On the mechanism of long-range orientational order of fibroblasts

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Long-range alignment ordering of fibroblasts have been observed in the vicinity of cancerous tumors and can be recapitulated with in vitro experiments. However, the mechanisms driving their ordering are not understood. Here, we show that local collision-driven nematic alignment interactions among fibroblasts are insufficient to explain observed long-range alignment. One possibility is that there exists another orientation field coevolving with the cells and reinforcing their alignment. We propose that this field reflects the mechanical cross-talk between the fibroblasts and the underlying fibrillar material on which they move. We show that this long-range interaction can give rise to high nematic order and to the observed patterning of the cancer microenvironment.

Fibroblasts are spindle-shaped cells that are highly motile and are involved in many critical biological processes, such as wound healing (1, 2). Recently, their major role in shaping the local microenvironment around a growing tumor was shown in numerous studies (3–5). As a result, fibroblasts can affect the ability of cancer cells to metastasize (6–8) and conversely, the ability of the immune system to find and attack those cells (9).

An isolated fibroblast moves back and forth on coverslips for over 60 h without significant change of the direction of its major axis (10). Typically, those fibroblasts are in a spindle shape with an aspect ratio from two to five. Apart from steric constraints, fibroblasts barely interact with each other (10). Curiously, imaging of tumor microenvironments often indicates long-range ordering of fibroblasts. This order often takes the form of circumferential alignment of the cells in a region surrounding the cancer cells (11, 12). The mechanisms that lead to this ordering are not well-understood.

Notably, a similar ordering can be observed for underlying collagen fibers (6, 13). Collagen fibers are the main structural component in the extracellular space of various normal connective tissues and play a significant role in local cancer cell invasion and in metastasis (14). Aligned fibers perpendicular to the boundary cancer cell clusters facilitate local invasions of cancer cells, and conversely, aligned fibers parallel to the tumor boundary may restrict the migration of cancer cells. Furthermore, the circumferential collagen fiber structure has been hypothesized to be responsible for the observed separation between immune and cancer cells (13). Specifically, ex vivo assays indicate that the migration of tumor-killing CD8+ T cells is reduced where dense collagen fibers form conduit-like structures (15). Therefore, understanding the mechanism of the pattern formation of fibroblasts and collagen fibers in the cancer microenvironment is important to strike a balance between constraining cancer cell from invasion and enabling the infiltration of cancer-killing immune cells.

In vivo experimental observation of this pattern formation dynamically is technically challenging. On the other hand, long-range ordering of fibroblasts can be recapitulated in 2D cell culture experiments. In particular, Duclos et al. (10) generated quantitative measurements of cluster orientation as fibroblasts move, collide, and proliferate (grow and divide) on coverslips in 2D. To quantify the collective alignment of fibroblast, the authors used their data to compute the population-averaged nematic order parameter \( Q = \sqrt{ \langle \cos 2\theta_i \rangle^2 + \langle \sin 2\theta_i \rangle^2 } \), which increases from zero for randomly oriented cell population up to the value of one for perfectly aligned cells. The results indicate that, as cell density increases because of cell growth, the cells become more aligned, and the order parameter increases. After roughly 80 h, cell motions become significantly limited because of jamming, and the nematic order parameter freezes at a fixed value. Fibroblasts grown in a relatively narrow channel (100 cells across) robustly display almost perfect nematic alignment (order parameter \( Q \approx 1 \)).

In this work, we aim to understand the underlying mechanisms of the long-range nematic alignment of fibroblasts. Noting that steric interactions between fibroblasts may lead to local alignment, we ask if these interactions are sufficient to explain the experimental results described above. The results indicate that perfect long-range alignment cannot emerge from models treating fibroblasts as apolar active particles with hard collision interactions (active nematics). Therefore, we further test models in which nonlocal interactions between fibroblasts are introduced. Biologically, these models are motivated by the observations (16–19) that fibroblasts are able to deposit and reorganize fibrillar materials around them. Using this model, we investigated whether coupling between cells and their mechanical environment can recapitulate patterns of fibroblasts both in vitro and in vivo.

Results

“For Fibroblasts” Interacting Through Collisions Cannot Produce the Long-Range Alignment. We first set out to test whether the steric interactions between fibroblasts are sufficient to explain the

Significance

Long-range alignment patterns of fibroblasts have been observed both in vivo and in vitro. However, there has not been much understanding of the underlying mechanism. In this work, we show that these patterns cannot be simply explained by their steric interactions with one another during collisions. Instead, we propose that fibroblasts may collectively align through nonlocal interactions arising from their modification of an underlying ECM. The proposed mechanism explains the observed coalignment between fibroblasts and collagen fibers around tumors and can be tested in future experiments that can image the dynamics of this pattern formation in vivo or in vitro.

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long-range alignment of cells observed in the experiment. To proceed, we start with a Monte Carlo simulation framework, in which fibroblasts are represented as hard elliptic particles. Particles move back and forth and reorient after overlapping with neighbors (Fig. 1A). To mimic the fibroblast proliferation observed in experiments (10), new particles are added in front of randomly selected particles and with the same orientation as the selected particle. In addition, we also studied another model that considers explicit forces acting on particles. In this model, each fibroblast cell is represented by a 2D spherocylinder (Fig. 1B).

Implementation details of various cell processes are described in Materials and Methods.

With the collision-like interaction, our simulations (Fig. 2) did not reproduce the perfect alignment of fibroblasts (particles in the model) in a channel-like structure, where the width of the channel is 60 fibroblasts across. In our simulations, while those particles right next to channel boundaries align parallel to boundaries, this alignment disappears for cells in the channel center (Fig. 2A and B). The spatial-dependent alignment is further quantified in SI Appendix, Fig. S18. In contrast, experiments in ref. 10 showed nearly perfect alignment of fibroblasts to the channel boundaries up to a channel width of 500 µm (which is equivalent to about 100 cells across given that a cell width is around 5 µm) (Fig. 3B). We note that, while changes in the particle geometry (e.g., aspect ratio; ratio between length and width of a particle) can affect the alignment, perfect nematic alignment is never observed for biologically relevant parameter values.

We have investigated a number of other modifications of our basic model (SI Appendix). For example, we tested the effects of nonreversal motion, channel width, flexibility of fibroblasts, initial cell density, initial alignment orders, etc. However, we did not observe significant change to our major conclusions. We, therefore, conclude that additional mechanisms are needed to explain emergent nematic ordering.

Mutual Alignment Between Fibroblasts and Fibers Can Generate Perfect Alignment in Narrow Channels. Motivated by ref. 20, we propose a mechanism in which there exists an orientation field coupled to nearby particles and that this field can, in turn, affect the orientation of those particles. For fibroblasts, this idea is supported by the fact that cells secrete materials for ECM, such as collagen (16, 17). Furthermore, as fibroblasts move, they contract and align ECM fibers depending on their direction of motion (18, 19). Recent experimental results also show that a fiber network under strain over a threshold time can change its structural and properties in a nonreversible fashion (21). As a result, the fiber network at some point in space can "remember" the average orientation of the particles that have previously traversed close to that location (22). We hypothesize that mutual alignment of ECM network and fibroblasts drives the emergence of the patterns in cancer tissue specimens and the high nematic order in culture.

Rather than formulate an extremely detailed mechanical model of ECM-fibroblast co-orientation, we have opted for a simple proof-of-principle phenomenological approach. Because of the qualitative similarity between the Monte Carlo simulation and the Newtonian force model, we only implement and study the mechanism using the Monte Carlo simulation framework. The local orientation field at a point on a 2D surface is affected by the orientations of particles \( \theta_i \), within a radius of \( R_s \). However, as the orientation field gradually changes in response to nearby fibroblasts, it maintains a memory of its previous state. On the other hand, each particle gradually turns to align with the direction of the local orientation field and moved according to this new orientation. Detailed implementation of the model can be found in Materials and Methods.

The model implementing the above-described mechanism shows a higher order of particle alignment. Specifically, nearly perfect nematic alignment can be realized for the channel-like confinement setup, where the width of the channel is 60 particles across. In this case, cells close to the hard walls move around and orient themselves to align with the wall. Then, this orientation propagates into the channel center. The time-dependent configurations of particles are shown in Fig. 3A. These features are quite similar to those reported by Duclos et al. (10) (Fig. 3B) and quantified in Fig. 3C. We should note that, for the simulations in the channel case, even with mutual alignment interactions, there are still two cases of eight simulations which stay at a high but not nearly perfect orientational order. In those cases, defects developed close to the boundary and did not resolve before the particle density increased to a level that the jamming effect kicked in. As expected, with a bigger channel width, clusters of fibroblasts which are not parallel to the boundary will develop, and the orientational order decreases (SI Appendix, Fig. S19).

We also performed simulations with periodic boundary conditions in both x and y directions, which mimic the fibroblast culture experiment on a large 2D surface instead of a channel-like confinement setup. We show that the nonlocal alignment interaction between fibroblast facilitated by the "fiber" field is
Mutual Alignment Between Fibroblasts and Fibers Results in the Long-Range Circumferential Alignment of Fibroblasts and Fibers Around Obstacles. To investigate whether the mutual alignment model can explain the in vivo results, we now add obstacles to our simulation mimicking the effect of the cancer cell clusters. We found that the ability of these simulations to produce long-range alignment around the obstacles depends on the rules of fibroblast proliferation. If new elliptic particles are introduced into the simulation box with the same probability everywhere (i.e., if fibroblast proliferation rate is not location-dependent), simulations often result in clusters of particles pointing toward an obstacle (SI Appendix, Fig. S21). Because it takes some time before particles align to the boundary of an obstacle and reorganize the orientation field around an obstacle, jamming effects caused by high particle density can hinder the circumferential alignment. Therefore, we concluded that it is important to ensure that particles around an obstacle do not jam before they align with the boundary.

To avoid this possible jamming, we introduce one other feature, namely that new elliptic particles be introduced with a higher probability in a range close to the obstacles. This assumption is based again on the observations of ref. 10: that fibroblasts closer to boundaries proliferate faster. This may also be a feature of the cancer microenvironment, where fibroblasts can be stimulated by growth factors released by the tumor.

With this new ingredient applied in the simulation, we can now generate the pattern in which the ellipses as well as orientation field align azimuthally around obstacles. These results are shown in Fig. 4 A and B.

We should note that, without the coupling between particles and the orientation field in our simulations, the circumferential pattern of particles can also emerge in the region immediately around the obstacle (SI Appendix, Fig. S22). However, as we move away from the obstacle, the circumferential pattern disappears, and the particles are not well-organized as shown in the mutual alignment model (Fig. 4 A and B).

Experimental Results Confirm the Predicted Circumferential and Long-Range Alignment of Fibroblasts and Collagen Fibers Around Tumor Cell Clusters. When we consider the mutual alignment between fibroblasts and fibers, our model predicts a long-range circumferential alignment of both fibroblasts and collagen fibers around tumor cell clusters. To confirm the prediction in the patient cancer tissue specimens, we simultaneously imaged cancer-associated fibroblasts and collagen fibers in the same region of patient tumor specimens as shown in Fig. 4 C and D. The experimentally observed patterns of fibroblasts and fibers closely match the simulation results, which suggest that our model can capture the underlying mechanism of the patterns of fibroblasts and fibers in cancer tissue. We should note that the pattern observed in the experiment shown in Fig. 4 C and D is a projection of the actual 3D structure on a 2D section. Although we have not yet been able to perform a 3D simulation, we argue that the mechanism proposed should be important for the long-range alignment order in 3D cancerous tissues.

Discussion

In this paper, to understand the underlying mechanism of the long-range alignment pattern of fibroblasts observed both in vitro and in vivo, we developed computational frameworks to test the roles of steric interactions among neighboring cells and mutual interactions between cells and their ECM network. Our results indicate that mutual alignment between fibroblasts and collagen fibers is critical to recapitulate the observed long-range alignment pattern of fibroblasts.

We showed that treating fibroblasts as apolar active particles with hard collision interactions (active nematics) cannot recapitulate the long-range alignment of fibroblasts in vitro (Fig. 2). Our results are not totally surprising in light of some previous studies. For point-like particles with nematic interactions described by a Vicsek-type model (25), there can be high nematic order in a low-rotational noise regime. However, the results are different for hard elliptic particles. Shi and Ma (26) performed kinetic Monte Carlo simulations of such particles moving back and forth and colliding on the 2D surface, which mimic experiments carried out by Narayan et al. (27). The results indicate that, unlike particles performing Brownian motions, nematic order for active nematics gradually decreases with increase in particle density. Direction reversal of self-propelled particles can also play a key role, because it can disrupt nematic order or cell clustering (20, 28). There also has been recent analytical work (29, 30) showing that highly ordered state can be unstable in active nematics. We also tested a competing idea, where fibroblasts can secrete matrix metalloproteinases, which can degrade fiber network and...
that directly image deposited biopolymers in the culture experiments. The model would predict the coevolution in the orientation of fibroblasts and corresponding ECM networks. On the other hand, if the accumulation of ECM materials or the interaction between fibroblasts and underlying ECM networks is significantly disrupted, the model would predict a diminished long-range alignment order of fibroblasts. Future efforts should focus on more detailed quantitative modeling of these mechanical interactions.

Materials and Methods

Modeling Details of the Kinetic Monte Carlo Simulations of Elliptic Particles. Motion of an elliptic particle along its major and minor axes. The lengths of the major and minor axes of an elliptic particle are 2a and 2b, respectively. Aspect ratio is \( r = a/b \). Along its major axis, a particle can move a distance \( v \cdot dt \) in one simulation step. In our simulations, \( dt = 1 \), and \( v \) is a constant velocity along its major axis. At each time step, a particle can reverse its velocity with a probability \( p = 0.025 \). If possible, one particle is introduced into the system every simulation time step, and one simulation time step corresponds to 0.05 h in real time. This mimics the cell density increase observed in experiments. For simulations of elliptic particles, the self-propelled velocity is chosen as \( v = 0.1, 0.1667, 0.2333, 0.3 \) for different aspect ratios \( r = 3, 5, 7, 9 \), respectively.

Along its minor axis, a particle can randomly move with a step size \( v' \cdot dt \); \( v' \) is the velocity along its minor axis and is a random number between \( -v/2 \) and \( v/2 \). In our simulations, varying the amplitude of \( v' \) does not give rise to a perfect alignment of particles in a wide channel (SI Appendix, Fig. S13). In addition, according to the movies in ref. 10, an isolated fibroblast does not significantly translocate along its minor axis compared with its motion along its major axis.

Detecting overlaps between particles. In our kinetic Monte Carlo simulations, an elliptic particle is described by points on its boundary. If we define the angle \( \theta_{\text{old}} \) as the angle between the semimajor axis and the line formed by a point on the boundary and the center of an ellipse, the angle difference \( \Delta \theta_{\text{old}} \) between adjacent points is the same.

In our simulations, the numbers of points for an elliptic particle with the aspect ratio \( r = 3, 5, 7, 9 \) are taken to be equal to 120, 240, 360, and 480, respectively. To detect whether there is any overlap between two particles, we simply calculate (i) the distance \( d_j \) between each point \( j \) on particle 1 and the center of particle 2 or (ii) the angle \( \theta_j \) between point \( j \) and the reference major axis of particle 2. If \( d_j \) is smaller than the distance \( d_j^\text{old} \) of the point on particle 2 with the same angle \( \theta_j \), the two particles should overlap with each other. We also do the same calculations for each point on particle 2 to further improve the accuracy of our detection. A detailed illustration of this procedure can be found in SI Appendix, Fig. S10.

We should note that our method is not exact. However, it works well for the system that we studied, which can ensure there are no particle configurations with any overlap between two particles in our simulations.

Rules of the interaction between channel boundaries and elliptic particles. After a proposed translational motion of a particle, if the particle moves out of the channel boundary and the particle is moving toward the boundary, first the particle will retreat back to its original location. Then, a new orientation is proposed according to Eq. 1:

\[
\theta_{\text{new}} = \frac{k - 1}{k} \theta_{\text{old}} + \frac{1}{k} \theta_b, \quad |\theta_b - \theta_{\text{old}}| \leq \frac{\pi}{2},
\]

or

\[
\frac{\pi}{2} < |\theta_b - \theta_{\text{old}}| \leq \frac{3\pi}{2},
\]

where \( \theta_b = \pi/2 \) or \( 3\pi/2 \) for the left channel boundary or the right one, respectively; \( k \) is a random number evenly distributed between 2 and 10. In the simulation, we further check whether the particle with \( \theta_{\text{new}} \) at its original location will overlap with its neighbors or not. If not, \( \theta_{\text{new}} \) will be accepted and updated. If yes, all proposed motions fail, and the particle simply keeps its original orientation and stays at its original location.

Rules of the reorientation after collisions between elliptic particles. When we examine a pair of particles with their proposed locations, if the two particles overlap with each other, first both particles will retreat back to their original locations. Then, new orientations are proposed to reverse or rotate according to Eq. 2 with equal probability 0.5:

\[
\theta_{\text{new}} = \frac{k - 1}{k} \theta_{\text{old}} + \frac{1}{k} \theta_b, \quad |\theta_b + \text{sgn}(\theta_{\text{old}} - \theta_b) \cdot \pi|,
\]

where \( \text{sgn} \) is the sign function.
where \( \theta_{\text{norm}} \) is the direction of the normal vector of the overlapped surface if the overlapped surface is in the head part of a particle; if the overlapped surface is in the tail part of a particle, \( \theta_{\text{norm}} \) is the opposite direction of the normal vector of the overlapped surface. An illustration on how we decide \( \theta_{\text{norm}} \) can be found in SI Appendix, Fig. S11.

Furthermore, in SI Appendix, Fig. S12, we show that varying the noise level \( \delta/4 \) to \( \delta/2 \), or \( \delta/8 \) cannot give perfect alignment, where \( \delta \) is an evenly distributed random number between 0 and 1.

**Simulation procedures.**

i. Propose new locations for all particles along their major and minor axes.

ii. For simulations carried out in a channel-like structure, we check whether each particle with the proposed location will run into boundaries. If yes, the particle retreats to its original location and rotates according to Eq. 1. If the particle will not overlap with its neighbors, the proposed orientation will be accepted and updated. If there is an overlap, the particle will simply keep its original orientation and location.

iii. We check whether particles will run into their neighbors. For particles with no neighbors around, proposed positions will be accepted and updated. For those with neighbors, it is then checked pair by pair in a sequential fashion whether particles will run into each other. If yes, both particles in a pair retreat to their original locations and then reverse or rotate according to Eq. 2 separately, so that the two particles will be less likely overlapped further.

iv. With newly proposed orientations for the two particles in a pair, we then check whether there is any further overlap between the first particle (\( P_1 \)) selected in the pair and all of its neighbors (in their original positions and orientations). If there is no overlap, \( P_1 \) will get its new orientation accordingly. If there is an overlap, \( P_1 \) will keep its original orientation and location. Then, the second particle (\( P_2 \)) in the pair will be checked to see whether it will overlap with \( P_1 \) (in the already updated orientation) and all other neighbors. If there is no overlap, \( P_2 \) will get its new orientation accordingly. If there is an overlap, \( P_2 \) will keep its original orientation and location.

We perform procedures iii and iv within one simulation step in a sequential fashion for all pairs, and we randomly permuted the sequence of particle interactions after each simulation step.

**Formulation of the orientation field and its effect on particles.** To study the evolution of the fiber orientation, we first divide the 2D space into grids. The orientation field \( \theta \) of each grid point will be affected by the orientations of particles \( \theta_i \) within a radius \( R_c \) surrounding that grid point in a time-dependent manner. Specifically, the orientation field of each grid point will be updated according to Eq. 3:

\[
\frac{d\theta}{dt} = \frac{\alpha}{M} \sum_{i=1}^{M} \sin(2(\theta_i - \theta)),
\]

where \( M \) is the number of particles within a radius \( R_c \) of each grid point. Fig. 5 illustrates the parameters involved. For small \( \alpha \) (field update strength), the orientation field slowly responds to nearby fibroblasts and has a long memory of previous motion in its vicinity.

For a given updated orientation field, a new orientation of each particle is proposed to be equal \( \theta_i' \), where \( \theta_i' = \theta \) if \( |\theta_i' - \theta| < \pi/2 \), \( \theta_i' = \pi \) if \( \pi/2 < \theta_i' - \theta < \pi \) or \( \theta_i' = \pi - \theta \) if \( \pi < \theta_i' - \theta < 3\pi/2 \). This is the orientation of its closest grid point of the orientation field.

The parameter \( R_c \) gives the range in which the fiber orientation will be affected by the contraction of a fibroblast. There has been evidence showing that the contraction of fibroblasts can align neighboring fibers to the extent of a few times the cell body length (19, 32). Based on this, we choose \( R_c \) as three times the length of the semimajor axis of the fibroblast for the simulation in the channel-like confinement setup. We also tested the effects of varying \( R_c \) on the nematic order of fibroblasts in a box with periodic boundary condition shown in SI Appendix, Fig. S20.

**Spherocylinder Cell Model for Fibroblasts.**

**Cell movement.** Here, fibroblasts are represented by a rectangle and two circles at two ends (i.e., a 2D spherocylinder). The particle width or the diameter of the circles at two ends is \( 2b \), and the particle length is \( L_c = 2rb \), where \( r \) is the aspect ratio. Each particle moves on a 2D surface because of self-generated motility forces (\( F^\text{mot} \)) acting at the particle center in the direction of its current orientation \( \theta \). Furthermore, particles periodically reverse (with time scale \( \tau_a \)) their travel direction by flipping their current orientation by \( \pi \) (180°).

**Viscous drag.** Fibroblasts are surrounded by liquid medium, and thus, their motion is hindered by its viscous drag. We approximate the motility of cells as motion of particles in the overdamped limit (low Reynolds number regime) (33). We apply corresponding viscous drag forces (\( F^\text{d} \)) on particles given by \( F^\text{d} = -\gamma v \), where \( v \) is the current velocity of the particle.

**Cell-cell collisions.** We simulate excluded volume interactions during fibroblast movement through cell-cell collisions. Collisions in simulations are defined by the overlap of particle bodies during their movement. We resolve particle collisions by adding appropriate collision resolution forces (\( F^\text{c} \)) to each of the colliding particles at the point overlap in the direction of their collision normal (\( \hat{n} \)).

**Equation of motion.** We solve the following equation of motion based on Newtonian dynamics under overdamped limit [linear (\( \gamma \)) and angular (\( \zeta \)) drag coefficients] for each particle in the system subject to the forces acting on it and obtain its position (\( \hat{r}_i \)), velocity (\( \hat{v}_i \)), orientation (\( \theta_i \)), and angular velocity (\( \hat{\omega}_i \)) at each step of the simulation:

\[
\begin{align*}
\hat{r}_i &= \hat{r}_i - \frac{1}{\gamma} \sum_j \hat{T}_j, \\
\hat{v}_i &= \hat{v}_i - \frac{1}{\gamma} \sum_j \hat{T}_j, \\
\theta_i &= \theta_i - \frac{\omega_i}{\zeta}, \\
\hat{\omega}_i &= \hat{\omega}_i - \frac{\omega_i}{\zeta},
\end{align*}
\]

where summation is over various forces (\( F \)) and torques (\( T \)) acting on a particle. We use the Box2D (34) physics library to solve the equation of motion and resolve collisions in our simulation. All simulation parameters are given in SI Appendix, Table S1.

**Cell growth.** Fibroblast cell density in experiments increased linearly with time when growing in channel structures (10) (except at initial and final stages of experiment). We replicate this linear cell density increase in simulations by adding new particles into simulation at a constant rate \( \gamma \) equivalent to rate of cell density increase in experiments. We add new particles into the simulation by selecting an existing particle randomly within the simulation region and initializing a new particle with same orientation as the selected particle. This process ensures that the added new particle does not disrupt the existing order in the system and imitates the cell division process in experiments (10), where daughter cells move with same orientation as the parent cell. The new particle position \( \hat{p}_i \) is initialized randomly within a radius of \( L_c/2 \) from the selected particle’s center. Additionally, cell density remained stationary in experiments after the density reached above 0.9 (10). We follow this process by stopping the addition of new particles into the simulation region when the system packing fraction (\( \alpha \)) reaches close to one.

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In the supplementary material, variations of our original models are described and studied. The overall conclusion from those studies is that long-range alignment order cannot be achieved with short-range interactions only.

First, we present more details about our Newtonian-dynamics model simulation, such as simulation set-up and values of parameters used. For the parameters chosen based on experimental values, nearly perfect nematic order cannot be achieved in a channel-like structure of 60 cells across.

Second, detailed descriptions of the kinetic Monte-Carlo simulations are presented and several modifications of the simulations reported in the main text are investigated. For example, investigation of the effect of initial ordering showed that as long as the initial density does not give rise to a jamming state, the effect of initial ordering gradually decays away. In addition, simulations with fixed particle density are performed.

To summarize, we show that without non-local interactions for active nematics, a near-perfect long-range alignment cannot emerge from an initial condition with a lower order.

Modeling details of Newtonian-dynamics cell simulation with rod-shaped cells and excluded volume interactions

Our Newtonian-dynamics particle-based simulation framework allows us to study the nematic ordering among fibroblast cells in more detail taking into account excluded cell volume interactions, realistic cell mechanical interactions, and the resulting cell dynamics under the
influence of the forces and viscous drag from surrounding fluid. Additional implementation
details for this model are given below.

**Simulation setup of the spherocylinder cell model for fibroblasts**

![Fig. S1: Schematic of simulation region with rigid and periodic boundaries](image)

We simulate the motility and interaction behavior of particles in a rectangular simulation
region of dimensions $L_{\text{sim}} \times W_{\text{sim}}$ with rigid (horizontal) and periodic boundaries (vertical)
as shown in Fig. S1. We start the simulation with $N_0$ particles positioned randomly in
the simulation region with orientations chosen randomly from $[0, 2\pi]$ range with uniform
probability. Particle positions $\bar{p}$, orientations $\theta$, internal cell length $L$ and a snapshot of the
simulation region are recorded for every 300 simulation steps for later analysis and for easy
visualization.

**Simulation parameters**

To efficiently use the Box2D framework, we employ following scaling for simulation parameters: 10 min of real time = 300 simulation steps; 1 m real space = 1 length unit of simulation.
Variations of the Newtonian-dynamics cell model simulation

Using our particle-based simulation framework we investigated the how mechanical interactions among cells influence the ordering behavior of cells and the overall nematic order of the system in rectangular channels (similar to the experimental setup [1]). We studied the particle motility behavior and overall system order under different system configurations (variation of particle aspect ratios, initial packing fractions of system, initial particle ordering, channel width etc.) to explore the possibility of producing perfect-nematic order among particles similar to the experimental observations [1]. We observed that none of the proposed variations of the simulations result in the perfect nematic order. Results of these simulations are presented below.

Sample simulation run
Fig. S2: Snapshots of the simulation region at different times with rod-shaped $(r=5)$ non-reversing particles. Particles are initialized with random orientations at random positions inside the simulation region. Particles are colored based on their nematic orientation value (see color bar). We observe that with time particles started forming ordered clusters through neighbor collisions. These clusters stabilized and grew in size to much larger ordered regions. Specifically, we observe that particle clusters started growing from the rigid boundaries at sides of the simulation region indicating the crucial role of boundaries in evolution of overall order in the system. Evolution of the system order parameter, $<Q>$, and packing fraction, $\eta$, values corresponding to this simulation are shown in Fig. S3. There can be overlaps between particles at $t=0$ and when a new particle is introduced. But the overlaps will resolve by the resultant repulsive force eventually under the Box2D framework.
Fig. S3: Evolution of system order parameter, $\langle Q \rangle$, (red) and cell packing fraction, $\eta$, (green) values with time for rod-shaped ($r=5$) non-reversing particles. We observe that system order parameter increases, from an initial small value, and reaches a steady state value shortly after the simulation region is completely covered with particles ($\eta = 1$). The time displayed on the x-axis is in the scale of real time, which is converted from simulation steps.
Effect of particle aspect ratio

Next we studied the effect of particle aspect ratio on overall nematic order of the system. We varied the particle aspect ratio over a wide-range ($r = 3-19$, see Fig. S4) and measured the average order parameter $<Q>$ of the system for both reversing ($\tau_R = 3600$ steps) and non-reversing particles. Our simulations show that with increase in particle aspect ratio, avg. order parameter of the system increased for both reversing and non-reversing particles. Further, avg. order parameter values consistently lower for reversing particles compared to non-reversing particles at each particle aspect ratio, consistent with results of previous studies [2]. However, even for very large particle aspect ratio ($r = 19$) values, our simulation did not produce the perfect nematic order among particles as observed in experiments.

![Graph](image)

Fig. S4: Average order parameter $<Q>$ of the system for rod-shaped particles with different aspect ratios, $r$, (Std. dev. values indicate variation across different simulation runs, $n=5$). The order parameter value of each simulation run is averaged over last 18000 simulation steps (= 1 hr real time).

Effect of particle packing fraction

We studied the effect of the initial packing fraction on steady-state nematic ordering of particles (see Fig. S5, S6). We have performed simulations at fixed growth rate of particles (Fig. S5) and also for a fixed packing fraction values (Fig. S6). We observe that at a fixed growth rate, the steady-state order parameter of the system $<Q>$ remained relatively constant for different starting packing fractions values. This indicates that initial packing fraction of system plays only a minor role in determining steady-state order of the system under fixed growth rate. However, with under fixed packing fraction values with no particle growth, steady-state order parameter of the system decreased with increasing system packing fraction. Here, at high packing fraction, particles are not able to reorient and align with neighbors. Finally, we observe that again our simulations fail to produce perfect nematic order under different initial packing conditions of the system.
Fig. S5: Average order parameter $<Q>$ of the system for rod-shaped particles for different initial packing fraction values $\eta$ with particle growth. Standard Deviation values indicate variation across different simulation runs, $n=5$. The order parameter value of each simulation run is averaged over last 18000 simulation steps.

Fig. S6: Average order parameter $<Q>$ of the system as a function of particle packing fraction, $\eta$, (constant through-out simulation). Standard Deviation values indicate variation across different simulation runs, $n=5$. The order parameter value of each simulation run is averaged over last 18000 simulation steps. Aspect ratio of the particle in this simulation is 5.
**Effect of initial ordering of particles**

Particles in our simulations form ordered clusters through mechanical collisions and subsequent reorientation of particles parallel to each other. However, the density of particles in the system increases due to particle growth, thus limiting the movement of particles and hindering their reorientation upon collisions. Thus final steady-state order achieved by the system is limited by the particle growth rate. It is possible that initializing the system with partially ordered particles instead of fully random orientations can increase the final order of the system. So we initialized a fraction of particles ($f_{\text{initial}}$) particles parallel to channel walls ($\theta_i = \pi/2$) and measured the steady-state order achieved by the system. Our results show that initial ordering of system is has negligible effect on the final order of the system (see Fig. S7); even initializing the particles with perfect initial order ($f_{\text{initial}} = 1$) resulted much lower steady-state final order ($< Q > \approx 0.35$).

![Fig. S7: Avg. order parameter, $< Q >$, as a function of fraction of particles initialized parallel to the channel (ordered cell fraction, $f_{\text{initial}}$). Values are averaged over 5 independent simulation runs. Aspect ratio of the particle in this simulation is 5.](image)

**Effect of channel width**

Fibroblast cells moving in narrow channel-like regions formed large-scale perfect nematic ordered regions that spanned whole channel area [1]. Further Duclos et al. showed that this perfect nematic ordering of cells increased with decrease in channel width (perfect nematic ordering achieved as long as cell correlation length is larger than channel width). So we investigated the effect of variation in channel width on system order in our simulations (see Fig. S8). We observe that, similar to experiments, decrease in channel width increased the steady-state avg. order parameter values in simulations. However, even for a very narrow channel case (simulation region dimensions $4rb \times 180b$) particles in our simulation did not reach perfect nematic ordering as observed in experiments.
Fig. S8: Average order parameter $<Q>$ of the system as a function of channel width $W_{sim}$. Values are averaged over 5 independent simulation runs. Aspect ratio of the particle in this simulation is 5.

**Effect of particle flexibility**

Fibroblast are flexible cells and it was shown previously that flexibility can be important in the ordering of cells through mechanical collisions [2]. So we investigated the effect of particle flexibility on steady-state ordering of particles (refer to Balagam et al. [3] for flexible particle model implementation of rod-shaped cells). We observe that both reversing and non-reversing flexible cells did not produce the perfect nematic order in the system (see Fig. S9), and in fact flexibility reduces the order parameter increase seen with large aspect ratio cells. This further reinforces our result that there is no path towards complete ordering without a new mechanism.
Fig. S9: Average order parameter $< Q >$ of system for flexible rod-shaped particles for different aspect ratios. Refer to Balagam et al. [3] for details of flexible particle model.

**Modeling details of the kinetic Monte-Carlo simulations of elliptic particles**

**Detecting overlaps between particles**

In our kinetic Monte Carlo simulations, an elliptic particle is described by points on its boundary. If we define the angle $\theta_{pt}$ as the angle between one of the semi-major axis and the line formed by a point on the boundary and the center of an ellipse, the angle difference $\Delta \theta_{pt}$ between adjacent points chosen to be constant—see Fig S10.

In our simulations, the number of points for an elliptic particle with the aspect ratio $r = 3, 5, 7, 9$ are 120, 240, 360, and 480, respectively. To detect whether there is any overlap between two particles, we simply calculate i) the distance $d_j$ between each point $j$ on particle 1 and the center of particle 2; ii) the angle $\theta_j$ between point $j$ and the reference major axis of particle 2. If $d_j$ is smaller than the distance $d_j'$ of the point on particle 2 with the same angle $\theta_j$, the two particles should overlap with each other. We also do the same calculations for each point on particle 2 to further improve the accuracy of our detection.

We should note that our method is not exact. But we have tested this approach extensively, and it works well for the system we studied, which can make sure there are no particle configurations with any overlap between two particles in our simulations.

**Rules of the reorientation after collisions between elliptic particles**

When we examine a pair of particles with their newly proposed locations, if the two particles overlap with each other, first the particles will retreat back to their original locations. Then new orientations are proposed according to Eq. 1:
θ_{\text{new}} = θ_{\text{old}} + \frac{\delta}{4}(θ_{\text{norm}} - θ_{\text{old}}), |θ_{\text{norm}} - θ_{\text{old}}| \leq \pi,

or θ_{\text{old}} + \frac{\delta}{4}(θ_{\text{norm}} - θ_{\text{old}} + \text{sgn}(θ_{\text{old}} - θ_b) \cdot 2\pi), |θ_{\text{norm}} - θ_{\text{old}}| > \pi,

(1)

where $θ_{\text{norm}}$ is the direction of the normal vector of the overlapped surface if the overlapped surface is in the head part of a particle; if the overlapped surface is in the tail part of a particle, $θ_{\text{norm}}$ is the opposite direction of the normal vector of the overlapped surface. Fig. S11 illustrates how we decide $θ_{\text{norm}}$. 

Fig. S10: This figure illustrates how we determine whether two elliptic particle overlaps with each other.
Fig. S11: $\theta_{\text{norm}}$ in Eq. 1 depends on where particles overlap.

Furthermore, in Fig. S12 we show that varying the noise level from $\delta/4$ to $\delta$, $\delta/2$, or $\delta/8$ cannot give rise to a perfect alignment, where $\delta$ is an evenly distributed random number between 0 and 1.

Fig. S12: Changing the noise level from $\delta/4$ in Eq. 1 to $\delta$, $\delta/2$, or $\delta/8$ does give rise to a perfect alignment. Other parameters are $v = 0.1667$, $p = 0.025$ and $r = 5$. 
Effects of different velocity along the short axis of a “fibroblast”

Here in this section, we vary the parameter $v_{mi}$, which is the velocity of a fibroblast along its short axis, and show that it does not generate the perfect alignment of fibroblasts, for the parameters we test. According to the movies in [1], there is no significant trans-location of the fibroblast along its short axis; hence we only tested a few cases where $v_{mi}$ is smaller than the velocity along its long axis.

Fig. S13: Three simulations are performed for each $v_{mi} = 2v/r$, $v/r$, $v/2r$, where $v = 0.16667$ is the velocity of a “fibroblast” along its long axis and $r = 5$ is the aspect ratio of a “fibroblast”. In all simulations, the number of “fibroblasts” increase from 300 (packing fraction 0.11) to about 1600 (packing fraction 0.59).

Long-time simulations with fixed number of particles does not give rise to the nearly perfect alignment without non-local interactions between particles.

Here in this section, we show that even if the particle number in a channel is low (no large-scale jamming) and does not change, there is still no nearly perfect alignment to the channel boundaries in our simulations. Furthermore, as the particle density increases, the nematic order in the long-time limit decreases.
Fig. S14: In this example, the aspect ratio for elliptic particles is 5. Other parameters are $v = 0.1667$ and $p = 0.025$. Number of particles are fixed as 300 (packing fraction 0.11). (a) Evolution of the order parameter Q in the long-time limit averaged over 3 numerical realizations. (b) Snapshot of the particle configuration at simulation step 36000.

Fig. S15: In this example, the aspect ratio for elliptic particles is 5. Other parameters are $v = 0.1667$ and $p = 0.025$. Number of particles are fixed as 600 (packing fraction 0.22). (a) Evolution of the order parameter Q in the long-time limit averaged over 3 numerical realizations. (b) Snapshot of particle configurations at simulation step 32000.
An initial perfectly aligned order decreases without non-local interactions between particles.

Here in this section, we show that even if the initial particle alignment is perfect, the order parameter will gradually decay away from 1 as particles move around and the number of particles continuously increases. For the case where initially 1/4 of the channel is filled with perfectly aligned particles, the initial perfect order will gradually drop to around 0.8 as shown in Fig. S17.
Quantification of the alignment to the channel boundary in the model with steric interactions only

Fig. S18: A quantification of the absolute angle difference between fibroblasts and their nearest boundary for the Monte Carlo simulations with only steric interactions between fibroblasts. There are 9 samples in total, which correspond to the configurations of fibroblasts at simulation step 1900 (95 hrs, last data points of the black lines in Fig. 3C of main text). The distance between a fibroblast to its nearest boundary is binned at b, 3b, 5b, etc., which corresponds to the width of 1 cell, 2 cells, 3 cell, etc., respectively. The absolute angle difference between a fibroblast and its nearest boundary in each bin is averaged for each sample. The error bars represent the standard deviation of the average of the 9 samples. In the center of the channel, which is 60 cells across, the average angle difference is 45 degrees, which means that the orientation of fibroblasts is random. In the distance within 2 cells to the boundary, the angle difference is less than 10 degrees, which means the fibroblasts are aligned with the boundary. And this alignment gradually decreases for fibroblasts further away from the boundary.
Lower nematic order for wider channels with mutual alignment between fibroblasts and fibers

Fig. S19: (a) For the channel width as 150 cells across, the alignment of fibroblasts to the channel boundary is significantly different from perfect for some numerical realizations. The width of the channel is 300b, which corresponds to the 150 cells across. The simulation window in vertical axis is set as 150b and the boundary condition in the vertical axis is periodic. Other parameters are the same as those used for the red lines in Fig. 3C of main text. Particularly, $R_c = 15b$. (b) The configuration of fibroblasts in the channel, which corresponds to the red dot in (a). The color code for the fibroblasts is the same as Fig. S17 (c) For some of the numerical realization with high nematic order, the orientation of most fibroblasts is not aligned to the boundary. The example configuration corresponds to the blue dot in (a).
Large aligned domains of “fibroblasts” in the model with mutual alignment of fibroblasts and fibers resemble experimental results.

Fig. S20: Simulations considering the mutual alignment of fibroblasts and fibers can generate long-range alignment order in a box with periodic boundary condition. The number of particles gradually increases from 300 to over 1500 in the simulations, i.e., the packing fraction increases from 0.12 up to 0.58. The aspect ratio of particles in these examples is chosen as 5. $\alpha$ (orientation field update strength, see Material and Methods) is chosen as 0.1 in those simulations. (a) A snapshot of the particle configuration of one simulation at step 1600 for periodic boundary condition, which corresponds to nematic order of the blue dot in (b). (b) Different solid lines represent evolution of the nematic order by varying the range of interaction $R_c$ (see Material and Methods). Dash-dotted line is the sample average given in the paper by Duclos et al. [1] and dashed lines are the standard deviations. It is interesting to notice that increasing $R_c$ does not always give rise to a higher order.
Jamming can hinder the circumferential alignment of fibroblasts

Fig. S21: Monte Carlo simulations in a box with periodic boundary condition and one obstacle in the center. The aspect ratio of particles in these examples is chosen as 5. Color scheme for different orientations of particles is as indicated. This particular simulation shows that if a new particle is introduced anywhere in the open space at each simulation step, there will be regions where particles point towards the obstacle instead of circumferentially aligning around its boundary. Basically, the particles become jammed before they can align with the obstacle boundary.
Less organized pattern of fibroblasts without considering the mutual alignment between fibers and fibroblasts

![Image](a) Orientation

![Image](b)

Fig. S22: Monte Carlo simulations in a box with periodic boundary condition and one obstacle in the center. The aspect ratio of particles in these examples is chosen as 5. Color scheme for different orientations of “fibroblasts” is as indicated. As described in the main text, we only introduce new “fibroblasts” in the region close to the obstacle. (a) A snapshot of the particle configuration with only collisional interactions between “fibroblasts”. (b) A snapshot of the particle configuration of our enhanced model, i.e., with interactions between “fibroblasts” and the orientation field.

**Microchannels created by fibroblasts may not be able to reproduce the perfect alignment of fibroblasts in the channel-like structure**

It is possible that because of the secretion of Matrix metalloproteinases (MMPs), fibroblasts can degrade the fiber network and create channel for themselves to move through. Therefore, a fibroblast has a preferential direction to move and another nearby fibroblast may tend to re-orientate and move inside of these created channels. We here describe how we modeled this micro-crack based mechanism and concluded that it was not capable by itself of generating the observed ordering.

In the simulation, we assume that as a fibroblast moves, it will change the orientation field into its moving direction only in the area covered by the fibroblast and only as long as the area has not been explored by any fibroblast before. If a new fibroblast moves to the area with this orientation field, the new fibroblast will re-orientate according to the direction of orientation field in a nematic alignment fashion. On the other hand, there is a small probability (p=0.1) that the orientation will be modified by this new fibroblast. Via this set of rules, the new fibroblast will keep its original direction and create new microchannels ahead for it to follow. This mimics the effects of microchannel creating and microchannel following of fibroblasts. However, this mechanism alone is not able to generate the perfect alignment of fibroblasts in the channel-like structure either, as shown in the following figure.
Fig. S23: Monte Carlo simulations in a channel-like structure with the implementation of the mechanism of microchannel-creating and following. The simulation set-up and parameters are the same as the Monte Carlo simulations with steric interactions only, which are shown in Fig. 2A and black lines in Fig. 3C in the main text.

**Immunohistochemical Labeling**

Anti-alpha-smooth muscle actin ($\alpha$-SMA, clone 1A4, Dako) and anti-pan cytokeratin (clone AE1/AE3, Dako) immunostains were performed on 3 $\mu$m-thick formalin fixed paraffin-embedded (FFPE) breast cancer tissue sections. By following the multiplex IHC opal method (Stack et al. 2014 [4]), $\alpha$-SMA and pan-cytokeratin visualization was accomplished by OPAL-520 TSA and OPAL-690 TSA (both 1:50; PerkinElmer Opal kit), respectively. Samples were further counterstained with DAPI to visualize the nuclei of all cells. Prior imaging, the tissue sections were coverslipped with ProLong® Gold Antifade mounting media (Cat. # P36930, Life Technologies).

**Image Acquisition and Analysis**

Images in Fig. 4C and Fig. 4D were acquired by a commercial two-photon microscope (Ultima Intra-Vital, Brunker Corporation) using a 10X air objective (NA=0.3; working distance=10 mm, Olympus). For $\alpha$-SMA, and pan-cytokeratin imaging a Coherent Chameleon Ultra II laser (tunable from 680-1080 nm; 3000mW at 800 nm) was set at 900 nm and output power at 20% to image collagen using a second harmonic generation (SHG) technique. For the DAPI channel, the laser was set at a wavelength of 730nm and the power output was lowered to 5%. The DAPI emission and the SHG signals were collected at the range of 435-485nm. Green emission was collected between 500-550nm and for the far-red channel of pan-cytokeratin the wavelengths between 640-670nm were collected (all channels, PMT gain between 800-900).

For visual clarity, for the $\alpha$-SMA and SHG images, the pixels, which have an intensity higher than 1200 in the DAPI channel, were first set as zero intensity. Then pixels with intensity at top 30%, 10% and 10% of the non-zero-intensity pixels were selected to show
for pan-cytokeratin, α-SMA, and SHG images, respectively. To further filter out the small clusters inside an image for visual clarity, top 30 and 500 connected clusters in size are selected for pan-cytokeratin and SHG images, respectively. Finally, the processed images were merged together with the background color chosen as red. Fig. 5C is a merge of pan-cytokeratin and αSMA images; Fig. 5D a merge of pan-cytokeratin and SHG images.

References


