A Dual-Assay Platform for High-Throughput Screening of Circadian Modulators Using FDA-Approved Small Molecules

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Abstract

Between 50 and 70 million Americans live with chronic sleep disorders, underscoring the urgent need for treatments that can safely modulate human circadian rhythms. These rhythms arise from internal biological clocks with an approximately 24-hour period that regulate key physiological and behavioral processes, including the sleep–wake cycle. Traditional drug-screening approaches often rely on single-endpoint assays, which are incompatible with assessing circadian phenotypes that require continuous monitoring over multiple days, to detect drug-induced changes in period or phase. To address this limitation, we implemented a scalable drug-repurposing strategy using FDA-approved small molecules in the fungal model organism *Neurospora crassa*. This system offers a rapid and cost–effective platform for identifying compounds capable of altering circadian function, enabling high-throughput screening of **2,600** small molecules for their effects on clock regulation.

Circadian Feedback Model & Clock Mechanism

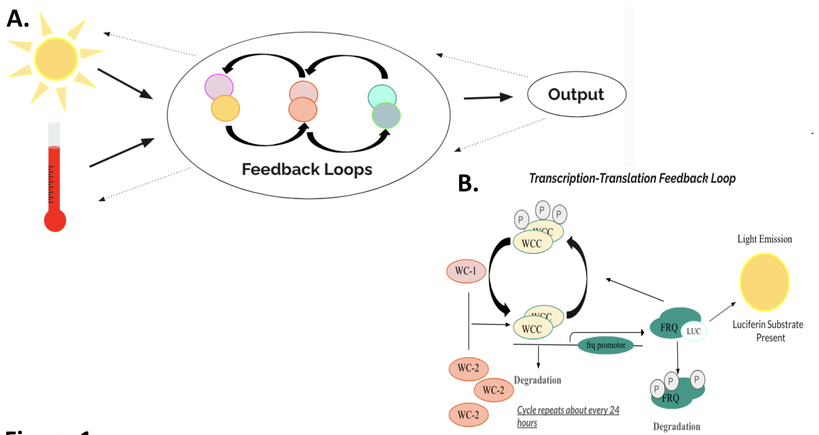

Figure 1.
A) A model of the circadian clock network of multiple feedback oscillators is presented by solid color lines and circles. The external input (light and temperature: Zeitgeber's) signal for clock entrainment is represented by solid black arrows pointing towards the feedback loops. The variation in the model's result is in both directions (seen by dotted arrows going in the opposite direction to the solid arrows). A direct observation of an output effect is: Protoperithecia Production Assay (PPA). **B)** Core Molecular Mechanism of the Circadian Clock. The *frq* promoter is activated by White Collar complex (WC-1/WC-2). The activation causes transcription of the *frq* luciferase gene. When this fusion protein is present, with Luciferin substrate, this chemically reacts, creating a quantitative glow (luminometer chamber detects).

Materials and Methods

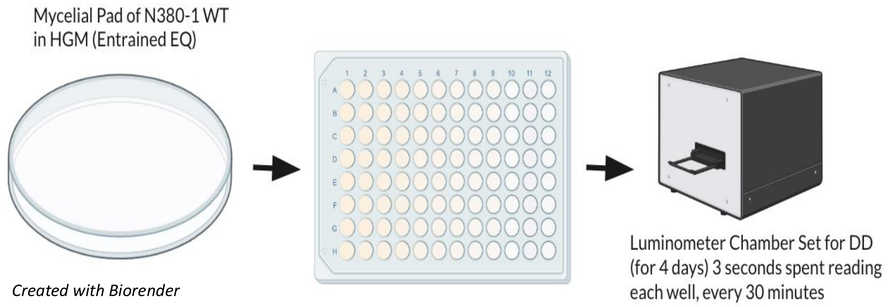

Figure 2. The FRQ:LUC reporter assay procedure: 190 µL plating media (LGM and Luciferin), 1 mm hole punch of mycelial pad (Strain WT: N380-1), 10 µL of treatment (4 Replicates of Each: LGM Control, DMSO, Drugs # (resuspended in original well plate with 0.5% DMSO "Dimethyl Sulfoxide" solvent and DI water) Sealed wells and poked a hole in seal for oxygen flow.

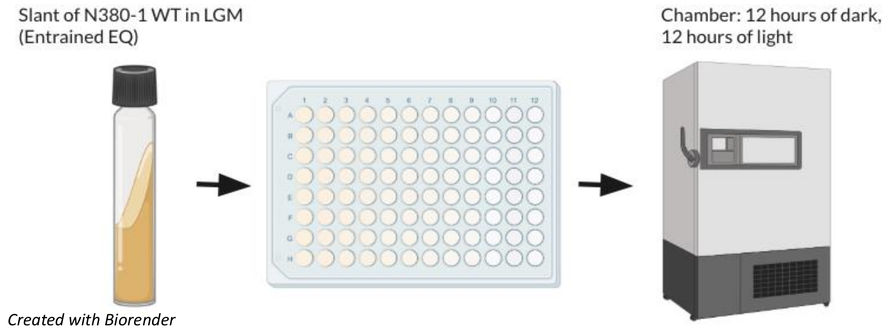

Figure 3. Protoperithecia Production Assay procedure: 150 µL synthetic crossing media into each well, 10 µL of treatment in according wells, 10 µL of WT N380-1 suspension (1 mL H2O suspension) into each well, the well plate is placed 25ºC chamber for 7 days (EQ Condition). Remove conidia from the top of the well using a microscope to picture and count protoperithecia.

Background

We developed and optimized a secondary reporter assay in *Neurospora crassa* using a **FREQUENCY (FRQ)–luciferase fusion protein** driven by the **frq** promoter, enabling real-time monitoring of circadian dynamics through rhythmic bioluminescence over multiple days. To complement this molecular readout, we incorporated a phenotypic analysis using the **Protoperithecia Production Assay (PPA)**, which quantifies protoperithecia formation under varying photoperiods as a proxy for the organism's ability to measure day length. Together, these assays provide a comprehensive platform for evaluating how small molecules affect both the core circadian oscillator and the organism's quantitative photoperiod-sensing function.

Results

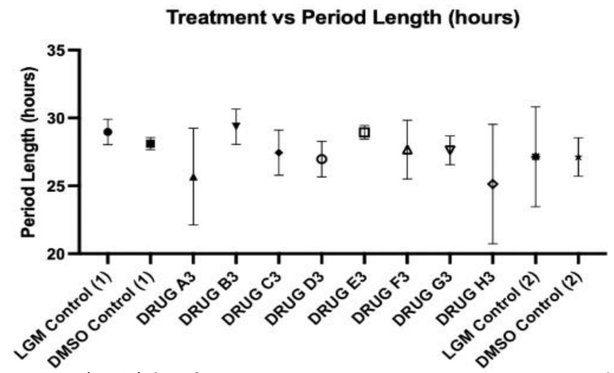

Figure 4. A (FRQ)-luciferase reporter assay experimental results comparing the period length (hours) and the treatment component: Drugs or Control group. One control is Low Glucose Media and the other is the DMSO (which is what is used to resuspend the drugs)>

Direction for Future Research

These two methods of screening were adapted and optimized for high-throughput screening, enabling an efficient, unbiased technique for identifying small molecules that modify circadian rhythmicity in *N. crassa.* Additionally, the completion of these screenings will provide the research community with a tangible platform for the rapid identification of circadian modulating compounds, which have the potential to enhance mechanistic studies and enable the repurposing of FDA–approved drugs. More broadly, this work supports the development of new strategies that may ultimately improve treatments for sleep disorders and other circadian–linked health conditions.

Acknowledgements

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References

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Comprehensive Modelling of the Neurospora Circadian Clock and Its Temperature Compensation - Scientific Figure on ResearchGate. Available from: https://www.researchgate.net/figure/Simplified-representation-of-the-Neurospora-circadian-clock-Transcription-factors-WHITE_fig1_223977888 [accessed 25 Nov 2025]

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