Title: Single cell analysis of circadian dynamics in tissue explants

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Abstract
Tracking molecular dynamics in single cells \textit{in-vivo} is instrumental to our understanding of how cells act and interact in tissues. Current tissue imaging approaches have focused on short term observation and typically non-endogenous or implanted samples. Here, we developed an experimental and computational setup that allows for single cell tracking of a transcriptional reporter over a period of more than a week, in the context of an intact tissue. We focused on the peripheral circadian clock as a model system and measured the circadian signaling of hundreds of cells from two tissues. The circadian clock is an autonomous oscillator whose behavior is well described in isolated cells, but \textit{in situ} analysis of circadian signaling in single cells of peripheral tissues is as yet uncharacterized. Our approach allowed us to investigate the oscillatory properties of individual clocks, to determine how these properties are maintained among different cells, and to assess how they compare to the population rhythm. These experiments, using a wide field microscope, a previously generated reporter mouse, and custom software to track cells over days, suggest how many signaling pathways might be quantitatively characterized in explant models.

Introduction
Studies of the dynamics of proteins in single cells have revealed the behavior and heterogeneity of many key signaling pathways (Locke and Elowitz, 2009; Purvis and Lahav, 2013). The behavior of signaling molecules and the extent of variation among cells when they are organized
into tissues and organs is rarely explored, mainly due to the complexity of studying protein dynamics in live tissues. Approaches to bridge this gap have used organoid systems and tissue slices – particularly neural slices – to study the effects of cell identity and environment on signal transduction (Gogolla et al., 2006; Williams et al., 2013). While our understanding of tissue architecture and spatial regulation of signaling within tissues is considerable, it usually represents a static view with little information about fidelity or how signals might propagate among cells over time. The recently developed intravital imaging approach has improved our ability to analyze live cells within tissues, although to date it has been mainly used to study non-endogenous structures, and it is limited to an observation time of several hours (Ellenbroek and van Rheenen, 2014; Pittet and Weissleder, 2011). Extending this picture requires long-term observation at single cell resolution of tissues expressing florescent reporters of a type that has thus far been largely limited to neural slices (Cheng et al., 2009).

There are multiple unique challenges to imaging single cells in live tissues. Direct in situ observation of individual cells in a tissue context requires imaging environments that offer ultra-stable culture conditions while permitting constant observation. Additionally, the complexity of analysis in the crowded and often highly auto-florescent environment requires sophisticated computational approaches to correct for aberrations and permit exposures that minimize the photon dose to the sample.

One system that has drawn particular attention in the efforts to move towards near in vivo imaging is the circadian clock. Most organisms have circadian clocks, molecular oscillators that drive rhythmic processes in physiology and behavior (Gachon et al., 2004). In mammals, circadian clocks are cell-autonomous and built on a transcription-translation negative feedback loop, where the transcription factor BMAL1-CLOCK drives the rhythmic expression of its own inhibitors, PER and CRY (Takahashi et al., 2008). Circadian clocks are found in the cells of most organs (Yamazaki et al., 2000), and the circadian clock in each organ drives a unique subset of rhythmic processes. Decades of work have empirically defined the molecular components of the circadian clock and how these components combine to construct robust oscillations (Gekakis et al., 1998; Lee et al., 2001; Preitner et al., 2002; Busino et al., 2007; Godinho et al., 2007;
Siepka et al., 2007; Asher et al., 2008; Duong et al., 2011; Padmanabhan et al., 2012; Lande-Diner et al., 2013; Kim et al., 2014)

The activity of circadian clocks in peripheral organs has been studied extensively at the population level, mainly by measuring bioluminescent reporters of circadian transcription in cultured organ explants (Yamazaki et al., 2000). At present, there is no quantitative information about the dynamic behavior of circadian clocks at the single-cell level in any intact peripheral tissue. Here we developed experimental and computational approaches to quantify and analyze the dynamics of circadian clocks in live mouse tissue explants at the single cell level and to characterize the diversity of the clock dynamics among cells and between pulses within a cell. Long-term imaging of individual circadian clocks in the context of tissue explants, as we have developed and demonstrated here, will facilitate the characterization of the individual properties of each clock, its organization and its contribution to the overall population rhythm. More globally, our approach can be used to study similar complex dynamical systems, where the overall output is derived from the integration of many individual signals.

**Results and Discussion**

*Circadian rhythms of Per1-YFP can be measured and quantified in tissue explants*

We developed a system that allows long term imaging of the circadian clock in individual cells, in the context of their tissue of origin. We explanted tissues from a previously described transgenic mouse ubiquitously expressing Per1-YFP, a validated fluorescent reporter of circadian clock activity (Cheng et al., 2009). For the purpose of this study, we chose to focus on the activity of clocks in osteocytes from the calvarial bone, and tenocytes from the tail tendon. In these tissues, the relatively large distance between nuclei eliminates the need to deconvolve neighboring cells, and the nature of the extracellular matrix restricts cellular motility (Figure 1A, B). The long observation times and the limited axial resolution of our microscopy was well suited to the relatively immobile and co-planar cells in the bone and tendon, enabling long term analysis that would be challenging in other tissues. Microscopy modalities that provide greater 3D imaging depth such as light sheet microscopy might make analysis of other tissues more tractable.
We cultured explanted tissues in a temperature, CO₂, and humidity-controlled microscope, using a B27-supplemented transparent medium that maintained cell viability for more than a week. This approach allows for single cell imaging and long term observations of tissue pieces in a near-natural tissue environment. Using this setting we were able to image tissues for 6-8 days, sampling more than 40 fields of view every 30 minutes. Acquisition time per each field of view was less than 5 seconds, much faster than typical luciferase based single cell imaging that requires at least several minutes (Welsh and Noguchi, 2012).

We acquired brightfield images of bone and tendon explants, which capture the unique architecture of each tissue. Osteocytes were well separated and regularly distributed across the tissue surface (Figure 1A) and tenocytes (Figure 1B) were arranged in stripes. We measured the fluorescence intensity of Per1-YFP in individual cells over time and observed oscillations in both tissues (Supplemental Movies S1, S2). Visual analysis of the traces suggested an oscillatory pattern with a period of ~24h (Figure 1C, D), as expected from a circadian signal.

**Individual cell oscillators have stable periods in the circadian range**

We then collected data from five separate mice and developed software that identify cells within each image, quantify the YFP intensity, and string images together to form tracks of single cells over days of observation. We recorded and quantified the levels of Per1-YFP in more than 600 cells from the calvarial bone and ~150 from the tendon. Inspection of the extracted single cell traces of the circadian dynamics in both tissues indicates that cells show pronounced oscillations in the expected circadian range (Figure 2A, B). Circadian oscillations of similar characteristics were previously observed in single cell traces from SCN slices (Liu et al., 2007) as well as dissociated fibroblasts (Welsh et al., 2004; Bieler et al., 2014).

Visual analysis of the single cell traces suggests that cells exhibit synchronous circadian behavior at the beginning of the experiment, as indicated by the distinct stripes of signal in the heatmap, but lose coherence as the experiment advances. This was true for both tissues tested. The initial synchrony we observed could represent the default state in the intact living tissue or a resetting effect of post-mortem extraction and mounting of the tissue. The latter involves growth...
serum shocks and temperature fluctuations, both strong circadian synchronizing agents (Balsalobre et al., 1998).

Analysis of the intervals between subsequent peaks revealed a strong circadian peak of approximately 24hrs and substantial variability in the waveform of the traces with a long tail and a small peak at roughly 48hrs, suggesting some cells either skip a period or we fail to detect it (Figure 2 C, E). To quantify the periodicity in the YFP signal, we computed the Fourier transform from the pooled data of each organ. Reassuringly, using either the mode of the peak-to-peak intervals or the Fourier transform we obtained a period of between 22-25hrs for both tissues (Figure 2D, F). These values are within the range of those typically reported for bioluminescence population circadian rhythms (Yamazaki et al., 2000; Yoo et al., 2004).

We then considered whether there is mouse-to-mouse variability in the oscillatory period or de-phasing of the circadian signal. Comparing data collected on the calvarial bone for three different mice we observe negligible differences in the autocorrelation curves, arguing that the measured period and de-phasing are highly reproducible (Figure 2G). Similarly, comparison of the autocorrelation of the osteocytes and tenocytes from either the calvarial or tail tendon show a slightly lengthened period in the tendons, but overall similar behaviors across these tissues (Figure 2H). The autocorrelation curves (Figure 2 G, H) suggested a period of 23hr, consistent with our other analyses (Figure 2C-F).

To test for potential sources of systematic error in our measurements, we adapted a method developed to compare a measured circadian regulated activity – such as movement in animals – measured with some presumed error with the underlying clock fidelity by asking if the sequential periods are negatively correlated in length (Pittendrigh & Daan 1976a). A negative correlation suggests that the measurement has less fidelity than the underlying clock. Reassuringly, in neither the tenocyte or osteocyte data do we observe a significant negative correlation (Figure 2I; t-Test p-value > 0.05).

*Circadian signaling fidelity drops over the course of the experiment*
Given that we acquired measurements across at least seven days of oscillations for the osteocytes, we next asked whether the period and phase change over time. We compared the mean peak-peak interval for the first and last day of observation. We found that while the mean itself was not statistically different, the variability was greatly expanded by the eighth day of the experiment (Figure 3A). One possible explanation for this is that the fidelity of the oscillator begins to break down in some cells. Alternatively, the computational signal estimates may become more difficult as the tissue begins to decay. Consistent with a drop in fidelity and a general loss of synchrony, as implied by the autocorrelation function, the phase distribution of osteocytes expands monotonically as the experiment progresses (Figure 3B).

Variation in circadian amplitude is constrained within a cell and unrelated to period

The amplitude of circadian signaling has typically achieved less interest and study than the period. This is in part due to ease of measurement and also a lack of studies using single cells with uniform integrated reporter constructs. We quantified the amplitude of each oscillation at its peak for the osteocyte data and found a distribution of intensities ranging over roughly eight-fold (Figure 4A). Further, unlike the period, we found that the variability of the amplitude doesn’t increase with time, as both the mean amplitude and its variability were preserved from the first to the last oscillation (Figure 4A, inset). We then compared the overall variability in amplitude to the internal variation in a single cell. Interestingly, we found that on average individual cells shows lower variability in amplitude between their peaks than the variability observed between peaks in all cells (Figure 4B). This suggests that intrinsic factors that regulate the amplitude of circadian oscillations are preserved in cells throughout multiple days. The potential role of extrinsic factors, such as the local environment, remains to be explored.

Finally, we asked if there was a relationship between period length and amplitude. We tested if, for example, longer periods grant additional time for the Per1-YFP to accumulate, or conversely, if high circadian signaling might drive a faster period. Comparing the peak height to the subsequent interval between peaks we find no substantial correlation (Figure 4C, Pearson <0.1), suggesting that in this system period is buffered against variation in promoter activity of target genes. The conservation of period length among cells in a tissue is what one might expect if the system prioritizes the phase angles of individual cellular oscillators with respect to a common
entrainment signal, much as precise period lengths of behavioral activity over time assure a consistent phase angle of activity with respect to the light-dark cycle (Pittendrigh & Daan, 1976b). In a tissue composed of weakly-coupled cellular oscillators, this property would promote coherent and properly-phased rhythmic function of the tissue as a whole.

**Materials and Methods**

*Tissue collection and sample preparation*

Mice were housed in 12:12-h light/dark cycle and sacrificed in accordance with a protocol approved by the Harvard Medical School Standing Committee on Animals. Organs were collected and immersed in ice-cold HBSS supplemented with Penicillin and Streptomycin. Bone and tendon tissues were dissected using a Leica 2000 stereomicroscope, to obtain ~1mm x 1mm pieces. Explants were placed on Mat-Tek 35mm glass-bottomed dishes coated with 100 μl of 1.5% collagen (CellMatrix) and incubated for 5 minutes at 37°C. 50 μl of 1.5% collagen were added to cover the tissue, followed by a 10’ incubation at 37°C. 2 ml of transparent DMEM/F12 media, supplemented with Penicillin, Streptomycin and B-27 (Gibco) were then added to each dish. The collagen coating immobilizes the tissue while allowing for nutrients and oxygen to penetrate. Calvarial-derived bone and tail tendons have a naturally thin cross section and this feature enabled proper focusing of the objective on the cells. The depth of field of the microscope utilized was 3500 μm (see below). When accounting for the thickness of the collagen layer, we found that in order to allow for proper visualization, samples needed to be in the range of 150 μm. We also found that tissues such as liver, where cell density is high and nuclei are closely packed, were challenging to measure, as resolving individual nuclei proved to be difficult. Furthermore, tracking cells from tissues with high mitotic activity, such as lymph nodes, was difficult over the number of frames analyzed in one single experiment. In our experience, tissues in which nuclei are separated by an extracellular matrix constitute the best samples for this assay as nuclei are well spaced and constrained in their movement. Additional tissues that meet these requirements, and may be analyzed using similar approaches, are adipose tissue, cartilage and blood capillaries.

*Time-Lapse Microscopy*
Tissues were imaged using the VivaView system (Olympus). This system consists of a fully integrated and motorized inverted microscope with a cooled CCD camera that allows high quality, long-term time-lapse imaging in a constant and optimized environment. The microscope has a rotating platform that can accommodate up to eight 35mm dishes, and that allows for imaging of multiple positions in each one of the dishes. We used a magnification of 20x to capture the largest possible number of nuclei. Tissues were maintained in humid conditions (~95% RH), 37°C and 5% CO₂ for the entire imaging period. DIC, Venus (Per1-YFP) and RFP (auto-fluorescence) images were taken every 30 minutes for the duration of the experiment. Images were acquired using MetaMorph software (Molecular Devices).

The microscope uses an X-Cite exacts Fluorescent Hg lamp, and a UPLSAPO20X objective (Numerical Aperture (NA) 0.95, WD (working distance) 0.18mm). Venus filter set (EYFP/Venus/Citrine): excitation: 495nm, emission: 540nm. RFP filter set (TexasRed/mCherry/AlexaFluor 594): excitation: 560nm, emission: 635nm.

Image Analysis
Images were processed and analyzed with custom matlab code, which can be provided upon request. Briefly, each plane of a Z-Stack was background subtracted followed by max-intensity projection. Images were down-sampled 2-fold to improve signal. If substantial drift had occurred over the course of imaging sequential timepoints were aligned using a local cross correlation metric [Guizar-Sicairos et al., 2008]. Images were then smoothed on the time axis using a Kalman filter. The First 5-10hrs of data was then discarded as unreliable as the tissue was often ‘settling’ on the stage. Typically 5-7 days of good quality data was obtained from a given experiment.

Each position was manually visualized in ImageJ to determine if it was of sufficient quality for further analysis. Components that went into this decision were: number of visible cells, stability of the tissue over time (as accessed by DIC structure), and auto-fluorescence over time (as measured by RFP and YFP signal).
Nuclei were identified as objects with a given YFP intensity, shape and size and tracked through subsequent frames, intensity was computed as the mean of the brightest 10 pixels within an identified region. Identified cells were connected across frames using a nearest neighbor algorithm resulting in single cell traces; cell traces which did not persist for more than 80% of frames were discarded. Missing data (typically small numbers of frames in a trace where a cell was missed by the automated analysis) was interpolated from a spline fit to the trace as a whole. Raw traces and the objects that gave rise to them were manually examined for correct tracking and cell-like properties (e.g. some degree of non-monotonic changes in intensity and visually verifying the cell track appeared continuous), typically 30-50% of automated traces were discarded. Traces were then smoothed (averaging filter, width of 2.5hrs) and baseline subtracted (48hr trend line was subtracted) for peak identification and period analysis, amplitude analysis used non-baseline subtracted data.

Period was estimated by three methods: Autocorrelation, peak-to-peak distance, and Fourier analysis. Briefly autocorrelation was computed for each single cell independently and averaged across all cells. Peaks were identified by smoothing the trace and identifying peaks using the inbuilt Matlab algorithm, distance was then calculated for sequential pairs of peaks, finally peaks from all cells were pooled to estimate the distribution of periods. The same peak positions were used to measure the peak amplitude. To estimate the Fourier transform, single cell traces were Fourier transformed and the result averaged across cells, the peak of this was taken as the frequency.

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FIGURE 1: A Per1-YFP reporter allows for single cell quantification of circadian rhythms in a mouse organ explant system. (A, B) Brightfield and fluorescence images of bone from the calvarium (A) and tendon from tail (B). (C, D) Images and quantification of three bone (C) and three tendon (D) cells. Cells were imaged every 30 minutes. Frames every eight hours are shown. Quantification of YFP intensity over time shows an oscillatory pattern with a periodicity of ~24h.
FIGURE 2: Long term time lapse imaging and automated segmentation and analysis allow for large scale acquisition of single cell circadian data in tissues. (A, B) Heatmap and representative line plots showing Per1-YFP oscillations in calvarial cells (A) and tendon (B) drawn from three (A) or two (B) mice. (C-F) Distributions of peak-to-peak intervals and frequencies for calvarial cells (C, D) and tendon cells (E, F). (G) Average autocorrelation of Per1-YFP signal in three mice shows near identical period and decay rate (N=343, 129, and 221). (H) Comparison of calvarial (N=693) and tail tendon (N=108) signals shows similar average autocorrelation. (I) Scatter plots showing that the length of each individual period is independent of the preceding period (p-value=0.78, 0.31 by Student’s t-test).
FIGURE 3: Circadian signals in tissues show an initial synchrony that decays over time. (A) Period, measured by peak-peak interval, was calculated for the first and last circadian periods. Note that both measurements have comparable median values but the last period has a wider variation. (B) The phase distribution of cells from the calvarial bone is plotted after 1,2,3,4, and 5 days (N=334 cells in both panels).
FIGURE 4: The Amplitude of Per1-YFP is constrained within a cell but varies across the population and is independent of the period. (A) Distribution of Per1-YFP amplitude across cells in the calvarial bone. Note that the average intensity of YFP across the population does not change over the duration of the experiment (inset). Error bars represent standard deviation. (B) The coefficient of variation of Per1-YFP amplitude was computed across cells and within single cells across periods. The distributions are significantly different (KS-test, p-value=$10^{-54}$). (C) No correlation is observed between Per1-YFP amplitude and the period of the subsequent oscillation (N=254 cells in all panels).