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Topological defects in confined populations of spindle-shaped cells.

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9 Most spindle-shaped cells (smooth muscles, sarcomas etc...) routinely organize in vivo in wellaligned "nematic" domains 1^{-3} . This organization implies intrinsic topological defects that may be 10 11 used to probe the behavior of these active nematic systems. Active non-cellular nematics have been shown to be dominated by activity, yielding complex chaotic flows ^{4,5}. However, the regime in which 12 13 live spindle-shaped cells operate, and in particular the importance of cell-substrate friction, remains 14 largely unexplored. By designing model in vitro experiments, we show that these active cellular 15 nematics operate in a regime where activity is effectively damped by friction and therefore that the 16 interaction between defects is controlled by the system's elastic nematic energy. Due to the activity of the cells, these defects behave as self-propelled particles and pairwise annihilate until all 17 displacements freeze as cell crowding increases ^{6,7}. When confined in mesoscopic circular domains, 18 19 the system evolves toward two identical +1/2 disclinations facing each other. The most likely 20 reduced positions of these defects are found to be independent of the size of the disk, the cells' 21 activity or even the cell type. These results are well described by equilibrium Liquid Crystal theory. 22 Therefore, these cell-based systems operate in a regime more stable than other active nematics, 23 which is likely to be required for their biological function.

Ever since Otto Lehman⁸ and Georges Friedel⁹ have shown that the microscopic structure of nematic or smectic phases could be inferred from a careful observation of their characteristic structural defects, the study of liquid crystals has been going hand in hand with a characterization of the topological defects – i.e. singular points in the orientation field - that affect the long range orientational order¹⁰.

Far-from-equilibrium active nematics are no exception to that rule. These systems are composed of interacting elongated self-propelled particles that actively align with their neighbors, in a nematic or polar phase ¹¹. These collective behaviors are observed in a wide variety of systems and length-scales, and share common universal properties ¹¹. For example, actin microfilaments or microtubules associated with their molecular motors ^{4,5,12,13}, elongated motile cells ^{1,3,14} or bacteria ¹⁵ or more macroscopic systems such as fish swimming in schools ¹⁶ exhibit strong long-range alignment.

Spindle-shaped cells spontaneously form nematic arrangements in vivo in diverse situations such as smooth muscles or pathological tissues such as sarcomas. We ³ and others ^{1,2} have shown that, when plated on a surface at sufficiently high density, these cells self-align in a bidimensional nematic phase. As time goes on, the cell density increases via cell proliferation, which increases the nematic ordering but also gradually freezes the cell movements ^{6,17–20}, resulting in a juxtaposition of large, perfectly ordered domains whose directors are not aligned. These domains are separated by topological defects trapped in the structure ³.

On the theoretical side, several recent works have addressed the behaviors of defects in active nematics by adding active terms to the hydrodynamic equations of liquid crystals. Although there are differences between these approaches ²¹, they all converge in showing that active systems differ from passive nematics by the spontaneous motion of the +1/2 topological defects that behave as selfpropelled particles ^{22,23}, the creation/annihilation of pairs of defects of opposite signs ^{22–24}, or the associated velocity correlations in the bulk ^{25,26}. Most of the active systems that have been identified so far operate in a regime where activity is large and viscous damping dominates cell-substrate friction

⁴. However, live cells are known to develop adhesion with their substrate yielding a large friction ²¹.
Here, by studying the topological defects in a nematic cell monolayer, we propose to assess the
contribution of friction in these systems.

52 We first present experimental results dealing with proliferating spindle-shaped NIH-3T3 mouse 53 embryo fibroblasts that interact via steric hindrance when plated on fibronectin-coated glass 54 substrates (Supplementary Materials and Methods). Retinal Pigment Epithelial (RPE1) cells and C2C12 55 mouse muscle myoblasts behaved very similarly. To study the structural defects in this system, we 56 dynamically mapped the orientation field and the velocity field after confluence, defined as the point where the surface is entirely covered by cells (Fig. 1A-C). The root-mean-square (rms) velocity, taken 57 as an indirect measurement of the cells' activity ²⁵ (Supplementary Fig. 1), decreased over time and 58 59 reached very low values associated with local rearrangements (Fig. 1D). The velocity correlation length 60 increased from 50 μ m to 300 μ m (the latter corresponding to about 20 cell sizes) in typically two days ¹⁹, as the system evolved from an isotropic to a nematic state (*Fig. 1D, insets*). 61

At high cell density, the fusion of the well-oriented domains was hindered by topological defects characteristic of nematic phases (*Fig. 1B, Fig. 2A*). According to the usual classification ¹⁰, these disclinations were either of topological charge s=+1/2 or s=-1/2 (*Fig. 2A*).

With time, the number of defects decreased because of pairwise annihilation as described below (*Fig. 2B, Supplementary Fig. 2*). The density of defects then stabilized after typically 40 h since the proliferation-induced increase in cell density caused the displacements of the cells to gradually freeze.

68 This final steady-state corresponded to a distance between defects of typically 650 μ m (*Fig. 2B*).

Investigating the individual dynamics of these defects, we observed that -1/2 defects did not move significantly, as expected from symmetry arguments. In contrast, +1/2 defects were animated by a directed motion with their "comet tail" forward (*Fig. 2C*), which is the signature of an active contractile system ^{23,24}. In contractile systems, pairs of defects can in principle be created via a splay instability ^{25,27}. However, in practice, we didn't observe the creation of defects in our experiments; their high

number at confluence resulted from local ordering at the Isotropic-Nematic transition ^{1,28}. This observation suggests that the system is dominated by a large cell-substrate friction ²¹, which is consistent with our estimate of the hydrodynamic screening length of about one cell size (*Supplementary Note 1*).

This active migration of the $\pm 1/2$ defects was accompanied by large-scale collective displacements around the defect itself in the monolayer (*Fig. 1C*). Since the system is dominated by friction, these flows are attributed to the gradients of active stress. The situation is therefore different from other active nematic systems characterized by a high activity and a low friction, and resulting in more unstable systems exhibiting complex chaotic flows and creation of many defects ^{4,5}.

Very interestingly, the directed movement of the +1/2 defects took place along lines of "kinks" that separated perfectly ordered domains (*Supplementary Fig. 3*). Similar lines have previously been observed in numerical simulations for nematics in a regime dominated by friction ^{25,29}. The local order parameter therefore mirrors an effective energy landscape where the lines of kinks correspond to valleys confining the migration of the disclinations.

88 The microscopic origin of the decrease in the number of defects could be traced to pairwise annihilations between defects of opposite topological charges (Supplementary Fig. 4A)¹⁰. When +1/2 89 and -1/2 defects came closer than typically 150 µm, they moved toward each other with symmetrical 90 91 trajectories until they merged and annihilated at time t_a . Their separation d close to their annihilation point was compatible with a law $d \propto \sqrt{t_a - t}$ (*Supplementary Fig. 4B*), as predicted in the case where 92 activity is secondary to pairwise topological attraction ^{22–24,30}. Of note, the ratio of the cell-substrate 93 friction to the activity defines a time $\tau \sim 1 - 10$ h (*Supplementary Note 1*) that compares well with the 94 95 experimentally measured annihilation time of 4 h for defects initially separated by 150 μm 96 (Supplementary Fig. 4B).

To model our system, we relied on Liquid Crystal theory. Dealing with a two-dimensional nematic system, and using the classical one-constant approximation, the Frank-Oseen elastic free energy \mathcal{F} reads:

100
$$\mathcal{F} = \frac{K}{2} \int_{FOV} |\nabla \boldsymbol{n}|^2 dx dy = \frac{K}{2} \int_{FOV} \left[\left(\frac{d\varphi}{dx} \right)^2 + \left(\frac{d\varphi}{dy} \right)^2 \right] dx dy \qquad (eq. 1)$$

101 where **n** is the local director, K is the Frank constant and φ is the polar angle ¹⁰.

102 The elastic energy decreased over time as the tissue reshaped itself during its development and the 103 cells' activity decreased (*Supplementary Fig. 5*). The positions of the defects correlated well with the 104 local maxima of the free energy (*Supplementary Fig. 5*).

For a better control of the defects localization and the associated cellular collective motion, we confined the cells in micropatterned adhesive disks of various radii R_0 ranging from 250 µm to 400 µm. As previously noted, edge cells aligned with the boundary of the domains, which mathematically fixed the total charge of the nematic tissue to +1. In two dimensions, however, the stable configuration consists in two defects of charge +1/2¹⁰ when the domain's radius is smaller than the orientation correlation length (typically 500 µm³).

111 Similarly to the boundary-free situation, several randomly distributed defects were initially present in 112 each domain when the cells reached confluence. As the density increased, the defects pairwise annihilated until two facing +1/2 defects remained (Fig. 3A-D, Fig. 4A,B, Supplementary Fig. 6, 113 114 Supplementary Video 1), as predicted. Their most likely radial position r then scaled with the domain's radius: $r = \alpha R_0$ with $\alpha = 0.67 \pm 0.02$ (Standard Error) (Fig. 4D,E). These radial positions were 115 116 pairwise-uncorrelated (Pearson coefficient < 0.2 for all disks' radii). Remarkably, the same experiments 117 performed in presence of Blebbistatin that affects myosin contractility and therefore the activity of the 118 cells (and possibly their Frank constant) or even with another cell line presenting similar nematic 119 alignment (C2C12 myoblasts), gave the exact same result qualitatively and quantitatively (Fig. 4D, Supplementary Fig. 7). Therefore, the position of the defects appears to be a robust characteristic of 120

the nematic order adopted by spindle-shaped cells. Finally, the two defects positioned themselves
preferentially along a diameter as expected from symmetry arguments (*Fig. 4A,B,F*).

To understand this behavior, we minimized the Frank-Oseen free energy (eq. (1)) with imposed circular boundary conditions and a perfect alignment with the boundary ³¹ (*Supplementary Note 2*). The position of the two defects then resulted from a balance between the alignment of the cells at the boundary and the repulsion between these two defects of identical charge.

127 We determined the nematic orientation field under these conditions and the corresponding energy of 128 the system for defects at polar positions (r, θ) and (r, θ) (Fig. 3C). The system minimizes its energy for θ = π and $r = 5^{-1/4} \cdot R_0 \sim 0.668 \cdot R_0$ (see Supplementary Note 2, Fig. 4C). This solution is in excellent 129 130 agreement with the experimental results (Supplementary Fig. 8). Importantly, this most likely position 131 of the defects is predicted to be independent of both the activity and the Frank elastic constant, explaining why the same position was experimentally observed for different cell types and in presence 132 133 of Myosin inhibitor (Supplementary Fig. 7). Of note, taking into account the finite size of the defects 134 does not significantly affect these conclusions (Supplementary Note 2).

135 Cells being active particles, the positions of the two defects are described by distributions about their 136 most likely positions (Fig. 4A,B,E,F). Computing the energy landscape allowed to define an effective temperature T corresponding to the widths of these distributions (Supplementary Fig. 9, 137 138 Supplementary Note 2). Fitting model to data in each experimental condition, we consistently 139 measured small temperatures in units of K: 0.1 < T/K < 0.2, independently of the size of the domain 140 (Fig. 4G). K being the only energy scale in this system, this result confirms that, at high cell density, the activity of these cellular nematics, although at the origin of the self-induced motion of the +1/2 141 142 defects, is effectively damped by cell-substrate friction. The interaction between defects in nematic 143 cell layers is thus controlled by the elastic nematic energy.

144 To conclude, we have shown that spindle shaped cells self-assemble as active nematics. Upon 145 confinement, the distribution of the topological defects is extremely robust with respect to

- confinement's size, activity or even cell type. The description of our results by a simple nematic disk model confirms that activity is effectively damped by cell-substrate friction and their robustness suggests that this conclusion might be generalized to other biological tissues made of spindle-shaped cells. These observations show that these cell monolayers operate in a regime more stable and less chaotic than other active nematics. The extrapolation of these results should shed new light on the behavior and dynamics of spindle-shaped tissues including sarcoma.
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218 Author contributions

- 219 G.D. and P.S. designed the research. G.D. performed the experiments and C.E. developed the theory.
- 220 P.S. and J.-F.J. supervised the research. All authors analyzed the data and participated to writing the
- 221 manuscript.
- 222
- 223 Supplementary Information is available in the online version of the paper
- 224

226 Material and methods

227 Cell culture

NIH 3T3 fibroblasts (kind gift from Dr Karine Laud-Duval, Institut Curie), C2C12 myoblasts (kind gift 228 229 from Dr Clotilde Théry, Institut Curie) and RPE1 (kind gift from Dr Maxime Dahan, Institut Curie) cells 230 were cultured in Dulbecco's modified Eagle's medium (High glucose + GlutaMAX, Gibco) supplemented 231 with 10% FBS (Sigma) and 1% antibiotics solution [penicillin (10,000 units/mL) + streptomycin (10 232 mg/mL); Gibco] at 37°C, 5% CO2, and 90% humidity. Cells were initially plated at low density on a 233 microscope glass slide. When indicated, Fibronectin (Invitrogen) was used at a concentration of 234 10µg/mL to coat the glass surface. Blebbistatin (Sigma) was used at concentrations of 1µM and 3µM 235 that significantly affected the activity (Supplementary Fig. 7). Low-calcium medium (Calcium-free 236 DMEM, FBS 10%, Penstrep 1%, 50 µM Calcium) was used to stop cell division. The cells were initially 237 cultured in regular medium and then re-plated with low calcium medium.

238 Time-lapse microscopy

239 Time-lapse multifield experiments were performed in phase contrast on an automated inverted 240 microscope (Olympus IX71) with 10X objectives and equipped with thermal and CO₂ regulations. 241 Typical field of view (FOV) was 1.5mm x 1.5mm. The displacements of the sample and the acquisitions 242 with a CCD camera (Retiga 4000R, QImaging) were controlled through Metamorph (Universal Imaging) 243 software. The typical delay between two successive images of the same field was set to 10 min. High resolution fluorescence images on fixed cells was performed with an inverted spinning disk confocal 244 245 microscope (Nikon) with a 40X oil immersion objective. Cells were fixed in 4% PFA, permeabilized in 246 0.1% Triton X-100 and blocked in 10% FBS in PBS. Actin was labeled using Alexa 546 phalloidin (Life 247 Technologies, 1:500). Alpha-Tubulin was labeled using anti-alpha Tubulin Alexa Fluor 488 (eBioscience, 1:1000). Hoescht 33342 (Sigma, 1:10000) was used to mark the nuclei. 248

249 Image Processing.

Most of the image processing was performed using the ImageJ public domain software ³². The orientation field was obtained by computing the local structure tensor with ImageJ's plugin OrientationJ ³³. For representation purposes, we used a Line Integral Convolution (LIC) ³⁴ under Matlab. The local order parameter Q_{loc} map was obtained by computing the order parameter over squared sub-windows Ω of size 8 µm: $Q_{loc} = \sqrt{\langle cos 2\theta \rangle^2_{(x,y) \in \Omega} + \langle sin 2\theta \rangle^2_{(x,y) \in \Omega}}$. Q_{loc} varies between 0 and 1.

Q_{loc} was used to detect and track the defects after thresholding. The map of the order parameter
 (Supplementary Note 2) was determined from immunofluorescence images on actin after fixation.

The velocity field in the monolayer was mapped by particle image velocimetry (PIV) analysis ³⁵. Stacks of images were analyzed with a custom made PIV algorithm based on the MatPIV software package for MatLab (MathWorks Inc.). The time between successive analyzed images was 60 min. The window size was set to 128 pixels = 95 µm with a 0.5 overlap. The normalized velocity correlation function was classically defined as $\langle \frac{\vec{V}(\vec{R}) \cdot \vec{V}(\vec{0})}{|\vec{V}(\vec{0})|^2} \rangle$.

262 Micropatterning technique

Clean glass substrates were first uniformly coated with a protein-repellent layer (interpenetrated gel of acrylamide and polyethylene glycol) 36,37 . A photoresist mask was then deposited on top of the layer by classical photolithography methods and air plasma was used to locally etch the protein-repellent coating through this mask. Substrates were incubated in Fibronectin (10 µg/mL) after which the photoresist was removed yielding a cell repellant substrate where fibronectin domains have been defined.

269 Statistical analysis

270 Statistical analysis was performed with Matlab (Mathworks) or Origin (Originlab). Experiments were 271 performed in at least 3 replicas, with more than 130 disks and up to 430 disks per condition. Unless

- 272 stated otherwise, distributions were compared using a two-sample t-test. Error bars represent the SDs
- 273 over all the FOVs or disks analyzed (pooling all experiments in a single set).
- 274 The most likely radial position of the defects was initially obtained by fitting a bigaussian function to
- the distribution.
- 276 Modeling of the nematic disk and minimization of the Frank-Oseen free energy in disks was performed
- with Matlab (MathWorks, v. R2014b).
- 278 The data that support the plots within this paper and other findings of this study are available from the
- 279 corresponding author upon request.

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297

298 Figure captions

299

Figure 1: Orientation field in a NIH-3T3 monolayer. A/ phase contrast image 40h post-confluence. B/ LIC representation showing the field lines of the orientation field. Defects are outlined with colored circles (-1/2=cyan, +1/2=orange), C/ complex velocity flows developing in the monolayer in the vicinity of the +1/2 defects. D/ The rms velocity decreases with time until reaching very low values (red curve, colored area is the SD, N=17). At the same time, the velocity correlation length ξ_{vv} increases (green curves).

306

307 Figure 2: Topological defects in the NIH-3T3 monolayer. A/ Identification of the two types of defects 308 that can be observed. Left: orientation field superimposed to actin fluorescence images. Center: same 309 area where actin and tubulin have been labeled. Right: schematics of the +1/2 and -1/2 defects. B/ Time evolution of the number of defects in the monolayer. A finite density is reached at long times. 310 311 Note that the small positive fluctuations are measurement artifacts: In our experiments, defects are 312 not created. (colored area is the SD, N = 137 FOV from 9 independent experiments) C/ +1/2 and -1/2313 defects have very different dynamics. +1/2 defects have a clear directed motion whereas -1/2 diffuse 314 about their initial position.

315

Figure 3: Effect of confinement in a disk. A/ With time, the number of defects decreases and reaches a stable position. B/ Identification of the two +1/2 defects in LIC representation (white triangles). C/ Schematic of the defects positions and convention. There is a unique way to position the two defects in the disk with one defect on the horizontal axis and [R₁>0, R₂>0, $0<\theta<\pi$]. D/ At long times, there remains only two defects in the disk (Colored area is the SD, N_{disks}=150). All panels: NIH-3T3 cells, R₀=350µm.

Figure 4: Defects' positions in the disks. A/ Point representation of the defects. Each point 323 corresponds to a defect and each color to a different domain (N_{disks} =486). NIH-3T3 cells, R_0 =400 μ m. B/ 324 325 Same where the colors code for the probability density of defects. C/ Simulation of the probability density of defects with $\frac{T}{K} = 0.2$ (N_{defects}=20000). D/ The radial defects' position r scales with the radius 326 of the pattern R₀. Green points: NIH-3T3 cells. The red line is a linear fit: $r = 0.67 \cdot R_0$. Note that this 327 328 positioning is independent of activity (red points are in presence of Blebbistatin) or even cell type (blue 329 point refers to C2C12 cells). Error bars are SEMs. E,F/ Distributions of the positions of the defects along 330 R (E) and θ (F). The outcome of the nematic disk model is superimposed (black line). R₀=400 μ m, NIH-3T3 cells, N=486. G/ The effective temperature (in units of K) in various conditions remains much 331 332 smaller than 1, demonstrating the small contribution of activity in these systems. Error bars are 333 standard errors, N>276 disks for each point.





100 µm







