlap. First, two sequential spheroids from the library are selected and centered with random orientations. They are then translated along the $\vec{e}_x$ axis until their boundaries touch. The minimum translation required for geometric contact is computed; if it exceeds a critical threshold, the spheroids are moved further apart and the process is repeated. If it is below the threshold, the spheroids are moved to achieve contact and the iteration ends. Finally, the simulation is centered at the origin. For parameter combinations that resulted in numerical instability, we only retained results if at least 5 runs completed successfully; otherwise, the condition is reported as `failed to converge.' All simulations were carried out in Python using the Mpacts particle-based simulation framework."

and also state this in the Figure caption of Fig 3-4.

Is there sufficient statistical power to support the claim that "Fusion time-scale first increases with pa at low motility, while it decreases with pa at higher levels of motility"? Fig. 3G seems rather noisy, such that the statement on the dependency on pa seems unsupported (assuming each pixel in the plot shows a single simulation).

Each pixel in the plot shows not a single simulation but the average across 10 random repeats. The reason this plot appears noisy is that the fusion time is the inverse of a relaxation rate. In the extremely granular case ($\alpha \to 1$), the relaxation rate becomes close to zero, and the fusion time as well as its variability between repeats becomes very high. However, for all other cases, the trend is highly consistent, as shown in the plot below which is added as the **new Supplementary Fig. 6**. This shows the median and interquartile range values of the fusion angle (left) and fusion time (right) when varying activity (x-axis) for varying α, excluding the two highest values of α with very granular behavior.

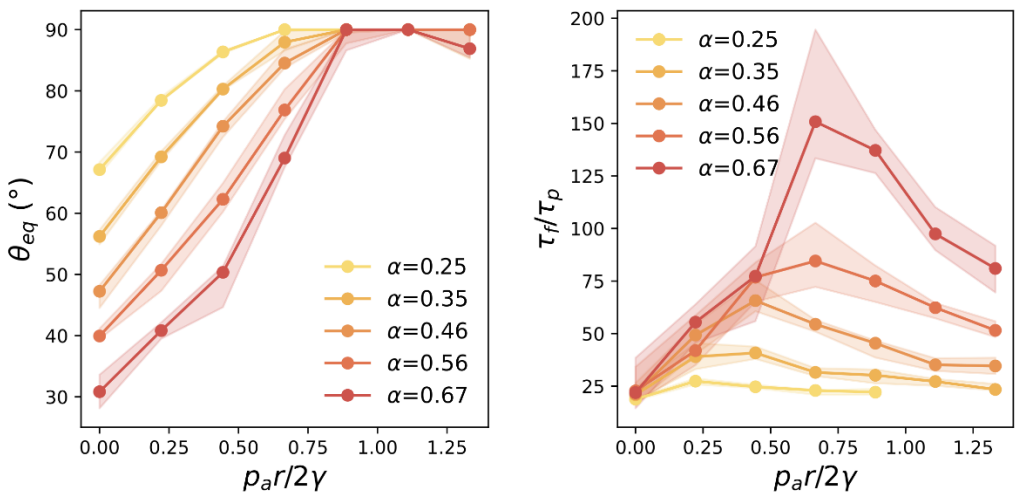


Fig. New Supplementary Fig. 6, showing fusion angle and fusion time for varying cell activity at different values of relative cell-cell tension, excluding the two highest studied values which contain granular behavior.

The statement that "fusion time-scale first increases with pa at low motility, and then decreases with pa at higher motility levels" is strongly supported by this data. The trend is robust and reproducible across different α values, as demonstrated in the supplementary figure. While statistical significance testing could be performed, we believe it adds limited value here since the differences are visually and numerically clear, and the trend remains consistent across conditions. Nonetheless, we included the supplementary figure to provide transparent and comprehensive support for our claim.

Apparently based on the data shown in Fig. 3c, the authors state on page 6 that "the critical active pressure to fluidize the tissue material scales as pa,crit ∝α^{−1/2}". This strong statement is not sufficiently supported by the data. It looks like it may be approximately correct, given the coarse data, but this is not conclusive by any means. Verifying a power-law relationship as claimed here would require fine data over at least about two orders of magnitude, and a fit that yields an exponent that is statistically consistent with the claimed one. As I'm assuming the authors do not have such data, the statement should be weakened clearly to reflect the uncertainty.

Based on Fig. 4C, we indeed only conclude that the critical pressure to unjam *appears* to scale as $p_{a,crit} \propto \alpha^{-1/2}$. We therefore also weakened this statement on p6 to:

> "The critical active pressure to fluidize the tissue material appears to scale as $p_{a,\text{crit}} \propto \alpha^{-1/2}$."

However, we are quite confident about this scaling based on the analysis in Fig. 4E. We refer the referee to our reply on their remarks on Fig. 4E below.

The term "Normalized unique contacts" is not clearly defined. The caption of Fig. 4 writes that it is the total number of unique contacts during fusion divided by average number of contacts. Unique in what way? How is "during fusion" defined? Averaged over what? Time, or the cells? This makes it hard to interpret Figs. 4C,E,F,H.

This is the total number of unique contacts during a fusion simulation, divided by the average number of contacts in that simulation over the whole simulation runtime (set to 24 h, although time is non-dimensionalized in most cases, so corresponding to 864 τ_p unless stated otherwise). The precise meaning or scaling of 'Normalized unique contacts' is not important, just that it serves as a clear order parameter for tissue fluidity – in that it sorts correctly simulation samples according to fluidity. That this is the case can be observed by comparison to other metrics of tissue fluidity, for example $MSRD_{60}$ (the ensemble-averaged mean squared relative displacement for a lag time of 60 min), as can be seen in Fig. 4F (inset), or by comparing Supplementary Fig. 9C and F.

In the revised manuscript, we now define the normalized unique contacts more clearly in the caption of revised Fig. 4 on p7:

> "Normalized unique contact number (total number of distinct cell-cell contacts formed over the full simulation, divided by the time-averaged number of contacts) for varying \$\alpha\$ and \$p_a\$. Dashed lines show the scaling \$\alpha \propto (p_a r / 2\gamma)^2\$."

The origin of the dimensionless activity parameter A is not explained. Where does this scaling come from? Is it just a phenomenological observation that the authors came up with by trial and error? Is there an intuitive derivation or interpretation for it? Are the exponents -1/2 and 1/4 exact, or just guessed? This needs to be clarified to allow the readership to judge its credibility.

The observation of data collapse stems from phenomenological observation (one of the strengths using model for this kind of purpose, as these mechanisms are intricate and their scaling relation not straightforward). However, there is a theoretical interpretation of this scaling that is both intuitive and consistent.

The derivation of this theoretical scaling is added as the **new Methods section "Theoretical scaling of tissue fluidity"**. Briefly, the rationale for this scaling is based on the analogy between active protrusions and cell protrusions generated in a micropipette aspiration experiment. Such

a protrusion is unstable (i.e., it will grow indefinitely), if the protrusion length exceeds its radius, a classical result from micropipette aspiration (see Figure below).

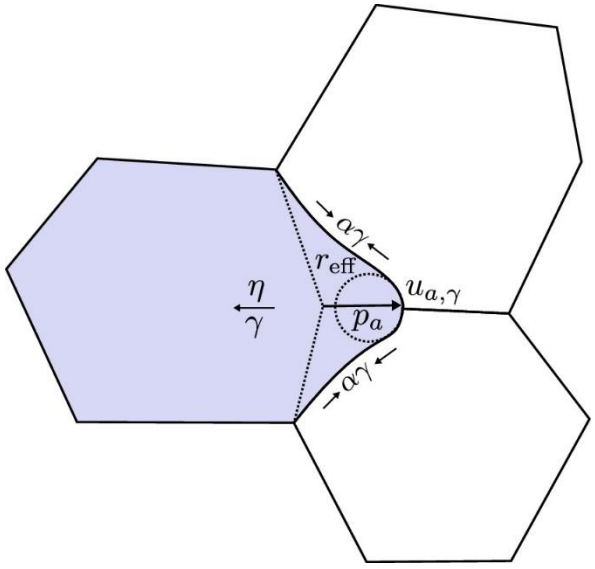


Figure: Schematic representation of active protrusions driving topological transitions in our active foam model

In our case, protrusions are generated by active pressure p_a, while they are resisted by the tension at the interface, $\alpha\gamma$. At long times, we can approximate the protrusion length of a given protrusion using an Ornstein-Uhlenbeck process, with relaxation time $\tau_\gamma = \frac{\eta}{\gamma}$ and some effective diffusivity of the front D_a. Given that the cell is performing a persistent random walk, we can assume that $D_a \propto v_a^2 \tau_p$ at long timescales, with active velocity $v_a \propto p_a r^2/\eta$. This yields a characteristic protrusion length $L_a^* \propto \frac{p_a r^2}{\gamma}\sqrt{\frac{\tau_p}{\tau_\gamma}}$. Meanwhile the radius of the protrusion is determined by Young-Laplace law, $r_a^* \propto \frac{\alpha\gamma}{p_a}$. Since the instability criterion of a protrusion is $\frac{L_a^*}{r_a^*} > 1$, we obtain an activity parameter that controls tissue fluidity, $\mathcal{A}^2 \propto \frac{L_a^*}{r_a^*}$, which is the observed scaling. In this derivation, we assumed that surface effects are important (since the relaxation time is τ_γ and not $\frac{\tau_\gamma}{\alpha}$. Otherwise, we predict a scaling $p_{a,crit} \propto \alpha^{\frac{3}{4}}$ which should hold for 3D bulk tissues. In the revised manuscript, we now state on p6:

> "The observed scaling can be rationalized by a minimal model in which active protrusions must exceed a critical length $L_{a,y}^* > r_{a,\alpha\gamma}^*$ to grow, with $r_{a,\alpha\gamma}^* \propto \alpha\gamma / p_a$ (see Methods), akin to a micropipette aspiration threshold \cite{hochmuth2000micropipette}. Modeling protrusion length as an Ornstein–Uhlenbeck process with relaxation time

τ_γ and effective diffusivity $D_a \propto v_a^2 \tau_p$, where the active front velocity scales as $v_a \propto p_a r^2 / \eta$, yields a characteristic protrusion length $L_{a,y}^* \propto (D_a \tau_\gamma)^{1/2}$. This leads to the dimensionless activity scaling as $\mathcal{A}^2 \propto L_{a,y}^*/r_{a,\alpha\gamma}^*$."

We also performed a statistical analysis to estimate the coefficients of the scaling law. This procedure is described in the **new Method section "Scaling exponents of tissue fluidity"**. Here we determine the exponents a_i for dimensionless groups i, that minimize a loss function based on Spearman's rank correlation coefficient (i.e., we want to obtain a scaling that preserves the rank and minimizes the standard deviation between successive differences). The result of this analysis is presented in the **new Supplementary Fig. 8**. Here we arrive at the following coefficients:

Coefficient	Optimized	Theoretical / Estimated
$\boldsymbol{a_1}$	-0.516	-1/2
$\boldsymbol{a_2}$	0.274	-1/4
$\boldsymbol{a_3}$	0.070	0

On page 7, it is written that the activity parameter A scales differently from the effective temperature in an active vertex model, citing ref. 16. For clarity, it would be helpful to explain why the authors consider this comparison meaningful, even though the quantities (A vs. temperature) are different. Moreover, the cited reference 16 mentions the scaling Teff~v0^2 as a conjecture, not a conclusive result, but from the authors' text it appears as though this was a fact.'

In the revised manuscript, we removed this interpretation altogether and leave this interpretation to the reader.

On page 7, the statement "the tissue viscoelastic timescale sharply increases with activity" is made. It is not clear to me how this is supported with data, as I can't find this relationship shown anywhere.

Our original statement was:

> "Jamming coincides with arrested coalescence, where θ_{eq} and, correspondingly, the tissue viscoelastic timescale τ_ϵ, sharply increase with activity."

1. The observation that in this regime, theta_eq increases with activity is clear from the **revised Fig. 4f**, where we now show the $MSRD_{60}$ in a logarithmic scale.
2. In our visco-elastic fusion model, the visco-elastic timescale τ_ϵ is directly related to the equilibrium fusion angle as explained in the methods section "Visco-elastic model for tissue spheroid fusion", since $\theta_{eq} = \arccos(-\beta + \sqrt{\beta^2 + 1})$, which monotonously increases with β and $\beta \propto \tau_\epsilon$.

   In the revised manuscript (), we omitted the word 'sharply' since while the trend of τ_ϵ with θ_{eq} is obvious from the math, the sharpness of this trend is not.

Fig. 4g is unclear. What is x? Are the curves from individual simulations, or averaged?

These are averaged over 10 random repeats, like the other simulation results. This has been clarified in the revised manuscript, in the caption of revised Fig. 4.

Fig. 5 introduces another time τm that doesn't appear elsewhere in the manuscript (only in the caption of Extended Data Fig. 2).

In the revised manuscript, we have removed the reference to the symbol τ_m in the main text to avoid confusion, and instead refer more generally to the "mixing time", which is now clearly explained in the caption of Fig. 5. The mixing time can be understood as the minimum lag time at which the mean squared relative displacement (MSRD) exceeds r^2. It is closely related to the inverse of the MSRD60 value shown in Fig. 4. However, we note that in the jammed regime, particles do not undergo relative rearrangements, and mixing does not occur. Consequently, the mixing time formally diverges in this regime and cannot be reliably extracted from simulation data.

In Eq. 4, the symbol T is undefined, and the integral should probably be a definite integral.
In the section "3D active foam model" of the Methods section,

We agree that the previous definition was not precise enough. We have revised this method section to more precisely state how the MSRD was obtained in simulations and experiments.

the definition of the internal reaction traction Tc,i (which seems to be a vector) appears to be a scalar integral without direction.

In this expression d**S**_i is the outward oriented surface element, which is a vector. Hence, the total reaction traction is also a vector. We adapted this method section to better explain that d**S**_i is an outward oriented surface element and also use the closed integral to emphasize the integration over a closed surface (the cell).

In Eq. 4 of the Supplementary Note, the volume strain history term is unexplained. What is its purpose, and how did the authors ensure that their solutions are not history-dependent (or is such a dependence intended)?

We want to model the cytoplasm as an incompressible fluid. Ideally, one would model this as hard constraint for convergence for every timestep. However, such an approach would be numerically very complicated and require at least the use of a quadratic programming approach. Alternatively, one could just model a compressible cytoplasm with a bulk modulus K. However, as $K \to \infty$ (roughly, water), such an approach is numerically unstable. Hence, we opt for an 'engineering solution', where we consider the cytoplasmic volume as a control parameter that needs to be maintained around its equilibrium volume with sufficient accuracy. For this, we use a PI-controller, which adds an integrative term τ_I on top of an effective bulk modulus K (proportional term). As long as this timescale is smaller than physical timescales of interest, the volume is approximately kept constant, and there are no noticeable history effects. This is discussed as well in

Cuvelier, M., Vangheel, J., Thiels, W., Ramon, H., Jelier, R., & Smeets, B. (2023). Stability of asymmetric cell division: A deformable cell model of cytokinesis applied to C. elegans. *Biophysical Journal*, *122*(10), 1858-1867.

and the supplementary information thereof.

Of course, we are aware that cells are not truly incompressible materials (in that water may flow in and out of the cell as a result of pressure). However, assuming near-incompressibility removes this effect as a parameter affecting observed behavior.

The authors do not provide a link to a code repository. I would strongly encourage them to make their source code publicly available.

In this revision, we include a link to a GitLab repository containing all relevant simulation code and implementations associated with this project. The repository also provides the backend framework for running particle-based simulations in Python. This reflects our commitment to making our code as openly accessible as possible.

Writing errors:
Line below Eq. 1: "visco-elastic" -> "visco-elastic time"

Corrected, thanks!

The figures were not always correctly referenced, e.g. where it says figure 3c, figure 4c seems to be meant.

We assume the referee refers to:

“resulting in the loss of distinct boundaries between the original tissues (Fig. 3c).”

Here we wanted to refer to the visualization of the simulation to highlight the loss of distinct boundaries between the tissues .

Also in the supplementary table for the forth condition the units for ROCKi say micro instead of micromolar

Corrected, thanks!

Page 6: “Fig. 3k” -> “Fig. 3d”

Corrected, thanks!

Discussion: “tissue shapes an decreased” -> “tissue shapes and decreased”

Corrected, thanks!

Page 9: “This procedure performed” -> “This procedure was performed”

Corrected, thanks!

Below the definition of τf in the Methods, the spheroid radius before fusion, R0, is mistakenly called a0.

Corrected, thanks!

First line of page 12: “between between”

Corrected, thanks!

There are some formatting issues in the bibliography, such as missing volume numbers, wrong capitalization (“Iscience” instead of “iScience”) and so on.

We reviewed all references in the bibliography, hopefully all these issues are fixed.

Figure 2b: description says four conditions but there are only three displayed, TGF-b is missing.

Indeed we opted to show only 3 conditions, since TGF- did not look visually distinct from C8 (as in most of our results), see also Fig 2A. The caption was corrected.

> “\textbf{B}: Circular approximation of spheroid periphery, showing contour radii and contact angles for conditions C8, ROCKi$^-$, and Bleb/ROCKi$^+$.”

Figure 2c: in the description C8 condition is forgotten.

Corrected, thanks.

Caption of Extended Data Fig. 2: “time affect fusion” -> “time affects fusion”

Corrected, thanks.

Caption of Extended Data Fig. 5: “shape index ¿ 5.41”

Corrected, thanks.

Reviewer #3 (Remarks to the Author):

Ongenae et al. developed a 3D computational model based on the recently introduced "active foam" framework to capture the dynamics of spheroid fusion. Unlike earlier versions, this approach successfully replicates complex 3D cell dynamics and incorporates a novel parameter—cell-cell friction—which the authors highlight as a critical factor for accurately modeling tissue fusion. Notably, the study reveals that jamming and arrested coalescence can arise even when cells exhibit an "unjammed" shape index. The use of high cell-cell friction to explain these phenomena is particularly compelling.

We thank the reviewer for this encouraging assessment of our work.

While the computational model is robust, the biological alignment with it remains somewhat unclear to me. However, I view this ambiguity as a strength, as it opens the door to extensive experimental exploration. For instance, what role does cellular granularity play? And why can fusion occur without substantial cell rearrangement? These are intriguing questions that could inspire follow-up studies.

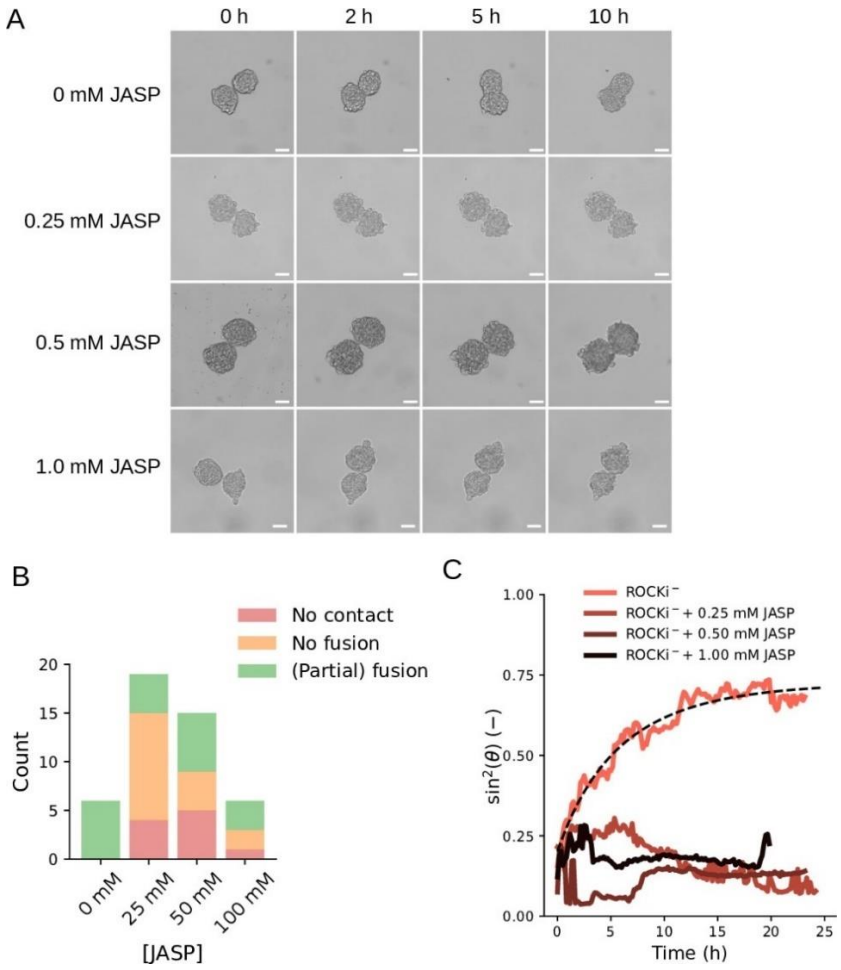
We strongly agree with the reviewer

Overall, I find the study to be meticulously executed. I have only one major concern: is ROCKi exerting only the expected effects, or could some of the "unexpected" outcomes be due to off-target effects? I suggest the authors explore alternative inhibitors targeting actin to clarify this. Additionally, it would be valuable to examine the effects of enhanced actomyosin contractility

using drugs like calyculin or jasplakinolide. Testing these conditions could significantly strengthen the manuscript and won't take too long.

We agree with the reviewer that addition of ROCK inhibitor (ROCKi) could lead to various unintended outcomes due to potential off-target effects. Notably, ROCKi is routinely included in the C8 chondrogenic differentiation medium, as it has been found to enhance chondrogenic differentiation. This effect may stem from direct ROCK inhibition or from downstream alterations in the mechanical microenvironment and associated mechanotransduction pathways.'

To further probe the role of actin dynamics independently of ROCKi, we tested treatment with jasplakinolide (JASP), a compound known to stabilize F-actin and inhibit actin depolymerization. By effectively "freezing" the cytoskeletal architecture, JASP prevents dynamic remodeling of the actin network. We applied varying concentrations of JASP in the absence of ROCKi, to avoid potential interaction effects. The results, presented in the **new Supplementary Fig. 1** (also shown below), show that JASP strongly inhibits spheroid fusion.



This is consistent with our model predictions: In our activity parameter $\mathcal{A}$, actin remodeling is captured through the relaxation timescale τ_γ, which increases when remodeling is inhibited. Furthermore, cell stiffening induced by JASP is expected to increase γ, which also reduces the activity parameter.

We discuss these findings in the revised manuscript, where we now state on p3,

> "Finally, we inhibited actin disassembly with jasplakinolide. This strongly impaired spheroid fusion, indicating that cytoskeletal remodeling is essential for spheroid fusion (Supplementary Fig. 1)"

I strongly recommend this paper for publication in Nature Communications. However, I encourage the authors to make the text more accessible for readers less familiar with computational modeling and tissue mechanics, as this could broaden the work's impact.

We agree with the assessment of the reviewer that some parts of the paper could be hard to follow through, especially since we aim to be precise on the modeling, necessitating the use of mathematical symbols and dense language to describe the modeling methodology in a concise yet correct manner.

For example we changed the opening sentence of the results section on modeling to:

> "To study the physical behavior of multicellular aggregates during tissue fusion, we developed a 3D active foam model in which each cell is treated as a soft, deformable object. The model mimics how cells interact, move, and deform under mechanical forces, similar to how bubbles behave in a foam."

Moreover, we changed the intro section where these types of models are introduced to

> "More recently, these models have been extended into ``active foam'' frameworks that represent cells as soft, space-filling bubbles capable of generating internal forces […]. This approach allows si mulation of both shape and movements of cells, including the formation of dynamic intercellular spaces. Foam-like models have also proven effective for inferring mechanical forces from the 3D geometry of cells in tightly packed aggregates […]."

Minor Comment:

The statement, "Elimination of either ROCKi or TGF-β1 resulted in deceleration of fusion," warrants reconsideration. Figure 1c shows a small difference between C8 and TGF-β1, but this is

not reflected in Figure 1d. Based on this discrepancy, it may be inaccurate to claim that the elimination of TGF-β1 significantly decelerates fusion.

This is a mistake on our part, and we thank the reviewer for pointing this out. We corrected this in the revised manuscript as:

> "Elimination of ROCKi resulted in deceleration of fusion (Fig. 1c)."