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Experimental and Modeling Approaches for Understanding the Effect of Gene Expression Noise in Biological Development

David M. Holloway [1,2]*

[1] Mathematics Department, British Columbia Institute of Technology, Burnaby, BC, Canada, [2] Biology Department, University of Victoria, Victoria, BC, Canada

Biological development involves numerous chemical and physical processes which must act in concert to reliably produce a cell, a tissue, or a body. To be successful, the developing organism must be robust to variability at many levels, such as the environment (e.g., temperature, moisture), upstream information (such as long-range positional information gradients), or intrinsic noise due to the stochastic nature of low concentration chemical kinetics. The latter is especially relevant to the regulation of gene expression in cell differentiation. The temporal stochasticity of gene expression has been studied in single celled organisms for nearly two decades, but only recently have techniques become available to gather temporally-resolved data across spatially-distributed gene expression patterns in developing multicellular organisms. These demonstrate temporal noisy "bursting" in the number of gene transcripts per cell, raising the question of how the transcript number defining a particular cell type is produced, such that one cell type can reliably be distinguished from a neighboring cell of different type along a tissue boundary. Stochastic spatio-temporal modeling of tissue-wide expression patterns can identify signatures for specific types of gene regulation, which can be used to extract regulatory mechanism information from experimental time series. This Perspective focuses on using this type of approach to study gene expression noise during the anterior-posterior segmentation of the fruit fly embryo. Advances in experimental and theoretical techniques will lead to an increasing quantification of expression noise that can be used to understand how regulatory mechanisms contribute to embryonic robustness across a range of developmental processes.

Keywords: transcription, *Drosophila*, mRNA, stochastic model, embryo development, gene regulation, spatial pattern formation, tissue differentiation

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***Correspondence:**
David M. Holloway
david_holloway@bcit.ca

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THE CHALLENGE OF DEVELOPMENT: COORDINATED TISSUE DIFFERENTIATION FROM CELL-SPECIFIC GENE EXPRESSION

Development of a multicellular organism's body depends on the reliable differentiation of cells into tissues. Differentiation must be coordinated in space, defining the extent of the tissue, and in time, for cells to acquire the proper identity at the correct time. Genetically, cell type is defined by expression of a unique subset of the genome. Gene expression has multiple levels, including the

binding of DNA by regulatory transcription factors, transcription from DNA to mRNA, splicing variation, and translation of mRNA to protein. Protein transport, degradation and interactions with other proteins and cellular components then form the molecular biology, structure and functionality of a specific cell type.

Variability exists in all of these processes. Extrinsic variability can be in temperature or moisture; the timing of a hormone signal; or the spatial distribution of a transcription factor, for example the anterior-posterior Bicoid (Bcd) gradient in the fruit fly (*Drosophila*) which activates different tissues depending on its concentration (**Figure 1A**, purple). Gene expression also has intrinsic variability (noise), since it occurs at low enough concentrations to exhibit stochastic kinetics (e.g., [1]).

In single cells, intrinsic variability can contribute to population heterogeneity that may be exploited for evolutionary adaptivity to environmental changes (e.g., [2–5]). In the development of multicellular metazoans, however, such heterogeneity must generally be much lower to produce distinct tissue types with clear boundaries. Anterior-posterior segmentation of the insect body must achieve 1–2 cell positional accuracy to avoid developmental errors. This implies variability-limiting mechanisms at most, if not all, steps of cellular differentiation. This Perspective addresses how gene regulation can affect the variability due to intrinsic transcription noise, particularly in *Drosophila*.

REGULATORY MECHANISMS FOR ROBUST GENE EXPRESSION

Several dozen genes are involved in early *Drosophila* anterior-posterior segmentation (e.g., [6, 7]). Many of these code for transcription factors which regulate other segmentation genes, creating a highly interconnected gene regulatory network. Hierarchically, long-range maternal gradients (e.g., Bcd) activate broad gap-gene patterns (e.g., *hunchback*, *hb*; *Krüppel*, *Kr*; *Giant*, *Gt*; *knirps*, *kni*; **Figure 1A**), which then regulate the finer-scale pair-rule genes (e.g., *even-skipped*, *eve*). Stripes of pair-rule expression, each several cells wide, form in the long 14th interphase after fertilization (**Figure 1B**); these are the first manifestation of the fly's future body segments.

This coordinated spatially-patterned expression of genes has been extensively studied with mathematical models. For example, parameter searches with the gene interaction matrix approach of Mjolsness et al. and Reinitz and Sharp [8, 9] found classes of networks for wild-type [10] and mutant [11–13] gap-gene patterns. Such potential mechanisms can be further screened for robustness to extrinsic variability, such as to Bcd gradient variability [11, 12].

Such deterministic models are not appropriate, however, for the intrinsic noise generated during gene transcription and translation: they can guide the development of stochastic models, but only to the extent that terms can be converted to elementary probabilistic events. New types of data are also required for quantifying intrinsic noise and model validation.

PIONEERING WORK IN SINGLE CELLS; GENE EXPRESSION IS INTRINSICALLY NOISY

While the effects of noise in gene expression had been discussed in the 1990's (e.g., [14–19]), technological advances in the early 2000's allowed for the first experimental measurements of noise in living cells, in bacteria (e.g., [1, 20]) and in yeast (e.g., [21]). In *E. coli*, for instance, genes for cyan and yellow fluorescent proteins were incorporated into the genome: when a cell experienced fluctuations in extrinsic factors, the transgenes expressed together, producing an equally-blended color; deviations from this revealed the intrinsic noise at each gene [1]. Advances in this period include finding how noise scales [22], and that prokaryotic expression exhibits noisy bursts in translation while eukaryotic expression, with slower initiation kinetics, is dominated by transcriptional bursting (see review [23]; also [24] regarding transcription factor concentrations and burst dynamics).

TRANSCRIPTION NOISE IN MULTICELLULAR SPATIALLY-PATTERNED TISSUES

While fluorescent protein transgenes have been used extensively in metazoans, the long maturation times (∼30 min) of those developed for *Drosophila* segmentation genes (e.g., *bcd* [25] and *eve* [26]) tend to obscure the rapid (∼10 min) pattern changes during interphase 14. Gene expression noise studies in *Drosophila* have therefore focused on transcription, measuring RNA rather than protein.

Static Snapshots

High resolution FISH (fluorescent *in situ* hybridization) can image sub-nuclear RNA transcription centers (or "dots," corresponding to individual gene copies on the DNA) and even single RNA molecules (smFISH). Fixed embryos can be assigned within several-minute developmental stages. Stochastic transcriptional processes can be inferred from the distribution of RNA in these staged snapshots.

Data

Different FISH probes can visualize different stages of RNA production, from nascent (actively transcribing from the DNA) to nuclear (dots) to cytoplasmic. Intronic probes for nascent transcripts were used to demonstrate transcriptional bursting in segmentation genes [27]. Similar to a unicellular population, early Bcd-activated *hb* expression can be heterogeneous [28], but becomes more synchronized as spatial pattern matures [29]; this may be aided by a persistence of the transcriptional state through cell divisions [30]. Correlation between Bcd and *hb* intronic signal [31] was used to calculate the number of Bcd binding sites (BSs) in the *hb* cis-regulatory element (CRE; the DNA region to which transcription factors bind). *hb* Bcd-dependence decays quickly in early interphase 14 [32], after which gap-gap interactions become important. Noise

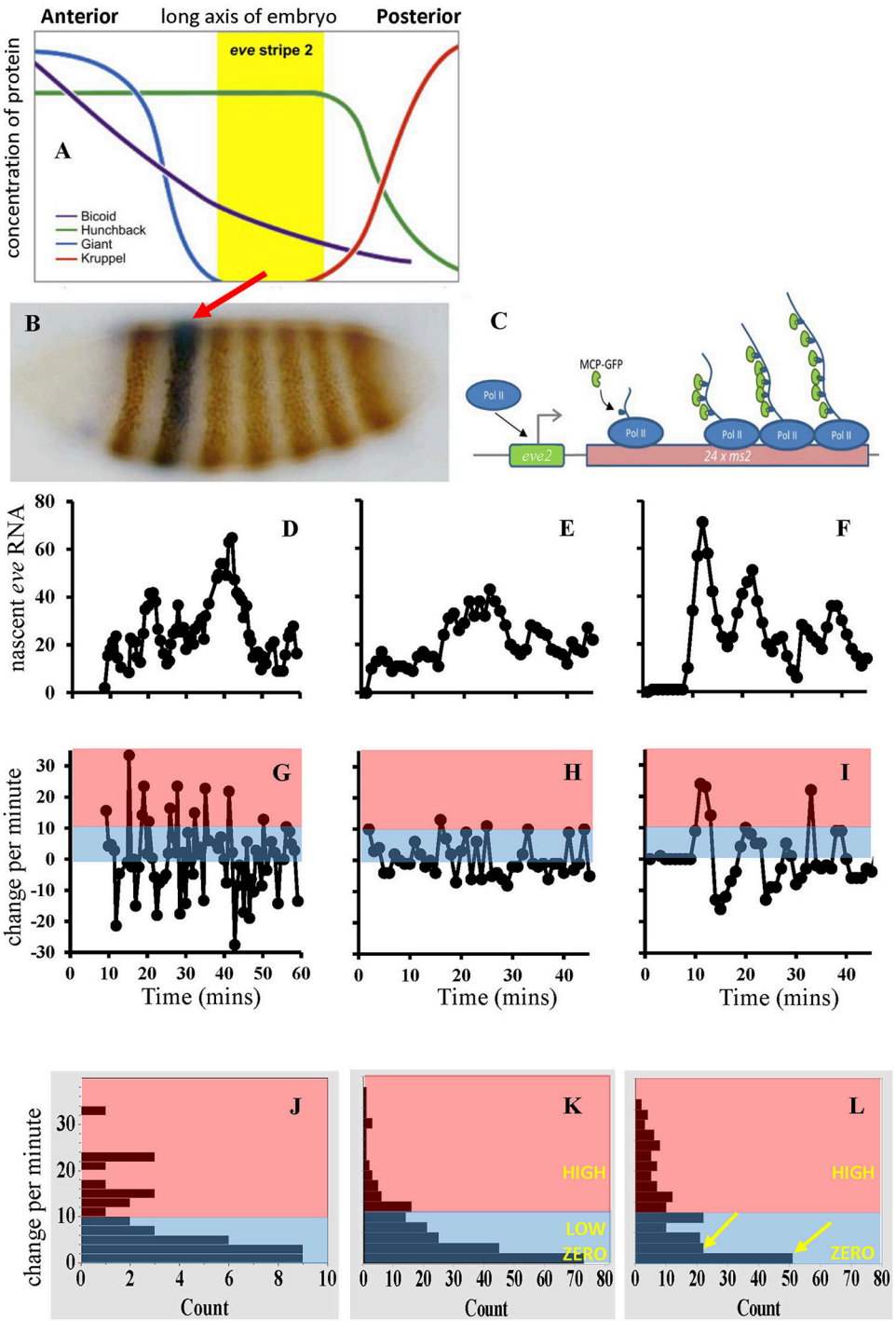
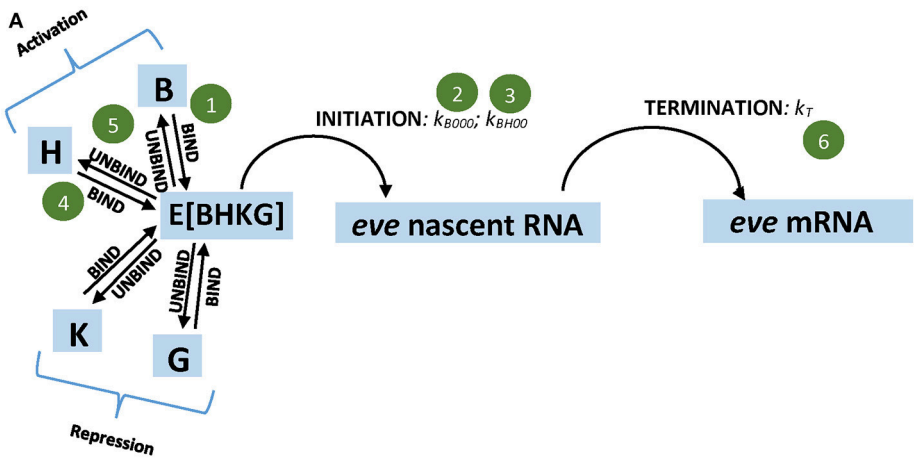


FIGURE 1 | (A) Spatial expression of several *Drosophila* segmentation genes. **(B)** *eve* gene expression stripes on an interphase 14 embryo. Red arrow, *eve* stripe 2. **(C)** Schematic of the MCP/MS2 system. "*eve2*" represents the CRE controlling transcriptional initiation (right-angle arrow); see **Figure 2A** for an expanded view and model of this *eve* MSE. PolII transcribes multiple MS2 loops in addition to the target gene. MS2 loops in the nascent RNA bind MCP tagged with green fluorescent protein (GFP). **(D)** Experimental time series from a nucleus in the center of *eve* stripe 2, from Bothma et al. [54]. **(E)** A stochastic simulation for OFF-LOW-HIGH *eve2* transcription (model in **Figures 2A,B**). **(F)** A simulation of simple OFF-ON transcription. Experimental time series exhibit a 10 min initial lag, simulation dynamics start at time 0; time axes same as **(G–I)**, vertical scale (number of transcript molecules) shared **(D–F)**. **(G–I)** Corresponding change per minute for the data **(G)**, and for the OFF-LOW-HIGH **(H)**, and OFF-ON **(I)** simulations. Pink, minutes with high initiation; blue, minutes with low to zero initiation. **(J–L)** Distributions of change-per-minute for data (**J**, from **G**), OFF-LOW-HIGH (**K**, 10 pooled simulations) and OFF-ON (**L**, also 10 simulations; yellow arrows indicate dichotomy in low to zero rates not seen in **J,K**). Vertical bin ranges identical for **(J–L)**. LOW corresponds to k_{B000} in **Figure 2**, HIGH to k_{BH00}. **(A,B)** adapted from Levine [79], **(C)** from Desponds et al. [53], **(D–L)** from Holloway and Spirov [66].



B

CRE binding	transcription
$E[0xxx] + B \underset{k_{UNBIND}=3.3e-2}{\overset{k_{BIND-B}=2.6e6}{\rightleftharpoons}} E[Bxxx]$ (1, 5)	$E[B000] \xrightarrow{k_{B000}=9e-2} E[B000] + eve_{nascRNA}$ (2)
$E[x0xx] + H \underset{k_{UNBIND}=3.3e-2}{\overset{k_{BIND-H}=1.8e5}{\rightleftharpoons}} E[xHxx]$ (4, 5)	$E[BH00] \xrightarrow{k_{BH00}=5.6e-1} E[BH00] + eve_{nascRNA}$ (3)
	$eve_{nascRNA} \xrightarrow{k_T=3.97e-3} eve_{mRNA}$ (6)

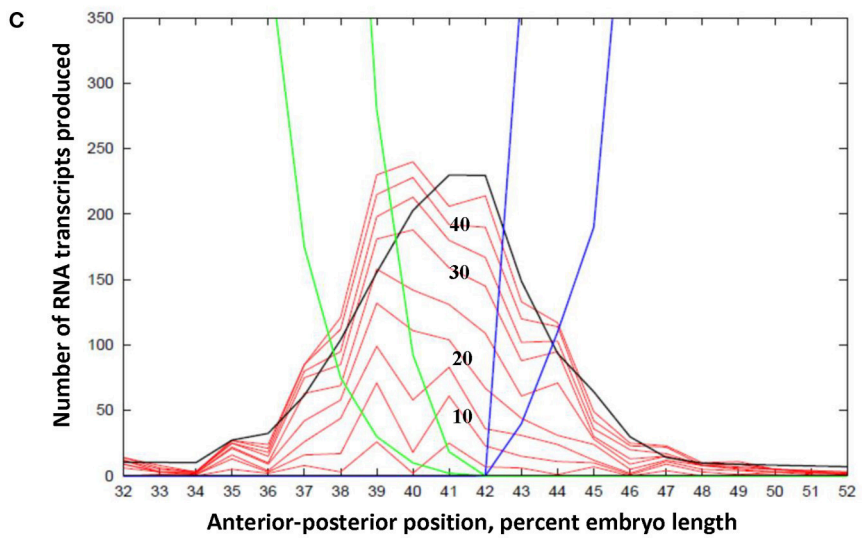


FIGURE 2 | (A) States (blue) and events (arrows) of the stochastic *eve2* transcription model [66]; shown as elementary reactions in **(B)**, solved as master equations [80, 81]. E, the *eve2* MSE, can bind four transcription factors (B, Bcd; H, Hb; K, Kr; G, Gt); transcriptional initiation occurs at a LOW (green circle 2) or HIGH (circle 3) rate. Experimental signal corresponds to *eve* nascent RNA. K, G repression not shown in **(B)**, see [66]. **(C)** Modeled spatial patterning of *eve2*: numbers of transcripts against position. Red, stochastic solutions at 5 min intervals (times numbered in black); black line, deterministic solution at 45 min; blue (Kr) and green (Gt), early (outer) and late (inner) repressor patterns. Adapted from Holloway and Spirov [66].

damping and synchronization can be aided by a "paused" state, in which the transcriptional machinery (RNA polymerase II complex, PolII) is assembled and ready, but not actively transcribing [33, 34]. Having multiple CREs for a gene may also reduce transcription noise [35, 36]. While *hb*, *gt*, *Kr*, *kni* are noisy during transcription, cytoplasmic mRNA levels

can be smoother, which may indicate spatiotemporal averaging [37].

Modeling

With FISH data, the substantial theory from unicellular noise research can start to be applied to multicellular gene expression. For instance, Boettiger et al. [38] used a Markov chain approach (see, for example, [39–41]) to compare initiation-regulated and elongation-regulated transcriptional dynamics and show how the latter could produce the more consistent patterns of paused genes observed in Boettiger and Levine [33]. Xu et al. [42] recently derived probability distributions for the number of nascent transcripts and corroborated these against smFISH signal for *hb* activated by high, medium and low levels of Bcd.

FISH also provides the resolution to check stochastic versions of long-range spatial patterning models. For instance, a stochastic model of Bcd and Hb (self) activation of the anterior *hb* expression domain [43], developed from an earlier deterministic version [44], predicted that reporter constructs with less than wild-type numbers of Bcd BSs should show increased variability of the mid-embryo boundary, and that loss of *hb* autoregulation should decrease correlation of the FISH signal between the two *hb* gene copies (dots). See also Sanchez et al. and Monteoliva et al. [40, 45] on the effect of the number of BSs on transcriptional noise. For later patterning, we modeled the gap-gap interactions producing the mid-embryo Hb concentration peak necessary for the future thorax [46]. Stochastic simulations indicated that *hb-Kr* interactions reduce expression noise and contribute to the reliability of mid-embryo development, predicting that FISH dot-dot correlation should decrease in Kr^- mutants.

Live Time Series

More recently, technology has been developed for the visualization of transcription in live embryos, using the MS2/MCP system originally developed in Bertrand et al. [47] (see also [48] for a recent application in yeast). A viral coat protein (MCP) and its corresponding RNA stem loop (MS2) is introduced into a host reporter gene. MCP, tagged with green fluorescent protein, binds an MS2 loop during transcription of the reporter, producing signal for the nascent transcript (**Figure 1C**). The first application in *Drosophila* was to visualize mature *nanos* RNA [49]. While the technique has inherent background issues compared to FISH (see extensive review in Ferraro et al. [50]), it is increasingly being used to visualize live transcription in embryos at timescales on the order of 1 min. This resolution allows for the corroboration of stochastic models and data along time series, as well as spatially. The time dimension provides new constraints for screening potential regulatory mechanisms.

Data

Time series were first measured in *Drosophila* for *hb* [51, 52]. The MCP signal was sampled every 30–60 s and calibrated to the number of active PolII molecules. Peak transcription (up to 100 transcripts being made) can occur within 1–2 min after nuclear division [52]. *hb* output corresponds well to transcriptional initiation rates up through interphase 13, but the large increase in output entering interphase 14 indicates an additional contribution from whether a nucleus is active or not [51]. Though fluctuations are observed, particularly in longer traces (e.g., Figure 1D of Garcia et al. [51]), the short cell cycles prior to interphase 14 make quantification challenging. A new autocorrelation technique for short sampling periods has been used to detect *hb* transcriptional bursts in interphase 13 [53].

Live pair-rule gene transcription has been measured [54] with MS2 driven by a 1.7 kb CRE of *eve* [55, 56] which expresses in stripes 2 and 7 (**Figure 1B**). A 480 bp minimal stripe element (MSE) within this sequence controls expression at *eve* stripe 2 (*eve2*). The MSE has BSs for Bcd, Hb, Gt and Kr [57–59]. The activators, Bcd and Hb, are high throughout the anterior of the embryo; *eve2* forms in a trough between the repressor patterns, with Gt to the anterior and Kr to the posterior (**Figure 1A**). Bothma et al. [54] sampled individual nuclei in stripe 2 over nearly 60 min time series at ∼1 min resolution (**Figure 1D**). These show bursts in *eve2* transcription, with "peaks" of some 50–60 nascent transcripts interspersed with "troughs" of about 10–20 nascent transcripts. The authors suggested this indicated two distinct ON rates (i.e., transcriptional initiation could be OFF, LOW, or HIGH). Lower expression at stripe-edge nuclei indicated repression from Gt and Kr.

Using Spatio-Temporal Stochastic Modeling to Find Gene Regulatory Mechanisms From Noisy Time Series

Expression noise has primarily been modeled with simple OFF-ON mechanisms, with transcriptional initiation ON at random intervals, at a characteristic mean rate, and OFF otherwise. The ON intervals can include many initiation events, producing bursts of transcripts (see review in Munsky et al. [60]). An OFF-ON model was recently used to study the effects of transcription noise on *eve2* stripe border variability [61]. A number of systems have now been characterized, however, which display multiple distinct ON rates [62–64]. The proposal in Bothma et al. [54] that *eve2* has multiple ON rates could have a mechanistic basis in the dual activation of the MSE by Bcd and Hb: removal of the Hb BS leaves reduced Bcd-only activated expression of *eve2* [57, 65]. However, to determine whether this regulatory feature can be extracted from time series, it must first be determined whether output from simple OFF-ON and multiple-ON mechanisms can be distinguished.

We, Holloway and Spirov [66], developed a stochastic model of the *eve2* MSE, with BSs for Bcd, Hb, Gt, and Kr (**Figures 2A,B**), and parameters calibrated to experimental data (see also [67, 68] for deterministic MSE models). Sets of time series were generated for a multiple-rate OFF-LOW-HIGH mechanism (**Figure 1E**, at stripe center, where repression is minimal) and for a simple OFF-ON mechanism (**Figure 1F**, also stripe center), both producing the observed total number of transcripts in interphase 14. Both mechanisms produced "bursty" peaks in number of transcripts qualitatively like the data (**Figure 1D**). A more direct measure of initiation rates, however, is the minute-to-minute change in number of transcripts (**Figures 1G–I**): any increase in signal over the previous minute indicates at least that number of transcripts initiated. The distribution of these minute-to-minute changes is

closer between the data (**Figure 1J**) and the OFF-LOW-HIGH mechanism (**Figure 1K**) than between the data and the OFF-ON mechanism (**Figure 1L**), particularly in the low addition range (2-3 initiations per minute). The two models produce distinct distributions (χ^2, $p < 0.05$ [66]). Furthermore, OFF-ON simulations show significant autocorrelation in the minute-to-minute changes (particular initiation rates are maintained over multiple minutes), which is not seen with the data or the OFF-LOW-HIGH mechanism (initiation rates are not maintained from minute-to-minute; e.g., see the net *loss* in the "burst peak" at minute 40 in **Figure 1D**). This indicates that features of the time series do support a multiple ON rate mechanism. This corresponds to BS knockouts exhibiting lower *eve2* expression for Bcd-only activation than for Bcd+Hb co-activation [57, 65]. Without the LOW rate, the OFF-ON model switches between OFF and HIGH intensity intervals; we suggest that biologically the Bcd-only LOW rate steadies a basal production, while the Bcd+Hb HIGH rate allows for more total transcript. Spatially, the model produces the observed *eve2* stripe sharpening in time (**Figure 2C**), and indicates that time series from nuclei under repression at the stripe edges should be distinguishable from low expression due to reduced activation (e.g., Hb BS knockout).

This approach indicates that a combination of live imaging, data analysis and stochastic modeling can be used both to find regulatory mechanisms and to understand how they affect transcription noise. Transcription factor binding and initiation kinetics must be slow enough to produce bursting and not time-average output [66]. The time series also need to be long enough, but this can be shortened: Desponds et al. [53] simulated Bcd-activated *hb* expression, comparing OFF-ON transcription to a mechanism with two OFF states (corresponding to different inactive states of the DNA); they reported that with their new autocorrelation technique time series of 20 min should be sufficient to distinguish these alternatives.

FUTURE PROSPECTS

Live MS2 imaging has been extended to an increasing number of genes in *Drosophila*, including *kni* and, in dorsal-ventral patterning, the ventrally expressed *snail* [69, 70] and its targets *brinker* and *short gastrulation* [71]. These provide new insights into temporal aspects of regulation and patterning, but also allow for new noise analysis, particularly how transcriptional noise control may vary between genes or between tissues. For instance, while single CREs can exhibit multiple initiation rates (e.g., *eve2*), a broader level of control may arise with the multiple enhancers (CREs) seen for many genes: [72] reported a correlation between enhancer strength and transcriptional burst frequency in *snail*, *rhomboid*, *Abdominal-B* and *Kr*. In addition to *Drosophila*, MS2 has been introduced into zebrafish [73] and mice [74] to visualize patterning dynamics and transcriptional bursting in vertebrate development.

Discerning regulatory mechanisms from time series will be facilitated in several ways. First, while bursts can be defined theoretically (e.g., [19, 61]), quantifying the duration and amplitude of bursts in data series can be challenging. Comparison of experiment and theory is likely to be more robust using time series statistics such as autocorrelation [53, 66]. Next, numerical simulations currently offer a way to find characteristics of multi-state regulatory processes: e.g., **Figures 1**, **2**; also see [75] for a systematic method for fitting putative models to single-cell expression. Analytical approaches have been developed for stochastic transcription of a spatial pattern with OFF-ON initiation [61]; and for steady-state distributions of nascent signal for intronic FISH, addressing the effect of time lags due to placement of the probe [42]. Extensions of such approaches to multi-state initiation and time-series analysis from cells in a developing spatial pattern may give a more complete treatment of MS2 signal dynamics and better predictive power for inferring regulatory mechanisms.

Characterizing the stochastic dynamics of transcription provides insight into how biology may exploit different regulatory mechanisms. For instance, the *eve2* modeling indicates that multiple ON-state mechanisms may support smoother mRNA output compared to OFF-ON mechanisms. Recent examples from single-cell work include modeling dosage regulation after DNA replication as a reduced probability of the ON state [76]; and an experimental and theoretical demonstration that in HIV production a positive feedback slows promoter toggling and uncouples mean from variance, allowing for stochastic active/latent switching at high output which increases viral fitness [77]. Recent single-cell transcriptomes in mice indicate noise regulation is under selective pressure in metazoans as well: low expression noise is associated with gene expression regulators and highly-networked genes (such as *Drosophila* segmentation genes); while higher noise is associated with stress response, which could aid adaptivity [78]. A more complete picture of the evolution of the gene regulatory networks controlling spatial pattern formation in metazoans will ultimately combine quantification of expression variability with the experimental and theoretical characterization of the stochastic dynamics of gene expression, such that we can understand how regulators interact with the target gene structure to provide both the stability and adaptivity needed for robust development over successive generations.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and approved it for publication.

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