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Streamlined single-molecule RNA-FISH of core clock mRNAs in clock neurons in whole mount *Drosophila* brains

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Circadian clocks are ~24-h timekeepers that control rhythms in almost all aspects of our behavior and physiology. While it is well known that subcellular localization of core clock proteins plays a critical role in circadian regulation, very little is known about the spatiotemporal organization of core clock mRNAs and its role in generating ~24-h circadian rhythms. Here we describe a streamlined single molecule Fluorescence *In Situ* Hybridization (smFISH) protocol and a fully automated analysis pipeline to precisely quantify the number and subcellular location of mRNAs of *Clock*, a core circadian transcription factor, in individual clock neurons in whole mount *Drosophila* adult brains. Specifically, we used ~48 fluorescent oligonucleotide probes that can bind to an individual *Clock* mRNA molecule, which can then be detected as a diffraction-limited spot. Further, we developed a machine learning-based approach for 3-D cell segmentation, based on a pretrained encoder-decoder convolutional neural network, to automatically identify the cytoplasm and nuclei of clock neurons. We combined our segmentation model with a spot counting algorithm to detect *Clock* mRNA spots in individual clock neurons. Our results demonstrate that the number of *Clock* mRNA molecules cycle in large ventral lateral clock neurons (LLNvs) with peak levels at ZT4 (4 h after lights are turned on) with ~80 molecules/neuron and trough levels at ZT16 with ~30 molecules/neuron. Our streamlined smFISH protocol and deep learning-based analysis pipeline can be employed to quantify the number and subcellular location of any mRNA in individual clock neurons in *Drosophila* brains. Further, this method can open mechanistic and functional studies into how spatiotemporal localization of clock mRNAs affect circadian rhythms.

KEYWORDS

circadian rhythms, single molecule RNA-fluorescence *in situ* hybridization (smFISH), machine learning-based analysis pipeline, spatiotemporal organization of clock mRNAs, *Drosophila* clock neurons

Introduction

Almost all organisms have evolved internal clocks to tell time. Circadian clocks are cell-autonomous timekeepers that can be entrained by light (Stanewsky et al., 1998) or temperature (Sehadova et al., 2009; Buhr et al., 2010; Yadlapalli et al., 2018) cycles. These clocks generate ~24-h oscillations in the expression of many genes and control rhythms in sleep-wake cycles, metabolism, immunity (Hardin et al., 1990; Aronson et al., 1994; Sehgal et al., 1994; Patke et al., 2020). While past studies highlight the critical role of subcellular localization of core clock proteins in controlling circadian rhythms (Curtin et al., 1995; Shafer et al., 2002; Cohen et al., 2014; Zhao et al., 2015; Xiao et al., 2021; Yuan et al., 2022), what remains poorly understood is how clock mRNAs are organized over the circadian cycle and the role of such organization in controlling circadian rhythms. A major drawback of techniques such as qPCR and single-cell RNA-sequencing currently used to study circadian gene expression is lack of subcellular spatial information (Zhang et al., 2014; Ma et al., 2021). Single-molecule RNA Fluorescence *In Situ* Hybridization (smFISH) has recently emerged as a powerful technique which enables quantitative measurement of the number as well as the subcellular location of mRNA molecules in individual cells (Raj et al., 2008; Trcek et al., 2017). In one recent study, smFISH was adapted to visualize core clock transcripts in *Neurospora crassa* and it was reported that core clock mRNAs are clustered in the perinuclear cytoplasmic region in a time-of-day dependent manner (Bartholomai et al., 2022).

Drosophila melanogaster is a powerful model system for understanding spatiotemporal regulation of circadian rhythms, as it has a highly conserved clock along with powerful genetic and molecular tools (Dubowy and Sehgal, 2017; Patke et al., 2020). Further, the *Drosophila* clock network is relatively simple consisting of ~150 clock neurons (Nitabach and Taghert, 2008; Dubowy and Sehgal, 2017) (Figure 1A). These clock neurons express the key clock proteins—CLOCK (CLK), CYCLE (CYC), PERIOD (PER) and TIMELESS (TIM), that form the core of the molecular clock (Patke et al., 2020) (Figure 1B). CLK and CYC act as the positive transcription factors and PER and TIM act as the core clock repressors. During the activation phase, CLK and CYC drive transcription of hundreds of target genes, including *per* and *tim*, by binding to the E-boxes in their promoter regions. PER and TIM, after a time delay, enter the nucleus and inhibit CLK/CYC activity thereby silencing their own expression as well as the expression of other clock-regulated genes. PER and TIM are then degraded leading to the end of the repression phase and the start of the activation phase of a new cycle (Patke et al., 2020). In addition to this core feedback loop, there exists a second interlocked feedback loop that controls rhythmic expression of *Clk* mRNA (Cyran et al., 2003; Glossop et al., 2003) (Figure 1B). CLK/CYC proteins bind to the E-boxes of the transcription factors, Vriille (VRI) and PAR domain protein 1ε (PDP1ε), creating rhythms in their mRNA with peak levels during early night and trough levels during early

day. Interestingly, VRI and PDP1ε protein levels peak at different times during the night, with VRI peaking during the early evening and PDP1ε later in the night. VRI and PDP1ε proteins bind one after the other to the VRI/PDP1ε-binding boxes in the *Clk* enhancer to repress or activate *Clk* transcription, respectively, thus resulting in rhythmic *Clk* mRNA expression.

Here, we describe a streamlined single molecule FISH (smFISH) protocol and a fully automated analysis pipeline to quantify the number and localization of mRNAs of *Clock*, a key clock transcription factor, in individual clock neurons in whole-mount *Drosophila* adult brains. We developed a simple, robust smFISH protocol that takes 3 days from brain dissections to slide preparation. Next, we generated an accurate three-dimensional segmentation model of clock neurons using machine learning-based approaches by adopting a pretrained decoder-encoder convolutional neural network model. Using these tools, we visualized and quantified *Clock* mRNA distribution in Large ventral lateral clock neurons (ILNVs), one of the main *Drosophila* clock neuron groups. Our results show that *Clock* mRNA levels cycle in ILNVs, consistent with past biochemical studies and single-cell RNA-seq studies (Ma et al., 2021), with peak levels at ZT4 (ZT-Zeitgeber time, ZT0-time of lights on, ZT12-time of lights off) with ~80 molecules per neuron and trough levels at ZT16 with ~30 molecules per neuron. Our study describes a powerful technique to directly visualize and quantify the number and subcellular location of core clock mRNAs in individual neurons, which can open new investigations into the role of spatiotemporal localization of mRNAs in controlling circadian rhythms. The methods described here can be easily adapted to directly visualize gene expression at the single-cell level in *Drosophila* brains.

Results

To directly visualize the subcellular localization and quantify the number of core clock mRNAs in individual clock neurons, we adapted single molecule RNA-Fluorescence *In Situ* Hybridization (smFISH) method for use in *Drosophila* clock neurons and developed a machine-learning based analysis pipeline to analyze the data. To validate our smFISH protocol and data analysis pipeline, we chose to focus on visualizing *Clock* mRNAs in ILNVs, one of the main groups of clock neurons. We note that the same methods can be applied to visualize mRNAs in any other clock neurons. Below, we describe the smFISH method and analysis pipeline in detail and present our findings.

Procedure overview

Our smFISH procedure consists of five parts: smFISH sample preparation, image acquisition, data analysis, quality control check, and final plot generation (Figure 1C). For sample

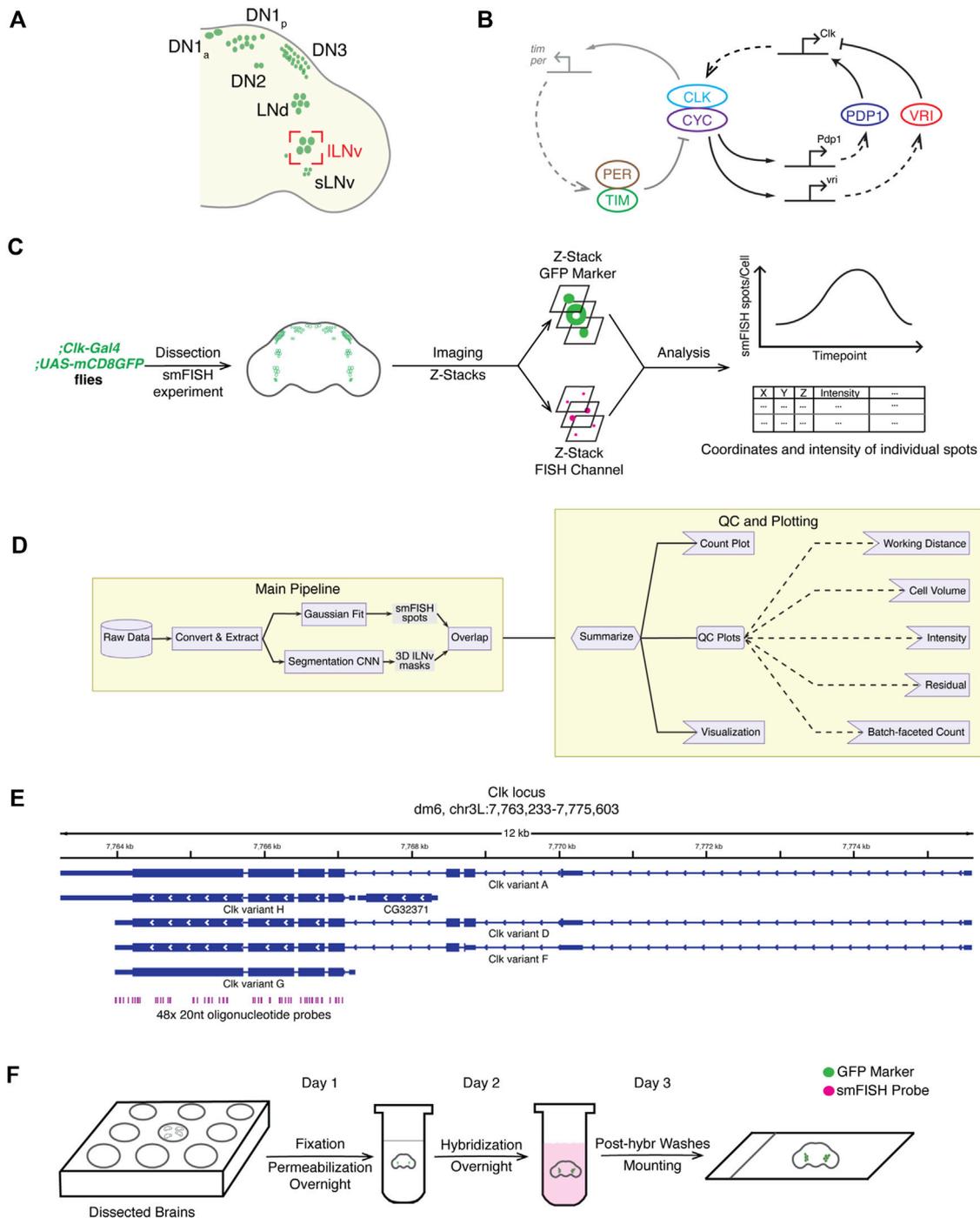


FIGURE 1

Overview of the smFISH method and the data analysis pipeline. **(A)** Schematic of different clock neuron groups in *Drosophila melanogaster* brains. Large ventrolateral neurons (ILNvs) are circled in red. **(B)** Schematic of the molecular clock in *Drosophila*. The *Drosophila* circadian clock is composed of two interlocked negative feedback loops. **(C)** Overview of the full workflow including smFISH experiment and data analysis pipeline. **(D)** Schematic of the data analysis pipeline. To quantify *Clock* smFISH spots in ILNvs, two tasks are performed in parallel: Detection of *Clock* smFISH spots and segmentation of ILNvs. **(E)** Genomic locations of oligonucleotide probes in the *Clock* locus. Probes are designed to hybridize with the shared exon sequences to visualize mRNAs of all annotated *Clock* transcript variants. **(F)** Schematic of the smFISH procedure.

preparation, *Drosophila* brains are dissected, fixed, and hybridized with smFISH probes with the whole protocol taking 3 days requiring little hands-on time (~1 h/day). For imaging, we used a glycerol-based mountant for tissue clearing and acquired images using a high-resolution Airyscan confocal microscope, which enabled us to acquire well defined diffraction-limited spots corresponding to individual mRNA molecules. To automate the process of identification of the cytoplasm and nucleus of ILNvs, a group of clock neurons, we developed a custom convolutional neural network (CNN) segmentation model to generate three-dimensional ILNv clock neuron masks. Performance of our CNN segmentation model was validated by manual inspection of CNN-generated masks on a testing dataset. To quantify the intensity and visualize the subcellular location of individual mRNA spots, we used a published spot detection algorithm, AIRLOCALIZE (Lionnet et al., 2011). Finally, we developed an integrated pipeline in which all data analysis workflows are automated by the workflow manager Snakemake (Molder et al., 2021) (Figure 1D). After the data analysis step, we generated quality control plots to verify the consistency of our results, identify noisy image outliers, and optimize analysis parameters. The analysis can be rerun with optimized parameters in a fully automated and reproducible manner. In the next subsections, we describe individual steps of the protocol in detail.

Fluorescent oligonucleotide probes for smFISH experiments

To enable detection of *Clock* mRNA transcripts, we ordered commercial fluorescent oligonucleotide probes from Biosearch Technologies, Inc. First, we designed ~48 20 nt short oligonucleotide probes that span across the full-length of all annotated *Clock* mRNA isoform species covering all exons using the online Stellaris RNA-FISH Probe Designer (Figure 1E). For genes with alternative splicing isoforms, shared exons can be used to design smFISH probes to ensure detection of all isoforms. The designer provides masking of repetitive sequences to improve probe specificity, and we generally start with the most stringent masking level. In our experience, thirty probes are generally adequate, but the minimum number of probes that can yield high signal-to-noise ratio images is likely dependent on microscope setup (e.g., objective numerical aperture, detector sensitivity etc.). If the target mRNA is short or contains repetitive sequences, it might not be possible to design ~48 smFISH probes targeting the mRNA. In such a case, masking level can be adjusted to increase the number of smFISH probes. Each of the 20 nt probe is conjugated at the 5' end to a fluorophore and collaborative binding of ~48 probes to an individual mRNA molecule enables detection of a single mRNA spot by fluorescence microscopy. Upon receipt of the probe pool, we prepare aliquots of 20 mM probe stock in 10 mM Tris-Cl, pH 8.5 and store them in -20°C.

Sample preparation for smFISH experiments

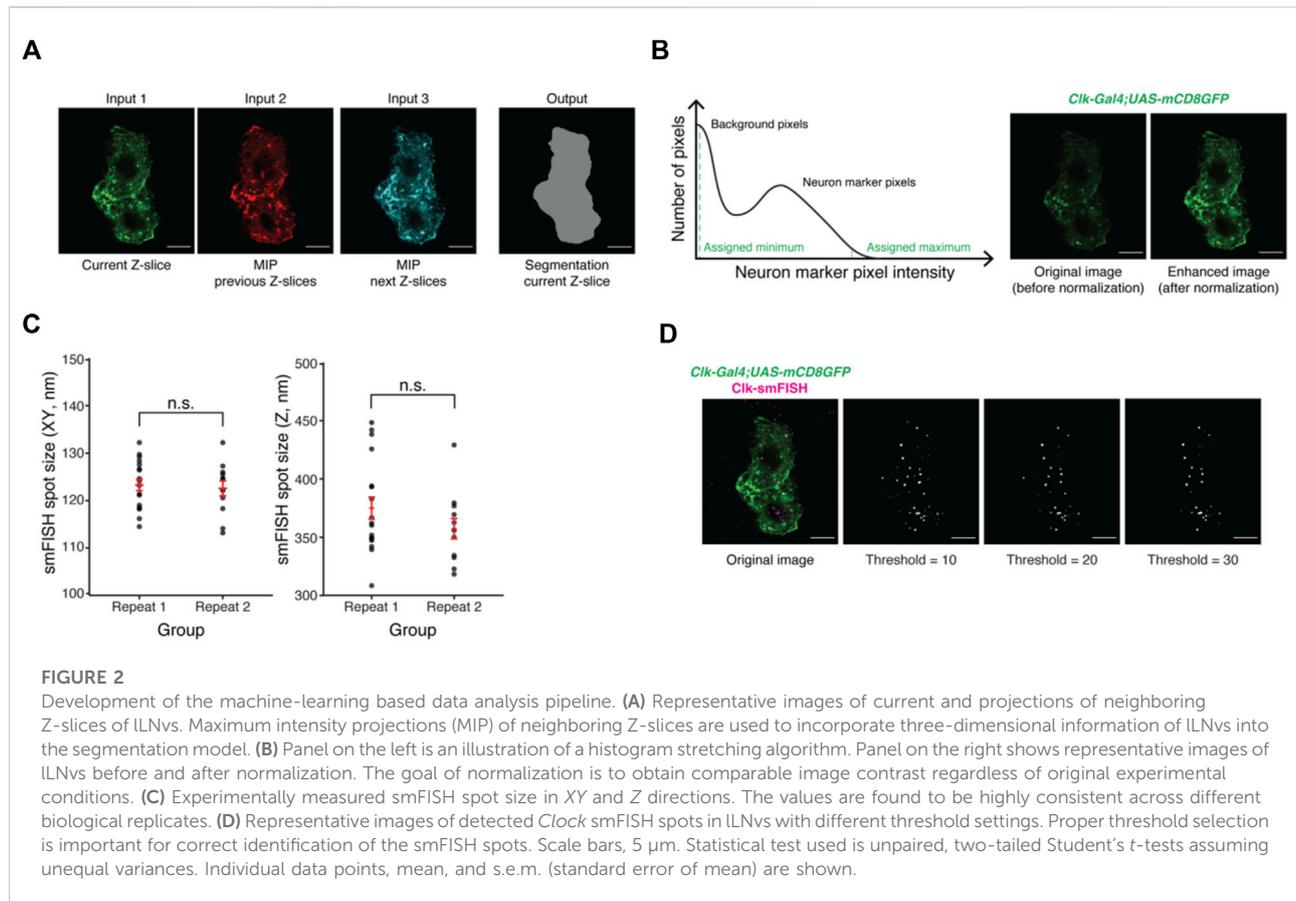
In our smFISH experiments, we typically use transgenic flies in which cell membranes are fluorescently labeled. Specifically, we drive the expression of CD4-tdTomato or CD8-GFP (Han et al., 2011) exclusively in clock neurons by crossing them with pan-clock neuron driver flies, *Clk856-GALA* (Gummadova et al., 2009). On day 1, *Drosophila* brains are dissected, fixed in 4% paraformaldehyde, and permeabilized in 70% ethanol at 4°C overnight (Figure 1F). Fixation is a crucial step in smFISH experiments, as both under-fixation as well as over-fixation of tissues can result in samples in which cellular morphology is not well preserved. Permeabilization with 70% ethanol is another necessary step to allow the smFISH probes to pass through the cell membrane and enter the cell. On day 2, brains are washed with PBS and then incubated in hybridization solution with probes overnight at 37°C. On day 3, brains are washed with PBS to remove the hybridization solution and mounted on a slide using a glycerol-based mountant for tissue clearing (see Methods section for a detailed protocol). This smFISH protocol is robust, simple to implement, taking 3 days from brain dissections to slide preparation, and produces highly reproducible results across experimental repeats (Figure 1F).

Microscope setup for image acquisition

The goal is to obtain clock neuron images where smFISH spots and the neuron cell membrane marker are clearly visible and can be distinguished from the background. In general, image acquisition parameters should be optimized taking into account both the clock neuron marker and smFISH probe channels, and the same settings need to be reused across all experimental conditions and repeats to ensure data consistency. Filter settings must be compatible with the fluorophore used and appropriate laser power and detector gain should be determined. While larger laser power and detector gain will yield higher pixel intensities, high laser power will lead to significant bleaching during acquisition and high detector gain will lead to increased random noise. Finally, to further suppress random noise, pixel averaging can be performed to obtain distinct diffraction-limited spots in the smFISH probe channels and clear cell boundaries in the neuron marker channel across all Z-slices.

Segmentation of clock neurons

The goal of the segmentation model is to assign a classification label to each pixel of the Z-stack image, i.e., to determine whether a pixel can be assigned to a 'clock neuron' or to the background. The clock neuron marker used in our studies, CD8-GFP, not only labels the plasma membrane but also the components of the secretory pathway intracellularly. Therefore, CD8-GFP cell marker intensity varies significantly throughout



the cytoplasm, making conventional segmentation methods such as simple thresholding [e.g., Otsu method (Otsu, 1979)] ineffective. To solve this issue, we adopted a publicly available pretrained encoder-decoder convolutional neural network (CNN) model based on the U-Net architecture (Ronneberger et al., 2015) for semantic segmentation of clock neurons. In short, the CNN model computes complex features based on the original clock neuron marker signal (i.e., encoding) and then assigns classification labels based on the computed features (i.e., decoding). Publicly available pretrained CNN models are typically developed with two-dimensional image datasets (Ronneberger et al., 2015). To provide an accurate segmentation model of 3-dimensional clock neurons, for each Z-slice we input the following images to the CNN model: 1) current Z-slice, 2) a maximum intensity projection (MIP) of neighboring Z-slices within 0.4 μm in one direction, 3) a MIP of neighboring 0.4 μm slices in another Z-direction. (Figure 2A).

Further, during model training, we found that CD8-GFP intensity level may vary significantly across different experimental conditions, which hindered the performance of the model. To address this issue, we adopted a MATLAB-based histogram stretching algorithm (MATLAB and Image Processing Toolbox, 2021) to normalize the levels of CD8-GFP intensities among different images. Briefly, the distribution of cell marker

pixel intensities follows a bimodal distribution; most pixels will show near-zero values (background pixels) while some show significantly higher values (true CD8GFP signal) (Figure 2B, left panel). We then take the 2% dimmest and brightest intensities and assign them to be the minimum and maximum values. Figure 2B panel on the right shows the original image, in which CD8-GFP signal is dim, and the enhanced image after normalization.

One of the caveats to adopting complex CNN models to generate segmentation masks is the limited training set size which may lead to suboptimal models. To efficiently train our CNN image segmentation model, we augmented (i.e., increased the size of) our training dataset by computationally generating images with varying brightness levels from the original training data set (Dong et al., 2017). We note that ILNv clock neurons are typically contiguous to each other, which makes it difficult to identify individual ILNvs using our segmentation model. In fact, our segmentation model provides an outline of all the ILNvs in each hemi-brain (Supplementary Video S1). We verified the performance of the segmentation model by visual inspection of testing datasets, and we were able to generate segmentation masks of the cytoplasm and the nucleus of ILNvs with ~ 10 manually annotated Z-stacks (Supplementary Video S1).

smFISH spot detection

We adopted the publicly available spot counting package, AIRLOCALIZE (Lionnet et al., 2011), for mRNA spot detection and quantification. In short, diffraction-limited spots are modeled as a three-dimensional Gaussian function with lateral (i.e., XY) symmetry. Parameters of the Gaussian function, essentially sizes of the spot in both XY and Z directions, can be measured using a graphical interface provided by the AIRLOCALIZE package. Using our Airyscan confocal microscope, we found that the spot sizes in both XY (~120 nm) and Z (~400 nm) directions are highly reproducible (Figure 2C).

To detect smFISH spots in an image, the AIRLOCALIZE algorithm first applies a global threshold to reject noise in the image. Then, the algorithm finds candidates of smFISH spots by identifying locally brightest pixels. Finally, Gaussian fitting is performed using the identified candidate spot locations and a list of “true” smFISH spots is obtained. In Figure 2D, we show the effect of applying different threshold settings on smFISH spot detection. In this example image, the number of detected spots in ILNvs changes with threshold setting. At the minimum threshold tested, we were able to detect ~70 smFISH spots. At the maximum threshold tested, ~30 spots were detected (Figure 2D). The global threshold therefore affects smFISH spot detection results and should be set empirically. Typically, we find that the same threshold can be used for all images acquired in one experimental batch. Moreover, our analysis pipeline (discussed in the next section) provides spot visualization capabilities, which can be used to optimize the threshold parameter (Supplementary Video S1).

Data preprocessing and integration of the workflows

To ensure reproducibility and aid optimization of analysis parameters, we developed an automated analysis pipeline using the Snakemake workflow management system (Molder et al., 2021) (Figure 1F). First, the raw data is converted to a lossless, open-source OME-TIFF format by the Bio-Formats package (Linkert et al., 2010). Next, smFISH and cell marker images are used for spot detection and cell segmentation, respectively. To obtain the list of smFISH spots within clock neurons, we only consider smFISH spots whose centroids overlap with the ILNv segmentation masks. Finally, quality control plots and images of all intermediate outputs (segmentation masks and detected smFISH spots within clock neurons) are generated together with the final output plots. In the final output plots, we report the number of detected smFISH spots per clock neuron and the locations and intensities of smFISH spots (Figure 1F). Quality control plots are discussed in more detail in the following section. The data analysis pipeline with detailed documentation is

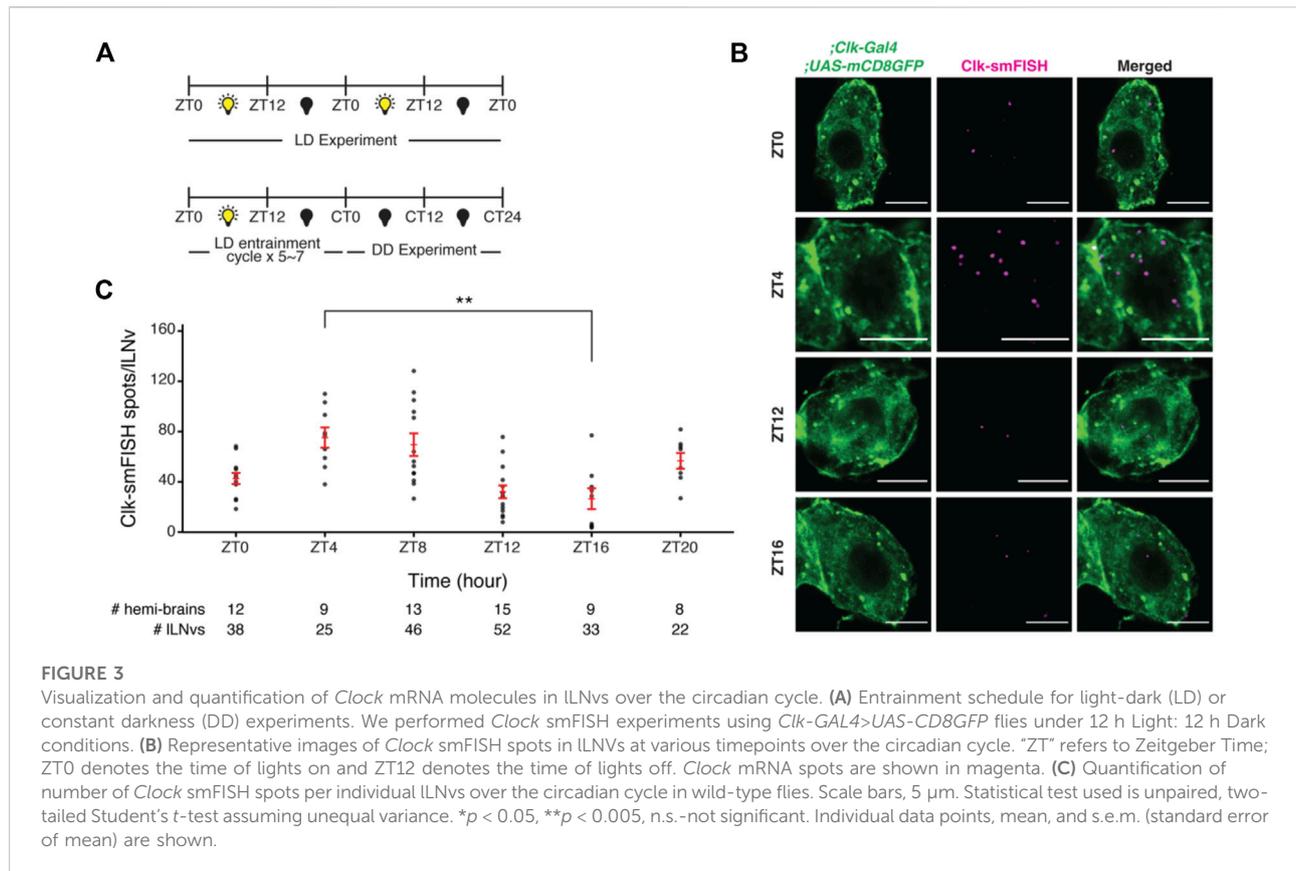
publicly available online at Github repository (https://github.com/yeyuan98/rna_fish_analysis).

Visualization and quantification of *Clock* mRNAs in ILNv neurons

Using our smFISH method and data analysis pipeline, we performed smFISH experiments to detect *Clock* mRNA transcripts in large ventral lateral neurons (ILNvs) in whole-mount *Drosophila* brains across the circadian cycle. We entrained the flies to 12 h Light: 12 h Dark cycles (“ZT0” refers to time of lights on and “ZT12” refers to time of lights off) and performed smFISH experiments at various timepoints across the circadian cycle (Figure 3A). We observed ~80 *Clock* mRNAs per ILNv at ZT4 (peak) and ~30 mRNAs per ILNv at ZT16 (trough) (Figures 3B,C; Supplementary Figure S1), consistent with past qPCR and single-cell RNA-seq studies (Ma et al., 2021).

Quality control is an essential component of our fully automated data analysis pipeline, enabling us to verify the validity of our experimental results. As a large number of Z-stack images are generated in each experiment, unbiased consistency verification and identification of noisy images are vital for our workflow. Towards this end, we developed a series of quality control plots. First, we generated intensity histogram plots of all detected smFISH spots in individual images. As individual mRNAs are expected to be bound by a similar number of fluorescent oligonucleotide probes, the histogram plots of smFISH spot intensities are expected to show predominantly a single peak with consistent distribution among different samples. The individual and merged intensity histogram plots generated from our experimental data show that *Clock* smFISH signal intensities in ILNvs from wild-type flies are highly consistent (Figure 4A). Next, as we usually scan ~10 μm in the Z-axis for imaging ILNvs in a hemi-brain, we examined how smFISH spot intensity varies across Z position in the acquired images (Figures 4B,D). Again, as individual mRNAs are bound by a similar number of fluorescent oligonucleotide probes, if working distance is sufficient smFISH spot intensity should not depend on Z-position. Indeed, >80% of our acquired images show no significant correlation between smFISH spot intensity values and Z position of the spots. Finally, equivalent diameters of segmentation masks of ILNvs demonstrate consistent volumes expected for ILNv neurons (Figures 4C,D). With these quality controls, we were able to confirm the consistency and validity of our experimental data. Intermediate results of the pipeline including segmentation masks and identified smFISH spots are readily available for visual inspection (Supplementary Videos S1,S2).

To further validate our findings, we examined how the number of *Clock* mRNAs are affected over the circadian cycle in various mutants. We found that *Clock* mRNA transcript numbers do not cycle in per null mutant flies (Konopka and



Benzer, 1971) and remain low throughout the circadian cycle (Figures 5A,C; Supplementary Figure S2). These results are consistent with past studies which reported that CLK protein fluorescence levels are lower in *per* mutant flies compared to control flies (Andreazza et al., 2015). Further, we tested how *Clock* mRNA levels are affected upon clock neuron-specific RNAi-knockdown of PDP1 and VRILLE, transcriptional regulators of *Clock*. Our smFISH results demonstrate that knockdown of PDP1 abolished *Clock* mRNA rhythms with lower number of *Clock* mRNA molecules at different timepoints over the circadian cycle, consistent with PDP1's function as a *Clock* activator (Figures 5B,D; Supplementary Figure S3). As expected, knockdown of VRILLE, which is a transcriptional repressor of *Clock*, led to a higher number of *Clock* mRNA molecules over the circadian cycle (Figures 5B,E; Supplementary Figure S3).

Discussion

Here, we present a streamlined single molecule RNA-fluorescent *in situ* hybridization (smFISH) protocol and a machine-learning based data analysis pipeline that enables direct visualization of the subcellular location of individual mRNAs and

precise quantification of the total number of mRNA molecules in clock neurons. Using this method, we show that *Clock* mRNAs cycle in ILNvs with peak levels (~80 molecules/ILNv) at ZT4 and trough levels (~30 molecules/ILNv) at ZT16. We validated our smFISH experimental protocol and data analysis pipeline by analyzing *Clock* mRNA levels in wildtype and mutant conditions.

In addition to transcriptional regulation, post-transcriptional regulation plays a prominent role in regulating gene expression. Localization of mRNAs to specific subcellular structures (e.g., P-bodies, stress granules, nuclear speckles) has been shown to be vital for post-transcriptional regulation in many different biological processes (Decker and Parker, 2012; Fei et al., 2017; Hubstenberger et al., 2017). To visualize mRNA localization, RNA fluorescence *in situ* hybridization (RNA-FISH) methods have been widely adapted in the past in many model systems (Lawrence and Singer, 1986; Femino et al., 1998; Fowlkes et al., 2008). In general, RNA-FISH technique uses a number of oligonucleotide probes that can specifically bind to specific sequences of a mRNA, and direct detection (as shown in our *Clock* smFISH experiments) or amplification-based methods (e.g., hybridization chain reaction, HCR (Schwarzkopf et al., 2021)) can be used to visualize the mRNAs. While amplification-based methods offer more flexibility with regard to target length and yield better signal-to-noise ratios, direct

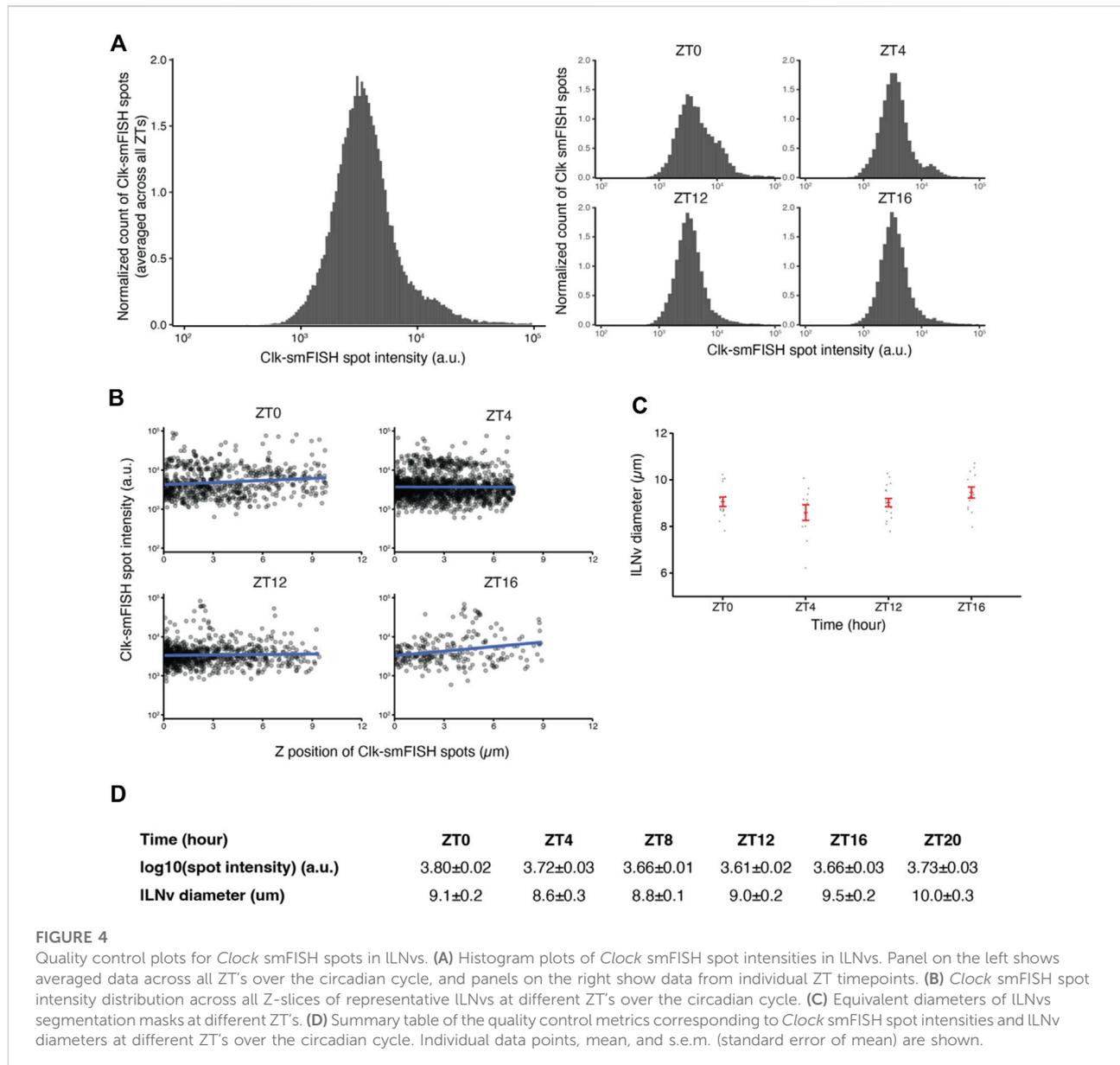


FIGURE 4

Quality control plots for *Clock* smFISH spots in ILNvs. **(A)** Histogram plots of *Clock* smFISH spot intensities in ILNvs. Panel on the left shows averaged data across all ZT's over the circadian cycle, and panels on the right show data from individual ZT timepoints. **(B)** *Clock* smFISH spot intensity distribution across all Z-slices of representative ILNvs at different ZT's over the circadian cycle. **(C)** Equivalent diameters of ILNvs segmentation masks at different ZT's. **(D)** Summary table of the quality control metrics corresponding to *Clock* smFISH spot intensities and ILNv diameters at different ZT's over the circadian cycle. Individual data points, mean, and s.e.m. (standard error of mean) are shown.

detection methods generate individual diffraction-limited spots for each single mRNA molecule with well-defined intensity and shape profiles.

smFISH protocol requires hybridization of tens of ~20 nt oligonucleotides to the same RNA molecule of interest. Therefore, a relatively long transcript (~1.5 kb) is required for adequate signal-to-noise ratio (SNR). If short transcripts or specific parts of transcripts (e.g., individual exons or introns) need to be visualized, amplification-based methods such as hybridization chain reaction (HCR) may be adopted (Schwarzkopf et al., 2021). However, while spatial information is typically retained in amplification-based methods, precise quantification of the number of

mRNA molecules might require optimization as signal amplification kinetics must be determined empirically. While smFISH and HCR protocols involve fixing the sample and hybridizing it with fluorescent oligonucleotide probes, MS2-MCP system enables live imaging of individual mRNA molecules with high temporal and spatial resolution (Lionnet et al., 2011; Tutucci et al., 2018). Briefly, RNA loops derived from the bacteriophage MS2 can be inserted in the 3'UTR of an mRNA of interest and co-expressed with MCP, which is a MS2 coat protein that can tightly bind to MS2 binding sites, fused to fluorescent proteins. While the MS2-MCP system enables live imaging of individual mRNA molecules, however, addition of stem-loops to the mRNA

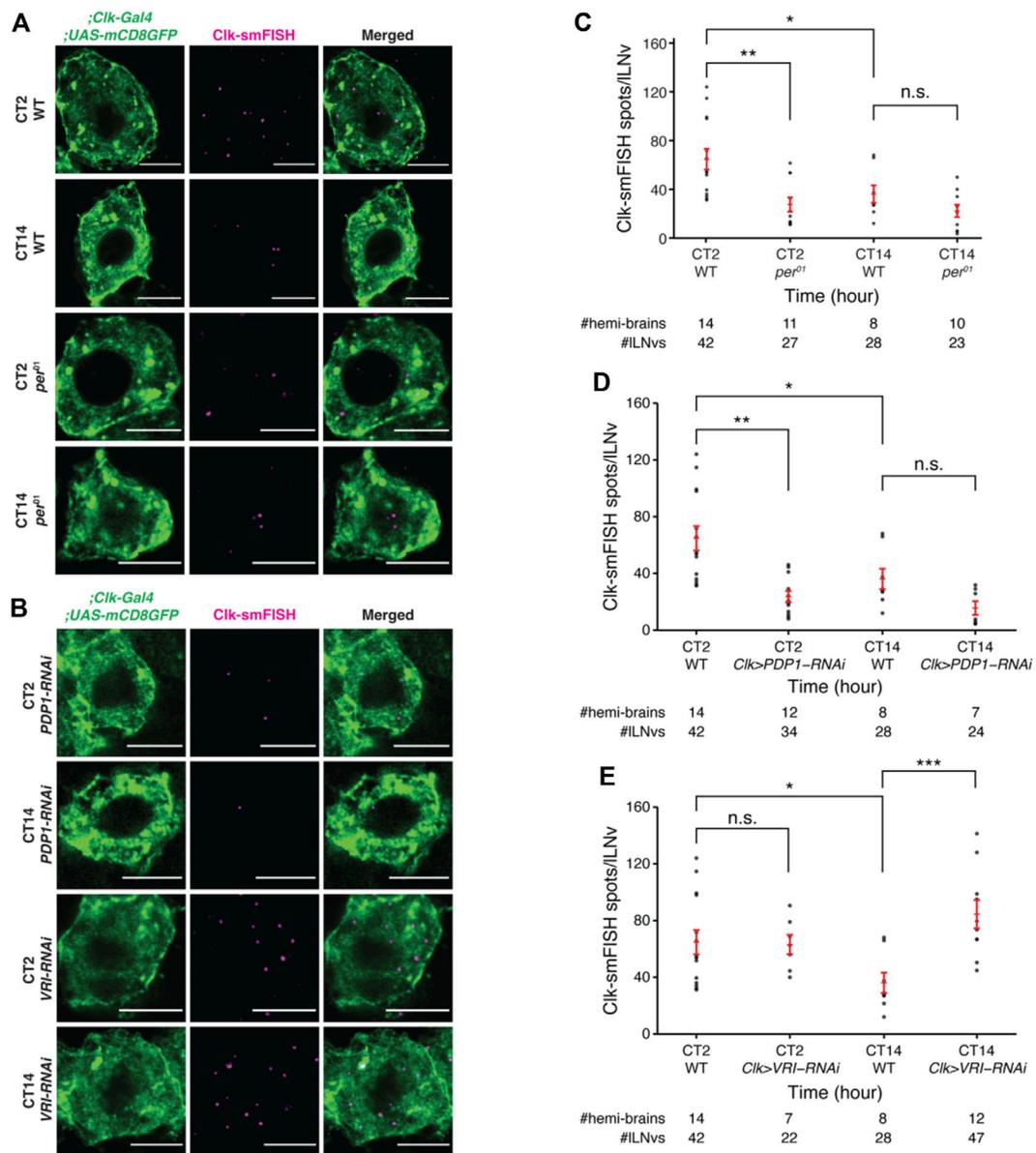


FIGURE 5

Clock mRNA rhythms are disrupted in *per⁰¹* null mutant and clock neuron specific-knockdown of PDP1 and VRI. (A) Representative images of *Clock* smFISH spots in ILNvs from control and *per⁰¹* mutant flies in constant darkness conditions. "CT" refers to Circadian Time. *Clock* smFISH spot count is lower at both the timepoints over the circadian cycle in *per⁰¹* mutants. (B) Representative images of *Clock* smFISH spots in ILNvs from *Clk-Gal4; UAS-PDP1-RNAi* and *Clk-Gal4; UAS-VRI-RNAi* flies at different CT's over the circadian cycle. (C) Quantification of number of *Clock* smFISH spots per individual ILNvs over the circadian cycle in *per⁰¹* mutant flies. (D,E) Quantification of the number of *Clock* smFISH spots per ILNv in *PDP1-RNAi* (D), and *VRI-RNAi* (E) flies at different CT's. Scale bars, 5 μ m. Statistical test used is unpaired, two-tailed Student's *t*-test assuming unequal variance. **p* < 0.05, ***p* < 0.005, ****p* < 0.0005, n.s.-not significant. Individual data points, mean, and s.e.m. (standard error of mean) are shown.

molecule of interest may affect its function and/or transcription/degradation kinetics. In conclusion, our smFISH method and the data analysis pipeline presented here is a powerful tool that can be employed to study subcellular organization of any clock mRNA molecule in *Drosophila* clock neurons.

Materials and methods

Data reporting

No statistical calculations were used to pre-determine sample sizes. Samples were not randomized for the smFISH experiments

and blinding was not used during the data analysis step. We reported the sample sizes for each of our experiments in the figures.

Drosophila strains

Flies were raised on cornmeal-yeast-sucrose food under a 12:12 Light:Dark cycle at 25°C and 60–70% humidity. The following flies used in the study were previously described or obtained from the Bloomington Stock Center: *Clk-GAL4* (Gummadova et al., 2009) (expressed in all clock neuron classes), *per⁰¹* (Konopka and Benzer, 1971), *UAS-CD8GFP* (Han et al., 2011), *UAS-Pdp1-RNAi* (BL-40863), *UAS-vri-RNAi* (BL-40862). Specific genotypes of flies used in the experiments are presented in the figures.

Solutions used for smFISH

Prepare all solutions with RNase-free tubes and reagents and filtered pipette tips. Aliquot reagent stocks to reduce chance of contamination.

Fixation solution: 4% formaldehyde in 1X phosphate-buffered saline (PBS) and is made from 16% EM-grade formaldehyde (Electron Microscopy Sciences 15710) and 10X PBS (Invitrogen AM9625) in RNase-free water.

Permeabilization solution: 70% ethanol diluted from 200 proof ethanol (Thermo Scientific T038181000) in RNase-free water.

Hybridization solution: 2X saline-sodium citrate (SSC, Invitrogen AM9763), 10% dextran sulfate (Thermo Scientific AC433240050), 1 mg/mL *E.coli* tRNA (Roche 10109541001), 2 mM ribonucleoside vanadyl complex (RVC, New England Biolabs S1402S), 0.5% bovine serum albumin (BSA, Roche 10711454001), 10% formamide (Sigma, F9037), and 5 μM oligonucleotide probes conjugated to fluorophores (Stellaris, LGC Biosearch Technologies) in RNase-free water.

Wash solution: 2X SSC and 10% formamide in RNase-free water.

We prepare 20% (weight/volume) dextran sulfate stock from powder and store at 4°C. Fixation and permeabilization solutions are prepared fresh on day of experiment. RVC and formamide aliquots are used one-time once thawed.

smFISH protocol

1. Prepare fixative solution. For each sample, put 500 μL of fixative to a well of a clean glass dish and leave on ice.
2. Dissect at least 10 brains in 1X PBS for each sample. Put dissected brains directly into the fixative on ice in the glass dish. Dissections should be completed within 30 min.

3. Place the glass dish on a nutator and incubate for 20 min at room temperature.
4. Remove the fixative and perform three quick rinses with 1X PBS.
5. Remove solution and wash twice with 1X PBS, 5 min per wash at room temperature with nutation.
6. Remove 1X PBS completely and add 500 μL of permeabilization solution to the glass dish. With a P1000 tip, carefully transfer the brains into a protein low-bind tube. Avoid damaging the brains during transfer.
7. Fill the tubes with extra permeabilization solution to a final volume of 1 ml. Incubate the sample overnight at 4°C with nutation, protected from light.
8. Prepare hybridization solution, 100 μL per sample and leave on ice protected from light. Prepare wash solution, 1 ml per sample and leave at room temperature. Hybridization solution is viscous and should be well-mixed by pipetting (>10 times).
9. Aspirate permeabilization solution and add 1 ml of wash solution. Incubate 5 min at room temperature with nutation.
10. Aspirate the wash solution completely and add 100 μL of hybridization solution containing probe(s). Gently pipette to make sure all brains are collected in the solution.
11. Incubate overnight in a 37°C incubator, protected from light.
12. Add 1 ml of wash solution without removing the hybridization solution. Gently pipette once to mix without touching the brains. Incubate at 37°C for 30 min.
13. Aspirate off the solution and wash one more time with 1 ml of wash solution at 37°C for 30 min. Supplement the wash solution with 1 μg/ml DAPI for nucleus staining.
14. Remove the wash solution and mount the samples in Prolong glass mountant. Make sure the brains are mounted in the appropriate orientation to visualize the clock neurons. Seal the samples immediately after mounting and leave at room temperature overnight protected from light.
15. The samples are ready for imaging the next day.

Imaging protocol

Flies were entrained to Light-Dark (LD) cycles with lights on for 12 h and off for 12 h for 5–7 days, and then released into complete darkness (DD) for 6–7 more days. Zeitgeber Time (ZT) 0 marks the time of lights on, and ZT12 marks the time of lights off. Circadian time (CT) refers to times in complete darkness (DD) and CT0 is the start of the subjective light phase and CT12 is the start of the subjective dark phase, the times when the light transitions would have occurred had the LD cycle continued.

All flies used for smFISH experiments were placed in density-controlled food vials (4 females and 4 males) and entrained for 5–7 days in incubators. We performed all our smFISH

experiments on 5–7 day old male or female flies, and did not notice any differences in our experimental results. The fly genotype and ZT was as described in the figure legends. We used the GAL4/UAS system to express transgenes in clock neurons in the brain. We imaged our samples using a Zeiss LSM800 laser scanning confocal microscope with AiryScan super-resolution module (125 nm lateral and 350 nm axial resolution). We have acquired our images using a 63 × Plan-Apochromat Oil (N.A. 1.4) objective and 405, 488, and 561, and 647 nm laser lines.

Statistical tests

All experiments were repeated at least twice independently. All statistical significance tests presented are unpaired, two-tailed Student's *t*-tests assuming unequal variances.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Author contributions

YY, Conceptualization, Data curation, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing—original draft, Writing—review and editing; M-AP and DC, Formal analysis, Visualization; SY, Conceptualization, Investigation, Supervision, Funding acquisition, Methodology, Writing—original draft, Writing—review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphys.2022.1051544/full#supplementary-material>

SUPPLEMENTARY FIGURE S1

Zoomed-out representative image of *Clock* smFISH spots in all ILNvs. Here we show a representative Z-slice of all four ILNvs observed in a hemi-brain at ZT4. Scale bars, 5 μ m.

SUPPLEMENTARY FIGURE S2

Quality control plots for *Clock* smFISH spots in ILNvs from *per⁰¹* null mutant flies. (A) Histogram plots of *Clock* smFISH spot intensities in ILNvs from *per⁰¹* (left) and wildtype (right) flies at different CT's over the circadian cycle. (B) Equivalent diameters of ILNvs segmentation masks from *per⁰¹* (left) and wildtype (right) flies at different CT's. (C) *Clock* smFISH spot intensity distribution across all Z-slices of representative ILNvs from *per⁰¹* (left) and wildtype (right) flies at different CT's. Statistical test used is unpaired, two-tailed Student's *t*-test assuming unequal variance. **p* < 0.05, n.s.-not significant. Individual data points, mean, and s.e.m. (standard error of mean) are shown.

SUPPLEMENTARY FIGURE S3

Quality control plots for *Clock* smFISH spots in ILNvs from *Clk>Pdp1-RNAi* and *Clk>vri-RNAi* mutants. (A,B) Histogram plots of *Clock* smFISH spot intensities in ILNvs at different CT's over the circadian cycle in PDP-RNAi (A) and VRI-RNAi (B) conditions. (C,D) Equivalent diameters of ILNvs segmentation masks at different CT's in PDP-RNAi (C) and VRI-RNAi (D) flies.

SUPPLEMENTARY VIDEO S1

Representative segmentation mask of ILNv neurons. A Z-stack video showing a group of 4 ILNv clock neurons marked with CD8-GFP (left panel) and representative segmentation masks from our CNN model (right panel). Scale bar: 4 μ m.

SUPPLEMENTARY VIDEO S2

Visualization of detected *Clock* smFISH spots in ILNvs. A Z-stack video showing *Clock* mRNA probe signals in magenta (Quasar 670 fluorophore) and clock neurons in green (CD8-GFP) in the left panel. Right panel shows visualization of detected *Clock* smFISH spots in ILNvs using AIRLOCALIZE algorithm. Scale bar: 4 μ m.

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