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Editorial

Gene Expression of the Clock Gene in Moving Mice in Multiple Tissues

Francesco Clerici*

Department of Oral and Maxillofacial Radiology, Radiology Center, Kagoshima University Hospital, Kagoshima, Japan

Introduction

In animals, a master clock in the Supra Chiasmatic Nucleus (SCN) regulates circadian rhythms in physiology and behavior SCN. Clock genes play an important role in the expression of cellular circadian rhythms, which are principally controlled by a cell-autonomous molecular feedback loop1. Clock gene expression is not restricted to the SCN, as it can be seen in a wide range of organs. However, outside of the SCN, the expression pattern of clock genes is poorly understood, partly due to a lack of tools for simultaneously monitoring circadian clock gene rhythms in specific tissues and detecting output functions in the absence of physical limitations.

The bioluminescent reporter enzyme firefly Luciferase (luc) and its substrate D-luciferin have been successfully adapted to whole-body imaging of clock genes in anaesthetized mice using a Charge-Coupled Device (CCD) camera, and have been used to generate optical imaging signals with high sensitivity in living animals. Anesthesia, on the other hand, has been shown to change the expression of the clock gene. Saini used a CCD camera outside the body to assess rhythmic bmal1 expression solely in the liver of a freely moving mouse.

A bioluminescent reporter has also been used to monitor clock gene expression in *vivo*, either with an optical fiber in the SCN or with a photon detector outside the body. Bioluminescence emitted by tissue-specific reporters was efficiently gathered using a conical wall that funnels photons toward a Photomultiplier Tube (PMT), and the emitting tissue was identified using a tissue-specific reporter in the latter scenario. These approaches, while promising, are not without flaws.

Monitoring clock gene expression via an implanted optical fiber, for example, only allows for a limited amount of focus on the target area in a small space, and insertion of an optical fiber may cause damage to the area surrounding the target. If the target is relatively large and located in deep locations inside the body, tissue-specific bioluminescence may need to be quantified when employing a PMT exterior to the body for bioluminescent detection. In addition, signal strength must be calibrated in three dimensions (3D).

We present a method for quantifying clock gene expression in multiple locations of freely moving mice over lengthy periods of time. The task at hand was to calibrate the intensity of bioluminescence in moving targets. We developed a Dual-Focal 3d Tracing (DuFT) technology and a Signal-Intensity Calibration Technique (SICT) to overcome the technical challenge of intensity changes with time of day and distance from the recording apparatus, and combined these two systems into a software application for analyzing gene expression that we call 'Mouse Tracker.'

With these tools, we can track the circadian rhythm of clock gene expression in several organs simultaneously in unrestrained animals. In reaction to a long-duration light pulse, the Olfactory Bulb (OB) moves faster than other tissues (right and left ears and cortices, and skin).

Discussion

The current study offers a new method that allows for simultaneous, continuous, real-time monitoring of gene expression in many places in freely moving animals in 3D space over several days. For behavioral study in two dimensions, tracking techniques for small, unmarked animals employing pattern-matching techniques with one camera have recently been deployed. The focus point of the imaging space in the recording cage was successfully identified and tracked using pattern-matching algorithms applied to 3D imaging in the current work. The determination of the direction of travel of the targets and the simultaneous quantification of gene expression in numerous places over a lengthy period of time were made possible by tracking the position of markers on an animal in 3D.

The current system has a spatial resolution of 1.63 mm, which means it can distinguish between two unique spots that are >1.63 mm away. As seen by a dark region clearly demarcating the two target points, the influence of lights scattering from an adjacent target area or from each of the triangle locations is insignificant. The impact of dispersed light has been investigated. In few pixels (20–30 m3), the light intensity decreases dramatically with distance from an illuminating spot to background levels.

The bioluminescent intensity changes seen are dependent on the time of day, the position of the target in the observation chamber's 3D space, and the angle of the CCD camera relative

***Corresponding Author:** Clerici F,Department of Oral and Maxillofacial Radiology, Radiology Center, Kagoshima University Hospital, Kagoshima, Japan, E-mail: Francescoclerici@gmail.com

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to the ROI. The DuFT approach uses stereo photography principles to determine the 3D position of a target, while the SICT algorithm calibrates the signal strength against a calibration table. In unrestrained mice, these techniques allowed us to track the circadian rhythms of clock gene expression in numerous parts of the body, as well as their responses to intrinsic and extrinsic stimuli.

The Per1 reporter's bioluminescence was sufficient for detection with an EM-CCD camera with only a 0.5-s exposure, demonstrating that real-time investigation of the link between gene expression and behavior is conceivable. Only locations on or near the body surface were chosen as monitoring targets in this investigation. The use of strong tissue-specific reporters in the future may allow for the monitoring of many targets throughout the body, regardless of their location.

In *vivo* monitoring of clock gene expression in freely moving animals has hitherto proved difficult. The current strategy has three advantages over prior investigations. The first is the capacity to quantify gene expression with 3D calibration, which was previously unavailable in investigations. The multi-region monitoring capability, which was used here, allows for the investigation of correlations between tissues or cell-specific changes/responses in gene expression. The capacity to statistically monitor behavior in 3D space is the third advantage, as it allows for the investigation of gene expression in relation to specific behaviors, such as drinking and eating, as well as gene expression in a specific place. Thus, the present in *vivo* monitoring system represents a novel analytical tool for examining the relation between circadian rhythms in clock gene expression and physiological outputs.

The luciferin delivery mechanism, which restricts the recording period, is the fundamental constraint of the current technique. Connecting animals to a delivery pump (iPRECIO) with a feed line or implanting an osmotic pump is required for luciferin delivery. Furthermore, the intracellular luciferin concentration must be sufficient to enable the luciferin–luciferase reaction in order to estimate the transcription rate from bioluminescent intensity. We administered luciferin at a rate that resulted in a plasma concentration of >0.2 g l1 at all times. However, additional advancements in substrate delivery technologies might make such research easier.

In addition, the kinetics of distinct phase-shifts of circadian rhythms in six different tissues was shown. A minor secondary peak seen in five tissues between the first and second days suggests that a phase-delaying light pulse caused an internal transitory desynchronization among the peripheral tissues, a phenomenon seen in jet lag, shift-work-related illness, hypoinsulinemia, and diabetes.

In summary, we anticipate that advances using remote tracking devices, detecting low-intensity signals, eliminating noise and making improvements in automated algorithms will be forthcoming and will be widely applied to many areas of biomedical research, as well as finding applications beyond medicine. Our technology is by no means limited to circadian-related disease and research. Nevertheless, any other applications will need to investigate the sensitivity and spatial resolution if the tissue environment or source depth differs from what has been used in the present work.