Fluctuations, geometry and non-equilibrium thermodynamics of living epithelial tissue

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We introduce a measure of the entropy production in a living functional epithelial tissue. We do this by extracting the functional dynamics of development while at the same time quantifying fluctuations. Using the translucent Drosophila melanogaster pupal epithelium as an ideal tissue for high resolution live imaging [1], we find surprisingly, irreversible dynamics without entropy production. This is done using a detailed analysis of the dynamics of the shape and orientation of individual cells which enables separation of local and global aspects of the tissue behaviour.

A tissue is a group of similar cells that function together as a unit. Hence there is a hope that ideas and techniques to describe many particle systems from condensed matter physics will be helpful to understand their function [2]. There have already been some significant successes following this line of reasoning. The mechanical influences on the dynamic interplay between cells in epithelial tissues have been shown to be important for a diverse array of biological processes from embryonic development and growth [3,4] through to healing of wounds [5,7] and other pathologies like cancer [8].

However self-sustaining tissue is different from a collection of cells (its constituent parts) due to a variety of stochastic [9, 10] feedback processes and information flow essential for life to function. More is different but in not quite the same way as in traditional condensed matter [2]. To consider the physical constraints on biological function in the most realistic context, our focus here is quantifying functional living tissue in-vivo. By doing this we aim to contrast with recent work on collective cell behaviour in in-vitro sheets of cells [12–17] or ex-vivo tissue extracts [1]. We explore this difference by quantifying the fluctuations as well as the dynamics of various geometric quantities in living tissue at cellular scales looking for signatures of functionality [15]. From analysis of the fluctuations we measure the system’s entropy and how it evolves in time. The fact that entropy increases for irreversible adiabatic processes (the 2nd law of thermodynamics) is one of the touchstones of modern physics. The question of how entropy evolves in living multicellular organisms which of course are not adiabatic, nor at equilibrium remains an open question which we address here.

We do this by precisely quantifying a well-known analogy [19] to the breaking of rotational symmetry occurring in the transitions from an isotropic to an ordered mesophase in a liquid crystal [20] and the global shape and orientational order occurring in regions of developing tissue [21]. We map the shape and orientations of cells in the epithelium to a model liquid crystal. While easy to see for in-vitro sheets of confluent elongated cells like fibroblasts [12, 13], it is harder to make this analogy for the more isotropic cells found in functional tissue. This also addresses the question whether the cells show nematic [22] or polar (ferroelectric) [23] liquid crystalline order. We show unambiguously that epithelial cells show nematic order on large scales but polar order on very small scales which quickly decays to zero over a correlation length comparable to the size of a single cell. Furthermore, the irreversible process [21,25] of epithelial growth is indicated by the amount of liquid crystal (nematic) order increasing with time while at the same time surprisingly, the associated observed (information) entropy [26, 27] remains constant.

We study epithelial tissue at a well characterised stage of development focusing on the fluctuations on the cellular scale. We show that throughout a one hour period of observation, despite the increasing average orientational order that the probability distribution of cell shapes and orientations follows a universal form which remains steady, but that individual cells on average evolve in a precise deterministic way according to an equation of motion which we are able to obtain. This implies that the average dynamics are tightly coupled to the fluctuations and vice versa providing a special type of steady state where the density of states remains constant even while the system is evolving continually in time [25]. This suggests that zero entropy production, in the presence of irreversible dynamics, can be a way to identify healthy functional living tissue. Using this entropy, we are able to separate the fluctuations [9, 29] in the tissue at the cellular level from the large scale changes occurring that lead to development, growth and morphogenesis.

Drosophila is highly genetically tractable and is well characterised as a model of embryonic development and disease [30]. Here we study the pupal stage of its life cycle because pupae are translucent and immobile, (see fig 1a). More specifically, we investigate pupal wings, (see fig 1b and fig 1d) which enable viewing of a flat 2D surface of epithelial cells, to gather data rich high resolution in-vivo images with relative ease [1]. These images of the wing provide us with a unique opportunity for a level of analysis that is currently not possible in other systems due to their opacity and the difficulty in obtaining equivalent quality in-vivo data.

Confocal movies were captured from 18 hours post pu-
pal formation. The pupae are first dissected and removed from their puparium. This allowed us to directly view the wing, (see fig 1a), and to gather confocal time-lapse movies of the living wing tissue, details of which are in the supplementary. An example image from a movie is given in fig 1b, with a cross-sectional view in fig 1f. From the experimental data we generate binary images (as shown in fig 1d [31, 34]. The cell boundaries are fitted to polygons (∼5-16 edges) suitable for efficient mathematical analysis (see fig. 1g).

![Image](image_url)

**Figure 1:** a) Translucent Drosophila pupa after dissection from its puparium [1, 35]. b) Low magnification of the entire transgenic pupal wing; the total wing length is 800µm and the width is 250µm. The green is "Ecadherin-GFP" which labels the cell’s "Adherens Junctions" and the red is "Histone-RFP" which labels the cell nuclei. c) Pupal wing tissue. d) A high magnification (binary) view of cell boundaries. e) High magnification cross-section of the wing. f) A schematic diagram of the cross-section of the wing. g) Geometric characterisation of a cell and its boundary. Scale bars as indicated.

**Analysis**  In this stage in development of the wing tissue, the cells are reducing their area at a linear rate. At the same time the number of cells is also increasing linearly.

**Shape Tensor** Each cell (polygon) can be described by a 2nd rank tensor which we call the shape tensor (S), a measure of the variance of the shape of a polygon from its centroid in different directions. Encoding information about the shape and orientation of each cell, it is similar to the texture tensors introduced in [36, 37]. To compare cells of different sizes, it is made dimensionless by dividing by the area squared (see supplementary).

**Cell Shape Factor** We can gain information about a cell’s shape from the difference between the eigenvalues of the shape tensors, which we call the shape factor. The shape factor lies between [0, 1]. When eigenvalues are similar this gives a low shape factor (close to 0); indicating an isotropic, non-oriented shape. A high shape factor implies an elongated shape with vastly different eigenvalues. For more details about shape tensors/factors see supplementary.

Figures 2a, 2b show the shape factor heat map and histogram respectively for a typical image. Figure 2c shows how the average shape factor evolves with time. There is clear linear increase with time. This means cells are becoming increasingly elongated as the tissue develops.

**Cell Orientation** We define the cell orientation as the angle, \( \theta \in [0, \pi] \) between the major axis of the cell and the x axis (see Fig 1g). The orientation (major) axis is the eigenvector of the shape tensor with the smallest eigenvalue. The orientation axis, invariant under reflection, is \( \pi \)-periodic, \( \theta = \theta + \pi \). Figure 2d shows a heatmap of the orientations from the same image as fig. 2a. It is clear from the heatmap there is a global orientation of this tissue. The most common cell colour, yellow-green indicates a mean orientation of \( \sim \pi/6 \).

The mean orientation, determined by orientation of the pupa wing on the slide, is arbitrary. However, the standard deviation of cell orientations around the mean remains consistent between samples at the same develop-
The shape factor of cells (elongated and thin shapes have high values; yellow, isotropic (non-oriented) shapes have low values: blue) b) The distribution of the shape factor. The black line shows the mean. c) The mean shape factor increases linearly with time. d) Heat map showing the orientation of each cell relative to the x-axis of a single sample. e) The distribution of orientations in the sample in part (c) but ‘rotated’ such that the mean is at $\frac{\pi}{2}$. The black line shows the mean. The standard deviation is in the subtitle. f) The standard deviation of the orientation averaged over 15 samples decreases linearly with time. (Bars are root mean square errors)

Cell Polarisation The shape tensor is unable to identify if a cell has any shape anisotropy along its main axes which requires a 3rd rank tensor. From this tensor we can define a polarisation vector, $p$ (see supplementary). The polarisation of a cell measures the skewness of its shape. The polarisation of each cell, $p$ can be decomposed into magnitude and direction: $p = |p| \hat{p}$, where $p = |p|$ and $\hat{p} = (\cos \phi, \sin \phi)$. When this magnitude is large, the shape is highly polarised and when the magnitude is small, the shape is not polarised. As above, to get an indication if the tissue has a global polarisation, it is instructive to compute the average of the polarisation vectors, $P = \langle p \rangle = \overline{P}$ and standard deviation ($\sigma_p$). $P$ is the magnitude of this average vector (how strong it is) and the unit vector, $\hat{P}$ indicates in which direction the tissue is polarised. If $\frac{P}{\sigma_p} \sim 1$ then the system is functionally polarised and if $\frac{P}{\sigma_p} \ll 1$ it is functionally isotropic. Typically, values of $\frac{P}{\sigma_p} \approx 0.021$ indicating isotropy with no polarisation.

Correlations of the orientation and polarisation We also consider the orientation and polarisation correlations of cells separated by distance $R$. 

$$C_p(R) = \frac{1}{N_R} \sum_{R < |r_i-r_j| < R+dR} \langle \hat{p}_i, \hat{p}_j \rangle$$

$$C_q(R) = \frac{1}{N_R} \sum_{R < |r_i-r_j| < R+dR} \langle \hat{q}_i, \hat{q}_j \rangle$$

For the $\hat{q}$ we use the Frobenius inner product. The functions, $C_p, C_q$ are plotted in Figure 2b. $C_p$ has a small anti-correlation at small distances after which the correlation function decays quickly to zero. It is negative at short distances which implies that if a cell is polarised in one direction its direct neighbours are likely to be polarised in the opposite direction but that cells further than one cell part are essentially uncorrelated. Therefore, while individual cells can be polarised, the tissue is not polarised at all. The fact that $C_q$ does not decay to zero for large distances demonstrates that there is strong orientational order throughout the tissue. Hence the tissue is orientated but not polarised.

Entropy production We also analyse the full distribution of cell shapes and orientations, its evolution in time and hence extract the flow of information (entropy) associated with it. At this point it is probably helpful to review some basic notions of thermodynamics and non-equilibrium statistical mechanics. We consider in general a macroscopic system plus its environment at fixed temperature which together form an isolated composite. From the 2nd law we have that the total entropy production is given by the sum of that produced by the system (sys) and its environment (env), $\dot{S}_{\text{tot}} = \dot{S}_{\text{sys}} + \dot{S}_{\text{env}} \geq 0$, with $\dot{S}_{\text{sys}} = \dot{S}_{\text{env}} = 0$ at equilibrium. For a non-equilibrium passive system evolving towards equilibrium, we usually expect that both $\dot{S}_{\text{sys}}, \dot{S}_{\text{env}} > 0$. We explore here what happens in a living functional tissue in which the system corresponds to the degrees of freedom associated with the cell shape and orientations.
Defining the probability $p_q$ of finding a cell with shape $q$, we can calculate the \textit{shape entropy}, the contribution to the entropy from shape fluctuations, $S_q(t) = -\sum q \rho_q \ln \rho_q$ and its evolution with time. We find that the entropy production is on average zero, i.e. entropy remain constant over the whole period of observation despite the increase in orientational order. This surprising observation of irreversible dynamics \textit{without} increase in entropy is an indication of a flux of energy into the degrees of freedom under observation \[38\]. We emphasize that this implies that while $\dot{S}_{n} > 0$ that $\dot{S}_{sys} = 0$ and the 2nd law is still satisfied. Constant entropy, however is ideal for accurate information processing and quick response to external perturbations.

\begin{itemize}
  \item \textbf{Theory} \hspace{1cm} We now develop a model of the development of tissue orientation along an axis with angle $\phi$ that can be compared quantitatively to what we observe in the experimental data. The aim is to describes the dynamics of the probability distribution of cell shapes encoded in the tensor $q = \begin{pmatrix} q_1 & q_2 \\ q_2 & -q_1 \end{pmatrix}$. All the data is consistent with a steady-state probability density given by

$$P_{ss}(q, q_0) = \frac{1}{Z} e^{-h(q; q_0)}$$

where $H(q, q_0) = \sum_{i,j=1}^{2} \frac{1}{2} \delta q_i A_{ij} \delta q_j + O(||\delta q||^3)$, where $\delta q = q - q_0 n$ with the orientation $n = (\cos \phi \sin \phi, \sin 2\phi)$, $(-n_1, n_2, -n_1)$. Note that $\min(h) > -\infty$, must be bounded for eqns.\[3\] to make sense. The most general form of the matrix $A$ which is rotationally invariant is given by $A_{ij} = A_0 \delta_{ij} + A_1 n_i n_j$. The fluctuating variables $q_0(t), q_1(t), q_2(t)$ capture the changes in cell shape which evolve \textit{on average} according to the deterministic dynamical system

$$\frac{d q}{d t} = -A \cdot (q - \bar{q} n)$$

The existence of the dynamical system (i.e. the fact that the rhs of eqns.\[4\] are not zero) means that the living epithelium cannot be mapped to an equilibrium system. There are non-zero currents $J_{ss} = (q, \bar{q}_0) P_{ss}$ which break detailed balance. Guided by the data, we will obtain values for $A_0, A_1, k_0$. This allows us to study statistically the behaviour of different trajectories (experimental samples). First coordinate axes are rotated so the orientation axis is along the $x$-axis (i.e. $n_1 = 1, n_2 = 0$). With this we can express the steady-state probability density in terms of the deviations from the typical values $q_0(t), q_1(t), q_2(t)$. From the data we can compute $\langle ||q|| \rangle$, $\langle (\delta q_1)^2 \rangle$ and $\langle (\delta q_2)^2 \rangle$, this is shown in Figure 3c-d, from which we can extract $k_0, A_0, A_1$ (see supplementary).

\item \textbf{Summary and Discussion} \hspace{1cm} The ability to visualise the dynamic evolution of the spatial distribution of specific proteins within individual cells in tissue provided by high resolution imaging holds promise for us eventually being able to extract the organising principles behind tissue function and repair. This biological function happens in the presence of large fluctuations, both chemical and mechanical, hence these principles, whatever they turn out to be, must be robust to noise. It is the implications of this robustness that we focus on in this letter. Here we have tracked the concentration of junction proteins to quantify the dynamics of cell shapes, orientations and polarisation measuring not only their averages but most importantly their fluctuations. The experimental data in total paint a consistent picture. We observe irreversible dynamics of the cells becoming more elongated and the tissue becoming more oriented along a particular direction $n$ without any associated entropy production. We emphasize that this implies that while the rate of entropy production of the environment is non-zero, $\dot{S}_{env} > 0$ that the rate of entropy production associated with those degrees of freedom in the tissue $\dot{S}_{sys} = 0$ (so the 2nd law is still satisfied). Given that $\dot{S}_{sys} \ll \dot{S}_{env}$, we note that it is
\end{itemize}

**Figure 3:** a) The entropy of the $q$ tensor. Each point is the mean entropy of the videos. b)The figure shows the correlation function off the polarisation and the orientation respectively. Distance by a typical cell length scale is defined by the square root of the mean area of the cells in each image. $C_p(R)$ at 1 cell length starts negatively and then decays exponentially to 0. $C_q(R)$ is correlated around 0.6 throughout the tissue. (Error bars are smaller then markers). c) $\langle (\delta q_1)^2 \rangle$ and $\langle (\delta q_2)^2 \rangle$ over the frames of the video (Error bars are smaller then markers). d) $\langle ||q|| \rangle$ over the frames of the video. This increased linearly over time and line of best fit is shown in black.
also possible that at different points in development, the entropy production in the tissue, \( \dot{S}_{\text{sys}} \) can be non-zero, even negative.

We also find no macroscopic shape polarisation of the tissue; i.e. we find nematic symmetry of the director axis, i.e. \( \mathbf{n} \) and \(-\mathbf{n}\) are equivalent. This can be explained by a model of cell shape and orientation that can be mapped to a non-equilibrium driven nematic liquid crystal. We find that while oriented, the tissues have no global polarisation and that cells that are polarised only affect other cells in their very close proximity. An observation from our data is that all these features are developed in the presence of strong local variation and fluctuations about the average behaviour. This indicates a large but constant information entropy. This suggests an intriguing possibility that should be investigated further by studying the statistical dynamics of other chemical and geometrical quantities in tissue to see if zero entropy production with irreversible dynamics is a signature of functionality and homeostasis in healthy living organisms. This characterisation of an unperturbed but dynamic, developing tissue will lay the groundwork for understanding what happens when these tissues are perturbed for example by cancer or wounding.

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