Probing the limits to positional information

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The reproducibility and precision of biological patterning is limited by the accuracy with which concentration profiles of morphogen molecules can be established and read out by their targets. We consider four measures of precision for the Bicoid morphogen in the Drosophila embryo: The concentration differences that distinguish neighboring cells, the limits set by the random arrival of Bcd molecules at their targets (which depends on the absolute concentration), the noise in readout of Bcd by the activation of Hunchback, and the reproducibility of [Bcd] at corresponding positions in multiple embryos. We show, through a combination of different experiments, that all of these quantities are ~ 10%. This agreement among different measures of accuracy indicates that the embryo is not faced with noisy input signals and readout mechanisms; rather the system exerts precise control over absolute concentrations and responds reliably to small concentration differences, approaching the limits set by basic physical principles.

INTRODUCTION

The macroscopic structural patterns of multicellular organisms have their origins in spatial patterns of morphogen molecules (Wolpert 1969, Lawrence 1992, Gerhart & Kirschner 1997). Translating the qualitative picture of morphogen gradients into quantitative terms raises several difficulties. First is the problem of precision (Figure 1): Neighboring cells often adopt distinct fates, but the signals that drive these decisions involve very small differences in morphogen concentration, and these must be discriminated against the inevitable background of random noise. The second problem is reproducibility: If cells “know” their location based on the concentration of particular morphogens, then generating reproducible final patterns requires either that the absolute concentrations of these molecules be reproducible from embryo to embryo or that there exist mechanisms which achieve a robust output despite variable input signals.

These problems of precision and reproducibility are potentially relevant to all biochemical and genetic networks, and analogous problems of noise (de Vries 1957; Barlow 1981; Bialek 1987, 2002) and robustness (LeMasson et al 1993; Abbott & LeMasson 1993; Goldman et al 2001) have long been explored in neural systems. One might even hope that maximizing reliability in the presence of noise or maximizing robustness to variation in protein copy number could provide “design principles” with which to understand or predict the structure and dynamics of these networks. Here we address these issues in the context of the initial events in fruit fly development.

The primary determinant of patterning along the anterior-posterior axis in the fly Drosophila melanogaster is the gradient of Bicoid (Bcd), which is established by maternal placement of bed mRNA at the anterior end of the embryo (Driever & Nüsslein-Volhard 1988a,b); Bcd acts as a transcription factor, regulating the expression of hunchback (hb) and other downstream genes (Struhl et al 1989; Rivera-Pomar et al 1995; Gao & Finkelstein 1998). This cascade of events generates a spatial pattern so precise that neighboring nuclei have readily distinguishable levels of expression for several genes [as reviewed by Gergen et al (1986)], and these patterns are reproducible from embryo to embryo (Crauk & Dostatni 2005; Holloway et al 2006).

In trying to quantify the precision and reproducibility of the initial events in morphogenesis, we can ask four conceptually distinct questions:

• If cells make decisions based on the concentration of Bcd alone, how accurately must they “measure” this concentration to be sure that neighboring cells reach reliably distinguishable decisions?

• What is the smallest concentration difference that can be measured reliably, given the inevitable noise that results from random arrival of individual Bcd molecules at their target sites along the genome?

• What level of precision does the system actually achieve, for example in the transformation from Bcd to Hb?

• How reproducible are the absolute Bcd concentrations at corresponding locations in different embryos?

The answer to the first question just depends on the spatial profile of Bicoid concentration (Houchmandzadeh et al 2002). To answer the second question about the physical limits to precision we need to know the absolute concentration of Bcd in nuclei, and we measure this using the Bcd-GFP fusion constructs described in a companion paper (Gregor et al 2006). To answer the third question we characterize directly the input/output relation between Bcd and Hb protein levels in each nucleus of
FIG. 1 Schematic of the readout problem for the Bicoid gradient. At left, the conventional picture. A smooth gradient of Bcd concentration is translated into a sharp boundary of Hb expression because Bcd acts as a cooperative activator of the *hb* gene. Although intended as a sketch, the different curves have been drawn to reflect what is known about the scales on which both the Bcd and Hb concentrations vary. Note that neighboring cells along the anterior-posterior axis experience Bcd concentrations that are very similar (differing by ~ 10%, as explained in the text), yet the resulting levels of Hb expression are very different. At right, we consider a larger number of cells in the mid-embryo region where Hb expression switches from high to low values. From direct experiments on simpler systems we know that, even when the concentrations of transcription factors are fixed, the resulting levels of gene expression will fluctuate (Elowitz et al 2002; Raser & O’Shea 2004), and there are physical limits to how much this noise can be reduced (Bialek & Setayeshgar 2005, 2006). If the noise is low, such that a scatter plot of Hb expression vs Bcd concentration is relatively tight, then the qualitative picture of a sharp Hb expression boundary is perturbed only slightly. If the noise is large, so that there is considerable scatter in the relationship between Bcd and Hb measured for individual cells, then the sharp Hb expression boundary will exist only on average, and not along individual rows in individual embryos.

RESULTS

Setting the scale

Two to three hours after fertilization of the egg, adjacent cells have adopted distinct fates, as reflected in their patterns of gene expression. At this stage, the embryo is ~ 500 µm long and neighboring nuclei are separated by ∆x ~ 10 µm. Distinct fates in neighboring cells therefore mean that they acquire positional information with an accuracy of ~ 2% along the anterior-posterior axis. Measurements of Bcd concentration by immunostaining reveal an approximately exponential decay along this axis, \( c(x) = c_0 \exp(-x/\lambda) \), with a length constant \( \lambda \sim 100 \mu m \) (Houchmandzadeh et al 2002). Neighboring nuclei, at locations \( x \) and \( x + \Delta x \), thus experience Bcd concentrations which differ by

\[
\Delta c(x) = \left| \frac{dc(x)}{dx} \right| \Delta x = \frac{1}{\lambda} c(x) \Delta x. \tag{1}
\]

The exponential form of the Bcd profile implies that the *fractional* concentration difference between neighbors is constant along the length of the embryo,

\[
\frac{\Delta c(x)}{c(x)} = \frac{\Delta x}{\lambda} \sim 0.1. \tag{2}
\]
To make decisions that reliably distinguish individual nuclei from their neighbors using the Bcd morphogen alone therefore would require each nucleus to “measure” the Bcd concentration with an accuracy of $\sim 10\%$.

**Absolute concentrations**

The difficulty of achieving precise and reproducibly functioning biochemical networks is determined in part by the absolute concentration of the relevant molecules: for sufficiently small concentrations, the randomness of individual molecular events must set a limit to precision (Berg & Purcell 1977; Bialek & Setayeshgar 2005, 2006). For a quantitative discussion, we thus would like to know the absolute concentration of Bcd molecules. Since Bcd is a transcription factor, what matters is the concentration in the nuclei of the forming cells. A variety of experiments on Bcd (Ma et al 1996; Burz et al 1998; Zhao et al 2002) and other transcription factors (Ptashne 1992; Pedone et al 1996; Winston et al 1999) suggest that they are functional in the nanoMolar range, but to our knowledge there exist no direct *in vivo* measurements in the *Drosophila* embryo.

In Figure 2A we show an optical section through a live *Drosophila* embryo that expresses a fusion of the Bcd protein with the green fluorescent protein (GFP), as described in a companion to this paper (Gregor et al 2006). There we established that this Bcd-GFP fusion quantitatively reproduces the functions of the native Bcd. In scanning two-photon microscope images we identify individual nuclei to measure the mean fluorescence intensity in each nucleus, which should be proportional to the protein concentration. To establish the constant of proportionality we bathe the embryo in a solution of purified GFP with known concentration and thus compare fluorescence levels of the same moiety along the same optical path (see Methods).

Some of the observed fluorescence is contributed by molecules other than the Bcd-GFP, and we test for this background by imaging wild type embryos under exactly the same conditions. As shown in Figure 2B, this background is almost spatially constant and essentially equal to the level seen in the Bcd-GFP flies at the posterior pole, consistent with the idea that the Bcd concentration is nearly zero at this point.

Figure 2B shows the concentration of Bcd-GFP in nuclei as a function of their position along the anterior-posterior axis. The maximal concentration near the anterior pole, corrected for background, is $c_{\text{max}} = 55 \pm 3$ nM, while the concentration in nuclei near the midpoint of the embryo, near the threshold for activation of *hb* expression (at a position $x/L \sim 48\%$ from the anterior pole), is $c = 8 \pm 1$ nM. This is remarkably close to the dissociation constants measured *in vitro* for binding of Bcd to its target sequences in the *hb* enhancer (Ma et al 1996; Burz et al 1998; Zhao et al 2002).

**FIG. 2 Absolute concentration of Bcd.** A: Scanning two-photon microscope image of a *Drosophila* embryo expressing a Bcd-GFP fusion protein (Gregor et al 2006); scale bar 50 µm. The embryo is bathed in a solution of GFP with concentration 36 nM. We identify individual nuclei and estimate the mean Bcd-GFP concentration by the ratio of fluorescence intensity to this standard. B: Apparent Bcd-GFP concentrations in each visible nucleus plotted vs. anterior-posterior position $x$ (reference line in A) in units of the egg length $L$; red and blue points are dorsal and ventral, respectively. Repeating the same experiments on wild type flies which do not express GFP, we find a background fluorescence level shown by the black points with error bars (standard deviation across four embryos). In the inset we subtract the mean background level to give our best estimate of the actual Bcd-GFP concentration in the nuclei near the midpoint of the embryo. Points with error bars show the nominal background, now at zero on average.

**Physical limits to precision**

Our interest in the precision of the readout mechanism for the Bcd gradient is heightened by the theoretical difficulty of achieving precision on the $\sim 10\%$ level. To begin, we note that 1 nM corresponds to $0.6 \text{ molecules/µm}^3$. Thus the observed concentration of Bcd in nuclei near the midpoint of the embryo is $c = 4.8 \pm 0.6 \text{ molecules/µm}^3$, which corresponds to 690 total molecules in the nucleus [with diameter $d \sim 6.25 \umu m$ (Gregor et al 2006)] during nuclear cycle 14. A ten percent difference in concentration thus amounts to changes of $\sim 70$ molecules.

Berg and Purcell (1977) emphasized, in the context of
bacterial chemotaxis, that concentration measurements are limited by fundamental physical noise sources that derive from the random character of diffusion and binding at the level of single molecules. This noise is determined not by the total number of available molecules, but by the dynamics of their random arrival at their target locations. Consider a receptor of linear size a, and assume that the receptor occupancy is integrated for a time T. Berg and Purcell argued that the precision of concentration measurements is limited to

\[
\delta c / c \sim \frac{1}{\sqrt{DacT}}.
\]

(3)

where c is the concentration of the molecule to which the system is responding and D is its diffusion constant in the solution surrounding the receptor. Recent work shows that the Berg-Purcell result really is a lower limit to the noise level (Bialek & Setayeshgar 2005, 2006): the complexities of the kinetics describing the interaction of the receptor with the signaling molecule just add extra noise, but cannot reduce the effective noise level below that in Equation (3). These theoretical results encourage us to apply this formula to understand the sensitivity of cells not just to external chemical signals (as in chemotaxis) but also to internal signals, including morphogens such as Bcd.

The measurements above show that the total concentration of Bcd in nuclei is \( c = 4.8 \pm 0.6 \text{ molecules/μm}^3 \) near the point where the “decision” is made to activate Hb. In a companion to this paper we show that Bcd diffuses slowly through the dense cytoplasm surrounding the nuclei with a diffusion constant \( D < 1 \text{ μm}^2/s \) (Gregor et al 2006), which is similar to that observed in bacterial cells (Elowitz et al 1999), and we take this as a reasonable estimate of the effective diffusion constant for Bcd in the nucleus (see Methods for more details on the estimates of c and D).

Receptor sites for eukaryotic transcription factors are \( \sim 10 \) base pair segments of DNA with linear dimensions \( a \sim 3 \text{ nm} \). Multiple sites might make the effective size slightly larger, but wrapping of the DNA around the nucleosome could effectively convert a longer sequence of sites into a two dimensional patch of sites with the same linear dimensions, and we know from the Berg-Purcell analysis and its generalization that such a patch acts as one effective site in setting the limiting noise level.

The remaining parameter, which is unknown, is the amount of time T over which the system averages in determining the response to the Bcd gradient; the longer the averaging time the lower the effective noise level. One way to express our results, then, is to ask for the minimum averaging time required to reach a precision of \( \delta c / c \sim 10\% \). With the parameters listed above,

\[
\frac{\delta c}{c} \sim \left[DacT\right]^{-1/2}
\]

\[
= \left[(1 \text{ μm}^2/s)(3 \text{ nm})(4.8 / \text{μm}^3)T\right]^{-1/2}
\]

\[
\sim 0.1,
\]

implies that this minimum time is \( T \sim 115 \text{ min} \), or nearly two hours. This is almost the entire time available for development from fertilization up to gastrulation, and it seems implausible that downstream gene expression levels reflect an average of local Bcd concentrations over this long time; given the enormous changes in local Bcd concentration during the course of each nuclear cycle (Gregor et al 2006), it is not even clear that there are mechanisms which could achieve this integration.

We emphasize that our discussion ignores all noise sources other than the fundamental physical process of random molecular arrivals at the relevant binding sites; additional noise sources would necessitate even longer averaging times. Already the minimum time required to push the physical limits down to the \( \sim 10\% \) level is implausible given the pace of developmental events.

Input/output relations and noise

The fact that neighboring cells can generate distinct patterns of gene expression does not mean that any single
step in the readout of the primary morphogen gradients achieves this level of precision. Indeed, the discussion in the previous section makes clear that any simple picture in which the expression levels of downstream genes provides a “measurement” of the Bcd concentration in single nuclei will run up against fundamental physical limits if we ask for precision sufficient to distinguish reliably among neighboring nuclei. To test the plausibility of these ideas more directly, we focus on the transformation from Bcd to Hb, one of the first steps in the generation of anterior-posterior pattern.

In Figure 3A we show confocal microscope images of a *Drosophila* embryo fixed during nuclear cycle 14 and immunostained for DNA, Bcd and Hb; the fluorescence peaks of the different labels are sufficiently distinct that we can obtain independent images of the three stains. The DNA images allow us to locate automatically the centers and outlines of the nuclei (see Methods). In a typical image of this sort we identify \( \sim 1200 \) nuclei; manual inspection shows that misidentifications occur at less than the 1% level, and these are easily corrected. Given these outlines we can measure the average intensity of Bcd and Hb staining in each nucleus (Figure 3B).

It is conventional to assume that immunofluorescent staining intensity is proportional to protein concentration; more precisely, that in each pixel the observed intensity \( I \) is proportional to the concentration \( c \) plus some non-specific background, so that \( I = Ac + B \), where \( A \) and \( B \) are constant in a single image. Recently we have been able to test this assumption quantitatively using flies that express a Bcd-GFP fusion protein (Gregor et al 2006), showing that the patterns of fluorescence from GFP, anti-GFP and anti-Bcd antibodies all are consistent with one another as expected if staining is related to concentration through this linear model. With this linearity, a single image provides more than 1000 points on the scatter plot of Hb expression level vs Bcd concentration, as in Figure 3C.

Scatter plots as in Figure 3 contain information both about the mean “input/output” relation between Bcd and Hb and about the precision or reliability of this response. Indeed, we can think of these data as the generalization to multicellular, eukaryotic systems of the input/output scatter plots measured for engineered regulatory elements in bacteria [e.g. Figure 3B of Rosenfeld et al (2005)]. To analyze these data we discretize the Bcd axis into bins, grouping together nuclei which have very similar levels of staining for Bcd; within each bin we compute the mean and variance of the Hb intensity. We normalize the input/output relation by measuring the Hb level in units of its maximal mean response, and the Bcd level in units of the level which generates (on average) half maximal Hb.

Mean input/output relations between Bcd and Hb are shown for nine individual embryos in Figure 4A. We see that the results from different embryos are very similar; differences among embryos are smaller than the output noise (see Methods for discussion of normalization across embryos). Pooling the results from all embryos yields an input/output relation that fits well to the Hill relation,

\[
Hb = Hb_{\text{max}} \frac{\text{Bcd}^n}{\text{Bcd}^n + \text{Bcd}^m_{1/2}}. \tag{5}
\]

We find that the best fit is with \( n = 5 \). This is consistent with the idea that Hb transcription is activated by cooperative binding of effectively five Bcd molecules, as expected from the identification of seven Bcd binding sites in the *hb* promoter (Struhl et al 1989; Driever & Nüsslein-Volhard 1989).

In Figure 4B we show the standard deviation in Hb levels as a function of the apparent Bcd concentration. We see that the fractional output fluctuations are below 10% when the activator Bcd is at high concentration, similar to what has been found in direct measurements on artificially constructed regulatory modules (Elowitz et al 2002; Raser & O’Shea 2004). If we think of the Hb expression level as a readout of the Bcd gradient, then we can convert the output noise in Hb levels into an equivalent level of input noise in the Bcd concentration. This is the same transformation as ‘referring noise to the input’ in specifying the performance of an amplifier or sensor, and the mathematical transformation is the same as for the propagation of errors: we ask what level of error \( \delta c \) in Bcd concentration would generate the observed level of variance in Hb expression,

\[
\sigma_{Hb}^2(Bcd) = \left| \frac{d[Hb]}{d[Bcd]} \right| (\delta c)^2, \tag{6}
\]

or

\[
\frac{\delta c}{c} = \sigma_{Hb}^2(Bcd) \left| \frac{d[Hb]}{d\ln[Bcd]} \right|^{-1}. \tag{7}
\]

It is this “equivalent fractional noise level,” shown in Figure 4C, that cannot fall below the physical limit set by Equations (3) and (4). For individual embryos we find a minimum value of \( \delta c/c \sim 0.1 \) near \( c = c_{1/2} \).

It should be emphasized that all of the noise we observe could in principle result from our measurements, and not from any intrinsic unreliability of the underlying molecular mechanisms. In particular, because the input/output relation is very steep, small errors in measuring the Bcd concentration will lead to a large apparent variance of the Hb output. In separate experiments (see Methods) we estimate the component of measurement noise which arises in the imaging process. Subtracting this instrumental variance from the inferred concentration noise results in values of \( \delta c/c \sim 0.1 \) on average (circle with error bars in Figure 4C). The true noise level could be even lower, since we have no way of correcting for nucleus-to-nucleus variability in the staining process.

Before proceeding it is important to emphasize the limitations of our analysis. We have treated the relationship between Bcd and Hb as if there were no other factors involved. In the extreme one could imagine (although
we know this is not true) that both Bcd and Hb concentrations vary with anterior-posterior position in the embryo, but are not related causally. In fact, if we look along the dorsal-ventral axis, there are systematic variations in Bcd concentration (as can be seen in Figure 3), and the Hb concentrations are correlated with these variations, suggesting that Bcd and Hb really are linked to each other rather than to some other anterior-posterior position signal. It has been suggested, however, that \( hb \) expression may be responding to signals in addition to Bcd (Howard & ten Wolde 2005; Houchmandzadeh et al 2005; McHale et al 2006). If these signals ultimately are driven by the local Bcd concentration itself, then it remains sensible to say that the Hunchback concentration provides a readout of Bcd concentration with an accuracy of ~10%. If, on the other hand, additional signals are not correlated with the local Bcd concentration, then one might expect that collapsing our description onto just the Bcd and Hb concentrations would treat other variables as an extrinsic source of noise; in this case the true reliability of the transformation from Bcd to Hb could be even better than what we observe.

FIG. 4 Input/output relations and noise. A: Mean input/output relations for 9 embryos. Curves show the mean level of Hb expression as a function of the Bcd concentration, where we use a logarithmic axis to provide a clearer view of the steep, sigmoidal nonlinearity. Points and error bars show, respectively, the mean Hb level and standard deviation of the output noise for one of the embryos. Inset shows mean Hb output (points) and standard errors of the mean (error bars) when data from all embryos are pooled. The mean response is consistent with the Hill relationship, Equation (5), with \( n = 5 \), corresponding to a model in which five Bcd molecules bind cooperatively to activate Hb expression (red line). In comparison, Hill relations with \( n = 3 \) or \( n = 7 \) provide substantially poorer fits to the data (green lines). B: Standard deviations of Hb levels for nuclei with given Bcd levels. C: Translating the output noise of (B) into an equivalent input noise, following Equation (7). Blue dots are data from 9 embryos, green line with error bars is an estimate of the noise in our measurements (see Methods), and red circles with error bars are results after correcting for measurement noise. D: Correlation function of Hb output noise, normalized by output noise variance, as a function of distance \( r \) measured in units of the mean spacing \( \ell \) between neighboring nuclei. Lines are results for four individual embryos, points and error bars are the mean and standard deviation of these curves. We have checked that the dominant sources of measurement noise are uncorrelated between neighboring nuclei. The large difference between \( r = 0 \) and \( r = \ell \) arises largely from this measurement noise. Inset shows the same data on a logarithmic scale, with a fit to an exponential decay \( C \propto \exp(-r/\xi) \); the correlation length \( \xi/\ell = 5 \pm 1 \).
Noise reduction by spatial averaging?

The observed precision of $\sim 10\%$ is difficult to reconcile with the physical limits [Equation (4)] given the available averaging time. If the precision cannot be increased to the observed levels by averaging over time, perhaps the embryo can achieve some averaging over space: If the Hb level in one nucleus reflects the average Bcd levels in its $N$ neighbors, the limiting noise level in Equation (4) should decrease by a factor of $\sqrt{N}$.

If we imagine that communication among nuclei is mediated by diffusion of a protein with diffusion constant comparable to that of Bcd itself, then in a time $T$ it will cover a radius $r \sim \sqrt{4DT}$ and hence an area $A \sim 4\pi DT$. But at cycle 14 the nuclei form an approximately regular lattice of triangles with side $\ell \sim 8.5 \mu$m, so the area $A$ contains $N \sim \frac{8\pi}{\sqrt{3}} \frac{DT}{\ell^2}$ (8) nuclei. Putting all the factors together (with $D \sim 1 \mu$m$^2$/s from above), we find that in just four minutes it should be possible to average over roughly 50 nuclei. Since averaging over time and averaging over nuclei have the same effect on the noise level—reducing the noise in proportion to the square root of time or number—averaging over fifty nuclei for four minutes is the same as each nucleus acting independently but averaging for two hundred minutes. More generally, with communication among nuclei the physical limit becomes

$$\frac{\delta c}{c} \sim \frac{1}{\sqrt{DacTN}} = \left[ \frac{\sqrt{3}}{8\pi ac} \right]^{1/2} \frac{\ell}{DT}$$ (9)

$$\sim \frac{20s}{T}$$ (10)

Thus 10% precision is possible with mechanisms that integrate for only $\sim 200$s, or $\sim 3$ minutes, rather than hours. In particular, it would be possible to make measurements with this precision over the course of a single nuclear cycle.

If each nucleus makes independent decisions in response to the local Bcd concentration, then noise in the Hb levels of individual nuclei should be independent. On the other hand, if Hb expression reflects an average over the nuclei in a neighborhood, then noise levels necessarily become correlated within this neighborhood. Going back to our original images of Bcd and Hb levels, we can ask how the Hb level in each nucleus differs from the average (along the input/output relation of Figure 4A) given its Bcd level, and we can compute the correlation function for this array of Hb noise fluctuations (see Methods). The results, shown in Figure 4D, reveal a component with a correlation length $\xi = 5 \pm 1$ nuclei, as predicted if averaging occurs on the scale required to suppress noise.

Reproducibility in live embryos

The results of Figure 4 indicate that individual embryos can “read” the profile of Bcd concentration with an accuracy of $\sim 10\%$. A particular value of the Bcd concentration thus has a precise meaning within each embryo, raising the question of whether this meaning is invariant from embryo to embryo. Such a scenario would require control mechanisms to insure reproducibility of the absolute copy numbers of Bcd and other relevant gene products. The alternative is that the spatial profiles of Bcd vary from embryo to embryo, but other mechanisms allow for a robust response to this variable input. A number of groups have argued for the latter scenario (von Dassow et al 2000; Houchmandzadeh et al 2002; Spirio et al 2003; Jaeger et al 2004; Howard & ten Wolde 2005; Holloway et al 2006). In contrast, the similarity of Bcd/Hb input/output relations across embryos (Figure 4A) suggests that reproducible outputs result from reproducible inputs.

To measure the reproducibility of the Bcd gradient, we use live imaging of the Bcd-GFP fusion construct, as in Figure 2. To minimize variations in imaging conditions, we collect several embryos that are approximately synchronized and mount them together in a scanning two-photon microscope. Nucleus-by-nucleus profiles of the Bcd concentration during the first minutes of nuclear cycle 14 are shown for 15 embryos in Figure 5A (see Methods). Qualitatively it is clear that these profiles are very similar across all embryos. We emphasize that these comparisons require no scaling or separate calibration of images for each embryo; one can compare raw data, or with one global calibration (as in Figure 2) we can report these data in absolute concentration units.

We quantify the variability of Bcd levels across embryos by measuring the standard deviation of concentration across nuclei at similar locations in different embryos, and expressing this as a fraction of the mean. The results, shown in Figure 5B, are consistent with reproducibility at the $10-20\%$ level across the entire anterior half of the embryo, with variability gradually rising in the posterior half where Bcd concentrations are much lower. Thus the reproducibility of the Bcd profile across embryos is close to precision with which it can be read out within individual embryos.

The average concentration profile $\bar{c}(x)$ defines a mapping from position to concentration; the basic idea of positional information is that this mapping can be inverted so that we (and the embryo) can “read” the position by measuring concentration. But then if a cell at position $x$ “sees” a Bcd concentration that deviates slightly from the average appropriate to that location, $c(x) = \bar{c}(x) + \delta c(x)$, then the readout will produce an error $\delta x$ in the position signal defined by

$$\bar{c}(x + \delta x) = \bar{c}(x) + \delta c(x)$$ (11)

$$\Rightarrow \delta x \approx \delta c(x) \left[ \frac{dc(x)}{dx} \right]^{-1}.$$ (12)
FIG. 5 Reproducibility of the Bcd profile in live embryos. A: Bcd-GFP profiles of 15 embryos. Each dot represents the average concentration in a single nucleus at the mid-sagittal plane of the embryo (on average 70 nuclei per embryo). All nuclei from all embryos are binned in 50 bins, over which the mean and standard deviation were computed (black points with error bars). Scale at left shows raw fluorescence intensity, and at right we show concentration in nM, with background subtracted, as in Figure 2. B: For each bin from A, standard deviations divided by the mean as a function of fractional egg length (blue); error bars are computed by bootstrapping with 8 embryos. Grey and black lines show estimated contributions to measurement noise (see Methods). C: Variability of Bcd profiles translated into an effective rms error σ(x) in positional readout, as in Equation (13); error bars from bootstrapping. Green circles are obtained by correcting for measurement noise.

Through this relation we can convert the measured standard deviations or rms errors δc(x) in the concentration profiles into an effective rms error σ(x) in positional information,

\[ \sigma(x) = \delta c(x) \left| \frac{d\bar{c}(x)}{dx} \right|^{-1}. \]  

(13)

This is equivalent to drawing a threshold concentration \( \theta \) and marking the locations \( x_\theta \) at which the individual Bcd profiles cross this threshold; \( \sigma(x) \) is the standard deviation of \( x_\theta \) when the threshold is chosen so that the mean of \( x_\theta \) is equal to \( x \). We find (Figure 5C) that the Bcd profiles are sufficiently reproducible that near the middle of the embryo it should be possible to read out positional information with an accuracy of \( \sim 2\% \) of the embryo length, close to the level required to specify the location of individual cell nuclei.

We emphasize once again that what we characterize here as variability still could result from imperfections in our measurements (see Methods for details). In Figure 5B we show the summed contributions from noise in imaging and from errors in locating the nuclei, as well a separate contribution from variations in the location of the nuclei relative to our plane of focus. Another source of variation is imperfect synchronization of the embryos; during the course of nuclear cycle 14 the Bcd concentration in nuclei is dropping systematically at a rate of \( \sim 2\%/\min \) as a result of nuclear swelling (Gregor et al 2006), so if we compare embryos that differ by just a few minutes in the time since initiation of this cycle we make a significant contribution to the apparent variability of the concentration. There also is a significant variation of Bcd concentration along the dorsal-ventral axis (as can be seen in Figure 3, for example), and so variations in the orientation of the embryo will lead to apparent variability of the Bcd concentration measured in nuclei at the rim of the mid-sagittal plane. The conservative conclusion is that nuclear Bcd concentration profiles are at least as reproducible as our measurements, which are in the range of \( 10 - 20\% \). In Fig 5C we correct for those sources of measurement error that we have been able to quantify, and we find that the resulting reproducibility translates into specifying position with a reliability \( \sim 1 - 2\% \) of the embryo length.

It is interesting to note that the absolute variability in the concentration profile is largest near the anterior pole. Although some of this effect could result from variations in the imaging geometry near the embryo poles, it also could arise if the degree of localization of the Bcd mRNA varies in addition to variations in the total mRNA copy number (see Methods, where we explore this idea in a simple model). Functionally, this means that measurements of Bcd alone cannot provide reproducible positional information to cells within the anterior 10 – 20\% of the embryo. Consistent with this view, patterning genes with boundaries in the anterior head region depend substantially on the torso terminal system and do not appear to be as dependent on Bcd (Furriols & Casanova 2003).

Quantifying reproducibility via antibody staining

Previous work, which quantified the Bcd profiles using fluorescent antibody staining (Houchmandzadeh et al 2002), concluded that these profiles are quite variable from embryo to embryo, in contrast to our results in Figure 5. We argue here that the discrepancy arises because of the specific normalization procedure adopted in the earlier work, and that with a different approach to the data analysis the two experiments (along with a new set of data on immunostained embryos) are completely
As discussed above, the fluorescence intensity at each point in an immunofluorescence image is related to the concentration through $I(x) = A_n c(x) + B_n$, where $A_n$ and $B_n$ are scale factors and backgrounds that are different in each embryo. There is no independent measurement of these parameters, and so we have to chose them in such as way as to make meaningful comparisons across images of different embryos. Houchmandzadeh et al (2002) set these parameters for each embryo so that the mean concentration of the 20 points with highest staining intensity would be equal to one, and similarly the mean concentration of the 20 points with lowest staining intensity would be equal to zero. This is equivalent to the hypothesis that the peak concentration of Bcd is perfectly reproducible from embryo to embryo.

If we suspect that profiles in fact are reproducible, we can assign to each embryo the values of $A_n$ and $B_n$ which result in each profile being as similar as possible to the mean. We will measure similarity by the mean square deviation between profiles, and so we want to minimize

$$
\chi^2 = \sum_{n=1}^{N} \int dx \left| I_n(x) - [A_n \bar{c}(x) + B_n] \right|^2 ,
$$

where $\bar{c}(x)$ is the average concentration profile. The choice of scaling and background for each embryo defines an estimate of the concentration profile $c_n(x) = [I_n(x) - B_n]/A_n$, and then $\bar{c}(x)$ is the mean of these profiles across all the $N$ embryos in our ensemble. Reanalyzing the data of Houchmandzadeh et al (2002) in this way produces Bcd profiles that are substantially more reproducible (Figure 6B vs 6A), down to the $\sim 10\%$ level found in the live imaging experiments (Figure 6C).

It should be emphasized that the difference between Figures 6A and B is not just a mathematical issue. In one case (A) we interpret the data assuming that the peak concentration is fixed, and this ‘anchoring’ of the peak drives us to the conclusion that the overall profile is quite variable, especially near the mid-point of the embryo. In the other case (B) we allow that the peak concentration can fluctuate, or more precisely that there is nothing special about the peak relative to any other point along the profile; this allows us to find an interpretation of the data in which the overall profile is more reproducible.

We have collected a new set of data from 47 embryos which were fixed during early cycle 14 and stained for both Bcd and Hb; processing a large group of embryos at the same time and imaging them side-by-side, we minimized spurious sources of variability. We confirm the 10% reproducibility of the Bcd profiles, and find that $\sigma(x)$ is even slightly smaller than in the earlier data, consistent with the live imaging results. Using the same methods to analyze the Hb profiles, we find that the reproducibility $\sigma(x)$ inferred from Bcd and Hb are almost identical in the mid-embryo region, as shown by the red and cyan curves in Figure 6D. We conclude that, properly analyzed, the measurements of Bcd in fixed and stained embryos give results consistent with imaging of Bcd-GFP in live embryos. Further, the reproducibility of the Hb profiles is explained by the reproducibility of the Bcd input profile, at least across the range of conditions considered here.

**DISCUSSION**

The development of multicellular organisms such as *Drosophila* is both precise and reproducible. Understanding the origin of precise and reproducible behavior, in development and in other biological processes, is fundamentally a quantitative question. We can distinguish two broad classes of ideas (Schrödinger 1944). In one view, each of the individual steps involved in the process is noisy and variable, and this biological variability is suppressed only through averaging over many elements or through some collective property of the whole network of elements. In the other view, each step in the
process has been tuned to enhance its reliability, perhaps down to some fundamental physical limits. These very different views lead to different questions and to entirely different languages for discussing the results of experiments.

Our goal in this paper has been to locate the initial stages of Drosophila development on the continuum between the ‘precisionist’ view and the ‘noisy input, robust output’ view. To this end we have measured the absolute concentration of Bcd proteins, and used these measurements to estimate the physical limits to precision that arise from random arrival of these molecules at their targets. We then measured the input/output relation between Bcd and Hb, and found that Hb expression provides a readout of the Bcd concentration with better than 10% accuracy, very close to the physical limit. The mean input/output relation is reproducible from embryo to embryo, and direct measurements of the Bcd concentration profiles demonstrates that these too are reproducible from embryo to embryo at the ∼10% level. Thus, the primary morphogen gradient is established with high precision, and it is transduced with high precision.

Several points which arise along the path to our main results are worth noting. First, our analysis of the Bcd/Hb input/output relations is similar in spirit to measurements of noise in gene expression that have been done in unicellular organisms (Elowitz et al 2002; Raser & O’Shea 2004; Rosenfeld et al 2005). The morphogen gradients in early embryos provide a naturally occurring range of transcription factor concentrations to which cells respond, and the embryo itself provides an experimental “chamber” in which many factors that would be considered extrinsic to the regulatory process in unicellular organisms are controlled. Perhaps surprisingly, coupling classical antibody staining methods with quantitative image analysis makes it possible for us to make progress on the characterization of noise in the potentially more complex metazoan context. This approach should be more widely applicable.

A second point concerns the analysis of immunofluorescence data. Although there are many reasons why antibody staining might not provide a quantitative indicator of protein concentration, our results [see also Gregor et al (2006)] indicate that the fluorescence intensity from fixed and stained embryos indeed is proportional to the concentration, and that with proper normalization such experiments can reveal precision at the 10% level or better. Different approaches to the analysis of these data can lead to very different conclusions, however, and it is important to link the assumptions of such analyses to their corresponding hypotheses about the underlying mechanisms. Thus, although one might have expected the peaks of morphogen profiles to be highly reproducible, in fact these peaks vary in absolute amplitude; in terms of fractional accuracy, the peak concentration is not privileged relative to other features of the profile. As discussed in the Methods, this could result from variability in the degree of localization of the maternal mRNA.

A third point concerns the matching of the different measures of precision and reproducibility. We have seen that, near its point of half-maximal activation, the expression level of hb provides a readout of Bcd concentration with better than 10% accuracy. At the same time, the reproducibility of the Bcd profile from embryo to embryo, and from one cycle of nuclear division to the next within one embryo (Gregor et al 2006), is also at the ∼10% level. Importantly, these different measures of precision and reproducibility must be determined by very different mechanisms. Our results suggest that these mechanisms are matched so that no single step dominates the overall noise level. For the readout, there is a clear physical limit which may set the scale for all steps. This limiting noise level is sufficient to provide reliable discrimination between neighboring nuclei, in effect providing sufficient positional information for the system to specify each “pixel” of the final pattern.

A fourth point concerns the problem of scaling. Previous work has shown that the Bcd profile scales to compensate for the large changes in embryo length across related species of flies (Gregor et al 2005), but evidence for scaling across individuals within a species has been elusive, perhaps because the relevant differences are small. We find that the Bcd profile is sufficiently reproducible that it can specify position along the anterior-posterior axis within 1 − 2% when we express position in units relative to the length of the embryo (Figures 5C & 6D). But embryos, for example in our ensemble of 15 that provide the data for Figure 5, have a standard deviation of lengths 6rms/L = 4.1%. Even if the Bcd profile were perfectly reproducible as concentration vs position in microns, this would mean that knowledge of relative position would be uncertain by 4%, more than what we see. This suggests that the Bcd profile exhibits some degree of scaling to compensate for length differences. New experiments will be required to test this more directly.

A fifth point concerns the possibility of spatial averaging in order to increase the readout precision to the observed levels. Our results suggest that if nearby nuclei communicate through a diffusible “messenger,” so that the Hb concentration in one nucleus reflects an average over Bcd concentrations in the neighborhood that can be spanned by the messenger, then we can reconcile the observed precision with the theoretically derived physical limit. In the simplest model, the messenger could be Hb itself, since in the blastoderm stages the protein is free to diffuse between nuclei and hence the Hb protein concentration in one nucleus could reflect the Bcd-dependent mRNA translation levels of many neighboring nuclei. This model predicts that precision will depend on the local density of nuclei, and hence will be degraded in earlier nuclear cycles unless there are compensating changes in integration time. Such averaging mechanisms might be expected to smooth the spatial patterns of gene expression, which seems opposite to the goal of morpho-
genesis; the fact that Hb can activate its own expression (Margolis et al 1995) may provide a compensating sharpening of the output profile. There is a theoretically interesting tradeoff between suppressing noise and blurring of the pattern, with self-activation shifting the balance.

At a conceptual level our results on Drosophila development have much in common with a stream of results on the precision of signaling and processing in other biological systems. There is a direct analogy between the approach to the physical limits in the Bcd/Hb readout and the ability of the visual system to count single photons (Rieke and Baylor 1998; Bialek 2002). In both cases the reliability of the whole process is such that the randomness of individual molecular events dominates the reliability of the macroscopic output. An even better analogy may be to bacterial chemotaxis, where the response to small concentration gradients is so sensitive that it approaches the limits set by random arrival of molecules at the cell’s surface receptors (Berg & Purcell 1977). There are several examples in which the reliability of neural processing reaches such limits (de Vries 1957, Barlow 1981, Bialek 1987), and it is attractive to think that developmental decision making operates with a comparable degree of reliability. More concretely, the approach to physical limits has led to successful predictions regarding the mechanisms of neural signal processing, and parallel considerations should generate interesting predictions regarding the dynamics of signal processing in morphogenetic systems. In this direction, we need to understand how signals can be integrated for the required time without adding significant amounts of additional noise, and we need independent experiments to test for the spatial averaging which we have proposed is essential in reconciling the observed precision with the physical limits.

The reproducibility of absolute Bcd concentration profiles from embryo to embryo literally means that the number of copies of the protein is reproducible at the ∼10% level. As noted above, this fits with the fact that Hb output varies significantly in response to 10% changes in the Bcd input. Understanding how the embryo achieves reproducibility in Bcd copy number is a significant challenge. Again there is a conceptual connection to ideas in neuroscience. Models for the electrical dynamics of neurons—generalizations of the classical Hodgkin-Huxley model (1952)—predict that even the qualitative behavior of the system depends sensitively on the number of copies of the different ion channel proteins, suggesting that there must be mechanisms which regulate these copy numbers, or at least their ratios (LeMason et al 1993; Abbott & LeMason 1993; Goldman et al 2001); such mechanisms have since been discovered experimentally (Turrigiano et al 1994). The analogous mechanisms which might insure the reproducibility of Bcd copy number remain to be explored.

Finally, we note that the precision and reproducibility which we have observed in the embryo is disturbingly close to the resolution afforded by our measuring instruments.

METHODS

Bcd-GFP imaging in live embryos

Bcd-GFP lines are from Gregor et al (2006). Live imaging was performed in a custom built two-photon microscope (Denk et al 1990) similar in design to that of Svoboda et al (1997). Microscope control routines (Pologruto et al 2003) and all our image analysis routines were implemented in Matlab software (MATLAB, MathWorks, Natick, MA). Images were taken with a Zeiss 25x (NA 0.8) oil/water-immersion objective and an excitation wavelength of 900 − 920 nm. Average laser power at the specimen was 15 − 35 mW. For each embryo, three high-resolution images (512 × 512 nm pixels, with 16 bits and at 6.4 µs per pixel) were taken along the anterior-posterior axis (focussed at the mid-sagittal plane) at magnified zoom and were subsequently stitched together in software; each image is an average of 6 sequentially acquired frames (Figures 2 and 5). With these settings, the linear pixel dimension corresponds to 0.44 ± 0.01 µm. At Bcd-GFP concentrations of ∼ 60 nM and a raw intensity value of ∼ 400, we counted 36 ± 5 photons/pixel in a single image.

Calibrating absolute concentrations

GFP variant S65T, a gift of HS Rye (Princeton), was over-produced in Escherichia coli (BL21) from a trc promoter and purified essentially as described by Rye et al (1997). Absolute protein concentration was determined spectrophotometrically. The S65T variant of GFP has optical absorption properties nearly identical to the eGFP variant used to generate transgenic Bcd-GFP fly (Patterson et al 1999; Gregor et al 2006). Living Drosophila embryos expressing Bcd-GFP were immersed in 7.15 ± 0.05 pH Schneider’s insect culture medium containing 36 nM GFP. Embryos were imaged 15 min after entry into mitosis 13, focusing at the mid-sagittal plane. Nuclear Bcd-GFP fluorescence intensities were extracted along the edge of the embryo as described below (see next section). At each nuclear location a reference GFP intensity was measured at the corresponding mirror image of the respective nucleus in the external immersion solution, using the vitelline membrane as a mirror.

Identification of nuclei in live images

For each embryo, nuclear centers were hand selected and the average nuclear fluorescence intensity was computed over a circular window of fixed size (50 pixels). Embryos imaged at the mid-sagittal plane contained on average 70 nuclei along each edge. In our high-resolution images nuclei have, on average, a diameter of 150 pixels. Towards the posterior end, where nuclei merge into the background intensity, virtual nuclei were selected by keeping the same approximate periodicity of the anterior end.
Which diffusion constant?

In the Berg-Purcell analysis and its generalization, the concentration \( c \) in Figure 3 refers to molecules that are free in solution and hence can diffuse up to the receptor site. In practice, when we measure concentrations using fluorescence, as in Figure 2, we are sensitive to a total concentration that includes both free molecules and those bound to other sites. Similarly, the diffusion constant \( D \) refers to the diffusion of free molecules, but when we measure a diffusion constant using optical methods we see an effective diffusion constant \( D_{\text{eff}} \) that includes the effects of binding and unbinding to less mobile sites. What we would really like to know, then, are \( c_{\text{free}} \) and \( D_{\text{free}} \), but what we measure is \( c_{\text{total}} \) and \( D_{\text{eff}} \). To understand the connections among these quantities, consider a model in which Bcd molecules are free in solution at concentration \( c_{\text{free}}(\vec{x},t) \) and diffuse with diffusion constant \( D_{\text{free}} \). Let there be binding sites for Bcd that are fixed (non-diffusing) at density \( \rho \); and let the second order binding rate be \( k_+ \) and the unbinding rate be \( k_- \). Then

\[
\frac{\partial c_{\text{free}}(\vec{x},t)}{\partial t} = D_{\text{free}} \nabla^2 c_{\text{free}}(\vec{x},t) - \rho \frac{\partial f(\vec{x},t)}{\partial t} \quad (15)
\]

\[
\frac{\partial f(\vec{x},t)}{\partial t} = k_+ c_{\text{free}}(\vec{x},t)[1-f(\vec{x},t)] - k_- f(\vec{x},t),
\]

where \( f(\vec{x},t) \) is the fraction of binding sites occupied by Bcd at position \( \vec{x} \) and time \( t \); the classical discussion of this problem is for calcium diffusion in neurons (Hodgkin & Keynes 1957). In steady state, the total concentration of Bcd molecule, bound and free, is given by

\[
c_{\text{total}} = c_{\text{free}} + \rho c_{\text{free}}/K, \quad (17)
\]

where the dissociation constant \( K = k_-/k_+ \). If we consider a perturbation around the steady state, \( c \to c + a(t) \sin(\vec{k} \cdot \vec{x}) \), where the wavelength of the perturbation \( \lambda = 2\pi/|\vec{k}| \), then for long wavelengths it can be shown from Equations (15) and (16) that the amplitude of the concentration profile decays as \( a(t) \propto \exp(-t/\tau) \), where \( \tau = 1/(D_{\text{eff}}|\vec{k}|^2) \). This dependence on wavelength is the same as for ordinary diffusion, so the binding sites change the effective diffusion constant to

\[
D_{\text{eff}} = \frac{D_{\text{free}}}{1 + \rho K/(c_{\text{free}} + K)^2}. \quad (18)
\]

In the limit that the binding sites are weak, \( c_{\text{free}} \ll K \), we have \( c_{\text{total}} = c_{\text{free}}(1 + \rho/K) \) and \( D_{\text{eff}} = D_{\text{free}}/(1 + \rho/K) \), so that \( c_{\text{total}} D_{\text{eff}} = c_{\text{free}} D_{\text{free}} \). Thus we can compute the Berg-Purcell limit [Equation 3], which depends only on the product Dc, from measured quantities alone, without corrections.

Antibody staining and confocal microscopy

All embryos were collected at 25°C, heat fixed, and subsequently labeled with fluorescent probes. We used rat anti-Bcd and rabbit anti-Hb antibodies (Reinitz at al 1998), gifts of J Reinitz (Stony Brook). Secondary antibodies were conjugated with Alexa-488, Alexa-546 and Toto3 (Molecular Probes), respectively. Embryos were mounted in AquaPolymount (Polysciences, Inc.). High-resolution digital images (1024 × 1024, 12 bits per pixel) of fixed eggs were obtained on a Zeiss LSM 510 confocal microscope with a Zeiss 20x (NA 0.45) A-plan objective. Embryos were placed under a cover slip and the image focal plane of the flattened embryo was chosen at top surface for nuclear staining intensities (Figures 3 and 4) and at the mid-sagittal plane for protein profile extraction (Figure 6D). All embryos were prepared, and images were taken, under the same conditions: (i) all embryos were heat fixed, (ii) embryos were stained and washed together in the same tube, and (iii) all images were taken with the same microscope settings in a single acquisition cycle.

Automatic identification of nuclei in fixed embryos

Images of DNA stainings (Toto3, Molecular Probes, Eugene, OR) were used to automatically identify nuclei. To pre-process the images, we examine each pixel \( x \) in the context of its \( 11 \times 11 \) pixel neighborhood. Let the mean intensity in this neighborhood be \( I(x) \) and the variance be \( \sigma^2(x) \); we then transform the intensity \( I(x) \) into a normalized form \( \psi(x) = |I(x) - \bar{I}(x)|/\sigma(x) \). These normalized images are smoothed with a Gaussian filter (standard deviation 2 pixels) and thresholded, with the threshold chosen by eye to optimize the capture of the nuclei and minimize spurious detection. Locations of nuclei were assigned as the center of mass in the connected regions above threshold. For each embryo a region of interest was hand selected to avoid misidentification due to geometric distortion at the embryo edge, yielding an average of 1300–1500 nuclei per embryo. Residual misidentification was at the 1% level.

Analysis of input/output relations

The raw data from images such as Figure 3 consist of pairs \( \{I_{\text{Bcd}}(n; k), I_{\text{Hb}}(n; k)\} \), where \( I_{\text{Bcd}} \) and \( I_{\text{Hb}} \) refer to anti-Bcd and anti-Hb fluorescence intensities, respectively; \( n \) labels the nuclei in a single embryo, and \( k \) labels the embryo. We expect that \( I_{\text{Bcd}}(n; k) = A_{\text{Bcd}}(k)c_{\text{Bcd}}(n) + B_{\text{Bcd}}(k) \), and similarly for the Hb data, where \( A_{\text{Hb}}(k) \) and \( B_{\text{Hb}}(k) \) are the scaling and background that relate Bcd concentration to fluorescence intensity in the \( k \)th embryo. Note that if we analyze input/output relations in single embryos, the choice of scale factors \( A_{\text{Bcd}} \) and \( A_{\text{Hb}} \) is entirely a matter of convention. To ask if all of the input/output relations can be placed consistently on the same axes, however, we need to choose these scale factors more carefully.

Initial guesses for \( A \) and \( B \) are made by assuming that the smallest concentration we measure is zero and that the mean concentration of each species is equal to one in all embryos. If we know the values of these parameters, we can turn all of the intensities into concentrations, and we merge all of these data into pairs \( \{c_{\text{Bcd}}(n), c_{\text{Hb}}(n)\} \), where the index \( n \) runs over all the nuclei in all the embryos. We characterize this merged data set by computing the mutual information \( I(c_{\text{Bcd}}; c_{\text{Hb}}) \) between \( c_{\text{Bcd}} \) and \( c_{\text{Hb}} \), being careful to correct for errors due to the finite size of the data set; see, for example, Slonim et al (2005). If the shapes of the input/output relations are very different in different embryos, or if we choose incorrect values for the parameters \( A \) and \( B \), then \( I(c_{\text{Bcd}}; c_{\text{Hb}}) \) will be reduced. We use an iterative algorithm to adjust all four parameters for each embryo until we maximize the mutual information. Once this process has converged we can use the
merged data to compute the mean input/output relation by quantizing the $c_{Bcd}$ axis and estimating the mean value of $c_{Hb}$ associated with each bin along this axis; we then normalize $c_{Hb}$ so that the minimum (maximum) of this mean output is equal to 0 (1), and we normalize $c_{Bcd}$ relative to the value which produces half-maximal mean output. Computing the standard deviation of $c_{Hb}$ values in each bin gives the output $Hb$ noise. We can then compute input/output relations for the individual embryos and verify that they are the same within the error bars defined by the output noise (Figure 4).

### Measurement noise in the input/output relations

Four fixed and antibody-stained embryos were imaged 5 times in sequence using confocal microscopy as above. For each identified nucleus the mean and standard deviation across the 5 images was computed. All embryos were normalized as above and their data sets merged to generate a quantized $c_{Bcd}$ axis. For each bin along this axis we computed average $c_{Bcd}$ measurement standard deviations by averaging the measurement variances of all the nuclei in the given bin. The same procedure was used to estimate measurement noise in the $Hb$ images, although this was found to be much less significant.

### Correlation function of $Hb$ noise

Let $c_{Hb}^n$ be the observed concentration of $Hb$ in nucleus $n$, and similarly for the $Bcd$ concentration $c_{Bcd}^n$; these are the coordinates for each point in the scatter plot of Figure 3C. For individual embryos the contribution to the correlation coefficient from two nuclei $i$ and $j$ was computed as

$$C_{ij} = \frac{c_{Hb}^i - \bar{c}_{Hb}(c_{Bcd})}{\sigma_{Hb}(c_{Bcd})} \cdot \frac{c_{Hb}^j - \bar{c}_{Hb}(c_{Bcd})}{\sigma_{Hb}(c_{Bcd})},$$

where $\bar{c}_{Hb}(c_{Bcd})$ is the mean input/output relation and $\sigma_{Hb}(c_{Bcd})$ is the standard deviation of the output, as in Figures 4A and 4B, respectively; we use the same binning of the $Bcd$ concentration as in Figure 4 to approximate these functions. The correlation function is the ensemble average over these coefficients, $C(r) = \langle C_{ij} \rangle$, where $\langle \cdot \cdot \rangle$ averages over all nuclei that are distance $r$ apart, with $r$ quantized into bins of size equal to the spacing between neighboring nuclei.

### Measurement noise in live images

We identified 4 different sources of measurement noise: 1. Imaging noise. Small regions of individual embryos were imaged 5 times in sequence, 3s per image, with the same pixel acquisition time as in actual data. The variances across those 5 images for identified nuclei constitute the instrumental or imaging noise. 2. Nuclear identification noise. To estimate the error due to mis-centering of the averaging region over the individual nuclei, we computed the variances across 9 averaging regions centered in a 3 x 3 pixel matrix around the originally chosen center. Gray line in Figure 5B stems from the sum of Imaging noise and Nuclear identification noise, which are uncorrelated. 3. Focal plane adjustment noise. For each individual embryo the focal plane has to be hand adjusted before image acquisition. We adjusted the focal plane to be at the mid-sagittal plane of the embryo but estimate our accuracy to be 6 $\mu$m, or one nuclear diameter. Due to power attenuation over that distance (~10% on average) we estimate our error by computing the variances of nuclear intensities across 7 images taken at consecutive heights spaced by 1 $\mu$m in a single embryo (black line in Figure 5B). 4. Rotational asymmetry around the anterior-posterior axis. Embryos are not rotationally symmetric around the anterior-posterior axis, and $Bcd$ profiles are significantly different along the dorsal vs. the ventral side of a laterally oriented embryo. An obvious error source arises from our inability to mount all embryos at the same azimuthal angle. We are unable to measure this potentially large error source accurately, but we estimate an upper bound by the difference of dorsal vs. ventral gradients. Between 10–50% egg length the upper bound for this contribution of the fractional error is $\sim 13.5\%$.

### Sources of variability

Consider the simplest model for the $Bcd$ profile in which $Bcd$ molecules diffuse with diffusion constant $D$ and are degraded through a first order reaction with lifetime $\tau$. Restricting our attention to the one dimension along the anterior-posterior axis, the equation for the concentration profile is

$$\frac{\partial c(x,t)}{\partial t} = D \frac{\partial^2 c(x,t)}{\partial x^2} - \frac{1}{\tau} c(x,t) + S(x),$$

where $S(x)$ describes the source of protein which results from translation of the maternal mRNA. The usual assumption is that this source is confined to the anterior pole, $x = 0$. A slightly more realistic assumption is that the source is localized within some distance $\Delta$, perhaps as $S(x) = (R/\Delta \exp(-x/\Delta))$, where $R$ is the total rate at which $Bcd$ molecules are synthesized. Then the steady state profile is

$$c(x) = \frac{R \lambda}{\lambda^2 - \Delta^2} \exp(-x/\lambda) - \frac{R \tau \Delta}{\lambda^2 - \Delta^2} \exp(-x/\Delta),$$

where $\lambda = \sqrt{D/\tau}$ and we assume that the embryo is long, $L \gg \lambda, \Delta$. We see that if the typical value of $\Delta$ is smaller than $\lambda$, then for $x \gg \Delta$ the dependence of the concentration on $\Delta$ is $\sim (\Delta/\lambda)^2$. On the other hand, near $x = 0$ there is a term $\sim \Delta/\lambda$. Thus the concentration near the anterior pole will be more sensitive to variations in $\Delta$ from embryo to embryo.

### Spatial profiles of Bcd and Hb in fixed and stained embryos

$Bcd$ and $Hb$ protein profiles were extracted from confocal images of stained embryos by using software routines that allowed a circular window of the size of a nucleus to be systematically moved along the outer edge of the embryo (Houchmandzadeh et al 2002). At each position, the average pixel intensity within the window was plotted versus the projection of the window center along the anterior-posterior axis of the embryo. Protein concentration measurements were made separately along the dorsal and ventral sides of the embryo; for consistency, we compared only dorsal profiles.
Minimizing $\chi^2$

As written, the minimization of $\chi^2$ in Equation (14) suggests an iterative process: compute the mean concentration profile, adjust the parameters $\{A_n, B_n\}$ so that the data from each embryo matches the mean as well as possible, recompute the mean, and iterate. In fact $\chi^2$ is quadratic in both the parameters $\{A_n, B_n\}$ and in the mean profile $\bar{c}(x)$, so one can make analytic progress. To begin, $\chi^2$ is minimized when each $B_n$ is chosen so that all of the profiles $c_n(x)$ have the same mean value when averaged over $x$; a convenient first step is to choose the $B_n$ so that this mean is zero. The hypothesis that most of the remaining variance can be eliminated by proper choice of the $A_n$ is equivalent to the statement that the singular value decomposition of the unnormalized, zero mean profiles is dominated by a single mode, and this mode will be the mean profile $\bar{c}(x)$. Thus we perform a singular value decomposition of the profiles and choose $A_n$ so that the projection of each profile onto the dominant mode is normalized to unity, and this provides the minimum $\chi^2$. Once the parameters have been set in this way we still have the freedom to add a constant background (so that the concentration falls to zero on average at the posterior of the egg) and an overall scale (so that the mean concentration profile has a maximum value of one).

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