

Nanog Fluctuations in Embryonic Stem Cells Highlight the Problem of Measurement in Cell Biology

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ABSTRACT A number of important pluripotency regulators, including the transcription factor Nanog, are observed to fluctuate stochastically in individual embryonic stem cells. By transiently priming cells for commitment to different lineages, these fluctuations are thought to be important to the maintenance of, and exit from, pluripotency. However, because temporal changes in intracellular protein abundances cannot be measured directly in live cells, fluctuations are typically assessed using genetically engineered reporter cell lines that produce a fluorescent signal as a proxy for protein expression. Here, using a combination of mathematical modeling and experiment, we show that there are unforeseen ways in which widely used reporter strategies can systematically disturb the dynamics they are intended to monitor, sometimes giving profoundly misleading results. In the case of Nanog, we show how genetic reporters can compromise the behavior of important pluripotency-sustaining positive feedback loops, and induce a bifurcation in the underlying dynamics that gives rise to heterogeneous Nanog expression patterns in reporter cell lines that are not representative of the wild-type. These findings help explain the range of published observations of Nanog variability and highlight the problem of measurement in live cells.

INTRODUCTION

Fluorescence has been used to report expression of gene products in live cells since green fluorescent protein (GFP) was first cloned and utilized as a tracer (1,2). Live cell fluorescence imaging and analysis techniques allow investigation of temporal changes in protein expression and have consequently become an essential tool in modern molecular biology (3). However, their proper use requires the reporter signal to be representative of expression of the protein of interest at the scale of interest. In particular, if the reporter is to be used as a proxy for protein expression within a single cell, then, to be able to draw accurate conclusions, the reporter signal should be representative of protein expression in that particular cell. This issue is particularly relevant when functional assays are performed after cell sorting based upon reporter signal intensity, and can present a significant problem if the long-term outcome of any

subsequent assays are driven by rare subpopulations of misidentified cells. As interest in single cell biology has increased, some generalized concerns about the fidelity of standard live-cell reporter strategies have been raised (4,5). However, the ways in which the genetic manipulations involved in generating reporter cell lines affect endogenous gene expression kinetics are not well understood.

Here, we explore how commonly used fluorescent reporter strategies can fail to accurately represent protein expression at the single cell level. For systems that use nonlinear feedback control mechanisms, we find that the introduction of reporter constructs can perturb important endogenous regulatory kinetics and induce qualitative changes in the protein expression patterns they are intended to measure. Because predicting when these problems will occur requires a priori knowledge of the underlying regulatory control mechanisms of the system under study—which is typically the knowledge that the reporter was introduced to provide—our results highlight a basic measurement problem in cell biology, reminiscent of that encountered in quantum physics (6,7), in which the act of measuring disturbs the

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system being measured. To illustrate these ideas, we consider the complications that can arise when using fluorescent reporters to monitor the expression dynamics of Nanog, a central element in the pluripotency regulatory network in mouse embryonic stem (ES) cells.

It has been widely observed that expression of a number of important pluripotency-associated transcription factors appears to fluctuate stochastically in individual ES cells (8–13). Although this heterogeneity has been linked to functional variability, its full developmental significance is still not well understood (14–16). The most widely studied of these fluctuating factors is Nanog, a core member of the regulatory network for pluripotency that is able to maintain pluripotency *in vitro* in the absence of Leukemia Inhibitory Factor (LIF), a cytokine normally required for the maintenance of self-renewal and prevention of differentiation (17–20).

Interest in Nanog expression variability began with a 2005 study by Hatano et al. (21) in which it was observed by direct immunostaining that mouse ES cell cultures displayed markedly heterogeneous patterns of Nanog protein expression, with a significant proportion of Oct4 positive pluripotent cells being negative for Nanog. A corresponding bimodal expression pattern was also observed via fluorescence in the first Nanog reporter ES cell lines (8,21). Subsequent studies indicated that Nanog levels appear to fluctuate stochastically in individual cells, thus providing a putative mechanism for the observed heterogeneity in expression (8,9,16,22). Importantly, during times of transient high Nanog expression, cells were observed to be resistant to differentiation cues; yet during times of transient low Nanog expression, cells became sensitive to differentiation-inducing stimuli. These results suggested that Nanog fluctuations are central to its role as molecular gatekeeper for pluripotency (8,9,23–26).

Many articles have followed up on this line of thought, and fluctuations in other factors have been investigated using similar strategies (10,11,27,28). Because these studies have focused on nuclear factors, they have generally employed fluorescence reporters to measure protein abundances, and it is typically implicitly assumed that the reporter signal is representative of expression of the factor of interest within individual cells, and, furthermore, that the observed patterns of expression in reporter cell lines are representative of those in (genetically unperturbed) wild-type cells. However, some concern has been raised that commonly used reporter strategies may not be representing Nanog expression patterns as accurately as they should. Faddah et al. (4) observed low correlation of reporter and Nanog mRNA levels in the original heterozygous reporter constructs; and both Faddah et al. (4) and Filipczyk et al. (5) have argued that observed heterogeneity of Nanog may be, at least in part, a reporter artifact.

To gain a more nuanced understanding of Nanog dynamics, more recent studies have employed a range of nonreporter (29,30) and live-imaging techniques (31–33), as well as more sophisticated protein and mRNA reporters (26,31,32).

These methods include: construction of reporter constructs that use self-cleaving 2A peptide linkers and do not disrupt the Nanog coding region (4,19); fusion constructs in which Nanog is directly fused to a fluorescent protein (5,32); and bacterial artificial chromosome (BAC) transgenes, which carry a plasmid with the reporter gene under the Nanog promoter, leaving the wild-type Nanog alleles unchanged (22,26). Dual allele reporter systems have also been used to compare allele-specific Nanog abundances and assess total Nanog expression (4,19). Although self-cleaving, fusion, and BAC lines typically report more consistent Nanog expression patterns than the original heterozygous knock-in (21,34) and loss-of-function (8) reporter lines, there is still no consensus concerning the extent to which observed Nanog expression variability depends upon reporter type, cellular genetic background, and culture conditions.

Here, we address these issues using a combination of mathematical modeling and experiment. In the first part of the article we use a mathematical argument to show why it should not generally be expected that reporters will faithfully reflect gene expression dynamics at the single cell level, and why reporter accuracy depends strongly upon regulatory context. Surprisingly, this analysis also suggests that expression noise can improve, rather than degrade, reporter accuracy. To illustrate these general results, we then consider the case of Nanog, and find that a range of commonly used reporter strategies can alter the kinetics of endogenous Nanog regulatory control mechanisms and can induce a bifurcation in the underlying dynamics that gives rise to heterogeneous Nanog expression patterns in reporter lines that are not representative of the wild-type. We finish with a discussion of the general relevance of these results, and some suggestions for designing more effective reporters.

MATERIALS AND METHODS

Cell culture

Pluripotent mouse embryonic stem cells were cultivated in Dulbecco's Modified Eagle Medium with 1% Penicillin/Streptomycin, further supplemented with 10% fetal bovine serum, 1× Modified Eagle Medium nonessential amino acids, 1× GlutaMAX (GIBCO/Thermo Fisher Scientific, Waltham, MA), and 50 μM β-Mercaptoethanol. LIF was added at a dilution of 1:1000 (produced in-house). This is Oi culture medium. For 2i culture medium, Oi medium was supplemented with 1:10,000 10 mM PD0325901 (Cat. No. 4197; Tocris Bioscience, Bristol, UK) and 1:3000 10 mM CHIR99021 (Cat. No. 27-H76; Reagents Direct, Encinitas, CA). After transfer from Oi media, cells were adapted to 2i media over six passages. Cells were initially cultured on 0.1% gelatin-coated tissue culture plates preseeded with γ-irradiated mouse embryonic fibroblasts. After two passages, cells were cultivated on 0.1% gelatin-coated tissue culture plates without mouse embryonic fibroblasts. Cells were maintained at 37°C, 5% CO₂, routinely passaged every other day using Trypsin/EDTA detachment, and media was replaced every day. The wild-type male embryonic stem cell line v6.5 was purchased from Novus Biologicals (Cat. No. NBP1-41162; Littleton, CO). Nanog reporter cell line NHET was kindly provided by Jianlong Wang (Icahn School of Medicine, New York, NY). In this cell line,

originally generated by Maherali et al. (34) using the design of Hatano et al. (21), the endogenous Nanog open reading frame has been substituted by a gene cassette containing GFP in series with a Puromycin resistance cassette, separated by an internal ribosome entry site (IRES). For 0i and 2i cultures, three technical replicates were assessed for Nanog and GFP distributions by flow cytometry and image analysis at passage number 11 (v6.5s) and passage number 20 (NHETs, also day 0 in time-course experiments). For undirected differentiation time-course experiments, three replicates from each initial condition (0i and 2i) were cultured separately for seven days after withdrawal of LIF from culture media on day 0. Cultures were passaged every two days and assessed by flow cytometry and image analysis on days 0, 1, 2, 3, 5, and 7.

Immunocytochemistry and flow cytometry

Cells for flow cytometry were detached using Trypsin/EDTA. Cells cultures for imaging were briefly washed in PBS. All cells were fixed for 20 min at room temperature (RT) in 4% Paraformaldehyde in PBS and washed three times with PBS. Cell and nuclear membranes were permeabilized using 0.1% Triton-X-100 in PBS for 10 min at RT. Unspecific antibody binding was blocked with 0.1% Triton-X-100 in PBS with 10% fetal bovine serum for 45 min at RT. Blocked cells were washed three times with blocking solution and resuspended in blocking solution containing either primary antibodies overnight at 4°C. Cell suspensions were under continuous agitation and cell plates were under continuous gentle motion. All experimental results in the main article used directly conjugated primary antibodies: Mouse anti-mouse Nanog (1:200, Cat. No. 560279, Alexa Fluor 647; Thermo Fisher Scientific, Waltham, MA), mouse IgG1 κ isotype control (Cat. 557732, Alexa Fluor 647; Thermo Fisher Scientific), rat anti-histone H3 (pS28) (Cat. 560606, Alexa Fluor v450; Thermo Fisher Scientific), and rat IgG2a κ isotype control (Cat. No. 560377, Alexa Fluor v450; Thermo Fisher Scientific). Samples were washed three times with PBS and for cell imaging, nuclei were incubated with 20 μ g/mL DAPI (Invitrogen, Carlsbad, CA) for 15 min before imaging. The following nonconjugated primary antibodies were also used: Mouse anti-Oct3/4 (c-10) (Cat. No. SC5279; Santa Cruz Biotechnology, Dallas, TX) and murine IgG2b isotype control (Cat. No. SAB4700729; Sigma-Aldrich, St. Louis, MO). After incubating with primary antibodies overnight, these samples were washed three times with blocking solution and incubated with secondary antibodies for 1 h at RT. Secondary antibodies: goat anti-mouse (IgG H&L) (Cat. No. abA11017, Alexa Fluor 488; Abcam, Abcam, Cambridge, UK) and goat anti-mouse IgG (Cat. No. 405322, Alexa Fluor 647; BioLegend, San Diego, CA). Images were recorded using an Eclipse Ti microscope (Nikon, Melville, NY). Cell suspension samples were analyzed using a BD FACS Aria II fluorescence activated cell sorting (FACS) device and BD FACSDIVA software (Becton-Dickinson, Oxford, UK). Flow cytometry analysis was performed using the softwares FlowJo (FlowJo LLC, Ashland, OR) and MATLAB (The MathWorks, Natick, MA), and the R programming language (35,36). Nanog and GFP fluorescence were quantified in terms of molecules of equivalent soluble fluorophore (MESF) units using Quantum Alexa Fluor 488 and 647 MESF calibration beads (Bangs Laboratories, Fishers, IN). Fluorescence probability distributions for nondirected differentiation experiments were aligned at the first percentile of Nanog and GFP observations between days.

Image analysis

Image analysis was carried out on grayscale fluorescence image sets using the software CellProfiler (<http://cellprofiler.org/>) (37). Each image set consisted of a 5 \times 5 grid of adjacent images from a given cell culture. Nuclei were identified automatically based on DAPI signal and hand curated to exclude mitotic cells, unresolved or split nuclei, and those at the image edge. Spatially variable background fluorescence for each fluorescence channel was accounted for by determining the average illumination correc-

tion function per image set (38). The illumination correction function for each image was calculated by finding the minimum value pixel within a given block size (75 pixels) and then smoothed using a polynomial fit smoothing function. The average illumination correction function for each image set was subtracted from the Nanog and GFP signal images before measurement of the mean fluorescence intensity per nuclear area.

Model fitting

Nanog expression distributions from FACS and image analysis were fitted to a Gaussian mixture model with one or two components using expectation maximization. Model selection was conducted using Bayes information criterion. When fitting to two-component mixtures, to ensure that robustly bimodal distributions were identified, we required both components to have a weight >0.1 and excluded those models for which the peak probability density of one component was less than the probability density of the other component at the same point.

Mutual information calculation

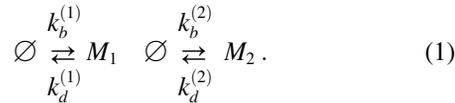
Mutual information (MI) was estimated using the James-Stein-type shrinkage estimator (39). For MI between GFP and Nanog expression levels, discretization of each variable at each time point was performed separately via the Bayesian blocks method (40). Because MI is invariant to smooth reparameterization, we worked with the aligned, rescaled, log-transformed fluorescence values.

RESULTS

Regulatory noise and reporter accuracy

The first generation of Nanog studies used knock-in reporters to assess Nanog dynamics (8,21). Due to their simplicity, these and similar reporter designs are still widely used to assess expression fluctuations of Nanog and other key transcription factors in ES cells. In these constructs, one of the alleles for the gene of interest (for example, *Nanog*) is replaced with a reporter gene, often encoding for a fluorescent protein, perhaps with additional features such as an antibiotic selection cassette (3). Due to the loss of one gene copy, these are often described as heterozygous loss-of-function reporters. For such constructs to be effective at the single cell level, the fluorescence signal driven from the reporter allele should accurately represent protein expression from the wild-type allele. We therefore begin by considering a simple model of transcriptional coactivity, to explore the conditions under which two alleles that are subject to the same regulatory control may either synchronize or decouple in their activity, and thereby the conditions under which the output of one allele may be used to report on the other. For simplicity, we will focus on mRNA dynamics, but similar reasoning may also be extended to the protein level, and the general conclusions that we draw are not limited to heterozygous reporters (see [Supporting Material](#) and the following section for details). We will start with a simple model of a pair of constitutively active alleles before moving onto more realistic models of Nanog expression in the following sections.

Consider the transcriptional dynamics of two alleles of the same gene in a single cell. Let M_1 and M_2 denote the mRNA products of alleles 1 and 2, respectively; let $m_1(t)$ and $m_2(t)$ denote the number of mRNA transcripts in the cell associated with alleles 1 and 2, respectively, at time t ; and assume that expression from both alleles is governed by linear birth-death processes with production rates $k_b^{(1)}, k_b^{(2)}$ and decay rates $k_d^{(1)}, k_d^{(2)}$. Thus, we are concerned with the dynamics of the following system of reactions:



This is clearly a simplistic view of transcription, yet it suffices to illustrate some of the essential issues regarding the reliability of reporters and is analytically tractable. Because the alleles are not coupled, they act independently and the stationary joint probability mass function (PMF) for this process is the product of two Poisson distributions (Fig. 1 A), as follows:

$$p(m_1, m_2) = \frac{\lambda_1^{m_1}}{m_1!} e^{-\lambda_1} \times \frac{\lambda_2^{m_2}}{m_2!} e^{-\lambda_2}, \quad (2)$$

where $\lambda_i = k_b^{(i)}/k_d^{(i)}$ for $i = 1, 2$. As we have not allowed for coregulation of expression, this model is rather artificial. In reality, we expect that if the alleles are both under the same promoter control then they will be regulated by the same upstream factors, and this coregulation may coordinate their dynamics. To couple the alleles, we allow the transcription

rates to be driven by a shared upstream regulator X . Let x denote the concentration of X and let $\rho(x)$ be the stationary probability density function for x . Assuming that the mRNA birth rate is now given by $k_b^{(i)}x$, the stationary joint PMF is then obtained from Bayes' theorem, as follows:

$$p(m_1, m_2) = \int_0^\infty p(m_1, m_2 | x) \rho(x) dx, \quad (3)$$

$$= \int_0^\infty \frac{(\lambda_1 x)^{m_1}}{m_1!} e^{-\lambda_1 x} \times \frac{(\lambda_2 x)^{m_2}}{m_2!} e^{-\lambda_2 x} \rho(x) dx.$$

Because the joint PMF $p(m_1, m_2)$ depends upon the distribution of the upstream regulator, an appropriate form for $\rho(x)$ must be chosen. It is commonly observed that protein concentrations are Gamma-distributed, so this is a natural choice (41). In the case $x \sim \text{Gamma}(r, \theta)$, and the joint PMF is as follows:

$$p(m_1, m_2) = \frac{\Gamma(m_1 + m_2 + r)}{m_1! m_2! \Gamma(r)} (1 - p - q)^r p^{m_1} q^{m_2}, \quad (4)$$

where $p = \lambda_1 \theta / [1 + \theta(\lambda_1 + \lambda_2)]$, $q = \lambda_2 \theta / [1 + \theta(\lambda_1 + \lambda_2)]$, and Γ is the Gamma function. The marginal distributions for the two allele products are then negative binomials (NB) and the joint PMF is a bivariate negative binomial distribution (BNB), as shown in Fig. 1, B and C. If the two alleles are kinetically identical ($\lambda_1 = \lambda_2$), then the marginal distributions will be identical, and the product of either allele may be used to report on the other at the population level assuming that the same dynamics occur within each cell in the population. However, this does not guarantee any

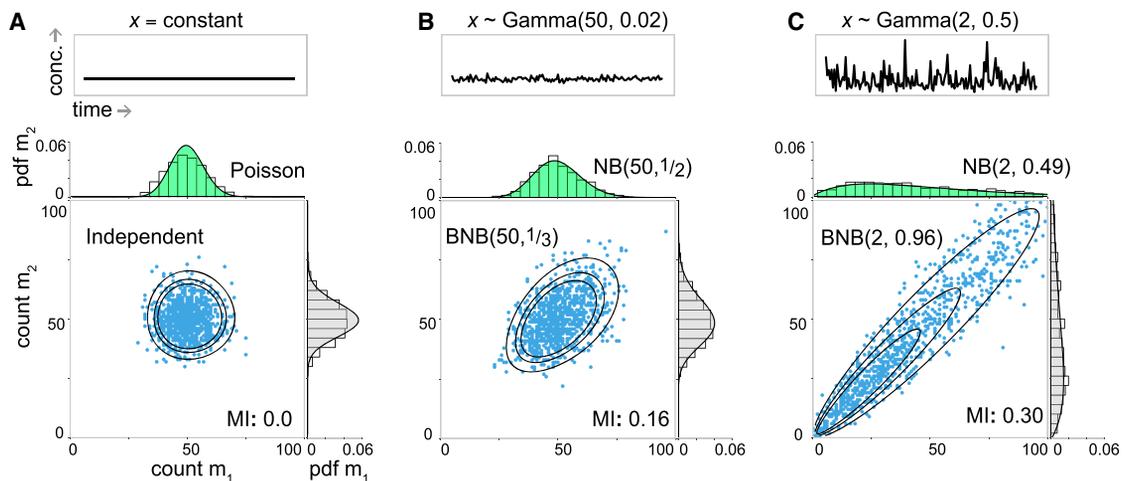


FIGURE 1 Reporter accuracy depends upon regulatory context. Identical alleles of the same gene produce mRNA molecules M_1 and M_2 . Alleles 1 and 2 behave independently when there is no common upstream regulator or regulator concentration (x) is constant, yet become coupled if the upstream regulator fluctuates. (Top panels) Shown here are fluctuations of upstream regulator concentration. Panels show constant x (A) and $x \sim \text{Gamma}(r, \theta)$, for low regulator dispersion $\theta = 0.02$ (B) and high regulator dispersion $\theta = 0.5$ (C). (Bottom panels) Shown here are joint and marginal distributions for m_1 and m_2 . Joint distributions are the product of two Poisson distributions (A, Eq. 2) and bivariate negative binomials, $\text{BNB}(r, p)$, with $p = q$ for identical alleles (B and C; Eq. 4). All distributions use $\lambda = 50$ for both alleles and contours show probabilities: 0.0001 inner, 0.0003 middle, and 0.0005 outer. $\text{NB}(r, p/(1-p))$ denotes “negative binomial”. Scatter plots, histograms, and MI (in nats) are shown for a random sample of 1000 draws. The same scales apply to all comparable plots. To see this figure in color, go online.

association between the allelic outputs at the individual cell level. To measure the degree of association between alleles within an individual cell, we must consider the covariance between their outputs, which is easily calculated in this case (see [Supporting Material](#)) and has a particularly simple form, given as follows:

$$\text{Cov}(m_1, m_2) = \lambda_1 \lambda_2 \text{Var}(x). \quad (5)$$

Thus, the covariance between the two allele products is proportional to the variance of the upstream regulator and the sensitivities of the two alleles to the upstream regulator. Whereas the form of joint PMF given in [Eq. 4](#) depends upon the upstream regulator being Gamma-distributed, [Eq. 5](#) holds for any upstream probability distribution $\rho(x)$, including, for example, the Gumbel distribution, which has been used to characterize extrinsic noise (42) (see [Supporting Material](#)). A comparable result may be obtained when transcription from each allele occurs at rate $k_b^{(i)}f(x)$, for any smooth function $f(x)$ (see [Supporting Material](#)). Similarly, it may also be shown that the correlation between the two alleles depends in a monotonic positive way on the Fano factor (or index of dispersion) of the upstream regulator (see [Supporting Material](#)).

Because the covariance between the two alleles is proportional to the variance of the upstream regulator, these results indicate that regulatory noise upstream can increase the coordination of alleles downstream and therefore improve reporter accuracy. Thus, although this model is the simplest possible to account for stochastic transcription from two alleles, it nevertheless provides insight into the effect of extrinsic fluctuations in a common upstream regulator on the coordination of two alleles. However, this model does not account for the fact that transcription from each allele typically occurs in bursts (43–46), and therefore cannot account for the effects of intrinsic noise on reporter accuracy.

A simple model of bursty transcription from a pair of alleles may be obtained by modifying this basic model to allow each allele to have two transcriptional states—one with a high rate of transcription, the other with a low rate of transcription—that they switch between stochastically at constant rates (45). In this case, the effective rates of transcription from each allele are now themselves stochastic processes and the mRNA coexpression dynamics are therefore described by a doubly stochastic process, which has accordingly more complex solutions (47). However, if the rate of switching between transcriptional states is slow with respect to mRNA degradation rate, then approximate solutions to this system may be obtained, and the covariance between alleles may again be found analytically (see [Fig. S1](#) and [Supporting Material](#) for details). In particular, assuming that transcription from both alleles is driven by the same upstream regulator X and both alleles are kinetically identical, the covariance is as follows:

$$\text{Cov}(m_1, m_2) = (w\lambda_+ + (1-w)\lambda_-)^2 \text{Var}(x), \quad (6)$$

where w is the proportion time each allele spends in the active state, and $\lambda_+ + \lambda_-$ are the effective transcription rates from the active and inactive states, respectively. If $w = 1$, then both alleles are constitutively active, and [Eq. 6](#) reduces to [Eq. 5](#) with $\lambda_+ = \lambda_1 = \lambda_2$. However, for $w < 1$ this equation highlights the different effects that intrinsic and extrinsic noise can have on allelic coordination. Because the covariance between alleles is again proportional to the variance of the upstream regulator, extrinsic fluctuations upstream always increase the covariance between alleles downstream and thereby improve reporter accuracy, as before. By contrast, because $w\lambda_+ + (1-w)\lambda_- < \lambda_+$, intrinsic noise due to transcriptional bursting always decreases the covariance between alleles and so always compromises reporter accuracy. These observations are in accordance with previous work in which dual and single allele reporters were used to assess the relative effects of intrinsic and extrinsic noise on gene expression (48–53).

Taken together, these results indicate that a reporter's accuracy depends on the regulatory context in which it is placed, here represented by the dynamics of the upstream regulator X , which are external to its design. In a complex regulatory environment, these external factors may not be fully (or even partially) known, and their effect on the reporter may be correspondingly hard to predict or control. Furthermore, because extrinsic regulatory factors are not usually constitutively expressed (as assumed in this basic model) but rather are themselves regulated by complex mechanisms—often involving feedback with the product of the gene being monitored—the dynamics of the regulatory environment may be intrinsically coupled to that of the target gene and its reporter(s). In this case, the insertion of a reporter construct may have unforeseen effects on endogenous regulatory kinetics, and change the dynamics of the system being studied in unpredictable ways. Because the ways in which such perturbations may arise will depend upon the particular details of system under study, it is helpful to consider a specific example. Here we examine the case of Nanog, an important transcriptional regulator of pluripotency in ES cells, which is known to be regulated by a complex network of direct and indirect feedback loops and for which different reporters have given different assessments of Nanog dynamics.

Reporter perturbation of Nanog dynamics

It has been widely observed that Nanog expression fluctuates stochastically in individual ES cells (8,9,11,26,29,32,33). However, different reporter constructs have given different assessments of the strength and developmental significance of these fluctuations (4,8,14–16,19,22,25) and some concerns have been raised that the use of reporters may be introducing artifacts that are confounding, rather than clarifying, our understanding of pluripotency (4,5). To address this issue, we

will consider a simple mathematical model of Nanog dynamics in ES cells in the presence of different kinds of reporter constructs. Nanog levels are regulated in pluripotent cells by a complex network of molecular interactions that involve both protein-protein and protein-DNA interactions (54–57). Given the complexity of this regulation, the fully stochastic framework used in the section above is not practical because all but the simplest stochastic processes are analytically intractable (58). So we instead use an ordinary differential equation approach. A number of groups have adopted a similar strategy when modeling these dynamics mathematically (9,59,60). At the core of this extended regulatory network is a series of nonlinear positive feedback loops that are dependent on Nanog for their function (24,61). Because these feedback loops are central to Nanog regulation, and to maintain a tractable mathematical model of general relevance, we will focus on this aspect of Nanog regulation here. Such positive feedback mechanisms naturally give rise to switchlike dynamics; they are correspondingly central to many kinds of cell fate decisions (62–64). Therefore, the model of positive feedback that we outline below is of general relevance to the design of reporters for other similarly regulated lineages specifying master transcription factors.

Mathematical model

We consider the following set of ordinary differential equations as a simple model of Nanog protein dynamics in wild-type cells:

$$\frac{dn_1}{dt} = c_b + \frac{c_f n^H}{K^H + n^H} - c_d n_1, \quad (7)$$

$$\frac{dn_2}{dt} = c_b + \frac{c_f n^H}{K^H + n^H} - c_d n_2, \quad (8)$$

where n_i denotes the concentration of the Nanog protein output of allele $i = 1, 2$. The first terms on the right-hand sides of these equations account for baseline production at a constant rate c_b ; the second terms are Hill functions that account for feedback-enhanced production at a rate dependent on total Nanog concentration $n = n_1 + n_2$, up to a maximum rate c_f ; and the third terms account for Nanog protein decay at constant rate c_d . Hill functions are commonly used to model feedback processes, and may be derived as the effective production rate from a directly autoregulated two-state gene with stochastic transcriptional bursting (65), or as the result of more complex indirect feedback mechanisms (66). These equations therefore implicitly account for both direct Nanog autoregulation (61) and indirect feedback mechanisms in the core ES cell circuit (24,67) and thereby the effects of auxiliary factors such as other transcriptional regulators via their effect on the model rate constants, but for mathematical simplicity, the expression of these factors is not modeled explicitly. Adding these

equations, we obtain an ordinary differential equation for the total Nanog protein concentration n , as follows:

$$\frac{dn}{dt} = 2c_b + 2 \frac{c_f n^H}{K^H + n^H} - c_d n. \quad (9)$$

To better understand the model dynamics, it is convenient to nondimensionalize this equation. Doing so, using the scalings $n = 2c_f c_d^{-1} \bar{n}$, and $t = c_d^{-1} \tau$, we obtain the following:

$$\frac{d\bar{n}}{d\tau} = \alpha + \frac{\bar{n}^H}{\gamma^H + \bar{n}^H} - \bar{n}, \quad (10)$$

where \bar{n} is the dimensionless total Nanog concentration and τ is dimensionless time. The dimensionless constants $\alpha = c_b/c_f$ and $\gamma = \gamma_{wt} = c_d K/2c_f$ describe the relative strength of the basal and positive feedback enhanced production rates, respectively. Equation 10 has either one or two stable equilibrium solutions depending on the relative sizes of α , γ , and the Hill coefficient H . In particular, for $H > 1$, two bifurcation curves in the $\alpha\gamma$ plane may be found (see Supporting Material). The case $H = 2$ suffices to illustrate the general structure of the resulting classification diagram (see Fig. 2 A). In this case, the bifurcation curves are as follows:

$$\gamma_{\pm}(\alpha) = -\left(\alpha^2 - \frac{5}{2}\alpha - \frac{1}{8}\right) \pm \left(\frac{1}{4} - 2\alpha\right)^{\frac{3}{2}}. \quad (11)$$

If the model parameters fall inside the region enclosed by these curves, then Nanog expression dynamics are bistable; if the model parameters fall outside this region, then Nanog expression dynamics are monostable. In the presence of molecular noise, which is inherent to the intracellular microenvironment, bistability can give rise to coexisting subpopulations of phenotypically distinct cells within an isogenic population under the same environmental conditions (63). Thus, both homogeneous and heterogeneous Nanog expression patterns are allowed by this model, depending on whether the underlying dynamics are monostable or bistable. It should therefore be expected that Nanog expression patterns in ES cell populations will vary substantially under different experimental conditions, as is commonly observed (4,19,23), depending on how they stimulate Nanog feedback mechanisms. More significantly, it should also be expected that any genetic interventions that perturb the kinetics of Nanog feedback have the potential to push the dynamics in or out of the bistable regime, thereby affecting a qualitative change in expression patterns.

To see this, consider the case of a heterozygous knock-in reporter, in which one allele produces an inert reporter and one allele is left intact. In this case, the wild-type kinetics described by Eqs. 7 and 8 are modified as follows:

$$\frac{dn}{dt} = c_b + \frac{c_f n^H}{K^H + n^H} - c_d n, \quad (12)$$

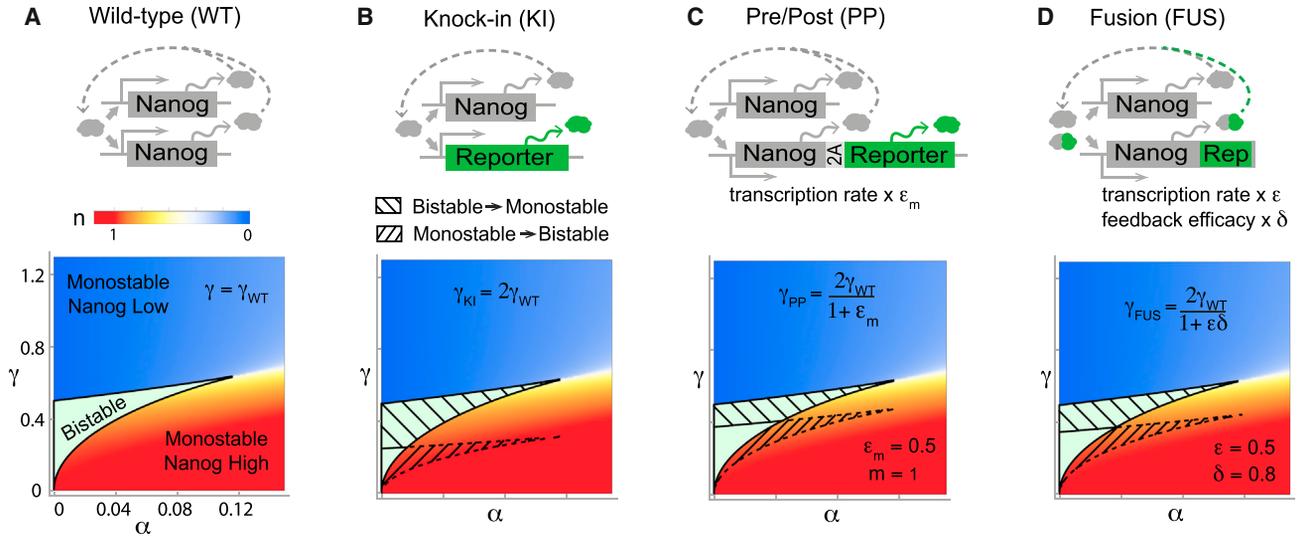


FIGURE 2 Perturbation of Nanog dynamics by reporters. (A) Given here is the wild-type: Nanog protein is produced from both alleles. Monostable or bistable dynamics can occur depending on α and γ . (B) Given here are knock-in reporters: one allele is left intact and one allele produces an inert reporter protein. Loss of one Nanog allele reduces Nanog production by a factor of 2, thereby doubling γ . (C) Given here are pre-/postreporters: both alleles encode for Nanog; m copies of a self-cleaving reporter protein are also transcribed from one allele. In the case shown, the transcription rate from the reporter allele is reduced by a factor $0 \leq \epsilon_m \leq 1$, thus increasing γ by a factor $2/(1 + \epsilon_m)$. If ϵ_m decreases with m , these reporters become more prone to systematic errors with each additional insert. (D) Given here are fusion reporters: one allele encodes for Nanog; the other for a fusion of Nanog and a reporter protein. Transcription rate from the reporter allele is altered by factor $0 \leq \epsilon \leq 1$ as for the PP reporter, but the reporter fusion also reduces Nanog feedback functionality by a factor $0 \leq \delta \leq 1$. Overall, this increases γ by a factor $2/(1 + \epsilon\delta)$. Hatching shows regions of the parameter plane at risk of qualitative changes in behavior when the reporter is introduced to the wild-type system. The upper-hatched regions are areas of parameter space for which the wild-type system is bistable, but the reporter system with the same underlying values of c_b , c_f , c_d , and K is monostable. The lower-hatched regions are areas of parameter space for which the wild-type system is monostable and the knock-in system is bistable. To see this figure in color, go online.

$$\frac{dr}{dt} = c_b + \frac{c_f n^H}{K^H + n^H} - c_d r, \quad (13)$$

where r is the reporter protein concentration. For simplicity we have assumed that the reporter and Nanog protein half-lives are perfectly matched. However, this assumption may be relaxed without altering conclusions qualitatively. Details of the dynamics when decay rates are mismatched are given in the [Supporting Material](#). The dimensionless equation for total Nanog concentration in the reporter line is as follows:

$$\frac{d\bar{n}}{d\tau} = \alpha + \frac{\bar{n}^H}{\gamma_{ki}^H + \bar{n}^H} - \bar{n}, \quad (14)$$

where $\alpha = c_b/c_f$ as before, but $\gamma_{ki} = c_d K/c_f = 2\gamma_{wt}$ (see [Supporting Material](#) for details). In this case, the loss of Nanog production from one allele diminishes the Nanog production rate by a factor of 2, which weakens the endogenous feedback mechanisms and thereby doubles the parameter γ . Because for fixed α the magnitude of γ determines if the dynamics are monostable or bistable, and therefore if Nanog is homogeneously or heterogeneously expressed in the population, this change can induce a heterogeneous Nanog expression pattern in the reporter cell line that is not found in the wild-type (or vice versa). Areas in the $\alpha\gamma$ plane for which the map $(\alpha, \gamma) \mapsto (\alpha, 2\gamma)$ crosses

one of the bifurcation curves in [Eq. 11](#) are at risk of this kind of perturbation. Importantly, this problem is not restricted to knock-in lines: similar issues arise with a wide range of other reporters, in both single-allele and dual-allele reporter systems. [Fig. 2](#) summarizes similar analyses for some other reporters. See [Supporting Material](#) for full details of calculations for these and a range of other reporter constructs.

For example, instead of replacing the Nanog protein coding region on one allele by that of a fluorescence reporter, the reporter construct may be inserted immediately pre/post the Nanog gene using a 2A self-cleaving peptide or internal ribosome entry site. If this insertion alters the Nanog mRNA transcription rate from that of the allele, then the resulting mismatch in transcription rates can lead to a perturbation similar to that of the knock-in. As there are many factors that influence transcription rate—including gene length, proximal pausing, and recruitment of RNA polymerase and cofactors ([46,68](#))—it is reasonable to assume that insertion of a reporter construct has the potential to alter transcription rate through interference with one or more of these factors in a manner that is likely to be context-specific for each given gene. In this case, assuming that the transcription rate from the reporter allele is reduced by a factor of $0 \leq \epsilon \leq 1$, the insertion changes γ by a factor of $2/(1 + \epsilon)$ (see [Fig. S2](#) and [Supporting Material](#) for full details). Thus, so long as the insertion does not completely

block transcription from the reporter allele (in which case, $\epsilon = 0$), pre-/postreporters are less likely than knock-in reporters to induce qualitative changes in Nanog expression dynamics, yet they are still subject to a similar type of systematic risk (compare the at-risk regions in Fig. 2, B and C). Furthermore, if multiple (m) reporters are inserted then this affect is compounded, assuming that the transcription rate from the reporter allele changes by a factor of $0 \leq \epsilon_m < \epsilon$. Thus, although multiple reporter additions may improve fluorescent signal, the risk of inducing a qualitative change in dynamics is increased with each additional reporter insert (see [Supporting Material](#) for more details). Similarly, fusion reporters, which encode a fusion of Nanog and a reporter protein from the reporting allele(s), are also susceptible to related problems. Assuming that the fusion reporter alters the transcription rate from the reporter allele by a factor of ϵ , and fusion of the reporter protein to Nanog reduces its functional efficacy by a factor $0 \leq \delta \leq 1$, fusion reporters change γ by a factor of $2/(1 + \epsilon\delta)$ (see [Supporting Material](#) for full details). In this case, the risk of a qualitative perturbation to the dynamics increases with both the extent to which the reporter perturbs transcription rate (ϵ) and the extent to which the attachment of the reporter protein to Nanog compromises Nanog function (δ).

Taken together, these theoretical considerations suggest that both technical and systematic errors can arise when using genetic reporters for Nanog. Technical errors occur due to the inevitable temporal mismatch between Nanog and reporter expression within individual cells, due, for example, to the effects of intrinsic noise on gene expression (as described in the previous section); systematic errors occur when unforeseen interactions between the reporter construct and the endogenous pluripotency regulatory circuitry induce qualitative changes in dynamics in the reporter cell line that are not representative of the wild-type (as described above).

Experimental results

To determine the extent to which these issues arise in experiment, we compared Nanog expression patterns in wild-type (male v6.5) mouse ES cells to those in a heterozygous knock-in reporter ES cell line with the same male v6.5 genetic background, in which the Nanog coding sequence was replaced with a GFP-IRES-puro reporter on one allele (34) (designated NHET cells). Cells were cultured in standard culture conditions (0i, serum plus LIF) and 2i conditions (0i conditions with the addition of mitogen-activated protein kinase and glycogen synthase kinase with three inhibitors), which maintain “ground state” pluripotency (69). Homogeneous Oct3/4 expression was confirmed via immunostaining in all culture conditions (Fig. S3). Nanog expression in individual cells was assessed via fluorescence immunolabeling and quantified by flow cytometry (Fig. 3) and image analysis (Fig. S4). Substantial variability in Nanog expression was observed in both v6.5 and NHET lines in 0i conditions (Fig. 3 A; Fig. S4). In accordance

with previous reports, substantially less variability was observed in 2i conditions (Fig. 3 A; Fig. S4) (69). Distinctly bimodal GFP fluorescence was observed for NHET reporter cells in 0i cultures, with cell clusters containing both GFP high cells and GFP low cells present in abundance. In both conditions a clear mismatch between Nanog and GFP expression levels was observed in a substantial proportion of cells (Fig. 3 B). This was most apparent in 0i conditions, where, of the highest 20% of Nanog-expressing cells, 23% were GFP low and of the lowest 20% of Nanog-expressing cells, 11% were GFP high. In 2i conditions the percentage of GFP low cells was consistent across the Nanog distribution, suggesting that GFP status was not representative of Nanog expression. In addition, within the GFP high subset there was no clear association between Nanog and GFP expression levels (Fig. 3 B). Although 2i conditions showed more consistent Nanog-GFP coexpression patterns, there was a clear bias toward high GFP levels independently of Nanog expression (Fig. 3 B). These observations are indicative of technical errors due to expression noise (as described in the first section of this article) and we caution that some substantial contamination should be expected subsequent to cell sorting based on GFP signal as a proxy for Nanog when using such lines.

To determine whether Nanog expression was perturbed by introduction of the knock-in reporter, we compared Nanog distributions between NHET and wild-type v6.5 cell lines using immunostaining and flow cytometry. In NHET cells, the Nanog distribution in 0i conditions exhibited a wide, flattened distribution. Fitting of this data to a Gaussian mixture model with one or two components revealed that the two-component model best described the data, suggesting the presence of two coexisting subpopulations of cells characteristic of bistability in the underlying dynamics (Fig. 3 B). By contrast, in wild-type v6.5 cells, the Nanog distribution in 0i conditions was less broad and was better fit by a single-component model, suggesting monostability in the underlying dynamics (Fig. 3 B). To establish the robustness of these results, we also assessed Nanog expression using image analysis, and these broad conclusions were confirmed (Fig. S4 A). Taken together, these analyses suggest that the bimodal expression patterns observed in 0i conditions using the NHET line may be due to systematic perturbation of the Nanog regulatory network by the reporter, as predicted by theory. By contrast, both wild-type and NHET cells expressed similar, more compact, Nanog distributions in 2i conditions, with neither showing evidence of bimodality. This suggests that in 0i conditions, the wild-type system lies within the at-risk region of the $\alpha\gamma$ parameter plane, whereas in 2i conditions, the system lies outside the at-risk region.

To further investigate the extent to which environmental changes might affect the fidelity of the reporter output, we also sought to assess the association between Nanog and GFP during the process of cellular differentiation. Starting

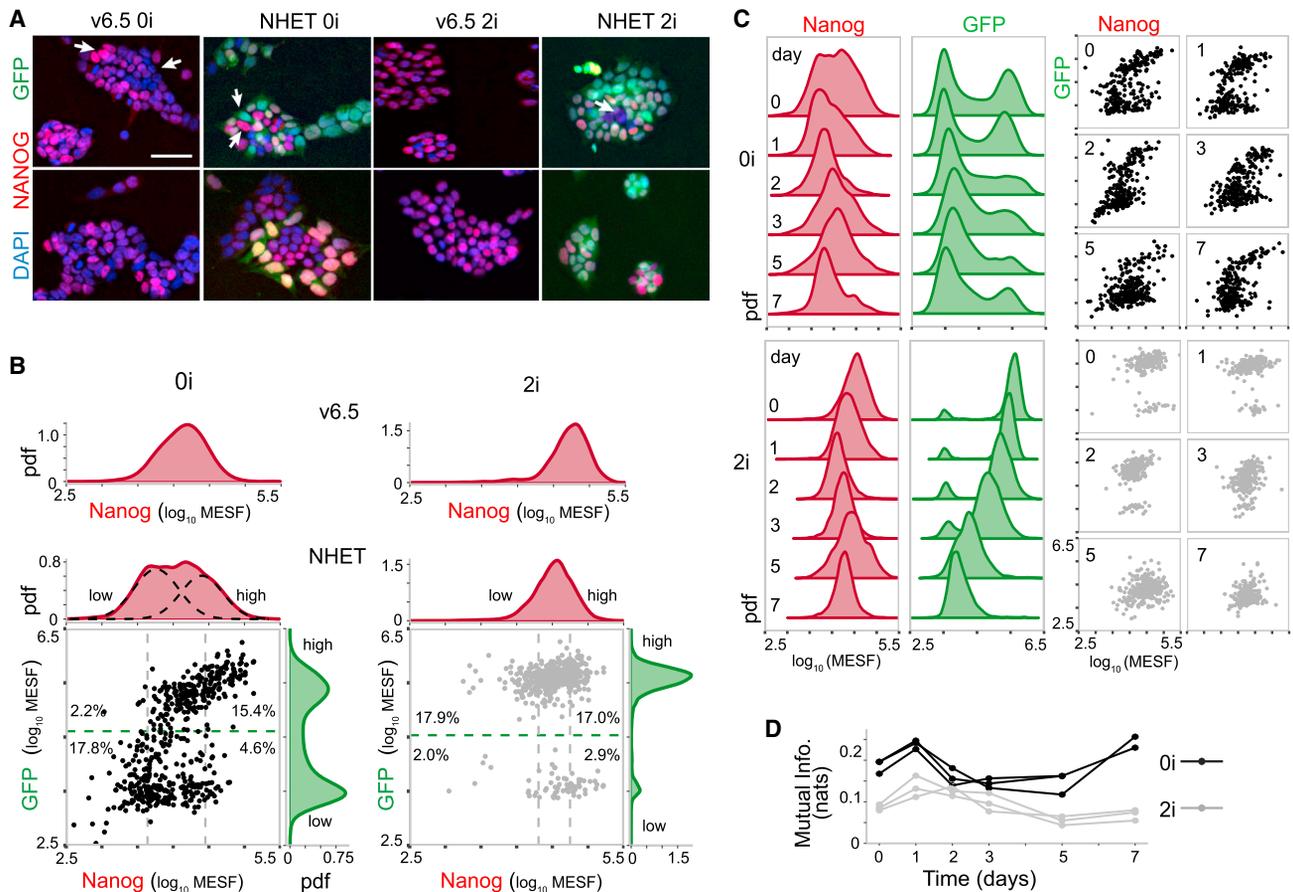


FIGURE 3 Nanog expression in wild-type and reporter cell lines. (A) Shown here are wild-type and Nanog reporter (NHET) cell cultures in 0i and 2i conditions. Nanog immunofluorescence is in red, and direct GFP fluorescence is in green. White arrows indicate Nanog low/high cells (wild-type) or cells in which there is a Nanog-GFP mismatch (NHET). Grayscale fluorescence signals are shown Fig. S3. Scale bar represents 50 μm . (B) Given here are representative flow cytometry distributions of Nanog in v6.5 wild-type cells (top) and Nanog-GFP joint distributions in NHET cells (bottom). Dashed-black lines show components of fit to a two-component Gaussian mixture model. Dashed-gray threshold lines indicate regions of Nanog high/low expression (highest 20% and lowest 20% of cells). Dashed-green lines indicate regions of GFP high/low expression (minimum between two peaks). Percentages show proportions of cells in the relevant subpopulations. (C) Shown here are changing Nanog and GFP distributions during undirected differentiation subsequent to LIF withdrawal starting from 0i (top) and 2i (bottom) cultures. Data from days 0, 1, 2, 3, 5, and 7 are shown. (D) Given here is MI between GFP and Nanog during differentiation. Results of three experimental repeats are shown. To see this figure in color, go online.

in 0i or 2i conditions, NHET cell cultures were allowed to undergo undirected differentiation by withdrawing LIF for a period of seven days. Fig. 3 C and Fig. S4 B show the evolving joint distributions for Nanog and GFP coexpression. From 0i conditions, the proportion of cells in the GFP high population gradually decreased over time and the corresponding Nanog distribution concomitantly evolved from an initial broad, flat distribution to a narrow distribution with lower average expression, indicating gradual loss of Nanog expression. From 2i conditions, both GFP and Nanog levels gradually declined over time without qualitative change in distribution shape. To quantify the association between Nanog and GFP levels, we calculated the mutual information (MI) between their expression patterns as follows:

$$I(n,r) = \int_0^\infty \int_0^\infty p(n,r) \log\left(\frac{p(n,r)}{p(n)p(r)}\right) dn dr, \quad (15)$$

where $p(n,r)$ is the joint probability density function for Nanog and GFP coexpression, and $p(n)$ and $p(r)$ are the marginal probability density functions for Nanog and GFP expression, respectively. Mutual information is a powerful generalization of traditional measures of association, such as correlation, which is able to identify nonlinear relationships between variables (70). In this context, the MI provides an unbiased measure of the amount of information that knowledge of a cell's GFP status provides about its Nanog status (zero MI indicates complete independence; low values indicate near independence; high values indicate strong association). In all cases, the mutual information between Nanog and GFP exhibited a mild transient increase, indicating a slight increase in strength of association during the early stages of differentiation (Fig. 3 D; Fig. S4 C; compare the MI values in these plots with those in Fig. 1 for an informal assessment of their relative size). However,

mutual information was always low, indicating that Nanog and GFP signals are only weakly related both in and out of equilibrium (Fig. 3 C).

DISCUSSION

The advantages of genetic reporters are substantial: they provide a means to investigate expression dynamics of hard-to-monitor proteins and enable live cell observation, tracking, and selection. By assessing expression directly via fluorescence rather than indirectly via immunolabeling they also provide a more transparent way to assess protein activity, free of the reproducibility issues associated with the use of antibodies. However, it is generally accepted that genetic reporter systems are not perfect: quantification is normally relative, reporter fluorescence is an imperfect proxy-measurement for the variable of real interest, and it is known that wild-type dynamics may be compromised, for example by fusing cumbersome fluorescent proteins to (often relatively small) proteins of interest (71). To assess the importance of these issues, the advantages and disadvantages of different types of reporter are usually considered purely in terms of their technical characteristics, or with only limited concern for their regulatory context, for instance to match reporter half-life to that of the protein of interest (72). Here, we have explored how intrinsic and extrinsic noise and reporter interactions with endogenous regulatory mechanisms affect reporter accuracy, focusing on Nanog as an example. Although technical issues relating to noise and reporter protein mismatch are generally well accepted, the systematic limitations we have identified, have not been well appreciated. Yet, our results show that if such limitations are not taken into account then confounding results can follow. The example of Nanog shows how a whole field of study can become complicated by these issues.

Taken together, this work suggests several practical guidelines to help prevent unforeseen issues with reporter observations: first, the scale at which the reporter is used should be considered. In particular, for assays involving cell sorting based upon reporter signal, accuracy should be tested at the single cell level before subsequent functional assays. Second, systematic limitations of reporters, due to interactions between the reporter and its regulatory context, should also be considered. The most appropriate reporter strategy will be determined by a trade-off between the type of spatial and temporal information required, the strength of the reporter signal required, and the likelihood that the reporter chosen will qualitatively perturb the endogenous kinetics. For example, reporters that produce multiple copies of a fluorescent protein per copy of the protein of interest naturally produce a stronger fluorescence signal, yet their construction involves greater genetic intervention, so they also carry a correspondingly higher systematic risk (see Fig. 2; Supporting Material). Before designing or using

such reporters the benefits of increased signal should therefore be weighed against the increased possibility of systematic errors. For genes that are regulated by positive feedback mechanisms—which includes many developmentally important factors (24,59,62,64)—the risk of systematic failures is greatest for knock-in reporters and least for BAC reporters, and single allele reporters carry less systematic risk than dual allele reporters (see Supporting Material).

Because systematic perturbations depend on the details of the regulatory kinetics of particular system under study, it is difficult to determine a priori when they will occur. One potential strategy is to engineer two separate reporter cell lines for the same factor: one in which expression of the gene of interest is monitored in one color, and expression of an inert downstream target of the gene of interest (which does not affect the dynamics of the upstream regulator either directly or indirectly) is monitored in a different color; and a second in which only the downstream gene is monitored and the gene of interest is left unperturbed. Potential systematic perturbations to the dynamics of the upstream gene may then be identified by careful comparison of the reporter distributions for the downstream target in the two reporter cell lines. In all cases, because reporter accuracy depends intimately on regulatory context, and the same reporter in the same cells may fail in some experimental conditions and succeed in others, quality controls should be conducted for all experimental conditions under consideration.

SUPPORTING MATERIAL

Supporting Materials and Methods, four figures, and two tables are available at [http://www.biophysj.org/biophysj/supplemental/S0006-3495\(17\)30509-X](http://www.biophysj.org/biophysj/supplemental/S0006-3495(17)30509-X).

AUTHOR CONTRIBUTIONS

R.C.G.S. and B.D.M. wrote the article. R.C.G.S. and P.S.S. did the experiments. R.C.G.S., P.S.S., S.J.R., and B.D.M. did the mathematical modeling. R.C.G.S., A.S., S.F., and H.A.H. analyzed the experimental data. R.C.G.S. and B.D.M. designed the study.

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