Analysis and design of single-cell experiments to harvest fluctuation information while rejecting measurement noise

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Abstract

Despite continued technological improvements, measurement errors will always reduce or distort the information that any real experiment can provide to quantify cell signaling dynamics. This problem becomes even more serious in the context of cell signaling studies that are specifically designed to quantify heterogeneity in single-cell gene regulation, where important RNA and protein copy numbers are themselves subject to the inherently random fluctuations of biochemical reactions. It is not clear how measurement noise should be managed in addition to other experiment design variables (e.g., sampling size, measurement times, or perturbation levels) to ensure that collected data will provide useful insights on signaling or gene expression mechanisms of interest. To address these fundamental single-cell analysis and experiment design challenges, we propose a computational framework that takes explicit consideration of measurement errors to analyze single-cell observations and Fisher Information Matrix-based criteria to decide between experiments. Using simulations and single-cell experiments for a reporter gene controlled by an HIV promoter construct, we demonstrate how our approach can analyze and redesign experiments

to optimally harvest fluctuation information while mitigating the effects of image distortion.

1 Introduction

Heterogeneity in signaling and gene expression at the single-cell level has wide-ranging biological and clinical consequences, from bacterial persistence ([40, 24, 25, 15]) and viral infections ([72, 85, 62, 8]) to tumor heterogeneity ([7, 46]). Beside genetic and environmental factors, a significant degree of heterogeneity is caused by biochemical noise ([47, 60, 16, 3]). Therefore, even genetically identical cells grown in the same experimental conditions may display variability in their response to environmental stimuli. This variability, often termed *intrinsic noise* when it originates within the pathway of interest or *extrinsic noise* when it originates outside the pathway of interest, obscures the underlying mechanisms when viewed through the lens of deterministic models and bulk measurements ([51]). Yet, this so-called noise can be highly informative when examined through the lens of single-cell measurements coupled with the mathematical modeling framework of the Chemical Master Equation (CME) ([26, 1, 75]). Through this joint experimental and modeling approach, mechanisms of signaling and single-cell gene expression can be explained, predicted ([52, 53, 51]), or even controlled ([50, 49, 5, **?**]).

In this paper, we are concerned with how and when stochastic models based on the CME could be inferred with high confidence from single-cell experiments such as flow cytometry or optical microscopy (such as single-molecule *in situ* hybridization, smFISH), from which data sets are produced in the form of fluorescence intensity histograms ([33, 42, 87, 44]) or molecular count histograms ([19, 58, 41]). These measurements, like any other experimental technique, can be corrupted by errors arising from imprecise detection or data processing methods. In flow cytometry, variations in the binding efficiency, binding specificity, or intensity of individual fluorescent reporters or probes, or the existence of background fluorescence, are unavoidable perturbations that may obscure the true copy number of RNA or protein ([52, 64, 80]). While smFISH data sets are generally considered to provide the'gold standard' for measuring transcriptional heterogeneity, the estimation of molecular copy numbers in single cells depends heavily on image segmentation and spot counting algorithms ([4, 56, 59, 35]) that involve several threshold parameters, which are set *ad*

hoc and typically vary from one expert to another. In addition, the sensitivity of smFISH measurements can be heavily affected by the length of the target mRNAs, the number of probes ([70]), or the number of hybridization steps ([86]).

The inescapable fact of measurement noise motivates intriguing questions on the design of single-cell experiments. For instance, under what conditions can detailed statistical measurement noise models and cheap single-cell measurements combine to replace accurate, yet more expensive experimental equipment? How approximate or coarse (and therefore fast) can image processing be while still faithfully retaining knowledge about parameters or mechanisms of interest? These questions are becoming more pressing as new advances in fluorescence tagging and microscopy technology are leading to more sophisticated experimental protocols to produce ever-increasing data on complex signaling and gene expression networks. Addressing these challenges is non-trivial, as it requires careful consideration of the potentially nonlinear combination of measurement noise and the biological question of interest.

The key contribution of this study is to provide new model-driven experiment analysis and design approaches (Fig 1) that include explicit consideration of probabilistic measurement errors in single-cell observations. The process begins with one or more hypotheses written in the form of stochastic gene regulation models (Fig 1A) with uncertain guesses for parameters or mechanisms. Predictions from these biophysical models are then coupled with empirically determined or physically estimated statistical models, known as *Probabilistic Distortion Operators* (PDOs), that explicitly estimate the effects of different types of measurement errors that could be temporal, discrete, non-symmetric, non-Gaussian, or even the result of their own stochastic process dynamics (Fig 1B). During model inference, the biophysical and measurement distortion models are simultaneously optimized to best explain the data (Fig 1C, right) and to quantify uncertainty in their respective parameters (Fig 1C, left). During experiment design (Fig 1D), sensitivity analysis is used to estimate which combinations of experimental conditions, sampling procedures, or measurement strategies can be expected to provide the most information to constrain the current set of hypotheses, and the procedure is iterated in subsequent rounds of experimentation and model refinement.

To address the specific question of model-driven single-cell experiment evaluation and design (Fig 1D), we adopt the framework of the Fisher Information Matrix (FIM). The FIM is the basis for a large set of tools for optimal experiment design in a myriad of science and engineering fields ([57, 17, 13, 63, 12, 83]),

and it has been employed in the study of identifiability and robustness of deterministic ODEs models in system biology ([29, 81]) as well as for designing optimal bulk measurement experiments ([18, 23, 38]). As an early application of the FIM to stochastic modeling of gene expression, Komorowski et al. ([37]) devised a numerical method to compute the FIM based on the Linear Noise Approximation (LNA) of the CME, which they used to demonstrate the different impacts of time-series, time snapshots, and bulk measurements to parameter uncertainties. There have been subsequent work on approximating the FIM with moment closure techniques ([63, 64]), with demonstrable effectiveness on designing optimal optogenetic experiments([65]). Under an assumption of ideal measurements, Fox and Munsky ([21]) recently extended the Finite State Projection (FSP) algorithm ([21]) to compute a version of the FIM that allows for time-varying and non-linear models that result in discrete, asymmetric, and multi-modal single-cell expression distributions. By extending the FSP-based FIM ([21]) to also account for realistic measurement errors, our new approach can help scientists to decide which combinations of experimental conditions and measurement assays are best suited to reduce parameter uncertainties and differentiate between competing hypotheses.

To verify our proposed approaches, we simulate data for a bursting gene expression model under many different types of measurement errors, and we show that the FIM correctly estimates the effects that measurement distortions have on parameter estimation. To demonstrate the practical use of our approaches, we apply them to analyze single-cell data for the bursting and deactivation of a reporter gene controlled by an HIV promoter construct upon application of triptolide (Trp). We show that the iterative use of FSP to fit distorted experimental data, followed by FIM analysis to design subsequent experiments can lead to the efficient identification of a well-constrained model to explain and predict gene expression.

2 Methods

2.1 Stochastic modeling of gene expression

The expression dynamics of gene or groups of genes in single cells are often modeled by stochastic reaction networks ([48, 26, 1]). For these, the time-varying molecular copy numbers in single cells are treated as a Markov jump process $\{\boldsymbol{X}(t)\}_{t\geq 0}$ whose sample paths $\boldsymbol{x}(t) = (x_1(t), \ldots, x_N(t))$ take place in discrete

multi-dimensional space, where $x_i(t)$ is the integer count of species i at time t. Each jump in this process corresponds to the occurrence of one of the reaction events R_k ($k = 1, \ldots, M$), which brings the cell from the state $\boldsymbol{x}(t_-)$ right before event time t to a new state $\boldsymbol{x}(t_+) = \boldsymbol{x}(t_-) + \boldsymbol{\nu}_k$, where $\boldsymbol{\nu}_k$ is the stoichiometry vector corresponding to the k-th reaction. The probabilistic rate at which each reaction occurs is characterized by its propensity (or reaction intensity) function, $\alpha_k(t, \boldsymbol{x}|\boldsymbol{\theta})$. The vector $\boldsymbol{\theta} = (\theta_1, \ldots, \theta_d)$ is a d-dimensional vector of *model parameters*. Intuitively, we interpret $\alpha_k(t, \boldsymbol{x}|\boldsymbol{\theta})\Delta t$ as the probability for reaction k to fire during the waiting interval $[t, t + \Delta t)$ for a sufficiently small waiting time Δt. The probability distributions $\boldsymbol{p_X}(t; \boldsymbol{\theta})$ of single-cell gene product copy numbers model what is often termed *intrinsic noise* in gene expression. These distributions are the solution of the chemical master equation (CME)

$$\frac{d}{dt}\boldsymbol{p_X}(t; \boldsymbol{\theta}) = \boldsymbol{A}(t, \boldsymbol{\theta})\boldsymbol{p_X}(t; \boldsymbol{\theta}), \tag{1}$$

where $\boldsymbol{A}(t; \boldsymbol{\theta})$ is the infinitesimal generator of the Markov process described above (see Supplemental Text S1 for detailed definition of $\mathbf{A}(t, \boldsymbol{\theta})$). Extrinsic noise can be modeled by assuming a probabilistic variation for the model parameters, then integrate (1) over that distribution ([65]). However, we focus on intrinsic noise for the current investigation.

2.1.1 Computing the likelihood of single-cell data

Consider a data set $\mathbf{D}$ that consists of N_c independent single-cell measurements $(t_1, \boldsymbol{c}_1), \ldots, (t_{N_c}, \boldsymbol{c}_{N_c})$ where $\boldsymbol{c}_i$ is the vector of molecule counts of cell i measured at time t_i. The likelihood function of the biophysical parameters $\boldsymbol{\theta}$ given the data set $\mathbf{D}$ is given by

$$L(\boldsymbol{\theta}|\mathbf{D}) = \prod_{i=1}^{N_c} p_{\boldsymbol{X}}(t_i, \boldsymbol{c}_i; \boldsymbol{\theta})$$

where $p_{\boldsymbol{X}}(t, \boldsymbol{x}; \boldsymbol{\theta})$ is the probability of observing molecular counts $\boldsymbol{x}$ at time t, obtained by solving the CME (1). Taking the logarithm of both sides, we have the log-likelihood function

$$\log L(\boldsymbol{\theta}|\mathbf{D}) = \sum_{i=1}^{N_c} \log p_{\boldsymbol{X}}(t_i, \boldsymbol{c}_i; \boldsymbol{\theta}), \tag{2}$$

which is mathematically and numerically more convenient to work with.

2.1.2 Sensitivity analysis

Taking the partial derivative of both sides of the CME (1) with respect to parameter θ_ℓ ($\ell = 1, \ldots, d$), we get

$$\frac{d}{dt}\left(\frac{\partial \mathbf{p}_{\mathbf{X}}(t;\theta)}{\partial \theta_\ell}\right) = \frac{\partial \mathbf{A}(t,\theta)}{\partial \theta_\ell}\mathbf{p}_{\mathbf{X}}(t;\theta) + \mathbf{A}(t,\theta)\frac{\partial \mathbf{p}_{\mathbf{X}}(t;\theta)}{\partial \theta_\ell}.$$

When the state space of the Markov process is finite, we can collect the equations above for $\ell = 1, \ldots, d$ along with the CME (1) to form a joint system involving the CME solution $\boldsymbol{p}_{\boldsymbol{X}}$ and its partial derivatives $\boldsymbol{s}_\ell \equiv \frac{\partial}{\partial \theta_\ell}\boldsymbol{p}_{\boldsymbol{X}}$, given by

$$\frac{\partial}{\partial t}\begin{bmatrix} \mathbf{p}(t;\theta) \\ \mathbf{s}_1(t;\theta) \\ \vdots \\ \mathbf{s}_d(t;\theta) \end{bmatrix} = \begin{bmatrix} \mathbf{A}(t,\theta) & \mathbf{0} & \ldots & \mathbf{0} \\ \partial_{\theta_1}\mathbf{A}(t,\theta) & \mathbf{A}(t,\theta) & \ldots & \mathbf{0} \\ \vdots & & \ddots & \\ \partial_{\theta_d}\mathbf{A}(t,\theta) & \mathbf{0} & \ldots & \mathbf{A}(t,\theta) \end{bmatrix}\begin{bmatrix} \mathbf{p}(t;\theta) \\ \mathbf{s}_1(t;\theta) \\ \vdots \\ \mathbf{s}_d(t;\theta) \end{bmatrix}.$$

This forward sensitivity system can be solved numerically with any standard ODE solver. When the state space is infinite, a truncation algorithm based on extending the Finite State Projection ([21]) can be applied to approximately solve the forward sensitivity system (see SI Section 1 for more details).

Knowing the sensitivity of the distribution to parameter changes then allows us to compute the sensitivity of the log-likelihood function with respect to biophysical parameters $\theta_1, \ldots, \theta_d$ using the formula

$$\frac{\partial}{\partial \theta_\ell}\log L(\boldsymbol{\theta}|\mathbf{D}) = \sum_{i=1}^{N_c}\frac{s_{\boldsymbol{X},\ell}(t,\boldsymbol{c}_i;\boldsymbol{\theta})}{p_{\boldsymbol{X}}(t,\boldsymbol{c}_i;\boldsymbol{\theta})},$$

where $s_{\boldsymbol{X},\ell}(t,\boldsymbol{x};\boldsymbol{\theta}) \equiv \partial p_{\boldsymbol{X}}(t,\boldsymbol{x};\boldsymbol{\theta})/\partial\theta_\ell$.

2.1.3 Modeling distortion of measurements

Let $\boldsymbol{y}(t)$ be the multivariate measurement made on a single cell at time t, such as the discrete number of spots in an smFISH experiment, or the total fluorescence intensity, such as from a flow cytometry experiment. Because of random measurement noise, $\boldsymbol{y}(t)$ is the realization of a random vector $\boldsymbol{Y}(t)$ that is the result of a random distortion of the true process $\boldsymbol{X}(t)$. The probability

mass (density) vector (function) $\boldsymbol{p}_{\boldsymbol{Y}}(t)$ of the discrete (continuous) observable measurement $\boldsymbol{Y}(t)$ is related to that of the true copy number distribution via a linear transformation of the form

$$\boldsymbol{p}_{\boldsymbol{Y}}(t;\boldsymbol{\theta}) = \boldsymbol{C}_{\boldsymbol{X}\to\boldsymbol{Y}}(t;\boldsymbol{\theta})\ \boldsymbol{p}_{\boldsymbol{X}}(t;\boldsymbol{\theta}). \tag{3}$$

Mathematically, $\boldsymbol{C}_{\boldsymbol{X}\to\boldsymbol{Y}}(t;\boldsymbol{\theta})$ functions as a Markov kernel, and we shall call it the *Probabilistic Distortion Operator* (PDO) to emphasize the context in which it arises. Considered as a matrix whose rows are indexed by all possible observations $\boldsymbol{y}$ and whose columns are indexed by the CME states $\boldsymbol{x}$, it is given entry-wise as

$$\boldsymbol{C}_{\boldsymbol{X}\to\boldsymbol{Y}}(t;\boldsymbol{\theta})(\boldsymbol{y},\boldsymbol{x}) := \Pr(\boldsymbol{Y}(t) = \boldsymbol{y}|\boldsymbol{X}(t) = \boldsymbol{x},\boldsymbol{\theta}), \tag{4}$$

where Pr stands for probability mass if $\boldsymbol{Y}$ is discrete or probability density if $\boldsymbol{Y}$ is continuous. Together, equations (1) and (3) describe single-cell measurements as the time-varying outputs of a linear dynamical system on the space of probability distributions on the lattice of N-dimensional discrete copy number vectors. The output matrix of this dynamical system is the PDO $\boldsymbol{C}_{\boldsymbol{X}\to\boldsymbol{Y}}$. If the observations $\boldsymbol{c}_1,\ldots,\boldsymbol{c}_{N_c}$ in the dataset $\boldsymbol{D}$ are assumed to be distorted according to the PDO $\boldsymbol{C}$, then the log-likelihood function (2) is changed into

$$\log L(\boldsymbol{\theta}|\mathbf{D}) = \sum_{i=1}^{N_c} \log p_{\boldsymbol{Y}}(t_i,\boldsymbol{c}_i;\boldsymbol{\theta}) \tag{5}$$

where $p_{\boldsymbol{Y}}(t,\boldsymbol{y};\boldsymbol{\theta})$ are point-wise probabilities of the distribution $\boldsymbol{p}_{\boldsymbol{Y}}(t;\boldsymbol{\theta})$ defined in (3).

There are many different ways to specify the PDO, depending on the specifics of the measurement method we want to model. In this paper, we demonstrate various examples where the PDO is formulated as probabilistic models that use simple distributions as building blocks (see Results and SI Section S3.2), or as deterministic binning/aggregation (Results and SI Section S3.2). One could even use a secondary CME to model the uncertain chemical kinetics of the measurement process, such as the random time needed to achieve chemical fixation in smFISH experiments or the dropout and amplification of mRNA that occurs during single-cell RNAseq experiments (SI Section S3.3), or distribution convolution to describe cell segmentation noise (SI Section S3.4). Despite the variety of ways these measurement noise models can be derived, they all lead to

the same mathematical object (i.e., the PDO), which allows computation of the FIM associated with that particular noisy single-cell observation approach, and the effects of all PDO can be analyzed using the same computational procedure as we describe next.

2.1.4 Computation of the Fisher Information Matrix for distorted experimental measurements

In practice, when closed-form solutions to the CME do not exist, a forward sensitivity analysis using an extension of the finite state projection algorithm can be used to evaluate the probability distribution $\boldsymbol{p_X}(t;\boldsymbol{\theta})$ and its partial derivatives $\partial_{\theta_j}\boldsymbol{p_X}(t;\boldsymbol{\theta})$ with respect to the kinetic parameters ([21]. Using eq. (3), we can transform these into the distribution $\boldsymbol{p_Y}(t;\boldsymbol{\theta})$ of $\boldsymbol{Y}(t)$. Furthermore, the sensitivity indices $\boldsymbol{s}^j_{\boldsymbol{Y}}(t;\boldsymbol{\theta}) = \partial_{\theta_j}\boldsymbol{p_Y}(t;\boldsymbol{\theta})$, of the observable $\boldsymbol{Y}$ are computable by back-propagating the sensitivities of the noise-free measurement distributions through the PDO,

$$\frac{\partial}{\partial\theta_j}\boldsymbol{p_Y}(t;\boldsymbol{\theta}) = \boldsymbol{C_{X\to Y}}(t;\boldsymbol{\theta})\frac{\partial}{\partial\theta_j}\boldsymbol{p_X}(t;\boldsymbol{\theta}) + \partial\theta_j\boldsymbol{C_{X\to Y}}(t;\boldsymbol{\theta})\boldsymbol{p_X}(t;\boldsymbol{\theta}). \tag{6}$$

Then, the Fisher Information Matrix (FIM) $\boldsymbol{F_{Y(t)}}(\boldsymbol{\theta})$ of the noisy measurements $\boldsymbol{Y}(t)$ at time t is computed by

$$[\boldsymbol{F_{Y(t)}}(\boldsymbol{\theta})]_{i,j} = \sum_{\boldsymbol{y}} \frac{s^i_{\boldsymbol{Y}}(t,\boldsymbol{y};\boldsymbol{\theta})s^j_{\boldsymbol{Y}}(t,\boldsymbol{y};\boldsymbol{\theta})}{p^{\boldsymbol{Y}}(t,\boldsymbol{y};\boldsymbol{\theta})}. \tag{7}$$

Details of the numerical approximation can be found in SI Section S3.1. In this formulation, we only need to solve the (usually expensive) sensitivity equations derived from the CME once, then apply relatively quick linear algebra operations to find the FIM corresponding to any new PDO for specific microscope, fixation protocol, or probe designs.

To convert the FIM between parameters defined in linear space to the same parameters in logarithmic space, we apply the transformation:

$$[\boldsymbol{F_{Y(t)}}(\log\boldsymbol{\theta})]_{i,j} = \theta_i\theta_j[\boldsymbol{F_{Y(t)}}(\boldsymbol{\theta})]_{i,j}. \tag{8}$$

2.1.5 Parameter estimation and uncertainty quantification

Models are fit to experimental data in Matlab, (R2021b, [**?**]) using an iterated combination of the builtin "fminsearch" algorithm (an implementation of the

Neldar-Mead simplex method, [**?**]) to get close to the MLE (i.e., to maximize the likelihood in Eq. 5) followed by a customized version of the Metropolis Hastings (MH) sampling routine,"mhsample" ([**?**]). All parameter searches were conducted in logarithmic space, and model priors were defined as lognormal with log-means and standard deviations as described in the main text. For the MH sampling proposal function, we used a (symmetric) multivariate gaussian distribution centered at the current parameter set and with a covariance matrix proportional to the inverse of the Fisher Information matrix (calculated at the MLE parameter set) and scaled manually during an initial 1000-sample run to achieve an approximately 20-40% proposal acceptance rate. MH chains were run for a burnin of 3,000 samples followed by 30,000 samples that were thinned by a factor of three. Convergence was checked by computing the autocorrelation function and verifying that the effective sample size was at least 1000 for every parameter in every MH chain. All data, model construction, FSP analysis, FIM calculation, parameter estimation, and visualization tasks were performed using the Stochastic System Identification Toolkit (SSIT) available at: https://github.com/MunskyGroup/SSIT using manuscript specific scripts provided in folder "CommandLine/Vo_et_al_2023".

2.2 Single-cell Labeling and Imaging

2.2.1 Cell Culture

The experiments presented here were performed on Hela Flp-in H9 cells (H-128). The generation of the H-128 cell line has previously been discussed ([78]). Briefly, Tat expression regulates the MS2X128 cassette-tagged HIV-1 reporter gene in H-128 cells. The HIV-1 reporter consists of the $5'$ and $3'$ long terminal repeats, the polyA sites, the viral promoter, the SD1 and SA7 splice donors, and the Rev-responsive element. Additionally, the MS2 coating protein conjugated with a green fluorescent protein (MCP-GFP), which binds to MS2 stem loops when transcribed, is expressed persistently by H-128 cells. Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific, 11960-044) supplemented with 10% fetal bovine serum (FBS, Atlas Biologicals, F-0050-A), 10 U/mL penicillin/streptomycin (P/S, Thermo Fisher Scientific, 15140122), 1 mM L-glutamine (L-glut, Thermo Fisher Scientific, 25030081), and 150 μg/mL Hygromycin (Gold Biotechnology, H-270-1) in a humidified incubator at 37°C with 5% CO_2.

2.2.2 smiFISH and Microscopy

Single-molecule inexpensive fluorescence *in situ* hybridization (smiFISH) was performed following a protocol previously described ([82, 30]). This technique is known as inexpensive, because the primary probes consist of a region binding the transcript of interest plus a common sequence established as FLAP, which is bound by a complementary FLAP sequence conjugated with a fluorescent dye following a short PCR-cyle (the primary and FLAP-Y-Cy5 probes used in this study were purchased from IDT, see Table 1). To perform smiFISH, H-128 cells were plated on 18 mm cover glasses within a 12-well plate ($\sim 10^5$ cells/well), 24 h before the experiment. Some samples were exposed to 5 μM triptolide (Sigma-Aldrich, 645900) for different incubation periods. Immediately after this drug treatment periods, samples were washed out twice with RNAse free 1XPBS, and fixed in 4% PFA at RT for 10 min, followed by 70% ethanol permeabilization at 4°C for least 1 h. After washing each sample with 150 μL of wash A buffer (Biosearch Technologies, SMF-WA1-60) for 5 min, each cover glass was set on a droplet (cells facing down) consisting of 45 μL hybridization buffer (Biosearch Technologies, SMF-HB1-10) and 1 μL of the duplex smiFISH probes (MS2-transcript-binding probe mix + FLAP-Y-binding region annealed to FLAP-Y-Cy5) in a humidified chamber at 37°C overnight. The following day, samples were placed in a fresh 12-well plate, and the cells (facing up) were incubated twice in wash A buffer at 37°C for 30 min, first alone, and then containing DAPI. Finally, cells were incubated with wash B buffer (Biosearch Technologies, SMF-WB1-20) at RT for 5 min, and then mounted on a 15 μL drop of Vectashield mounting medium (Vector Laboratories, H-1000-10), and sealed with transparent nail polish.

Fluorescent images were acquired with an Olympus IX81 inverted spinning disk confocal (CSU22 head with quad dichroic and additional emission filter wheel to eliminate spectral crossover) microscope with 60x/1.42 NA oil immersion objective. Confocal z-stacks (0.5 μm step-size, 27 stacks in each channel) were collected. Each field of view was imaged using four high-power diode lasers with rapid (microsecond) switcher (405 nm for DAPI, 488 nm for MS2-MCP-GFP reporter, 561 nm for cytosol marker, and 647 nm for smiFISH MS2-Cy5, exposure time of 100 ms for all except smiFISH channel that was 300 ms) when samples had cytosol marker or three lasers for samples without the marker. The system has differential interference contrast (DIC) optics, built-in correction for spherical aberration for all objectives, and a wide-field Xenon light source. We used an

EMCCD camera (iXon Ultra 888, Andor) integrated for image capture using Slide book software (generating 60× images with 160 nm/pixel, and 100x images with 96 nm/pixel). The imaging size was set to 624 x 928 pixels^2.

2.2.3 smiFISH image processing

In this study, we employed Python to implement an image processing pipeline comprising three steps: cell segmentation, spot detection, and data management (as previously described in [66]). Nuclear segmentation on the DAPI channel (405nm) was carried out using Cellpose ([76]) with a 70-pixel diameter as an input parameter. Spot quantification for both the MS2-MCP-GFP reporter channel (488 nm) and the smiFISH MS2-Cy5 channel (647 nm) was performed independently, using BIG-FISH software ([32]). The spot quantification procedure employed a voxel-XY of 160 nm, a voxel-Z of 500 nm, and spot radius dimensions of 160 nm and 350 nm in the XY and Z planes, respectively. This process included the following steps: i) the original image was filtered using a Laplacian of Gaussian filter to improve spot detection. ii) Local maxima in the filtered image were then identified. iii) To distinguish between genuine spots and background noise, we implemented both an automated thresholding strategy and a manual approach involving the use of multiple threshold values between 400 and 550. The intensity thresholds (550 for MS2-MCP-GFP and 400 for smiFISH MS2-Cy5) that resulted in the highest number of co-detected spots in both channels were chosen. Data management involved organizing all quantification data into a single dataset, including information on the specific image and cell in which the spots were detected. All image processing codes are available from `https://github.com/MunskyGroup/FISH_Processing`.

3 Results

Many models have been used to capture and predict observations of single-cell heterogeneity in gene expression ([52, 74, 14, 45, 53, 73, 69]). When selecting an experimental assay to parameterize such models, one is faced with several choices, each with its own characteristic measurement errors ([61]). Here, we start by introducing several mathematical forms for probabilistic distortion operators (PDOs) that can quantify these measurement errors. We then use a model and simulated data to show how different measurement errors can affect model identification, and we show how this can be corrected through consideration of

the PDO in the estimation process. Next, we show how models and PDOs can be used in the framework of Fisher Information in iterative design of single-cell experiments for efficient identification of predictive models. Finally, we illustrate the practical use of the PDO, model inference, and FIM based experiment design on the experimental investigation of bursting gene expression from a reporter gene controlled by an HIV promoter.

3.1 Distorted single-cell measurements sample a probability distribution that is the image of their true molecular count distribution through a linear operator

Most parameters needed to define single-cell signaling or gene expression models cannot be measured directly or calculated from first principles. Instead, these must be statistically inferred from datasets collected using single-molecule, single-cell experimental methods such as smFISH ([58, 27]), flow cytometry ([42, 44]), or live-cell imaging ([77, 22, 20]). In this work, we focus on the former two experimental approaches in which collected data consists of independent single-cell measurements taken at different times. Here, we consider measurement distortion effects corresponding to probe binding inefficiency and spot detection for smiFISH experiments, and in the supplemental text, we extend this to consider effects of reporter fluorescence intensity variability in flow cytometry experiments (Section S3.1), data binning (Section S3.2), effects of competition with non-specific probe targets (Section S3.3), and effects of segmentation errors (Section S3.4).

We first consider five formulations to define the measurement distortion matrix (cf. eq. (4) in Methods, and illustrated in Fig 2), corresponding to scenarios in which experimental errors arise from either inefficient mRNA detection, additive false positives, or combinations. Specifically,

1. The first model supposes that $\tilde{y}$ is obtained from a "lossy" spot-counting process applied on images taken in an smFISH experiment. We model $y|\boldsymbol{x} = n$ with a binomial distribution $B(p_{\text{detect}}, n)$, where each spot has a chance $p_{miss} := 1 - p_{\text{detect}}$ of being ignored by the counting algorithm, resulting in underestimation of the true mRNA copy number. In the context of optical microscopy, such a distortion might result from quantifying spots at a single plane, where p_{detect} might represent the fraction of the imaged section compared to the full volume of the cell, but similar error models

have also been proposed in the context of single-cell RNA sequencing ([39, 6]). We choose $p_{\text{detect}} = p_{miss} := 0.5$ in this example, but it is straightforward to generalize to different values (see Supplemental section S3.2). We call this distortion "Missing Spots". Its PDO is illustrated in Fig 2A.

2. The second model is a simple variation of the first model in which p_{detect} varies with the number of mRNA molecules n, specifically, $p_{detect} := 1.0/(1.0 + 0.01n)$. This formulation implies that spot detection rate degrades as the number of mRNA molecules in the cell increases, which may correspond to the effect of co-localization and/or image pixelation which could cause the undercounting of overlapping spots. We call this model "Missing spots with varying rate", and its PDO is illustrated in Fig 2B.

3. The third model assumes that $\tilde{y}$ is the output of a spot-detection process contaminated by false positives, e.g., due to background fluorescence noise in the image that can appear to be spots. We model these false positives by additive Poisson noise, making $\tilde{y} = \tilde{n} + \tilde{e}$ where $\tilde{e} \sim \text{Poisson}(\lambda)$. We choose $\lambda := 10$ for illustration. We call this model "Poison Noise." Its PDO is illustrated in Fig 2C.

4. The fourth model is a simple extension of the third model in which the number of detected spots given a true number, n, is a Poisson distribution where λ varies with n. Specifically, $\lambda := \max(0, \lambda_0 + \lambda_1 n)$, where λ_0 is Poisson noise, and λ_1 is the Poisson detection rate. We call this model "Poisson Observation", and its PDO, $\mathbf{C}_{\text{PO}}$, is illustrated in Fig 2D.

5. The fifth model concerns fluorescent intensity integration measurements (such as those used in flow cytometry). We use the model proposed in ([52]), in which $\tilde{y} = \kappa \cdot \tilde{n}_{RNA} + \eta_{\tilde{n}_{RNA}} + \tilde{\varepsilon}_{BG}$, where $\tilde{\eta}_{\tilde{n}_{RNA}} \sim \mathcal{N}(0, \tilde{n}_{RNA}\sigma^2_{probe})$ models fluorescent heterogeneity and $\tilde{\varepsilon}_{BG} \sim \mathcal{N}(\mu_{BG}, \sigma^2_{BG})$ is the background noise. For all simulations, we use $\mu_{BG} = 200$, $\sigma_{BG} = 400$, $\kappa = \sigma^2_{probe} = 25$. The PDO is a hybrid matrix with discrete columns (i.e., its domain are probability vectors over discrete CME states) and continuous rows (its range consists of continuous probability density functions over the range of fluorescence intensities). This PDO, which we label "Integrated Intensity" is illustrated in Fig 2E.

We stress that these PDOs are provided as just a few of many possible distortions that could be modeled using the proposed framework. Other, more complex

distortion operators are discussed in Supplemental Text S3, including one where a secondary CME is employed to model the uncertain chemical kinetics of the measurement process (Section S3.3) and another to describe cell merging due to image segmentation errors (Section S3.4).

3.1.1 Measurement noise introduces bias and uncertainty into model identification

To illustrate the impact of measurement distortion on parameter estimation and our use of the PDO formalism to mitigate these effects, we begin with an analysis of the random telegraph model (Fig 3A), one of the simplest, yet most commonly utilized models of bursty gene expression ([55, 58, 77, 67, 68, 39]). In this model, a gene is either in the inactive or active state, with transition between states occurring randomly with average rates k_{ON} (to activate the gene) and k_{OFF} (to deactivate the gene). When active, the gene can be transcribed with an average rate k_r to produce mRNA molecules, each of which degrades with an average rate γ.

To demonstrate how measurement distortions affect parameter identification, and why explicit measurement error modeling is necessary, we use the bursting gene expression model to simulate mRNA expression data where each cell is distorted by the "Missing spots with varying rate" effect above (PDO reproduced in Fig 3B). Each data set consists of five batches of 1,000 independent single-cell measurements that are collected at five equally-spaced time points $j\Delta t, j = 1, 2, 3, 4, 5$ with $\Delta t = 30$ minutes. We considered two methods to fit the telegraph model to these data based on the Maximum Likelihood Estimator (MLE): one in which the likelihood function ignores measurement noise, and one where measurement noise modeled by the PDO is incorporated into the likelihood function (see Supplemental text S2 for their formulations). If one fails to account for measurement uncertainty, these fits produce strongly biased estimates for the RNA production and degradation rates as seen in Fig 3C,D (red). Because this bias is inherent to the measurement technique, it cannot be corrected simply by averaging over more experiments. On the other hand, using the distortion correction method, Fig 3C,D (dark green) shows that the inaccurate spot counting procedure can be corrected by explicitly accounting for measurement uncertainty in the modeling phase.

Figures 3C,D also demonstrate that the Fisher Information Matrix computed for the noisy measurement (see eq. (7)) produces a close approximation to the

covariance matrix of the MLE (compare magenta and black ellipses). This suggests that the FIM can provide a relatively inexpensive means to quantify the magnitude and direction of parameter uncertainty, a fact that will be helpful in designing experiments that can reduce this uncertainty, as we will explore next.

3.1.2 Fisher Information Matrix Analysis reveals how optimal experiment design can change in response to different measurement distortions

Having demonstrated the close proximity between our FIM computation and MLE uncertainty for simulated analyses of the bursting gene expression model, we next ask whether the sampling period Δt could be tuned to increase information from measurements. Recall that our simulated experimental set-up is such that measurements could be placed at five uniformly-spaced time points $t_j = j\Delta t, \;\; j = 1, 2, 3, 4, 5$, with the sampling period Δt in minutes, and that at each time point we collect an equal number n of single-cell measurements, chosen as $n := 1000$. We find the optimal sampling period Δt for each measurement method, and compare the most informative design that can be achieved for each class. Here, we define 'optimal' in terms of the determinant of the Fisher Information Matrix, the so-called D-optimality criterion, whose inverse estimates the volume of the parameter uncertainty ellipsoid for maximum likelihood estimation ([2]). Figures 4A,B shows the information volume to five kinds of noisy measurement described above (Fig 2), in addition to the ideal noise-free smiFISH, at different sampling rates. We observe that every probabilistic distortion to the measurement decreases the information volume (but to different extents), and that each measurement method results in a different optimal sampling rate. In Fig 4C-D, we plot the three-sigma (i.e., 99.7%) confidence ellipsoids of the asymptotic distribution of MLEs projected on the $\log_{10}(k_{ON}) - \log_{10}(k_{OFF})$ plane and the $\log_{10}(k_r) - \log_{10}(\gamma)$ plane.

3.2 FIM and PDO Analysis of Experimental Measurements for HIV-1 Promoter Bursting Kinetics

To provide a concrete example for the use of the FIM and PDO in practice, we performed single-cell measurements to quantify the relative measurement distortion between different single-mRNA labeling strategies in HeLa (H-128) cells (see Methods). We expressed a transcription reporter gene with 128 repeats of the MS2 hairpin, and we simultaneously used both MCP-GFP (green labels

in Fig 5A) and smiFISH MS2-Cy5 (magenta labels in Fig 5A) to target the MS2 repeats. In each cell, both approaches detect similar patterns for the number and spatial locations of mRNA within the nuclei (Fig 5B-G). For our particular choice of image processing algorithm ([66, 76, 32]) and intensity threshold for spot detection (see Methods) and a two-pixel (x,y,z) Euclidean distance threshold for co-localization detection, after analyzing 155 cells in steady-state conditions, we found that 34.8% of mRNA spots (12,231 out of 35,151 total) were detected in both channels (e.g., spots denoted by white triangles in Fig 5A). However, many spots (27.7%, 9,750 spots) are detected only using smiFISH (e.g., those denoted with magenta triangles) and 37.5% (13,170 spots) are only detected using the MS2-MCP labels (e.g., green triangles). Due to differences in label chemistry and image analysis, the quantified distribution of mRNA expression depends heavily upon which assay is utilized (compare Figs 5B-D to 5E-G) a fact that suggests a need for consideration of measurement distortions in subsequent analysis.

3.2.1 PDO measurement noise parameters can be calibrated using single-cell experiments with multiple measurement modalities

To parameterize a PDO, we took total, MCP-GFP-only, and smiFISH-only spot count data for 155 cells at t=0 and 66 cells at t=300 min after Trp. We then define the total of all detected spots as the "true" measurements $\{x_1, \ldots, x_{N_c}\}$ and consider the smiFISH or MCP-GFP labeling results as two different groups of distorted observations $\{y_1, \ldots, y_{N_c}\}$. Using the simple "Poisson Observation" PDO from above, $\boldsymbol{C}_{\mathbf{X}\to\mathbf{Y}}(\lambda_0, \lambda_1)$, the log-likelihood to observe $\{y_i\}$ given $\{x_i\}$ is computed as

$$\log L(\{y_i\}|\boldsymbol{C}, \{x_i\}) = \sum_{i=1}^{N_c} \log C_{\boldsymbol{X}\to\boldsymbol{Y}}\left(y_i, x_i|\lambda_0, \lambda_1\right).$$

Using this equation, we find the likelihood of the observations is maximized for parameters ($\lambda_0 = 0$,$\lambda_1 = 0.70$) for MCP-GFP data and ($\lambda_0 = -1.6$,$\lambda_1 = 0.65$)[1] for the smiFISH data. It is instructive to note that in each case, λ_1 matches closely to the fraction of spots detected in the corresponding channel (0.72 for MCP-GFP and 0.63 for smiFISH). Figures 5B,E show the contours of the corresponding PDO for the MCP-GFP and smiFISH data, respectively, and Figs

[1] A negative value for $\lambda = \lambda_0 + \lambda_1 n$, which can occur when n is small and λ_0 is negative, is defined to result in a Poisson distribution with a small positive mean of 10^{-7}.

5C,D,F,G show the PDF and CDF for the data at t=0 (blue lines) compared total mRNA data (red lines). From the figures, we find the distortion model does an excellent job to calibrate between the total mRNA and MCP-GFP- or smiFISH-detected spot counts (compare blue and green lines). We next verified that the PDO remains constant by showing that the same model and same parameters also accurately reproduce the difference in total mRNA and MCP-GFP or smiFISH measurements at a held out time of 18 min after application of 5 μM Trp (see Fig 6).

3.2.2 The PDO allows estimation of predictive bursting gene expression model parameters from distorted data

We next asked if using the estimated PDO while fitting the smiFISH- or MCP-GFP-only data would enable the identification of an appropriate model to predict the total spot count. Based on previous observations ([20]), we proposed a 3-state bursting gene expression model (Fig 7A) where each of two alleles can occupy one of three states: (S1=OFF) where no transcription can occur; (S2=Poised) where the promoter is ready to begin transcription; and (S3=Active) where transcripts are produced in rapid bursts of mRNA expression. The model contains six parameters: k_{ON} and k_{OFF} are the promoter transition rates between the OFF and Poised states; ω and k_{Ex} are the burst frequency and burst exit rates; and k_r and γ are the transcription and mRNA degradation rates, respectively. Based on previous observation ([20]) that triptolide (Trp) represses transcription after an average of 5-10 min needed for diffusion of Trp to the promoter and completion of nascent mRNA elongation and processing, we model the Trp response as a complete inactivation of transcription ($\omega \to 0$) that occurs at t=5 min.

To specify a prior guess for the model parameters, we considered published values from ([20]), where we used live-cell imaging of ON and Poised transcription sites to determine the burst frequency $\omega \approx 0.2$ min^{-1} and that k_r and k_{Ex} are too fast to be estimated independently, but are related by a burst size of $k_r/k_{\mathrm{Ex}} = \beta \approx 7.1$ mRNA/burst. Also, based on observations in ([20]) that transcription sites remain in the OFF or Poised/Active states for long periods of 200 minutes or more, we assumed that k_{ON} and k_{OFF} would be too slow to estimate except under much longer experiments. We therefore estimated $k_{\mathrm{ON}} = k_{\mathrm{OFF}} = 10^{-4}$ min^{-1}, but we sought only to estimate the relative rate for k_{OFF}. We estimated a typical mammalian mRNA half-life of 60 min yielding a rate $\gamma = 5.8 \times 10^{-3}$ min^{-1}. With these baseline values as rough estimates, we

assumed a log-normal prior distribution with a standard log deviation of one-half order of magnitude from the literature-based values for parameters $\{\omega, \beta, \gamma\}$ and a full order of magnitude for the more approximate value for $k_{\text{OFF}}\}$. These initial parameter guesses and prior uncertainties are summarized in Table 2(column 3).

Having specified parameter priors, we then took our single-cell data for 155 cells at steady state (t=0) and 66 cells at t=300 min after application of 5μM Trp, and we applied the Metropolis-Hasting (MH) algorithm (10,000 samples) to estimate rates and uncertainties for the four free parameters. As above, we assumed that the total spot count analysis provided the 'true' spot count, and we considered three estimation problems using either the total spot counts or only the smiFISH or MCP-GFP data, each using the empirically estimated distortion operators from before (e.g., Fig 5B for MCP-GFP or Fig 5E for smiFISH). Table 2 presents the MLE parameter values after this initial stage, and Figs 7B-D compare the resulting fits of the model to the data. Figures 7E,F show the scatter plots of parameter uncertainties for the parameter identification using either the total spot count or the smiFISH data with the empirical distortion operator (results were similar for MCP-GFP). From Figs 7B-D and Table 2, one can see that all fits do an excellent job of matching their intended data, and when fit using the PDO, parameters and model predictions based only on the smiFISH and MCP-GFP data match well to the total spot count analysis.

3.2.3 The PDO-corrected FIM accurately estimates parameter uncertainty after experimental analysis of the bursting gene expression model

We next used the best fit model to compute the FIM for the corrected MCP-GFP and smiFISH measurements. Given that we only collected a single experimental data set for each time point, it is not possible to directly compare the FIM to the spread of MLE estimates like we could for simulated data. However, in Figs 7E,F for the total spot count and PDO-corrected smiFISH data, we can compare the uncertainty predicted by the FIM analysis to the posterior uncertainty of our parameters given our data. This comparison shows that the FIM does an excellent job to estimate the direction and magnitude of parameter uncertainties (compare overlapping black and magenta ellipses in Figs 7E,F.

3.2.4 PDO-corrected FIM analysis accurately ranks designs for most informative transcription-repression experiment

To demonstrate the practical use of FIM for experiment design, we next asked what design for a Trp-based transcription repression experiment would be best to improve our model of mRNA expression identified in Fig 7. We restricted the set of possible experiments to the previous data set (155 cells at t_1=0 min and 66 cells at t_2=300 min) plus an additional set of 100 cells at a new time t_3 after Trp application, where t_3 could be any time in the allowable set $t_3 \in [0, 6, 12, 18, \ldots, 1200]$ min.

We drew 20 random parameter samples from the previous 10,000-sample MH chains that were estimated using data at t_1=0 and t_2=300 min for total spots and PDO-corrected smiFISH and MCP-GFP measurements. We computed the FIM for each parameter set and for every potential choice for t_3. Figure 8A shows the determinant of the expected covariance of MLE parameters ($\Sigma \approx \mathbf{FIM}^{-1}$) versus t_3 assuming perfect observations of all spots (left) or distorted observations of just MCP-GFP (middle) or smiFISH (right). As expected, distortion always increases expected uncertainty (compare Figs 8A(left) with 8A(middle or right)). We also find that the optimal time for the experiment can be highly dependent on the particular assay. In particular, larger distortions require earlier sampling times to prevent mRNA expression from falling below the noise introduced by the distortion.

Although full validation of these designs would require a host of experiments that are beyond the scope of the current investigation, we analyze a new data set of 106 cells taken at $t_3 = 18$ min to compare experiment designs with time combinations of (0,18) min, (0,300) min, and (0,18,300) min. Figure 8B shows FIM predictions of uncertainty using parameters from the original fit to the (0,300) min data (blue) compared to the uncertainty estimate using parameters fit to the final data set with all three time points (red). The original and final FIM are in excellent agreement for all three experiment designs (different groups of blue and red bars) and for observations of total spots (left) or for only MCP-GFP spots (middle) or smiFISH spots (right). Moreover, upon using the MH to estimate the posterior parameter distributions, Fig 8C shows that both the original and the final FIM correctly predict the trend of parameter uncertainties for each of the different experiment combinations. For example, the FIM correctly predicts the result that a set of (155,66) cells at (0,300) min is far more informative than the larger set of (155,106) cells at (0,18) min. Finally,

Fig 8D shows the joint parameter estimation for the different combinations of fitting times and measurement modality (fits to the time set (0,18) min result in much greater uncertainty and bias as predicted by the FIM and are not shown). Once again Fig 8D confirms that including the $t = 18$ min data provides only a small improvement in parameter estimation (compare magenta and black ellipses), a result that was correctly predicted by the FIM in Figs 8A,B.

4 Discussion

Parameter estimation is a major step in constructing quantitative models for all physical and biological processes, and many such models for gene regulation and cell signaling are now being inferred from quantitative single-cell imaging experiments. Such measurements are subject to errors in labeling and image processing, for which different labels or different image processing can yield different measurement values (e.g., see Figs 5 and 6). We have demonstrated that these experimental distortions can can be mathematically described using a general class of Markov kernels that we dub *Probability Distortion Operators* (PDOs, Figs 2,5B,E), and that these PDOs lead to changes in the estimated parameters that are reflected not only in the magnitudes but also in the directions of their uncertainties (Figs 4,**??**E,F). We have introduced a new computational framework to systematically account for these noise effects, and provide a first step on how this could be integrated into the interpretation of single-cell experiments (Figs 5-8). Our results indicate that an appropriate statistical analysis coupled with a careful tuning of experimental design variables can meaningfully compensate for measurement noise in the data. For example, our results indicate that, when used iteratively with small sets of experimental data (e.g., as few as a couple hundred cells at only two points in time), FIM analysis can correctly predict which subsequent experiments are most likely to be informative and which are unlikely to provide additional insight into model parameters (Fig 8). Insight provided by such an integration of models and experiments could allow for better allocation of experimental resources first by helping to eliminate estimation biases that are due to experimental noise and second by helping to identify specific experimental conditions that are less prone to be impacted by those measurement artifacts.

Although the FIM is a classical tool for optimal experiment design, which has been used extensively in myriad areas of science and engineering ([57, 17,

13, 63, 12, 83]), it has not seen widespread adoption in the investigation of biological processes, which are heavily subject to heterogeneities. In the context of gene expression modeling, ([37]) proposes a method to approximate the FIM for single-cell experiment data based on the Linear Noise Approximation (LNA). Alternatively, the FIM could be approximated by using moment closure techniques ([63, 64]). These approaches work well in the case of high molecular copy numbers, but they break down when applied to systems with low molecular copy numbers ([21]). Moreover, it is not clear if or how such approximations can be modified to consider measurement noise and data processing noise that are non-additive, asymmetric, or non-Gaussian as is the case for many biological distortions. To circumvent these issues, our alternative framework directly analyses the probability distributions of the noisy measurements. Explicitly modeling the conditional probability of the observation given the true cell state allows us to express the observation distribution as a linear transformation of the true process distribution that is computable using the finite state projection. This leads to a systematic way to develop composite experimental designs that combine measurements at different fidelity and throughput levels to maximize information given a budgetary constraint.

Adjustments are easily made to the formulations proposed here for the probabilistic distortion operators of smFISH measurements to capture many different aspects of experimental error. For example, through experimental analysis of mRNA counts using different modalities (smiFISH and MCP-GFP labeling), we demonstrated how one could construct the PDO based on empirical samples of the joint distribution for the two modalities (Fig 8). Similarly, one could examine different microscopes, different cameras, different laser intensities, different image processing pipelines, or any of a number of permutations to compare and quantify differences between high-fidelity and noisy measurements of signaling or gene expression phenomena. In the current work, we have relied on specific parameterized statistical distributions (e.g. binomial or Poisson as examined in Figs 3,4,5 or data binning or categorization in Section S3.2) or mechanistic distortion models (e.g., integrated fluorescence intensity in Section S3.1, stochastic binding kinetics in Section S3.3, or segmentation errors in Section S3.4) to formulate the PDO. However, one could also envision parameter-free statistical methods based on recent probabilistic machine learning methods (e.g., normalizing flows ([36]) for modeling the conditional distribution $p(y|x)$ of a noisy label y given a vector x of features. In either case, one would need only to estimate the PDO once for each combination of labeling, microscopy, and

imaging techniques and then be ready to apply that PDO to many different biological processes, models, parameter sets or experiment designs. In the current investigation, we have assumed for simplicity that the PDO is known and constant in time. However, the formulation of the PDO and the FIM (Eqs. 3-7) are sufficiently general such that both of these assumptions can be relaxed. With these relaxations, the FIM could be used either to guide experiment designs to aid the simultaneous identification of both the parametrized PDO and the gene regulation model itself, or provide clear guidance that such distortions lead to degeneracy in the FIM and indicate which non-identifiable parameter combinations (i.e., the null space of the FIM) are most in need of model reduction. A full exploration of these more complex considerations is left for future investigations.

Although the presented approach is versatile and can in principle be applied to any stochastic gene regulatory network, its practical use depends on the ability to compute a reasonable approximation to the solution of the chemical master equation (CME) as well as its partial derivatives with respect to model parameters. Fortunately, there are now many relevant stochastic gene expression models for which exact or approximate analytical expressions for the CME solution are available ([55, 71, 79, 31, 10, 84]). Furthermore, the FSP and similar approaches have been used successfully to solve the CME for many non-linear and time-inhomogeneous regulatory models for which closed-form solutions do not exist ([74, 14, 45, 53, 73, 69]). Admittedly, given the complexity of gene regulatory networks in single cells, there are always stochastic gene regulatory models whose direct CME solutions are beyond reach. Nevertheless, continued advancements in computational algorithms ([34, 9, 27, 43, 11, 28, 54]) are enlarging the set of tractable CME models, which in turns can help accelerate the cycling between data acquisition, model identification, and optimal experiment design in single-cell studies.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

Conceptualization: BM. Theory and computational modeling: HDV and BM. Performed experiments/collected data: LSFQ. Image processing: LU. Writing: HDV, LSFQ, LUA, and BM. Resources, supervision, and funding acquisition: BM.

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Data Availability Statement

Python code for the simulation and FIM analysis of the two-state bursting gene expression model is available at:

https://github.com/voduchuy/NoisyMeasurementFIM

Python code for processing single-cell images is available at:

https://github.com/MunskyGroup/FISH_Processing

Matlab code for analyzing Tryptolide data using the three-state model is available at:

https://github.com/MunskyGroup/SSIT/,

where all data and codes for fitting and visualization of results are available in the folder "CommandLine/Vo_et_al_2023".

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Tables

Table 1: **smiFISH probe sequences.** Each primary probe has an added common FLAP-Y binding sequence (TTACACTCGGACCTCGTCGACATGCATT)

Probe name	Sequence	Type
MS2-P1	ATCCGTTCAAAGGCCTATTGGTCCTTTGC	Primary
MS2-P2	ATCCTCATGTACTAGCTTCCGAGTAATCT	Primary
MS2-P3	AGCTTGGGTTATTACTCCAAGATCACCGC	Primary
MS2-P4	TCCTGATAGGCTGTACTCATGCCTAC	Primary
MS2-P5	GGGTAATCATTCTAGTGATATGATTCTGTGCC	Primary
MS2-P6	GATCATACCGTATTCGTGTATGATTACATGGG	Primary
MS2-P7	TAATTGTGCGGTCGCTGACTGATACTTCTA	Primary
MS2-P8	TATTTCTCTCTGATACGCTGCGTACTCG	Primary
MS2-P9	ACCCTAATGGTGTTTACAAATGGTGGTAGTCC	Primary
MS2-P10	TGTTATTCTAATCCGTCACTATTGTTGACGGG	Primary
MS2-P11	AAGCCTTACTGATGCTTCCGGTCCATT	Primary
MS2-P12	ATCCTCATGTACTAGAGGCTCGGTACTC	Primary
FLAP Y-Cy5	AA TGC ATG TCG ACG AGG TCC GAG TGT AA	Secondary

Table 2: **Parameter priors, MLE estimates, Uncertainties upon Initial Fit**. Initial estimates after fitting to data at t =(0,300) min. MH results are from a chain of 10,000 samples. All parameter values and standard deviations values are shown in $\log_{10}$.

Parameter	Quantity	Prior	Total	MCP-GFP	smiFISH
k_{OFF} (s^{-1})	$\log_{10}(\cdot)$	-4.0	-3.856	-3.606	-3.630
	$\sigma_{p/MH}$	1.0	0.058	0.059	0.052
	σ_{FIM}	-	0.039	0.051	0.046
ω (s^{-1})	$\log_{10}(\cdot)$	-0.70	-1.705	-1.366	-1.162
	$\sigma_{p/MH}$	0.5	0.088	0.152	0.104
	σ_{FIM}	-	0.078	0.108	0.120
β (s^{-1})	$\log_{10}(\cdot)$	0.85	1.931	1.679	1.493
	$\sigma_{p/MH}$	0.5	0.084	0.146	0.103
	σ_{FIM}	-	0.060	0.113	0.115
γ (s^{-1})	$\log_{10}(\cdot)$	-2.2	-2.181	-2.186	-2.223
	$\sigma_{p/MH}$	0.5	0.027	0.036	0.021
	σ_{FIM}	-	0.022	0.025	0.023

Table 3: **Parameter priors, MLE estimates, Uncertainties upon Final Fit**. Final estimates after fitting to data at t =(0,18,300) min. MH results are from a chain of 10,000 samples. All parameter values and standard deviations values are shown in $\log_{10}$.

Parameter	Quantity	Prior	Total	MCP-GFP	smiFISH
k_{OFF} (s^{-1})	$\log_{10}(\cdot)$	-4.0	-3.805	-3.623	-3.638
	$\sigma_{p/MH}$	1.0	0.046	0.046	0.042
	σ_{FIM}	-	0.036	0.041	0.037
ω (s^{-1})	$\log_{10}(\cdot)$	-0.70	-1.655	-1.432	-1.212
	$\sigma_{p/MH}$	0.5	0.091	0.144	0.094
	σ_{FIM}	-	0.045	0.094	0.094
β (s^{-1})	$\log_{10}(\cdot)$	0.85	1.902	1.711	1.501
	$\sigma_{p/MH}$	0.5	0.083	0.140	0.098
	σ_{FIM}	-	0.050	0.089	0.093
γ (s^{-1})	$\log_{10}(\cdot)$	-2.2	-2.178	-2.195	-2.236
	$\sigma_{p/MH}$	0.5	0.024	0.035	0.019
	σ_{FIM}	-	0.020	0.025	0.024

Figures

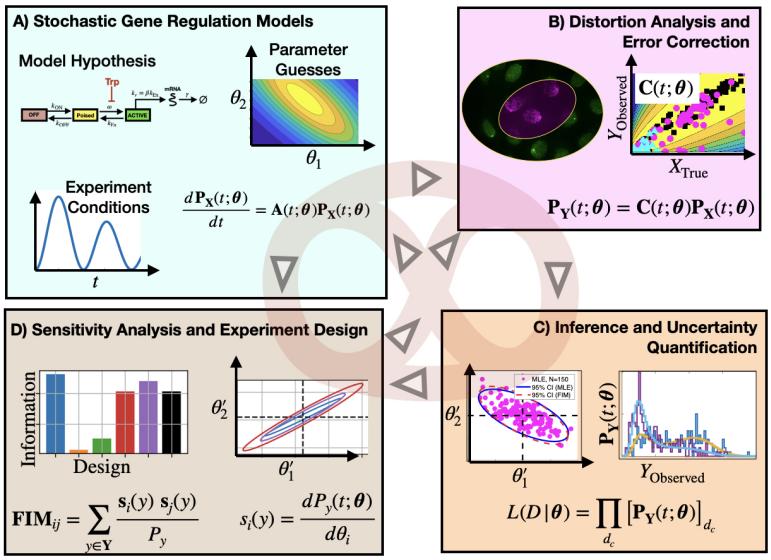

Figure 1: **Proposed approach to analyze and design single-cell experiments under measurement distortion. Box (A):** One or more mechanistic model hypotheses are proposed to describe gene regulation processes and are associated with prior parameter guesses. These models are combined with experiment design considerations (e.g., environmental, sampling, and measurement conditions) and the chemical master equation framework is used to predict statistics of single-cell gene expression. **Box B**: Empirical data and physical models are used to define a probabilistic distortion operator **C** to estimate how measurement errors (e.g., labeling inefficiencies, resolution limitations, or image processing errors) affect observations. **Box C**: Searches over model and PDO parameter space are conducted to identify model and parameter combinations that maximize the likelihood of the observed (i.e., distorted) data. **Box D**: Sensitivity analysis and the Fisher Information Matrix are computed and used to analyze the information content for different mechanistic models, distortion effects, or experiment designs. By estimating parameter uncertainties in the context of different forms of measurement distortion, subsequent experiment designs can help to alleviate measurement noise distortion effects (return to **Boxes A**, **B**, and **C**).

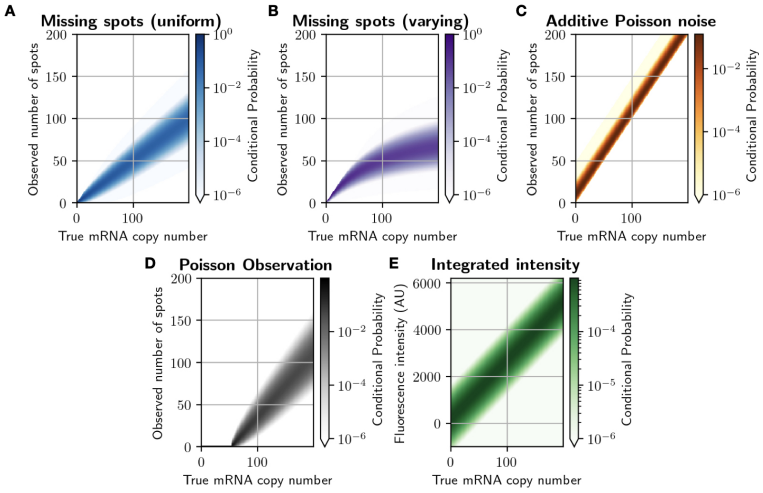

Figure 2: **Probabilistic distortion operators**. **(A)** The "Missing Spots" distortion model where spots can randomly go missing. **(B)** The "Missing Spots with Variable Rate" where the probability of missing a spot increases with spot density. **(C)** The "Poisson Noise" model where false positive spots are added to the counted number of spots. **(D)** A "Poisson Observation" model where the detected spots follow a Poisson distribution with mean proportional to the true number. **(E)** The "Integrated Intensity" model where only a perturbed version of the total fluorescence is recorded per cell. In each heat map, the color at point $(\tilde{x}, \tilde{y})$ is the conditional probability mass/density of the measurement y having value $\tilde{y}$ given that the true copy number x has value $\tilde{x}$.

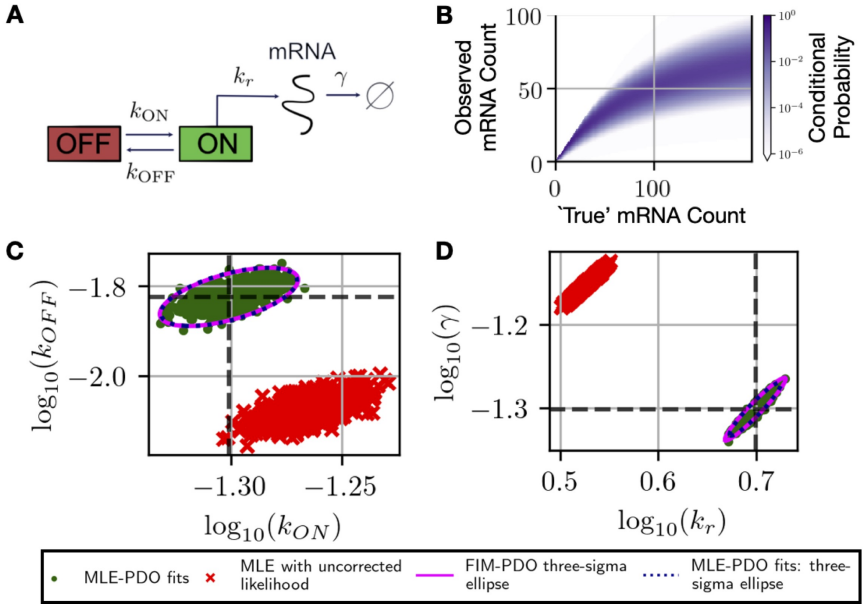

Figure 3: **Estimating and correcting for how measurement distortion affects model identification.** **(A)**: Schematic of the random telegraph gene expression model. Parameter values: gene activation rate $k_{ON} = 0.015$ events/minute, gene deactivation rate $k_{OFF} = 0.05$ events/minute, transcription rate $k_r = 5$ molecules/minute, degradation rate $\gamma = 0.05$ molecules/minute. **(B)**: A submatrix of the PDO for missing spots with varying rates, restricted to the domain $\{0, \ldots, 200\} \times \{0, \ldots, 100\}$. **(C) and (D)**: Maximum likelihood fits to validate FIM-based uncertainty quantification for observed mRNA distributions under distortion model shown in (B). We simulated $1,000$ datasets and perform maximum likelihood fits to these datasets using either a likelihood function that ignores measurement noise (red, labeled 'MLE with uncorrected likelihood'), or one whose measurement noise is corrected by incorporating the PDO (dark green, labeled 'MLE-PDO fits'). Also displayed are the three-sigma confidence ellipses computed by the FIM-PDO approach or from sample covariance matrix of the corrected MLE fits. Panel (C) shows the results in $\log_{10}(k_{OFF}) - \log_{10}(k_{ON})$ plane, while panel (D) shows them in the $\log_{10}(k_r) - \log_{10}(\gamma)$ plane. The intersection of the thick horizontal and vertical lines marks the location of the true data-generating parameters.

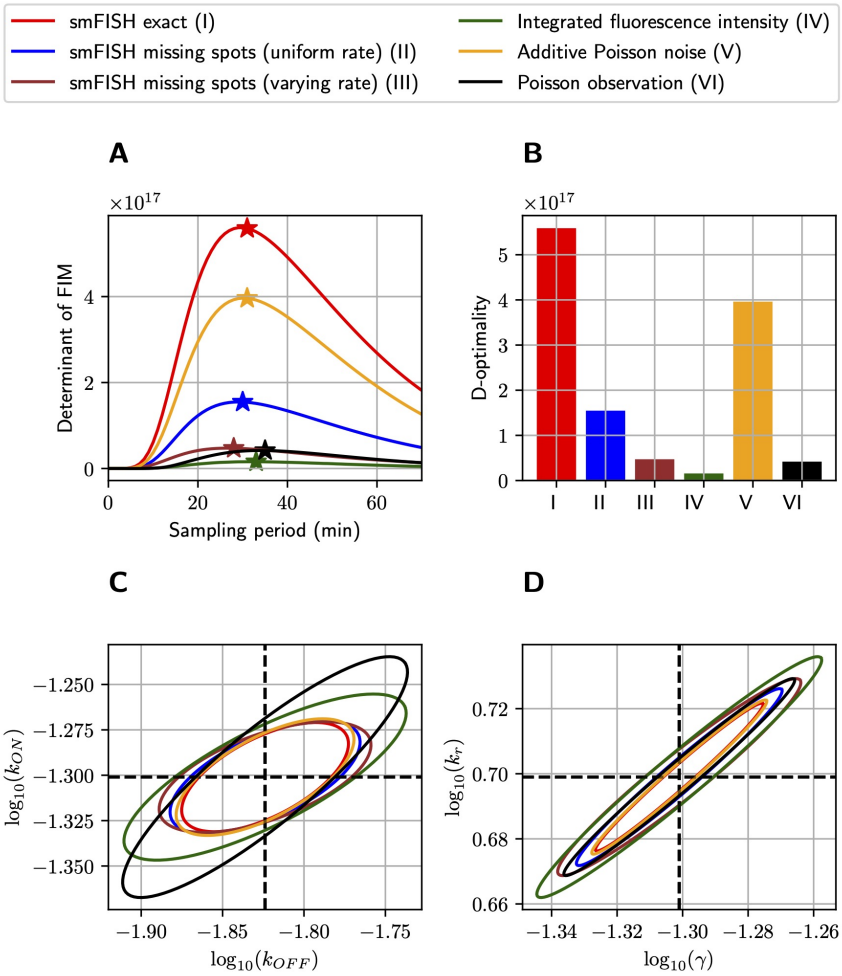

Figure 4: **Optimizing experiment sampling rate under different measurement distortion effects. (A)**: Comparison of D-optimality criteria in single-cell experiments with different types of measurement noise and at different sampling intervals (Δt). In this settings, independent measurements are collected at five equally-spaced time points $k\Delta t,\ k = 1, 2, 3, 4, 5$ with 1000 measurements placed at each time-point. The $\star$ symbol marks the optimal point of each curve and the bar charts in **(B)** visualizes the relative differences in D-optimal achievable by the measurement methods at their respective optimal sampling rates. **(C) and (D)**: The three-sigma confidence ellipses projected onto the $\log_{10}(k_{ON}) - \log_{10}(k_{OFF})$ and $\log_{10}(k_r) - \log_{10}(\gamma)$ planes. These ellipses are computed by inverting the FIMs of different measurement noise conditions at their optimal sampling rates. All analyses use the model and parameters from Fig 3.

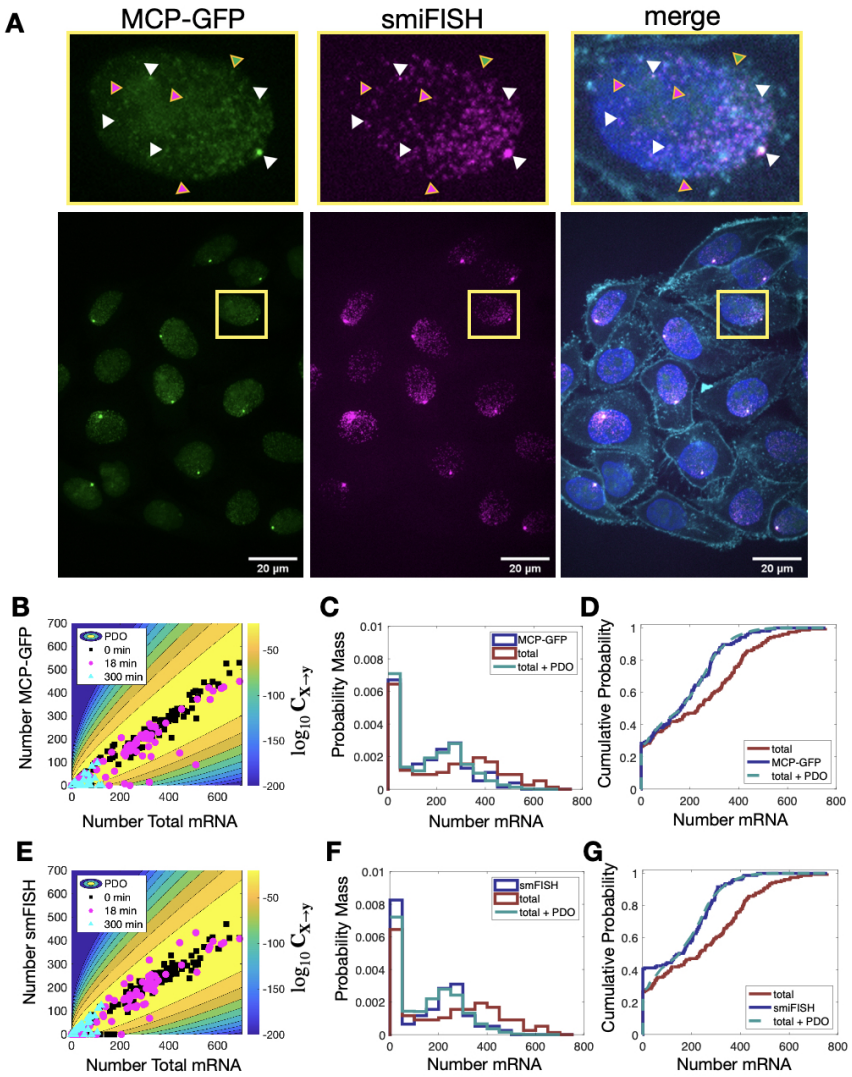

Figure 5: **Effect of labeling strategy on quantification of single-cell mRNA expression. A**) Example image of a single-cell population expressing a reporter gene controlled by an HIV-1 promoter and containing 128XMS2 stem-loop cassette, in which the mRNA was simultaneously measured using MCP-GFP labeling (green) and with smiFISH probes against MS2-Cy5 (magenta). A higher resolution image of the indicated single nucleus is shown at the top; merged image on the right includes DAPI and MemBrite cell surface stain 543/560 to denote the cell nucleus and plasma membrane, respectively. Triangles denote example mRNA that are detected in both channels (white, 34.8%), only in the MCP-GFP channel (green, 37.5%) and only in smiFISH (MS2-Cy5) channel (magenta, 27.7%). **B**) Scatter plot of the total spot count using both smiFISH and MCP-GFP versus using only MCP-GFP for data collected at t =0 min (black squares), 18 min (magenta circles), and 300 min (cyan triangles). Shading and contour lines denote the levels of the PDO ($\log \boldsymbol{C}$) that has been determined empirically from the data. **C,D**) Empirical probability mass (C) and cumulative distributions (D) for total mRNA detection (red) or only with MCP-GFP (blue). Predicted MCP-GFP observations using the total mRNA measurements and the estimated PDO are shown in green. **E-G**) Same as (B-D) but for smiFISH measurements.

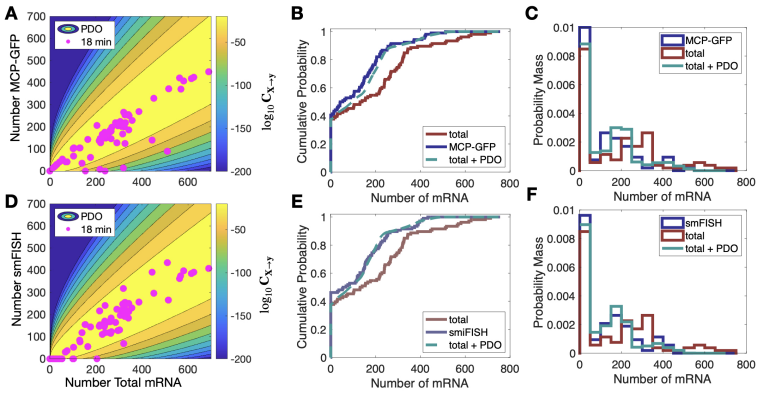

Figure 6: **Validation of PDO on held out data. A**) Scatter plot of total and MCP-GFP spot counts for data collected at t =18 min and contour of PDO determined from data at $t = (0, 300)$ min. **B,C**) PDF and CDF for total mRNA detection (red) or only with MCP-GFP (blue) and MCP-GFP-based PDO-prediction of total mRNA (green). **D-F**) Same as (B-D) but for smiFISH measurements.

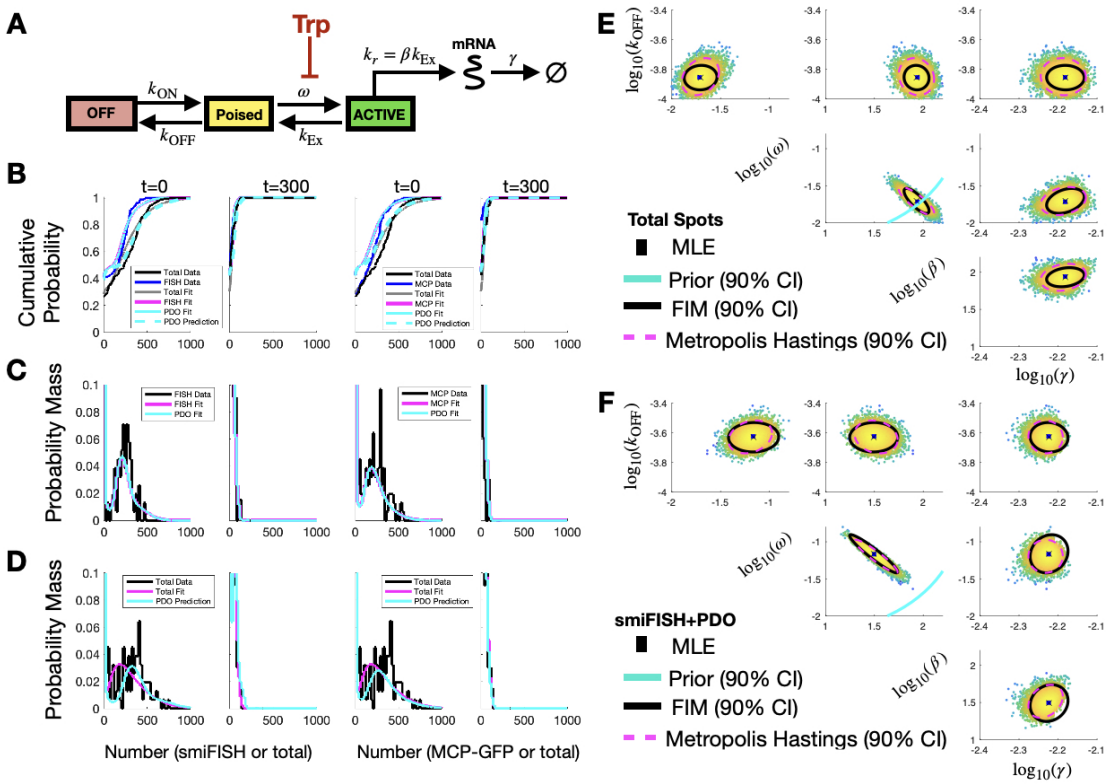

Figure 7: **Identification of stochastic model for MS2X128 cassette-tagged HIV-1 reporter gene. (A)** Schematic of the 3-state bursting gene expression model. **(B)** Measured and model-predicted cumulative probability distribution function (CDF) for the number of mRNA spots observed using smiFISH (left) or MCP-GFP (right) with and without consideration of the PDO at at times 0 and 300 min. **(C)** Measured histogram (black) for the number of mRNA spots using smiFISH (left) or MCP-GFP (right) and corresponding MLE-based PDF fit with (cyan) and without (magenta) PDO. **(D)** Histograms for measured (black), fit (magenta) and predicted (cyan) number of total mRNA. **(E,F)** Joint parameter uncertainty for model estimation using total spot count (E) or smiFISH spot counts with the PDO analysis (F). In each case, 90% CI for prior is shown in cyan; Metropolis Hastings samples (N = 10,000) are shown in dots; 90% CI for posterior is shown in magenta; and FIM-based estimate of 90% CI is shown in black.

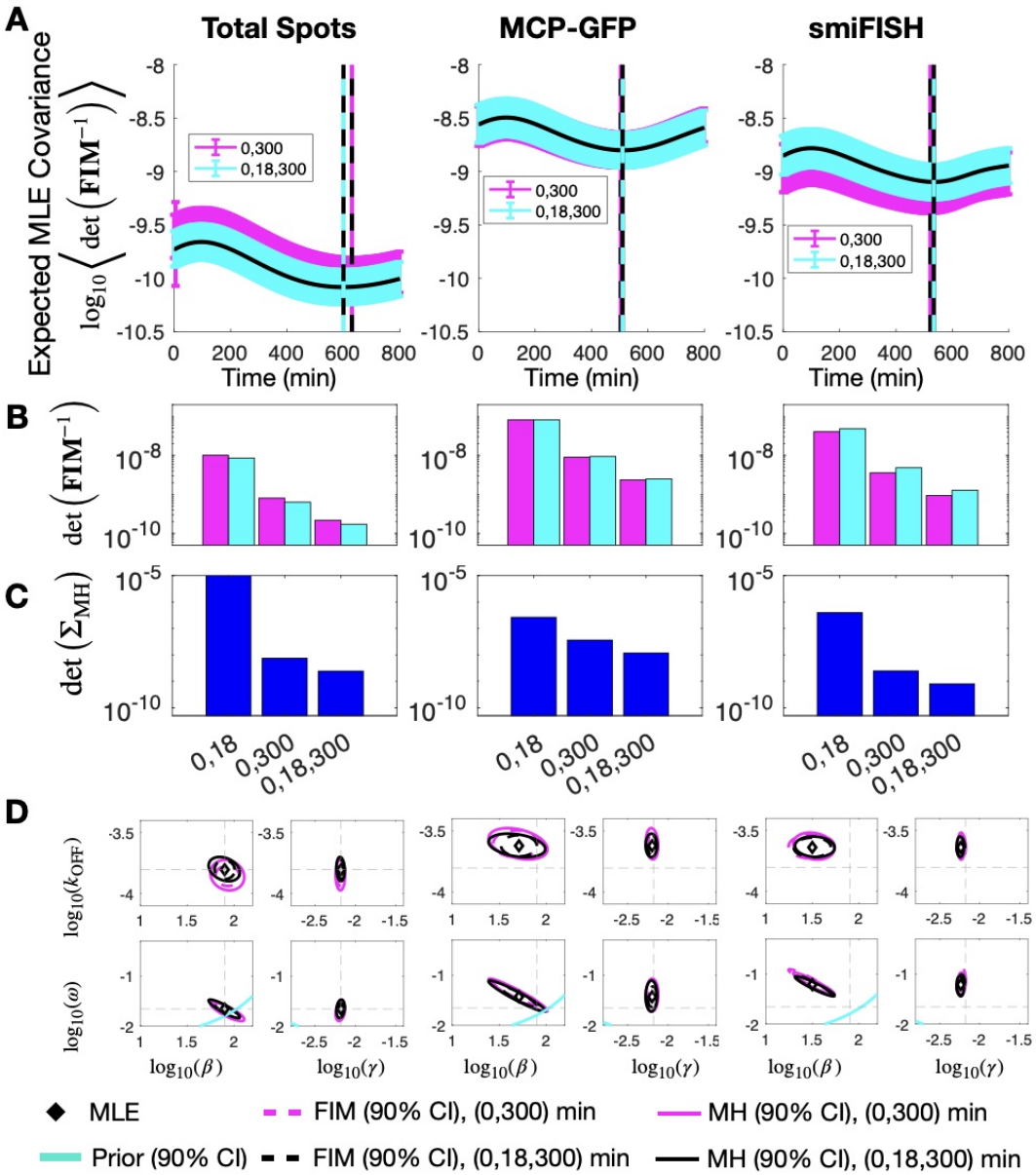

Figure 8: **Design of Subsequent Experiment for MS2X128 cassette-tagged HIV-1 reporter gene.** **(A)** Expected volume of uncertainty ($\det(\mathbf{FIM}^{-1})$) versus time of third measurement assuming 100 cells and measurement of all spots (left), MCP-GFP only (middle), or smiFISH only (right). Error bars denote mean ± SD for 20 parameter sets selected from MH chains after fitting initial data (magenta, t =(0,300) min) or final data (cyan, t =(0,18,300) min). Vertical lines denote the optimal design for the third experiment time. **(B)** Expected volume of MLE uncertainty ($\det(\mathbf{FIM}^{-1})$) for different sets of experiment times and measurement modalities and averaged over 20 parameters sets sampled from the MH chains for initial fit (magenta) or final parameter estimates (cyan). **(C)** Volume of MLE uncertainty ($\det(\Sigma_{\mathrm{MH}})$ estimated from MH analysis in the same experiment designs as **B**. **(D)** Expected (dashed lines, using FIM) and actual (solid lines from 10,000 MH samples) posterior parameter uncertainty (90% CI) for different combinations of measurement modality and available data and for times (0,300) min in magenta and (0,18,300) min in black. Each MH analysis contains 10,000 samples. Measurements include 155 cells at $t = 0$, 106 at $t = 18$, and 66 at $t = 300$.