

Inferring three-body interactions in cell migration dynamics

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In active matter and living matter, such as clusters of migrating cells, collective dynamics emerges from the underlying interactions. A common assumption of theoretical descriptions of collective cell migration is that these interactions are pairwise additive. It remains unclear, however, whether the dynamics of groups of cells is solely determined by pairwise interactions or whether higher-order interaction terms come into play. To investigate this question, we use time-lapse microscopy to record the dynamics of three cells interacting together in a linear three-site geometry. We collect a large number of cellular trajectories and develop an inference scheme to infer both pairwise and potential three-body cell-cell interactions. Our results reveal evidence of three-body interactions in one of the two cell lines tested. However, these three-body interactions only introduce minor corrections to the overall dynamics. Our work provides a methodology to infer the existence of three-body interactions from trajectory data and supports the commonly assumed pairwise nature of cell-cell interactions.

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I. INTRODUCTION

Many animal cells have the remarkable ability to migrate in a coordinated and collective manner. Collective cell migration is central in biological processes, including embryonic development, wound healing, and cancer metastasis [1–3]. Multicellular systems are also a prominent example of organized active matter [4–6], and there has been an effort to build physical models that capture their dynamics [7–9]. One popular approach uses “active particle models,” where each cell is treated as a point particle subject to active forces accounting for its motility and interactions with neighboring cells [7,9]. Despite their apparent simplicity, active particle models have been able to provide a quantitative description of migrating cells in various contexts [10–17]. However, it remains challenging to determine the exact nature of cell-cell interactions in such models from the underlying biomolecular machinery [8,18,19]. These interactions are therefore often constructed on phenomenological grounds with limiting assumptions.

A central assumption in most models is that cell-cell interactions are pairwise additive [9–11,14–16]. However, since cellular interactions integrate physical forces and biochemical signaling mechanisms [3,18,19], it is unclear whether higher-order interactions, involving three or more cells, may be at play. Higher-order interactions could affect the emergent properties of multicellular systems, as in other biological and soft matter systems [20–27]. Clarifying their presence and im-

portance is therefore crucial for understanding the dynamics of multicellular systems [11,15,17,19,28].

Detecting higher-order interactions from noisy experimental trajectories is challenging [25]. For colloidal suspensions at thermal equilibrium, image-based data have successfully been used to derive the three-body interaction between particles [29]. For cell migration, inference methods have proven to be a powerful tool for determining cell-cell interactions from measured cellular trajectories [19]. A popular approach to infer cell-cell interactions is to confine the cells in a microenvironment [12,14,17,19,30–32]. In particular, Brückner *et al.* employed micropatterns as “cell colliders” and used underdamped Langevin inference (ULI) [33] to systematically infer pairwise cell-cell interactions from experimental data of two interacting cells [12]. Furthermore, they demonstrated that the inferred interactions accurately capture cell motion.

Here, we develop a high-throughput method to study the complex dynamics of groups of three cells on confining micropatterns. We generalize the ULI framework [33] to infer three-body cell-cell interactions directly from the measured cellular trajectories. We examine two breast-derived cell lines: a noncancerous epithelial cell line (MCF10A) and a cancerous mesenchymal cell line (MDA-MB-231). Interestingly, we find that the dynamics of the first is fully captured by pairwise interactions, while the second exhibits distinct three-body interactions. Importantly, however, multicellular dynamics remains largely dominated by pairwise interactions, even for the cancerous cells. Our approach can be applied to other systems and geometries, where three-body interactions may play a more dominant role.

II. METHODS

A. Recording the dynamics of three interacting cells

To investigate the presence of three-body interactions in migrating cells, we record the trajectories of three cells

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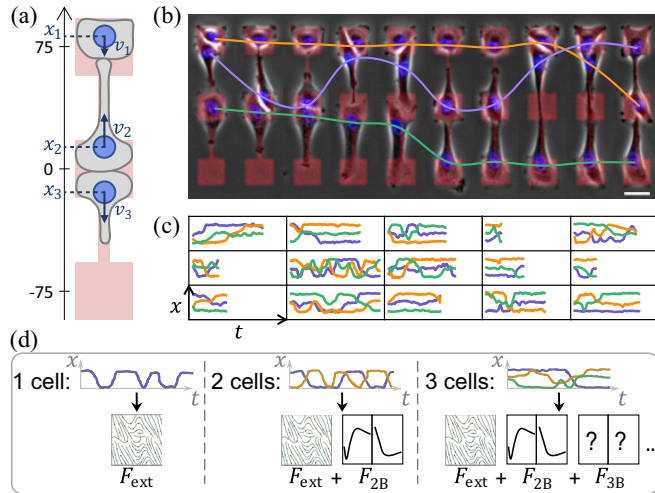


FIG. 1. Stochastic dynamics of three interacting cells. (a) Sketch of three cells confined on a three-site micropattern (axis unit: μm). (b) Time-lapse microscopy images for MDA-MB-231 cells. Micropatterns are labeled in red and cell nuclei in blue. Time interval between two images: 40 min. Scale bar: 30 μm . (c) Sample set of cellular trajectories as a function of time ($0 < t < 20$ h). (d) Schematic of the inference framework applied to systems with one [32], two [12], and three cells migrating on a confining micropattern. Underdamped Langevin inference is used to infer cell interaction with the micropattern (F_{ext}), two-body interactions (F_{2B}), and potential three-body interactions (F_{3B}).

interacting within a confined microenvironment consisting of three square islands connected by two thin bridges [Fig. 1(a)]. This geometry is a natural extension of the one previously used to infer two-cell interactions [12], enabling us to build on the existing framework. We produce large arrays of confining micropatterns coated with fibronectin, a protein that promotes cellular adhesion. The outer area is passivated with poly(L-lysine)-poly(ethylene glycol) (PLL-PEG), which prevents cells from adhering.

We select patterns in which three cells adhere and record their dynamics using time-lapse microscopy for up to 48 h. We observe that cells regularly transition between the square sites of the pattern, resulting in frequent cell-cell encounters. When two or three cells occupy the same site, cell-cell contacts form and can be partially maintained when a cell moves to an adjacent site [see Fig. 1(b) and movies in the Supplemental Material [34]]. This protocol thus allows us to study dynamics of a minimal multicellular system in which three-body interactions could manifest.

We compare the epithelial cell line MCF10A and the cancerous mesenchymal-like cell line MDA-MB-231, both derived from human breast tissue. We observe a qualitative difference in the collective behavior of the two cell lines: While MCF10A cells favor configurations where cells cluster together, often sharing the same site, MDA-MB-231 cells prefer to occupy separate sites. Accordingly, cell-cell contacts appear to be less stable for MDA-MB-231 cells, which is characteristic of mesenchymal-like cells [35]. The MDA-MB-231 cells also exhibit faster dynamics than the MCF10A cells, moving more frequently from site to site and reaching higher velocities (see movies in the Supplemental Material [34]).

To quantify this multicellular behavior, we use a coarse-grained approach and reduce the rich morphodynamics of the cells to one-dimensional trajectories. For each cell, we use the position of the nucleus center to determine its position $x(t)$ along the pattern's long axis [Fig. 1(c)]. For each cell line, we collect over 1500 h of trajectories of three cells interacting under standardized conditions.

B. Stochastic equation of motion

Previous works showed that the dynamics of migrating cells can be captured by an underdamped stochastic equation of motion [10,12,36–38]. In this framework, the acceleration of a cell i is given by

$$\frac{dv_i}{dt} = F_i(\mathbf{x}, \mathbf{v}) + \sigma \eta_i(t), \quad (1)$$

where $\mathbf{x} = \{x_1, x_2, x_3\}$ and $\mathbf{v} = \{v_1, v_2, v_3\}$ are the positions and velocities of the three cells along the x axis. F_i represents a deterministic effective force and η_i represents a Gaussian white noise [$\langle \eta_i(t) \rangle = 0$, $\langle \eta_i(t) \eta_j(t') \rangle = \delta(t-t') \delta_{ij}$] with amplitude σ . For single cells migrating within a confinement, $F_i = F_{\text{ext}}$ describes the dynamics arising from the interaction with the confinement [36,39,40]. For two interacting cells, a cell-cell interaction term F_{2B} is added [12].

In a multicellular system with three—or more—interacting cells, a natural extension would be to sum the cell-cell interaction term F_{2B} over each pair of cells, as assumed in many models [9–11,14–16]. However, there is no *a priori* reason for such a living system to behave in a pairwise additive manner. Indeed, cell-cell interactions rely on a complex integration of physical forces and signaling mechanisms [3,18]. Therefore, higher-order interactions such as three-body interactions may be necessary to fully capture their dynamics. We define a three-body interaction as an interaction that depends on the positions and velocities of all three cells and cannot be decomposed into a sum of pairwise interactions.

Then, the global effective force becomes a superposition of three terms: the first describing the cells' intrinsic motility within the environment; the second describing the cells' pairwise interactions; and a three-body interaction term

$$F_i(\mathbf{x}, \mathbf{v}) = F_{\text{ext},i} + \sum_{j \neq i} F_{2B,ij} + \sum_{j \neq i, k \neq i, j < k} F_{3B,ijk}. \quad (2)$$

C. Inferring three-body interactions

We use ULI, which enables a rigorous and robust inference of deterministic effective forces and noise amplitude from experimental trajectories [33]. It has previously been used to infer F_{ext} in the case of single-cell migration [33], and to simultaneously infer both F_{ext} and F_{2B} from data of two interacting cells [12]. We generalize this approach and adapt ULI to simultaneously infer F_{ext} , F_{2B} , and F_{3B} directly from our experimental data [(Fig. 1(d))].

ULI employs projections of the accelerations onto a set of basis functions and corrects for systematic errors arising from finite sampling [33]. Given a basis expansion for F_{ext} , F_{2B} , and F_{3B} , ULI infers the associated expansion coefficients such that the resulting function best explains the data. An intrinsic trade-off of the ULI method is that the inference gets

less reliable when the set of basis functions gets larger. This makes the choice of the basis both subtle and critical [19]. For F_{ext} and F_{2B} , we use the basis functions that were previously established [12].

For F_{3B} , we reduce the dimensionality of the inference by considering the symmetries of the system. Specifically, we restrict ourselves to functions $F_{3B}(\Delta x_{ij}, \Delta x_{ik})$ where $\Delta x_{ij} = x_i - x_j$, which satisfy the following conditions: $F_{3B}(-\Delta x_{ij}, -\Delta x_{ik}) = -F_{3B}(\Delta x_{ij}, \Delta x_{ik})$ (symmetry under reversal of the x axis) and $F_{3B}(\Delta x_{ik}, \Delta x_{ij}) = F_{3B}(\Delta x_{ij}, \Delta x_{ik})$ (identical cells). We further reduce dimensionality by using simplifying ansatzes that consider three-cell interactions in various distinct configurations of cells. This reduces the inference to that of a one-variable function, as detailed in Appendix C and in the Supplemental Material [34].

We use bootstrapping to estimate 3σ -error bars for the terms inferred by ULI [41,42]. To test the accuracy of the three-body interaction inference, we simulate trajectories with a given three-body interaction, perform inference with bootstrapping, and verify that the original input three-body interaction is encompassed by the 3σ -error bar [Fig. S1(a) of the Supplemental Material [34]]. We repeat this process with independent, randomly chosen three-body interactions and find that the input interaction lies within the bootstrap error bar in at least 95% of cases. This demonstrates that we can reliably reinfer a three-body interaction, despite large error bars [Figs. S1(b) and S2 of the Supplemental Material [34]]. Based on these results, we consider a three-body interaction to be significant if its 3σ -error bar does not encompass zero.

III. RESULTS

A. Two-body interactions are conserved in a three-cell system

Using our experimental data of three interacting cells, we infer $F_i(\mathbf{x}, \mathbf{v})$ [Eq. (2)]. We first examine the two-body cell-cell interaction F_{2B} . For two cells interacting on a two-island pattern, it was shown that the cell-cell interaction term can be written as a sum of two contributions:

$$F_{2B,ij} = f(|\Delta x_{ij}|)\Delta x_{ij} + \gamma(|\Delta x_{ij}|)\Delta v_{ij},$$

where $\Delta x_{ij} = x_i - x_j$ and $\Delta v_{ij} = v_i - v_j$. The function f can be interpreted as an effective attraction and γ as an effective cell-cell friction. Both are found to vary between different cell lines [12,17].

Notably, we find that for both cell lines, the two-body interactions f and γ as inferred from our three-cell systems are almost identical to the ones inferred for two cells interacting in a two-island pattern [17] [Figs. 2(a)–2(d)]. More specifically, we infer short-range repulsion and friction for the MCF10A cells, and short-range attraction, long-range repulsion, and effective antifricition for the MDA-MB-231 cells. This antifricition observed for MDA-MB-231 cells was shown to be linked to their antialignment of polarities and lack of adherens junctions, in contrast to MCF10A cells, which align their polarities and build adherens junctions [17]. These inferred interactions are consistent with those originally inferred by Brückner *et al.* [12] (see Fig. S3 of the Supplemental Material [34]), although there are some quantitative differences, which we attribute to the use of a different micropatterning technique. The term F_{ext} and the noise am-

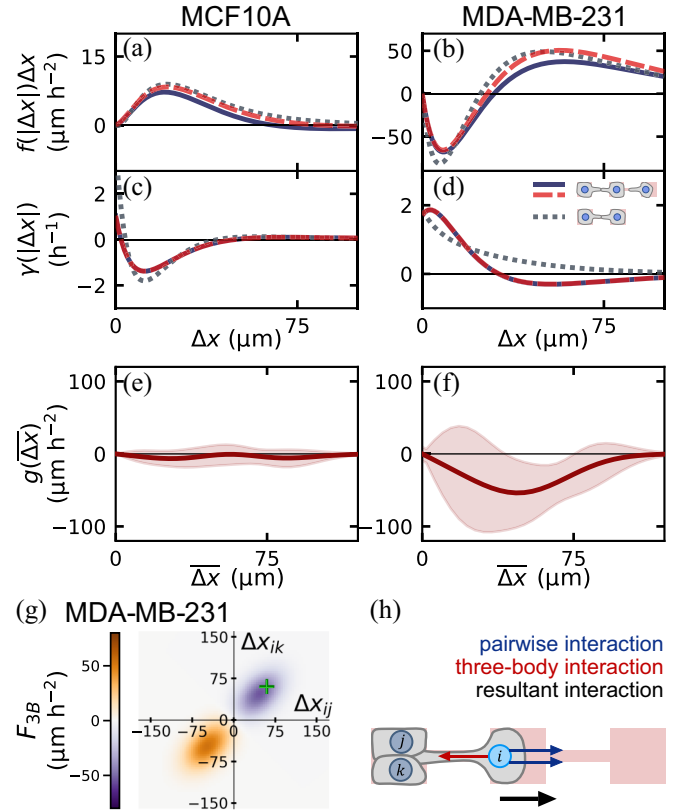


FIG. 2. Inferred pairwise and three-body interactions. (a), (b) Pairwise attractive/repulsive and (c), (d) pairwise frictional interaction terms, inferred for MCF10A and MDA-MB-231 cells. Solid blue lines are the interactions inferred from a purely pairwise model and dashed red lines when the three-body interaction is included. Gray dotted lines are the interactions inferred for two cells interacting in a two-island confinement [17]. (e), (f) Inferred three-body interaction for MCF10A and MDA-MB-231 cells, with bootstrap 3σ -error bar (light red area). (g) Two-dimensional (2D) representation of the three-body interaction inferred for MDA-MB-231 cells. (h) Schematic of the configuration indicated by the green cross in panel (g).

plitude σ are also similar to the ones inferred for two cells on the two-island pattern (Fig. S4 of the Supplemental Material [34]). Finally, we note that the two-body interactions inferred from three-cell systems remain nearly unchanged if a three-body interaction is included in the inference [Figs. 2(a)–2(d)].

B. A three-body interaction is detected for MDA-MB-231 cells

Next, we consider the inferred three-body interactions. For simplicity, we first present the results obtained with one of the simplest ansatzes for three-body interactions, for which the inferred interaction is also the most significant. This ansatz describes a three-body interaction that arises when two cells are in close proximity and together affect the third cell. In this configuration, the repulsive or attractive forces exerted by each cell may no longer be pairwise additive. The deviation from pairwise additivity can be captured by a three-body interaction of the form $F_{3B,ijk} = g\left(\frac{\Delta x_{ij} + \Delta x_{jk}}{2}\right)e^{-\frac{\Delta x_{jk}^2}{2w^2}}$. When cells j and k are close to each other (i.e., Δx_{jk} is small), the function

g describes the positive or negative amplitude of the deviation as a function of the average distance $\overline{\Delta x}$ between cells j and k , and cell i . A Gaussian decay is used for Δx_{jk}^2 as a standard symmetric decay function.

We find two qualitatively different results for the two cell lines: For the MCF10A cell line, we find no significant three-body interactions [Fig. 2(e)]. In contrast, for the MDA-MB-231 cell line, we reliably infer a nonzero three-body interaction [Fig. 2(f)]. This three-body interaction is attractive in nature and can be interpreted as a correction to the additivity of two-body cell-cell repulsion. It comes into play when two cells are colocalized, for example, when cells 1 and 2 are colocalized at a distance r from cell 3. The additive contribution of the two pairwise repulsions may be written as $F_{\text{pair}}(r) = F_{2B}(r_{13}) + F_{2B}(r_{23}) \sim 2F_{2B}(r)$. When including the three-body interaction, the effective interaction instead becomes $F_{\text{eff}}(r) = 2F_{2B}(r) + F_{3B}(r)$. Since $F_{3B}(r) < 0$, the three-body term partially cancels the pairwise repulsions. The collective repulsion exerted by two colocalized cells is therefore weaker than the sum of the pairwise repulsions [Fig. 2(g)].

Our inference results suggest that the dynamics of MCF10A cells is driven by two-body interactions, whereas a three-body interaction is present for MDA-MB-231 cells. This raises the question of how this three-body interaction impacts their collective dynamics.

C. Cellular dynamics is dominated by two-body interactions

To check the predictive power of our inferred model and assess the role of three-body interactions in MDA-MB-231 cells, we simulate Eq. (1) with the inferred terms and compare experimental and simulated statistics. Since the inference uses only projections of cell accelerations, the predictive power of the model must be tested using different, longer-term statistics. For the MCF10A cells, based on our inference results, we simulate a simple pairwise interaction model. For the MDA-MB-231 cells, we compare a model including the inferred three-body interaction to a purely pairwise model.

We compare the statistical properties of simulated and experimental trajectories, such as the steady-state distributions of positions, velocities, and dwell times [Figs. 3(a)–3(f); Figs. S5 and S6 of the Supplemental Material [34]]. The position distribution has three peaks matching the three islands of the pattern [Figs. 3(a) and 3(b)]. The velocity distribution deviates slightly from the exponential distribution reported for freely migrating cells [43], presumably due to confinement [Figs. 3(c) and 3(d)]. For the MCF10A cell line, all experimental statistics are well captured by a model including only pairwise interactions. For the MDA-MB-231 cell line, we observe little difference between the model that includes the three-body interaction and the pairwise model, and both models provide a good match to the statistics.

To better assess the influence of the three-body interactions, we consider two additional statistics that we expect to be particularly sensitive to cell-cell interactions. The first one is the distribution of relative positions, also called triplet correlation function, which has been shown to be sensitive to three-body interactions for colloidal suspensions [29]. It is defined as the probability of finding three particles at

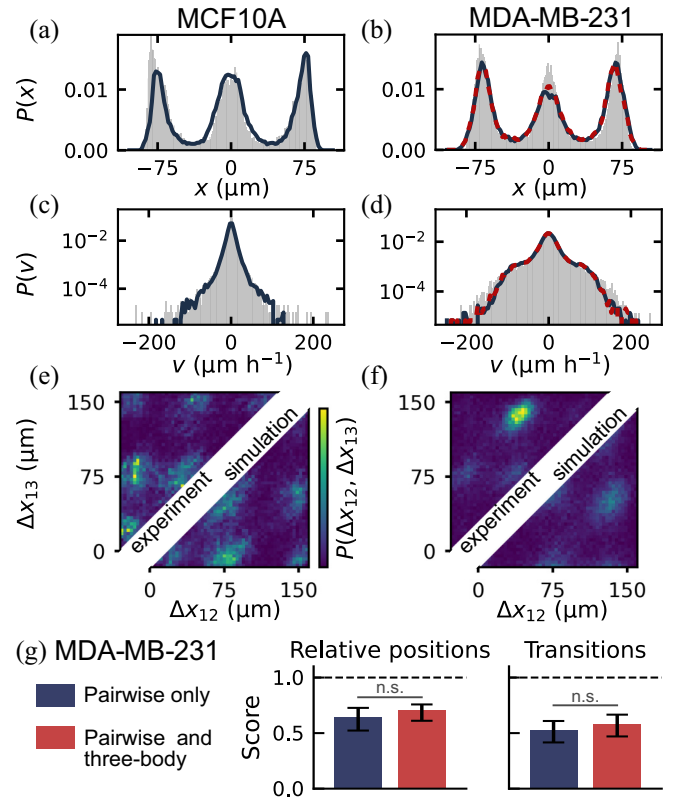


FIG. 3. Comparison of simulated and experimental data: (a), (b) Cell positions and (c), (d) cell velocities (semilog plot) for MCF10A and MDA-MB-231 cells. The gray areas show the experimental distribution, and continuous blue and red dashed lines show the distributions from the pairwise interaction model and from the model including the three-body interaction, respectively. (e), (f) Joint distribution of the relative cell positions. The upper (lower) triangle shows the distribution of experimental (simulated) data, for MCF10A cells (pairwise interaction model) and MDA-MB-231 cells (model with three-body interaction). (g) Scores of how well the relative positions and transitions between cellular configurations are captured by the models, for MDA-MB-231 cells. Higher scores indicate better agreement between simulation and experiment. The improvement in the scores is not statistically significant.

distances r_{12} , r_{13} , and r_{23} from each other. In one dimension, it reduces to two relative positions: Δx_{12} and Δx_{13} ($\Delta x_{23} = \Delta x_{13} - \Delta x_{12}$). The second statistic is the transition rate between different coarse-grained cellular configurations (Fig. S8 of the Supplemental Material [34]) and quantifies cell rearrangements.

We find that these two statistics are reasonably well captured for both cell lines [Figs. 3(e) and 3(f); Figs. S7 and S8 of the Supplemental Material [34]]. We observe a qualitative difference in the distribution of relative positions between the two cell lines. While MCF10A cells are often colocalized on the same pattern site (with peaks at $\Delta x_{12} \approx 0$), MDA-MB-231 cells tend to occupy three pattern sites, as indicated by sharp peaks at $\Delta x_{12} = 75 \mu\text{m}$ and $\Delta x_{13} = 150 \mu\text{m}$. This difference between the cell lines can be explained by pairwise interactions alone (Fig. S7 of the Supplemental Material [34]).

For the MDA-MB-231 cell line, we evaluate the improvement gained by including a three-body interaction in the

model by quantifying how well cell relative positions and cell transitions are captured [Fig. 3(g)]. We measure the energy distance between the relative positions and the Frobenius distance between the matrices of transitions between configurations. These distances are then normalized into scores defined as $1 - d/d_{\text{ref}}$, with d_{ref} the distance for a reference simulation without cell-cell interactions. A score of 1 indicates perfect alignment with the experiment, while a score of 0 indicates no improvement compared to the reference simulation. We do not observe a statistically significant improvement, and therefore conclude that the three-body interaction detected in MDA-MB-231 cells constitutes a minor correction to the dynamics.

D. Other three-body interactions

We explore several other possible ansatzes for three-body interactions, allowing for distinct degrees of structure in the interactions. For the MCF10A cells, we find no significant three-body interactions for any of the considered ansatzes (Fig. S9 of the Supplemental Material [34]). However, for the MDA-MB-231 cells, we infer other significant three-body interactions, which are shown in Fig. 4. When considering a configuration in which cells j and k are one island apart, we infer a three-body repulsion exerted on cell i that arises when cell i is located to the left [Fig. 4(b)]. Additionally, when considering a configuration in which cells i and j are colocalized, we infer a three-body attraction exerted on cell i , with a maximum when cell k is located $55 \mu\text{m}$ to the left [Fig. 4(b)]. Other three-body interactions inferred using different ansatzes are equivalent to the ones cited (Fig. S9 of the Supplemental Material [34]). As before, including these three-body interactions does not significantly improve the scores measuring how well the relative positions and transitions between cell configurations are captured [Fig. 4(c)]. The same applies to all the other ansatzes we tried, including a direct inference of a general two-variable function of cell distances and three-body interactions that depend linearly on the cellular velocities (Figs. S10 and S11 of the Supplemental Material [34]). We conclude that, for both cell lines, the dynamics is dominated by two-body interactions, and that the three-body interactions detected in the MDA-MB-231 cell line are minor corrections to the dynamics.

IV. DISCUSSION

We investigated the existence and significance of three-body interactions in a biological system. For this purpose, we developed a method that allows us to infer the presence of three-body interactions between cells based on their stochastic trajectories. We applied this method to two different cell lines and detected significant three-body interactions only for the cancerous mesenchymal cell line (MDA-MB-231). These inferred three-body interactions introduce a correction to the pairwise additivity of two-body interactions and can be attractive or repulsive depending on the configurations. In particular, we found that the repulsion felt by a cell neighboring two colocalized cells is weaker than the sum of the individual pairwise repulsions. Biologically, this means that the cell does not integrate the repulsive signal in an additive manner; in

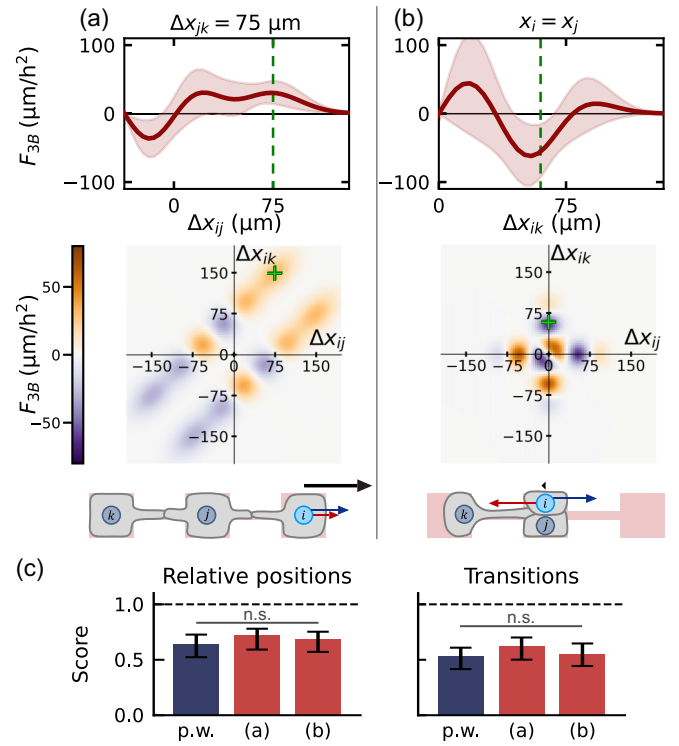


FIG. 4. Inference results for different implementations of three-body interaction, for MDA-MB-231 cells. (a), (b) For two additional implementations, the first line shows the inferred function F_{3B} with bootstrap 3σ -error bar, the second line shows a 2D representation of $F_{3B}(\Delta x_{ij}, \Delta x_{ik})$, and the sketches illustrate how the three-body interaction (red arrow) adds to the pairwise interaction (blue arrow) and contributes to the resultant interaction (black arrow) acting on cell i , in the configuration indicated by the green dashed line and cross. (c) Scores of relative positions and transitions between cellular configurations of simulated data are compared to experiments, for a pairwise interaction model (p.w.) and for models including three-body interactions depicted in panels (a) and (b), respectively. The differences are not statistically significant.

other words, the cell cannot count the number of neighboring cells. More generally, cell-cell interactions include not only mechanical forces, but also signaling contributions involving the polarity machinery, which may result in interactions that are not pairwise additive. However, we found that the multicellular dynamics is well captured by a model that only considers pairwise interactions, indicating that two-body interactions dominate the behavior.

We considered a simple geometry of linearly arranged sites and cannot exclude the possibility that higher-order cellular interactions play a more dominant role in two- or three-dimensional geometries involving more interfacial contact between cells. Further studies could adapt our method to investigate the presence and role of three-body interactions in cell migration under different geometric constraints.

In summary, we examined the role of three-body interactions in cell migration confined to a controlled geometry and concluded that the assumption of pairwise additive interactions is justified in this context. Therefore, our results corroborate the common use of the pairwise-interaction assumption in the field of cell migration. Our finding that

pairwise interactions dominate the dynamics is reminiscent of other physical systems, such as atoms interacting via van der Waals forces [44,45] or colloidal suspensions [29], in which three-body interactions appear to be minor corrections to the collective dynamics. The methodology we developed could be adapted to explore three-body interactions in a broader range of active matter systems undergoing stochastic dynamics.

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DATA AVAILABILITY

The experimental data supporting the findings of this article are openly available [46]. The custom scripts used for data analysis and simulations can be found at the same link.

APPENDIX A: EXPERIMENTAL METHODS

1. Micropatterning

The patterns are printed using a subtractive photopatterning technique. Ibidi μ -Dishes ibiTreat (ibidi GmbH, ref. 81156) are plasma treated with O_2 for 1 min, and then incubated with a 100 μ g/ml PLL solution (Sigma-Aldrich, ref. P8920, diluted in water) for 30 min. The dishes are then washed with 0.1 M HEPES (pH 8.3–8.5) and incubated for 1 h with 100 mg/ml mPEG-SVA (Laysan Bio, MPEG-SVA, MW 5000) dissolved in HEPES (pH 8.3–8.5). The dishes are profusely washed with water and left to air dry. For each dish, 3 μ l of the photoactivatable reagent PLPP Gel (Alvéole) is diluted in 60 μ l of 99.9% ethanol. The gel is spread onto the dish surface and allowed to dry in the dark. Patterns are printed with a PRIMO device (Alvéole) through a 20 $\times$ objective on a Nikon Ti inverted microscope using the Leonardo software (Alvéole), with a dose of 15 mJ/mm². The dishes are profusely washed with water, and then incubated in PBS for 5 min. The dishes are then incubated for 15 min with 20 μ g/ml of fibronectin (YO Proteins, 663 Fibronectin (human) 5 mg, resuspended in PBS), with a ratio 1:3 of fibronectin labeled with Alexa FluorTM 647 NHS-Ester (Invitrogen, ref. A37573). The dishes are washed with PBS and stored in the fridge overnight. The patterns consist of 35 μ m $\times$ 35 μ m squares linked by 40 μ m $\times$ 7 μ m bridges.

2. Cell culture

MCF10A cells (ATCC, ref. CRL-10317) are cultured at 37 °C and 5% CO_2 in DMEM/F-12 with GlutaMAX supplement (Gibco, ref. 10565-018), additionally supplemented with 5% horse serum (Sigma-Aldrich, ref. H1270), 10 μ g/ml insulin (Sigma-Aldrich, ref. I9278), 500 ng/ml hydrocortisone (Sigma-Aldrich, ref. H6909), 100 ng/ml cholera toxin (Sigma-Aldrich, ref. C8052), and 20 ng/ml human epidermal growth factor (Sigma-Aldrich, ref. E9644). MDA-MB-231 cells stably transfected with H2B mCherry (gift from Betz

Lab, University of Göttingen, Germany) are cultured at 37 °C in L-15 medium with GlutaMAX supplement (Gibco, ref. 31415-086) with 10% FBS (Gibco, ref. 10437028). The cells are kept in T25 flasks (Sarstedt AG, ref. 83.3910.300) and passaged every two to three days. For passaging, the cells are washed with PBS and incubated with Accutase (Invitrogen, ref. 00-4555-56) until they all detach. Culture medium is added and the solution is centrifuged at 500 rcf for 6 min for MCF10A cells or 800 rcf for 3 min for MDA-MB-231 cells. The cell pellet is resuspended in culture medium and approximately 5×10^5 cells are added to the new flask.

3. Cell seeding on micropatterns

For experiments, approximately 15 000 cells are added per μ -dish and left to adhere in the incubator in culture medium. Once the cells have adhered to the patterns (after up to 5 h), the medium is exchanged. For the MCF10A cells, the medium is replaced by culture medium with 25 nM Hoechst 33342 for nuclear staining. For the MDA-MB-231 H2B mCherry cells, the medium is replaced by L-15 without phenol red (Gibco, ref. 21083-027) supplemented with 10% FBS. During experiments, MCF10A cells are kept in a 5% CO_2 atmosphere and both MCF10A and MDA-MB-231 H2B mCherry cells are kept at 37 °C.

4. Image acquisition

Time lapses are acquired over 48 h with a Nikon Eclipse Ti inverted microscope equipped with a Perfect Focus System using a 10 $\times$ objective. The samples are placed in a temperature and CO_2 control chamber (Okolab). Fields of view where three cells are adhered onto the same pattern are selected. A fluorescence image of the patterns (made visible by the fibronectin labeled with Alexa 647) is acquired prior to starting the time lapse. During the time lapse, phase contrast and fluorescence images of the cell nuclei (made visible by the Hoechst stain for MCF10A cells, and by the H2B mCherry for the MDA-MB-231 cells) are acquired for each field of view, every 10 min.

5. Two-cell data

The data for the two cells interacting in a two-island pattern used in Fig. 2 were obtained using the same methods. These data were previously used in the publication [17].

APPENDIX B: IMAGE ANALYSIS

Patterns containing three adhered cells are manually selected in space and time using a tailor-made program. If two of the three cells originate from the division of one cell, the selection begins once the two daughter cells have visibly spread and adhered to the pattern. If one of the three cells undergoes division during the experiment, the selection is stopped 60 min before the cell rounds up. We exclude patterns where one or more cells exhibit visible abnormalities, such as multiple or disrupted nuclei.

For each selection, the pattern is automatically detected and the centers of the cell nuclei are tracked using a second tailor-made Python program. Pattern detection uses the

OpenCV library, while cell tracking uses the Trackpy library [47]. The results of the tracking algorithm are examined one by one and any errors made by the program (e.g., two cells being exchanged during tracking) are manually corrected. A total of 182 (resp. 73) trajectories were collected for the MDA-MB-231 (resp. MCF10A) cell line, with a total length of 1669 (resp. 2185) h.

APPENDIX C: INFERENCE METHODS

We infer the terms of Eq. (2) from the measured cell trajectories using the underdamped Langevin inference method extensively described in Ref. [33]. In practice, we use the package UnderdampedLangevinInference, which is available on GitHub [48]. We add new basis functions to the code, in order to infer three-body interactions.

1. Single-cell dynamics

Before performing the inference on the data of three interacting cells, we verify that we can capture the dynamics of single cells migrating on our new three-island pattern. This confirms that Eq. (1) is suited to describe single-cell dynamics on this new pattern, despite the fact that it does not include a memory kernel. Memory is needed to capture the dynamics of freely migrating cells, but is not required to capture the dynamics of a cell migrating in a small, confining environment [36].

2. Single-cell and two-body terms

For the system with three interacting cells, we first optimize the inference for a purely pairwise interaction model. The acceleration of each cell is then given by

$$\frac{dv_i}{dt} = F_{\text{ext}}(x_i, v_i) + \sum_{j \neq i} [f(|\Delta x_{ij}|) \Delta x_{ij} + \gamma(|\Delta x_{ij}|) \Delta v_{ij}] + \sigma \eta_i(t).$$

As in Ref. [12], we infer the single-cell term $F_{\text{ext}}(x_i, v_i)$ using a basis consisting of trigonometric functions of x_i and polynomials of v_i :

$$F_{\text{ext}}(x_i, v_i) \approx \sum_{n=1}^N \sum_{m=1}^M \left[A_{nm} \cos\left(\frac{2\pi n x_i}{w}\right) + B_{nm} \sin\left(\frac{2\pi n x_i}{w}\right) \right] v_i^m$$

and the pairwise interaction terms using a basis consisting of exponential decays:

$$f(|\Delta x_{ij}|) = \sum_{k=1}^K C_k \exp\left(-\frac{|\Delta x_{ij}|}{k r_0}\right),$$

$$\gamma(|\Delta x_{ij}|) = \sum_{k=1}^K D_k \exp\left(-\frac{|\Delta x_{ij}|}{k r_0}\right).$$

We compare the statistics obtained for simulations after inference with the experimental statistics for different values of N , M , w , K , and r_0 . We select a set of parameters

for which the statistics are best captured: $N = M = K = 3$ (the same value as in Ref. [12]), $w = 225 \mu\text{m}$ (reflecting the size of our pattern), and $r_{\text{max}} = K r_0 = 25 \mu\text{m}$. We keep these values for the inference with an additional three-body interaction.

3. Three-body interaction term

In principle, in our framework, a three-body interaction induced by cells j and k on cell i could be represented by any function $F_{3B}(x_i, x_j, x_k, v_i, v_j, v_k)$. However, due to technical limitations of the inference method and visualization issues, it is infeasible to infer a function with six dependencies. We therefore take the following constraining assumptions:

(1) The first constraint enforces mirror symmetry of the system and its invariance under a reversal of the x axis. Reversing the x axis gives $F_{3B}(-x_i, -x_j, -x_k, -v_i, -v_j, -v_k) = -F_{3B}(x_i, x_j, x_k, v_i, v_j, v_k)$.

(2) The second constraint is that all cells are treated as identical. When acting on cell i , cells j and k play an identical role, so $F_{3B}(x_i, x_j, x_k, v_i, v_j, v_k) = F_{3B}(x_i, x_k, x_j, v_i, v_k, v_j)$.

(3) We assume translational symmetry and only consider three-body interactions that depend only on the relative positions and velocities of the cells. We can therefore rewrite $F_{3B}(x_i, x_j, x_k, v_i, v_j, v_k) = \tilde{F}_{3B}(\Delta x_{ij}, \Delta x_{ik}, \Delta v_{ij}, \Delta v_{ik})$, where $\Delta x_{ij} = x_i - x_j$ is the distance between the cell nuclei, and $\Delta v_{ij} = v_i - v_j$ is the difference in velocities. Note that $\Delta x_{jk} = \Delta x_{ik} - \Delta x_{ij}$ and therefore does not need to be explicitly included in the arguments of the function (the same applies to Δv_{jk}).

(4) We further assume that the interactions are local, such that they go to zero when either Δx_{ij} or Δx_{ik} goes to infinity.

We test different ansatzes in accordance with these assumptions. All the ansatzes and parameters tested are listed in the Supplemental Material [34].

APPENDIX D: INFERENCE ACCURACY AND ERROR BARS

We estimate error bars for the terms inferred by ULI with bootstrapping, as proposed in Ref. [41]. To do so, we create 100 bootstrap samples of the experimental data by resampling with replacement, and we perform inference on each sample. Then, we compute the standard deviation of the inferred term at each point of the function's domain. We define the error bar as ± 3 standard deviations of the inferred interaction.

To check the accuracy of the inference method, we use simulations of trajectories with randomly chosen three-body interactions. Random three-body interactions F_{3B} are constructed by assigning a random coefficient to each function in the basis set according to a uniform distribution $\mathcal{U}([-a, a])$. The maximum value a is roughly estimated from the coefficients obtained when inferring the three-body interaction from the MDA-MB-231 experimental data, using the unbiased estimator for the maximum of a uniform law (also known as the German tank problem). As the absolute value of the largest of the four inferred coefficients is 30, we use $a = 40$ ($\approx 30 + 30/4$). The simulations are performed using the functions F_{ext} and F_{2B} and the noise amplitude σ inferred from

a pairwise interaction model for each cell line. We simulate cellular trajectories according to Eqs. (1) and (2). For each cell line, we simulate trajectories of the same length as those in our experimental dataset. We repeat this process 100 times, each time using a new randomly generated three-body interaction. For the ansatz presented in Fig. 2, we find that in at least 95% of cases, the original three-body interaction lies within the bootstrap error bar (see Figs. S1 and S2 of the Supplemental Material [34]).

APPENDIX E: SIMULATIONS

We simulate trajectories according to Eqs. (1) and (2) using the Verlet integration method with a time step of $\Delta t = 10$ s. We subsample the resulting trajectories to obtain the same sampling rate and trajectory length as the experimental data. We initiate the simulation using random cell positions, and allow for a 2-h prerun before data collection. We exclude simulations in which one or more cells exit the boundaries of the pattern.

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