Machine Learning-Assisted Decoding of Temporal Transcriptional Dynamics via Fluorescent Timer

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Abstract:

Investigating the temporal dynamics of gene expression is crucial for understanding gene regulation across various biological processes. The Fluorescent Timer protein (Timer) offers a valuable tool for such studies, exemplified by our Foxp3 Timer-of-cell-kinetics-and-activity (Tocky), which facilitates the analysis of Foxp3 dynamics at the single-cell level. However, the complexity of Timer fluorescence profiles has limited its application, necessitating novel analytic approaches. Here, we introduce an integrative method that combines molecular biology with machine learning (ML) to elucidate enhancer-specific regulation of transcriptional dynamics. Our approach is demonstrated by mutating the enhancer, Conserved Non-coding Sequence 2 (CNS2), of the Foxp3 Timer transgene in Foxp3-Tocky embryos via CRISPR. Technologically, we have developed machine learning tools that employ Random Forest and Convolutional Neural Networks with image conversion techniques, including Gradient-weighted Class Activation Mapping. These independent ML models effectively elucidated CNS2-specific regulation of Foxp3 transcription and underscored the roles of CNS2 in regulating the autoregulatory loop of Foxp3 transcription. In conclusion, our study reveals previously unrecognized roles of CNS2 in Foxp3 transcriptional dynamics, showcasing the potential of the CRISPR Tocky assay as an advanced method to understand transcriptional dynamics in vivo.

Introduction:

Understanding the temporal dynamics of gene expression is fundamental to comprehending gene regulation's functional significance, cellular differentiation, and development across various disciplines in molecular, cellular, and systems biology. Single-cell level analyses of diverse tissues—including developing embryos[1], differentiating cells in vitro[2], developing thymic T-cells[3], tumour-reactive T-cells[4], and retrovirus-infected T-cells undergoing leukemic transformation [5] —have utilized trajectory analyses to reconstruct in vivo activation or differentiation processes. However, trajectory analysis inherently relies on similarity analysis through dimensional reduction, which is not a direct measurement of temporal dynamics in vivo. Thus, the current challenge lies in accurately capturing and measuring temporal elements of these processes within individual cells.

Fluorescent Timer proteins offer a unique solution to this challenge. Previous approaches, such as mathematical modelling of Fluorescent Timer expression using tandem timers, have provided insights into cell population dynamics [6]. However, the full integration of single-cell techniques with a Timer-based approach has yet to be fully realized. We previously developed the Timer-of-cell-kinetics-and-activity (Tocky) system for single-cell flow cytometric analysis of cellular activities and transcription[7]. Tocky uses a mutant mCherry fluorescent timer protein, Fast-FT, which spontaneously and irreversibly changes its chromophore from blue to a mCherry-type red form [8]. Using this Tocky approach, we have developed Foxp3-Tocky and Nr4a3-Tocky mice, enabling us to analyse the temporal dynamics of Foxp3 transcription and transcriptional activities downstream of T cell receptor (TCR) signalling, respectively [7].

Biologically, we focus on the transcriptional regulation of Foxp3, a key transcription factor for regulatory T-cells (Tregs) [9]. The Foxp3-Tocky system has revealed the highly

dynamic nature of Foxp3 expression during both inflammation and homeostasis, uncovering an autoregulatory loop where Foxp3 transcription is modulated by the Foxp3 protein itself [10]. A critical element in this regulatory framework is the interaction between Foxp3 and Runx1, which not only influences Foxp3 protein function during Treg differentiation and suppressive activity [11] but also affects Foxp3 transcription [12]. Zheng et al showed that both Foxp3 and Runx1 bind to a key enhancer in the Foxp3 gene, Conserved Non-Coding Sequence 2 (CNS2), which is conserved across mammals including humans and mice [13]. CNS2 plays key roles in the maintenance of Foxp3 expression through epigenetic modifications [13, 14]. What remains unclear, however, and what our study addresses, are the specific temporal phases of Foxp3 transcription that are regulated by CNS2. Consequently, our study aims to elucidate the CNS2-dependent, temporally dynamic mechanisms of Foxp3 transcription using the Foxp3-Tocky system and a CRISPR-based approach targeting CNS2.

To address this biological aim, our study has developed a data-driven approach that establishes a single-cell level Timer method suitable for flow cytometric analysis. Given the complex kinetics of involving gene expression through transcription and cellular dynamics, it is essential to establish a data-oriented method for quantitatively analysing Timer Blue and Red fluorescence at the single-cell level. To this end, our proposed approach uniquely combines molecular biology techniques, including CRISPR-mediated CNS2 knock-out (KO), with a novel machine learning (ML) toolkit tailored for flow cytometric Timer fluorescence data using supervised ML methods.

Supervised ML approaches have been successfully applied to genomic data [15, 16] and multidimensional flow cytometric data, especially in clinical studies. For example, a combination of flow cytometry-optimised self-organising map (FlowSOM) and Random Forest (RF) has been employed to analyse clinical data [17], while Convolutional Neural

Networks (ConvNets) have linked clinical flow cytometric data with patient responses [18, 19]. Additionally, ensemble methods integrating FlowSOM and ConvNet has been utilized for haematological B-cell neoplasm datasets [20]. The R package FlowCT, which provides various clustering and ML functions, is developed for large flow cytometric datasets [21]. However, these methods often require large sample sizes (hundreds to thousands) and extensive image data for training ML models, in contrast to the smaller datasets typically derived from flow cytometric Timer fluorescence in animal studies, which generally number less than ten or up to tens of samples. Moreover, existing methods designed for multidimensional data incorporating various marker expressions may not suitably analyse Timer fluorescence data without introducing bias.

In this study, we introduce a novel toolkit composed of two independent approaches: TockyKmeansRF, which integrates clustering with Random Forest (RF) analysis, and TockyConvNet, a Convolutional Neural Network approach utilizing a novel image conversion technique and Gradient-weighted Class Activation Mapping (Grad-CAM). Employed to analyse flow cytometric data from wild-type (WT) Foxp3-Tocky and CNS2 KO Foxp3-Tocky mice, this toolkit facilitates the identification of feature cells that characterize CNS2-dependent regulation of temporally dynamic Foxp3 transcription. Technologically, this approach advances beyond traditional strategies including pre-determined gating and unsupervised clustering in flow cytometry, enabling a more sophisticated ML-assisted, data-oriented analysis of flow cytometric data.

Results

Integrative Experimental Design for Studying Transcriptional Dynamics In Vivo Using Molecular Biology and Machine Learning

In this study, a new integrative approach was established to investigate transcriptional dynamics of Foxp3 in vivo that are controlled by a specific enhancer (**Figure 1a**). In Foxp3-Tocky, the BAC Foxp3-Timer transgene reports Foxp3 transcriptional dynamics by Fluorescent Timer protein, which irreversibly and spontaneously changes its emission spectrum from a blue fluorescence to a red fluorescence with the maturation half-life 4.1 hours [8]. The mature red-form Timer protein is stable and its decay rate is 122 hours [10]. The major aim of the Foxp3-Tocky project is to understand Foxp3 expression dynamics through analysing Timer profiles. Given that Foxp3 expression show dynamic changes [10], it is essential to understand the dynamics at the single cell level.

However, even with the use of the Foxp3-Tocky system, the static nature of flow cytometric data and the limited dynamic range of the Timer protein present significant challenges [22]. Additionally, there is no general mathematical solution for converting Timer profiles back to transcription dynamics. To address these issues, this study explores machine learning (ML) approaches, which are promising as they do not require mathematical or statistical assumptions about the nature of Foxp3 transcriptional dynamics or Timer profiles.

By generating a CRISPR mutant strain of the Foxp3-Timer transgene that lacks a specific enhancer region, we can use flow cytometric data from WT and KO Foxp3-Tocky to identify enhancer-dependent temporal Foxp3 expression. In this approach, independent training and testing datasets will be obtained by flow cytometric analysis of

samples with a sample size, n = 10 – 20. While this may not be considered large in typical machine learning contexts, it is substantial for immunological studies involving animal subjects. Additionally, this number is technically at the high end, considering the practical and logistical challenges inherent in such studies (**Figure 1a**). Next, an ML classifier will be trained to differentiate between WT and KO samples based solely on their flow cytometric Timer profiles. Importantly, the behaviour of the model will be monitored using an appropriate method, allowing for the identification of feature cells that the ML model uses to discriminate between the two groups. The characterization of these feature cells will uncover aspects of the temporal regulation of Foxp3 expression via the enhancer, which cannot be revealed by other methods.

Overview of Novel ML Approaches to Analyse Flow Cytometric Tocky Data

To implement the research framework as presented in **Figure 1**, we have developed a new ML toolkit designed to analyse flow cytometric Timer profiles and identify feature cells that distinguish between group classes (**Figure 2**). Data preprocessing includes normalization and a trigonometric transformation approach, which converts Timer blue and red fluorescence data into Timer Angle and Timer Intensity (norm), as previously described [7] and recently implemented as a computational package [23]. These normalized data serve as inputs for *TockyKmeansRF*, a tool that integrates a clustering approach with Random Forest (RF). Both clustering and RF are repeated to understand variations in the clustering process, which has inherent stochastic elements. We monitor model behaviour using the mean decrease Gini index, which assigns an importance score to each identified cluster, hence each single cell, during each iteration. By averaging the importance scores across iterations and applying a threshold, we enable a convex hull gating approach to identify feature cells. This convex hull gating can then be applied to independent test datasets to assess the generalizability of the findings.

In addition, we have established a ConvNet approach, *TockyConvNet*, for further analysis. In this approach, Timer Angle and Intensity data are converted into a two-dimensional image dataset through binning in two-dimensional space. This image conversion allows for the application of ConvNet for group classification. Model behaviour is monitored by *Grad-CAM*, which highlights significant regions in input images that contribute to the network's predictions [24]. These two ML approaches—*TockyKmeansRF* and *TockyConvNet*—provide independent insights into the properties of enhancer-dependent transcriptional dynamics as measured by Foxp3-Tocky.

A Novel Experimental Tool to Investigate the Roles of Conserved Non-Coding Sequence 2 (CNS2) in Regulating Temporal Dynamics of Foxp3 transcription

To identify a biologically significant enhancer sequence and establish a prototypic approach to studying Foxp3 transcriptional dynamics, we analysed Chromatin Immunoprecipitation sequencing (ChIP-seq) data. Our analysis demonstrated that both Foxp3 and Runx1 proteins uniquely bound to the CNS2 region (also known as the Treg-specific demethylation region) of the Foxp3 gene (**Figure 3a**) as reported previously [13]. Importantly, the analysis of temporal dynamics of Foxp3 transcription using Foxp3-Tocky revealed that Foxp3 protein is required for sustaining Foxp3 transcription [10] and that the CNS2 region is actively demethylated at the moment when Foxp3 transcription is sustained and persistent in the CD4 single-positive thymocytes [7]. Therefore, we hypothesised that CNS2 functions as a platform for critical transcription factors, including Foxp3 itself and Runx1, to dynamically regulate the Foxp3 transcriptional activities. Deleting the CNS2 sequence should elucidate the temporal phases of Foxp3 transcriptional regulation that are dependent of CNS2 (**Figure 3b**).

The deletion of a sequence within a bacterial artificial chromosome (BAC) transgene could be done in vitro, followed by the creation of a new mouse strain. However, this

approach is subject to between-founder variations, which are significant in BAC reporter approaches [25]. Moreover, modifying the endogenous Foxp3 sequence could make any output reporter measurement secondary to the modified dynamics of the Foxp3 protein. Therefore, it was essential to delete the CNS2 sequence within the BAC Foxp3-Tocky transgene only, without disturbing the endogenous Foxp3 gene (**Figure 3c**).

To achieve this ambitious aim, we utilized a CRISPR transgenic approach (**Figure 3d**). Fertilized eggs were obtained from Foxp3-Tocky mice, and electroporation-mediated CRISPR was performed (Materials and Methods), followed by founder screening and the establishment of a CNS2 KO Foxp3-Tocky colony. To facilitate the screening process, we used a single-stranded oligodeoxynucleotide (ssODN) that included homology arms to promote homologous recombination and replace the CNS2 sequence with a short oligo sequence (**Figure 3e**). Lastly, we established a stable line of CNS2 KO Foxp3-Tocky mice, inheriting the mutated Foxp3 Timer BAC transgene in a Mendelian manner, thus further excluding the involvement of the endogenous Foxp3 gene on the X chromosome.

Analysis of CNS2 KO Foxp3-Tocky Using Established Methods

Upon establishing the CRISPR mutant strain, we investigated the roles of CNS2 using established methods and performed flow cytometric analysis of $CD4^+$ T-cells from Foxp3-Tocky (WT) and CNS2 KO Foxp3-Tocky (KO) mice (**Figure 4a**). The overall expression patterns in Timer Blue (immature) and Timer Red (mature) fluorescence showed only modest changes between KO and WT upon initial inspection, which posed a challenge for analysis using conventional approaches including manual gating. Mean Fluorescence Intensity (MFI) analysis revealed no significant differences in Timer Blue fluorescence between WT and KO, while Timer Red fluorescence was significantly lower in the KO group, although the difference was moderate ($p < 0.01$, **Figure 4b**). These

findings suggest that overall Foxp3 transcriptional activities are modestly attenuated in T-cells from KO mice.

Next, we employed a previously reported method involving trigonometric transformation for quantitative analysis of Timer fluorescence profiles (**Figure 2**) [7]. After normalisation and transformation, the Timer Angle and Intensity plot showed moderate changes between the two genotypes (**Figure 4c**). To quantitative analyse Timer Angle dynamics, we used the categorisation method known as the Timer Locus approach [26] (**Figure 4d**). This approach categorises Timer Angle into five loci: New (0˚), Persistent (30˚ – 60˚), Arrested (90˚), and transitional regions including NPt (New-to-Persistent transitioning, 0˚ – 30˚) and PAt (Persistent-to-Arrested transitioning, 60˚ – 90˚). Notably, the percentages of cells in NPt, Persistent, and PAt were decreased in CNS2 KO Foxp3-Tocky, both in lymph nodes and the spleen (**Figure 4e**). These results indicate that active Foxp3 transcription, as determined by relative high Timer Blue fluorescence, is attenuated in cells that have accumulated Timer Red protein in CNS2 KO.

Collectively, the established methods did not fully elucidate the types of Timer profiles controlled by CNS2. The major limitations of the existing methods include the following: First, the methods rely on inherently one-dimensional analyses, which do not allow for the examination of two-dimensional Timer data in their original form or in the transformed Timer Angle and Intensity space.

TockyKmeansRF: A Combinatorial Approach Using Clustering and RF to Timer Fluorescence Data

One of the novel methods proposed in this study is the combinatorial approach using k-means clustering and RF. Our initial investigations indicated that methods using self-organizing map did not yield stable results by testing a wide range of cluster numbers.

Meanwhile, k-means provided superior and promising classification metrics. In the *TockyKmeansRF* model, both clustering and RF are iteratively repeated to mitigate the inherent randomness in the k-means clustering process (**Figure 5a**). This procedure utilizes independent training and test datasets. In each iteration, k-means is applied within the training dataset to identify clusters and determine the percentage of each cluster. Concurrently, k-means is independently applied to the test dataset with an identical number of clusters, and the percentage of each is similarly assessed. The clustering results are then cross-compared, and equivalent clusters between the datasets are matched using Euclidean distance analysis of the clusters' centroids. The cluster percentage data from the training dataset are then used to construct the RF model, which is subsequently evaluated against a similar cluster percentage table from the test dataset, allowing for a robust assessment of the model's performance. Importantly, throughout the iterative process, model behaviour is monitored, and the contribution of each cluster to the classification task is evaluated using the mean decrease Gini index [27], which aids in identifying feature cells as detailed below.

Next, we assessed the performance of TockyKmeansRF for analysing CNS2-dependet Timer profiles using datasets from WT Foxp3-Tocky and CNS2 KO Foxp3-Tocky mice (**Table 1**). The optimal number of k-means clusters was evaluated by Receiver Operating Characteristic (ROC) analysis (**Figure 5b**). Interestingly, a higher number of clusters generally showed better performance than a lower number. Area under the curve (AUC) analysis demonstrated a similar trend, with effectiveness saturating around the cluster number, k = 15 (**Figure 5c**). As higher cluster numbers can lead to less stable clustering, we assessed the stability of performance by reiterating modelling (n = 100) for each k-means cluster number. Confidence interval analysis of the ROC indicated that a cluster number around 15 (k = 15) performed most efficiently (**Figure 5d**). Using the optimized cluster number for the datasets, the importance scores for

individual cells were visualised in the Timer Angle and Intensity space, as shown in **Figure 5e**. The same feature cells were also visualised in the original Timer Blue and Red fluorescence space, identifying unique and distinct clusters of cells (**Figure 5f**).

Identification of Feature cells Across Datasets using Convex Hull Gating Approach for TockyKmeansRF

The importance score, as previously defined, is inherently unique to each TockyKmeansRF model. However, a convex hull gating of feature cells can be utilized to delineate areas of importance in the Timer Angle and Intensity space (**Figure 6a**). Given that this approach can be subject to variations between datasets, we utilized an independent test dataset to evaluate the model's performance across different datasets. In the training dataset, three visually distinct feature cell clusters exceeded a threshold importance score (**Figure 6b**). These feature cells were then further classified using a density-based clustering algorithm, *DBScan* [28], successfully segregating them into Clusters 1, 2, and 3. The clusters are visualized in the original Timer Blue and Red fluorescence space (**Figure 6c**). Cells in Cluster 1 were significantly increased in the KO group, whereas Clusters 2 and 3 showed higher frequencies in the WT group (**Figure 6d**).

Next, we employed a convex hull gating approach to unambiguously determine the boundaries of each identified feature cell cluster (**Figure 6e**). Using the training dataset, the convex hull gates for Clusters 2 and 3 effectively captured the cells that were significantly decreased in the KO group (**Figure 6f**). Notably, Clusters 2 and 3 exhibited unique marker profiles (**Figure 6g**): CD25 expression, a hallmark of Foxp3 function, was increased in these clusters. CD44, a memory marker, was uniquely elevated in Cluster 2. Both Timer Blue and Red expressions were higher in Clusters 2 and 3 compared to Cluster 1, with Cluster 2 displaying the most significant elevation in Timer Blue

expression and Cluster 3 the highest in Timer Red (**Figure 6g).** Similar results were replicated by applying the convex hull gates to an independent test dataset, confirming consistency in cluster percentage changes (**Figure 6h**) and marker expression profiles (**Figure 6i**).

These results collectively demonstrate that the TockyKmeansRF model effectively captured the feature cells critical for classifying the two genotypes, pinpointing those significantly diminished in the KO group. Importantly, the unique marker profiles of the clusters support their biological significance and validate the approach. Intriguingly, the diminished clusters, Clusters 2 and 3, exhibited elevated CD25 expression among Foxp3 Timer+ cells, suggesting enhanced Foxp3 functional activities in these clusters.

TockyConvNet: A ConvNet Approach Using Grad-CAM for Feature Cell Selection Based on Timer Fluorescence Data

Independently from the RF-based approach, we developed a ConvNet-based ML approach for classification-oriented feature cell selection, aiming to provide deeper insights into CNS2-mediated regulation of Foxp3 transcription. The success of the convex hull gating approach and the relatively high number of clusters (n = 15) optimized for TockyKmeansRF suggest that converting Timer fluorescence data into image data may open new opportunities for ML applications. Importantly, this image conversion allows us to harness the full potential of ConvNet. After conversion, dot plot and pseudocolour representations of the Timer data ensured that the overall characteristics were preserved (**Figure 7a – 7b**).

To prevent overfitting, we intentionally constructed a relatively small ConvNet model, comprising only two convolutional layers and two dense layers (**Figure 7c**). ROC analysis using two independent test datasets demonstrated satisfactory performance,

with AUC values exceeding 0.9 (**Figure 7d**). To monitor the model's behaviour, the last convolutional layer was examined using Grad-CAM [24, 29]. This analysis showed that Grad-CAM effectively visualized the areas of importance for the classification task. Notably, the WT Grad-CAM aligned well with the distribution of WT cells, and similarly, the KO Grad-CAM with that of KO cells (**Figure 7e**). These findings affirm that the trained ConvNet model successfully captures the features critical for classification. Heatmap visualizations of the differences between the two Grad-CAM results highlighted an area approximately in the centre of the Angle and Intensity image space (**Figure 7f**). Reverse conversion of the feature 'pixels' for WT samples into cells in the original Timer Angle and Intensity space allowed for the visualization of feature cells as a cluster in a unique region (**Materials and Methods**, **Figure 7g**). Furthermore, these WT feature cells, as shown in **Figure 7g**, were significantly reduced in the KO group (**Figure 7h**), exhibiting high CD25 expression similar to other Timer$^+$ cells and higher PD1 expression than other Timer$^+$ non-feature cells. Timer Blue fluorescence was significantly higher in the WT feature cell cluster compared to other cells, whereas Timer Red fluorescence was lower than in non-feature cells.

Collectively, these results support the utility of the TockyConvNet modelling approach for identifying the CNS2-dependent Foxp3 expression profile. Interestingly, the WT feature cells identified by the TockyConvNet model differed from those captured by the TockyKmeansRF model, both in terms of the Timer profile and the marker profile. This underscores the value of employing multiple analytical approaches and highlights the distinctions uncovered.

Discussion:

This study offers unique biological insights into the roles of CNS2 in Foxp3 regulation, underscoring its critical function in T-cell biology. The previously reported Tocky Locus approach, which noted a nuanced decrease of T cells in NPt – Persistent – PAt loci in Timer Locus dynamics (Figure 4e), contrasts with our current findings where two machine learning approaches identified key feature cells essential for efficient data-oriented classification of genotypes. Intriguingly, feature cells in both models were diminished in T cells from CNS2 KO Foxp3-Tocky mice, indicating that CNS2 uniquely influences distinct phases of Foxp3 transcriptional dynamics. Specifically, the TockyConvNet model identified cells between 50˚– 80˚ in Timer Angle and these cells were uniquely PD-1 high (Figure 7j). In addition, the WT feature cells identified by the TockyKmeansRF model were broader and uniquely distribute between 40˚ and 80˚ across the full range of Timer Intensity, highly expressing CD25 and PD-1 (Figure 6b). These findings support the hypothesis that CNS2 may be redundant at the new phase of Foxp3 expression but contributes to the maintenance of Foxp3 expression at the moment when T-cells actively transcribe Foxp3 (Persistent locus), and further enhances the transcriptional activities towards a high end of Foxp3 Timer Intensity profile.

Furthermore, the differential expression observed in the CNS2 KO versus WT groups, particularly regarding CD25 and PD1 expression, reveals the broader role of CNS2 in regulating Foxp3 activity and T-cell function. Importantly, CD25 constitutes the Interleukin (IL)-2 receptor and its expression can be induced by either T cell activation through T cell receptor signalling and/or IL-2 signalling [30]. In addition, Foxp3 promotes the transcriptional activities of CD25 [11]. In contrast, PD1 expression is induced by TCR signalling as a negative feedback mechanism for TCR signalling. Given these backgrounds, our findings support the hypothesis that CNS2 facilitates sustained Foxp3 expression in response to TCR signalling. Intriguingly, previous reports indicated the requirement of CNS2 for the maintenance of Foxp3+ cells in aged mice, Foxp3

expression over cell divisions in vitro [13], and the high levels of Foxp3 expression in activated Treg, or 'effector Treg' [14]. Furthermore, our previous work showed that, by analysing thymic CD4-single positive cells from Foxp3-Tocky, CpG islands within CNS2 (i.e TSDR) start to be demethylated in Persistent Foxp3 expressors but not in T cells in the New– NPt loci [10]. These two lines of studies support the possibility that the active usage of CNS2 at the moment of persistent Foxp3 transcription promotes the demethylation of the CNS2 CpG islands, which is considered a hallmark of 'stable Foxp3 expression' [31].

The distinct feature cells identified by the two modelling approaches likely arise from their algorithmic designs. TockyKmeansRF focuses on clustering and percentage of clusters, which may be more adept at identifying subtle differences in dense cell areas. Conversely, TockyConvNet, which is visually oriented, may overlook densely populated areas in favour of visually distinct regions. Optimizing clustering algorithms and the number of clusters is essential when applying these methods to new data types, an aspect addressed by our TockyMachineLearning toolkit, which facilitates adaptation to diverse datasets.

As supported by the flexibility in the identification of feature cells, TockyConvNet might offer greater flexibility and generalizability. However, in general, significant challenges persist in applying ConvNet approaches to flow cytometric data due to typically small sample sizes in immunological experiments using animal samples and considerable batch effects in flow cytometric analysis. To address these, a big experiment could be designed using many samples, but logistic issues are significant: sample preparation and cell staining well take several hours; each sample acquisition may require 3 – 10 min and rare cell populations may need a longer acquisition in general; and Tocky T cells must be analysed within a day, and as prompt as possible, to preserve the Timer

fluorescence. Future efforts should focus on developing robust normalization methods to ensure data consistency across experiments. The current normalization and data transformation method for Timer fluorescence demonstrates a satisfactory level of robustness but is not directly transferable to other fluorescence data. Addressing these challenges can extend the application of ML approaches to data sharing and cross-institutional analysis, potentially revolutionizing cooperative scientific endeavours and enhancing our understanding of gene regulation dynamics.

Materials and Methods

CRISPR-mediated mutagenesis of Foxp3-Tocky

The Foxp3-Tocky mouse strain (BAC Tg (*Foxp3*$^{\Delta Exon3\ FastFT}$) was generated by the Ono group and reported previously [7]. Briefly, for generating the transgenic construct, a bacterial artificial chromosome (BAC) approach was used to replace the first coding exon of the Foxp3 gene with Fast-FT [8] using a knock-in knock-out approach. Subsequently, the BAC construct was injected into pronucleus at the two-cell stage of embryos using C57BL/6J mice. All animal experiments were approved by the Animal Welfare and Ethical Review Body at Imperial College London and the Animal Experiment Committee at Kumamoto University.

The CRISPR-mediated mutagenesis of the Foxp3-Timer transgene in Foxp3-Tocky mice was performed using the previously reported CRISPR transgenic approach [32, 33]. Briefly, fertilised eggs were obtained from Foxp3-Tocky and were subjected to electroporation to introduce the Cas9 protein (317-08441, Nippon Gene, Toyama, Japan), tracrRNA (GE-002, FASMAC, Kanagawa, Japan), synthetic crRNAs, and a single-stranded oligodeoxynucleotide (ssODN). The CRISPR strategy was designed to replace the

CNS2 sequence with a short insert sequence, AAGTTTAAAC. Accordingly, the following synthetic crRNAs were used: (1) 5' crRNA targeting CCTGAGCTCCATTATGACAG (PAM: AGG) and (2) 3' crRNA targeting AGTTCCACAAGTATTTAAGG (PAM: AGG). The ssODN designed for homology-directed repair and the insertion of the short nucleotide sequence AAGTTTAAAC/GTTTAAACTTC was 5'-CTCTTTATGTTTGGTCAGAACTTATAAGAAATCTCCTCCT**GTTTAAACTTC**AGAGGATTGGAAAACCCTCTACTGTCCTGATCTGGGGTC-3' (**Figure 3e**).

ChIP-seq Analysis

ChIP-seq data analysis was performed using the NCBI SRA dataset DRP003376 [34]. Briefly, HISAT2 was used to align sequence reads to the mouse genome (*mm10*), followed by the data processing using samtools to produce sorted bam files. The sequence peaks were visualised by Integrative Genomics Viewer.

Timer Trigonometric Transformation and Normalisation

Data pre-processing for normalisation and trigonometric transformation was built on our previously established methods [7]. Briefly, Timer fluorescence data are normalised across the immature blue and mature red fluorescence data, so that the two types of Timer fluorescence are treated equally. Subsequently, Timer fluorescence data are thresholded for removing autofluorescence, which occupies a large part of flow cytometric data. This is followed by trigonometric transformation of normalised timer blue and red fluorescence, converting Timer data into Timer-Angle and Timer-Intensity [7]. The R package *flowCore* [35] was used for importing fcs data.

TockyKmeansRF: the combinatorial method using k-means and RF for Tocky data

The TockyKmeansRF is implemented in the *TockyMachineLearning* package, integrating data preprocessing methods with a k-means clustering by base R and Random Forest (RF) classification by *randomForest* [36]. Briefly, after the normalisation and trigonometric transformation of flow cytometric Timer fluorescence data, data are processed through k-means clustering. For both training and testing datasets, we determine a predefined number of clusters. Each dataset undergoes clustering separately. Post-clustering, the centroid (geometric centers) of clusters in both training and test datasets are calculated based on the mean angle and intensity values. Clusters are thus matched between the training and test datasets, by analysing distances between cluster centroids in the Euclidean distance metric. After matching clusters, the percentage of cells in each cluster is calculated per sample, obtaining a feature table of cluster percentages. The Random Forest algorithm then uses these features to classify samples based on the relative proportions of cells in each cluster. Meanwhile, the relative contribution of each cluster (hence each cell) to the model is monitored using the mean decrease Gini index. To address variabilities during the k-means clustering step, these processes are iteratively performed, obtaining an averaged mean decrease Gini index for each single cell, which is used as the importance score that defines feature cells.

TockyConvNet: A Convolutional Neural Network Model for Flow Cytometric Timer Fluorescence Data

In this study, we developed a unique ConvNet model, TockyConvNet, to analyse two-dimensional Timer fluorescence flow cytometric data (**Figure 7c**). By integrating an image conversion technique, TockyConvNet model leverages image analysis techniques in ConvNets and thereby aims to improve the accuracy of classifying cellular states based on their transcriptional activity as reported by the Foxp3-Tocky system.

For data preprocessing, the blue-red normalisation and trigonometric transformation of Timer fluorescence data into Timer Angle and Intensity were employed. Subsequently, Timer Angle and Intensity data were converted into image data as follows. First, the global minimum and maximum values for the selected markers, corresponding to angle and intensity measurements, were computed. These values were employed to define uniform bin edges across all datasets, facilitating a standardized pixel representation. Specifically, the bin edges for angle and intensity were established using a sequence of 100 equidistant intervals spanning from the global minimum to the global maximum for each marker. Using these predefined bin edges, data were transformed into pixel-style representations, mapping the flow cytometric measurements into a 2D matrix. Each matrix element quantifies the count of data points within each bin, thereby converting the original flow cytometric data into a 100×100 pixel image where each pixel corresponds to a bin count. This method ensures a consistent and precise pixelated representation of the flow cytometric data for each sample.

To construct a ConvNet model tailored for Timer Angle and Intensity image data, TensorFlow and Keras were employed. The network features a series of convolutional layers enhanced with spatial attention mechanisms to focus processing on pertinent areas within the images. The input layer accepts batch image data of size 100×100 pixels with a single channel representing cell density. The initial convolutional layer, employing 16 filters of size 3×3 with 'same' padding, maintains dimensionality and is paired with a ReLU activation function to introduce non-linearity. The convolution is followed by a spatial attention block, comprising a 1×1 convolution with sigmoid activation to generate an attention map. This map is then multiplied element-wise with the input feature map to emphasise significant features. A subsequent max pooling layer reduces spatial dimensions, followed by a dropout layer at a rate of 0.2. This configuration, including a convolutional layer, an attention block, max pooling, and

dropout, is repeated twice. The convolutional layers feed into a flatten layer that transitions the data for classification. This is followed by a fully connected dense layer with ReLU activation and a final dropout at the rate of 0.5 before reaching the softmax output layer, which classifies the cells into two categories based on their transcriptional activity.

The model training utilized the Adam optimizer. To address potential class imbalances, class weights were calculated using the *compute_class_weight* function from scikit-learn [37], ensuring equitable training by weighting the influence of each class according to its representation in the dataset. These computed weights were then integrated into the training process by passing them to Keras, enhancing the model's focus on underrepresented classes. Given the relative small sample size, a three-fold cross-validation method was used during the training phase to ensure the robustness and generalizability of the model. For each fold, the model was compiled with the binary cross-entropy loss function and accuracy as the metric. Training was conducted over 12 epochs with a batch size of 8 and the learning rate 0.002, and the validation data for each fold was used to monitor model performance. The training histories were recorded for each fold to analyse model behaviour across different iterations.

Implementation of Grad-CAM in TockyConvNet

Grad-CAM [24] was employed to identify critical feature cells within flow cytometric Timer fluorescence data. This method aids in distinguishing between WT Foxp3-Tocky and CNS2 KO Foxp3-Tocky samples using a pre-trained TockyConvNet model. To implement Grad-CAM, a new gradient model was created using TensorFlow's Keras API. This model shares the same inputs as the original but outputs both the activations from the last convolutional layer and the model's final output. The *tf.GradientTape* function records operations for automatic differentiation and computes gradients of the

model's output with respect to the last convolutional layer. These gradients indicate the importance of each neuron in the convolutional layer for class prediction. By averaging the gradients across the width, height, and depth of the feature map, a single scalar value per feature map channel is obtained. Subsequently, a weighted combination of the feature maps with the pooled gradients is calculated, resulting in a 2D array. Each element in this array is the product of the feature map activation and the importance of that feature map, thereby creating a raw heatmap. A ReLU function is applied to the heatmap to retain only positive values, which are then normalized to a range between 0 and 1 by dividing by the maximum value in the heatmap. After generating individual heatmaps for each image in the KO and WT groups, the heatmaps were summed within each group to aggregate activation signals across multiple images. Each aggregated heatmap then underwent Gaussian smoothing to suppress noise and enhance the visibility of predominant activation patterns. Finally, a differential matrix was calculated by subtracting the KO group's Grad-CAM heatmap from the WT group's Grad-CAM heatmap. This differential heatmap effectively highlights regions with distinct activation between the groups, identifying feature cells that contribute to the classification task performed by TockyConvNet.

Acknowledgements: MO was supported by a CRUK Programme Foundation Award (DCRPGF\100007) and the MRC grant (MR/S000208/1). This research was also supported by KAKENHI research grants from the Japan Society for the Promotion of Science (JSPS) (JP21K07082, JP21H00433, and JP24K10259 to MO), JSPS Core-to-core program to MO and YS (JPJSCCA2020008 to MO and YS), and Japan Agency for Medical Research and Development (24gm1810001s0103 to MO), Advanced Animal Model Support (AdAMS) (JP16H0627601 and JP22H04922 to KA).

Conflicts of Interest

A patent associated with the ML method in this study has been filed.

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Figure Legends

Figure 1. Experimental Design of the Current Study.

This study aimed to identify Conserved Non-coding Sequence 2 (CNS2)-dependent Foxp3-Timer cells in the Foxp3-Tocky system. To this end, CRISPR-mediated mutagenesis was used to delete CNS2 in Foxp3-Tocky mice. Performing flow cytometric analysis of T-cells from WT Foxp3-Tocky and CNS2 KO Foxp3-Tocky, Timer fluorescence data were acquired in a batch manner. Note that Fluorescent Timer protein is expressed in a synchronised manner with Foxp3 transcription in Foxp3-Tocky mice and each Fluorescent Timer protein spontaneously and irreversibly changes its emission spectrum from immature blue fluorescence to mature red fluorescence. The maturation half-life of this protein is 4.1 hours, and the red-form protein has a decay half-life of 122 hours. A training dataset was employed to train each machine learning model to classify KO and WT samples, with model behaviours monitored using novel methods. This process enables the identification of feature cells that determine group identity, thereby establishing a CNS2-dependent Timer profile in a data-oriented manner.

Figure 2. Overview of Machine Learning Approaches in the Current Study.

The current study introduces TockyMachineLearning, a novel machine learning toolkit. The data preprocessing involves Timer Blue-Red normalization and a trigonometric transformation, which converts Timer Blue and Timer Red fluorescence into Timer Angle and Timer Intensity, as previously reported [7] and recently implemented as a computational package. Subsequently, the TockyKmeansRF method applies an iterative cycle of K-means clustering and Random Forest (RF) analysis, with model behaviours monitored by the mean decrease in Gini. This iteration facilitates the single-cell-level identification of feature cells, which contribute to the classification of the wild-type (WT) and knockout (KO) groups. The Convex Hull gating approach enables cross-dataset comparisons. For the Convolutional Neural Network (ConvNet) method, TockyConvNet, tailored for Timer Angle and Intensity data, a novel image data conversion bins Timer Angle and Intensity data into a 2D image reflecting cell density. These image-converted data are then batch-analysed by the ConvNet, with model behaviours monitored using Gradient-weighted Class Activation Mapping (Grad-CAM), enabling the identification of feature cells.

Figure 3. Development of CRISPR-mediated CNS2 KO in Foxp3-Tocky Mice.

The Conserved Non-coding Sequence 2 (CNS2) of the Foxp3 gene was deleted specifically in the Foxp3-Timer transgene in Foxp3-Tocky using a CRISPR approach.

(a) Display of aligned sequence reads from ChIP-seq experiments using anti-Runx1 antibody in Foxp3-negative CD4+ T cells (Tconv) and Foxp3-positive CD4+ T cells (Treg), as well as anti-Foxp3 antibody in Treg. The RefSeq annotation data shows exons and introns. The CNS2 sequence is highlighted.

(b) The working model for CNS2-mediated Foxp3 transcriptional regulation. CNS2 is bound by both Foxp3 and Runx1 proteins, which cooperatively regulate Foxp3 transcription. Notably, this model illustrates an autoregulatory loop where the Foxp3 protein itself participates in regulating its own transcription through interactions at the CNS2 site

(c) The CRISPR mutagenesis strategy used to delete CNS2. In the Foxp3 Timer bacterial artificial chromosome (BAC) transgene within the genome of Foxp3-Tocky mice, CNS2 was specifically targeted and excised to produce the CNS2 KO Foxp3 Timer transgene, hereafter designated as CNS2 KO Foxp3-Tocky. Notably, the first coding exon of the Foxp3 gene is replaced with the Timer gene in Foxp3-Tocky mice.

(d) The CRISPR transgenic approach to establish CNS2 KO Foxp3 Tocky. As a new sub-strain of the parental Foxp3-Tocky colony, CNS2 KO Foxp3 Tocky mice were developed by electroporating fertilized eggs from Foxp3-Tocky mice with CRISPR components targeting CNS2. This method ensured comparable genetic background, ensuring a reliable comparison to the parental strain. Founder mice were then screened to establish the novel CNS2 KO Foxp3-Tocky colony.

Figure 4. Analysis of Foxp3 Timer Fluorescence Data Using Conventional Methods.

Flow cytometric Timer fluorescence data were obtained from Foxp3-Tocky mice (WT) and CNS2 KO Foxp3-Tocky mice (CNS2 KO).

(a) Representative flow cytometric 2D plot showing Timer Blue and Timer Red fluorescence from WT and CNS2 KO samples.

(b) Mean Fluorescence Intensity (MFI) analysis of KO and WT samples for Timer Blue (left) and Timer Red (right). Mann-Whitney test was used.

(c) Representative Timer Angle and Intensity plots after normalisation and trigonometric transformation of Timer Blue and Timer Red fluorescence, showing WT and CNS2KO samples.

(d) The Timer locus approach, categorising Timer Angle into the five loci: New (0˚); New-to-Persistent-transitioning (NPt, 0˚ - 30˚); Persistent (30˚ - 60˚); Persistent-to-Arrested-transitioning (PAt) (60˚ - 90˚); and Arrested (90˚).

(e) This analysis of cell distribution by the Timer locus approach. The plots show the percentage of cells within each Timer locus in the parent population, specifically in CD4+ T-cells from the spleen (left) and superficial lymph nodes (right). Statistical significance was assessed using the Mann-Whitney test, with p-values adjusted by the Benjamini & Hochberg method. Significance levels are indicated as follows: $p < 0.05$ (*) and $p < 0.01$ (**).

Figure 5. TockyKmeansRF: Combinatorial Approach using Clustering and Random Forest (RF).

(a) Flowchart of TockyKmeansRF. K-means clustering is performed on both training and test datasets with a predetermined number of clusters. The percentages of cells within each cluster are used to construct an RF model. A cluster-matching algorithm then facilitates testing with the test dataset. The importance score for each cluster is determined by the mean decrease in Gini, obtained from each iterative cycle of TockyKmeansRF, subsequently providing an importance score per single cell. These scores are averaged to identify feature cells.

(b) Receiver Operating Characteristic (ROC) Analysis, showing TockyKmeansRF performance across different cluster numbers.

(c) Area Under the Curve (AUC) Analysis of TockyKmeansRF across various numbers of clusters evaluates the model's accuracy.

(d) ROC analysis with confidence intervals across different cluster numbers to evaluate the stability of the model.

(e-f) Visualization of Importance Scores. In (e), importance scores are visualised on a Timer-Angle and Timer-Intensity 2D plot; in (f), they are shown on a Timer Blue and Timer Red fluorescence plot. The importance score is depicted using a colour scale, with grey indicating Timer-negative cells.

Figure 6. Cross-Dataset Analysis using TockyKmeansRF

(a) Flowchart for cross-dataset analysis using TockyKmeansRF and a convex hull approach.

(b-c) Identification of feature cells. TockyKmeansRF, conducted with 100 iterations, identified Clusters 1 – 3 through a density-based clustering algorithm. These clusters are displayed in Timer-Angle and Timer-Intensity 2D space (b) and Timer Blue and Timer Red 2D space (c).

(d) Violin plot analysis for feature cells. Feature cells identified from the training dataset are analysed, with the percentage of cells in each cluster displayed by a violin plot comparing WT and KO groups.

(e) Convex hull gating was applied to the identified feature cell clusters, defining gates C1, C2, and C3.

(f) The convex hull gates were applied to the training dataset, with the percentage of cells in each gate displayed by a violin plot comparing WT and KO groups.

(g) Mean Fluorescence Intensity (MFI) analysis of indicated markers in convex hull clusters, including Clusters 1 – 3 and Cluster 0 (Timer negative cells), was performed using the training dataset.

(h) The convex hull gates were applied to the test dataset, with the percentage of cells in each cluster shown by a violin plot comparing WT and KO groups.

(i) MFI analysis of indicated markers in the convex hull clusters, including Clusters 1 – 3 and Cluster 0 (Timer negative cells), was performed using the test dataset.

Figure 7. TockyConvNet: ConvNet Modelling Using Image Conversion and Gradient Analysis.

(a) Image conversion technique as a data preprocessing. Flow cytometric Timer fluorescence data are normalised and transformed via trigonometric transformation. Subsequently, Timer Angle and Intensity data are transformed into 2D image data, with a resolution of 100 x 100 pixels by binning, retaining cell density data.

(b) Image-converted Timer data are displayed by dot plots (upper) and pseudocolour plots (lower), with left plots showing concatenated Foxp3-Tocky (WT) samples and right plots showing concatenated CNS2 KO Foxp3-Tocky (KO) samples.

(c) The structure of the ConvNet designed specifically for analysing image-converted Timer data.

(d) Model training with learning curve. The TockyConvNet model is trained using a three-fold cross-validation method, focusing on constructing a CNS2-dependent Timer profile.

(e) Receiver Operator Curve (ROC) analysis for two test datasets.

(f) Gradient-weighted Class Activation Mapping (Grad-CAM) analysis of the TockyConvNet model response to WT and CNS2 KO samples. Heat colour is used to show the reactive pixels by yellow highlights in each group. The dot plots for WT or KO are overlayed to show area of overlap.

(g) Differential heatmap analysis displaying the WT-specific response areas identified by Grad-CAM, shown in red heatmap colours.

(h) Feature cell identification by the TockyConvNet model. Feature cells are visualised in the original Timer Angle and Intensity space (left) and Timer Blue and Red fluorescence space (right).

(i) Analysis of WT feature cells. Percentage of WT feature cells by in the WT and CNS2 KO groups form test dataset 1 are displayed in a violin plot. The Mann-Whitney test was used to assess statistical significance, $P < 0.001$ (****).

(j) Mean Fluorescence Intensity (MFI) analysis of indicated markers for the WT feature cluster, other Timer+ cells, and Timer-negative cells. The test dataset 1 was used. The Mann-Whitney test with p-value adjustment was applied, indicating statistical significance levels at $P < 0.01$ (**), $P < 0.005$ (***), $P < 0.001$ (****).

Figure 1

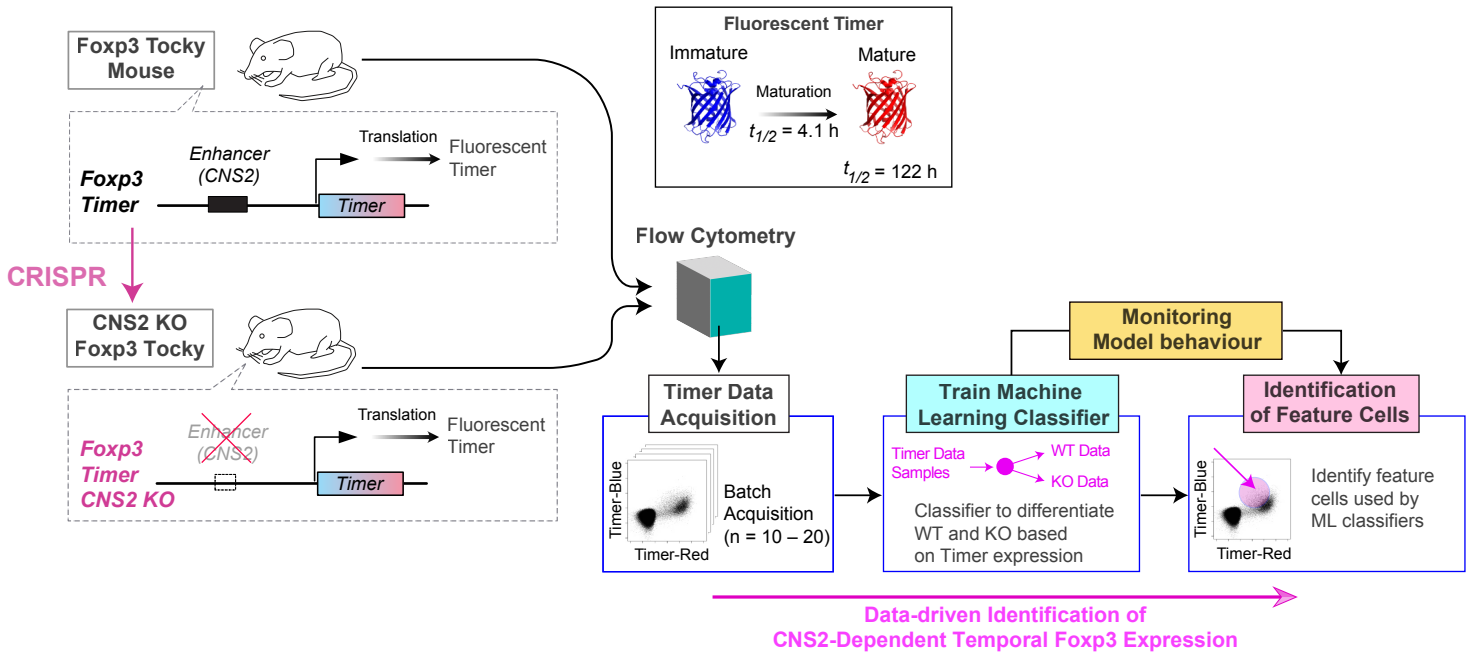

Foxp3 Tocky Mouse
Foxp3 Timer
Enhancer (CNS2)
Translation
Fluorescent Timer
Timer
CRISPR
CNS2 KO Foxp3 Tocky
Foxp3 Timer CNS2 KO
Enhancer (CNS2)
Translation
Fluorescent Timer
Timer
Fluorescent Timer
Immature
Mature
Maturation
$t_{1/2}$ = 4.1 h
$t_{1/2}$ = 122 h
Flow Cytometry
Monitoring Model behaviour
Timer Data Acquisition
Timer-Blue
Timer-Red
Batch Acquisition (n = 10 – 20)
Train Machine Learning Classifier
Timer Data Samples
WT Data
KO Data
Classifier to differentiate WT and KO based on Timer expression
Identification of Feature Cells
Timer-Blue
Timer-Red
Identify feature cells used by ML classifiers
Data-driven Identification of CNS2-Dependent Temporal Foxp3 Expression

Figure 2

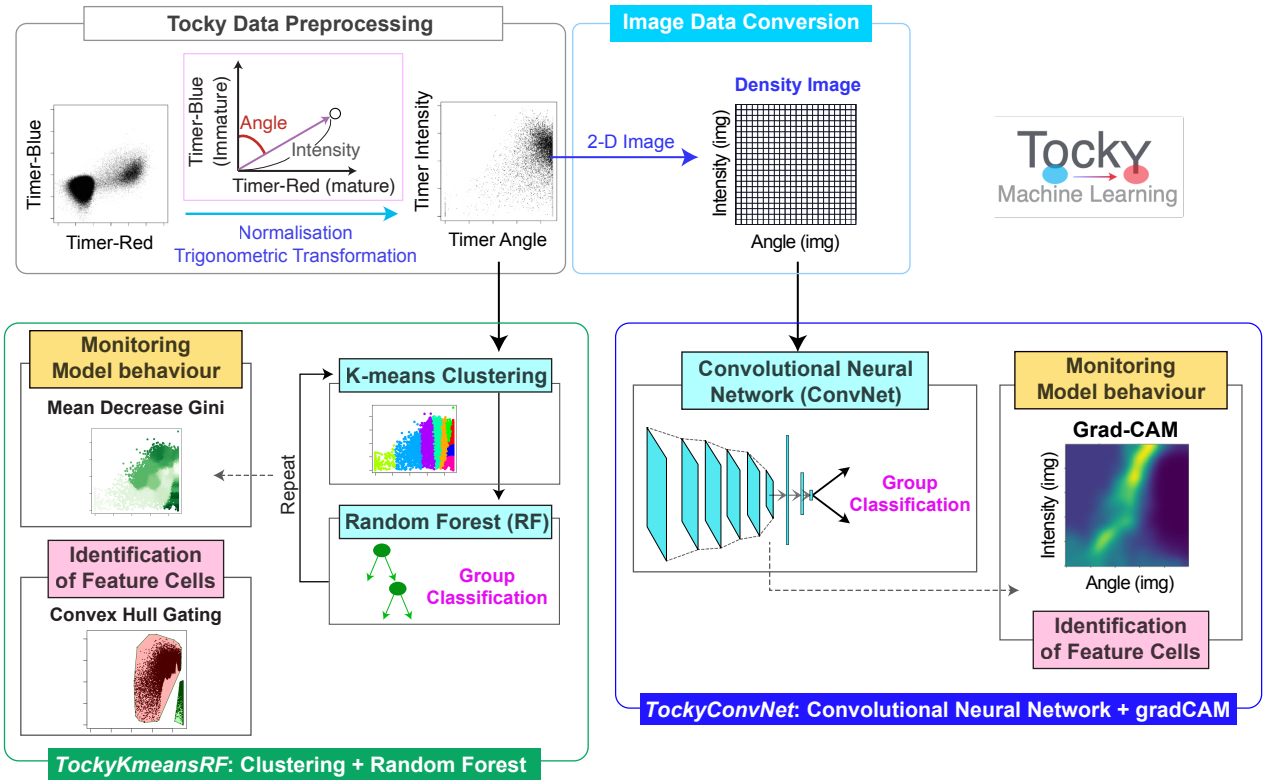

Tocky Data Preprocessing
Timer-Blue
Timer-Red
Timer-Blue (Immature)
Angle
Intensity
Timer-Red (mature)
Normalisation
Trigonometric Transformation
Timer Intensity
Timer Angle
Image Data Conversion
2-D Image
Density Image
Intensity (img)
Angle (img)
Tocky
Machine Learning
Monitoring Model behaviour
Mean Decrease Gini
Identification of Feature Cells
Convex Hull Gating
Repeat
K-means Clustering
Random Forest (RF)
Group Classification
TockyKmeansRF: Clustering + Random Forest
Convolutional Neural Network (ConvNet)
Group Classification
Monitoring Model behaviour
Grad-CAM
Intensity (img)
Angle (img)
Identification of Feature Cells
TockyConvNet: Convolutional Neural Network + gradCAM

Figure 3

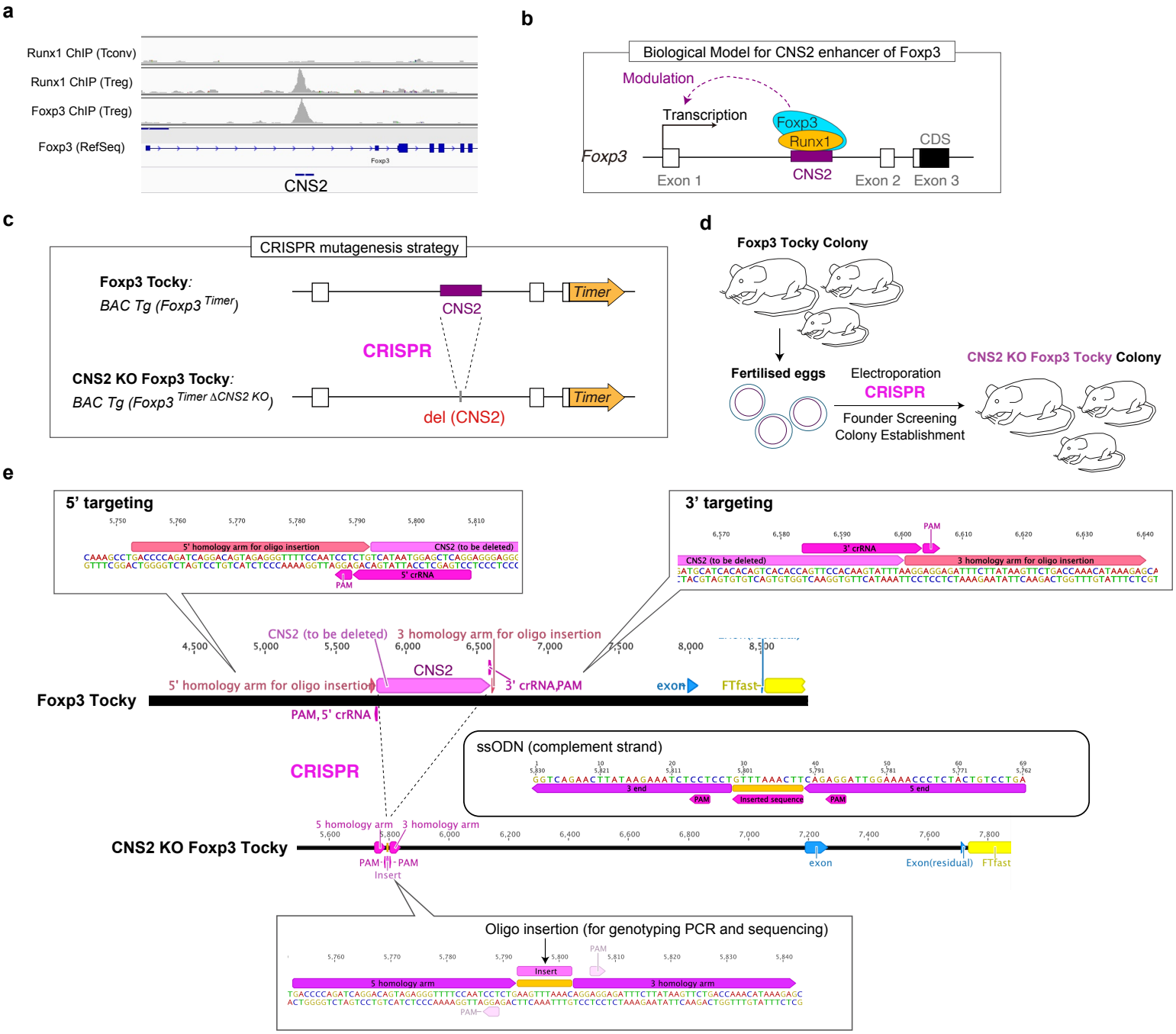

a
Runx1 ChIP (Tconv)
Runx1 ChIP (Treg)
Foxp3 ChIP (Treg)
Foxp3 (RefSeq)
CNS2
b
Biological Model for CNS2 enhancer of Foxp3
Modulation
Transcription
Foxp3
Runx1
CDS
Foxp3
Exon 1
CNS2
Exon 2
Exon 3
c
CRISPR mutagenesis strategy
Foxp3 Tocky:
BAC Tg (Foxp3 Timer)
CNS2
Timer
CRISPR
CNS2 KO Foxp3 Tocky:
BAC Tg (Foxp3 Timer ΔCNS2 KO)
Timer
del (CNS2)
d
Foxp3 Tocky Colony
Fertilised eggs
Electroporation
CRISPR
Founder Screening
Colony Establishment
CNS2 KO Foxp3 Tocky Colony
e
5' targeting
5' homology arm for oligo insertion
CNS2 (to be deleted)
5' crRNA
PAM
3' targeting
PAM
3' crRNA
CNS2 (to be deleted)
3 homology arm for oligo insertion
CNS2 (to be deleted)
3 homology arm for oligo insertion
CNS2
5' homology arm for oligo insertion
3' crRNA,PAM
exon
FTfast
Foxp3 Tocky
PAM,5' crRNA
CRISPR
ssODN (complement strand)
3 end
5 end
PAM
Inserted sequence
PAM
5 homology arm
3 homology arm
CNS2 KO Foxp3 Tocky
PAM
PAM
Insert
exon
Exon(residual)
FTfast
Oligo insertion (for genotyping PCR and sequencing)
PAM
Insert
5 homology arm
3 homology arm
PAM

Figure 4

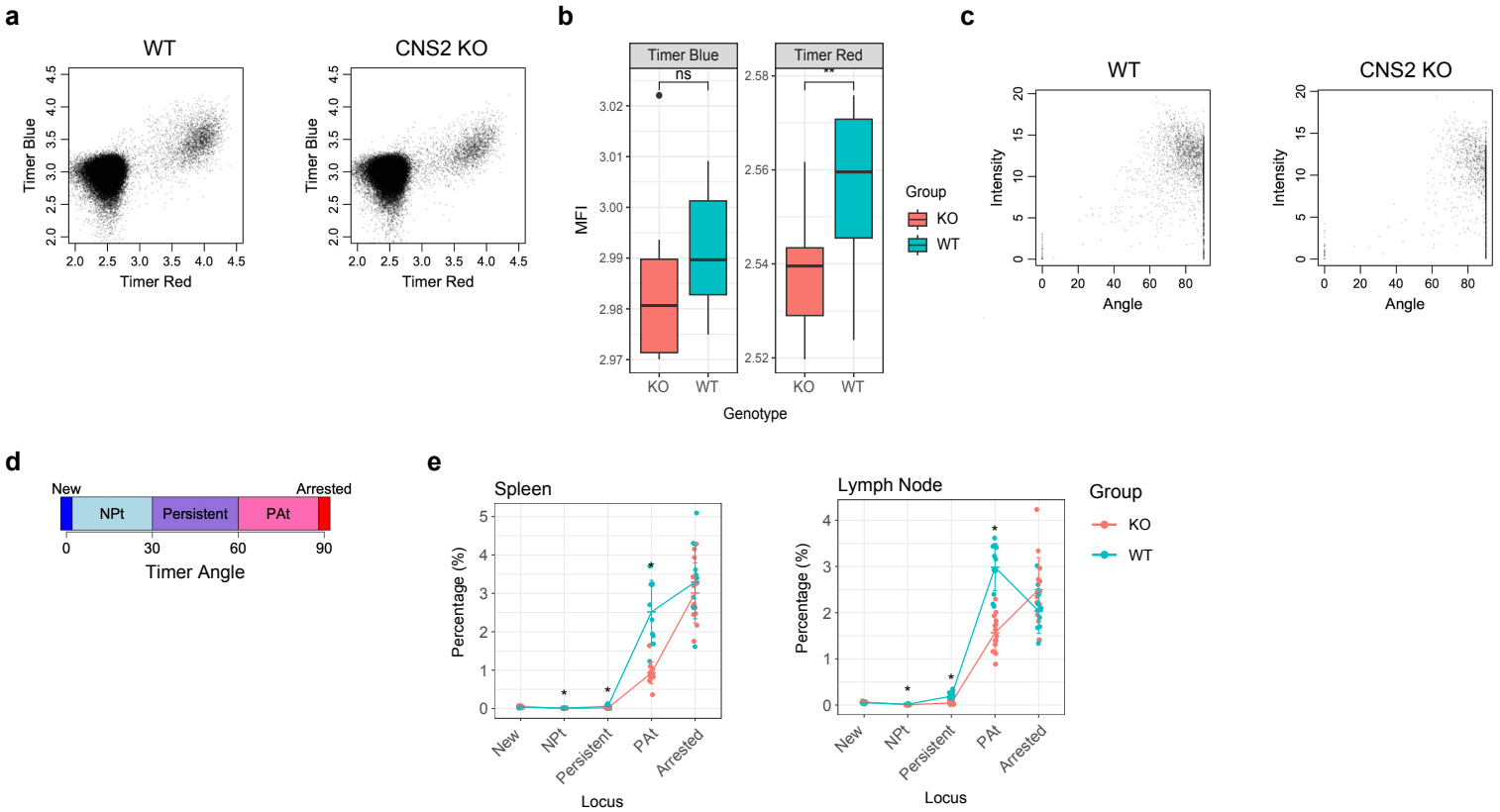

a
WT
CNS2 KO
Timer Blue
Timer Red
b
Timer Blue
Timer Red
ns
**
MFI
KO
WT
Genotype
Group
KO
WT
c
WT
CNS2 KO
Intensity
Angle
d
New
Arrested
NPt
Persistent
PAt
0
30
60
90
Timer Angle
e
Spleen
Lymph Node
Percentage (%)
New
NPt
Persistent
PAt
Arrested
Locus
Group
KO
WT

Figure 5

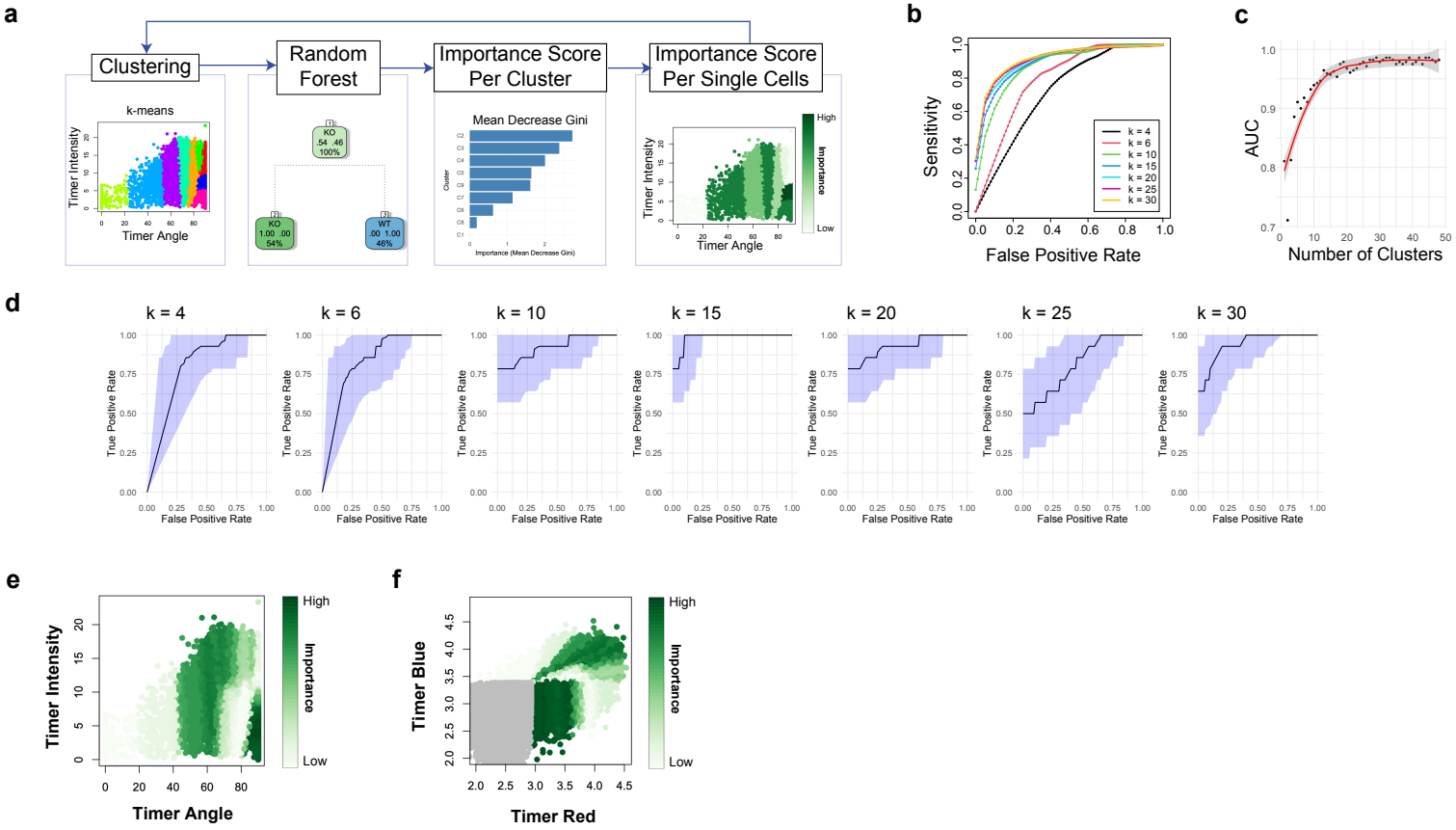

a
Clustering
Random Forest
Importance Score Per Cluster
Importance Score Per Single Cells
k-means
Timer Intensity
Timer Angle
KO .54 .46 100%
KO 1.00 .00 54%
WT .00 1.00 46%
Mean Decrease Gini
Cluster
Importance (Mean Decrease Gini)
High
Importance
Low
b
Sensitivity
False Positive Rate
k = 4
k = 6
k = 10
k = 15
k = 20
k = 25
k = 30
c
AUC
Number of Clusters
d
k = 4
k = 6
k = 10
k = 15
k = 20
k = 25
k = 30
True Positive Rate
False Positive Rate
e
Timer Intensity
Timer Angle
High
Importance
Low
f
Timer Blue
Timer Red
High
Importance
Low

Figure 6

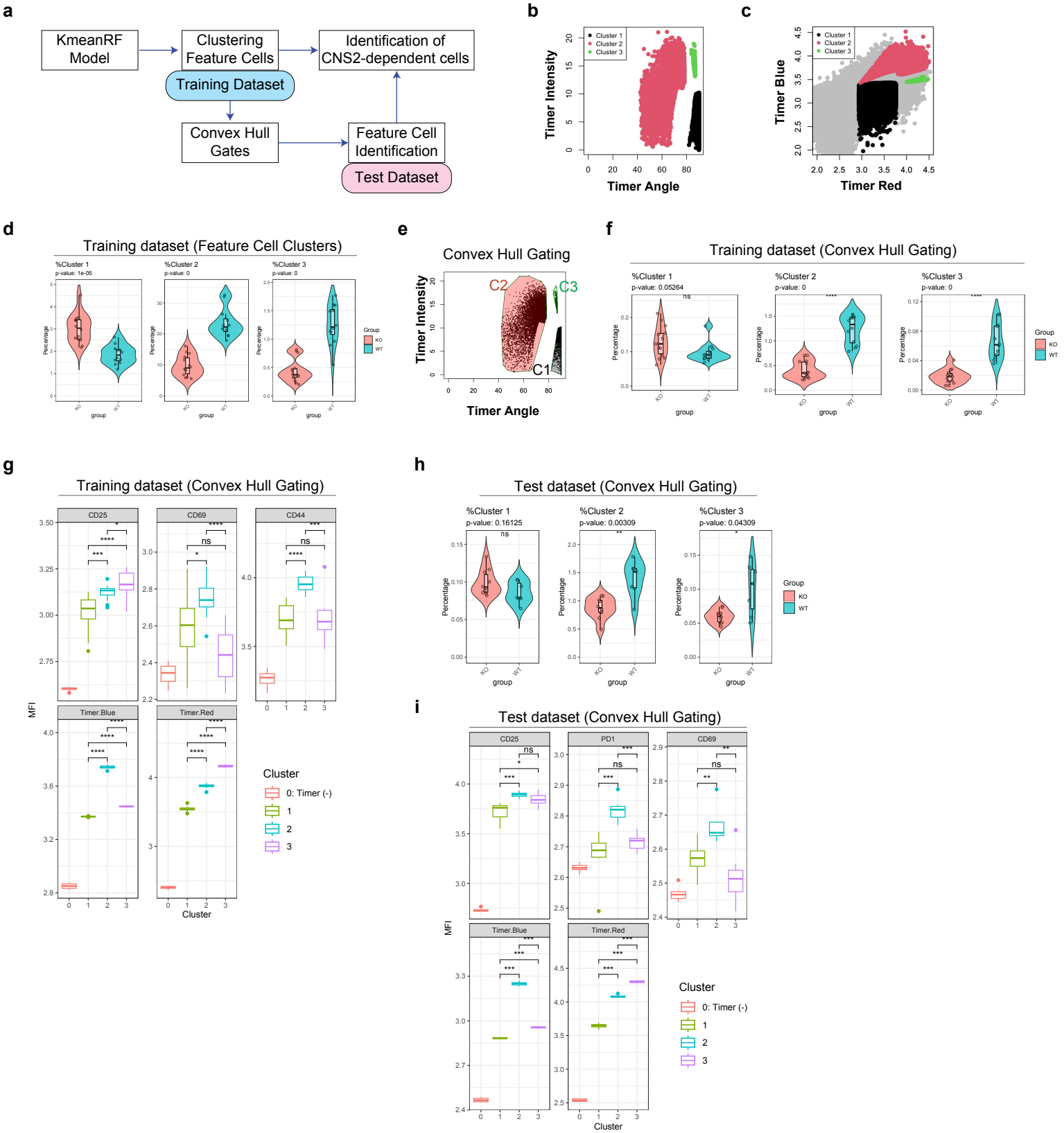
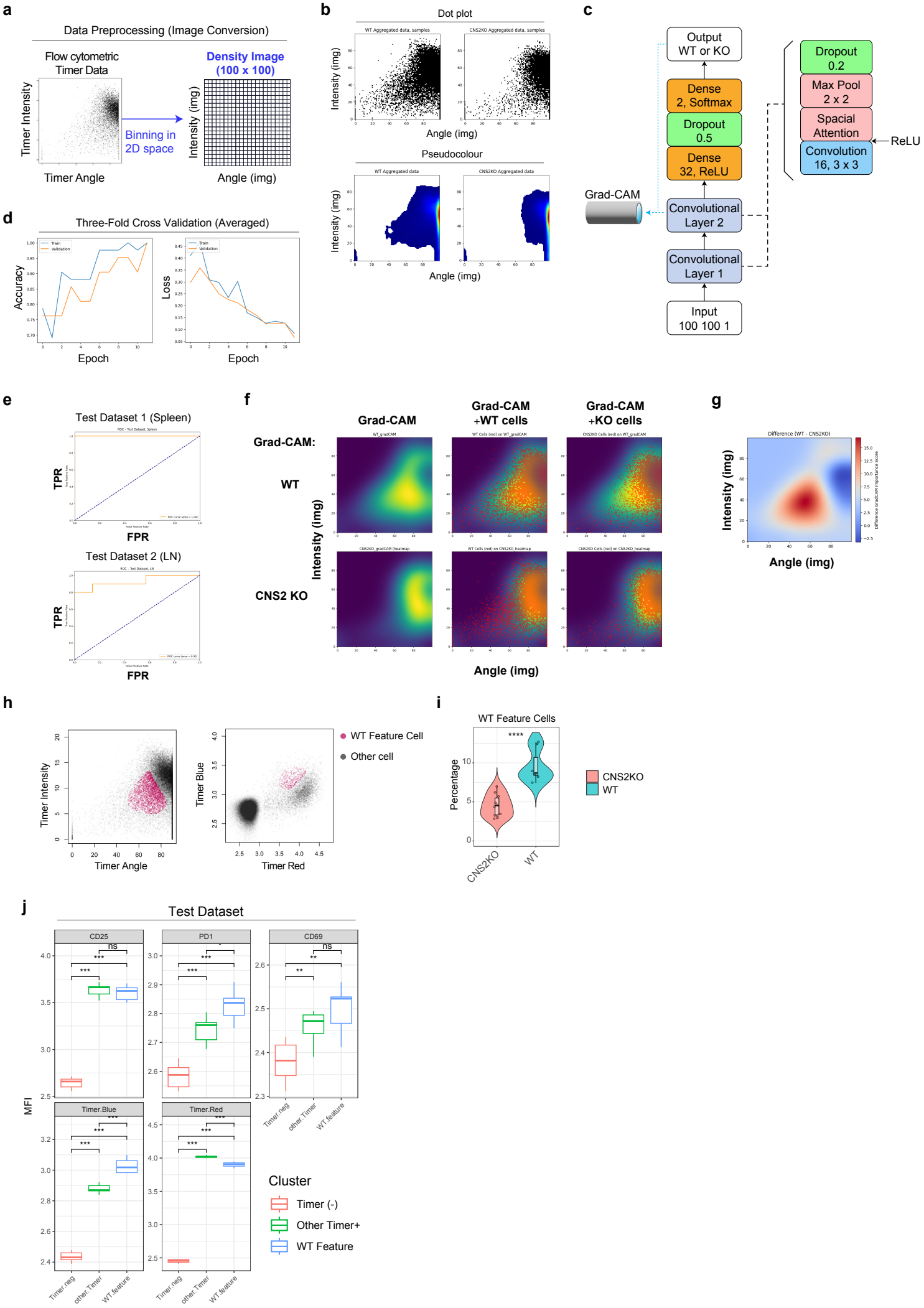

a
KmeanRF Model
Clustering Feature Cells
Identification of CNS2-dependent cells
Training Dataset
Convex Hull Gates
Feature Cell Identification
Test Dataset
b
Cluster 1
Cluster 2
Cluster 3
Timer Intensity
Timer Angle
c
Timer Blue
Timer Red
d
Training dataset (Feature Cell Clusters)
%Cluster 1
p-value: 1e-05
%Cluster 2
p-value: 0
%Cluster 3
p-value: 0
Percentage
group
KO
WT
Group
e
Convex Hull Gating
C1
C2
C3
Timer Intensity
Timer Angle
f
Training dataset (Convex Hull Gating)
%Cluster 1
p-value: 0.05264
%Cluster 2
p-value: 0
%Cluster 3
p-value: 0
g
Training dataset (Convex Hull Gating)
CD25
CD69
CD44
Timer.Blue
Timer.Red
MFI
Cluster
0: Timer (-)
1
2
3
h
Test dataset (Convex Hull Gating)
%Cluster 1
p-value: 0.16125
%Cluster 2
p-value: 0.00309
%Cluster 3
p-value: 0.04309
i
Test dataset (Convex Hull Gating)
CD25
PD1
CD69
Timer.Blue
Timer.Red

Figure 7


a
Data Preprocessing (Image Conversion)
Flow cytometric Timer Data
Timer Intensity
Timer Angle
Binning in 2D space
Density Image (100 x 100)
Intensity (img)
Angle (img)
b
Dot plot
WT Aggregated data, samples
CNS2KO Aggregated data, samples
Intensity (img)
Angle (img)
Pseudocolour
WT Aggregated data
CNS2KO Aggregated data
Intensity (img)
Angle (img)
c
Output WT or KO
Dense 2, Softmax
Dropout 0.5
Dense 32, ReLU
Convolutional Layer 2
Convolutional Layer 1
Input 100 100 1
Grad-CAM
Dropout 0.2
Max Pool 2 x 2
Spacial Attention
Convolution 16, 3 x 3
ReLU
d
Three-Fold Cross Validation (Averaged)
Train
Validation
Accuracy
Loss
Epoch
Epoch
e
Test Dataset 1 (Spleen)
TPR
FPR
Test Dataset 2 (LN)
TPR
FPR
f
Grad-CAM
Grad-CAM +WT cells
Grad-CAM +KO cells
Grad-CAM:
WT
CNS2 KO
Intensity (img)
Angle (img)
g
Difference (WT - CNS2KO)
Intensity (img)
Angle (img)
h
Timer Intensity
Timer Angle
Timer Blue
Timer Red
WT Feature Cell
Other cell
i
WT Feature Cells
Percentage
CNS2KO
WT
j
Test Dataset
CD25
PD1
CD69
Timer.Blue
Timer.Red
MFI
Timer.neg
other.Timer
WT.feature
Cluster
Timer (-)
Other Timer+
WT Feature