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High-resolution spatiotemporal analysis of gene expression in real time: *in vivo* analysis of circadian rhythms in *Neurospora crassa* using a FREQUENCY-Luciferase translational reporter

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Abstract

The pacemaker of the *Neurospora* circadian clock is composed of a transcriptional-translational feedback loop that has been intensively studied during the last two decades. Invaluable information has been derived from measuring the expression of the central clock component frequency (frq) under liquid culture conditions. Direct analyses of frq mRNA and protein levels on solid media -where overt circadian rhythms are normally visualized- have not been trivial due to technical issues. Nevertheless, a frq promoter-luciferase reporter has recently allowed the study of frq transcription under these conditions. It is known that FRQ undergoes extensive posttranslational modifications, and changes in its levels provide important information regarding the clockworks. Here we describe a FRQ-Luciferase translational fusion reporter that directly tracks FRQ levels, granting access to a better understanding and analysis of FRQ dynamics *in vivo*. More generally the method, which allows the investigator to follow continuous gene expression in real time in a spatially and temporally unrestricted manner, should be widely applicable to analyses of environmentally and developmentally regulated gene expression in ascomycete filamentous fungi as well as in basidiomycetes.

Keywords

Neurospora crassa; circadian; FREQUENCY; luciferase; bioluminescence

Circadian rhythms are biological oscillations that occur with a periodicity of approximately 24 hours. They have been reported in diverse organisms, from humans to bacteria, and in all cases they share the same basic design where a transcriptional-translation feedback loop (TTFL) is a central part of the circadian system (Dunlap, 1999). *Neurospora crassa* has served a premiere role in the understanding and molecular description of circadian clocks, and many of the findings described in this ascomycete have been extrapolated to other organisms including mammals. In *N. crassa*, the overt circadian rhythms in spore formation (conidial banding) and its analysis in race tubes, have been a great tool to access the status of the circadian oscillator (Baker et al., 2011; Lakin-Thomas et al., 2011; Liu and Bell-

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Pedersen, 2006). The *Neurospora* circadian oscillator is composed of the transcription factors -White Collar-1 (WC-1) and White Collar -2 (WC-2)- that drive the expression of the *frq* gene. The gene product, FRQ, is then capable of shutting down its own expression by inhibiting the White Collar Complex (WCC). FRQ is target of several different kinases in over 100 S/T sites, and its progressive and controlled phosphorylation is key in determining the periodicity of the system. As FRQ reaches a high degree of phosphorylation, the interaction of WCC and FRQ is disrupted, and the former can resume its activity. At the same time, hyperphosphorylated FRQ is degraded by the proteasome. Therefore, FRQ levels cycle daily (Baker et al., 2011).

Biochemical analyses of these oscillations in FRQ levels have been historically restricted to 52-70 hours due to the technical limitations of liquid culture conditions. It is assumed, nevertheless, that the changes in FRQ observed under these particular experimental setups are representative of what takes place on solid-medium race tubes. The feasibility to employ luciferase in *Neurospora* (Morgan et al., 2003), and lately the use of a fully-codon optimized luciferase gene, under the control of the *frq* promoter, has allowed to follow in great detail the dynamics of *frq* expression in solid media (Gooch *et al.*, 2008) partially confirming such assumptions. While other approaches such as mCherry-tagging of FRQ have allowed visualization of this protein under solid cultures conditions (Castro-Longoria *et al.*, 2010), the characteristics of the fluorescence-based microscopic analysis might hamper continuous circadian monitoring *in vivo*. In order to further access the molecular details of the circadian oscillator, we sought to create a strain that could directly and continuously report changes in FRQ levels, so that information of another main circadian state variable could be better analyzed *in vivo*.

FRQ-LUC levels oscillate *in vivo*

The codon-optimized luciferase sequence was fused to the C-terminal encoding region of *frq* by homologous recombination (Fig. 1A). The recombinant protein, in homokaryotic strains, shows oscillations under liquid culture conditions as confirmed by a standard western analysis (Fig. 1B). Importantly, circadian banding still occurs in the presence of FRQ-LUC, suggesting that this fusion protein can rescue clock function (Fig. 1C). CCD imaging of race tubes inoculated with the new *frq^{luc}*, reveals clear and sustained oscillations in luciferase levels, directly reflecting oscillations in FRQ amounts, with a great spatiotemporal resolution (Fig. 1D) (Video 1). The peak of FRQ-LUC expression is comparable to what has been described for FRQ in WT strains, exhibiting also a delay relative to the peak in *frq* mRNA levels (as inferred from a *frq_{promoter}-luc* reporter) (Gooch *et al.*, 2008) (not shown). The analysis of *frq^{luc}* strains on race tubes permits simultaneous examination of different regions, covering old and young tissues, allowing a dynamical view of the levels of this central clock protein (Fig. 1D, Video 1). While a similar reporter, involving a translational-fusion of a central clock component (PER2) and luciferase, has been described in mouse (Yoo *et al.*, 2004), those analyses fit more an *ex vivo* definition, since it requires examination of tissue culture or tissue slices. The *frq^{luc}* reporter, instead, allows for visual assessment and quantification of a central clock component in a less invasive manner, as the entire organism can be inspected as it grows under controlled conditions. As in the case of the *frq_p-luc*, *frq^{luc}* can track the status of the clock, independently from conidial banding. Therefore clock properties can be readily assessed in *ras-1^{bd}* or *ras-1^{wt}* strains (Belden *et al.*, 2007) (Video 1). While clear rhythms in FRQ-LUC levels are observed in both strains, the presence of high residual bioluminescence in the conidial bands is intriguing. This and other observations, including the expression of this reporter under different entrainment conditions –and in both genetic backgrounds– will be the subject of future studies. Importantly, now it is possible to directly quantify and follow FRQ under different culture conditions or in diverse genetic backgrounds, including ones where overt rhythms in conidiation cannot be

detected (e.g. 2% Glucose, etc.) (Data not shown). FRQ-LUC levels can also be informative of overall FRQ-half-life, as it can be inferred from the quantification of the bioluminescence signal during the first hours upon light to dark transition. Furthermore, such a strain could help assessing how local perturbations in FRQ levels can alter the rhythmicity of surrounding regions. Temperature resetting of the clock can also be nicely analyzed, following FRQ levels after a temperature step has been used to set the phase of the clock (Fig. 1E). In addition, fast increases in FRQ levels can be followed with great accuracy, as the ones produced after a light pulse has been given (Video 2).

While the fusion of LUC to the FRQ protein does not disrupt FRQ function, it does create a minor change in period, extending it in 1-1.5 h at 25°C. Nevertheless, the resulting strain is still a powerful tool to study in depth the spatiotemporal details of the clockworks by directly examining one of its state-variables *in vivo*. The comparative analysis of *frq* transcriptional and translational reporters might assist in the generation of stronger and extended data sets, helping the development of refined mathematical models and the understanding of the molecular events involved in the property of time-telling.

To our knowledge, this is the first time that luciferase is used as a translational reporter to study, *in vivo*, dynamic expression of an endogenous gene product in filamentous fungi. The strategy, described herein, could be easily adopted in *Neurospora* or other fungi, to follow the spatiotemporal expression of proteins whose levels are affected by environmental stimuli (nutrients, metals, metabolites or the interaction with another organism), or that are involved in other processes as sexual development, etc. Thus, the combination of real-time transcriptional and translational reporters should help in integrating existing biochemical and molecular data (acquired mainly from liquid cultures conditions) into a more holistic and dynamic view of gene expression in fungi. In *Neurospora* full-codon optimization was required in order to obtain high luciferase expression. While the *Neurospora*-optimized gene will be directly usable in many fungi, it may be that the same strategy will be needed in others; however, access to genomic information should allow prediction of whether optimization is necessary.

Methods

The *frq* locus was modified as described in (Larrondo *et al.*, 2009). Briefly, the coding sequence of the fully codon optimized luciferase sequence was fused to the last codon of the *frq* sequence, and 300 nt of the *frq* 3' region were added. Next to this region a *bar* selectable marker was included (Larrondo *et al.*, 2009). Homokaryons, expressing the fusion protein, were analyzed in race tubes confirming that circadian banding was maintained. High glucose (2%) liquid cultures were conducted under circadian conditions to assess the circadian expression of FRQ-LUC by western blots, as previously reported (Baker *et al.*, 2009). The imaging system and luminescence analysis was performed as recently described (Gooch *et al.*, 2008). A customized Excel Macro to process the images was developed and is available upon request. ImageJ was used for the 3D surface plot processing of video 2. Media and culture conditions, as well as western blot analysis can be found elsewhere (Baker *et al.*, 2009; Larrondo *et al.*, 2009).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

- Baker CL, et al. Quantitative proteomics reveals a dynamic interactome and phase-specific phosphorylation in the *Neurospora* circadian clock. *Mol Cell*. 2009; 34:354–63. [PubMed: 19450533]
- Baker CL, et al. The circadian clock of *Neurospora crassa*. *FEMS Microbiol Rev*. 2011
- Belden WJ, et al. The *band* mutation in *Neurospora crassa* is a dominant allele of *ras-1* implicating RAS signaling in circadian output. *Genes Dev*. 2007; 21:1494–505. [PubMed: 17575051]
- Castro-Longoria E, et al. Circadian rhythms in *Neurospora crassa*: dynamics of the clock component frequency visualized using a fluorescent reporter. *Fungal Genet Biol*. 2010; 47:332–41. [PubMed: 20051268]
- Dunlap JC. Molecular Bases for Circadian Clocks. *Cell*. 1999; 96:271–290. [PubMed: 9988221]
- Gooch VD, et al. Fully codon-optimized luciferase uncovers novel temperature characteristics of the *Neurospora* clock. *Eukaryot Cell*. 2008; 7:28–37. [PubMed: 17766461]
- Lakin-Thomas PL, et al. The genetics of circadian rhythms in *Neurospora*. *Adv Genet*. 2011; 74:55–103. [PubMed: 21924975]
- Larrondo LF, et al. Fungal Functional Genomics: Tunable Knockout-Knockin-expression and tagging strategies. *Eukaryot Cell*. 2009
- Liu Y, Bell-Pedersen D. Circadian rhythms in *Neurospora crassa* and other filamentous fungi. *Eukaryot Cell*. 2006; 5:1184–93. [PubMed: 16896204]
- Morgan LW, et al. Circadian and light-induced expression of luciferase in *Neurospora crassa*. *Fungal Genet Biol*. 2003; 38:327–32. [PubMed: 12684022]
- Yoo SH, et al. PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci U S A*. 2004; 101:5339–46. [PubMed: 14963227]

Generation of an efficient tool to study the dynamical changes of a main *Neurospora* circadian component.

Continuous and long-term monitoring and quantification of a *Neurospora* circadian clock core-component oscillations *in vivo*.

Dynamic analysis of FRQ levels under *in vivo* conditions and its responses to environmental cues

Proof of principle of the use of codon-optimized luciferase as a translational reporter in *N. crassa*.

Figure 1. A FRQ-LUC translational fusion rescues clock function in *Neurospora*

A) General strategy followed to obtain, by homologous recombination at the *frq* locus, a strain expressing a FRQ-LUC protein. The resulting strain expresses solely FRQ-LUC, and no unmodified FRQ, as observed by western blot. **B)** Western blot analysis of samples obtained from liquid cultures reveals oscillations in FRQ-LUC protein levels **C)** FRQ-LUC can sustain overt rhythmicity in conidiation, as observed by the classic race tube assay. **D)** Rhythms in FRQ-LUC levels can be monitored *in vivo* by use of a CCD-camera. The picture corresponds to a false-colored CCD-image taken at hour 155 in constant darkness (see video 1). Different levels of luminescence can be visualized based on pixel density (represented here as different colors from white (high levels) to blue (low levels)). Quantification FRQ-LUC expression can be performed in any region of interest as indicated by the three red rectangles (first three graphs) or in the entire growing culture (outer blue box, last graph). **E)** Quantification of FRQ-LUC levels in cultures grown in constant darkness at 25 °C, after they were held for 24 hours under constant darkness at 28 °C or 4° C, or constant light conditions (LL) and 25° C. Notice the antiphasic expression of FRQ-LUC in cultures coming from 4° C as previously reported using race tubes cultures by Liu et al., 1998 (reviewed in Baker *et al.*, 2011).