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Hypothesis testing via integrated computer modeling and digital fluorescence microscopy

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Abstract

Computational modeling has the potential to add an entirely new approach to hypothesis testing in yeast cell biology. Here, we present a method for seamless integration of computational modeling with quantitative digital fluorescence microscopy. This integration is accomplished by developing computational models based on hypotheses for underlying cellular processes that may give rise to experimentally observed fluorescent protein localization patterns. Simulated fluorescence images are generated from the computational models of underlying cellular processes via a “model-convolution” process. These simulated images can then be directly compared to experimental fluorescence images in order to test the model. This method provides a framework for rigorous hypothesis testing in yeast cell biology via integrated mathematical modeling and digital fluorescence microscopy.
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1. Introduction

As high-speed computing has become widely accessible to all disciplines, a unique opportunity now exists to integrate sophisticated digital light microscopy with mathematical modeling of cellular processes. Historically, integration of theoretical modeling with cell biology has been limited. Each field has developed independently with distinct fundamental tools and separate languages for communication. Here, we review an alternate approach to computational modeling in which experiments and theory are integrated to combine the sophistication of digital fluorescence microscopy with computer modeling of underlying cellular processes.

Quantitative modeling can contribute to experimental hypothesis testing in yeast cell biology both by providing for quantitative analysis of results and predictions, as well as constraining hypotheses to be physically plausible. Often, hypothesis testing via computer modeling will generate new or restructured models to explain experimental results. After building a computational model to better understand one set of experimental results, this model can then be used to design future experiments and predict results from these experiments. In this way, computational modeling can be used to assess the utility of proposed experiments, allowing for targeting of future experiments to those that may be most productive in producing meaningful conclusions.

Specifically, quantitative modeling can be used in combination with fluorescence microscopy in yeast to better understand protein dynamics (Box 1). The development of fluorescent protein fusions now allows sophisticated analysis of cellular components in living cells via digital fluorescence microscopy. The ability to quantitatively visualize fluorescent protein localization and dynamics provides strong clues to underlying cellular processes, and hypotheses are generally built on developing models for these processes. In using computational modeling to simulate an underlying cellular process, quantitative and qualitative

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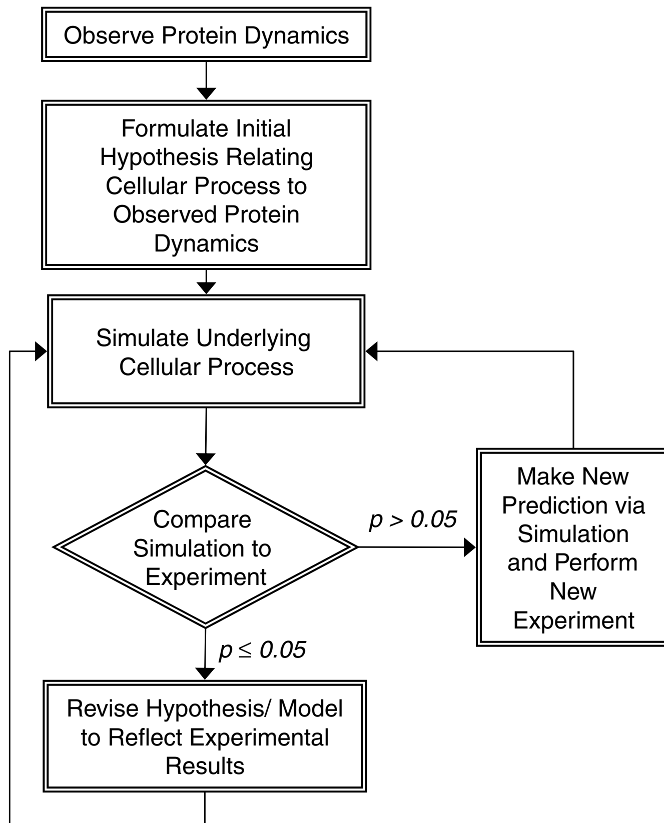

Box 1. A process for integrating computational modeling with digital fluorescence microscopy to better understand the underlying processes that regulate fluorescent protein localization and dynamics in yeast. Modeling provides a framework for iterative hypothesis testing and guiding of new experiments. A key to this process is the quantitative comparison of simulation results to experimental results, allowing for rigorous model evaluation.

predictions of the hypothesized model can be developed subject to a known set of model assumptions. Comparison of simulated predictions for fluorescent protein spatiotemporal dynamics to experimental images gathered via digital fluorescence microscopy then provides for quantitative model evaluation and the subsequent evolution and development of hypotheses.

2. Materials and methods

2.1. Modeling of green fluorescent protein (GFP) dynamics

In order to begin modeling any cellular process, the sub-system to be modeled must be carefully defined. A complex cellular process (e.g., mitosis), can generally be broken down into more manageable sub-systems for detailed quantitative modeling (e.g., the yeast mitotic spindle). This sub-system can then be defined by its structural and molecular components (e.g., chromosomes, kinetochores, etc.). The definition and scope of the sub-system require careful thought. An extremely complex and elaborate sub-system could result in a very computationally intensive model that is difficult to comprehend, even when it correctly predicts sub-system behavior. In contrast, a very narrow or simplified sub-system may ignore processes or components that may be critical to the experimentally observed fluorescent protein dynamics.

Once a sub-system is defined, a straightforward procedure can be followed for systematic development of a computational model.

(1) The particular process to be modeled should be selected based on the desire to answer a specific question. For example, modeling of the yeast mitotic spindle through the entire cell cycle may result in a highly complex model with a correspondingly long and tedious development time, whereas limiting the modeling effort to the specific stage being studied (e.g., metaphase) reduces the complexity, thus allowing for a more focused and potentially more fruitful modeling effort. Such restricted models can then serve to provide the framework for understanding preceding and subsequent processes.
(2) The physical structure of the sub-system to be modeled must be defined, and the sizes and relative physical locations of sub-system components should be scaled correctly. For example, in the case of the yeast mitotic spindle, high resolution structural data gathered via electron microscopy [1] can be used to define the spindle pole body sizes, as well as the relative locations of microtubule attachment to these structures. These sizes and locations are then integrated into the computer model [2].
(3) Explicit rules for the positioning of fluorophores in the cell during the cellular process need to be defined. For example, the localization of fluorescent kinetochore proteins in the mitotic spindle relies at least in part on the dynamics of individual microtubules attached to each kinetochore. Therefore, kinetochore-attached microtubule dynamics will govern the positions of simulated fluorescent kinetochore proteins. Thus, hypothesis testing via a computational model requires explicit rules and assumptions for how fluorophore positions are established in the underlying cellular process.
(4) Time scales and motion constraints must be established for the components of the underlying cellular process that establish fluorophore positions. For example, microtubule plus ends attached to fluorescent kinetochore markers grow and shorten over time. Rules for the rates of microtubule growth and shortening, as well as the frequency of changing states, will then govern the simulated fluorescent protein dynamics associated with the kinetochore.
(5) Assumptions inherent in the proposed model should be defined as explicitly as possible and critically reviewed. These assumptions define the limitations and scope of the model, and should thus be clearly articulated and examined. If modeling predictions are inconsistent with experimental results, then it is necessary to review and reassess the model assumptions.

For example, by modeling kinetochore protein dynamics based on the growth and shortening of microtubules, an assumption inherent to this model is that the kinetochore remains attached to the plus-end of the microtubule throughout the entire simulation. Therefore, events or mutations that may result in detachment of the kinetochore from its microtubule may not produce modeling results that match experimental observations.

2.2. Modeling methods and guidelines

2.2.1. Deterministic vs stochastic modeling methods

There are two basic classes of models that are commonly used to simulate cellular processes. The first type of model is a deterministic model, in which a system of mathematical differential equations is developed to completely characterize the underlying cellular process. Deterministic modeling can be used for characterization of biochemical cellular processes and networks [3–5]. There is a software modeling environment called "Virtual Cell" available on-line that is useful for modeling of biochemical interactions via numerical solutions to reaction-diffusion equations [6–8]. This software allows the user to build a biological model for a system based on its quantitative physical and chemical details. An advantage of deterministic modeling is that in developing mathematical equations to describe a cellular process, interactions and dependencies of system components can be explicitly understood.

The second commonly used method for simulation of cellular processes is stochastic modeling [9,10]. This method takes advantage of probabilities and computer random number generators in order to capture the stochastic aspects of a living system. For example, key aspects of a given cellular process may fluctuate due to normal variations in protein expression levels, such that fluorescent protein localization and dynamics would vary from cell to cell, and over time within a given cell. Stochastic modeling attempts to capture the range of results that may be expected from a given experiment simply due to the normal variation present in any living system [11]. There are many commonly used methods for stochastic modeling, including "Monte Carlo" simulations [9,10]. Stochastic models can be helpful in understanding how normal cell-to-cell variation in experimental results could affect the ability to detect subtle differences in cellular behavior. A disadvantage of stochastic modeling is that simulations need to be repeated many times in order to adequately sample the range of system fluctuations. Also, the quantitative relationships between system behavior and parameter values is often more obscure than it is when closed-form mathematical solutions are obtained.

2.2.2. Fundamental physical principles and observed physical phenomena

Once a sub-system is defined and a modeling method selected, a specific mathematical model can be built. Ideally, the model is based on fundamental physical laws and principles, such as conservation of mass, momentum, and energy. Rates of cellular processes should be constrained by fundamental physical processes as well, such as the known rates of molecular diffusion.

Experimentally observed phenomena may also be included in a model. For example, the dynamic instability behavior of microtubules is a phenomenon that, while not completely understood at the molecular level, has been widely observed in living cells and thus may be an important aspect of a given cellular process. Observed phenomena that are included in a model (i.e., "descriptive modeling") should be listed as model assumptions, since a detailed explanation for the behavior based on fundamental physical laws and principles may not be practical or even necessary.

2.2.3. Model parameters

Finally, once a model has been developed, there are often many model parameter values and constants, such as rate and diffusion constants, that must be defined and constrained in order to generate results. Where possible, parameter values should be experimentally measured or estimated from the existing literature in order to establish physically plausible ranges. In addition, all parameter value assignments should be carefully reviewed and listed as model assumptions. Often, model results will be more sensitive to some parameters than to others. It is important to understand which parameters have the most influence on the modeling results, and to test the physically plausible ranges of these parameters to ensure that modeling results and conclusions are not unduly dependent on the value of one particular parameter. Constraining a parameter to a very narrow range provides an excellent opportunity with which to more stringently test the model.

2.3. Simulation of digital fluorescence images (model-convolution)

Once a computational model for a particular cellular sub-system has been developed, a critical step in seamlessly integrating computer modeling with experimental cell biology is to develop an output for the simulation that can be conveniently compared to experimental results. In some cases, quantitative experimental data, such as relative sizes or time scales required to complete cellular activities, can be directly compared to simulation results. In a recent example of integrated modeling and experimental work, the time required for simulated kinetochore "capture" by microtubules was directly compared to experimentally observed prometaphase duration times [12,13].

In order to conveniently compare experimental fluorescent protein dynamics and localization data from living cells to simulation results, we have developed a method for simulating fluorescence images which we call "model-convolution" [2,14,15]. Using model-convolution, simulation results can be evaluated against experimental results by

direct comparison of simulated fluorescence images to experimental fluorescence images.

The model-convolution method takes advantage of the fact that experimental noise and the spreading of light inherent in the microscope imaging system (as characterized by the point spread function) can be quantified experimentally. By simulating fluorescent protein dynamics and localization, high resolution fluorescence images can be generated (Fig. 1A). Then, the high resolution simulated image is convolved with the experimentally measured microscope point spread function. Finally, after adding experimental background fluorescence and noise, the resulting simulated fluorescence image can be quantitatively compared to experimental images (Fig. 1B). In this way, model-convolution can provide an exact comparison of theoretical fluorescent protein distributions against experimental results, validating models within a known degree of statistical certainty [2]. The development of quantitative animations as an additional simulation output can increase the utility of the simulation by providing clear illustrations that are useful for visualizing and assimilating the emergent behaviors of the underlying model (Fig. 1C). In particular, very complicated models become increasingly difficult to "deconstruct," meaning that it is difficult to understand, and explain in words, why the model explains a given set of observations. As computing power increases and model construction becomes even more facile, it is expected that model deconstruction will become limiting to further progress. In this regard, we have found that animations aid in model deconstruction (for an example of the yeast spindle simulation animation, see http://www.tc.umn.edu/~oddex002/). In addition, creating simulated images and movies via the model-convolution method creates a model output that ideally "looks" like the real thing. This allows the experimenter to inspect simulated images and movies in the same way that the experimental images and movies are inspected.

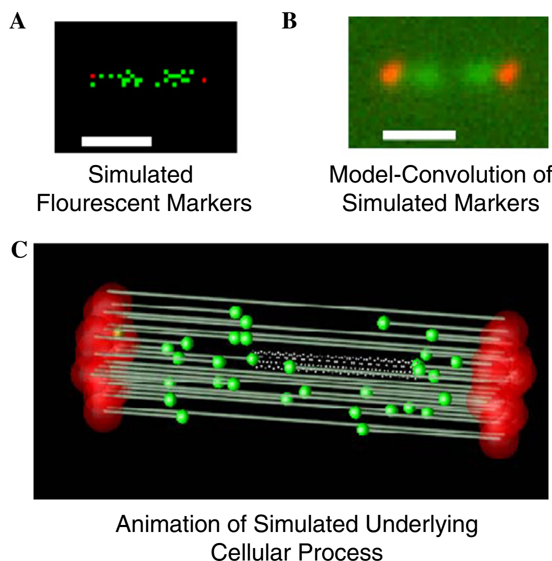

Fig. 1. The model-convolution process is used for rigorous model evaluation against experimental results [2,14]. (A) Simulated "sharp" images of fluorophore localization in the yeast mitotic spindle are convolved with the experimentally measured microscope point spread function and background in (B), scale bar 1000 nm. (red, spindle pole body markers, green, kinetochore-associated markers). (C) Quantitative animations provide for visualization of model components and predicted behaviors of underlying cellular processes.

2.4. Quantitative observation of experimental GFP dynamics

The appropriate collection of experimental images for comparison to simulation is critical to the success of integrated modeling and experimentation. In the yeast mitotic spindle example, image acquisition was carried out as described previously [16] on a Nikon TE2000 microscope with a 1.4 NA, 100× DIC oil immersion lens [17]. Images were acquired with an ORCA II ER cooled CCD camera. Cells were pipetted onto slabs of 25% gelatin containing minimal media ±2% glucose as described by Yeh et al. [18]. The microscope was modified for automated switching between fluorescence and differential interference contrast (DIC) by replacement of the camera mount with a filter wheel (BioPoint 99B100; Ludl Electronic Products Ltd.) containing the analyzer component of the DIC optics. The computer-controlled (MetaMorph 4.6 software) microscope executed an acquisition protocol taking fluorescence images at 1-μm axial steps and a single DIC image corresponding to the central fluorescence image. Fluorescence excitation through a 490/10-nm filter was normally attenuated to 1–10% of the available light from a 100 W mercury arc lamp. Fluorescence emission was collected through a 530/15-nm band pass filter. DIC images were made by rotating the analyzer into the light path and taking a 0.6-s exposure. The images corresponding to a single time-lapse point were projected to a single image by using only the brightest pixel at any one location. Registration of DIC and fluorescence images was verified by imaging of 1-μm fluorescent beads in DIC and fluorescence modes.

As simulated images generated via model-convolution account for the experimental microscope point spread function and noise, image processing techniques such as experimental deconvolution are not required. Specific experimental considerations for quantitative comparison of fluorescent images to modeling results are as follows.

(1) *Signal linearity:* It is important that the fluorescent signal remains at sub-saturation levels in order to properly quantify fluorescent protein localization.
(2) *Frame of reference:* In order to properly assess the orientation of the cell within the focal plane of the microscope, frame of reference points must be established. If possible, these points should be spaced appropriately to assess whether the region of interest remained in focus during the entire experiment. For example, in the yeast mitotic spindle example, spindle pole body markers provided a frame of reference for the length and orientation of the mitotic spindle. Image stacks at multiple planes of focus can be used to evaluate the orientation of the cell.

(3) *Noise:* As experimental noise will be incorporated into simulated images, it is necessary to quantify experimental noise levels on each image. The measurement method and location for evaluation of noise levels should be carefully considered to accurately reflect noise in the region of interest. For example, in the yeast mitotic spindle analysis, it was necessary that experimental noise be evaluated within the nuclear envelope just outside of the spindle area for the best approximation of noise levels in the spindle.
(4) *Sample numbers:* For quantitative comparison to simulation results, it is preferred that experimental sample sizes be as large as possible. In addition, images from control cells are required, so that controls can be simulated in parallel with experimental runs. Small sample size does not preclude hypothesis testing, but does make hypothesis rejection less likely.

2.5. Quantitative data analysis: comparison of experimental and simulated data

Close integration of experimental cell biology and computational modeling requires quantitative data analysis for the comparison of experimental data to simulated image data. Thus, the method for fluorescence image data analysis requires careful consideration. The fluorescence signal should be quantified as a function of spatial position, such that localized fluorescence signal is normalized over the total signal for the area of interest in each image. In order to account for small variations in yeast mitotic spindle length, we have developed a method for "binning" of fluorescence signal into a standard number of bins with a constant relative axial location for each bin [2,14]. For example, fluorescence intensity is analyzed by pixel for each spindle regardless of length. These "pixel" fluorescence intensity values are then recombined into a standard number of "bins" for each spindle. Thus, although absolute bin sizes will vary over different spindle lengths, the relative location of each bin should remain constant so that normalized signal by bin location can be averaged over many images and cells. The localization of various fluorescent protein positions can then quantified and compared relative to a standard "spindle bin number." Using this method, experimental normalized fluorescence signal for each bin can be quantitatively compared to simulation results.

We then calculate a probability of fit (p-value) to quantify the overall fit of the experimental data to the simulation data for a given set of parameter values. The p-value is an estimate of the probability that the deviations between the model and the experiment could have arisen by chance alone.

The p-value is calculated as follows:

(1) Multiple trials are simulated, and then normalized fluorescence signal is calculated by bin for each trial. A "grand average" is calculated for each bin by averaging values for a given bin over all trials.
(2) In order to assess the variation in fluorescence signal localization of each simulated trial against the "grand average" value over all simulated trials, the difference in mean signal (error) is calculated for each bin location against the "grand average" value for that bin over all trials. The error values for a given trial are then squared and summed over all bins to calculate the "sum-of-squares" error (SSE)[1] for each trial as compared to the "grand average" over all simulated trials.
(3) The SSEs for each simulated trial are then ranked from smallest to largest.
(4) The SSE for the experimental data is then calculated against the "grand average" value over all simulated trials, as described in step (2).
(5) The ranking of the experimental SSE in the list of simulated SSE values determines the likelihood that the model could have given rise to the experimental data (e.g., if the experimental SSE is less than 50 of 100 simulated trial SSEs, $p = 0.50$). If the SSE value for the experimental data is larger than all of the simulated trial SSE values, the experimental data does not lie within the range of simulation data, but rather falls outside of the range of variation between successive simulation runs (e.g., for 100 simulated trials the p-value would be less than 0.01). Thus the p-value would fall outside of the acceptable range, indicating that the simulation does not accurately predict the experimental results [2]. In comparing simulation data to experimental data, high p-values ($p > 0.05$) indicate that simulation results are not statistically different from experimental results.

In this way, a statistical comparison is made between the simulation and the experimental fluorescence image data. If the simulation does not accurately reproduce experimental results within the expected range of variability, then either the model hypothesis is incorrect or assigned model parameter values need adjustment. For this reason, a thorough search of potential parameter values is required prior to rejecting a model altogether.

3. Conclusions

Computational modeling is an iterative process which incorporates rigorous hypothesis testing and parameter value estimation. Ideally, models will be continuously challenged with new experiments and ideas. For example, a model that successfully reproduces an experiment that tests only fluorescent protein localization in static images may be significantly challenged by subsequently simulating an experiment that accounts for the temporal

[1] *Abbreviation used:* SSE, sum-of-squares error.

dynamics of fluorescent protein localization within a given cell [14]. To simulate the new experiment, one is constrained to use only the parameter values found to be valid for the first experiment. Subsequent experiments further constrain parameter space, and eventually may invalidate the model altogether. The budding yeast is a particularly appropriate organism in which to use this method because the experimental molecular tools are relatively advanced and the number of molecular components is relatively limited compared to other eukaryotes. The failure of a given model to reproduce experimental results generally leads to questions about the hypothesis used to develop the simulation. For example, simplifying assumptions should be reviewed, as well as processes or cellular components that were not considered in developing the original hypothesis. The process of developing a model that can successfully reproduce multiple experiments, each experiment testing a different aspect of a cellular process [14], is a journey requiring a close linkage between experiment and theory, resulting in a sharing of knowledge and technology that will facilitate our ability to achieve a more complete understanding of the cell.

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