

Nuclear Packing Sets Fluidity Along the Epithelial to Mesenchymal Spectrum

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Investigations of jamming in cancer have produced numerous phase diagrams intending to map fluidity across the epithelial-to-mesenchymal transition (EMT). Here, we use coalescence of multicellular spheroids to examine and carefully probe these phase spaces. Small changes in cellular EMT status result in full traversal of the solid-to-fluid continuum. Variations in fluidity as measured by degree of spheroid coalescence are captured by two-dimensional assessment of static cellular properties including internuclear spacing and nuclear shape, which can be determined in individual noninteracting spheroids. We combine these quantities into an effective nuclear packing metric that depends on nuclear occupancy and nuclear elongation. We find that dense nuclear packing impedes spheroid coalescence and that lowering effective nuclear packing by altering nuclear shape fluidizes an otherwise solidlike system. Furthermore, we employ heterotypic spheroids to tune effective nuclear packing and coalescence behavior, thus introducing a novel method of fluidity phase space exploration. Together, these findings reveal that nuclear occupancy and morphology act as important indicators and determinants of fluidity.

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I. INTRODUCTION

Cell migration is a key component of processes such as embryonic development, wound healing, and metastasis. It has been proposed that these processes, as well as cell migration generally, can be viewed through the framework of jamming transitions. For example, cells in solidlike tumors unjam and migrate during metastasis, bronchial epithelia compress and flow in asthma, and *Drosophila* development depends on a fluidlike transition during convergent extension [1–4].

Traditionally, jamming has been used to describe passive physical systems such as grains of sand [5]. The phase space that has been used to map jammed systems includes contributing factors of temperature, density, and load [6]. Such a jamming phase diagram can be mapped to biological systems, with active fluctuations, volume fraction, and supracellular stress as the relevant analogous parameters [7–9]. With the surge of studies of biological jamming in different contexts, other phase spaces have been proposed using parameters such as cell-cell adhesion, cell contractility, cell shape, and cell area [10–13]. These phase spaces have been mapped extensively with computational models [1,14–16], as well as with histological samples [10]. However, few studies have experimentally explored these proposed phase spaces, in part because tools are lacking to perturb these cellular features independently.

Of the many possible jamming parameters, cell shape has been shown in both experiments and simulations to have an important role in fluidity. Specifically, a jamming transition is observed as cells become rounder [2,17]. Beyond this, nuclear shapes and deformability have recently gained attention as playing a major role in mobility [10,11,18–24]. It has been reported that mobile cells have more elongated nuclei, as to navigate a crowded environment cells need to pass their nuclei between the surrounding cells or extracellular structures [25]. This leads to the idea that in tissues nuclear packing may play a role in mobility. Previously, confluent cellular systems were understood to have a packing density of one, as there is little to no intercellular space; however, given cell capacity to deform, there has been growing recognition that cellular packing density may not be the most relevant parameter. Indeed, recently it was shown that the area fraction of the nucleus to the cytoplasm plays a role in cellular mobility, with higher nuclear area fractions leading to denser packing and greater tissue stability [20]. However, there remains limited work that directly probes the role of the nucleus in fluidity of cellular systems either in computational studies or experimentally.

In this work, we use multicellular spheroid coalescence to probe the fluidity of different cell types and elucidate the role of the nucleus in jamming. Spheroid fusion has been used extensively to investigate cellular jamming, with macroscopic readouts such as the degree of fusion giving insight into cell mobility and mechanical properties such as surface tension [11,26–31]. We find that small changes in epithelial-to-mesenchymal transition (EMT) status can recapitulate the full solid-to-fluid range of coalescence. We show that both elongated cell and nuclear shapes and loose nuclear packing are correlated with higher degrees of fluidity. When nuclei—which are the stiffest cellular component—are tightly packed, they are an impediment to fluidity [32]. Altering

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nuclear shape, potentially through softening the nucleus, can fluidize a formerly solidlike system. Furthermore, we show that heterotypic spheroids can be used to actively tune the fluidity of the system, allowing for careful investigation of jamming phase spaces. Our results confirm the finding that cell and nuclear shapes determine the onset of fluidity of our system, with cell area having a small, independent additional contribution to the degree of fusion. Beyond this finding, we propose a metric that captures nuclear occupancy and shape, providing a single-parameter structural predictor of fluidity.

II. RESULTS

A. Small differences in EMT status are sufficient to drive a solid-to-fluidlike transition

To probe the mechanisms and significance of fluid-solid behavior in cancer progression, we employ an isogenic cell line pair consisting of MCF10A (10A) and MCF10DCIS.com (DCIS), a model normal breast epithelial cell line and a minimally transformed 10A derivative, respectively. In brief, DCIS cells are derived from a xenograft of premalignant MCF10AT cells injected into immunodeficient mice such that ductal carcinoma *in situ* lesions form [33]. In this way, DCIS cells represent an early, noninvasive (pre)cancer that may eventually progress into invasive ductal carcinoma. 10A and DCIS cells share many characteristics, including similar expression levels of epithelial and mesenchymal markers such as E-cadherin, N-cadherin, and vimentin [34–36]. As a result, both cell lines are relatively epithelial in character. This experimental system is in contrast to previous work that examines differences between cell lines on extreme ends of the EMT spectrum [11].

To interrogate the fluidity or solidity of each cell type, we utilize a multicellular tumor spheroid model, which has been used in studies of cancer progression and tissue dynamics [11,27–30,37–39]. Here, thousands of cells self-assemble into a microtissue roughly 200–300 μm in diameter. To assess the fluidity of a given cell type, two spheroids are placed side by side and allowed to fuse into a single aggregate, a bulk readout correlated to single-cell dynamics [11]. In some cases, the two spheroids coalesce completely into a singular circular aggregate [Fig. 1(a), top]. Here, surface area is minimized in an apparently surface-tension-driven process, as in Newtonian fluids; thus, the cells are presumed to be in a fluidlike state. Other spheroids begin to coalesce before stalling at an intermediate state. These systems exhibit behavior deviating from liquid droplet fusion and are often characterized as “solidlike” [Fig. 1(a), bottom] [11,26,29]. Qualitatively, this fluidlike vs solidlike behavior can be easily distinguished by examining the end state of spheroid fusion experiments [Figs. 1(a) and 1(b)]. Stalled fusions have an elongated geometry, and therefore larger aspect ratio ($\text{AR}_{\text{fusion}}$), while completed fusions are nearly perfectly circular and have an AR close to 1. To quantitatively distinguish between these cases, we defined coalescing spheroids with $\text{AR}_{\text{fusion}} \leq 1.2$ after 70 hours of coalescence as the threshold for a circular geometry and fluidlike behavior. When assessing fluidity of 10A and DCIS cells, we primarily consider the case in which cell division is absent by treating cells with the proliferation inhibitor mito-

mycin C [40,41] prior to spheroid formation (see Sec. IV), as proliferation may act to fluidize otherwise solidlike systems [42]. Nonproliferative 10A spheroids begin to fuse but stall at $\text{AR}_{\text{fusion}, 10A} = 1.63 \pm 0.07$, while DCIS spheroids quickly and completely fuse to an aspect ratio of $\text{AR}_{\text{fusion}, \text{DCIS}} = 1.11 \pm 0.05$ [Fig. 1(b)]. Surprisingly, despite the minimal transformation and noninvasive character of DCIS [34], we observe a dramatic increase in fluidity relative to 10A cells. This demonstrates that a minor change in EMT status, causing no change in invasiveness, can fluidize an otherwise solidlike tissue.

A variety of modeling approaches exist for quantifying fusion dynamics [11,27,29–31,38,43–45]. In the simplest and most popular model, tissues are modeled as passive Newtonian fluids. However, modeling fusion as viscous fluids driven by surface tension fails to describe stalled coalescence [11]. Furthermore, many liquid drop models use metrics that consider the neck length (the contact length between the two spheroids), but such models lack the ability to discern the difference between neck growth and meaningful increases in fusion. Given these issues, it is best to use a model that tracks the angle of fusion and can account for stalled coalescence.

Here, we treat the spheroids as homogenous incompressible Kelvin-Voigt materials and track the angle of fusion over time [Fig. 1(c)]. The Kelvin-Voigt model is the simplest model that has been successfully used to describe arrested coalescence in similar systems [30]. This fitting method considers spheroid fusion as driven by effective shear viscosity (η), shear modulus (μ), and surface tension (γ) (see Sec. IV). From fits, a fusion timescale, $\tau = \eta R_0 / \gamma$, and degree of fusion, $\beta = \mu R_0 / \gamma$, where R_0 is the initial spheroid radius, are obtained, with β reflecting the aspect ratio of postfusion spheroids (see Fig. 7 in Appendix A) [30]. Figure 7 shows that the aspect ratio cutoff of $\text{AR}_{\text{fusion}} \leq 1.2$, which denotes fluidity, corresponds to $\beta \leq 1.2$. Both τ and β show significant differences when comparing nonproliferative 10A and DCIS spheroids [Figs. 1(d) and 1(e)]. Characteristic of a jammed system, the 10A spheroids exhibit long relaxation timescales ($\tau_{10A} = 113 \pm 49$ h), much longer than the duration of the experiment. In contrast, the timescale obtained for DCIS spheroids ($\tau_{\text{DCIS}} = 38 \pm 9$ h) reflects the significantly faster fusion progression. The extracted degrees of fusion also decrease from $\beta_{10A} = 3.1 \pm 0.7$ to $\beta_{\text{DCIS}} = 0.7 \pm 0.2$, reflecting their stalled and fully coalesced states, respectively. Although we focus primarily on nonproliferative spheroids in this work, we note that in proliferative spheroids an increase in fusion is observed for 10A spheroids while little change in DCIS spheroid fusion is seen (see Fig. 8 in Appendix B). This finding suggests that, in a solidlike system, perturbations from proliferation facilitate fusion.

B. Cell shapes

To better understand the transition between fluidlike and solidlike behavior, we probe the internal structure of spheroids by investigating the shapes of cells in isolated spheroids. To this end, we fix and stain individual spheroids 24 h after initiating formation. Spheroids are then imaged using confocal fluorescence microscopy, and cells are segmented to obtain shapes (see Sec. IV). We note that in most cases only

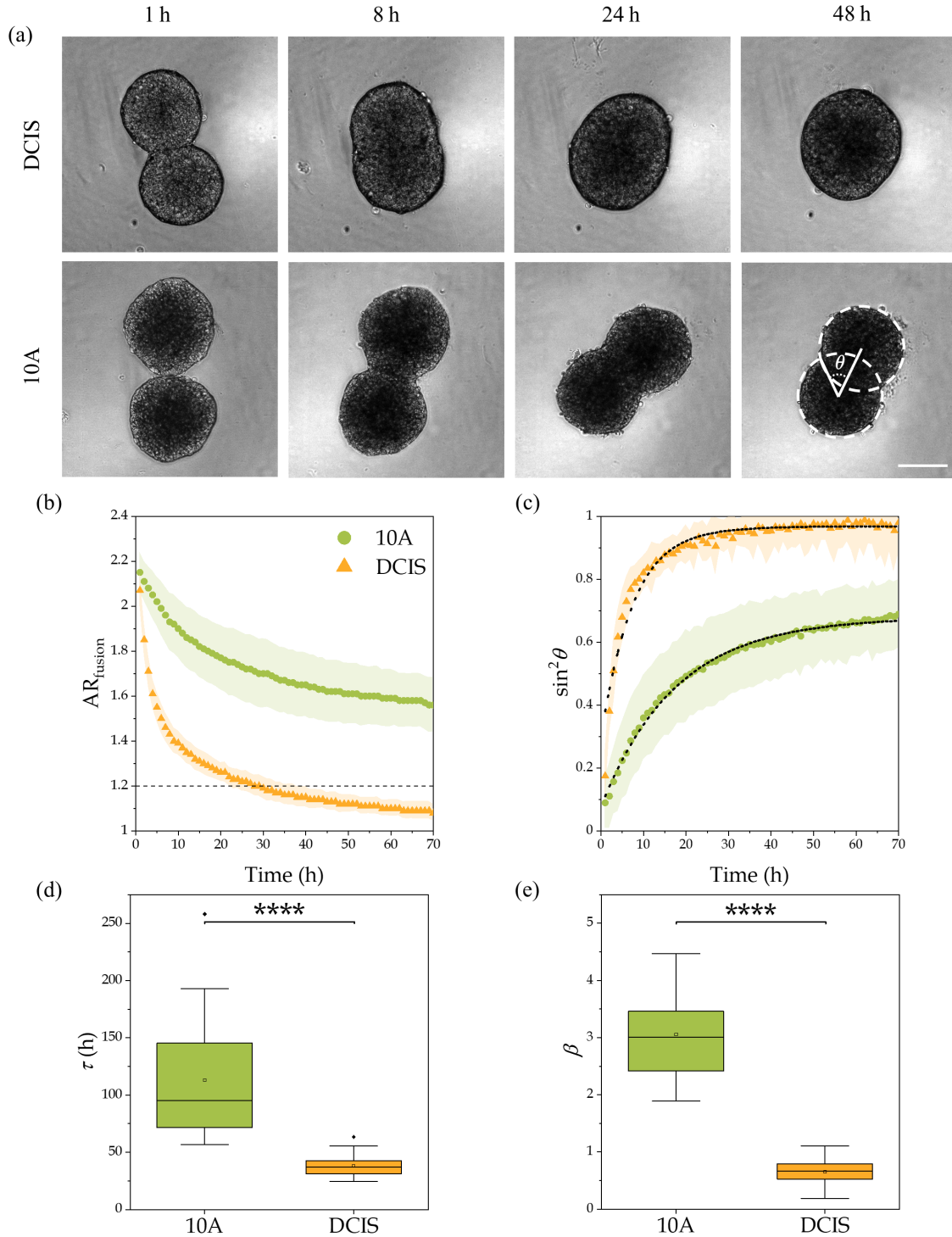


FIG. 1. (a) Top, MCF10DCIS.com (DCIS), and bottom, MCF10A (10A) spheroids during 1–48 hours of coalescence. Scale bar is 200 μm . (b) Aspect ratio (AR_{fusion}) of the fusing 10A (green circles) and DCIS (orange triangles) spheroids over the duration of the experiment. Dotted line indicates the aspect ratio cutoff of a liquidlike system, $AR_{\text{fusion}} \leq 1.2$. The $AR_{\text{fusion}} \leq 1.2$ cutoff is determined as point at which coalescing spheroids are visually indiscernible from a single sphere. (c) Evolution of $\sin^2 \theta$, with θ the angle of fusion between the two spheroids as shown in the inset of the bottom-right image in (a). Fits of the averaged data using the Kelvin-Voigt model (see Sec. IV) are shown by the dotted line. Standard deviations are shown as shaded regions. [(d), (e)] Box-and-whisker plots displaying τ and β obtained from fits of $\sin^2 \theta$ for individual sets of fusing spheroids. Box plots show mean (open symbols), median (lines), interquartile range (IQR) (boxes), and $1.5 \times \text{IQR}$ (whiskers). Outliers are those outside $1.5 \times \text{IQR}$ and are displayed as individual points. Both τ ($p_{\text{MW}} < 0.0001$) and β ($p_{t\text{-test}} < 0.0001$) are significantly different between 10A and DCIS spheroids. Legend in (b) applies to panels (b)–(e). For this figure, $n = 33$ 10A spheroid pairs and $n = 34$ DCIS spheroid pairs.

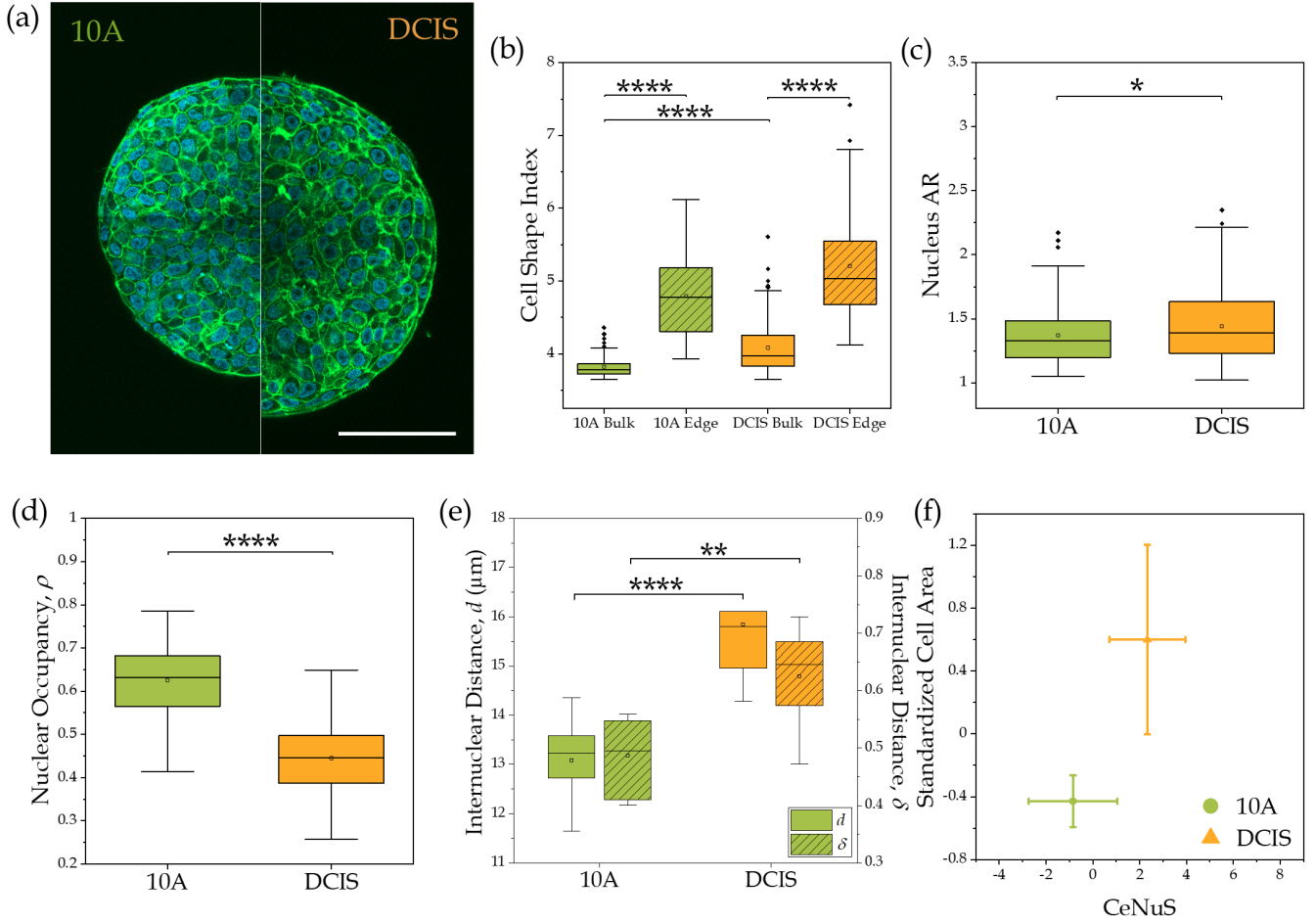


FIG. 2. (a) Actin (green) and DAPI (blue) stains of representative individual spheroids. Left, 10A spheroid; right, DCIS spheroid. Scale bar is 100 μm . (b) Cell shape index of 10A and DCIS cells in individual spheroids both in the bulk and at the periphery of the spheroids ($p_{\text{MW}} < 0.0001$, $n = 103$ bulk 10A cells, 46 edge 10A cells, 161 DCIS bulk cells, and 45 edge DCIS cells, each from $n = 3$ spheroids). (c) Nuclear aspect ratio (AR) in 10A and DCIS spheroids ($p_{\text{MW}} < 0.05$, $n = 114$ 10A cells and 151 DCIS cells). (d) Nuclear occupancy ($n = 43$ 10A cells and 86 DCIS cells, each from $n = 3$ spheroids) is higher in 10A cells compared to DCIS cells in individual spheroids ($p_{\text{t-test}} < 0.0001$). (e) Internuclear distance in absolute distance d ($p_{\text{t-test}} < 0.0001$) and normalized by nuclear size δ ($p_{\text{t-test}} < 0.01$) for 10A and DCIS cells in spheroids ($n = 9$ 10A and 6 DCIS spheroids). All box-and-whisker plots show mean (open symbols), median (lines), IQR (boxes), and (whiskers). Outliers are those outside $1.5 \times \text{IQR}$ and are displayed as individual points. (f) 10A and DCIS cells plotted in the cell area vs CeNuS phase space ($n = 175$ 10A and 156 DCIS cells from $n = 7$ 10A and 6 DCIS spheroids) with the pooled standardized set consisting of 184 cells of 8 (4 10A and 4 DCIS) of the evaluated spheroids. Error bars display standard deviations.

a single slice through the center of the spheroid is imaged; thus, cell shape and related quantities are two-dimensional (2D) measurements. As reported previously [46], we find that 2D and three-dimensional (3D) quantities are correlated (see Fig. 9 in Appendix C) and consider here whether 2D measurements can be used as a reporter of cell morphological characteristics to predict fluidity while avoiding some of the challenges of 3D cell segmentation [11,47]. Specifically, we quantify the 2D cell shape index, $q = \frac{p}{\sqrt{A}}$, where p and A are the perimeter and area of the cell, respectively. Cell shape index serves as an indicator of tissue fluidity and allows one to infer dynamic behaviors from static structure [3,17,48–50]. Specifically, elongated and therefore higher shape index cells have been found to be more mobile than round cells [10–12].

First, examination of fixed individual spheroids reveals that DCIS spheroids are larger than 10A spheroids [Fig. 2(a); see Fig. 10(a) in Appendix D]. Furthermore, examination

of the internal structure reveals that both 10A and DCIS spheroids are composed of elongated cells at the interface with the medium and more compact cells within the spheroid bulk, with $q_{10A, \text{edge}} = 4.79 \pm 0.59$ and $q_{\text{DCIS, edge}} = 5.21 \pm 0.79$ [Fig. 2(b)] corresponding to highly elongated cell geometry in both cases. We propose that this elongated outer rim of cells observed across both 10A and DCIS individual spheroids facilitates the early relatively rapid fusion when either 10A or DCIS spheroids are placed in near vicinity and come into contact initially, as also suggested by Grosser *et al.* [11]. However, as time passes and fusion progresses, the cells initially at the periphery become less elongated and rounder in shape, reflecting a transition from surface to bulk geometry (see Fig. 11 in Appendix E).

The interior bulk cells of the 10A and DCIS spheroids exhibit differences in shape index that are greater than that of edge cells and correlate more fully with observed

differences in fusion behavior. 10A bulk cells appear as relatively regular polygons with a shape index of $q_{10A} = 3.82 \pm 0.15$. This value corresponds closely with the shape index of 3.81 that has been identified in simulated monolayers as a shape threshold denoting the boundary between fluid and jammed solid phases [17]. DCIS spheroids, on the other hand, contain significantly more elongated cells, with a mean shape index of $q_{DCIS} = 4.08 \pm 0.35$. In sum, the studied system, with solidlike 10A spheroids consisting of rounder cells than the fluidlike DCIS spheroids, aligns with the previously identified link between cell shape and fluidity [17].

C. Nuclear contributions

Spheroids are densely packed microtissues with little to no empty space between cells and therefore have a packing fraction near unity. Additionally, the nucleus is known to be one of the stiffest and largest compartments of the cell [32]. As such, in order for a cell to navigate a crowded environment, such as the interior of a spheroid, the nucleus must be squeezed between surrounding cells [51]. Previous work has also shown that elongated mobile cells have elongated nuclei [10,11], indicating that nuclear shape could be another useful and perhaps more direct indicator of fluidity beyond cell shape.

We hypothesize two means through which the motility of cells in our system might be impacted by nuclei. First, we hypothesize that, similar to cell shapes, differences in nuclear shape would be observed between fluidlike and solidlike cells. Given that nuclei are more ellipsoidal and have less tortuous shapes than cells, for nuclei we measure aspect ratio as opposed to shape index, in accordance with prior studies [10]. We find that the more mobile and elongated DCIS cells have somewhat larger nuclear aspect ratios [Fig. 2(c)]. Second, barriers to cell mobility may be lower when packing fraction of the nuclei is lower. Examining the difference between 10A and DCIS cells, consistent with spheroid size, DCIS cells are larger than 10A cells, though so too are their nuclei [see Figs. 10(b) and 10(c) in Appendix D]. The cell size difference is larger than the nuclear size difference; thus, nuclear occupancy is significantly lower for DCIS cells than for 10A cells. While the nucleus occupies the majority of the cross-sectional cell area in 10A cells ($\rho_{10A} = 0.63 \pm 0.09$), for DCIS cells the nucleus occupies a significantly smaller proportion of the available space [$\rho_{DCIS} = 0.45 \pm 0.08$; see Fig. 2(d)], suggesting a lower nuclear packing density in DCIS spheroids.

To further probe nuclear packing density, we measure the absolute internuclear distance, d , by averaging the six nearest-neighbor internuclear distances of each cell within the bulk of each spheroid [Fig. 2(e)]. In accordance with similar work [20], we then normalize the absolute internuclear distance to provide a system-independent assessment of nuclear packing [Fig. 2(e)]. In brief, the absolute internuclear distance is normalized to the average minor axis length of the ellipsoidal nuclei for that specific spheroid, l , to obtain the normalized internuclear spacing, δ :

$$\delta = \frac{d}{l} - 1 = d \sqrt{\frac{0.25\pi AR_{nuc}}{Area_{nuc}}} - 1, \quad (1)$$

where l indicates the characteristic minor axis diameter of the nucleus for a given spheroid, which is a function of the aspect ratio, AR_{nuc} , and area of the nuclei, $Area_{nuc}$. Equation (1) shows the effective nuclear packing can be decreased by not only directly increasing the absolute internuclear distances, but also by reducing the space occupied by the nucleus or by elongating the nucleus, as depicted schematically in Figs. 12 and 13 in Appendix F.

When examining both the absolute and normalized internuclear distance, we find that the round 10A nuclei are densely packed, with typical nuclear spacing around a nuclear radius, $\delta_{10A} = 0.49 \pm 0.07$ nuclear diameters. In contrast, DCIS nuclei are more loosely packed, with both higher absolute and normalized internuclear spacing ($\delta_{DCIS} = 0.63 \pm 0.09$ nuclear diameters) [Fig. 2(e)]. The DCIS cells may thus be able to move more easily, without having to as substantially squeeze their nuclei between other nuclei as 10A cells.

To encapsulate the simultaneous influence of changes in cell shape, nuclear shape, and nuclear packing density, we turn to the shape metric proposed by Gottheil *et al.* [10]. This metric, CeNuS, which combines cell and nuclear shape, is defined as

$$CeNuS = \overline{q_{cell}} + \sigma_{cell}^2 + \overline{AR_{nuc}} + \sigma_{AR_{nuc}}^2 \quad (2)$$

and captures the structural information encoded in both cellular and nuclear shape distributions. Here, $\overline{q_{cell}}$ and σ_{cell}^2 are the standardized median cell shape index and the corresponding variance, respectively, and $\overline{AR_{nuc}}$ and $\sigma_{AR_{nuc}}^2$ are the standardized median nucleus aspect ratio and the corresponding variance, respectively. This aggregate metric, when plotted against the projected standardized cellular area, maps out a phase space that determines the fluidlike or solidlike behavior of the tissue. More fluidlike tissues have a larger CeNuS value and/or larger standardized cell area. Following the method described previously [10], we compute these parameters for individual 10A and DCIS spheroids (see Sec. IV). We note that with our internal normalization, CeNuS values span a range of -6 to 6 , a range comparable to those of previous studies, whereas our standardized cell area range is slightly smaller [10]. Independent of normalization metrics, 10A and DCIS spheroids are well separated in the cell area vs CeNuS phase space [Fig. 2(f)]. DCIS notably display an increased standardized cell area and CeNuS, both of which suggest enhanced fluidity.

Based on this area-shape phase space and our observations regarding nuclear packing, we propose that the nucleus is the bottleneck in 10A fusion, and 10A cells are unable to rearrange due to the dense packing of their stiff, round nuclei. While nuclei have been discussed as a potential barrier to cell mobility in confined spaces [52], the effects of nuclear packing have not been directly probed to our knowledge. We attempt to soften the nucleus—which we hypothesize could lead to, on average, a higher nuclear aspect ratio—by treating the cells with trichostatin A (TSA), an inhibitor of histone deacetylases [53]. In some contexts, TSA has been shown to soften nuclei, including in 10A cells [54], though this appears to depend on cell line, the time point at which cells are assessed, and the method of measuring nuclear stiffness [54–56]. While we do not directly measure nuclear stiffness, we observe that, upon TSA treatment, the previously

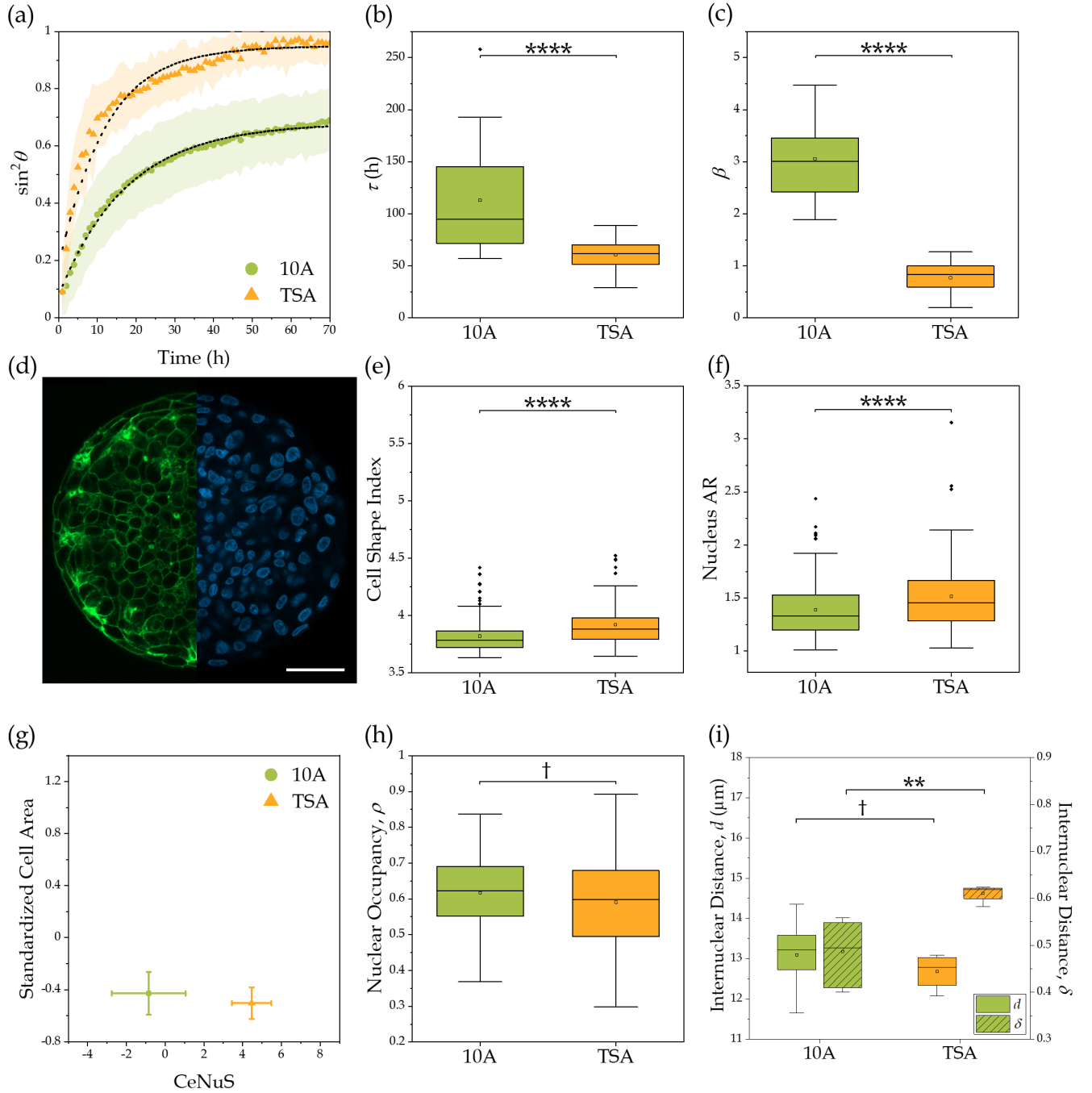


FIG. 3. (a) TSA-treated 10A spheroids (orange triangles) fuse more than control 10A spheroids (green circles). [(b), (c)] TSA-treated 10A spheroids (orange) fuse faster and more, as demonstrated by lower τ ($p_{\text{MW}} < 0.0001$) and β ($p_{t\text{-test}} < 0.0001$) values ($n = 33$ 10A spheroid pairs and $n = 16$ TSA spheroid pairs). (d) Actin (green) and DAPI (blue) stains of an individual 10A TSA-treated spheroid. Scale bar is 50 μm . (e) Cell shape index ($n = 176$ 10A and 98 DCIS cells from $n = 4$ 10A and 3 DCIS spheroids) and (f) nuclear AR ($n = 187$ 10A and 98 DCIS cells from $n = 4$ 10A and 3 DCIS spheroids) in 10A spheroids with and without TSA treatment ($p_{\text{MW}} < 0.0001$). (g) Cell area vs CeNuS plot reveals that cell and nuclear shape change with TSA treatment but cell area does not, such that this treatment moves cells along the CeNuS axis only ($n = 175$ 10A and 98 TSA cells from $n = 7$ 10A and 3 TSA spheroids). Error bars display standard deviations. (h) Nuclear occupancy, ρ , remains the same after TSA treatment ($p_{t\text{-test}} > 0.05$, $n = 114$ 10A and 116 TSA cells from $n = 7$ 10A and 3 TSA spheroids). (i) Internuclear distance in absolute distance d ($p_{t\text{-test}} > 0.05$) and normalized distance δ ($p_{t\text{-test}} < 0.01$) ($n = 9$ 10A and 4 TSA spheroids). Due to the elongated TSA nuclei, the normalized distance increases compared to control cells even though the nuclei remain the same size. All box-and-whisker plots show mean (open symbols), median (lines), IQR (boxes), and $1.5 \times \text{IQR}$ (whiskers). Outliers are those outside $1.5 \times \text{IQR}$ and are displayed as individual points.

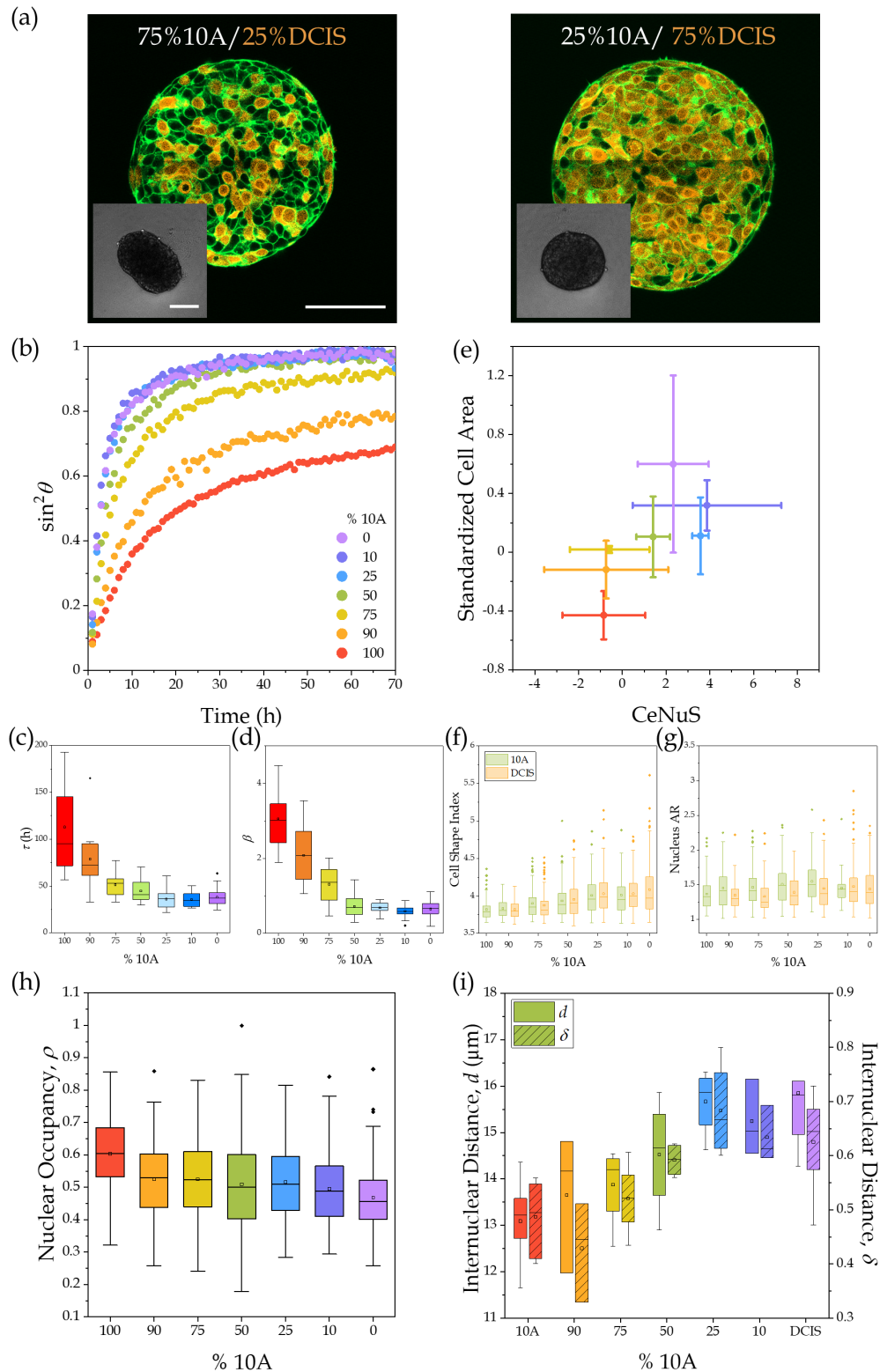


FIG. 4. (a) Individual heterotypic 10A and DCIS spheroids with actin (green) and CellTracker-Orange-labeled DCIS (orange) cells. Left spheroid contains 75% 10A cells and 25% DCIS cells; right spheroid contains 25% 10A cells and 75% DCIS cells. Scale bar is 100 μm . Inset: the corresponding fusion of heterotypic 10A/DCIS spheroids after 50 h of fusion. Inset scale bar is 200 μm . As the proportion of DCIS cells increases, fusion increases. (b) With increasing DCIS cells, fusion quickens and progresses further as demonstrated by (c) τ and (d) β values ($n = 33$ 10A spheroid pairs, $n = 34$ DCIS spheroid pairs, and $n = 17$ spheroid pairs on average for all other conditions). (e) Heterotypic 10A/DCIS spheroids in the cell area vs CeNuS phase space. There is a steady increase in both parameters as the fraction of DCIS cells increases (for each condition, $n \geq 3$ spheroids with an average of 36 cells per spheroid). Error bars display standard deviations. (f) Cell shape of individual cell types within heterotypic spheroids. Green and orange correspond to 10A and DCIS cells, respectively. (g) Nuclear shapes

solidlike 10A spheroids fuse significantly more and faster [Fig. 3(a)]. When treated with TSA, τ and β both decrease from a τ of 113 ± 49 h to 61 ± 14 h and β of 3.1 ± 0.7 to 0.8 ± 0.3 [Figs. 3(b) and 3(c)]. Moreover, both cells and their nuclei become more elongated, as can be seen via investigation of cells in individual spheroids [Figs. 3(d)–3(f)]. In the CeNuS phase space, TSA-treated 10A spheroids thus shift horizontally along the CeNuS axis, while there is little change in the cell area axis [Fig. 3(g)]. As a result of the conserved cell and nuclear area, the nuclear occupancy and the absolute nuclear spacing are also unchanged by TSA treatment [Figs. 3(h)–3(i)]. However, once nuclear spacing is normalized by the nuclear minor axis length, an increase in effective nuclear spacing for TSA-treated spheroids is apparent, increasing from a spacing of around a nuclear radius, $\delta_{10A} = 0.49 \pm 0.07$ nuclear diameters, to greater than a nuclear radius, $\delta_{TSA} = 0.61 \pm 0.02$ nuclear diameters [Fig. 3(i)]. This difference in spacing is dominated by changes in nuclear shape, as there is no change in nuclear (or cell) area. Thus, we see that although nuclear area fraction can be used as a proxy for absolute internuclear distances, nuclear shape must also be considered to accurately obtain nuclear spacing. For TSA-treated spheroids, the elongated nuclear shapes result in an increase in effective internuclear spacing and, in turn, an increase in fusion capability.

In sum, we suggest that even when nuclear occupancy is high in 10A spheroids, which would normally prevent fusion, TSA treatment, intending to soften the nucleus, results in elongation of cells and nuclei, allowing for fluidization and increased fusion. The increase in nuclear aspect ratio is a likely consequence of more deformable nuclei, which presumably allow increased cell-cell rearrangements. In contrast to 10A spheroids treated with TSA, TSA-treated DCIS spheroids remain relatively unchanged: they remain fluid, though fuse slightly faster, and there is little change in their shapes (see Fig. 14 in Appendix G). Cellular contractility has also been hypothesized to be a driver of fluidity [17,57,58]. However, consistent with previous reports [57], we find blebbistatin, which inhibits contractility [59], to have little effect on spheroid coalescence behavior (see Fig. 15 in Appendix H).

D. Heterotypic spheroids allow for traversal of fluidity phase space

1. Heterotypic 10A-DCIS spheroids

By employing the 10A-DCIS cell line system in combination with TSA treatment, we demonstrate that the CeNuS-area parameter space can be traversed, toggling between fluidlike and solidlike phases. For a more fine-grained map of this phase space, we next employ heterotypic spheroids composed of mixtures of fluidlike and solidlike cells. Indeed, physiological systems, in particular cancerous tumors, are highly

heterogeneous [26,60,61], and characterizing how the proportions of different cell types within tissues translate to bulk tissue dynamics is of significant interest. Prior computational and experimental work in heterotypic cellular monolayers has demonstrated that altering heterogeneity can tune the jamming properties of a system [62,63].

We first probe the fusion capacity of a mixed spheroid system containing varying proportions of 10A and DCIS cells [Fig. 4(a)]. Adding DCIS cells to a predominantly 10A spheroid leads to a gradual change in fusion ability [Fig. 4(b)]. Even a small proportion of DCIS cells causes the spheroids to fuse more, as spheroids with just 10% DCIS cells have dramatically lower τ and β values than homotypic 10A spheroids [Figs. 4(c) and 4(d)]. When the spheroid is composed of at least 50% DCIS cells, the spheroids completely fuse, reflected in τ and β values converging to the values observed in fusion of homotypic DCIS spheroids. Detailed examination of the CeNuS-area phase space reveals that heterotypic spheroids display a gradual and relatively linear increase in both CeNuS and cellular area as the proportion of DCIS cells increases [Fig. 4(e)], approaching the values observed in homotypic DCIS spheroids. Looking more closely, we find that the bulk shape index is not simply an average of varying proportions of two distinct cell shape populations. Instead, DCIS cells become rounder as the proportion of 10A cells in the spheroids increases [Figs. 4(f) and 4(g)]. Similarly, as the proportion of DCIS cells in the spheroid is increased, 10A cells and their nuclei become more elongated. Thus, the presence of a more fluidlike cell type can change the shape properties of otherwise solidlike cells.

The presence of the DCIS cells also loosens the packing of the nuclei, reflected in the increase of the nuclear spacing and decrease in the nuclear area fraction when DCIS cells are introduced into 10A spheroids [Figs. 4(h) and 4(i)]. Additionally, as nuclear aspect ratios increase with increasing proportion of DCIS cells, the resulting normalized internuclear distances reflect the same increase in spacing between nuclei as DCIS cells are added to the system [Fig. 4(i)]. Once the effective nuclear spacing exceeds $\delta = 0.6$ nuclear diameters, which occurs when 50% of the spheroid is comprised of DCIS cells, the system exhibits fluidlike behavior. In summary, we observe that in 10A-DCIS heterotypic spheroids, the DCIS-mediated fluidization process involves elongation of cells and nuclei as well as a reduction in effective nuclear packing that occurs through both absolute internuclear spacing increases and nuclear elongation.

2. Heterotypic 10A-TSA spheroids

To further investigate the cell area vs CeNuS phase space, we attempt to traverse just one axis of the phase space to examine whether the jammed 10A system could be fluidized

within heterotypic spheroids for each cell type. Same legend applies to (f) and (g). For each condition in (f) and (g), there are $n \geq 3$ spheroids with an average of 29 cells per spheroid, except the 10A cells within the 10% 10A condition with $n = 20$ cells from two spheroids. (h) The nuclear-to-cell area fraction steadily decreases as more DCIS cells are added to the spheroids (for each condition, $n = 167$ cells on average from $n \geq 4$ spheroids). (i) Both absolute, d , and normalized, δ , internuclear distances increase as DCIS cells are added to the spheroid ($n \geq 3$ spheroids). All box-and-whisker plots show mean (open symbols), median (lines), IQR (boxes), and $1.5 \times \text{IQR}$ (whiskers). Outliers are those outside $1.5 \times \text{IQR}$ and are displayed as individual points.

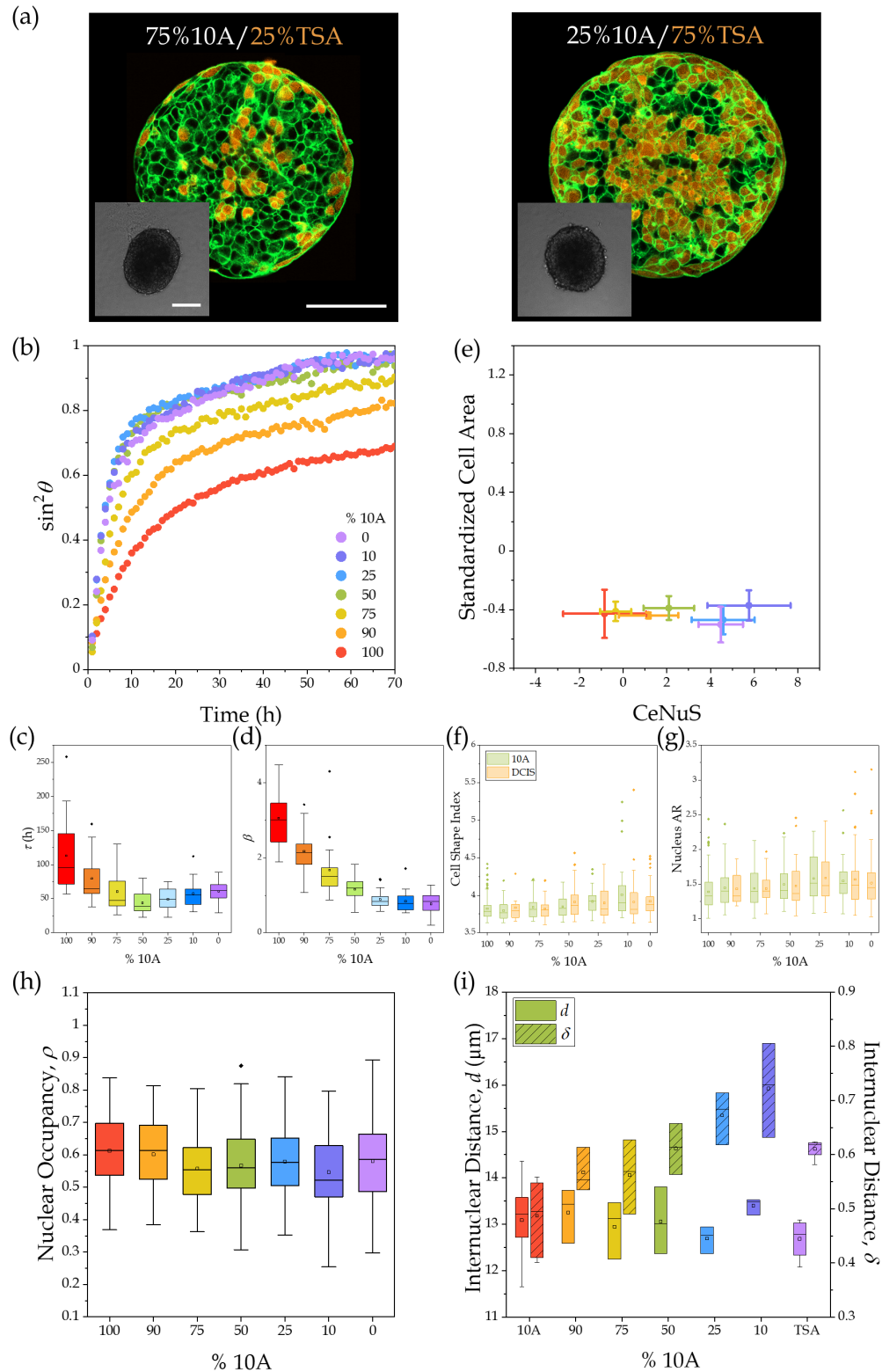


FIG. 5. (a) Individual heterotypic 10A and TSA-treated 10A spheroids with actin (green) and CellTracker-Orange-labeled TSA-treated 10A (orange) cells. Left spheroid contains 75% 10A cells and 25% TSA-treated 10A cells; right spheroid contains 25% 10A cells and 75% TSA-treated 10A cells. Scale bar is 100 μm. Inset: the corresponding fusion of heterotypic 10A and TSA-treated 10A spheroids after 50 h of fusion. Inset scale bar is 200 μm. As the proportion of TSA-treated 10A cells increases, fusion increases. (b) Fusion ability increases as more TSA-treated cells are added to 10A/TSA heterotypic spheroids. [(c), (d)] Adding TSA-treated cells to 10A spheroids quickens and progresses fusion ($n = 33$ 10A spheroid pairs, $n = 16$ TSA spheroid pairs, and $n = 18$ spheroid pairs for all other conditions). (e) Plotted in the cell area vs CeNuS phase space, heterotypic 10A/TSA spheroids change little in cell area but increase steadily in shape index (for each condition, there are $n \geq 3$ spheroids with an average of 32 cells per spheroid, except for the 90% 10A condition with $n = 2$ spheroids with an average of 25

without altering cell size and solely with changes to nuclear shapes. For this approach, we employ heterotypic spheroids composed of 10A cells and TSA-treated 10A cells [Fig. 5(a)]. As TSA-treated 10A cells are added to 10A spheroids, an increase in fusion is observed similar to that seen in 10A-DCIS mixed spheroids [Fig. 5(b)]. The corresponding τ and β values from fits decrease gradually as the proportion of TSA-treated cells increases [Figs. 5(c) and 5(d)], reflecting the quicker and more complete fusion. Like 10A-DCIS heterotypic spheroids, 10A and 10A-TSA heterotypic spheroids exhibit dramatic changes in fluidity with only a small proportion of the fluid cell type in the system. When examining 10A and TSA-treated 10A heterotypic spheroids, the cell area vs CeNuS phase space is traversed almost solely along the CeNuS axis [Fig. 5(e)], with little change in cell areas as TSA-treated cells are added. In contrast, elongation of all cells occurs with increasing proportion of TSA-treated cells. Looking at the individual cell types, cell shapes again change in the presence of another cell type [Fig. 5(f)], while nuclear aspect ratios increase steadily with increasing TSA-treated 10A cells [Fig. 5(g)].

Both cell and nuclear areas do not change with increasing proportions of TSA-treated cells, as exhibited by the constant nuclear-to-cell area fraction and absolute internuclear distance [Figs. 5(h) and 5(i)]. Instead, nuclei become more elongated in spheroids with increasing proportion of TSA-treated cells. As a result, though no change in the absolute internuclear distances is observed, an increase in the effective nuclear spacing as TSA-treated 10A cells are added is evident [Fig. 5(i)]. Again, as in the 10A-DCIS heterotypic spheroids, fluidlike fusion occurs when the normalized internuclear distance is greater than $\delta = 0.6$ nuclear diameters. However, unlike 10A-DCIS spheroids, the increase in effective nuclear spacing is due entirely to the elongated nuclei of TSA-treated 10A cells. This finding again supports the idea that, even if nuclear occupancy is high and nuclei are tightly packed, the addition of cells with more elongated, presumably softer, nuclei can cause a shape-driven increase the fusion ability of the system.

Examining all conditions on the cell area vs CeNuS phase space together, it is apparent that the fluidlike or solidlike state of the spheroids is primarily dictated by the shapes of the cells and their nuclei [Fig. 6(a)]. Specifically, fluidlike behavior is reflected in elongated cells and nuclei, which translate to high CeNuS values. However, cell area also plays a role in fluidity, as for a given CeNuS value, an increase in cell area leads to an increase in fusion ability. Because nuclear area and shape are correlated with cell area and shape, we can use the single metric of normalized internuclear spacing to

describe the systems studied here [Fig. 6(b)]. As normalized internuclear distance decreases, we observe a decrease in fusion ability, with the Pearson's correlation coefficient between δ and β of -0.75 . Additionally, we observe a demarcation around $\delta = 0.6$ nuclear diameters, below which the spheroids exhibit solidlike behavior and above which the spheroids exhibit fluidlike behavior. Furthermore, by scaling nuclear area occupancy by the aspect ratio of the nuclei, an analogous predictor of fluidity is obtained, without the need for laborious internuclear distance calculations [Fig. 6(c)]. In sum, we show that the system under study is dominated by shape-reflected and/or shape-driven changes in fusion ability. Elongated cells are better able to navigate a dense environment, not simply because of their elongated cell bodies, but also in part due to the effective increase in nuclear spacing that results from their elongated nuclei.

III. DISCUSSION

The results presented here agree with previous conjectures regarding the constraining effect of nuclei in cell migration, and to our knowledge are the first experiments probing nuclear occupancy and shape in multicellular spheroids. These results suggest that differences in fluidity emerge in part because stiff, large nuclei can act as bottlenecks to cellular rearrangements in a densely packed tissue. Thus, through TSA treatment, fluidization of an otherwise jammed system occurs. TSA treatment leads to more-deformable nuclei and subsequent changes to nuclear shape, which appear concurrent with changes to cell shapes. Therefore, both nuclear and cell shape serve as valuable readouts of the mechanical attributes of the cell and tissue.

In prior studies, nuclear size and occupancy (the fraction of space occupied by the nucleus within the cell) have been used as a proxy for nuclear packing density, with the underlying idea that higher nuclear occupancy leads to less intercellular space and thus smaller internuclear distances [10]. In this way, the arrangement of nuclei is essentially viewed as a packing of hard spheres [20]. We demonstrate that nuclear occupancy in our system does in fact strongly correlate with absolute internuclear spacing. However, nuclei are deformable, and the ability of a nucleus to squeeze through internuclear spaces depends not only on absolute internuclear distances but also on the shape that it assumes. A deformable nucleus can adopt ellipsoidal geometries with increasing aspect ratio, where the length of the minor axis becomes the limiting distance that must be traversed during cell rearrangements. By scaling internuclear distance according to this minor axis, we see that an effective increase in internuclear spacing can be achieved

cells per spheroid). Error bars display standard deviations. (f) Cell shape changes for individual cell types in heterotypic spheroids. Green and orange represent 10A and 10A-TSA cells, respectively. Little change in cell shape is observed until more than 50% of the spheroid is comprised of TSA cells. (g) Nuclear shapes elongate as more TSA cells are added to the spheroids. Same legend applies to (f) and (g). For each condition in both (f) and (g), there are $n \geq 3$ spheroids with an average of 29 cells per spheroid, except for the TSA-treated cells within the 90% 10A condition with $n = 8$ cells from one spheroid. (h) Little change is observed in nuclear-to-cell area fractions (for each condition, $n = 91$ cells on average from $n = 3$ spheroids). (i) Though there is no change in absolute distance between nuclei, d , once the nuclear minor axis length is used to normalize the distances, an increase in internuclear distance, δ , in heterotypic spheroids is apparent ($n \geq 3$ spheroids). For all box-and-whisker plots, whiskers display the range of data excluding outliers. All box-and-whisker plots show mean (open symbols), median (lines), IQR (boxes), and $1.5 \times \text{IQR}$ (whiskers). Outliers are those outside $1.5 \times \text{IQR}$ and are displayed as individual points.

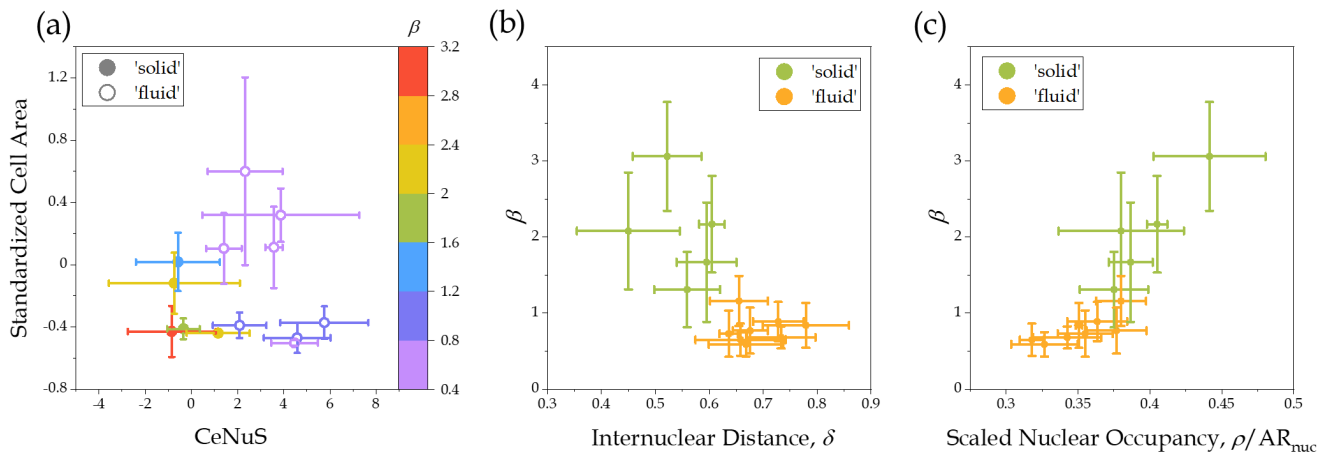


FIG. 6. (a) All conditions displayed on the cell area vs CeNuS phase space (for each condition, $n \geq 3$ spheroids with an average of 36 cells per spheroid, except for the 90% 10A condition with $n = 2$ spheroids with an average of 25 cells per spheroid). Coloring indicates β values and solid and open points indicate solidlike and fluidlike behavior, respectively. (b) The system is more fused as normalized internuclear distance, δ , increases ($n = 20$ spheroids on average for each β condition and $n \geq 3$ spheroids for each δ condition). (c) Scaled nuclear area occupancy also reflects fusion ability well, with lower occupancy correlating with more fusion ($n = 20$ spheroids on average for each β condition and $n = 4$ spheroids on average for each scaled nuclear occupancy condition). Error bars display standard deviations.

without increasing absolute internuclear distance, with a clear threshold denoting a regime of solidlike and fluidlike behavior. Given the correlation between nuclear occupancy and packing density, we devise a coarse-grained metric, normalized internuclear distance, that scales the nuclear occupancy by the nuclear shape, as a simple but accurate measurement of the effective packing density. We note that these quantities are all derived from 2D images of 3D samples; thus, the metrics examined and found to be of import here may also be relevant in histopathology. Indeed, nuclear morphology has long played a role in cancer grading, with recent findings linking nuclear heterogeneity to higher grade cancers [64–66].

The MCF10A and MCF10DCIS.com system explored here offers a useful model for probing the early stages of cancer progression by capturing the transition from a normal epithelial tissue to a preinvasive state. Surprisingly, we find that small changes in EMT status [34] lead to dramatic changes in tissue fluidity. Given that DCIS cells are generally noninvasive, fluidity is apparently acquired before invasive capacity. Additionally, a notable feature of DCIS cells is the increase in individual cell volumes relative to 10A. Recent work has pointed to volume expansion as a possible key step in advance of cancer invasion [67,68]. Volume expansion may provide a pathway for tissues to generate large forces that weaken the surrounding basement membrane, allowing for more efficient cell invasion. Such volume expansion may simultaneously fluidize the tissue through loosening of tightly packed stiff nuclei, readying its cells for invasion.

IV. METHODS AND MATERIALS

A. Cell lines and reagents

MCF10A cells are a gift from Professor Carol Prives (Columbia University, NY). MCF10DCIS.com are obtained from Wayne State University (Michigan, USA).

Ultralow attachment 96-well U-bottom Nunclon Sphera plates and methanol-free formaldehyde are from Thermo

Fisher Scientific (Waltham, MA). Glass bottom 35-mm culture dishes are from MatTek Dishes (Ashland, MA). Acetic acid (99.7%), bovine serum albumin (BSA), trichostatin A (TSA), mitomycin C, glycerol, Triton-X 100, hydrocortisone, cholera toxin, epidermal growth factor (EGF), insulin (10 mg/mL), ATTO-488 phalloidin, and DAPI are obtained from Sigma Aldrich (St. Louis, MO). Acid-solubilized (AS) rat tail collagen I (approximately 4 mg/mL solution) is obtained from Advanced BioMatrix (San Diego, CA). Phosphate buffered saline (PBS) is obtained from GE Healthcare HyClone (Logan, Utah). Accutase and 100 $\times$ penicillin-streptomycin-amphotericin B are obtained from MP Biomedicals (Santa Ana, CA). Horse serum and DMEM/F12 are obtained from Gibco (Grand Island, NY). CellTracker Orange CMRA is obtained from Invitrogen Life Technologies (Grand Island, NY). Para-nitroblebbistatin is obtained from Optopharma (Budapest, Hungary).

B. Cell culture

MCF10A and MCF10DCIS.com cells are cultured in DMEM/F12 growth medium containing 5% horse serum, 1% 100 $\times$ penicillin-streptomycin-amphotericin B, 0.5 μ g/ml hydrocortisone, 10 μ g/ml insulin, 0.1 μ g/ml cholera toxin, and 20 ng/ml EGF. Cells are maintained at 37 $^{\circ}$ C with 5% CO₂. Cells are detached at 80–90% confluence using Accutase prior to experiments and are not used beyond passage 40.

C. Spheroid preparation

Cells are pretreated with 4 μ g/ml mitomycin C for 2 h to inhibit proliferation. For TSA treatment, cells are treated with 500 ng/ml TSA for 20 h. TSA concentration is chosen to match the proliferation inhibition effects of mitomycin C.

For spheroid formation, 2000 cells are placed in wells of an ultralow-adhesion 96-well plate in media and centrifuged for 10 min at 1000g. The cells are allowed to compact for 24 h at 37 $^{\circ}$ C with 5% CO₂ to form spheroids.

D. Fusion microscopy

Two spheroids are placed into media-filled wells of an ultralow-adhesion 96-well plate. They are imaged every hour on a BioSpa Citation Imager using a 4× phase objective [numerical aperture (NA) 0.13]. The sample is kept at 37 °C and 5% CO₂ for the duration of the experiment (up to 70 h). For blebbistatin fusion experiments, spheroids are added to wells with 20 μM of blebbistatin.

E. Fusion analysis

The fusion movies are thresholded and a mask is created in ImageJ. Aspect ratio is the ratio of the major to minor axes lengths derived from the eigenvalues of the inertia tensor of the mask. A custom python script is written to fit the mask of the fusing spheroids to two circles [Fig. 1(a), bottom right], and the angle of their fusion, θ , is recorded. The angle of fusion, θ , is fit using a Kelvin-Voigt model as previously described [30]:

$$\dot{\theta} = \frac{2 \cot \theta}{\tau} \left(\frac{R_0}{R(\theta)} \right)^3 [f(\theta) - \beta g(\theta)], \quad (3)$$

where R_0 is the average initial radius of the fusing spheroids, $R(\theta)$ is the radius of the spheroids over time, $\tau = \eta R_0 / \gamma$ and $\beta = \mu R_0 / \gamma$, with η the viscosity, μ the modulus, and γ the surface tension. Additionally, $f(\theta)$ and $g(\theta)$ are defined as

$$f(\theta) = \frac{4}{(1 + \cos \theta)^2}, \quad (4)$$

$$g(\theta) = \frac{2}{\cos \theta (1 + \cos \theta)} \left[\frac{2R_0}{R(\theta)(1 + \cos \theta)} - 1 \right]. \quad (5)$$

Each pair of fusing spheroids is fit using this model to obtain the resulting fit parameters, β and τ , in python using SciPy's `curve_fit` function.

F. Labeling and imaging

Low-tolerance dishes are collagen coated with 50 μg/ml AS collagen for 1 h, then rinsed with PBS and water. Spheroids are fixed with 4% formaldehyde for 1 h, washed three times for 10 min each with PBS, permeabilized with 0.2% Triton-X 100 for 1 h, washed three times for 10 min each with 10% (w/v) BSA, and soaked in 10% BSA for 30 min. Spheroids are then placed on collagen-coated glass bottom dishes. The spheroids are then labeled with 1:400 ATTO 488 phalloidin to label actin and 1:500 DAPI to label nuclei in 10% BSA for 24 h at 4 °C. The spheroids are then washed with BSA, mounted in 80% glycerol with pH of 8.5, and left to clear for at least 24 h at 4 °C. For cells where cytosol labeling is desired, cells are incubated with CellTracker Orange for 45 min at a concentration of 2 μM prior to spheroid formation.

Fixed single slice spheroid imaging is performed around the spheroid bisecting plane on an inverted confocal laser-scanning microscope (Zeiss LSM800) in confocal fluorescence mode with a 63× oil (NA 1.4) objective, except for Fig. 11, where a 20× air (NA 0.75) objective is employed. For 3D quantification, 50-μm Z-stacks are collected with a step size of 0.5 μm using a 40× oil (NA 1.3) or 63× oil (NA 1.4) objective. ATTO 488 phalloidin (actin imaging), DAPI (nuclear imaging), and CellTracker Orange (cytosolic imaging)

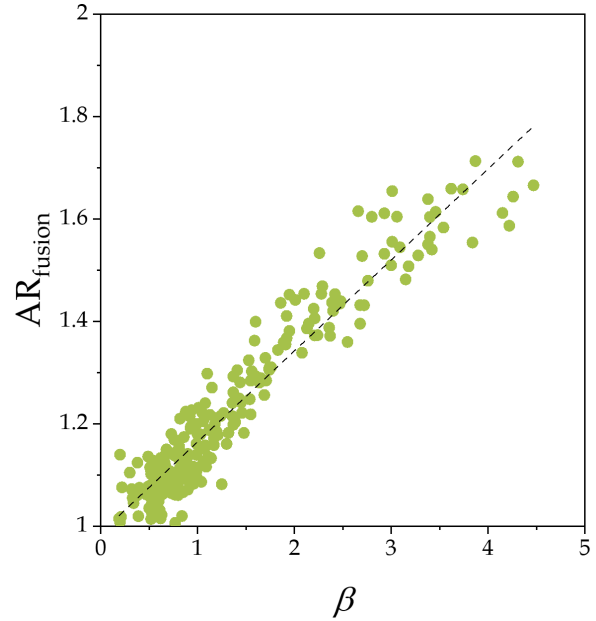


FIG. 7. Aspect ratio (AR_{fusion}) plotted against β from Kelvin-Voigt fits ($n = 255$ spheroid pairs). Dotted line displays fit from linear regression with $R^2 = 0.92$.

are excited in sequence with 488-, 405-, and 561-nm lasers, respectively, and emission is collected through appropriate filter sets.

G. Cell-shape quantification

For all cell and nuclei area and shape quantification except for that shown in Appendix C, segmentation and shape quantification are performed as follows. Within each spheroid, cells and their corresponding nuclei are chosen at random for segmentation from the bulk of the spheroid, except in cases where edge cells are being quantified. Segmentation of cells and nuclei in 2D images is performed manually in ImageJ using the freehand selection tool to draw regions of interest (ROIs) using phalloidin-labeled actin to delineate cell membranes and DAPI to delineate nuclei. Perimeters, p , and areas, A , calculated within ImageJ from the segmented cells are used to calculate the cell shape index, $q = \frac{p}{\sqrt{A}}$. Nuclear aspect ratio is also obtained in ImageJ, derived from the ratio of the minor and major axes of a fitted ellipse to the nuclear ROI.

For each spheroid, a single CeNuS parameter [Eq. (2)] is calculated by sampling a subset of cells within the spheroid. The values are standardized, as a z-score, to a random subset ($n = 4$ 10A spheroids and 4 DCIS spheroids with an average 23 cells each) of the mitomycin-C-treated 10A and DCIS cell data.

For data shown in Appendix C, both 2D and 3D cell segmentation and shape quantification are performed using one or more images from the Z-stacks described in Sec. IV F above. Spheroid images are first despeckled and bleach corrected in ImageJ [69]. All cells and nuclei in each spheroid are then segmented using CellPose-SAM [70]. Segmentations are quantified using a custom python script employing scikit-image [71]. 3D cell shape indices are calculated as $q_{3D} = S/(36\pi V^2)^{1/3}$, where S is the surface area and V the

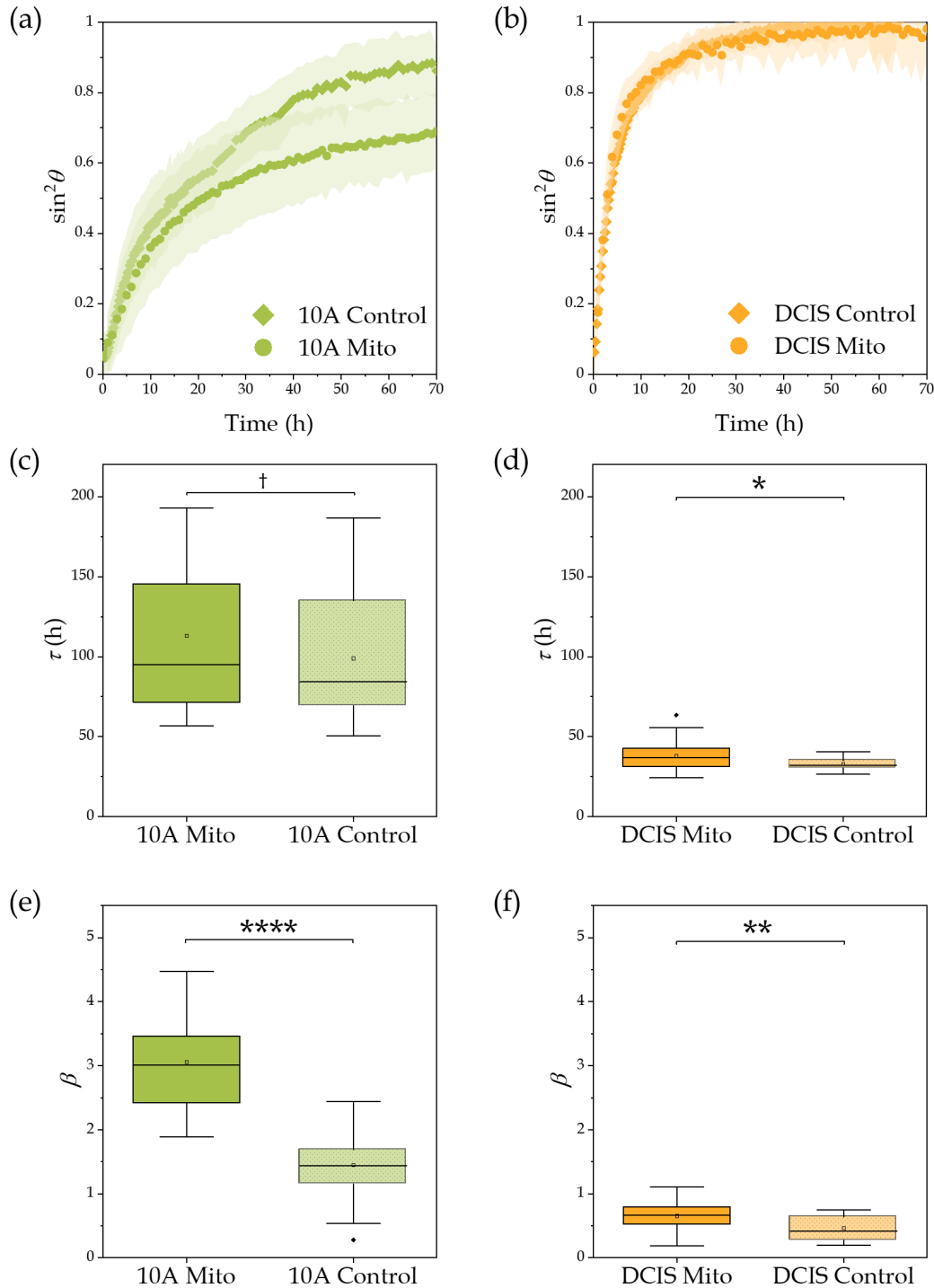


FIG. 8. [(a), (b)] Evolution of $\sin^2 \theta$, with θ the angle of fusion for (a) 10A and (b) DCIS spheroids treated with mitomycin-C vs control, with the mitomycin-C-treated data also shown in Fig. 1 in the main text. Standard deviations are shown as shaded regions. (c) Box plot displaying τ from Kelvin-Voigt fits in mitomycin-C-treated and control 10A spheroids ($p_{\text{MW}} > 0.05$, $n = 17$ 10A control spheroid pairs and $n = 33$ 10A mitomycin C spheroid pairs). (d) Box plot displaying τ from Kelvin-Voigt fits in mitomycin-C-treated and control DCIS spheroids ($p_{\text{t-test}} < 0.05$, $n = 18$ 10A control spheroid pairs and $n = 34$ 10A mitomycin C spheroid pairs). [(e), (f)] β from fits to the Kelvin-Voigt model for mitomycin-C-treated and control sets of (e) 10A ($p_{\text{t-test}} < 0.0001$, $n = 17$ 10A control spheroid pairs and $n = 33$ 10A mitomycin C spheroid pairs) and (f) DCIS spheroids ($p_{\text{t-test}} < 0.01$, $n = 18$ 10A control spheroid pairs and $n = 34$ 10A mitomycin C spheroid pairs). All box-and-whisker plots show mean (open symbols), median (lines), IQR (boxes), and $1.5 \times \text{IQR}$ (whiskers). Outliers are those outside $1.5 \times \text{IQR}$ and are displayed as individual points.

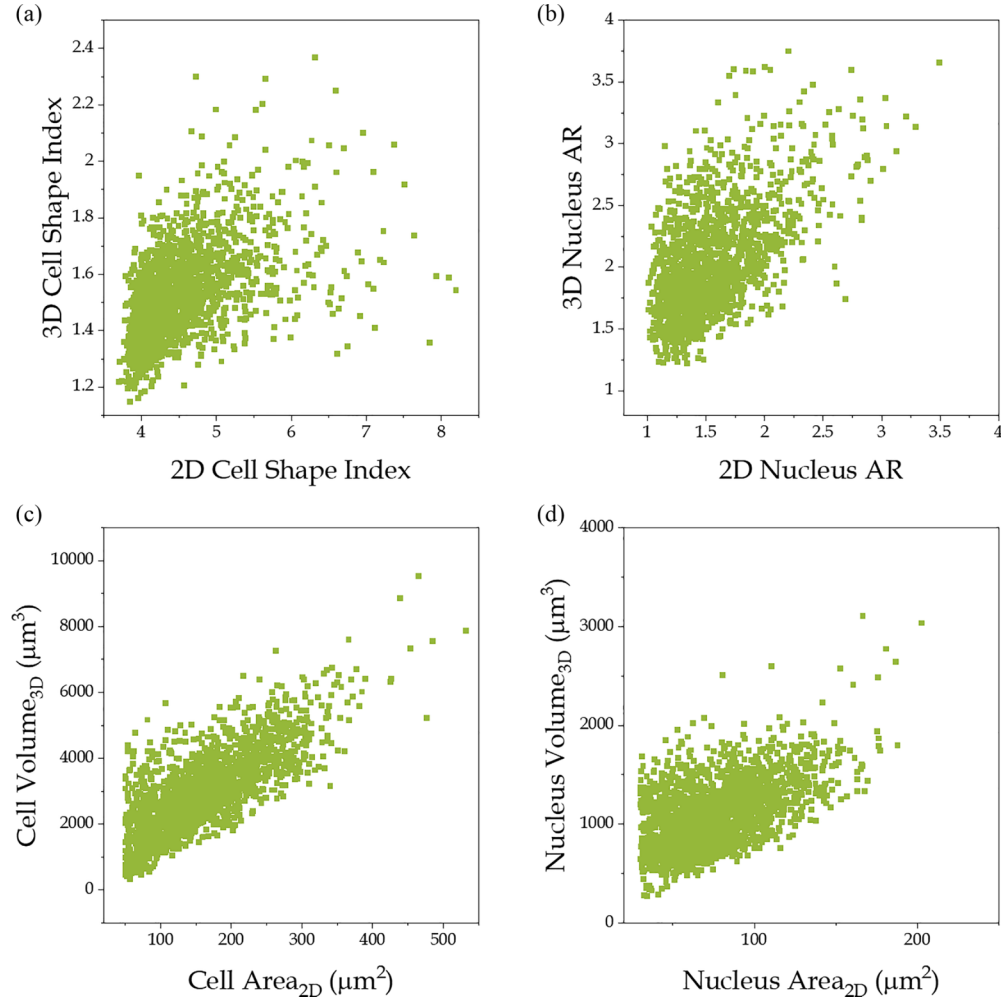


FIG. 9. 3D vs 2D comparison of Cellpose-SAM segmented (a) cell shape index $q_{3D} = S/(36\pi V^2)^{1/3}$ and $q_{2D} = \frac{p}{\sqrt{A}}$, (b) nuclear aspect ratio (ratio of the major to minor axis length), (c) cell area/volume, and (d) nuclear area/volume. For each plot, $n = 1804$ cells obtained from an average of $n = 200$ cells from each of three 10A spheroids, $n = 193$ cells from each of three DCIS spheroids, and $n = 208$ cells from each of four TSA-treated 10A spheroids.

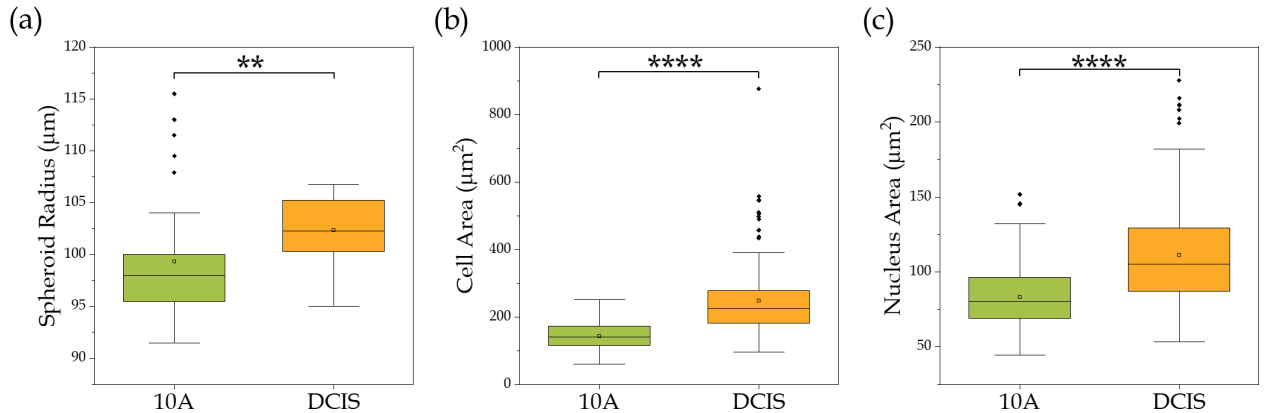


FIG. 10. (a) DCIS spheroids are significantly larger than 10A spheroids ($p_{MW} < 0.01$, $n = 34$ 10A spheroids and $n = 36$ DCIS spheroids). (b) DCIS cells are larger than 10A cells ($p_{MW} < 0.0001$, $n = 103$ 10A cells from four spheroids and $n = 161$ DCIS cells from six spheroids). (c) DCIS nuclei are larger than 10A nuclei ($p_{MW} < 0.0001$, $n = 123$ 10A nuclei from four spheroids and $n = 154$ DCIS cells from six spheroids). All box-and-whisker plots show mean (open symbols), median (lines), IQR (boxes), and $1.5 \times \text{IQR}$ (whiskers). Outliers are those outside $1.5 \times \text{IQR}$ and are displayed as individual points.

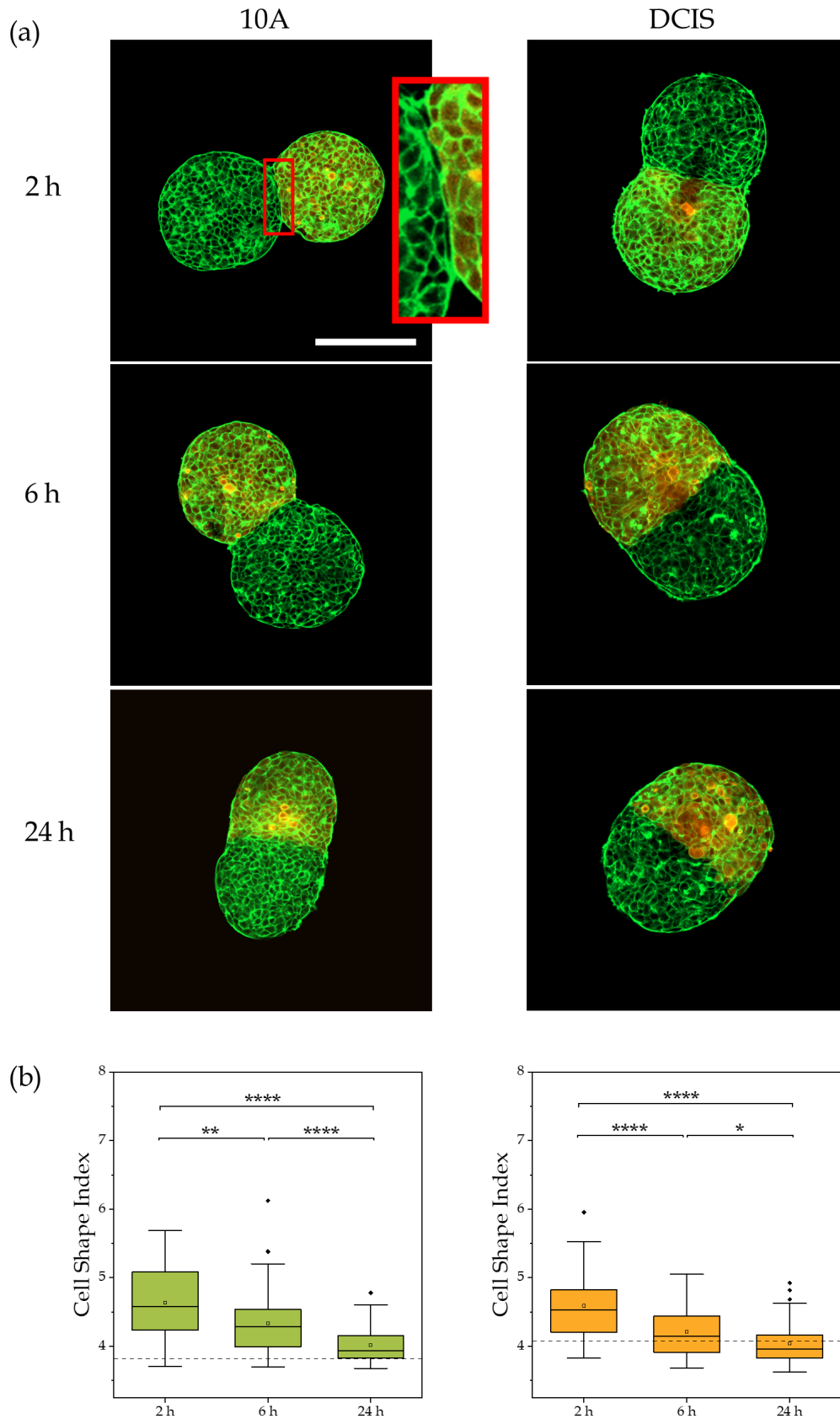


FIG. 11. (a) Fusion progression for (left) 10A and (right) DCIS spheroids at (top) 2 h, (middle) 6 h, and (bottom) 24 h. One spheroid is labeled only with phalloidin (green) while the other is labeled with phalloidin and CellTracker Orange (orange). Inset: higher-magnification image of neck cells in contact with the other spheroid. Scale bar is 100 μm . (b) Shape evolution of cells at the neck of fusing spheroids over time ($p_{\text{MW}} < 0.01$, $p_{\text{MW}} < 0.0001$, and $p_{\text{MW}} < 0.0001$ for 10A 2 h–6 h, 2 h–24 h, and 6 h–24 h comparisons, respectively; $p_{\text{MW}} < 0.0001$, $p_{\text{MW}} < 0.0001$, and $p_{\text{MW}} < 0.05$ for DCIS 2 h–6 h, 2 h–24 h, and 6 h–24 h comparisons, respectively. $n = 45, 62$, and 30 10A cells from three

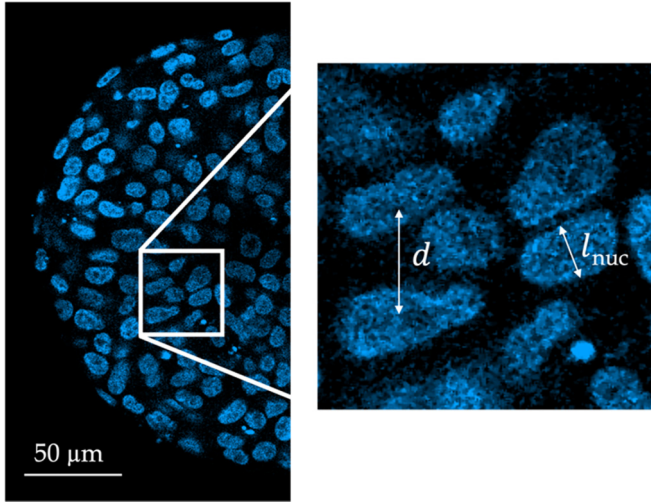


FIG. 12. Absolute internuclear distance, d , is centroid-to-centroid distance between nuclei. This quantity is then scaled by the average length of the minor axis of the nuclei, l , for each spheroid. The effective internuclear distance describing the space between nuclei can then be calculated as $\delta = \frac{d}{l} - 1$.

volume of each cell. Comparisons are made to 2D metrics by analyzing the same cells in a single confocal slice from the Z-stack at the bisecting plane of the spheroid. 2D shape indices are calculated as $q_{2D} = \frac{p}{\sqrt{A}}$, where p is the perimeter and A the area of each cell. For both 2D and 3D segmentations, aspect ratios of nuclei are taken as the ratio of the major to minor axes calculated from the axis lengths of an ellipse with the same normalized second central moment as the segmented nuclei. Cells and nuclei with 2D cross-sectional area less than 50 and 30 μm^2 , respectively, are excluded from analysis, as these are not well represented by the imaged 2D slice.

H. Nearest-neighbor calculation

Nuclear centroid positions are identified using the StarDist plugin in ImageJ [72]. A K-Means tree is constructed with all nuclei in the spheroid. The distances of the six nearest neighbors of each nucleus, excluding those on the edge of the spheroid, are calculated and averaged to obtain the average nuclear spacing for each spheroid, d . This quantity is then normalized by the average minor axis of the nucleus of that spheroid, l , using the aspect ratio and area of the nuclei, obtained from ImageJ to obtain the effective internuclear distance, δ [Eq. (1); see Figs. 12 and 13 in Appendix F]. Trends are found to be invariant to the number of nearest neighbors probed.

I. Statistical analysis

All data are tested for normality using Shapiro Wilks tests. If normal, t -tests are performed (indicated by $p_{t\text{-test}}$). Otherwise, Mann-Whitney tests are performed (indicated by p_{MW}).

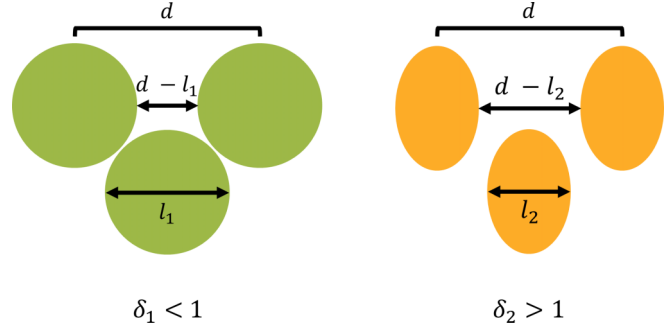


FIG. 13. Schematic depiction of how nuclear shape affects internuclear spacing. Though absolute internuclear spacing, d , remains the same at left and right, differences in nuclear shape affect the minor axis length, l , which then alters effective nuclear spacing, δ .

To test correlations, a Pearson's correlation test is performed. All tests are performed in OriginPro. Statistical significance is indicated by $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$. Lack of statistical significance is denoted by a dagger ($\dagger$).

ACKNOWLEDGMENTS

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DATA AVAILABILITY

The data that support the findings of this article are not publicly available. The data are available from the authors upon reasonable request.

APPENDIX A: CORRELATION BETWEEN FUSION ASPECT RATIO AND β

Linear regression is performed to assess the relationship between aspect ratio ($\text{AR}_{\text{fusion}}$) and β in OriginPro. The resulting fit has an adjusted R^2 value of 0.92 with p -values less than 0.01 for both the intercept and slope: $\text{AR}_{\text{fusion}} = 0.99 + 0.18\beta$. Thus, $\text{AR}_{\text{fusion}} = 1.2$ is equated to $\beta = 1.2$ (see Fig. 7).

APPENDIX B: PROLIFERATION EFFECTS

We investigate the effects of proliferation on coalescence. For 10A spheroids, coalescence is progressed by proliferation, while for DCIS spheroids little change is observed (see Fig. 8).

spheroids each for 2, 6, and 24 h, respectively. $n = 41, 52$, and 42 DCIS cells from three spheroids each for 2, 6, and 24 h, respectively). Dotted reference line displays the cell shape index of the bulk of the corresponding individual spheroids. All box-and-whisker plots show mean (open symbols), median (lines), IQR (boxes), and $1.5 \times \text{IQR}$ (whiskers). Outliers are those outside $1.5 \times \text{IQR}$ and are displayed as individual points.

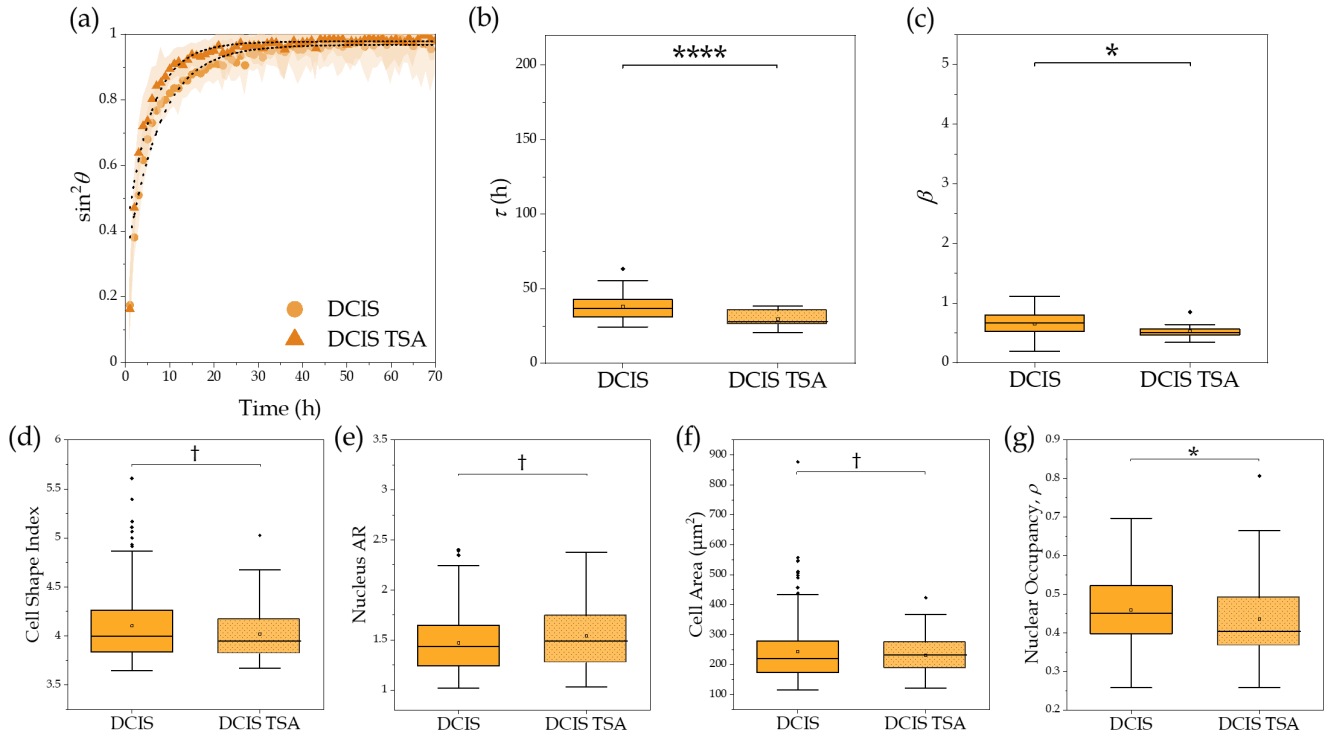


FIG. 14. (a) TSA-treated DCIS spheroids (triangles) fuse slightly more than control spheroids. Standard deviations are shown as shaded regions. [(b), (c)] TSA-treated DCIS spheroids fuse slightly faster and more, as demonstrated by lower (b) τ ($p_{t\text{-test}} < 0.0001$) and (c) β ($p_{t\text{-test}} < 0.05$) values ($n = 34$ DCIS spheroid pairs and $n = 18$ DCIS TSA spheroid pairs). [(d)–(f)] TSA treatment has little effect on (d) cell shape index ($p_{\text{MW}} > 0.05$, $n = 221$ cells from six DCIS spheroids and $n = 93$ cells from five DCIS TSA spheroids), (e) nuclear AR ($p_{\text{MW}} > 0.05$, $n = 210$ cells from six DCIS spheroids and $n = 93$ cells from five DCIS TSA spheroids), (f) cell area ($p_{\text{MW}} > 0.05$, $n = 153$ cells from six DCIS spheroids and $n = 93$ cells from five DCIS TSA spheroids), and (g) nuclear occupancy fraction ($p_{\text{MW}} < 0.05$, $n = 146$ cells from six DCIS spheroids and $n = 93$ cells from five DCIS TSA spheroids). All box-and-whisker plots show mean (open symbols), median (lines), IQR (boxes), and $1.5 \times \text{IQR}$ (whiskers). Outliers are those outside $1.5 \times \text{IQR}$ and are displayed as individual points.

APPENDIX C: TWO- VERSUS THREE-DIMENSIONAL COMPARISON

While this work focuses on metrics that can be obtained from 2D images, we assess correlation between 2D and 3D quantities of interest (see Fig. 9). Spheroids composed of 10A, DCIS, or TSA-treated 10A cells are prepared and imaged for 2D vs 3D comparisons, with 50- μm Z-stacks with a step size of 0.5 μm collected around the midplanes of these spheroids. Cellpose-SAM [70] is used on all slices within each Z-stack for 3D segmentation and on an individual slice for 2D segmentation. Phalloidin-labeled actin is used to segment cells and DAPI-labeled nuclei are used to segment nuclei. Segmentations are analyzed using a custom python script. 3D shape indices are defined as $q_{3D} = S/(36\pi V^2)^{1/3}$, where S is the surface area and V the volume of the cell. 2D shape indices are computed from a confocal slice of the spheroid and are defined as $q_{2D} = \frac{p}{\sqrt{A}}$, where p and A are the perimeter and area of the cell, respectively. 2D cell shape indices reported here are somewhat higher than reported elsewhere in the paper, where cell segmentation is performed by hand. We hypothesize this finding is due to both inclusion of spheroid edge cells in these quantifications and pixelation effects also noted in Ref. [46]. Aspect ratios of nuclei are taken as the ratio of the longest to shortest axes calculated from the axis lengths of an ellipse/ellipsoid with the same normalized second central moment as the segmented nuclei.

APPENDIX D: 10A VERSUS DCIS SIZE COMPARISON

Spheroids composed of 10A cells are smaller than those composed of DCIS cells, as driven by smaller cellular area (see Fig. 10). The nuclear area of 10A cells is also smaller than that of DCIS cells but nuclear occupancy is higher in 10A cells, as shown in Fig. 2 of the main text.

APPENDIX E: CELL SHAPES AT FUSION NECKS

We propose that rapid initial fusion is due in part to the elongated cells at the edge of the spheroids. These elongated borders come into contact at the neck of fusing spheroids (see Fig. 11).

APPENDIX F: SCALED INTERNUCLEAR SPACING

StarDist segmentation of nuclei is used to obtain centroid positions of all nuclei within a confocal slice of each spheroid. K-means clustering is then used to average the centroid-to-centroid distances between each cell in the bulk of the spheroid and its six nearest neighbors. For each spheroid, all of these average six nearest-neighbor distances are then averaged to obtain an absolute internuclear spacing metric, d , as displayed schematically in Fig 12. For each spheroid, the average nuclear minor axis length, l , is used to normalize the internuclear spacing to obtain the normalized internuclear

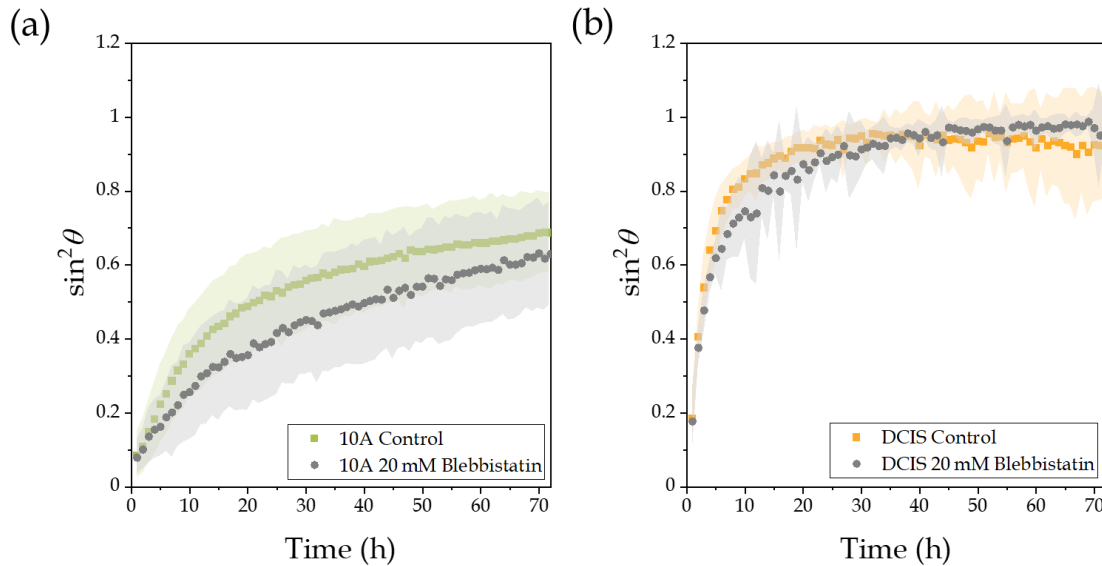


FIG. 15. (a) For 10A spheroids, 20 μ M blebbistatin slows but does not significantly alter the extent of coalescence compared to control ($n = 52$ 10A control spheroid pairs and $n = 17$ 10A blebbistatin spheroid pairs). (b) For DCIS, 20 μ M blebbistatin does not alter coalescence compared to control ($n = 54$ DCIS control spheroid pairs and $n = 17$ DCIS blebbistatin spheroid pairs). Standard deviations are shown as shaded regions.

spacing, $\delta = \frac{d}{l} - 1$. The normalized internuclear spacing reflects the unoccupied space between nuclei, which is sensitive to changes in nuclear size and shape, which are not captured by considering only absolute centroid-to-centroid nuclear spacing (see Fig. 13).

APPENDIX G: TRICHOSTATIN-A-TREATED DCIS SPHEROIDS

TSA treatment leads to a small decrease in fusion timescale and increase in fusion for DCIS spheroids. Little change in

the shapes and a small decrease in nuclear occupancy are observed (see Fig. 14).

APPENDIX H: CONTRACTILITY EFFECTS ON COALESCENCE

To probe contractility effects on fluidity, we monitor coalescence of nonproliferative spheroids (treated with mitomycin C) in the presence of 20 μ M blebbistatin to reduce actomyosin contractility. Though a minor slowdown is observed for 10A coalescence, the overall extent of fusion is unchanged, as 10A spheroid coalescence stalls while DCIS spheroids fully fuse (see Fig. 15).

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