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Review

Current Application of Modeling and Cell-Free System for Synthetic Gene Circuit Design

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Received: 1 July 2024; Accepted: 20 August 2024; Available online: 22 August 2024

ABSTRACT: The desire to harness nature's capability of precise gene expression regulation has motivated the pursuit of synthetic gene circuits. However, designing and building novel synthetic gene circuits with predictable dynamics is nontrivial. To facilitate the design, cell-free systems have emerged as an effective alternative testbed to living biological systems in characterizing and prototyping synthetic gene regulatory networks, given its relative simplicity and designability in terms of cellular contents. Meanwhile, as parameterizing and analyzing first principle-based models can shed light on the required kinetic parameter values, thus the specific regulatory components, for the desired dynamics, coupling mathematical modeling with cell-free experiments has become an effective approach in exploring novel synthetic gene circuits. In this mini-review, we provide an overview of current progress on using deterministic first principle-based mathematical modeling in conjunction with cell-free systems, in designing and characterizing novel gene circuits, as well as the standing challenges and issues with this approach.

Keywords: Cell-free system; Mathematical modeling; Gene circuits; Synthetic biology

1. Introduction

A gene regulatory network is composed of DNAs, RNAs, and proteins, and governs the expression of the target gene involved in the network. Living cells leverage gene regulatory networks, or collectively known as networks, to process information about the environment and to adapt to the surroundings by expressing genes accordingly. In synthetic biology, the main goal is to manipulate the inner networks of cells to achieve desired dynamics, thus enabling applications in diagnostics [1–3] biofuels [4,5], and large-scale protein synthesis [6,7]. Therefore, understanding the characteristics of these networks and being able to build networks that replicate the functionality of innate gene regulatory networks can empower us to manipulate native cell behavior and even manufacture synthetic cells with optimal performance for targeted applications. To achieve these objectives, it is necessary to first discover, create, characterize, and prototype the gene regulatory networks. Towards this end, synthetic biologists have excelled in designing and analyzing gene circuits, the synthetic counterparts of the native gene regulatory networks. Figure 1 gives an example of the widely used RNA-based gene regulation tools and some of the well-known natural and synthetic gene circuits.

https://doi.org/10.35534/sbe.2024.10013

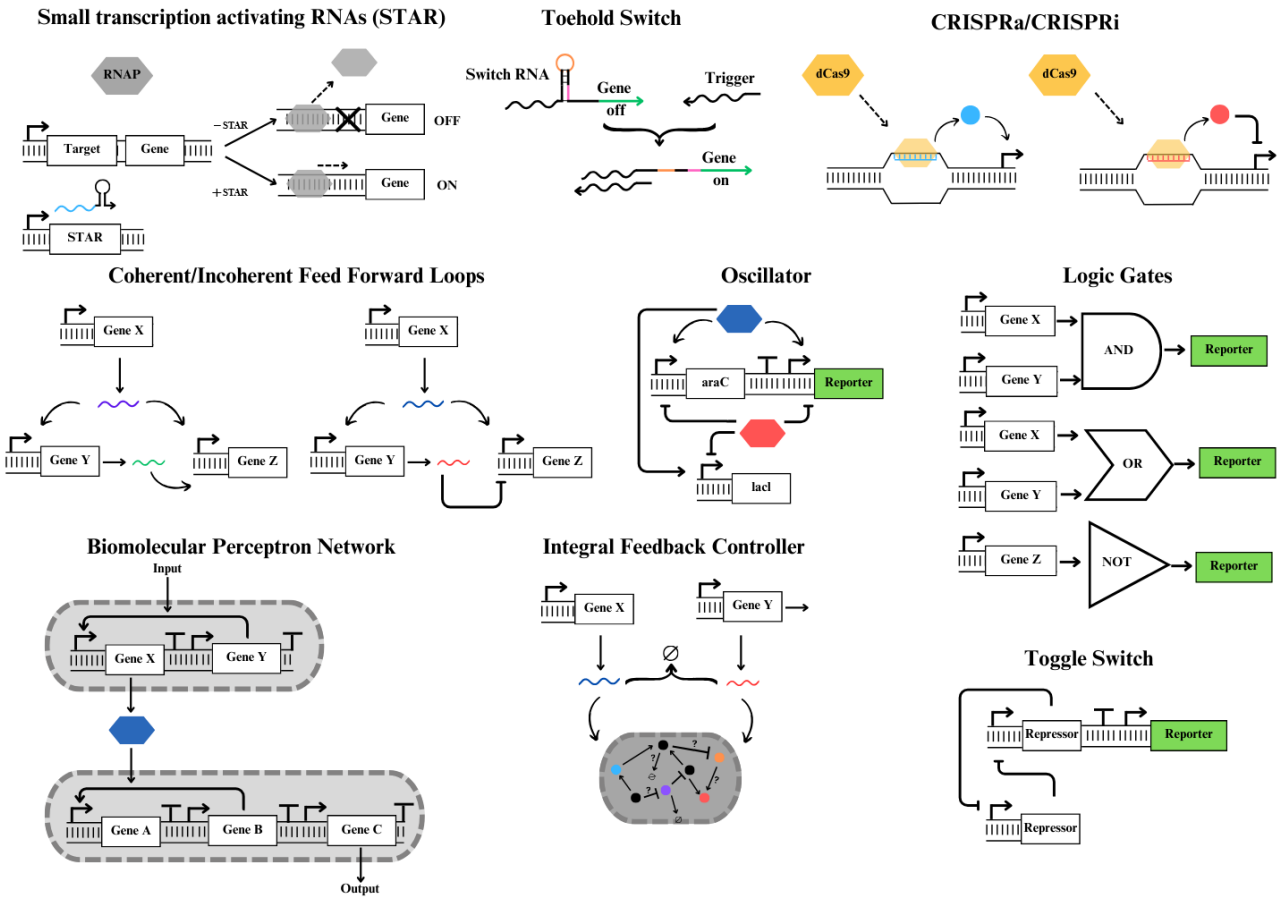

Figure 1. Popular RNA-based gene expression regulatory tools include the CRIPSR system [8], the small transcription activating RNAs (STAR) [9], and the toehold switches [10] are leveraged to realize the transcriptional and translational regulation in a gene circuit. These tools can enable the design and construction of a wide range of natural and synthetic regulatory circuits, spanning from feedforward loops [11], logic gates [12], and toggle switch [13] to advanced biomolecular perceptron networks [14], oscillator [15] and biomolecular integral feedback controller [16]. Note, all drawings are reconstructed based on the corresponding references.

The dynamic and complex cellular environment hampers studying gene circuits inside living cells. Beyond the primary task of protein synthesis, cells engage in several activities, such as metabolic processes and reproduction, necessitating considerable energy expenditure [17,18]. To overcome these obstacles and study gene regulatory networks independently, researchers have introduced cell-free systems. These systems mimic the complex cellular gene regulatory network outside living cells by segregating cellular growth and reproduction through the utilization of cellular extracts. Hence, the cell-free system has emerged as a promising alternative to *in vivo* gene expression techniques, affording precise control over cellular transcription and translation machinery [19–21]. The inherent characteristics of the cell-free system, such as fast performance, decoupled from cell growth, unbiased nature towards DNA templates, and easy tunability, make it an ideal system for understanding complex genetic networks [22].

While the advent of cell-free systems has significantly mitigated the challenges of performing *in vivo* experiments, conducting experiments can still be laborious, especially when exploring new designs, and this can be alleviated with mathematical modeling. In general, mathematical models can be developed with different approaches, such as first principle-based and data-based models. Data-based models learn the underlying dynamics of the system based on experimental observations and are particularly appealing when a theoretical understanding of the system dynamics is missing but massive experimental data is available. On the other hand, with the current understanding of fundamental principles, researchers can construct first principle-based models to predict the behavior of a given system, avoiding the requirement of extensive data collection. Furthermore, when coupled with experimental measurements, such first principle-based models can, in turn, help refine our understanding of the fundamental principles. Popular model types for gene circuits design normally take the form as ordinary differential equations (ODEs) [23–26], partial differential equations when considering the heterogeneity of cellular resource distribution (deterministic or stochastic) [27,28], or probabilistic models such as Markov state models [29,30]. Over the past decades, mathematical models have gained

increasing interest in the study of gene circuits. Table 1 summarizes the main model types for gene circuits as well as some of the corresponding works over the recent years.

Table 1. Summary of popular modeling types and corresponding noticeable works.

Model	Application	Mathematical Description	Work
Ordinary Differential Equations	Parameter estimation and optimization, RNA regulators, logic circuits, feed-forward loops, antithetic integral controller, gene networks dynamics	$\frac{dG_A}{dt} = \alpha \cdot \left[\frac{G_B^n}{k_p + G_B^n}\right] - \delta \cdot [G_A]$ where α, δ and k_p are transcription rate, degradation rate and Hill-function dissociation constant, respectivetely.	[11,23–25,31]
Partial Differential Equations	Diffusion of cellular resources to account for spatial heterogeneity in cells.	$J(x,y) = D(-\frac{\partial y(t,x)}{\partial x} v(x) + y(t,x)\frac{\partial v(x)}{\partial x})$ where *J* is the flux of the cellular species, *D* is the diffusion coefficient, *v* is volume, *y* is concentration per unit length, *x* is distance.	[27,28]
Markov State Models	Stochastic multistability and state-transitions in gene networks, parameter estimation.	$P(X_1, \ldots, X_P) = \prod_{i=1}^{P} P(X_i \mid P_\alpha(X_i)$ where gene i is represented as X_i $(i = 1, 2, \ldots, p)$	[29,30]
Artificial Neural Networks	Prediction of states and phase space of genetic circuits	$\frac{dx_i}{dt} = f_i(\sum(a_{ij}x_j - \theta_i)) - b_i x_i$ where x represents internal states, b_i and a_{ij} represent time constant and connection weights, respectivetelly.	[32]

In this review, we provide an overview of recent advances in using deterministic ODEs-based mathematical modeling in conjunction with cell-free systems in designing and characterizing novel gene circuits. Specifically, we focus on recent developments in how mathematical models and cell-free systems cooperatively facilitate the exploration of novel gene circuit designs for specific regulatory parts and desired output dynamics, as outlined in Figure 2.

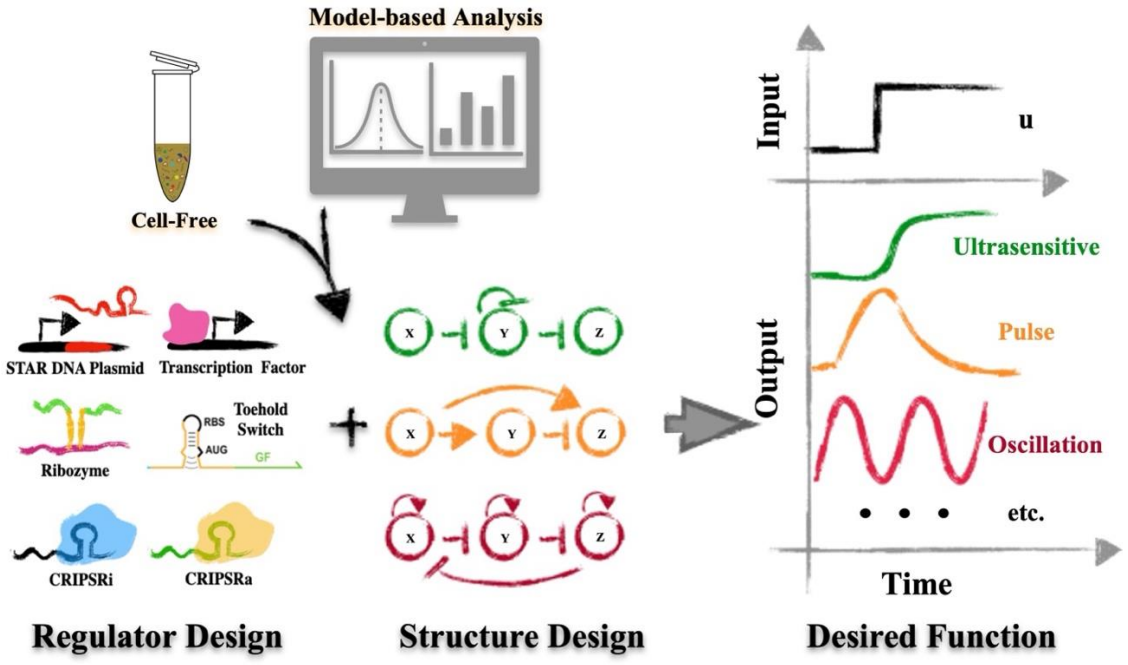

Figure 2. Cell-free systems and mechanistic mathematical modeling can cooperatively contribute to the efficient design and prototyping of novel synthetic gene circuits, with predictable dynamics.

2. Cell-Free and Whole Cell Experiments for Model Analysis

The cell-free system is an ideal system for genetic parts and circuits characterization due to its fast performance, focused analysis on the task at hand, and easy tunability. However, to fully unleash the potential of cell-free systems for rapid circuit characterization and construction, it is essential to understand the property of the system itself. To achieve this, besides direct experimental comparison with the systems, mathematical models offer a more economical alternative.

Marshall and Noireaux developed a useful, simple ODE model to describe an all-*E. coli* TX-TL (transcription-translation) system, controlled by different sets of promoters and DNA elements commonly used in gene circuits [33]. The model was able to capture the TX-TL regimes and saturations due to transcription and/or translational machinery depletion. They chose three sigma factor 70 promoters and UTRs (untranslated regions) of different strengths and combined them in nine combinations to predict protein synthesis rates. The model was sensitive to varying ribosome concentrations and mRNA degradation kinetics, less sensitive to total core RNA polymerase and deGFP maturation, but not sensitive to the Michaelis-Menten constant for transcription and translation and to the concentrations of sigma factor 70. Finally, they integrated these parameters to create a load calculator that calculates the burden on the TX-TL components, factoring in promoter and UTR strength and gene length. These findings reveal the relationship between the cell-free system environment and the kinetic parameters in the model, thus providing guidelines for future researchers in designing the cell-free system environment as well as the appropriate design of the gene circuit parts, such as promoters and UTRs [34,35], for predictable dynamics or to optimize the output dynamics given a specific gene circuit design [36].

Another type of modeling is known as constraint-based modeling, which estimates the performance of metabolic networks. The Palsson group initially designed the metabolic model for *E. coli* [37] and incorporated transcription and translation template reactions [38] before 2010. In 2012, the Voight group added other models to improve the accuracy at the DNA and protein sequence level, including promoter initiation [39]. Most recently, the Varner group tailored the model for the cell-free system by deleting growth-associated reactions and other modifications and named it sequence-specific constraint-based modeling [40]. For simulation, they chose to express the proteins chloramphenicol acetyltransferase (CAT) and dual emission green fluorescent protein (deGFP), calculating transcription rates and maximizing translation rates. The constraints applied depended on previously reported literature. For example, metabolic constraints could be applied for CAT production under a T7 RNA polymerase promoter, but not for deGFP under a P70a endogenous RNA polymerase promoter, because the metabolic reactions had not been recorded for the latter. However, the endpoint and dynamic measurements of the protein produced showed good agreement with the models for both CAT and deGFP. In this paper, they also tested how these models could provide deeper insights into metabolic processes by simulating reactions and obtaining all details, then experimentally testing the reactions to see if the protein output matched what was observed when the metabolic reaction was allowed to run as simulated, or when the part studied was inhibited or taken out completely. They found that some simulated metabolic reactions and component concentrations correlated better with experimental results than others. Identifying these discrepancies is crucial for refining models. They were able to design a model to predict system productivity and energy efficiency based on the carbon number of the protein, with and without amino acid supplementation in a glucose-supplied cell-free protein synthesis system, even for proteins not used in the original dataset of the simulations [40].

3. Cell-Free Experiments and Modeling for Gene Circuit Design

The performance of a novel gene circuit relies on the design of the interactions between constituent components (i.e., circuit topology), the specific realization of the design (i.e., parts selection), and the interactions between the circuit and the host system (e.g., resource competition) [41,42]. Based on understanding of the underlying mechanism, the dynamics of a given gene circuit can be simulated with mass action equations, which typically take the form of ordinary differential equation, using species concentration and reaction rate constants. Such a formation permits the additions of new parts and interactions upon existing designs, with complexity depending on the availability of experimental data. Examples of circuitry that can be mapped by an ODE, so far, include the response time of an RNA circuit and the concentration of components in the system at a given time [22]. Other important models include rate constants and steady-state kinetic parameters of certain activities in the systems. These have also been found for ClpXP protease [43,44], protein synthesis rate [43], mRNA concentration at a given time [43], transcription factor-based biosensor and its genetic circuit cascade [45].

Based on preliminary experimental data, researchers have also developed holistic mathematical models to further guide the design of new gene circuits. Examples include the construction of a pulse generator using the timescale difference between STARs and CRISPRi regulation in [46]; the characterization of a coherent feedforward loop as a noise filter in [47]; the quantification of an integral feedback controller in [48]; the parameterization of RNA genetic circuits in [24]; and the modeling and parameterization of transcription and translation processes in [47,49]. These works demonstrate the potential of combining modeling and cell-free systems to improve the efficiency of characterizing and designing gene circuits, thereby minimizing experimental efforts [47].

To further facilitate the experimental construction of circuits, automation of circuit design via software tools has thrived over the past decades. Examples include Cello, a Java-based software to design ordered and tagged DNA sequences for gene networks beyond *E. Coli* cells [50]; COPASI, a biochemical simulator based on numerical methods that facilitates the user to solve biological systems problems through different mathematical approaches [51]; iBioSim, a computational tool to design and simulate synthetic genetic circuits based on experimental data and biological knowledge from other genetic circuits [52]; Tellurium, a python-based software developed for biological systems examination including mathematical modeling, simulation and analysis tools [53]; promoter/RBS calculator, a multi-parameter model to predict transcriptional components such as sigma factors promoters with desired transcription rates [54]; and Galaxy SynbioCAD, a portal that integrates different computational tools to design and engineer metabolic pathways for specific desired chemical targets [55]. Efforts for cell-free systems have also started to attract attention. In 2021, the Murray group described a toolbox called *txtlsim* created to assist genetic circuit modeling developers [56]. The toolbox considers cell-free systems conditions, such as resource loading, consumption, and degradation, making it useful for modeling the dynamics of transcription-translation reactions. Additionally, to predict the in vitro behavior of several genetic circuits, the toolbox provides a library of parts that can be combined in different patterns. With a simple interface and straightforward commands, this tool allows the description of complex biological interactions and, hence, the dynamics of the system. Furthermore, the paper presents a multi-stage Bayesian inference procedure for characterizing its parameters, which were used to predict and experimentally validate the behavior of an incoherent feed-forward loop. Another example includes that the Murray group presented a full-stack modeling, analysis, and parameter identification pipeline to guide the modeling and design of gene circuits with specific functions. The authors integrated cell-free systems with mechanistic modeling and model reduction to characterize integrase and excisionase activity for the modeling and construction of gene circuits [57]. These tools further facilitate the rapid design and construction of novel gene circuits with predictable dynamics.

4. Discussion

The success of cell-free systems arises from its decreased complexity and constraints associated with *in vivo* gene expression, such as genetic variability, membrane barriers, cell viability, manipulation of living organisms, and cellular growth [21,58,59]. The power of this emerging tool has been explored for pollutants identification, RNA genetic circuits dynamics, enzyme expression, rapid field-portable diagnosis, and industrial applications [60–63]. An important feature of cell-free reactions is their ability to be lyophilized or dried and subsequently reactivated through controlled temperature adjustments and rehydration. This property enhances the temporal flexibility of experimental protocols and facilitates their application in non-laboratory settings [2]. Additionally, computational and mathematical tools have been utilized to improve the predictability of cell-free reaction behaviors. However, these tools have encountered challenges in accurately modeling the stochastic nature of genetic circuits [24,25,64].

While models characterized by kinetic parameters defined according to understanding of the biological systems, such as the ODE models, are useful for simulating circuit dynamics, these models are often overfitted and suffer from poor transferability, i.e., one set of fitted parameters can hardly be used to predict a new design or in new systems, despite the similarity in the components used. Several reasons could attribute to this phenomenon, such as model structural and parameter identifiability, optimization for parameterization, as well as resource competition or changes in cellular context.

Structural and parameter identifiability problem has been a long-standing topic in modeling, where structure identifiability refers to model structure issues that lead to indeterminable parameters, and practical identifiability refers to the lack of measurements to obtain precise parameters [65]. Notable works include the following, as examples. In [66], the authors presented a comparison of methods often used to describe non-linear dynamic models in systems biology, such as Taylor series, generating series method, similarity transformation, differential equations, test for reaction networks, among others. A MATLAB toolbox called STRIKE-GOLDD offers an efficient structural identifiability analysis by considering parameters as state variables and decomposing the model into sub models, this tool eliminates parameters already classified as identifiable to reduce the problem size [67]. A combination of sensitivity analysis and identifiability tests was proposed by [68], where linear correlations between parameters are obtained through a logical numerical approach. One future direction can be the investigation of probabilistic model fitting for a distribution of parameter values to increase the possibility of applying to new systems. However, such an approach might only provide a qualitative estimation rather than an accurate quantitative simulation. Alternatively, a physics-informed machine learning-based model, combined with transfer learning could be a potential solution [69]. In this

approach, a machine learning-first principal hybrid model would be developed and parameterized with experimental measurement. When applying this to a new system, additional yet small amount of experiments would be performed for model refitting using the previously fitted parameters, thereby reducing the cost of materials and computation. Transfer learning has shown great promise in many fields, with reduced amount of data for new systems. We expect its application to gene circuit modeling to facilitate future model development and circuit design [70–72].

Parameterization is another potential obstacle in developing ODE-based models for circuit simulation. Depending on the complexity as well as the topology of the model, the ODEs could have singular points, which could result in failure with current widely used integration solvers, that no accurate solutions are to be obtained. Furthermore, increased model complexity with an increased number of parameters generally requires more experimental measurements compared to a simpler model for accurate parameterization, but this comes with the risk of overfitting. The relationship between sample size, model complexity and parameterization accuracy has been constantly explored in the field of system identification, where researchers investigate the challenges of model estimation for dynamic systems [73]. This issue is frequently addressed using classical approaches, including the realization of linear state-space models and prediction-error method, as well as integrated methodologies that combine control and identification, such as adaptive control [74]. The applications of system identification extend well beyond control theory, and its application to other system could significantly enhance the development of models for gene circuits simulation [75]. In [76], the authors highlighted the challenge of applying system identification methods to biological systems given time scale measurements issues, where they presented a new identification method that uses unequally spaced sparse time series data with different time scales to describe PC12 cells growth *in vivo* system. Built upon these existing efforts, we anticipate future work with extensive experimental validations to further benefit the modeling of gene circuits for wide-range applicability. Given the fast-prototyping advantages of cell-free systems, and its flexibility in designing experimental conditions compared to *in vivo* experiments, we anticipate cell-free experiments will serve as an ideal system for such investigations.

Resource competition is a long-known culprit for the failure of newly designed circuits when implemented in host systems [77–81]. Therefore, understanding the robustness of the designed circuit to resource competition is critical to delivering predictable functions. The advantages of manipulating the resource condition in cell-free systems present an ideal testbed for quantifying the effect of resource competition to circuit dynamics. Despite existing work on characterizing and leveraging the negative impact of resource competition to circuit design and implementation [82–84], further efforts on this topic would still benefit the development of gene circuit design, and we expect cell-free systems and modeling to contribute significantly to this effort.

The success of machine learning in biology has also promoted its application in the design of gene circuits. In 2020, Church and Collins showed how decision trees and other machine learning algorithms could provide a better understanding of which attributes are the most influential on the design of cell-free systems [85,86]. This mathematical artifact allows for the behavior of the system to be characterized by the neural network without the need for a priori physical knowledge of the system. Angenent-Mari et al. demonstrated that Deep Neural networks (DNN's) are not only capable of predicting the functionality of components of the circuitry but also useful in providing a more "human understandable" behavior of their components [86]. However, training the model generally requires a considerable amount of data, which can be a barrier with *in vivo* experiments, but could potentially be addressed with cell-free systems.

Author Contributions

Y.-C.K. and X.T. conceived and supervised the project. T.R.S., A.L. and C.E.C. wrote the original manuscript. T.R.S., Y.-C.K. and X.T. revised and edited the manuscript. All authors contributed to the article and approved the submitted manuscript.

Ethics Statement

Not applicable.

Informed Consent Statement

Not applicable.

Funding

This work was supported by the National Science Foundation (Award No. 2223720), Louisiana Board of Regent (RCS, Grant No. LEQSF (2020-23) RD-A-01), and USDA National Institute of Food and Agriculture (HATCH, Accession No. 1021535, Project No. LAB94414).

Declaration of Competing Interest

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.

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