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Author for correspondence:
Thomas Gregor
e-mail: tg2@princeton.edu

†These authors contributed equally to the study.

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Fly wing vein patterns have spatial reproducibility of a single cell

Laurent Abouchar†, Mariela D. Petkova†, Cynthia R. Steinhardt and Thomas Gregor

Joseph Henry Laboratories of Physics, and Lewis Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ 08544, USA

Developmental processes in multicellular organisms occur in fluctuating environments and are prone to noise, yet they produce complex patterns with astonishing reproducibility. We measure the left–right and inter-individual precision of bilaterally symmetric fly wings across the natural range of genetic and environmental conditions and find that wing vein patterns are specified with identical spatial precision and are reproducible to within a single-cell width. The early fly embryo operates at a similar degree of reproducibility, suggesting that the overall spatial precision of morphogenesis in *Drosophila* performs at the single-cell level. Could development be operating at the physical limit of what a biological system can achieve?

1. Introduction

A key feature of multicellular developmental processes is their ability to reliably generate complex patterns during morphogenesis. As a general rule, morphogenesis proceeds sequentially, interpolating coarser patterns of previous processes to achieve more refined differentiation [1,2]. Despite the tendency during this sequential refinement process to amplify variability from one patterning layer to the next, the final structures are formed with high reproducibility, i.e. the bodies of insects are covered with patterns that are indistinguishable across individuals [3–5]. Morphogenesis has solved the reproducibility problem in non-equilibrium processes [6], but its strategy remains unclear.

The fruit fly *Drosophila melanogaster* is an ideal system to observe morphogenesis from embryo to adulthood and to identify quantitative rules of biological pattern formation. The body plan of the adult organism is specified during the first 3 h of embryogenesis by a molecular blueprint in the form of gene expression patterns [7,8]. The classic example of reproducibility in the 3-h old embryo is the location of the first morphologic mark on a uniform sheet of approximately 6000 cells (table 1), i.e. the cephalic furrow [13]. Its location is reproducible to within half a cell width along the body axis [12,14]. As few as six cells of this sheet—after dividing another 12–13 times over the course of 6 days [9]—generate a disc in the fly larva with approximately 50 000 cells that forms into the adult wing on the 10th day of development [15]. This process of wing morphogenesis is controlled by a multitude of genes [16] as well as by environmental factors such as developmental temperature [17], but the fly wings emerge with a highly stereotyped blade structure with a pattern of five longitudinal and two transverse veins (figure 1*a*). How reproducible is this macroscopic pattern, and how does it compare to the reproducibility in the early embryo?

The fly provides two independent handles for quantifying the patterning process: first, because the formation of the left and right wing is a reiteration of the same process under identical conditions [18,19], the two wings are an internal control for the *left–right* (*LR*) *precision* of the patterning process, i.e. a measure for bilateral symmetry [20]. Second, in randomly chosen left and right wings from a population of flies, wing formation is enacted under different conditions for each individual, allowing for a measure of the *inter-individual precision* of the patterns. Here, we quantify how the final wing architecture is modified across the naturally occurring genetic variation as well as by

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Table 1. Size comparison of features in wings and embryos.

wing features (developmental time)	cells
wing disc, embryo (3 h) [9]	~6
wing disc, embryo (10 h) [10]	~24
wing disc, larva (24 h) [11]	~50
wing disc, pupa (6 days) [11]	~50 000
wing blade, adult (10 days)	~20 000
features (wing 10 days; embryo 3 h)	**size (μm)**
wing length	~1900
wing cell size	13.0 ± 0.7
wing vein precision (inter-individual)	6.5 – 14.4
wing vein precision (LR)	7.4 ± 1.4
embryo length	~490
embryo cell size [12]	8.2 ± 1.0
embryo spatial reproducibility [12]	4.2 ± 0.7

developmental temperatures within the viable range. Thus, we carry out a performance analysis of wing morphogenesis and probe the limits of precision to assess developmental patterning fidelity.

2. Material and methods

The two most prominent features characterizing a wing are its size and its shape or pattern. Size variations depend on nutritional intake, which is difficult to control experimentally, but at least 75% of wing shape variation is independent of variations in size [21,22], thus allowing us to quantify variations in the final wing vein pattern. To measure variations in the principal pattern features, we apply a succession of affine transformations known as Procrustes transformations on each wing from a given dataset (electronic supplementary material, figure S1) [23,24], which maximally align the wings. The vein pattern for each wing is reduced to the configuration of seven landmark coordinates $\{x_i, y_i\}$, which demarcate wing vein crossing points (figure 1*a*); wing size is measured by the centroid size S of the configuration (electronic supplementary material, methods and figure S2*a*). The Procrustes alignment redistributes the variation among all landmarks, and the average linear (i.e. one-dimensional) variation in the spatial landmark location Σ is

$$\Sigma = \langle S \rangle \sqrt{\frac{1}{10}\left[\sum_{i=1}^{7} \mathrm{var}(x_i) + \sum_{i=1}^{7} \mathrm{var}(y_i)\right]},$$

where $\mathrm{var}(z) = \sum_{n=1}^{N} (z_n - \hat{z})^2$ is the variance in coordinate z and $\hat{z}$ is the average coordinate over all N wings in a dataset. Importantly, this transformation reconstructs a mean of centroid size $\langle S \rangle$, which allows us to measure departures from the mean pattern in absolute units and compare these to the physically relevant length scale of the system, i.e. the linear size of an individual wing cell (see the electronic supplementary material). Pattern variations in males and females are assessed separately due to systematic differences in the vein patterning between sexes [16].

After transformation, the landmark coordinates of unit sized wings can be superimposed, and the residual variation corresponds to variation in shape (figure 1*c*,*d*). Particularly surprising is the precision with which sets of right wings overlap in the least (figure 1*b*) and most (figure 1*c*) variable landmark distributions among all examined fly lines. In both cases, the spatial extent of the landmark distributions is comparable to the average linear dimension of an individual wing cell (i.e. approx. 13 μm; electronic supplementary material). Our independently measured experimental error in determining the location of individual landmarks only represents a small fraction of that, i.e. $\Sigma_{\mathrm{err}} = 1.9 \pm 0.3$ μm (figure 1*d*; electronic supplementary material), implying that we are measuring mostly true biological variation. Hence, the spatial variation of the landmarks demonstrates a remarkable level of scale invariance and a highly conserved vein pattern.

To measure precision, we superimpose pairs of transformed wings and quantify their spatial variations in terms of the differences between landmark locations. *LR precision* is assessed from the differences in landmark locations ($\Delta_{\mathrm{L-R}}$) between the left and right wing in the same fly, while *inter-individual precision* is computed from the differences in landmark locations of a pair of randomly chosen left and right wing from the entire fly population ($\Delta_{\mathrm{L-R'}}$, note the prime marking different individuals). The average within-individual variance $\Sigma_{\Delta_{\mathrm{L-R}}}$ is calculated from the differences in the landmark coordinates of pairs of wings ($\{x_i^{\mathrm{L}} - x_i^{\mathrm{R}}, y_i^{\mathrm{L}} - y_i^{\mathrm{R}}\}$), see the electronic supplementary material and figure S2*b*,*c*. Note that measuring high reproducibility or precision corresponds to low pattern variations (small Σ or Σ_{Δ}).

Finally, we use populations of flies with different levels of genetic heterogeneity: i.e. inbreds, crosses of inbreds or laboratory-bred Ore-R flies and wild-caught populations (see the electronic supplementary material). All fly populations are raised in identical environmental conditions and at room temperature (i.e. 22°C), except when effects of developmental temperature are directly explored for which we chose population-specific temperature set-points within the viable range (i.e. 14°C–30°C, see the electronic supplementary material, table S1). Temperatures at the edge of the system's viable range are considered stressful because of a decrease in viability [25] and an increase in the variability of morphological marks [26].

3. Results

In principle, phenotypic variation of the wing vein pattern can be explained as the interplay between various sources of variation, such as genetic differences, environmental effects, developmental errors and regulatory processes that buffer against such variation [27]. Therefore, the precision of the patterning process should be affected by changes in the system's genetic makeup [22], and when the system develops at sub-optimal growth temperatures [21]. However, when we assess patterning fidelity by quantifying LR precision for different genetic backgrounds (figure 2*a*) and for several growth temperatures from the natural viability range (figure 2*b*), we observe that LR precision for all conditions clusters around half a cell size (denoted as dotted lines in figure 2*a*,*b*), independent of genetic heterogeneity and non-optimal growth temperatures. In all examined fly lines, no systematic differences between the left and right wings are observed, and thus the measured variation reflects the fidelity of the developmental programme (electronic supplementary material, figure S4). Hence for a given genetic configuration and temperature set-point within the naturally available range, the system positions pattern features with half a cell precision.

Given that all fly lines display LR precision of approximately half a cell width, the difference in landmark distributions observed in sets of right wings such as in figure 1*b*,*c* must reflect a systematic inter-individual effect. The simplest way to assess inter-individual precision is by direct comparison

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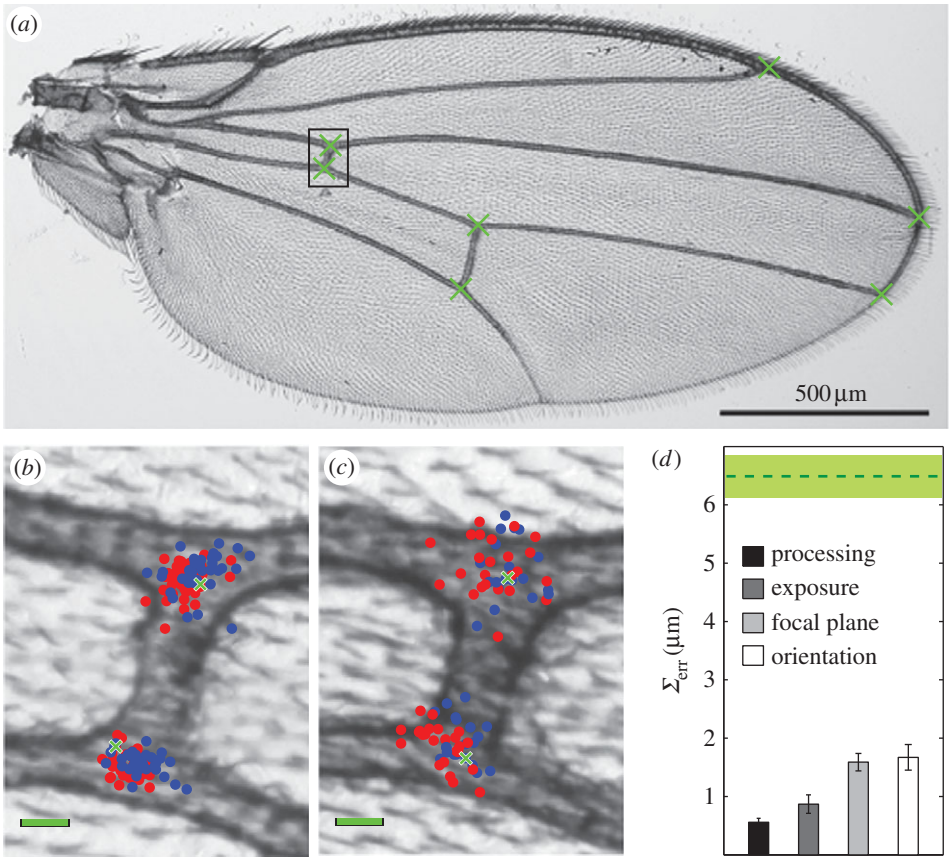

Figure 1. Wing vein patterns are naturally precise. (*a*) Right wing of a male *Drosophila* adult fly. The coordinates of seven wing vein crossings (landmarks) quantify principal pattern features. Green crosses indicate landmark positions of the depicted wing, respectively in all panels. Close-up of the two most proximal landmarks (black rectangle) with the Procrustes superimposed landmark distribution from right wings of (*b*) 41 flies raised under optimal conditions (OreR raised at 18°C) and (*c*) 22 flies from a natural population (caught in Cartagena, Colombia, raised at 22°C). Individual landmark locations are shown for females and males in red and blue, respectively. Green scale bar represents linear dimension of an average wing cell. Individual cells are marked by a bristle in the centre and cell size is measured from the distances between bristles. (*b*) Corresponds to the distribution where we observed the smallest landmark dispersion and (*c*) to the largest one. (*d*) Various systematic measurement errors (Σ_{err}) on the location of landmark locations (electronic supplementary material). Horizontal green line is the average spread of landmark distributions ($\langle\Sigma\rangle = 6.5 \pm 0.7$ µm) across male wings of all fly lines ($N = 143$), see text. (Online version in colour.)

to LR precision measurements in a scatter plot for both genetic and temperature scenarios (figure 2*c*,*d*). In both cases, we recover the above constancy of LR precision, which matches spatial accuracy to the linear dimension of a wing cell. Therefore to compare different fly lines, we normalized our measurements of precision by the average cell size of the respective fly line (see the electronic supplementary material, figure S5). Variations due to differences in the inherent length scale are thus excluded, tightening the spread on the LR precision *y*-axis (converging to half a cell size), but not on the inter-individual *x*-axis.

In each case, we observe conditions for which the wings in different animals are as similar to each other as the wings within an individual. These conditions are identified by data that cluster along the diagonal $\Sigma_{\Delta_{L-R'}} = \Sigma_{\Delta_{L-R}}$. For these data, the vein pattern cannot identify whether two random wings in a population stem from the same individual or not, suggesting that in principle wing patterning could proceed independently in left and right wings of a given fly, as long as the same high fidelity patterning programme operates in every single wing. Importantly, in the genetic case (figure 2*c*), these ideal conditions are attainable only for populations comprising individuals with identical genetic composition.

As individuals from a homogeneous population have indistinguishable vein patterns, the decrease in inter-individual precision in a heterogeneous fly population (spread on *x*-axis in figure 2*c*) must result from an increasing number of genetic compositions. Each composition corresponds to a particular mean landmark configuration, which when observed individually displays reproducibility at the half-cell width precision optimum. However, when many of such genetic compositions are mixed in a population, the reduced reproducibility results from accumulating landmark configurations with different means. Most remarkably, the bounds on the observed inter-individual precision are surprisingly small: they are as large as only a single wing cell and as low as half of that.

In the temperature case (figure 2*d*), we observe an optimum at 18°C for which vein patterns are indistinguishable within and across individuals even in a fly line that contains genetic heterogeneity. Inter-individual precision decreases for lower and for higher temperatures, possibly due to temperature-induced stress that affects the fidelity of the patterning process. However, contrary to that intuition, the constant LR precision across all temperature set-points indicates that the spread along the inter-individual axis probably results from temperature-induced amplification of the epigenetic differences in that fly population rather than from a decline in patterning fidelity. We test this conjecture directly in an inbred fly population where two subsets are raised at two non-optimal temperature conditions (e.g. 22°C and 28°C). In these populations, temperature has no effect on the overall variability in landmark positions: wings in the same animal are as similar as those in

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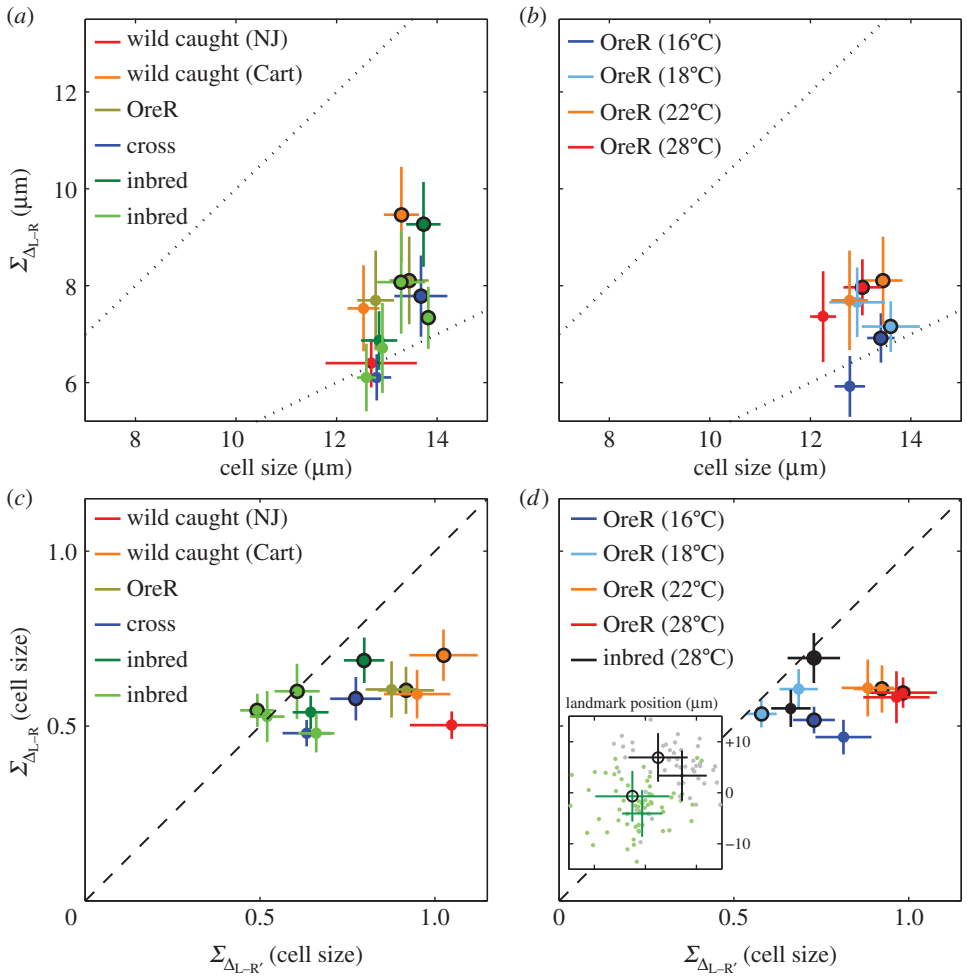

Figure 2. LR and inter-individual patterning precision range between 0.5 and 1 cell diameter for various genetic and environmental conditions. LR precision for fly populations of different genetic makeup (*a*) and at different growth temperatures (*b*) is measured as the spatial within-individual landmark variation ($\Sigma_{\Delta_{L-R}}$), and shown as a function of the average linear wing cell size of the population. Females are indicated by black circles. In (*a*), all flies are raised in a controlled environment at 22°C; in (*b*), identical genetic background (OreR) is used. In both cases, the LR precision of the vein pattern is unaffected and remains at half a cell size (dotted lines have a slopes of 0.5 and 1.0). (*c*,*d*) LR versus inter-individual precision under varying genetic (*c*) and temperature (*d*) conditions. LR precision (*y*-axis) and inter-individual (*x*-axis) precision are measured in units of the linear wing cell size. Flies are on average more symmetric than reproducible (dashed line with a slope of 1 for identity). However, under controlled genetic and environmental conditions (in the most inbred fly lines in (*c*) and at 18°C in (*d*)) the left and right wing of a fly are as similar to each other, as they are to the wings of other flies of the same line. Thus, inter-individual and LR patterning precision are equal. Inbreds raised at 28°C (black data in (*d*)) show no reduction in inter-individual precision. Inset in (*d*) shows the net difference in the location of landmark 2 between two inbred populations raised at 22°C (light green data in (*c*)) and at 28°C (black data in (*d*)), exemplifying that flies with the same genetic makeup raised at two different temperature set-points can have distinct average landmark configurations (green and black crosses indicating error bars), which remain within a single cell and with a single cell and with an LR precision of half a cell diameter. (Online version in colour.)

different animals (green and black data in figure 2*c*,*d*, respectively). For these conditions, the vein pattern is indeed generated with half a cell precision, but two distinct mean landmark configurations are observed (figure 2*d*, inset shows landmark 2 as an example, and electronic supplementary material, figure S6 shows all landmarks). Each configuration is generated with half a cell precision, and the inter-individual precision remains within a single cell.

4. Discussion

Our analysis of the *Drosophila* wing vein pattern measures wing-to-wing variations of less than the linear dimension of a single cell. Pattern variations between the left and the right wing of individual flies are as low as half of that. Thus, the intra-individual precision is at the level of half a wing cell, and it is stable under the range of naturally occurring genetic variations and environmental temperatures. Differences in either condition can lead to systematic shifts of the means of the landmark locations, and the spatial limit of these shifts is at the level of the size of a single wing cell, rendering landmark configurations remarkably conserved. Given that a single cell is the minimal physical unit at which tissue patterning can be realized, our findings suggest that wing patterning operates at the physical limit of the system. Furthermore, for a specific set of external conditions—when genetic fluctuations are minimized in inbred fly lines and at room temperature—we observe that inter-individual variation is as low as intra-individual variation, meaning that the vein pattern is as reproducible between individuals as it is symmetric within individuals, suggesting that the wing patterning programme operates with high fidelity, potentially independently in each wing.

From a functional point of view, the single-cell invariance of the vein pattern in both genetic and temperature scenarios

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might be surprising. However, symmetry between the left and right wing of an individual is functionally important for proper steering during flight [28] and for successful courtship [29]. Hence, some evolutionary pressure is expected on precision. Thus, while the functional role for single-cell reproducibility of the vein pattern is unknown, it is likely related to the animal's overall flight control. The vein pattern is geometrically important for aerodynamic properties [30], while the ratio of wing size to body size relates to overall flight capability [31]. Therefore, single-cell reproducibility might reflect the necessary level of scale invariance to ensure perfect aerodynamic control and the necessary level of size matching to maintain flight despite differences in body sizes. New measurements involving asymmetric wing configurations will be necessary to test these implications of reproducibility empirically.

It remains unclear what exactly the precision of certain features, such as the location of the intersection of two veins, implies for the fates of individual cells. Do cells migrate to their final locations with high precision? Do *all* cells in the fly wing end up at their final locations with the same precision? It should be possible to explore these questions by developing individual cell lineage tracing methods in growing wings to follow individual cells during their developmental path. Alternatively, the final precision of the wing shape could be independent on the precision of the individual cell. Instead there could exist some sensing mechanism—possibly related to cell-to-cell pressures or sensing of morphogens—that cause adjustments of inaccurately placed cells to change their locations until the final accuracy is achieved. Apoptosis is known to cause cells to self-destruct to correct local errors in the formation of the wing disc [32], and more recently global mechanical anisotropies [33] and local cell interactions [34] have been implicated in cell polarization in the wing blade and boundary formation in the wing disc. Therefore, wing vein precision in mutants affecting apoptosis and ablation experiments at different wing developmental stages could give some clues whether there exist error-correcting mechanisms that work towards positioning the vein pattern with high accuracy [35].

Overall, our observations support a straightforward strategy for the maintenance of bilateral symmetry during the wing generation process. Inter-individual precision being as low as LR precision indicates that left and right wings can in principle form independently. In particular, as long as the seed cells on the left and the right sides of the developing wing structures are symmetric, patterning can proceed independently with high fidelity on the left and right sides of the organism and result in highly symmetric wings. Thus, spatial decisions in the previous layers of the wing formation process have to be performed with a spatial precision of at least that of the final product, i.e. a single cell. Indeed, a similar situation has been observed in the early embryo where the precision of molecular patterns that are set up during oogenesis lead to precise molecular patterns in the early embryo all the way to the first macroscopic pattern, the cephalic furrow [12,14,36,37]. This connection between the early embryo and the final adult wing structure indicates that spatial reproducibility of morphogenetic features in the fly may be maintained throughout the entire 10 days of development.

In principle, morphogenetic processes in the fly could have reproducible outcomes by measuring at each stage the size of the relevant local 'building block' of the pattern (i.e. an individual cell) and determining position with spatial precision of half of that unit's size (table 1). This suggests that an error of half the size of the building block is a sufficient strategy for generating and maintaining spatial reproducibility from one patterning layer to the next. In particular, it seems to be sufficient to reproducibly generate a complex pattern comprised of as many as approximately 20 000 such units in the *Drosophila* wing. It will be important to test how these constraints affect current models for growth and patterning at different stages of the wing formation process [38–40].

Could such extreme reproducibility be a general feature of morphogenesis? Our findings suggest that it might at least be the case for the maintenance of symmetric features during developmental growth. Interpreting variations in absolute units allows us to recognize that wing patterning runs with a precision of less than a single cell. Because it is individual cells that make fate determining decisions, a cell is arguably the minimal physical unit at which tissue patterning can be realized. We identify here a signature of optimization in developmental processes, which seemingly perform at their physical limit in generating patterns. Our work thus represents a necessary first step towards an understanding of reproducibility in systems that have to cope with environmental fluctuations and noisy processes.

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