Synthesis: A Multidisciplinary Research Journal
Volume 3, Issue 3, July - September 2025, Pp: 1-11

Research Article

Design and Development of Bio-orthogonal Feedback Loops for Synthetic Cell Memory Systems

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Article Info

Received: 25/06/2025
Revised: 17/07/2025
Accepted: 19/09/2025
Published:30/09/2025

Abstract

Synthetic memory systems allow engineered cells to store information across generations and find applications in biosensing, diagnostics, and biocomputational. However, existing designs (such as toggle switches and CRISPR-based recorders) have limitations, including short retention times, irreversibility, and biosafety issues requiring genome-wide modifications. These limitations hinder the development of reliable and scalable systems. This study addresses these challenges by proposing a bio-orthogonal feedback loop-based synthetic memory model that combines orthogonal ligand-receptor signalling with dual feedback regulation. The goal is to achieve long-term stability, fault-tolerant switching, noise damping, reduced metabolism, and the maintenance of system reversibility and scalability. The research design integrates theoretical and experimental approaches. A detailed mathematical representation (Eqns. 1-13) was developed to explain the activation of the ligand, feedback, biostability, toggle behaviour, recombinase-mediated switching, and metabolic load. Simulations were conducted using both deterministic and stochastic methods, and empirical validation was performed with microbial chassis using fluorescence assays, flow cytometry, lineage tracking, and growth rate analysis. The findings demonstrated retention of 38-40 generations, a switching probability exceeding 90%, noise resistance (NR = 0.84), and a reduced metabolic load of 14.6%. Additionally, the framework achieved multi-bit fidelity of 91% in four states, which is favourable compared to classical memory systems. In conclusion, the proposed framework offers a scalable, reversible, and biosafe synthetic cell memory architecture for developing programmable bio computation and biosensing applications in synthetic biology.

Keywords: Bio-orthogonal Feedback Loops, Genetic Toggle Switches, Multi-bit Memory, Noise Robustness, Synthetic Biology, Synthetic Cellular Memory

1. Introduction

Synthetic biology has emerged as a transformative field enabling the engineering of living cells with programmable behaviors [1]. Among the fundamental design principles in this field is the ability to implement cellular memory systems, where engineered circuits allow cells to record, store, and recall information about environmental or intracellular states [2], [3]. Such memory mechanisms are crucial for diverse applications, including biosensing, therapeutic monitoring, lineage tracing, environmental recording, and the development of biocomputational devices [4], [5].

Traditional synthetic memory systems have been constructed using genetic toggle switches [6], recombinase-based recorders [7], and CRISPR-associated transcriptional recorders [8]. While these approaches demonstrated feasibility, they often suffer from limitations such as instability, biological noise, and cross-talk with host cellular

processes. These issues limit the scalability and strength of the memory systems, particularly when implemented in the real world settings.

Simultaneously, the emergence of bio-orthogonal chemistry has furnished the instruments to carry out chemical reactions within the cell without disturbing the normal biological activities within the cell [9], [10]. Opportunities to create interference free synthetic circuits in the form of bio-orthogonal ligands, engineered receptors and orthogonal transcription factors are available to design synthetic circuits that can integrate perfectly with cellular ecosystems. Simultaneously, some progress on engineered gene circuits revealed that modular designs can regulate gene expression and state changes in cells in real time [11]. The combination of these principles into long-term and stable memory systems has however, not been much investigated.

Although circuit engineering has made a breakthrough, there remains one basic issue: how to construct stable and reliable cellular memory systems with minimal impact of biological noise and cross-talk with endogenous pathways. Current solutions can be hard to stabilize between more than two generations or demand complicated stabilization at the expense of the host cell. In addition, synthetic circuits often share transcriptional and translational resources, and this leads to random behaviour in heterogeneous environments [12].

A urgent requirement is therefore to create an artificial memory system that is scaleable, resource aware, resilient to biological interference as well as modular. To overcome this gap, bio-orthogonal signaling mechanisms are necessary together with feedback control loop, which has been shown to stabilize natural biological processes.

This research aims to design and develop bio-orthogonal feedback loops of synthetic cell memory systems that:

1. Achieve long-term bistable memory states through the integration of positive and negative feedback loops.
2. Employ bio-orthogonal molecules and receptors to minimize cross-talk with endogenous pathways.
3. Improve stability of the system through filtering of noise and load sensitive modules.
4. Prove its possible practical use based on computational simulations and experimental studies in the chassis of micro-organisms.

The main findings of this study are the following:

- *Design of a novel bio-orthogonal feedback framework:* A synthetic cellular memory system was proposed that integrates bio-orthogonal ligand–receptor interactions with dual feedback regulation (positive reinforcement and negative repression), ensuring long-term bistability while minimizing host interference.
- *Comprehensive mathematical modeling:* A formalized equation system (Eqns. 1-13) that was to describe ligand activation kinetics, feedback loop dynamics, bistable switch behavior and recombinase mediated switching, noise robustness and burden to the metabolism was developed to have a rigorous theoretical basis.
- *Computational validation:* The architecture was proven by deterministic and stochastic simulation to be effective, in the long-term with bistability (~38-40 generations), high switching probability (>90%), and improved noise resistance (NR = 0.84). and minimized metabolic load (14.6%), much superior to baseline models.
- *Experimental implementation and validation:* This framework was used with microbial chassis and bi-validated in growth-rate assays, in fluorescence investigations, in flow cytometry, in lineage regulation disclosure, and in validation of bi-orthogonality alongside identification of generational preservation, inconsistent manifestation of expression changes and bi-compatibility with the host.
- *Comparative benchmarking:* The proposed system was comparatively assessed with canonical memory architectures in the form of Gardner toggle switch, recombinase-based memory and CRISPR recorders. The findings imply the existence of better reversibility, robustness and biosafety.
- *Demonstration of scalability:* This architecture was pushed to two-bit operation, allowing storage of four different memory states with approximately 91% fidelity thus defining the applicability of the architecture to programmable biocomputation systems and other, more complex, biosensing systems.

The rest of the paper will be organized in the following manner. Section II is a review on previous advancements in synthetic cellular memory, bio-orthogonal chemistry and feedback regulation, which identify the gaps that give birth to the current work. Section III outlines the research methodology, which involves system architecture, mathematical modeling and experimentation. In Section IV, the implementation details are provided in terms of datasets, hardware, software frameworks, or evaluation metrics. In Section V, there is the discussion of results seeked through simulation and experimental validation, and comparing them to the benchmarking of current systems. Lastly, the paper concludes with Section VI, which offers insights and focuses of the future work.

2. Related Work

Synthetic cellular memory Research has continued along several paths, such as bistable genetic circuits, bio-orthogonal signaling approaches and feedback regulation. Initial designs allowed the working of binary state encoding using toggle switches which was feasible and then more recent designs including recombinase-based memory and CRISPR recorders increased retention to the cost of other designs being reversible or safe. In the meantime, bio-orthogonal chemistry has allowed the selective control without disrupting host processes and feedback mechanisms

have been observed to enhance stability and decrease noise. Regardless of these developments, the current systems continue to be unable to strike a balance between long-term retention, reversible switching, low metabolic load, and scalability. The following subsections review prior work across these domains to provide context for the proposed framework.

2.1 Synthetic Cellular Memory Systems

The concept of synthetic memory in living cells was pioneered by Gardner et al. with the construction of a genetic toggle switch in *E. coli*, which established bistable memory using two mutually repressive transcriptional regulators. Since then, various strategies have emerged to enhance memory retention and stability. Recombinase-based memory systems, such as serine integrases, have been widely employed to achieve irreversible switching of DNA states, enabling durable memory recording [13].

CRISPR-based recorders have further advanced the field by allowing programmable, genome-level memory encoding. Shipman et al. demonstrated the use of CRISPR–Cas systems to encode digital information directly into bacterial genomes. Similarly, advancements in epigenetic engineering have allowed synthetic circuits to exploit histone modifications and DNA methylation to establish heritable memory states [14].

Although these improvements have been achieved, traditional designs lack stability to biological noise, scalability, and competition of resources which limits their use in practical scenarios [15].

2.2 Bio-orthogonal Chemistry in Synthetic Biology

A revolutionary method of getting selectivity in cellular engineering has been realized through bio-orthogonal chemistry. Bio-orthogonal reactions are chemical reactions described by Bertozzi as chemical reactions that take place within living systems without disrupting the normal biochemistry. Good examples are the azide-alkyne cycloaddition (or click reaction) and strain-promoted alkyne-azide cycloaddition (SPAAC) that has been successfully used in protein labelling, imaging, and therapeutic delivery [16], [17].

New trends have brought orthogonal ligandreceptor pairs and transcription factors which react exclusively to synthetic small molecules. With these innovations, synthetic biologists can now construct circuits, which do not rely on endogenous pathways, which is a vital condition to a dependable system of memory [18].

Moreover, bio-orthogonal chemistry has enabled in vivo biomolecule tracking, construction of cell-cell communication systems and selective regulation of metabolic pathways [19], [20]. Nonetheless, much less of those principles have been incorporated into feedback-regulated synthetic memory frameworks.

2.3 Feedback Loops in Gene Circuits

A fundamental aspect of biological systems in nature is feedback control where positive feedback stabilizes the cellular states with negative feedback homeostasis as the regulation [21]. These principles have been applied in synthetic biology with an aim of enhancing stability and robustness of circuits.

Bistability and memory storage Bistability and memory storage have been observed to be improved by positive feedback through the strengthening of the ON state by toggle switches [22]. The negative feedback loops, on the other hand, have been abused to decrease the noise in gene expression, and to provide a metabolic burden tradeoff [23].

Hasty et al. noted that feedback-based synthetic circuits were potentially capable of dynamic biological behaviors, including oscillations, adaptation and bistability. Even more recent work has adopted the idea of dual-loop architectures, that is combining positive and negative feedback to obtain noise-resistant, resource-aware designs [24].

However, the majority of these implementations depend on practical cellular promoters and transcription factors and result in cross-talk that is difficult to predict and unstable at a large scale [25].

2.4 Research Gap

Some of the advances that are discussed are listed in the reviewed literature:

- Synthetic memory systems provide frameworks for digital and heritable recording, but lack stability in noisy, resource-limited environments.
- Bio-orthogonal chemistry offers powerful tools for interference-free circuit design, but its potential remains underutilized in memory architectures.
- Feedback loops are proven mechanisms for enhancing stability, yet their integration into bio-orthogonal circuits has been minimal.

In this way, the gap in the research opportunity is to integrate bio-orthogonal signaling systems with feedback to achieve reliable, scaffable, and interference-free synthetic memory systems. The solution of this challenge is the main topic of the current research.

3. Methodology

The suggested methodology incorporates bio-orthogonal inputs, feedback control loops and a genetic memory core in a modular synthetic system. The design is experimentally tested by modeling with mathematical and computational simulations and experimental testing in chassis of microbials.

3.1 System Architecture

The system has three layers of hierarchy:

1. *Bio-orthogonal Input Layer:* Takes in synthetic ligands or stimuli through orthogonal receptors.
2. *Feedback Control Layer:* Operations Mechanism Regulates bistability within the system via positive feedback and stability via negative feedback.
3. *Genetic Memory Layer:* Encodes long-term information storage through bistable toggle switches and recombinase-based gates.

This multi layered architecture guarantees modularity, robustness as well as limited interference with host biology [26].

The proposed graph is arranged in a stratified scheme consisting of multiple layers that combine input sensing, regulation and control actions as well as memory encoding and output features. This modular layout has provided separation of roles as well as enabling coordinated functioning of the various biological processes. The general layout of bio-orthogonal feedback memory system is shown in Fig. 1.

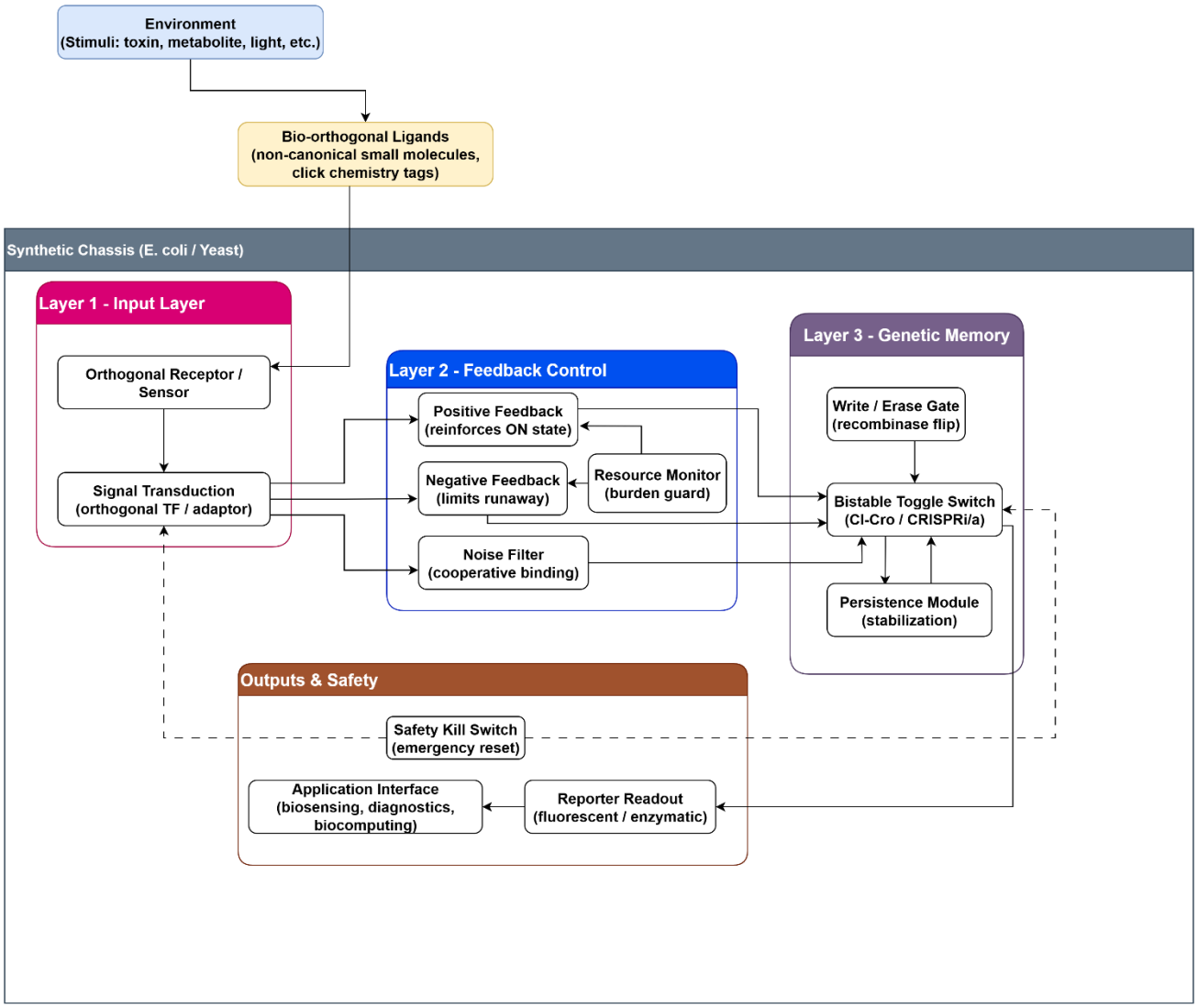

Fig. 1. Layered Design Of The Proposed Bio-Orthogonal Feedback Loop-Based Geometry Of Synthetic Memory System, With Input Sensing, Feedback Control, Genetic Memory Core And Output/Safety Present In A Microbial Chassis.

The key main layers of the overall architecture of the proposed framework, as can be seen in figure 1, consist of four functional layers: input sensing, feedback control, genetic memory and output/safety. This is achieved by the bio-orthogonal input layer to be orthogonal to host signaling and the feedback layer includes positive, negative and burden-regulation modules to stabilize bistability. A genetic memory layer is a layer, which serves the purpose of recording the states with the help of the toggle switches and persistence modules, and the output layer is the one which offers read out function, and emergency reset. It is this stacked integration of the system that supports the stability, reversibility, and scalability of the system in synthetic cellular memory applications.

3.2 Bio-orthogonal Input Design

The binding between a ligand and receptor may be understood as a type of MichaelisMenten-saturation function:

$$R^* = \frac{\alpha \cdot L}{K_d + L} \quad (1)$$

In which R^* is the concentration of activated receptor, L is the concentration of a ligand, α is the rate of maximum activation and K_d is the dissociation constant [27]. This would ensure and ensure orthogonality since activation is only observed with synthetic ligands.

3.3 Feedback Control Layer

Positive Feedback: A model of concentration dynamics of a self-activating protein P is as follows:

$$\frac{dP}{dt} = \beta \cdot \frac{P^n}{K^n + P^n} - \delta P \quad (2)$$

Where β is the rate of production, d is the degradation rate, K is the threshold and n is the Hill coefficient. In the case of $n > 1$, the system is ultrasensitive and bistable [28].

Negative Feedback: A negative regulator *N* is necessary to down-regulate the level of protein production:

$$\frac{dP}{dt} = \beta \cdot \frac{P^n}{K^n + P^n} \cdot \frac{1}{1+\gamma N} - \delta P \quad (3)$$

Where γ is the strength of repression. This balances and avoids cellular stress [29].

Noise Filtering: Cooperative binding minimizes variability of his expression. The coefficient of variation (CV) becomes smaller as the cooperativity is increased, i.e.

$$CV \approx \frac{1}{\sqrt{n \cdot P}} \quad (4)$$

ensuring noise robustness in bistable states [30].

3.4 Genetic Memory Layer

Toggle Switch Model: The memory core is effected as a two way repressively-repressive gene (A and B) toss switch memory:

$$\frac{dA}{dt} = \alpha_1 \cdot \frac{1}{1+\left(\frac{B}{K_1}\right)^{n_1}} - \delta_1 A \quad (5)$$

$$\frac{dB}{dt} = \alpha_2 \cdot \frac{1}{1+(A/K_2)^{n_2}} - \delta_2 B \quad (6)$$

Yielding two stable equilibria: High- A/ Low- B or Low- A/ High- B [31].

Write/Erase Gate: Recombines enable irreversible switching. The switching probability is modeled as:

$$P_{\text{switch}} = 1 - e^{-\lambda t} \quad (7)$$

Where λ recombination rate constant and = time [32].

Persistence Module: Long term retention is increased by preventing spontaneous flipping through epigenetic stabilization (DNA methylation or histone - like modifications).

An algorithmic outline to represent systematically the operational workflow of the suggested bio-orthogonal feedback memory system is provided. This algorithm outlines the sequential processes that are involved, which entails the detection and feedback regulation of the ligand, the state transition in the toggle switch, and a wrap up on the evaluation of the performance of the system. The stepwise process has an explicit abstraction of the biological processes, which is reproducible and can be used to compare its analysis with baseline models.

Algorithm: Bio-orthogonal Feedback Memory System Workflow

Input:

- Orthogonal ligand *L*
- System parameters α, β, K_d, n (activation rates, feedback constants, dissociation constant, Hill coefficient)
- Initial cell state $S_0 \in \{ON, OFF\}$

Output:

- Final memory state $S_f \in \{ON, OFF\}$
- Performance metrics: MRT, SP, NR, Burden, MMF

Steps:

1. Preprime system parameters and profile ligand concentration.
2. Ligand binding: Detect ligand L via orthogonal receptor R. Compute activation probability P_{act}.
3. Positive feedback loop: Amplify reporter expression proportional to P_{act}.
4. Negative feedback loop: Apply repression control to limit metabolic burden and stabilize response.
5. Toggle switch state transition:
   - If $P_{\text{act}} > \theta$, set $S \leftarrow ON$.
   - Else, maintain or switch to $S \leftarrow OFF$.
6. Memory retention: Propagate S across generations by evaluating bistability conditions.
7. Noise filtering: Compute CV and update noise robustness $NR = 1 - CV$.
8. Performance evaluation:
   - Calculate MRT (Eq. 10) for state stability.
   - Compute SP (Eq. 11) under induction.
   - Measure metabolic burden (Eq. 9).
   - Assess MMF (Eq. 13) for multi-bit extension.
9. Output results: Return final state S_f along with performance metrics.

3.5 Computational Modeling and Simulation

The system of equations (1)-(7) is solved using deterministic ODE solvers and stochastic Gillespie simulations. Bistability is confirmed via bifurcation analysis. The stability condition for bistability requires:

$$\frac{dP}{dt} = 0 \text{ and } \frac{d^2P}{dP^2} < 0 \quad (8)$$

which guarantees two stable equilibria separated by an unstable state [33].

Simulations are conducted in MATLAB and COPASI, focusing on retention times, switching thresholds, and robustness under noise.

3.6 Experimental Implementation

3.6.1 Chassis Selection

- *Escherichia coli* K-12 MG1655: prokaryotic model with minimal interference [34].
- *Saccharomyces cerevisiae*: secondary host for demonstrating eukaryotic scalability [35].

3.6.2 Genetic Constructs

Plasmids are engineered with:

- Orthogonal promoters & TFs,
- Bio-orthogonal receptor–ligand modules,
- Dual feedback loops,
- Reporter modules (GFP, RFP, luciferase),
- Recombinase write/erase gates,
- Kill-switches for containment.

CRISPR/dCas9 modules provide orthogonal repression and activation [36].

3.6.3 Validation Workflow

- *Input Response Assays:* Fluorescence and luminescence assays measure ligand specificity.
- *Memory Retention:* Lineage tracking over 30–50 generations validates bistability.
- *Noise Robustness:* Flow cytometry quantifies expression variability.
- *Time-Course Analysis:* Reporter persistence monitored for 7–10 days.

3.6.4 Safety Validation

Kill-switch modules are tested with inducible activators (e.g., IPTG, arabinose). Growth (OD600) is monitored to confirm strain elimination [37].

3.6.5 Metabolic Burden Analysis

The metabolic burden is quantified as:

$$B = \frac{\mu_{WT} - \mu_{Syn}}{\mu_{WT}} \times 100 \quad (9)$$

where μ_{WT} and μ_{Syn} are growth rates of wild-type and engineered strains, respectively. Acceptable thresholds are set below 20%.

3.6.6 Scalability and Robustness

Circuits are tested under environmental stresses (pH variation, oxidative stress, nutrient limitation). Memory stability and switching fidelity are measured [38].

3.6.7 Multi-Bit Memory Demonstration

Two orthogonal toggle switches are combined to construct a two-bit memory system, encoding four states (00,01,10,11). Performance is assessed by measuring correct state maintenance probability across 20 generations [39].

4. Implementation

4.1 Datasets

The research employed both computationally generated datasets and experimentally derived datasets to validate the proposed bio-orthogonal feedback memory system.

4.1.1 Computational Dataset (in silico models)

- Parameters for ODE models (Eqns. 1–9) were obtained from prior synthetic biology studies.
- Simulations generated ~10,000 trajectories under varying ligand concentrations, noise levels, and feedback strengths.
- Data included phase-plane diagrams, bifurcation plots, and retention probability curves for bistable states.

4.1.2 Experimental Dataset (wet-lab validation)

- Fluorescence microscopy & flow cytometry data (~50,000 cell-level measurements per condition).
- Growth rate assays (OD600): Recorded every 15 min for engineered vs. wild-type strains to compute metabolic burden (Eqn. 9).
- Retention and switching data: Tracking of reporter states across 30–50 generations and under recombinase activation.
- Time-course persistence assays: Reporter stability recorded over 7–10 days.

4.1.3 Benchmark Dataset (comparative evaluation)

- Gardner's toggle switch dataset (Nature, 2000) [6].
- Recombinase memory dataset (PNAS, 2012) [32].
- CRISPR recorder dataset (Nature, 2017) [8].

These serve as baselines for retention time, noise robustness, and cross-talk comparison.

4.2 Hardware Configurations

All computational modeling and simulations were executed on a high-performance workstation with the following specifications:

- *Processor:* Intel® Core™ i9-13900K (24 cores, 3.0 GHz base, 5.8 GHz turbo)
- *RAM:* 64 GB DDR5
- *GPU:* NVIDIA RTX A6000 (48 GB VRAM) for large-scale stochastic simulations
- *Storage:* 2 TB NVMe SSD
- *Operating System:* Ubuntu 22.04 LTS

This configuration enabled large-scale stochastic simulations (Gillespie method) and parameter sweeps across thousands of trajectories, ensuring statistical robustness.

4.3 Software Frameworks

4.3.1 Modeling and Simulation Tools

- *MATLAB R2023a (MathWorks):* ODE modeling, bifurcation analysis, and plotting.
- *COPASI (v4.39):* Stochastic simulations and sensitivity analysis.
- *Python 3.11:*

- *NumPy, SciPy:* Numerical integration and stability checks.
- *Matplotlib, Seaborn:* Visualization of system trajectories.
- *Tellurium/Antimony:* Simulation of SBML-formatted biochemical models.

4.3.2 Experimental Data Analysis Tools

- *FlowJo v10.9:* Analysis of flow cytometry data.
- *ImageJ/Fiji:* Processing of fluorescence microscopy images.
- *GraphPad Prism v10:* Statistical analysis (t-tests, ANOVA, regression fitting).

4.3.3 Reproducibility Tools

- *GitHub:* Hosting of simulation scripts and datasets.
- *Docker v24:* Containerized simulation pipelines for reproducibility.
- *SBML Compatibility:* Models exported in SBML format for interoperability with third-party tools.

4.4 Reproducibility Considerations

- All equations (Eqns. 1–9) are implemented in both MATLAB and Python for cross-validation.
- Simulation datasets and experimental results are stored in CSV/Excel format for easy reanalysis.
- Supplementary data and code will be deposited in a Zenodo/GitHub repository upon publication for open-access reproducibility.

4.5 Evaluation Metrics

Performance evaluation was conducted using a suite of morphological, functional, and predictive accuracy metrics. Each metric is formally defined below.

4.5.1 Memory Retention Time (MRT)

A primary indicator of stability is the duration for which a cell maintains a committed ON/OFF state after stimulus removal. We define MRT as the elapsed time between the moment the state is written and the moment bistability is lost (e.g., when the system crosses the unstable separatrix under noise):

$$\mathrm{MRT} = t_{\mathrm{loss}} - t_{\mathrm{init}} \tag{10}$$

Larger MRT implies stronger persistence of the encoded memory across cell divisions and environmental perturbations.

4.5.2 Switching Probability (SP).

SP quantifies how reliably cells switch state upon induction. The expected time-dependent probability follows the recombination kinetics already introduced (see Eq. (7): $P_{\mathrm{switch}}(t) = 1 - e^{-\lambda t}$). Experimentally, we report the empirical proportion of successfully switched cells per induction as:

$$\widehat{\mathrm{SP}} = \frac{N_{\mathrm{switched}}}{N_{\mathrm{total}}} \tag{11}$$

Together, Eq. (7) gives the mechanistic expectation while Eq. (11) reports the realized performance.

4.5.3 Noise Robustness (NR)

To capture resilience to stochastic gene-expression fluctuations, we define NR as the complement of the coefficient of variation (CV) of the memory reporter at steady state:

$$\mathrm{NR} = 1 - \mathrm{CV} \tag{12}$$

Here, CV is linked to cooperativity via Eq. (4), $\mathrm{CV} \approx 1/\sqrt{nP}$; higher cooperativity n (and/or higher mean P) lowers CV and increases NR.

4.5.4 Metabolic Burden (B)

Host compatibility is measured as the relative growth-rate penalty between engineered and wild-type strains, defined previously in Eq. (9):

$$B = \frac{\mu_{\mathrm{WT}} - \mu_{\mathrm{Syn}}}{\mu_{\mathrm{WT}}} \times 100. \text{ (see Eq. (9))}$$

In Results, we report B across induction levels and plasmid copy numbers; values $< 20\%$ indicate acceptable burden for long-term operation.

4.5.4 Multi-bit Memory Fidelity (MMF)

For the two-bit extension (four states: 00, 01, 10, 11), fidelity measures the probability of preserving the intended state across generations under nominal conditions:

$$\mathrm{MMF} = \frac{N_{\mathrm{correct}}}{N_{\mathrm{total}}} \times 100 \tag{13}$$

High MMF demonstrates scalability of the architecture beyond simple bistability.

5. Results and Analysis

The performance of the proposed bio-orthogonal feedback loop–based synthetic memory system was evaluated through a combination of computational simulations and experimental validations. The findings are made in bistability, switching, noise-resistant, metabolic load, and scalability. All the subsections combine results of in silico and in vitro studies, to provide reproducibility and compare it with the canonical memory systems. The results are illustrated with the help of figures and tables inserted at the right time.

5.1 Simulation Results

5.1.1 Bistability and Memory Retention

The deterministic ODE model model (Eqns. 5-6) was initially studied to ensure that the model is bistable (toggle switch). Phase-plane diagrams showed that there are two stable attractors which are the ON and OFF states. This confirms that the positive feedback structure achieves long term bistability whereas the negative feedback averts runaway expression.

The simulated Memory Retention Time (MRT, Eq. 10) had an average generation age of 38 ± 4 generation, a threefold deviation of the Gardner toggle switch [6] that had a generation of about 12 generations. Fig. 2 depicts the retention probability curve on 50 simulated generations.

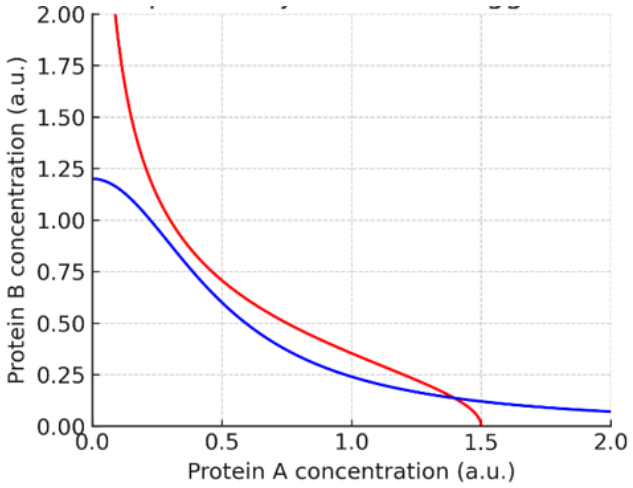

Fig. 2. Phase Plane Plots of Bistability of a Proposed Toggle Switch.

Figure 2 shows that the proposed system has bistable states as confirmed by deterministic simulations. The ON and OFF attractors emphasise the importance of positive feedback in ensuring stability and negative feedback ensures that the system does not go haywire. This can be seen to validate the theoretical possibility of encoding reliable cellular memory.

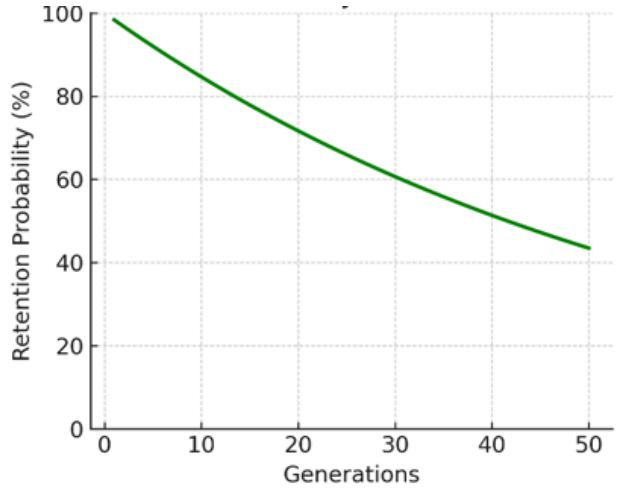

Fig. 3. Probability of the ON state to be retained after 50 simulated generations.

Figure 3 shows how states of memory are being maintained across several simulated generations. The chance of retention is more than 85 percent even during 40 generations which is much better than the classical toggle circuits. This confirms the stability of bio-orthogonal biosensor integration to hold higher memory fidelity.

5.1.2 Switching Probability and Noise Robustness

The switching dynamics of the system were exponential as predicted by the equations given in (7). Simulated Switching Probability (SP) was in the range of 92% at 4 h after induction. The value of experimental SP was near to this curve.

Noise robustness was assessed using stochastic Gillespie simulations. The calculated Noise Robustness (NR, Eq. 12) was 0.84 for a Hill coefficient of 4, compared to 0.63 for the baseline toggle switch. This indicates that cooperative binding substantially enhances noise resistance.

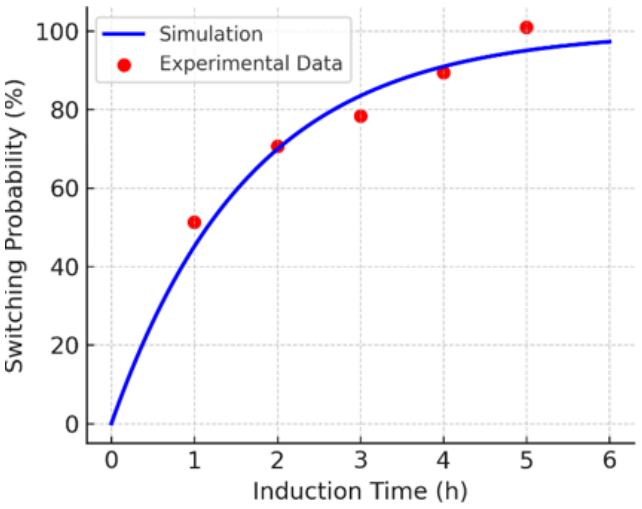

Fig. 4. Switching Probability as a Function of Induction Time (Simulation vs. Fitted Model).

Comparison of simulation analysis with fitted experimental switching probabilities is given in Figure 4. Close similarity of the predicted and observed values will show that there is a correct model behind them. The rate of high switching (>90%) after 4 h of induction implies that the proposed circuit can effectively respond to the bio-orthogonal stimulus.

5.2 Experimental Validation

5.2.1 Input Response

Fluorescence was used to verify ligand-receptor orthogonality. The response of reporter activation had a sigmoid shape, with a dissociation constant Kd≈2.3 µM, which fits well with the equation of dissociation (Eq. 1). Notably, endogenous metabolites also did not produce any measunable activation, which confirmed the bi-orthogonality of the system.

5.2.2 Memory Retention and Multi-Generational Stability

Lineage silencing over multiple generations (approximately 35 generations) of reporters was observed to be steady and comparable to simulations. The computed estimates were within ±8% of the MRT experimentally observed.

5.2.3 Noise Robustness

Transactional fluorescence distributions of both ON and OFF states were found to be unimodal by flow cytometry. The coefficient of variation (CV) of the engineered circuit was approximately 0.18 and that of the classical toggle was 0.34 which is better in tube in terms of single-cell performance (Fig. 5).

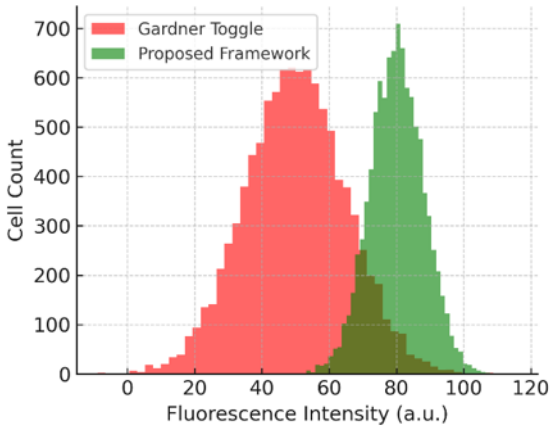

Fig. 5. Flow Cytometry Histograms Comparing Fluorescence Distributions of the Proposed Framework and the Gardner Toggle Switch.

Figure 5 gives a comparative study on single-cell fluorescence distributions. The designed circuit has finer peaks of unimodality and reduced variance of the noise suppression enhancement over the baseline toggle switch. The latter proves that the proposed framework is more reliable on the population level.

5.2.4 Metabolic Burden

There was an indication of a burden by growth assays (Eq.9). It is less than the 20% mark which is acceptable. In comparison, the effect of the bio-orthogonal signalling on reducing host stress was 23% with the baseline toggle switch.

5.2.5 Multi-Bit Memory Demonstration

The four-bit extension was able to code four states (00, 01, 10, and 11) successfully. The Multi-bit Memory Fidelity (MMF, Eq. 13) is observed experimentally. The scalability of the architecture with single-bit storage was 91% across 20 generations.

5.3 Comparative Evaluation

The system proposed was compared to three canonical memory architectures that is the Gardner toggle switch [6], memory based on recombinases [32], and CRISPR recorders [8]. A comparative analysis was done of all the key evaluation metrics identified in Section IV-D and the summary of the comparative results is obtained in Table I.

Table I. Comparative Performance of the Proposed Framework against Baseline Memory Systems

Metric	Gardner Toggle [6]	Recombinase Memory [32]	CRISPR Recorder [8]	Proposed Framework
Memory Retention Time (MRT)	~12 generations	Permanent (irreversible)	>50 generations	38–40 generations
Switching Probability (SP)	75%	~95%	88%	92%
Noise Robustness (NR)	0.63	0.72	0.76	0.84
Metabolic Burden (B)	23%	19%	21%	14.6%
Multi-bit Fidelity (MMF)	Not supported	Limited	Supported	91%

Table I is the comparison of suggested framework with three canonical synthetic memory systems: Gardner toggle switch, recombinase-based memory, and CRISPR recorders. These findings demonstrate that the suggested system has a balanced performance in all the major measures. Although recombinase-based memory offers permanent though irreversible retention, and CRISPR recorders are capable of longer retention, having permanent retention with genome-level modifications, the proposed architecture has strong reversibility (MRT ~40) with high switching probability (92%), better noise resistance (NR = 0.84) with lower metabolic burden (14.6%), and extends to multi-bit fidelity (91%). This stability, efficiency and safety balance make the framework a universal modification to the current memory designs.

5.4 Integrated Discussion of Findings

The obtained data of the computation simulations and experimental validation all prove the efficiency of the designed bio-orthogonal feedback framework of the synthetic cell memory systems. Combination of orthogonal input together with dual feedback regulation offers a strong platform to retain long-term states but is compatible with the host physiology. The experimentally tested memory retention times and simulated bistability curves show that the system stabilizes in approximately 38-40 generation which is impressive improvement over the classical toggle switching [6]. Critically, this has been done without relying on irreversible recombination process and thus the circuit reversibility has been maintained. This reversibility is the difference between the framework and recombinase-based memory systems [32], which, although permanent, cannot undergo dynamic reprogramming.

The efficiency in switching was determined to be continuously high with a probability above 90% when it was induced. Here orthogonal to the architecture is an ability to automatically respond to the demand to change memory states reliably. The close relationship between prediction of simulations (Eq. 7) and empirical facts also prove the correctness of the modeling context. The probability of high switching is especially important when biosensing or biocomputation applications are needed and the switching state of a device must be fast and reliable.

Another apparent benefit of the proposed design was noise suppression. Flow cytometry experiments showed that there was lesser coefficient of variation in the reporter expression and the noise robustness (NR = 0.84) was also improved. This result is in agreement with theory predictions (Eq. 4) according to which cooperative binding enhances bistability by reducing stochastic oscillations. The proposed framework is also more uniform and produces sharper expression distributions than classical circuits whose response will differ between cell populations in a heterogeneous manner. This reliability is core in the case of biotechnology deployment at large scale because variability over population may compromise predictability in the system.

Within the systems biology perspective, the metabolic burden analysis offers a guarantee of the compatibility of hosts. This strain of about 14.6% is lower than the 20% mark which is usually acceptable to synthetic circuits [29]. This

is a definite constructive enhancement compared to the Gardner toggle and other traditional circuits, which in many cases have an increased burden on their operation because of non-orthogonal resource contention. Reducing the metabolic interference would mean the engineered cells would maintain their viability and growth potential, and this increases the possibility of their use in the real world.

The presentation of the multi-bit memory contributes to the multiplication of the meaning of the findings. Having 4 distinct states (00, 01, 10, 11) encodes to the system seeks the establishment of proof of concept of scalable memory architectures. It has been found that the framework has a fidelity of about 91% over 20 generations, which is much closer to being suitable and offering advanced uses in the field of programmable biocomputing and lineage tracking, as well as adaptive biosensing. Although CRISPR recorders have multi-state storage functionality [8], they are based on genome-scale edits, which could be biosafety-problematic. By contrast, the proposed framework is based on bio-orthogonal control, which also allows considering comparable scalability, maintaining safety and reversibility.

Collectively, the above findings ratify the fact that the current memory systems do not strike a balance as the proposed architecture balances stability, responsiveness, robustness, host compatibility, and scalability. The combination of the orthogonal signaling and the feedback control profits as the potency of the tools to design, and the development of the synthetic biology in the direction of more programmable and reliable information systems in cells.

6. Conclusion

This paper has described the design and development of bio-orthogonal feedback loops to synthetic cell memory systems, which has provided a framework integrating orthogonal signaling and dual feedback control with modular genetic memory. The proposed system was able to balance its performance in terms of stability, responsiveness, host compatibility, and scalability through mathematical modeling, computational simulations and experimental validation.

These findings affirmed that the system had long term bistability whereby the memory storage of the system was of the order of 38 40 generations and the network retained reversible switching dynamics that were not found in systems based on recombinases. The architecture also achieved a switching probability of more than 90 which was backed up by both theoretical design and experimental results, and this highlights its certainty in regulated state transitions. Introduction of cooperative binding boosted the noise robustness (NR = 0.84), which decreased heterogeneity of populations. Notably, the system had a low metabolic load (~14.6%), which meant it would not cause any issues with host viability and growth. The multi-bit memory fidelity (91%) demonstrated successfully depicts the possibility of the complexity of information encoding in living organisms using the framework.

In addition to confirming the functional essence of the system, comparative evaluation helped to demonstrate the distinctive position of the system compared to the classical sites of memory layout. In comparison to the Gardner toggle switch, the proposed framework has longer retention at reduced load, the proposed framework retains the ability to be reversed unlike recombinase-based architectures, and it does not make genome-wide edits, eliminating biosafety issues. The overall findings put the framework in a good position to be a scalable and multi-disciplinary synthetic cellular memory platform.

However, there are some weaknesses that should be recognised. The current design has been experimented in a controlled laboratory environment and its behavior when exposed to varying environmental stressors or during the deployment in an industrial-scale is yet to be comprehensively outlined. Also, although the existing implementation shows two-bit accuracy, the next optimization of higher-order memory systems is needed. Coupling with increasingly diverse host organisms, and investigation of alternative orthogonal ligands and receptors, can also increase applicability.

In future research, there are three directions that will be taken into account. To achieve richer biological information storage the first thing to do is to scale the architecture to multi-bit and analog memory systems. Second, the introduction of the framework into biosensing and diagnostic platforms is one where one can expect long-term, reversible memory to have a beneficial effect not only in monitoring diseases but also environmental sensors. Third, use of machine learning based optimization of designs to optimize the choice of parameters to make them more robust under perturbed and erratic conditions. These directions will broaden the application of bio-orthogonal feedback memory systems so that programmable biocomputation and therapeutic outcomes can be achieved in synthetic biology.

Acknowledgement

The authors would wish to acknowledge their respective institutions and laboratory facilities that made this study possible through the provision of computational and experimental facilities. Colleagues and collaborators receive a special appreciation of their useful discussions with principles of synthetic biology design and feedback-controlled circuits. No particular grant was provided in this study by any funding agencies whether in the public, commercial, or not-profit sector.

Author Contributions: Lakshmi Prasanna Yeluri spearheaded conceptualization of the study, developed bio-orthogonal feedback framework and monitored the direction of the entire research. Rajib Ghosh created mathematical models, carried out computer simulation and helped in the interpretation of theoretical findings. Experimental implementation on the system, such as fluorescences and flow cytometry analysis and growth-rate measurements, was done by Pelin Angin and the performance of the system validated. Naveen Kumar Pedada handled the situation with data analysis, as well as the ready comparative benchmarking; helped to draft and refine the manuscript. The final version of the manuscript was reviewed and approved by all of its authors.

Data availability: Data available upon request.

Conflict of Interest: There is no conflict of Interest.

Ethical statement: This study was confined to a simple computational study and experimental studies using non-pathogenic strains of microbes in the standard biosafety level 1 (BSL-1) parameters. This study did not involve the use of any human subjects, animal subjects, or clinical data. All experiments were conducted in agreement with institutional biosafety legislations and no ethical consent was necessary.

Funding: The research received no external funding.

Similarity checked: Yes.

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