Supplementary Information: Multi-scale spatial heterogeneity enhances particle clearance in airway ciliary arrays

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CONTENTS

EXTENDED MATERIALS AND METHODS:

1. Image analysis

Calculations were performed in MATLAB using custom built code unless otherwise stated.

a. Measurement of cilia coverage fraction and wavelength from fixed samples

Coverage fraction and wavelength were measured from 2D binarized images of the entire trachea surface as the one shown in Extended Data Fig. 1A. The raw data from which these images were obtained was collected as described in Methods §1c (Multiciliated cell imaging). Binarized images were assembled from the raw data as follows.

First, the maximum intensity projection of the z-stack collected for each tile was calculated. Maximum intensity projections of all the tiles from a trachea were stitched together using the ImageJ [1] "Stitching" plugin [2], yielding an image as the one shown in Extended Data Fig. 1A.

Next, a bandpass filter was applied to stitched image to reduce noise. The filtered image was then binarized using the "imbinarize" function in matlab. "Imbinarize" computes a threshold for each pixel using the local mean intensity around the neighborhood of each pixel (Bradley's method). The sensitivity factor for the adaptive threshold can have values in the range [0,1]. A high sensitivity value leads to thresholding more pixels as foreground at the risk of including some background pixels. We chose a value of 0.99 since the spatial filtering was effective in discriminating the background from the signal. Visual inspection showed that the threshold was accurate at identifying multiciliated cells. However, the high value of sensitivity used introduced isolated white pixels that did not correspond to multiciliated cells. To remove these artifacts, objects with areas smaller than 30 pixels were excluded from the final binarized image (for reference a typical multiciliated cell is composed of $\sim$90 pixels). All binarized images were further inspected manually to validate the thresholding.

Finally, we note that a mask was used to exclude from the coverage fraction and wavelength calculations the

pixels at the edge of each stitched trachea image. These edge pixels are not part of the trachea. All masks were drawn manually.

b. *Measurement of cilia coverage fraction and wavelength from live samples*

Coverage fraction and wavelength were measured from 2D binarized images where regions with white pixels correspond to regions of cilia activity (See Extended Data Fig. 1C). The raw data from which these images were obtained was collected as described in Methods §1c (Cilia live imaging). Binarized images were obtained from the raw data as follows. The median intensity projection of each image stack was calculated. Subsequently, the median image was substracted from each frame of the original stack. From this resulting stack a maximum intensity projection was obtained. The maximum intensity projection was binarized in ImageJ using the default threshold. This method allowed for identification of regions where pixel values fluctuate due to ciliary beating, rendering a binarized image where the white pixels correspond to regions of cilia activity. Images were inspected manually to verify that only regions where cilia activity was observed were included. The coverage fraction, $\bar{\varphi}$, was calculated from binarized images according to equation M2. To obtain the wavelength, λ, we computed the correlation function defined in equation M4. From this function we extracted the wavelength, λ, by determining its first local maximum. Extended Data Fig. 1D shows a plot of S_2 for all the fields of view analysed. The squares mark the first local maximum of S_2, which corresponds to λ.

c. *Analysis of number and orientation of cilia*

The number and orientation of cilia was measured from maximum intensity projections of Centrin and Centriolin image z-stacks. Extended Data Fig. 1C shows an example of such images. The raw data from which the maximum intensity projection was obtained was collected as described in Methods §1c (Basal body imaging).

Cilia quantification: Each cilium extends from a single basal body, therefore the number of cilia in a cell corresponds to the number of basal bodies in a cell. Centrin-GFP images were analysed to identify individual basal bodies as follows. First, images were filtered using a bandpass filter. The center of bright puncta that corresponded to basal bodies was identified with subpixel resolution based on local maximum intensity and size. Basal bodies were binned by cell and counted to obtain the number of cilia in each cell. The boundaries of each cell was traced manually from Vangl1 staining. Figure 1F shows a histogram of the number of cilia per cell counted from 375 cells.

Cilia orientation measurement: The orientation of a cilium is defined by the unit vector, $\boldsymbol{p}_i$ that connects each basal body (marked by Centrin) with its corresponding basal foot (marked by Centriolin). For a pair of Centrin and Centriolin images, the orientation of each cilium was obtained as follows.

First, the center of each basal body was identified from the Centrin-GFP image as described in the previous section. The image was then binned in windows of 80x80 pixels centered around every identified basal body. The same windows were identified in the Centriolin image. For each pair of Centrin-Centriolin windows, the 2D crosscorrelation function of the images was calculated for all possible directions. The orientation of a cilium, $\boldsymbol{p}_i$ was defined as the direction where the crosscorrelation between Centrin and Centriolin windows was maximum (For an illustration of this see Extended Data Fig. 1B). Prior to computing the crosscorrelation, each image window was processed by substracting its mean and multiplying it by a gaussian kernel of size 80x80 pixels and standard deviation 20.
In some cases Centriolin staining was weak, and the analysis yielded a spurious direction. To correct for this, orientation vectors were filtered by calculating the maximum to mean ratio of each window. Vectors obtained from windows with a maximum to mean ratio less than 40 were excluded from further calculations. Once the orientations of all cilia, $\boldsymbol{p_i}$, in an image were obtained, their organisation was measured as follows.

To measure the cellular-scale organisation, we define the order parameter, m:

$$m = |\langle \boldsymbol{p}_i \rangle| \tag{S1}$$

where $\boldsymbol{p}_i$ is the unit vector that describes the orientation of the i-th cilium in a cell. The average is taken over all cilia in a cell. Values of m for all cells analysed are shown in Figure 1H (cell).

To measure tissue-scale cilia organisation we assign an orientation to each cell defined by:

$$\boldsymbol{P} = \langle \boldsymbol{p}_i \rangle \tag{S2}$$

Thus $\boldsymbol{P}$ is a unit vector obtained by averaging the unit orientation vectors of all cilia within a cell. The tissue-scale organisation of cilia is then measured by calculating the order parameter, M, according to:

$$M = |\langle \boldsymbol{P}_j \rangle|, \tag{S3}$$

where the average is calculated over all the cells in a field of view. All fields of view analysed have dimensions of 1584x1584 pixels and contain ~ 35 multiciliated cells. All values obtained are shown in Figure 1H (Tissue).

To determine how the orientation of cilia varies

across neighboring cells in the tissue, we compute the spatial correlation function of $\boldsymbol{P}$ defined as:

$$O(r) = \langle \boldsymbol{P}_j \cdot \boldsymbol{P}_{j+r} \rangle \tag{S4}$$

where $\boldsymbol{P}_j$ is the average orientation of cilia in the jth cell as defined by Eq. S2 and $\boldsymbol{P}_{j+r}$ is the average orientation of cilia in the cells at a distance less than or equal to r. The average is taken over the polarity vectors that correspond to all multiciliated cells in an image. This function is well approximated by an exponential decay of the form $O = 0.32e^{-n/r_0} + 0.68$ where $r_0 = 12.2\mu m$. While the orientation of cilia decays with a characteristic length scale of $r_0 = 1.13$ cells, we note that O decays to a constant value of 0.68, showing that orientation of cilia in cells remains highly correlated over the entire field of view.

d. *Analysis of flows*

Flow fields were measured from time-lapse imaging of tissues with tracer particles. The raw data was collected as described in Methods §1c (Flow imaging). Particle Image Velocimetry (PIV) fields were generated using "mpiv" [3]. Field parameters were optimized based on bead dilution used in flow field visualization experiments, and then held constant across all analyses to reduce systematic errors due to inconsistent discretization. Parameters used are given in table I. Using these parameters a velocity vector is obtained every 8 pixels (in the x and y directions). Therefore, the typical number of velocity vectors per field of view was $\sim 50,000$. Velocity fields were filtered using a standard filter and interpolated spatially using Kriging interpolation. Filtering and interpolation were done with "mpiv_filter" [3].

PIV type	'MQD'
Window size	128
Window overlap	0.5
Iterations	4

TABLE I. Parameters used for PIV analysis

To calculate the speed of the flow, velocity fields were averaged temporally. For images captured with a 4X objective temporal averaging= 5 minutes and time step=1 second. For images captured at 40X magnification, temporal averaging= 1 second and time step=0.3-0.13 seconds. Probability density functions (PDFs) of the velocity vectors of the time averaged fields were obtained (Figure 2F). From these PDFs the average speed, and the average values of the x (v_x) and y (v_y) components of the velocity were calculated (Figure 2E). To assign units to the simulated data, the average speed for all simulated flows was matched to the average speed in ten flow fields measured (Figure 2F).

e. *Analysis of ciliary beating*

The cilia beat frequency and wave velocity were measured from kymogrpahs of time-lapse imaging of ciliary beating with a temporal resolution of 250-350 frames per second. This data was obtained as described in Methods §1c (Cilia live imaging). Kymographs were obtained for lines drawn parallel or perpendicular to cilia in an image (Extended Data Fig. 2E). Lines were drawn along traces in the kymograph that correspond to cilia beat cycles. The slope of these lines was averaged over all lines in a kymograph, yielding a measurement of cilia beat frequency (parallel to cilium, blue line) or group wave velocity (perpendicular to cilium, green line).

f. *Literature survey of patchiness and coverage fraction*

Animal	Reference	φ	λ
chicken	[4]	0.37	-
		0.40	-
		0.64	6
dog	[5]	0.36	12.47
ferret	[6]	0.44	10.5
hamster	[7]	0.58	14.8
human	[8]	0.44	14.1
		0.48	16.5
pig	[9]	0.69	21.4
	[10]	0.57	11.3
rabbit	[11]	0.49	15.2
rat	[12]	0.38	14.6
snake	[13]	0.19	121.3

TABLE II. Measurements of φ and λ for different species.

We conducted a literature survey to find images of the airway tissue where the pattern formed by multiciliated cells could be identified. Scanning electron microscopy images were found in the literature for airway tissue from the animals shown in table II. Images were cropped from figures, to include only the region where the multiciliated tissue was present. Regions of the image containing multiciliated cells were identified and drawn manually. This generated a binary image where the white pixels corresponded to areas where multiciliated cells are present. Since image frame had a limited number of pixels, instead of binning we calculated coverage fraction over the entire image as:

$$\varphi = \frac{1}{N} \sum_{i=1}^{N} I(\boldsymbol{r}_i) \tag{S5}$$

where $I(\boldsymbol{r}_i)$ are the values of all the pixels in the binary image as defined in equation M1 and N is the number of pixels in the image. Wavelength was calculated by

computing S_2 according to equation M4. The wavelength λ corresponds to the local maximum of S_2. In the case of chicken, two images found were too small to measure λ, we indicate this by a dash in table II. Our measurements from this brief literature survey provide an estimation of φ and λ, however, large data sets for each species will be required to measure these parameters conclusively.

2. Size and number of images analysed in this paper

Table III lists the size of all regions of interest (ROIs) analyzed in this paper. For basal body imaging twelve fields of view of the size specified in the table were analysed. For cilia live imaging 60X, seven fields of view of the size specified in the table were analysed. All sizes are given in pixels.

For images of multiciliated cells and 4X Flow imaging each ROI is the trachea of a different animal. The rest of the ROIs were collected from at least 4 different animals.

3. Simulation

a. *Fundamental solution*

The fundamental solution of Stokes flow (also known as the Stokeslet or Green's function) in a liquid film was derived recently by Mathijssen *et al.* [14]. The confinement drastically changes the flow structure in these geometries, and therefore it is helpful to revise this briefly.

The hydrodynamics of an incompressible Newtonian fluid at low Reynolds number are described by the Stokes equations,

$$0 = -\boldsymbol{\nabla} p + \mu \nabla^2 \boldsymbol{v} + \boldsymbol{F}(\boldsymbol{r}, t), \qquad 0 = \boldsymbol{\nabla} \cdot \boldsymbol{v}. \tag{S6}$$

where $\boldsymbol{v}(\boldsymbol{r}, t)$ is the flow velocity field at position $\boldsymbol{r}$ and time t, $p(\boldsymbol{r}, t)$ is the pressure field, μ is the dynamic viscosity and $\boldsymbol{F}(\boldsymbol{r}, t)$ is a force acting on the liquid. In the absence of boundaries, the flow due to a point force, $\boldsymbol{F}_s = \delta^3(\boldsymbol{r} - \boldsymbol{r}_s)\boldsymbol{f}_s$, with magnitude $\boldsymbol{f}_s(t)$ and position $\boldsymbol{r}_s$, with boundary conditions $\boldsymbol{v} = 0$ as $|\boldsymbol{r}| \to \infty$, is

$$\boldsymbol{v}_s(\boldsymbol{r}, t) = \mathcal{J}(\boldsymbol{r} - \boldsymbol{r}_s) \cdot \boldsymbol{f}_s, \tag{S7}$$

where the Oseen tensor $\mathcal{J}_{ij}(\boldsymbol{r})$ in Cartesian components is

$$\mathcal{J}_{ij}(\boldsymbol{r}) = \frac{1}{8\pi\mu} \left\{ \frac{\delta_{ij}}{r} + \frac{r_i r_j}{r^3} \right\}, \tag{S8}$$

with $i, j \in \{x, y, z\}$ and $r = |\boldsymbol{r}|$. In a thin film, this flow is modified by the boundaries. At the bottom surface we enforce the no-slip condition, $\boldsymbol{v} = 0$ at $z = 0$, and at the liquid-air interface we enforce the no-shear condition, $\frac{\partial \boldsymbol{v}}{\partial z} = 0$ at $z = H$. These conditions can be satisfied exactly using a Fourier-transform method (page 56 in [14]) or approximated by truncating an infinite series of images (ibid. p. 37). The resulting expressions are rather complex, but analytically tractable. In the thin-film limit the solution simplifies to

$$\mathcal{F}_{\alpha\beta} = -\frac{3z}{\pi\mu}\left(1 - \frac{z}{2H}\right)\frac{z_s}{H}\left(1 - \frac{z_s}{2H}\right)\frac{1}{\rho^2}\left\{\frac{\delta_{\alpha\beta}}{2} - \frac{r_\alpha r_\beta}{\rho^2}\right\}, \tag{S9}$$

with horizontal components $\alpha, \beta \in \{x, y\}$ and relative distance $\rho^2 = (x - x_s)^2 + (y - y_s)^2$, but vertical flows are suppressed exponentially (ibid. p. 63). Here the thin-film limit is defined as $H \ll \rho$, which is equivalent to the far-field limit. The prefactors describe a half-parabolic flow profile in z, and the last part gives the horizontal flow structure (in curly brackets). A first difference with the Oseen tensor (Eq. S8) is that the flows decay faster, as $1/\rho^2$ rather than $1/\rho$, since the surfaces impart viscous dissipation. Secondly, the streamline structure is very different.

This is demonstrated in Extended Data Fig. 3, where we show the exact solution as a function of confinement, H, with the Stokeslet in the middle of the film, $z_s = H/2$. For weak confinement, we recover the bulk Stokeslet flow (Eq. S8), where the streamlines are open and oriented along the force direction. For strong confinement we recover the thin-film limit (Eq. S9), the forward flows are weaker because of surface dissipation. Moreover, a recirculation emerges with liquid moving backwards (red colours), and the streamlines all completely close onto one another. For intermediate confinement, a more complex flow structure arises with two vortexes that are separated a distance $\delta \sim H$ (green circles). Locally ($\rho < H$) the Stokeslet limit still holds, but globally ($\rho > H$) the far-field limit applies and all liquid recirculates.

Once the Green's function $\mathcal{F}_{\alpha\beta}$ is known, it is possible to calculate the flow generated by an active carpet by integrating the Green's function over the carpet architecture [15]. Puzzlingly however, because the liquid recirculates in the far-field in a film geometry, *the flux due to a Stokeslet through any cross-section of the film is actually zero*, for any finite film height H. Specifically, the flux through any yz plane due to a Stokeslet oriented along x is $J = \int_0^H \int_{-\infty}^{\infty} \mathcal{F}_{xx} dy dz = 0$. As pointed out by Liron, this is also true for channels with two parallel no-slip surfaces, or cylindrical pipes of finite radius R, or any double-sided confinement [16]. In an infinite or semi-infinite (single surface) geometry, however, the flux does not vanish. This apparent dilemma is resolved by realising that one may add a constant Poiseuille flow $\boldsymbol{v}_P(z) = -\frac{2H^2}{\mu}\frac{\partial p}{\partial x}\frac{z}{2H}\left(1 - \frac{z}{2H}\right)\hat{\boldsymbol{e}}_x$ to the solution without violating the boundary conditions on the film surfaces, at $z = 0, H$. The flux is then specified by the pressure gradient generated by the cilia. Therefore, if each cilium on average exerts a force F_c along x and if cilia are distributed with a density n_c per unit area, then the flux per unit length in y is $J = n_c F_c H^2/3\mu$. Note that other

Multiciliated cells	Basal Body	Flow		Cilia Live	
12983x5312 7788x6864 6688x4080 8000x7088	1584x1584	4X	40X	40X	60X
		1215x936 2048x1302 2048x1431 2048x1674 2048x1404	2048x1022 2048x1022 2048x2048 2048x2048 2048x2048 2048x2048 1024x1024 1482x1405 2048x2048 2048x2048	1095x2044 1088x406 994x1022 2048x2044 2048x2044 2048x2044 926x2042 1354x2042 1245x1088 2048x2048	1494x286

TABLE III. Size of all images analyzed. All sizes are given in pixels.

forces can also generate pressure gradients, such as gravity, $\boldsymbol{\nabla} p = -\rho \boldsymbol{g}$, which must be added to (or subtracted from) the total flux and flow profile.

b. *Envelope approach*

Here, we follow an alternative method inspired by the 'envelope approach' introduced by Lighthill and Blake [17, 18] to study ciliary propulsion. Instead of modelling the cilia with point forces, one can consider an envelope that covers the tips of numerous beating cilia that together form a continuous moving sheet. The no-slip condition on the bottom surface is then replaced by the motion of this envelope, $\boldsymbol{v}_e = U\boldsymbol{p}$, a tangential slip velocity that follows the orientation field $\boldsymbol{p}$ of the underlying cilia with an average local flow velocity U. This approximation is justified in the case when the cilia are close together, which is true for multiciliated cells with $N_{cilia} \sim 200$ cilia.

On the one hand, this approach coarse-grains the length scales smaller than the cell, so it cannot resolve the interesting flows around the individual cilia that can give rise to mixing and nutrient exchange [19, 20]. On the other hand, it is very tractable analytically (as seen in the study of micro-swimmers and active colloids [21]) and it is straightforward to implement numerically, as is discussed next, being suitable even for very large systems.

c. *CFD solver*

The flow velocity $\boldsymbol{v}(\boldsymbol{r})$ is simulated using a 3D computational fluid dynamics (CFD) solver for the incompressible Navier-Stokes equations. Throughout this paper we focus on Newtonian fluids, but in SI § 3 g we also consider viscoelastic fluids with a shear-dependent viscosity. The CFD solver is implemented in a custom-built MATLAB code, optimised for low Reynolds numbers by using an implicit Crank-Nicolson method for the viscous terms and an Adams-Bashforth method for the advection terms [22]. This algorithm is implemented on a staggered grid of size $N_x^g \times N_y^g \times N_z^g$ corresponding to a liquid film of size $L_x \times L_y \times H$ [μm] and with periodic boundary conditions in the x and y directions, where x is defined as the distal-to-proximal direction of the trachea. At the fluid-air interface we enforce the no-shear condition, $\frac{\partial \boldsymbol{v}}{\partial z} = 0$ at $z = H$. On the tissue surface we apply the no-slip condition in the absence of cilia ($c = 0$), and in the presence of cilia ($c = 1$) we impose a slip velocity set by the envelope model, which gives the boundary condition

$$\boldsymbol{v}(\boldsymbol{r}) = cU\boldsymbol{p} \quad \text{on} \quad z = 0. \tag{S10}$$

Here the cilia distribution $c(\boldsymbol{r})$ and orientations $\boldsymbol{p}(\boldsymbol{r})$ are either taken directly from experiments (SI § 3 e), or systematically generated *in silico* for different coverage fractions, wavelengths, and disorder (SI § 3 f). We simulate this system until the (unique) solution is reached at steady state, after which we save the three-dimensional velocity field and the pressure field of the ciliary flow.

d. *Total flux & clearance time*

Once the flow is solved we compute the total flux in the distal-to-proximal direction,

$$J = \int_0^H \int_0^{L_y} v_x dy dz, \tag{S11}$$

which due to incompressibility is the same for all planes perpendicular to x. Because of this condition, the flux is equivalent to the volume-averaged flow along x, since $\iiint v_x dxdydz = J\int_0^{L_x} dx = JL_x$. Using the linearity of the Stokes equations, the flux can also be rewritten as

$$J = \varphi H L_y U \langle p_x \rangle, \tag{S12}$$

where the coverage fraction is

$$\varphi = \int_0^{L_x}\int_0^{L_y} c(\boldsymbol{r})dxdy/(L_xL_y), \tag{S13}$$

which is equivalent to Eq. M2. We also define the z-averaged flow velocity, $\bar{\boldsymbol{v}} = \int_0^H \{v_x, v_y\}dz/H$, the mean longitudinal flow $\langle \bar{v}_x \rangle = \varphi U \langle p_x \rangle$, and the largest backflow, $\beta = \min_x \min_y \bar{v}_x$.

Subsequently, the clearance time is obtained by simulating particle trajectories that represent non-motile viruses, bacteria or other harmful pathogens. These particles are subject to the computed flow and also Gaussian fluctuations with diffusivity D. The Stokes-Einstein diffusivity due to thermal noise is $D \sim 10^{-1}\mu\text{m}^2/\text{s}$ for micron-sized particles in water of viscosity $\mu = 10^{-3}$ Pa·s. However, in mucus the diffusivity is generally smaller than water, $D \sim 10^{-1} - 10^{-4}$ $\mu\text{m}^2/\text{s}$ for micron-sized particles, because the mucus is much thicker than water with viscosities $\mu \sim 10^{-3} - 1$ Pa·s [23].

We run a Brownian dynamics (BD) simulation to find the trajectories for an ensemble of $N_1 \geq 10^3$ particles. Initially the particle positions $\boldsymbol{r}_p$ are uniformly distributed across the simulation box, and follow the Langevin equation,

$$\frac{\partial \boldsymbol{r}_p}{\partial t} = \bar{\boldsymbol{v}}(\boldsymbol{r}_p) + \sqrt{2D}\boldsymbol{\eta}(t), \tag{S14}$$

where the noise is defined by the correlation functions $\langle \eta_i(t) \rangle = 0$ and $\langle \eta_i(t)\eta_j(t') \rangle = \delta(t-t')\delta_{ij}$. The Péclet number is defined as

$$\text{Pé} = \frac{\lambda \varphi U}{D}, \tag{S15}$$

which is large if the effects of advection outcompete diffusion.

These equations are integrated with a fourth order Runge-Kutta scheme, again with periodic boundary conditions in the x and y directions. For each pathogen i we then record the time T_i taken to travel a distance L_x in the x direction, along the D-P axis. Hence, we define the clearance time $T_c = \langle T \rangle$ as the first-passage time averaged over an ensemble of N_1 independent particles. We verified that this quantity does not depend on individual initial positions or the simulation geometry. Rather than absolute values, we report a non-dimensional ratio in all figures, such as $\frac{\langle T \rangle(\varphi)}{\langle T \rangle(\varphi=1)}$ to analyse the effect of each parameter.

Importantly, this clearance time is not only a measure of the total flux, but also of the flow structure. In particular, it is a measure of the connectivity between streamlines. If the streamlines are open and connected with the trachea outlet, the larynx, then pathogens will follow the river and rapidly be cleared. If the streamlines are closed, however, the particles are trapped in recirculation zones for a long time. Therefore, even small fluctuations (i.e. a large Péclet) can have a significant effect on the clearance times.

To the best of our knowledge, evaluating streamline connectivity using the first-passage time is a new concept. Having said that, streamline topology is important for transport and percolation in plasma physics [24] and affects particle diffusion in turbulence [25]. Recent results also show that changes in streamline topology can greatly enhance the rate of heat and mass transfer from neutrally buoyant particles in a shear flow [26, 27].

e. *Experimental input*

Large scale flows: The entire trachea is imaged with a resolution of 7176 × 4496 pixels, corresponding to 3013.9×1888.3 microns, with the D-P axis aligned along x. Here the multiciliated cells are marked with Centrin [Fig. 1B], so from this fluorescence signal we identify the positions and shapes of approximately $3{\cdot}10^4$ multiciliated cells using the Mathematica built-in segmentation function `MorphologicalComponents[]`. This measurement is course-grained by a factor of 5 to generate a grid of 1476×940 points, which subsequently is binarised to determine the coverage field $c(\boldsymbol{r}) = 0, 1$. The orientation of the cilia cannot be measured in this assay, so for each cell we assign a stochastic direction $\boldsymbol{p}$ such that $\langle p_x \rangle = 0.8$, in accordance with Fig. 1H. The ciliary envelope velocity $\boldsymbol{v}_e = cU\boldsymbol{p}$ is then computed on each grid point of the bottom boundary in the CFD simulation. The resulting flows are shown in Fig. 2D, left panel. Note, this is not the same mouse as the one shown for the flow measurement in Fig. 2C, because that tissue is alive with beating cilia whereas the former tissue is fixed to identify the basal bodies from fluorescence.

Small scale flows: The tissue scale is imaged with fields of view of size $68 \times 68\mu$m. Here we identify the boundary of multiciliated cells by segmenting the membrane stain Vangl1, which determines the coverage field $c(\boldsymbol{r})$. Next, we identify each cilium and its orientation by linking the relative positions of its ciliary rootlet and basal foot [see SI § 1 c]. For each cell, we then interpolate between its $N_{cilia} \sim 200$ cilia to extract the orientation field $\boldsymbol{p}(\boldsymbol{r})$. The ciliary envelope velocity $\boldsymbol{v}_e = cU\boldsymbol{p}$ is then computed on each of the bottom boundary grid points of the CFD solver, with box size $L_x = L_y = 68\mu$m. The resulting flow from one field of view is shown in Fig. 2D, right panel. Again, this should not be compared directly with Fig. 2C because it shows a different organism.

Nonetheless, even if it is not possible to compare the experimental and simulated flow fields directly, we compare the correlation functions and flow distributions across an ensemble of different tissues, as shown in Fig. 2E-H, using the same definitions as in the experi-

ments, as described in SI § 1 d.

f. *Controlled variations*

Next, we systematically explore how the total flux and clearance time depend on the physical properties of the mucus flow [Fig. 3A,B]. One by one, we address (1) the ciliary coverage fraction, (2) the patchiness, (3) Brownian fluctuations, (4) the spatial heterogeneity, and (5) the orientational disorder.

1. The coverage fraction φ is varied while keeping the wavelength constant, with a square periodic box of size $\lambda = L = 128\mu$m and film height $H = 10\mu$m. The envelope model is enforced by discretising Eq. S10 on a grid (i, j) of 128×128 cells. A patch of cilia sits at the centre of this grid, with radius $R(n) = 64\frac{n-1}{24-1}$ where $n = 1, 2, 3, \ldots, 34$. The discretised coverage field is then generated with the function $c_{ij} = \Theta\left[R^2 - (i-65)^2 - (j-65)^2\right]$, where $\Theta(x)$ is the unit step function. Consequently, the coverage fraction is $\varphi = \sum_{i,j} c_{ij}/128^2$. All cilia are aligned along the trachea axis, $p_x = 1$, so we find the velocities $v^x_{ij} = c_{ij}U$ and $v^y_{ij} = 0$ at the tissue surface. The Stokes equations are solved with these boundary conditions (SI § 3 c) and the resulting flows are shown in the left panels of Fig. 3C. We then simulate an ensemble of $N_1 = 10^4$ particle trajectories, initially distributed randomly, and subject to this flow and Brownian diffusion. We non-dimensionalise our system to $\lambda^* = 1$, $U^* = 1$, $D^* = 10^{-4}$, so the Péclet number Pé $= \varphi 10^4$, which is equivalent to a diffusivity $D = 0.128\mu\text{m}^2/\text{s}$, $L = 128\mu$m and envelope velocity $U = 10\mu$m/s. In the right panel of Fig. 3C we show the resulting mean clearance times, $\frac{\langle T\rangle(\varphi)}{\langle T\rangle(\varphi=1)}$, as well as the total flux, $\frac{J(\varphi)}{J(\varphi=1)}$, both normalised with respect to the case of full coverage ($\varphi = 1$).

2. The patchiness λ/H is explored by varying the wavelength λ while keeping the film height $H = 10\mu$m constant, and also a constant periodic box size $L = 128\mu$m, coverage fraction $\varphi = 0.1$ and cilia alignment $p_x = 1$. We again use a grid (i, j) of 128×128 cells, on which we implement a square array of $n_p \times n_p$ ciliary patches, with wavelength $\lambda = L/n_p$ and radius $R(n_p) = L\sqrt{\varphi/n_p\pi}$, where $n_p = 1, 2, 3, \ldots, 24$. In other words, the same number of cilia are spread out in more and smaller patches. Using these boundary conditions the flows are simulated, which are shown in the left panels of Fig. 3D. For large patchiness a back flow emerges. We again simulate $N_1 = 10^4$ particle trajectories with constant diffusivity $D = 0.128\mu\text{m}^2/\text{s}$, $L = 128\mu$m and mean flow velocity $\langle \bar{v}_x\rangle = \varphi U\langle p_x\rangle = 10\mu$m/s. In the right panel of Fig. 3D we show the resulting mean clearance times, $\frac{\langle T\rangle(\lambda/H)}{\langle T\rangle(0)}$, as well as the total flux, $\frac{J(\lambda/H)}{J(0)}$, and the back flow, $\frac{\beta(\lambda/H)}{\beta(0)}$, all normalised with respect to the case of uniform coverage ($\lambda = 0$). In Extended Data Fig. 4 the 3D structure of the resulting flow for a square ciliary array is shown for patchiness $\lambda/H = 12.8$, with a top view and cross sections at and between the ciliated cells. As predicted by the Stokeslet flow solution (Eq. S9), the flows above a no-slip surface tend to follow a half-parabolic profile. However, the flows above an active surface have an inverted profile to satisfy the conservation of mass and momentum. There is also a vertical component to the flow, especially near the edges of the multiciliated cells, but this component along z is smaller than the components along x and y.

3. The geometric order is varied by considering the crystallinity γ, while keeping the coverage fraction $\varphi = 0.1$, the mean patchiness $\langle\lambda\rangle/H = 8$ and the cilia alignment $p_x = 1$ constant. We begin with a crystalline array of 4×4 cilia patches, as in SI § 3 f*2* above with $n_p = 4$. These patches are displaced spatially by a random vector (X, Y), where both components are drawn from a Gaussian distribution with standard deviation σ. The geometric order parameter is then defined as $\gamma = 1 - \frac{\sigma\sqrt{2}}{\lambda}$, the crystallinity. As before we implement these patches on a periodic 128×128 grid, but we also consider periodic image systems so that if (X, Y) leaves the grid, it appears on the other side. Moreover, we allow patches to overlap randomly, so the relation $\langle \bar{v}_x\rangle = \varphi U$ remains satisfied. We generate these initial conditions for an ensemble of $N_2 = 50$ epithelia, and for $N_3 = 16$ different values of the order parameter, so $N_2N_3 = 800$ flows are simulated. A few examples of these flow patterns are shown in Fig. 4A. For each configuration we then simulate $N_1 = 1000$ particle trajectories, with a Péclet number of Pé $= 10^3$, so $800,000$ trajectories total. In the right panel we show the resulting clearance times, now averaged over the ensembles N_1 and N_2, $\frac{\langle T\rangle(\gamma)}{\langle T\rangle(1)}$, and the total flux, $\frac{J(\gamma)}{J(1)}$, both normalised with respect to the case of crystalline order.

4. The effect of cilia alignment is examined by varying the orientational order parameter, $M = \langle p_x\rangle$, while keeping the coverage fraction $\varphi = 0.1$, the patchiness $\lambda/H = 8$ and the crystallinity $\gamma = 1$ constant. Again we begin with a crystalline array of 4×4 cilia patches, c_{ij}, as in SI § 3 f*2* with $n_p = 4$. Now each cilia patch k is given a random orientation $\boldsymbol{P}_k$, which is drawn from a circular Gaussian, the Von Mises distribution,

$$f(\theta \mid \alpha) = \frac{e^{\alpha\cos(\theta)}}{2\pi I_0(\alpha)}, \quad \alpha > 0, \tag{S16}$$

where α is the distribution width and the Bessel function of the first kind $I_0(\alpha)$ is just a normalisation factor such that $\int f(\theta)d\theta = 1$. Random numbers θ are drawn from this distribution using the Smirnov transform, i.e. solving the inverse of the cumulative distribution, such that $\langle\cos(\theta)\rangle = M \in [0, 1]$. Hence we compile a grid for the longitudinal and perpendicular surface velocities, $v^x_{ij} = c_{ij}U\cos(\theta_{ij})$ and $v^y_{ij} = c_{ij}U\sin(\theta_{ij})$, where $\theta_{ij} = \theta_k$ for each patch. These initial conditions are generated for an ensemble of $N_2 = 50$ epithelia, and for $N_3 = 16$ different values of the order parameter, $M(n) = 1 - \frac{n-1}{16}$. We again simulate $N_1 = 1000$ particle trajectories for

each configuration, with Pé $= 10^3$. In the right panel of Fig. 4B we show the resulting clearance times averaged over N_1 and N_2, $\frac{\langle T\rangle(M)}{\langle T\rangle(1)}$, and the total flux, $\frac{J(M)}{J(1)}$, both normalised with respect to the case of aligned cilia.

5. The effect of Brownian fluctuations is tested by varying the Péclet number, while keeping constant the wavelength $\lambda = L = 128\mu$m, the film height $H = 10\mu$m, the coverage fraction $\varphi = 0.1$, crystallinity $\gamma = 1$, and cilia alignment $p_x = 1$. We use the same ciliary arrangement v_{ij}^x as before, (SI § 3 f*2* above with $n_p = 1$), for which the flow is shown in the fourth panel of Fig. 3D. Using this flow, in Fig. 4C the trajectories of 50 particles are presented for each value of the Péclet number, Pé $= 100, 100, 10$, by varying the diffusivity D. We then gather statistics of $N_1 = 10^4$ trajectories for the Péclet number Pé $= 10^{4\frac{n-1}{10-1}}$, where $n = 1, 2, 3, \ldots, 10$. In the right panel we show the resulting mean clearance times, $\frac{\langle T\rangle(\text{Pé})}{\langle T\rangle(10^4)}$, and the total flux, $\frac{J(\text{Pé})}{J(10^4)}$, both normalised with respect to the case of weak fluctuations.

g. Shear-dependent viscosity

We verify the robustness of our results with respect to viscoelastic effects [28] by considering a power-law fluid with a shear-dependent viscosity [29]. Instead of simulating a Newtonian liquid, we generalise the Stokes equations of Eq. S6 to

$$0 = \boldsymbol{\nabla}\cdot\underline{\underline{S}}, \qquad 0 = \boldsymbol{\nabla}\cdot\boldsymbol{v}, \tag{S17}$$

where the stress tensor is given by a constitutive equation,

$$\underline{\underline{S}} = -p\underline{\underline{I}} + 2\mu(\dot\gamma)\underline{\underline{E}}, \tag{S18}$$

where $\underline{\underline{I}}$ is the identity matrix, and the shear rate is defined as $\dot\gamma = \sqrt{2\underline{\underline{E}} : \underline{\underline{E}}}$ in terms of the deformation tensor,

$$\underline{\underline{E}} = \frac{(\boldsymbol{\nabla}\boldsymbol{v}) + (\boldsymbol{\nabla}\boldsymbol{v})^T}{2}. \tag{S19}$$

The power-law for the shear-dependent viscosity is

$$\mu(\dot\gamma) = k\dot\gamma^{n-1}, \tag{S20}$$

where k is a constant relative to the properties of the fluid and the exponent n indicates whether the liquid is shear-thinning ($n < 1$) or shear-thickening ($n > 1$). The Newtonian flow is recovered in the case of $n = 1$. The resulting shear-dependent viscosities are shown in Extended Data Fig. 5A.

We implement this constitutive equation in our CFD solver following Neofytou [30]. First, we simulate the Newtonian flow solution as before, using the Newtonian viscosity μ_N, from which we determine the shear rate averaged across the volume of the liquid film, $\langle\dot\gamma_N\rangle$. Then, we simulate the viscoelastic flow for various values of the exponent n, where we non-dimensionalise the shear rate with respect to the Newtonian limit and set $k = \mu_N/\langle\dot\gamma_N\rangle^{n-1}$.

To validate this solver, we first simulate the Poiseuille flow between two parallel plates due a pressure gradient. The analytical solution is known [29, 31] exactly for this viscoelastic flow,

$$\boldsymbol{v}_P(z) = v_{\max}\left(1 - \left(\frac{|z|}{H}\right)^{\frac{n+1}{n}}\right)\hat{\boldsymbol{x}}, \tag{S21}$$

where $v_{\max}$ is the maximum flow speed at the centre line. Extended Data Fig. 5B compares the power-law CFD simulations (points) with this theoretical flow profile (lines). For the Newtonian case ($n = 1$, green) we recover the usual parabolic Poiseuille profile, for the shear-thickening fluid ($n = 2$, red) we recover the sharper 'cusp' flow and for the shear-thinning fluid ($n = 0.5$, blue) we recover the flatter 'plug' flow.

We then apply the power-law CFD solver to simulate the ciliary flows, using the boundary conditions set by the envelope approach as before, following the same methods as in SI § 3 f. Hence, we compare the flow structure due to an array of multiciliated cells for different values of the power-law exponent, n, with a constant coverage fraction $\varphi = 0.1$, patchiness $\lambda/H = 12.8$, crystallinity $\gamma = 1$ and the cilia are all aligned $\langle p_x\rangle = 1$. Perhaps surprisingly, both the shear-thinning and the shear-thickening fluids have very similar flow structures compared to the Newtonian flow [Extended Data Fig. 5C]. For the shear-thinning flow, the flow strength is slightly weaker compared to the shear-thickening flow, but the recirculation zones (red areas) are also slightly smaller. Still, in both cases, particles stuck in these eddies feature a large clearance time. We test this quantitatively in Extended Data Fig. 5D, where we show the total flux and the clearance time as a function of patchiness, repeating the analysis described in SI § 3 f2, for both a shear thinning ($n = 0.5$) and a shear-thickening fluid ($n = 2$). As in the Newtonian case, the clearance time is small for a homogeneous carpet (low λ) but large for patchy epithelia because of the emergence of recirculation. In Extended Data Fig. 5E we also vary the Péclet number, repeating the analysis described in SI § 3 f5. Again as in the Newtonian case, the noise reduces the clearance time.

In summary, we conclude that if multiciliated cells drive a film of a generalised Newtonian fluid with a shear-dependent viscosity, recirculation still emerges with patchiness, but the particle clearance can still be reduced by disorder. It would be interesting to generalise this further to other viscoelastic constitutive equations that include normal stress differences, a yield stress or memory effects. Then, the deformation of streamlines and recirculation zones could be larger, which may enhance particle clearance. Indeed, these complex fluids can be implemented using the CFD framework described here, which provides an interesting avenue for future research.

h. Hexagonal arrays

We also test for robustness with respect to ciliary lattice structure by simulating hexagonal arrays of multiciliated cells, in a Newtonian fluid, with three different orientations [Extended Data Fig. 6A-C]. Compared to the rectangular arrays, we still see back-flow (red areas) and streamline recirculation when the patchiness λ/H is large, which for large Péclet numbers still leads to increased clearance times: In Extended Data Fig. 6D we vary the wavelength λ, as in SI § 3 f2, and simulate $N_1 = 10^4$ particle trajectories with constant diffusivity $D = 0.128\mu\text{m}^2/\text{s}$, box size $L_x = 128\mu\text{m}$, film height $H = 10\mu\text{m}$, and mean flow velocity $\langle \bar{v}_x \rangle = \varphi U \langle p_x \rangle = 10\mu\text{m/s}$. In Extended Data Fig. 6E we also vary the Brownian fluctuations, as in SI § 3 f5, by varying the diffusivity D with constant patchiness, $\lambda/H = 12.8$. Compared to the square lattice, the clearance times are smaller because the recirculating areas are smaller, but qualitatively the results are identical.

i. Phase diagrams

Finally, we cross-compare these parameters in three phase diagrams that combine the coverage fraction, the patchiness, and the ciliary orientation. For all diagrams, we generate an ensemble of ciliary carpet structures starting from a crystalline array of $n_p \times n_p$ cilia patches, as in SI § 3 f2 with $\gamma = 1$. As before the coverage fraction is varied by changing the patch size R so that $n_p^2 \pi R^2 = \varphi L^2$, the patchiness is varied by the number of patches n_p and the patches are given a ciliary orientation with order parameter $M = \langle p_x \rangle$ using the Von Mises distribution.

1. For the $\varphi - \lambda$ diagram we simulate $N_2 N_3$ epithelia for $N_2 = 14$ values of coverage and $N_3 = 10$ values of patchiness. In the resulting flow profiles we simulate $N_1 = 10^3$ tracer trajectories with $\text{Pé}/\varphi = 10^4$ and constant $p_x = 1$, which together yield the mean clearance time. The clearance time is normalised with respect to an ideal carpet, $\varphi = 1$ and $\lambda = 0$.

2. For the $\varphi - M$ diagram we simulate $N_2 N_3 N_4$ flows for $N_2 = 14$ different values of coverage, $N_3 = 16$ different values of orientational order, and for each an ensemble of $N_4 = 20$ epithelia. In all the resulting flow profiles we simulate $N_1 = 10^2$ tracer trajectories with $\text{Pé}/\varphi = 10^3$ and constant $\lambda/H = 8$. The clearance time is normalised with respect to $\varphi = 1$ and $M = 1$.

3. For the $\lambda - M$ diagram we simulate $N_2 N_3 N_4$ flows for $N_2 = 16$ different values of patchiness, $N_3 = 14$ different values of orientational order, and for each an ensemble of $N_4 = 20$ epithelia. In all the resulting flow profiles we simulate $N_1 = 10^2$ tracer trajectories with $\text{Pé} = 10^4$ and constant $\varphi = 0.4$. The clearance time is normalised with respect to $\lambda = 0$ and $M = 1$.

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