Title: An engineered cellular Timer for analysing the time domain of cellular

differentiation in vivo

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Abstract

Understanding in vivo mechanisms of cellular differentiation is challenging because the temporal regulation of cellular signalling and differentiation in vivo is heterogeneous and highly dynamic. Here we establish a novel cellular technology, **T**imer **o**f **c**ell **k**inetics and activit**y** (Tocky [*toki*], the Japanese word for time). Tocky uses Fluorescent Timer protein, which spontaneously shifts its emission spectrum from blue-to-red, in combination with computer algorithms to reveal the timing of cellular signalling and differentiation events in vivo. Using a transcriptional target of T cell receptor (TCR)-signalling, we establish *Nr4a3*-Tocky to analyse the temporal dynamics of *in vivo* TCR signalling activity. *Nr4a3*-Tocky determines the temporal order of events driving regulatory T cell (Treg) differentiation and identifies their immediate developmental precursors. Furthermore, by generating *Foxp3*-Tock*y*, we for the first time reveal *in vivo* dynamics of DNA demethylation, showing that *Foxp3* transcription precedes demethylation of its enhancer region. Thus, Tocky empowers cellular biologists to ask previously inaccessible questions, revealing time-dependent differentiation mechanisms and developmental states.

Main Text

It is a central question in cell biology how cellular differentiation progressively occurs through the activities of temporally-coordinated molecular mechanisms (*1, 2*). It is, however, challenging to investigate in vivo mechanisms at the single cell level because individual cells are not synchronized and are heterogeneous, receiving key signalling at different times and frequencies in the body. No existing technologies can systematically analyse the temporal dynamics of differentiation and activities of individual cells in vivo. Intravital microscopy is useful for analysing cells in micro-environments (*3*) but is not suitable for systematically analysing cells which rapidly migrate through tissues, such as T cells. Single cell sequencing can provide 'pseudotime', but this is not the measurement of time, as the name implies, but is a measurement of the transcriptional similarities between samples at chosen analysis time points (*4*). Flow cytometry is suitable for determining the differentiation stage of individual cells, but current methods cannot be applied to investigate how individual cells sequentially differentiate into more mature stages, as data from individual cells do not currently encode time information (*5*). There is thus a great need for a new technology to experimentally analyse the passage of time following a key differentiation event, or *the time domain,* of individual cells in vivo. Such a new technology would benefit all areas of cellular biology, but would be particularly useful for the study of T-cells under physiological conditions in vivo, where both the time and frequency of signaling are critical to their differentiation.

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T cells migrate through the body (*6*), and their activation and differentiation statuses are almost exclusively determined by flow cytometric analysis (*7*). In T cells, T cell receptor (TCR) signaling triggers their activation and differentiation (*8*), and is the central determinant of thymic T cell selection (*1*) (including negative selection (*9*) and Treg selection (*10*)) and antigen recognition in the periphery (*8*). While the temporal dynamics of proximal TCR signaling, which are in the time scale of seconds, have been comprehensively and quantitatively analysed (*9, 11*), it is still unclear how transcriptional mechanisms for activation and differentiation respond to TCR signals over time *in vivo*.

TCR signalling activates NFAT, AP-1 and NF-κB, which initiate the transcription of immediate early genes within a few hours (*12*), but their effects on T cell differentiation over the time scale of hours and days are obscure. In order to analyse TCR signal strength, currently, *Nur77 (Nr4a1)-EGFP* reporter mouse is commonly used(*13*), but the long half-life of the reporter gene, EGFP (~56 h (*14*)) prevents its application for the analysis of the temporal dynamics of the events downstream of TCR signaling *in vivo*.

In this study, we have established a new Fluorescent Timer technology, **T**imer **o**f **c**ell **k**inetics and activit**y** (Tocky), which uniquely reveals the time and frequency domains of cellular differentiation and function in vivo. Fluorescent Timer proteins have been used to analyse *in vivo* protein dynamics and receptor turnover (*15, 16*), as well as identify progenitor cells, i.e. those cells expressing only immature fluorescence during embryogenesis and pancreatic beta cell development (*17-20*). However, those studies were qualitative and

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did not recognise the quantitative power of Fluorescent Timer. Here we develop a new Fluorescent Timer approach to quantitatively analyse the time and frequency domains of gene transcription within individual cells in vivo. By identifying a downstream gene of TCR signalling (*Nr4a3*) and developing a Fluorescent Timer reporter mice for the gene, we experimentally establish and validate the Tocky system for TCR signalling. Furthermore, we apply the Tocky approach to the lineage-specific transcription factor of regulatory T cells (*Foxp3*), revealing in vivo dynamics of Treg differentiation. Thus, Tocky technology reveals time-dependent mechanisms of in vivo cellular differentiation and developmental states following key signalling pathway or lineage commitment, which cannot be analysed by existing technologies.

Results

Design of Tocky system for analysing the time and frequency domains of signal-triggered activation and differentiation events

Given the long half-life of stable fluorescent proteins (FP), like GFP (56 h (*14*)), the dynamics of gene transcription cannot be effectively captured using conventional FPs expression as a reporter. We therefore chose to use Fluorescent Timer protein (Timer), which forms a short-lived chromophore that emits blue fluorescence (Blue), the half-life of which is ~ 7 h, before producing the mature chromophore that emits red fluorescence (Red), which has a decay rate longer than 20 h (*20*) (**Fig. 1A**).

We investigated *in silico* how two different transcriptional activities would influence the production of blue and red fluorescent form of the protein through time, and how the light emitted would be detected by flow cytometry. We compared the reporting of transient, pulse-like transcription with that of persistent transcription, both of which are relevant to TCR-mediated transcription (*21*) (**Fig. 1B**). By plotting fluorescence against time, we observed that Blue was a better readout for the real-time level of transcription than Red and GFP, which rather reported the cumulative activity of transcription. By plotting blue fluorescence against red fluorescence, there are thus 3 key loci in the Blue-Red plane (**Fig. 1C**). Firstly, when new transcription occurs in Timer-negative cells, Timer^{+} cells acquire pure Blue, and are identified in the New locus. Secondly, if transcriptional activity is persistent and/or sufficiently frequent, cells accumulate in and around the steady-state diagonal line (Persistent locus). Lastly, when transcription is diminished, cells lose Blue and stay in the Arrested locus, until the red protein decays (**Fig. 1C**). Importantly, Timer decay is unidirectional and irreversible as the chromophore matures from Blue to Red in a clockwise direction. In the case of individual Timer^{+} cells, movement from New to Persistent loci is also unidirectional and irreversible, because the half-life of red protein is longer than the half-life of the blue form to mature into red. Therefore transition from the New to the persistent loci captures the time domain of cellular differentiation. In contrast, cells in the Arrested locus could re-initiate transcription to express new Blue protein and move anti-clockwise back into the Persistent locus (**Fig. 1C)**. Thus movement between Persistent and Arrested loci more specifically captures how frequently transcriptional

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activities occur in mature cells. These three loci can be identified and quantified more effectively by analysis of the angle of individual cells from the blue axis, and the 2-dimensional blue vs. red Timer fluorescence data can be transformed by trigonometric data transformation to provide new variables: the angle from the blue axis is defined as Timer-Angle, while the distance from the origin is defined as Timer-Intensity (**Fig. 1D**). We therefore coined the system Timer of cell Kinetics and activity (Tocky; とき [toki] means "Time" in Japanese). Timer-Angle provides time-related composite information: the first phase (New → Persistent) is for analysing how differentiation mechanisms are regulated over time (i.e. time domain analysis); and the latter half (Persistent – Arrested) is for analysing the relative frequency of transcriptional activation (i.e. frequency domain analysis). Thus, we designate this composite time-axis as *Tocky-Time* (**Fig. 1E**).

Nr4a3-Tocky reveals the time domain of TCR signal-triggered activation and differentiation events

T cell differentiation is triggered by TCR signals. In order to track the *in vivo* dynamics of transcription downstream of TCR signal transduction, we firstly identified genes immediately downstream of TCR signalling using a data-oriented multidimensional analysis, Canonical Correspondence Analysis (CCA) (*22*) (**Supplementary Fig. 1A**). *Nr4a3* (*Nor1*) was identified as the gene with the highest correlation with anti-CD3 mediated T cell activation (which mimics TCR activation) and *in vivo* TCR signals in the thymus, while *Nr4a1* (*Nur77)* and *Rel* also scored highly (**Supplementary Fig. 1B and C**). In agreement, upon anti-CD3 stimulation, *Nr4a3/Nor1* was rapidly induced in

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T cells and peaked within 2 hours of stimulation (**Supplementary Fig. 1D**). Having established *in silico* that Tocky can temporally report transcription, we generated BAC transgenic reporter *Nr4a3*-Tocky mice (**Supplementary Fig. 2**), in which the transcriptional activity of the *Nr4a3* gene is reported by Timer proteins and used as an indicator of new transcription mediated by TCR signal transduction. Ova stimulation of CD4$^+$ T-cells from OTII *Nr4a3*-Tocky mice resulted in the upregulation of Blue within 4 h, with some cells acquiring Red at 8 h after stimulation (**Fig. 2A**). *Nr4a3*-Tocky T cells further increased both Blue and Red throughout the 48-h culture, while removal of TCR stimulation by anti-MHC II-treatment from 24 h onwards resulted in the rapid loss of Blue (**Fig. 2A**), which captured the reduction in frequency of TCR signalling.

In order to analyse effectively the continuous progression of Timer fluorescence in Fig. 2a, Timer fluorescence data were transformed into Angle and Intensity data (**Fig. 2B**). Importantly, the progression of Timer Angle becomes slower and cells accumulate as cells approach ~45˚ by density plots, which visualise the distribution of angle values, reflecting sustained or high frequency TCR signalling (**Fig. 2C**). In order to quantify the maturation of the Timer chromophore since first onset of its transcription, we grouped cells into 5 populations (i.e. data categorisation) according to their Angle values: from the 'New' population (Angle = 0˚) representing cells within the first 7 hours of initiation of TCR-mediated transcription to the 'Arrested' population (Angle = 90˚), which represents cells in which all Timer protein has matured to red, and transcriptional activity has decreased below cytometer detection thresholds (**Fig. 2D**). The area between 30˚ – 60˚ was defined as the

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Persistent locus. The areas between New and Persistent (NP-t) or between the Persistent and Arrested (PA-t) contained cells from the surrounding loci that are in the process of changing their frequency of *Nr4a3* transcription (**Fig. 2E**). The analysis of the percentage of cells in these loci (designated as Timer locus analysis) neatly captured the change in TCR-mediated transcription through time, as cells shifted from New to Persistent, which represent the time domain. Furthermore, it showed that removal of TCR signals lead to a complete loss of New, NP-t and Persistent signalling within 8 hours, and cells migrated to Arrested transcriptional dynamics, which represents sparse or no signalling activity (**Fig. 2E**). Thus, *Nr4a3*-Tocky recaptured the predicted kinetics of Timer-expressing cells, validating the Tocky model (**Fig. 1E**).

We hypothesised that Timer-Intensity reflects both the signal strength and the duration/ frequency of TCR signalling, as Timer proteins accumulate in individual cells in response to strong and/or repeated TCR signals. Timer Intensity in antigen-stimulated *Nr4a3*-Tocky T cells increased over time, and fell after removal of the TCR signal (**Fig. 3A**). Furthermore, Timer Intensity was increased in a dose-dependent manner by cognate antigen (**Fig. 3B**). CD28 signals did not have statistically significant effects on the progression of Timer Angle and Intensity upon TCR engagement using plate-bound anti-CD3 and anti-CD28 antibodies (**Supplementary Fig. 3**). In addition, Timer Intensity showed a high correlation to cell surface CD25 expression, which is a marker of activated T cells (R^2 = 0.93) (**Fig. 3C**). Thus, using *Nr4a3*-Tocky, Timer Intensity reflects the cumulative transcriptional outcome of signals in a given cell, as the reporter protein accumulates in response to sustained or highly frequent signals.

Tocky mice identify Treg developmental precursors

TCR signaling is the major determinant of regulatory T-cell (Treg) differentiation in the thymus, as T cells that have received strong TCR signals preferentially express CD25 and Foxp3 and differentiate into Treg (*23, 24*).

In order to demonstrate the quantitative power of the Tocky technology, we investigated the temporal sequence of thymic CD4 T cell differentiation following TCR signals by analysing the time domain of *ex vivo* T cells from *Nr4a3*-Tocky thymus. Timer expression occurred in the $CD4^+CD8^+$ double positive (DP), $CD4^+$ single positive (CD4SP), and CD8SP populations, with CD4SP displaying the highest frequency ($8.9 \pm 3.7\%$, **Fig. 4A**). The cell surface expression of CD69, which is highly expressed on immature CD4SP, was high in CD4SP cells in the New locus, and progressively declined as the Timer protein matures. In contrast, the majority of CD4SP cells in the Persistent locus expressed Foxp3 and CD25 (**Fig. 4B**). With the assumption that in neonatal mice, nearly all cells in the Persistent locus would be derived from the New locus, these results led to the hypothesis that Treg differentiation requires persistent TCR stimulation.

The sequence of thymic Treg development is controversial. Some studies suggest that $CD25^+Foxp3^-$ Treg are the major Treg precursors (*25, 26*) while other studies argue that $CD25^-Foxp3^+$ cells are Treg precursors (*27*) (**Fig. 4C**). Analysis of Timer expression in the different populations enabled us to distinguish between these 2 possibilities. The majority of Treg ($CD25^+Foxp3^+$)

were Timer$^+$ (89.0 ± 6.1%), and both of the proposed Treg precursor populations (CD25$^+$Foxp3$^-$ and CD25$^-$Foxp3$^+$) also had high proportions of Timer$^+$ cells (63.3 ± 18.6% and 57.5 ± 15.1%, respectively, **Fig. 4D**), indicating that these three populations have all received either strong or frequent TCR signals. To place the three populations in time following TCR signal transduction, we analysed thymi from various ages of *Nr4a3*-Tocky neonates, and measured Foxp3 and cell-surface CD25 expression, in addition to Nr4a3 Timer fluorescence, and applied our Timer locus analysis. Timer expressing CD25$^+$Foxp3$^-$ cells were mostly Blue+Red-, while those of CD25$^+$Foxp3$^+$ Treg and CD25$^-$Foxp3$^+$ cells were mostly Blue+Red+ (**Fig. 4E**). Density plot showed that CD25$^+$Foxp3$^-$ cells only had a remarkable peak of Angle 0, while CD25$^+$Foxp3$^+$ Treg showed a clear peak around 30-50° and CD25$^+$Foxp3$^-$ cells had a higher peak at 90° (**Fig. 4F**). The mean values of Timer-Angle in the CD25$^+$Foxp3$^-$ cells were significantly lower than the values in CD25$^-$Foxp3$^+$ cells and CD25$^+$Foxp3$^+$ Treg (**Fig. 4G**), indicating that the CD25$^+$Foxp3$^-$ population are the cells that have most recently signalled through the TCR, and therefore the earliest of these three subsets. The mean values of Timer-Intensity were lowest in CD25$^-$Foxp3$^+$, and highest in CD25$^+$Foxp3$^+$ Treg (**Fig. 4H**), indicating that the CD25$^-$Foxp3$^+$ subset had received the weakest and/or least frequent TCR signals among these subsets. Next, in order to define the temporal relationships between the three populations (CD25$^+$Foxp3$^-$, CD25$^-$Foxp3$^+$ and CD25$^+$Foxp3$^+$), we quantified the proportion of Timer$^+$ cells in each maturation stage of Timer fluorescence (**Fig. 4I**). This showed that most of the CD4SP cells in the New locus were CD25$^+$Foxp3$^-$ cells, while most of CD4SP cells in the Persistent and PA-t loci

were CD25$^+$Foxp3$^+$ Treg. The relative contribution of CD25$^-$Foxp3$^+$ was greatest in the Arrested locus, suggesting that these cells are enriched with those with aborted TCR signalling (**Fig. 4I).**

Collectively, these analyses demonstrate that the major pathway for Treg differentiation is from CD25$^+$Foxp3$^-$ Treg precursors, in which persistent TCR signals induce Foxp3 expression. The CD25$^-$Foxp3$^+$ subset is enriched with Foxp3$^+$ cells that have received relatively weaker and/or less sustained TCR signals. Following TCR signalling, cell surface CD69 expression peaks within 4 to 8 hours. CD25 expression is induced in this early phase in the Timer Blue (New) population and steadily accumulates as T cells receive TCR signals. Foxp3 expression is the most delayed and occurs most efficiently after T cells have persistently interacted with antigen (**Fig. 5**). Importantly, the Tocky system has for the first time directly shown these in vivo dynamics of Treg differentiation. Thus, our investigations show that *Nr4a3*-Tocky and Timer locus analysis effectively unravel the temporal dynamics of T cell differentiation following TCR signals.

Foxp3-Tocky *successfully identifies newly generated Treg*

Next, in order to address whether the Tocky system can be applied to another gene, and to validate the system, we developed *Foxp3*-Tocky mice using the same approach as *Nr4a3*-Tocky (**Fig. 6A**). By investigating Foxp3 protein staining (**Fig. 6B**) and *Foxp3*$^{IRES-GFP}$ *Foxp3*-Tocky double transgenic mice (**Fig. 6C**), Timer expression showed high correlation with GFP and with Foxp3 protein. As expected, when naïve CD4$^+$ T cells were stimulated in the

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presence of IL-2 and TGF-β (i.e. induced-Treg [iTreg] conditions), new Foxp3 expression (Blue$^+$Red$^-$) was induced at 22 h, which gained Red proteins by 50 h as the Timer chromophore matured (**Fig. 6D**). Trigonometric Timer data analysis showed *ex-vivo* splenic Foxp3$^+$ T cells from adult mice had high Timer-Angle values throughout the culture. In contrast, iTreg showed very low Angle values at 22 h, which slowly increased overtime (**Fig. 6E–6F**).

Foxp3-Tocky reveals that the initiation of Foxp3 transcription precedes the demethylation of the Foxp3 gene in vivo

Next, taking advantage of the Tocky system, we investigated the transcription and methylation of the *Foxp3* gene during Treg generation. Whilst it is known that demethylation can occur within a few hours in cultured cells (*28*), there is no available method to investigate the in vivo dynamics of demethylation in the mouse body.

It is well established that DNA demethylation of the Treg-Specific Demethylated Region (TSDR) is a hallmark of committed Treg (*29*). It is, however, unknown whether demethylation occurs before or after the initiation of *Foxp3* transcription. To address this question, differentiating thymic T cells were isolated by flow-sorting thymic Blue^{high} T-cells into Timer-Angle Low, Medium, and High (**Fig. 7A**). Analysis showed that the isolated populations exhibited distinct Timer Angles from a mean of 10 to 80 degrees (**Fig. 7B & C**). DNA was isolated from the population and their TSDR demethylation was analysed (**Fig. 7D**). Importantly, the newest Foxp3$^+$ cells (Low) were still mostly methylated and not significantly different from Timer(-) cells in the

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degree of TSDR demethylation, while rapid demethylation occurred as cells moved to the Persistent locus (**Fig. 7D**). Timer-Angles showed a strong correlation with TSDR demethylation rates (Spearman's correlation coefficient ρ = -0.81, **Fig. 7E**), indicating that the *Foxp3*-Tocky reporter successfully captures the demethylation dynamics of the *Foxp3* gene. Thus, Foxp3 expression precedes the demethylation of the TSDR region, and the most active demethylation process occurs when *Foxp3* transcription is sustained.

Collectively, the *Foxp3*-Tocky reporter successfully identified newly generated Treg *in vitro* and *in vivo*, ordered cells from new to relatively aged ones (by the time domain analysis), and distinguished them from pre-existing mature Foxp3^{+} Treg (by the frequency domain analysis), thus, demonstrating the general applicability of the Tocky system to studies of cellular biology and immunology.

Discussion

Here we have established two uses of the Tocky system. First, Tocky allows investigators to determine the relative temporal order of the molecular events following the activation of key signalling pathways, and thereby provides a new way to identify immediate precursors and relatively mature cells using the time domain analysis. Previous studies using Fluorescent Timer proteins identified newly generated cells, as they expressed solely immature fluorescence without mature fluorescence (*17-19*). In this study, in addition to the rigorous determination of New cells (Blue$^+$Red$^-$), we established a quantitative method to measure the relative age of cells which are maturing *in vivo*. This has allowed us to simultaneously investigate the temporal dynamics of the demethylation process and the transcriptional activities in vivo. Surprisingly, *Foxp3* transcription preceded the DNA demethylation of its enhancer region, which is considered to regulate the transcriptional activity (*30*). This means that the Tocky system provides an unprecedented mean to investigate the in vivo regulation of molecular mechanisms. Thus, we determined the temporal sequences of molecular events during thymic Treg differentiation after receiving TCR signals using *Nr4a3*-Tocky, and identified the major precursor of these cells as the earliest cells among the presumptive precursor populations. In addition, using *Foxp3*-Tocky, we identified and characterised the earliest stages of Foxp3$^+$ Treg differentiation.

Second, the Tocky system allowed identification of cells with persistent or high-frequency transcription of the reporter gene through frequency domain analysis. The persistent dynamics can be maintained if the interval of recent

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transcriptional activities is sufficiently smaller than the half-life of Blue. Importantly, when Timer proteins are produced in a persistent manner, the progression of Timer Angle becomes slower as cells approach the Persistent locus (i.e red-to-blue ratio gradually increases; Fig. 1D), as the speed of cells decreases in the Blue-Red plane, resulting in a *ritardando* towards a *pause*. Consequently, the accumulation of cells within a defined red-blue space is indicative of cells displaying a similar average frequency of transcription/ similar signalling patterns over time, as reflected by tissue-infiltrating antigen-specific T-cells. Therefore, we use the term 'frequency domain' to define not individual cells, but groups of cells with similar transcriptional patterns that can be related to biological functions. It is highly likely that at the individual cell basis, transcriptional frequencies are highly stochastic. We anticipate that the development of future fluorescent timers with a shorter maturation time will enable a more precise estimation of true transcriptional frequencies, which will be important for analysing transcriptional circuits occurring in the time frames of minutes.

In fact, using *Nr4a3*-Tocky, we showed that thymic T cells that have received sustained TCR signals differentiate into Treg. Consistent with these findings, thymic Foxp3^{+} cells show slower and more confined migration, compared to other thymic T cells by two-photon microscopy (*31*). These differentiating Foxp3^{+} cells may integrate TCR and other signals from thymic epithelial cells and other APCs. Since the affinity of TCR-ligand interactions determines the dynamics of proximal TCR signalling molecules (*32*), future studies should investigate whether and how different TCR affinities are translated into

different dynamics of transcription of downstream transcription factors, including Nr4a3.

In general, persistent signals may have distinct biological roles compared to transient signals (*21*), and the Tocky system is an effective tool to investigate these dynamics. For example, sustained TLR4 signalling induces *Il6* transcription effectively, in contrast to transient activities (*33*); sustained DNA damage, but not transient damage, activate p53 and induces p21 expression and cell cycle arrest (*34*). To date, such studies used *in vitro* time course analysis and/or mathematical modelling to analyse the sustained dynamics of transcription. The Tocky system will benefit studies in cell signalling by providing a means to directly identify and isolate cells receiving persistent signals.

Thus, the Tocky system can be used to dissect the temporal dynamics of cellular differentiation and activation of individual cells by analysing their time- and frequency domains, providing a measurement of 'Tocky-time' (**Supplementary Fig. 4)**, which is in a non-linear relationship with 'real' time, and represents a relative chronological readout for events occurring following a cellular differentiation cue. The advantages of the Tocky system over other gene reporters are summarised in **Supplementary Table 1**. In the future Tocky mice for key transcription factors and genes will be promising tools to reveal the *in vivo* dynamics of gene transcription and cellular differentiation (e.g. Bcl6, for T-follicular helper cells; Rag2, for TCR recombination; and, Oct4, for stem cell-ness); these cannot be investigated otherwise.

In summary, we have established the Tocky system as a pioneering tool to investigate cellular differentiation and gene dynamics *in vivo* in immunology, developmental biology and stem cell biology.

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Materials and Methods

Study Design

Two independent lines were established for both of the *Nr4a3*-Tocky and *Foxp3*-Tocky transgenic reporter strains. Both lines exhibited highly similar phenotypes and frequencies of Timer$^+$ cells.

Transgenesis and mice

The BAC clones RP23-122N18 and RP23-330D17 were obtained from the BACPAC Resources Center at Children's Hospital Oakland Research Institute (CHORI), and were used for generating *Nr4a3*-Tocky and *Foxp3*-Tocky, respectively. BAC DNA was modified by the BAC recombineering approach using the *SW106* strain bacteria (*35*).

We used a Timer knock-in knock-out approach for BAC transgenic reporter constructs. Precisely, for *Nr4a3*-Tocky, the first coding exon of the *Nr4a3* gene in RP23-122N18 was targeted and replaced with the transgene cassette containing the *Timer* gene (*20*), a poly-A tail and a floxed neomycin resistance gene (*neo*, Supplementary Fig. 2). For *Foxp3*-Tocky, the first coding exon of the *Foxp3* gene in RP23-330D17 was targeted and replaced by the same transgene cassette. Subsequently, *neo* was excluded by arabinose-inducible Cre expression in SW106 (*35*). BAC DNA was purified by the NucleoBond Xtra Midi kit (Macherey-Nagel), and microinjected into the pronucleus of one-cell embryos from C57BL/6 mice under the approval by the Gene Recombination Experiments Safety Committee of Kyoto University. Founders were screened by genomic PCR, and transgene-positive founders were

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mated with wild-type C57BL/6 mice. F1 mice were screened by flow cytometry for Timer expression, and subsequently bred to homozygosity with *Foxp3[IRES-GFP]* mice (B6.Cg-*Foxp3[tm1Mal]/J*, Jackson Laboratories, #018628), to generate *Nr4a3*-Tocky:*Foxp3[IRES-GFP]* double transgenic mice. *OTII- Nr4a3*-Tocky: *Foxp3[IRES-GFP]* mice were similarly generated by crossing *Nr4a3*-Tocky, B6.Cg-*Tg(TcraTcrb)425Cbn*/J, and B6.Cg-*Foxp3[tm1Mal]/J*. All animal experiments were performed in accordance with local Animal Welfare and Ethical Review Body at Imperial College London (Imperial) and University College London (UCL), and all gene recombination experiments were performed under the risk assessment that was approved by the review board at Imperial and UCL. The

In vitro Treg polarisation and mature Treg culture

CD4$^+$CD44^{lo}Foxp3$^-$ naïve T-cells from *Foxp3*-Tocky mice were isolated by cell-sorting and 1×10^5 cells cultured on anti-CD3 (clone 1452C11, 2 μg/ml) and anti-CD28 (clone 37.51,10 μg/ml; both eBioscience)-coated 96 well plates (Corning) in the presence of 100 U/ml rhIL-2 (Roche) and 2 ng/ml rhTGFβ (R&D) for 0 - 48 h in a final volume of 200 μL RPMI1640 (Sigma) containing 10% FCS and penicillin/streptomycin (Life Technologies).

Mature Foxp3$^+$ Treg from *Foxp3*-Tocky mice were isolated by cell-sorting and 1x10^5 cells cultured on anti-CD3 (clone 1452C11, 2 μg/ml) and anti-CD28 (clone 37.51,10μg/ml; both eBioscience)-coated 96 well plates (Corning) in the presence of 100 U/ml rhIL-2 (Roche) for 0 - 48 h in a final volume of 200

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μL RPMI1640 (Sigma) containing 10% FCS and penicillin/streptomycin (Life Technologies).

DNA methylation analysis

DNA was extracted from sorted samples using Qiagen DNeasy kit according to the manufacturer's instructions. 100ng of DNA was bisulfite treated using the Epitect Bilsulfite Kit (Qiagen), and used as a template for amplification of the TSDR. TSDR was amplified using the following primers (Foxp3 TSDR For: ATTTGAATTGGATATGGTTTGT; Foxp3 TSDR Rev: AACCTTAAACCCCTCTAACATC (*29*)) and cycling conditions: 94 °C 1min followed by 40 cycles of 94 °C 15 s / 54 °C 30 s / 68 °C 30 s and a final extension phase of 68 °C for 15minutes. PCR amplicons were purified and then underwent Sanger Sequencing using the reverse primer by Source BioScience. Sequence traces and CpG methylation rates were analysed and determined by ESME software (Epigenomics) (*36*).

***In vitro* T cell activation of *Nr4a3^Timer^* mice**

CD4$^+$ T cells from OTII *Nr4a3*-Tocky:*Foxp3^GFPKI^* mice were isolated by immunomagnetic cell separation, (StemCell Technologies) and 2×10^5 cells cultured with 3×10^5 (2:3) CD90.2-depleted splenocytes in the presence of 1, 10, 100 or 1000 nM Ova$_{(323-339)}$ peptide (Sigma) on 96 well plates (Corning) in a final volume of 200 μL RPMI1640 (Sigma) containing 10% FCS and penicillin/streptomycin (Life Technologies) and 55 μM beta mercaptoethanol (Gibco) for the stated time periods. At 24 h, some cells were washed three

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times and recultured on a fresh plate in the presence of 40 μg/ml anti-MHC Class II (clone M5/114, BioXCell) for a further 8-24 h before analysis.

Flow cytometric analysis and cell sorting

Following spleen or thymus removal, organs were forced through a 70 μm cell strainer to generate a single cell suspension. For splenocyte preparations a RBC-lysis stage was employed. Staining was performed on a V-bottom 96-well plate, or in 15 ml falcon tubes for cell sorting. Analysis was performed on a BD Fortessa III instrument. The blue form of the Timer protein was detected in the blue (450/40 nm) channel excited off the 405 nm laser. The red form of Timer protein was detected in the mCherry (610/20) channel excited off the 561nm laser. For all experiments a fixable eFluor 780-fluorescent viability dye was used (eBioscience). The following directly conjugated antibodies were used in these experiments: CD4 APC (clone RM4-5, eBioscience), CD4 Alexa-fluor 700, CD4 BUV395 (clone GK1.5 BD Biosciences), (clone RM4-5, Biolegend), CD8 PE-Cy7 (clone 53-6.7, Biolegend), CD8 BUV737 (clone 53-6.7, BD Biosciences) TCRβ FITC & Alexafluor 700 (clone H57-597, Biolegend), TCRβ BUV737 (clone H57-597, BD Biosciences), CD25 PerCPcy5.5 (PC61.5, eBioscience) or PE-Cy7 (PC61.5, Tombo Bioscience), CD44 APC (clone IM7, eBioscience) or Alexafluor 700 (clone IM7, Biolegend), CD69 APC (H1.2F3, eBioscience) and Foxp3 APC (clone FJK-16s, eBioscience). For *Foxp3*-Tocky validation, RBC-lysed splenocytes were stained with eFluor 780 viability dye, before staining for APC-conjugated CD4 and FITC-conjugated TCRβ. Live CD4$^+$TCRβ$^+$ were gated and then sorted

into four fractions: Timer(Blue$^+$Red$^-$), Timer (Blue$^+$Red$^+$), Timer(Blue$^-$Red$^+$), Timer(Blue$^-$Red$^-$). Cells were then fixed and labelled with APC-conjugated Foxp3 using the eBioscience Foxp3 fixation and permeabilisation kit according to the manufacturer's instructions.

Canonical Correspondence Analysis

The gene signature and sample scores in Supplementary Fig. 1 were calculated by a cross-dataset analysis using CCA (*22*). Briefly, the expression data of GSE15907 (*37*) was regressed onto the log2 fold change of activated CD4$^+$ T cells (2 h after activation) and naïve T cells from GSE48210 (*38*) as the explanatory variable, and Correspondence Analysis was performed for the regressed data and the correlation analysis was done between the new axis and the explanatory variable. CCA was performed by the CRAN package *vegan* as previously described (*22*). The analysis was undertaken using only transcription factor genes, which were selected by the Gene Ontology database by including the genes that are tagged with GO:0003677 (DNA binding) and GO:0005634 (nucleus) and not with GO:0016020 (membrane) using the Bioconductor package *GO.db*.

Timer data analysis

Sample data including a negative control were batch gated for T cell populations and exported by FlowJo (FlowJo LLC, OR) into csv files, including all compensated fluorescence data in the fcs file. The code developed in this study imports csv files into R. Data were preprocessed and scaled, the negative cloud was collapsed (i.e. set a threshold for each fluorescence), and

Timer^{+} cells were automatically identified. Trigonometric data transformation was performed to produce Timer–Angle and –Intensity data for individual cells in each sample. Basic procedures for flow cytometric data analysis have been previously described elsewhere (*7*).

Statistical analysis and data visualisation

Statistical analysis was performed on R or Prism 6 (Graphpad) software. Percentage data for Timer[+] and Timer locus analysis was analysed by Mann-Whitney U test or Kruskal-Wallis test with Dunn's multiple comparisons using the CRAN package *PMCMR*. Samples with fewer than 18 Timer^{+} cells were not included in the analysis. Student's t-test was used for comparison of two means. For comparison of more than two means, a one-way ANOVA with Tukey's post-hoc test was applied using the CRAN package *Stats*. Scatterplots were produced by the CRAN packages *ggplot2* and *graphics*. Dose response data in Fig. 2g were fitted to a dose-response curve using the three-parameter log-logistic function of the CRAN package *drc* as previously described (*39*). All computations were performed on Mac (version 10.11.6). Adobe Illustrator (CS5) was used for compiling figures and designing schematic figures. Variance is reported as SD or SEM unless otherwise stated. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Data and code availability

All R codes are available upon request. Data will be made available upon reasonable requests to the corresponding author.

Supplementary Materials

4 supplementary figures and 1 supplementary table accompany this manuscript

Author Contributions

M.O. conceived the Tocky strategy. M.O. designed and generated transgenic constructs. S.K., H.M., and M.O. established the transgenic founders. S.K., H.M., P.P.M, and D.B screened mouse lines. D.B., T.C, and M.O conceived and designed immunological experiments. D.B., P.P.M, and A.P. performed animal experiments. D.B., P.P.M., E.M., and M.L. performed *in vitro* experiments. M.O. conceived Timer data analysis, wrote computational codes and performed bioinformatics analysis and data visualisation. D.B., T.C., and M.O. wrote the manuscript.

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Figures

Figure 1: Flow cytometric analysis of Timer chromophore
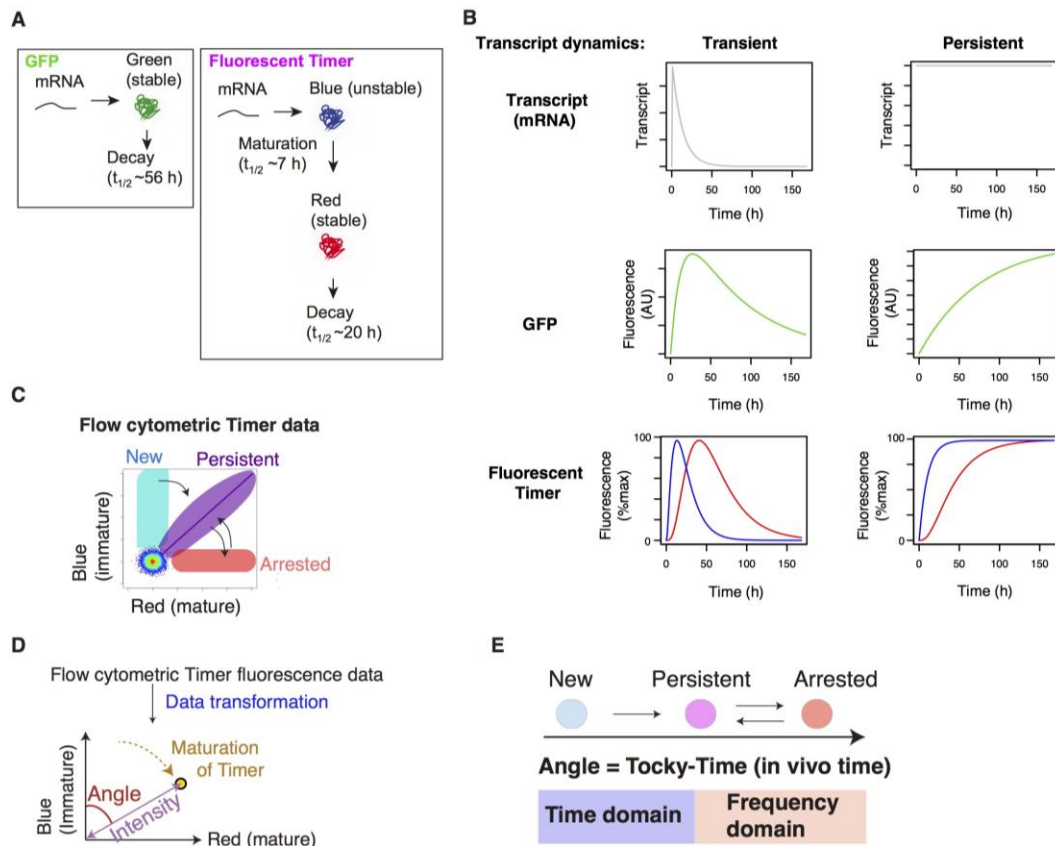
(**A**) Production and decay of GFP and Fluorescent Timer protein. (**B**) In silico analysis of GFP and Timer fluorescence by different transcriptional dynamics. Time course analysis of GFP or Blue and Red forms of Timer protein, simulated flow cytometric data, and Timer-Angle, given the constant influx of cells with the indicated transcriptional dynamics. Data from spike-like (transient) and constant transcriptional dynamics are shown. (**C**) Timer locus approach to translate flow cytometric Timer fluorescence data into transcriptional dynamics. (**D**) Schematic representation of Trigonometric data transformation of flow cytometric Timer data. Flow cytometric Blue and Red Timer fluorescence data were preprocessed and normalised, and subsequently transformed by a trigonometric function. (**E**) Model for analysing the time domain and frequency domain using Tocky-time analysis.

Figure 2: Establishment of the TCR signal-responsive reporter *Nr4a3-Tocky*
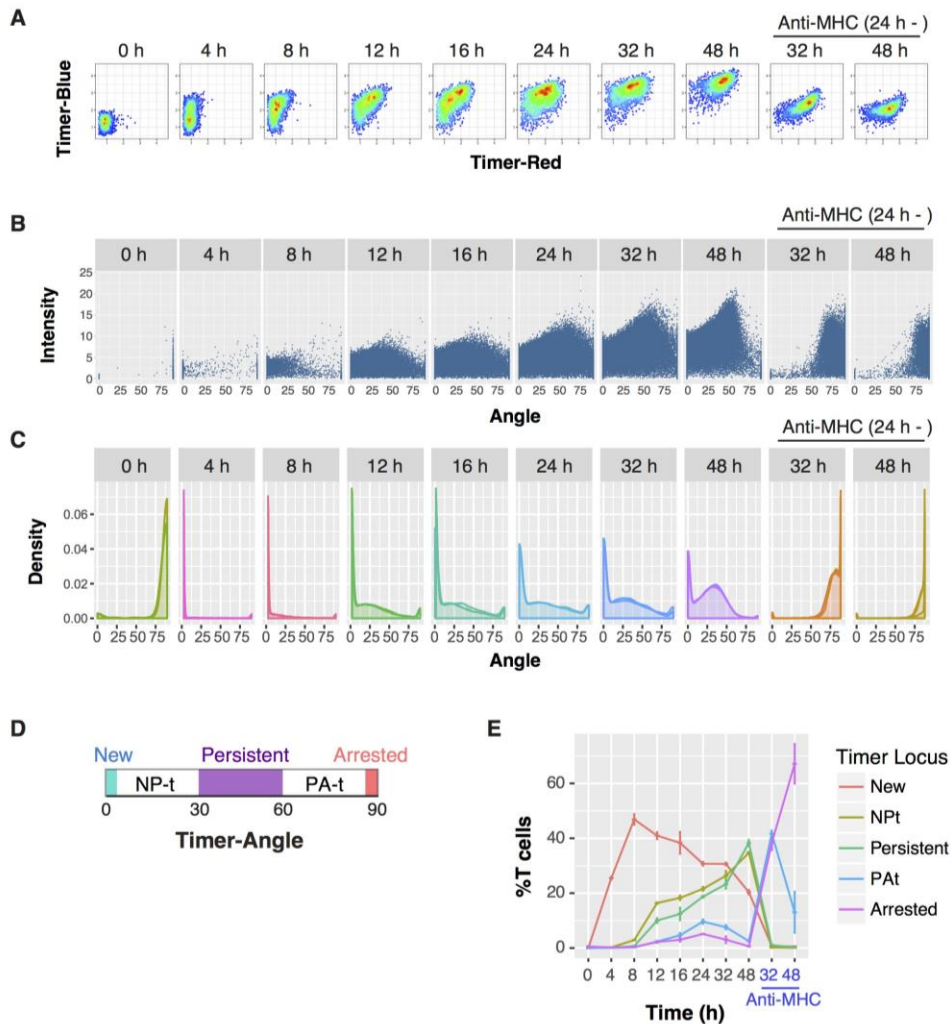
(**A**) Flow cytometric analysis of Blue and Red Timer raw fluorescence in antigen-stimulated OTII *Nr4a3*-Tocky T-cells. T cells were stimulated for the indicated time points with 1 µM Ova peptide. In some cultures, anti-MHC II antibodies were added at 24 h in order to terminate TCR signalling. (**B**) Trigonometric-transformed data of **a**. Individual cells are plotted against Timer-Angle and –Intensity. (**C**) Density plot of Timer-Angle from the transformed data. (**D**) The designation of the 5 Timer loci by Timer-Angle θ as follows: New ($\theta = 0°$), NP-t ($0° < \theta < 30°$), Persistent ($30° \leq \theta < 60°$), PA-t ($60° \leq \theta < 90°$) and Arrested ($\theta = 90°$). (**E**) Timer Locus analysis to show the frequency of cells within the 5 Timer loci defined in (C). Bars represent mean ± SD. N = 3 culture triplicates. Data are representative of at least two independent experiments.

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## Figure 3: Nr4a3 Timer intensity reflects strength of TCR signalling
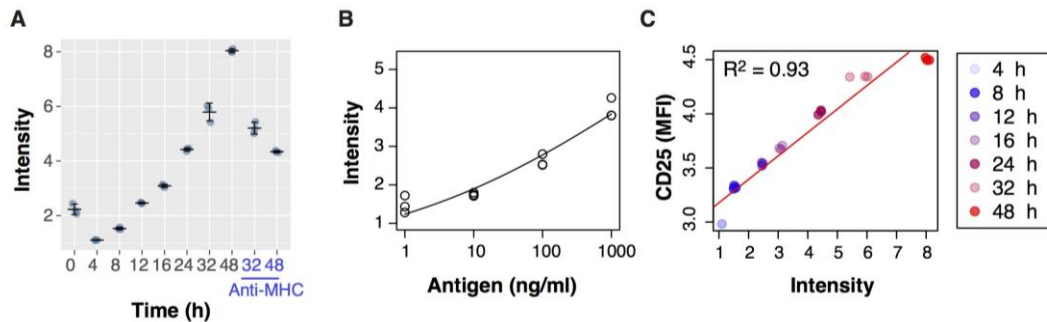
(**A**) Summary of Timer-Intensity in the cultures from **Figure 2A** over time. (**B**) Dose response curve of Timer-Intensity on stimulation with titrated doses of Ova peptide (Antigen). OTII *Nr4a3*-Tocky T-cells were stimulated for 22 h in the presence of 1, 10, 100 or 1000 nM Ova peptide and APCs. Data were fitted to a dose-response curve with a statistical significance by a lack-of-fit test (p = 0.014). (**C**) Scatter plot of Timer-Intensity vs cell surface CD25 expression. Linear regression analysis showed a strong correlation (R^2 = 0.93). See legend for sample identities. Bars represent mean ± SD. N = 3 culture triplicates. Data are representative of at least two independent experiments.

## Figure 4: *Nr4a3-Tocky* mice identify thymic Treg precursors
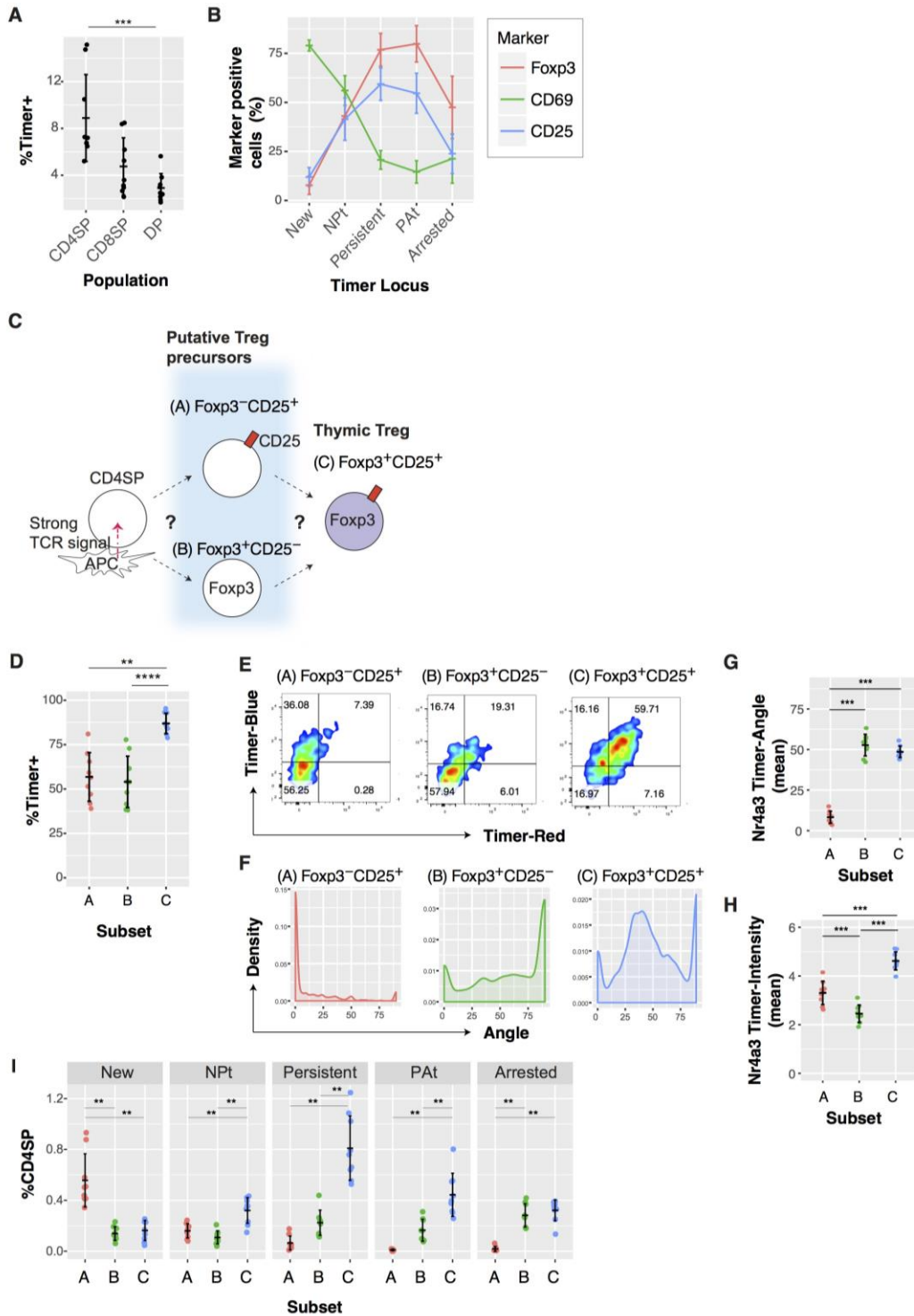
(**A**) Percentages of Timer^{+} cells in the indicated thymic T cell populations from *Nr4a3*-Tocky mice. (**B**) Mean percentages of CD4SP cells expressing CD69 (green), CD25 (blue) or Foxp3 (red) from the 5 Timer loci. (**C**) Current working model for thymic Treg differentiation. (**D**) Percentages of Timer^{+} cells in the indicated CD4SP subpopulations. (**E**) Timer Blue and Red fluorescence from

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D 7 CD4SP Thymic Treg subsets. (**F**) Density plot of Timer-Angle from the transformed data. Data from D 7, 9 and 13 were collected in three independent experiments and combined and thymic Treg subsets were analysed for the Timer Angle Mean, (**H**) or Intensity, (**H**) (A = CD25$^+$Foxp3$^-$, B = CD25$^-$Foxp3$^+$, C = CD25$^+$Foxp3$^+$). (**I**) Frequency within the CD4SP thymic population of each Timer locus in the three Treg subsets. Bars represent mean ± SD.

**Figure 5. New model for *in vivo* dynamics of thymic Treg differentiation.**
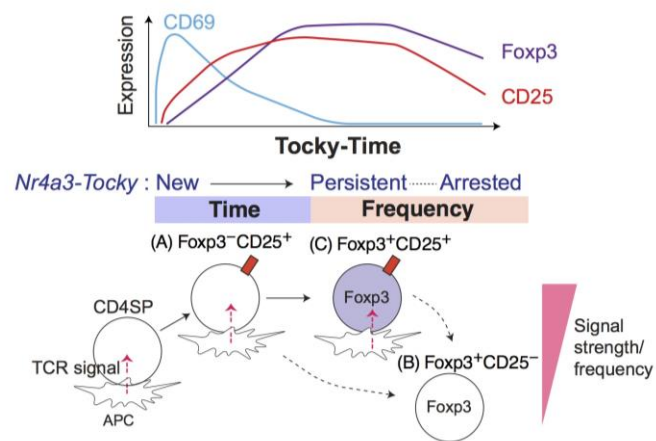
Figure 6. *Foxp3-Tocky* identifies newly generated Treg
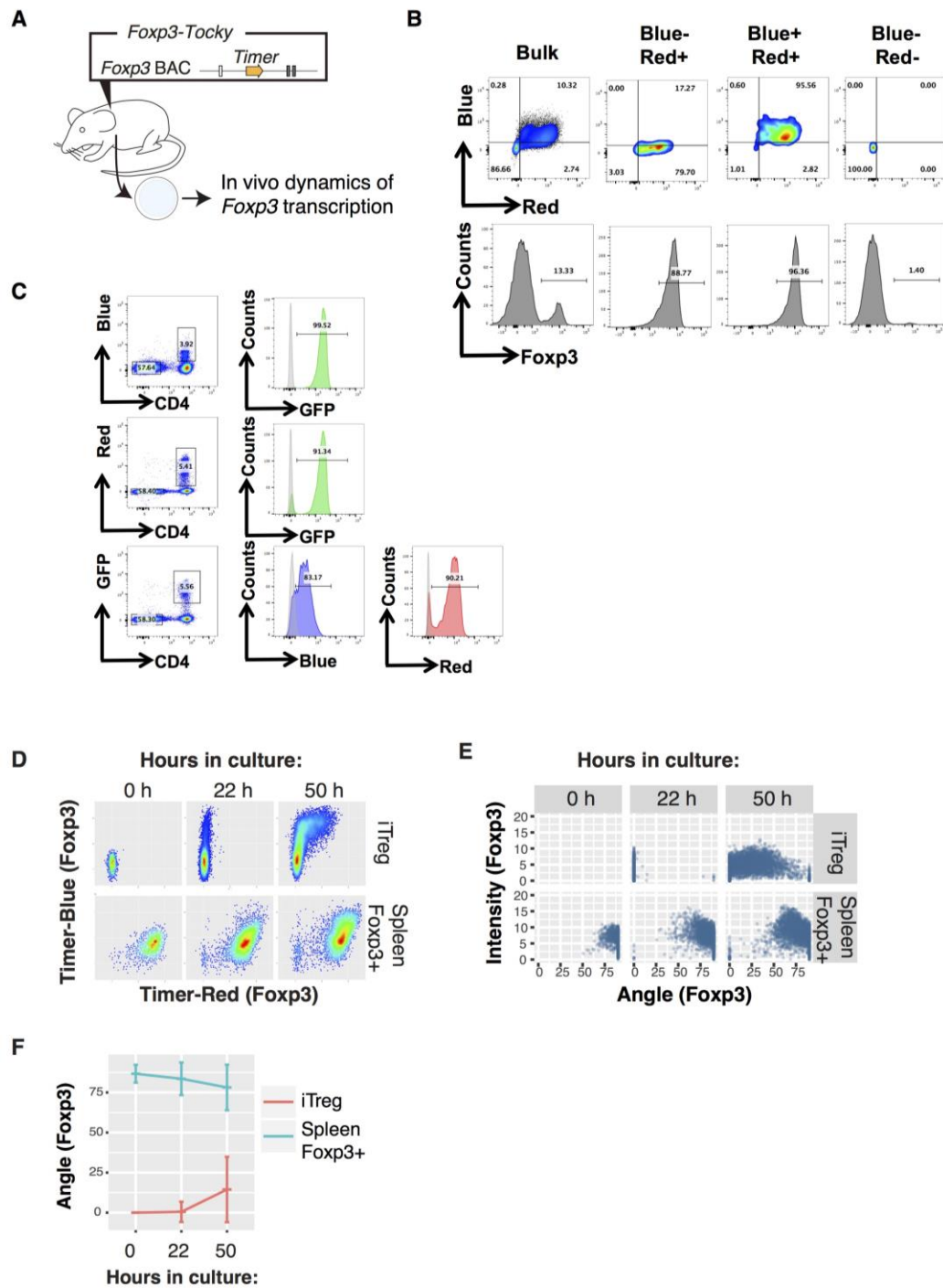
(**A**) Use of *Foxp3*-Tocky mice to investigate *in vivo* dynamics of *Foxp3* transcription. (**B**) Splenic T cells were sored into Blue-Red+, Blue+Red+, and Blue-Red- and analysed for intracellular Foxp3 proteins. (**C**) *Foxp3*-Tocky mice were crossed with Foxp3-IRES-GFP, and analysed for the co-expression of GFP and Timer. (**D**) Timer[−] naïve T-cells and splenic Timer[+] Treg from *Foxp3*-Tocky mice were isolated and stimulated by anti-CD3 and -

CD28 for 0, 22 or 50 h in the presence of IL-2 (and TGF-β for iTreg). Flow cytometry plots display raw Blue vs Red expression during the cultures. (**E**) Timer Angle vs. Intensity, or (**F**) Timer Angle vs. Time in the data from D.

Figure 7. *Foxp3-Tocky* reveals *in vivo* dynamics of demethylation of the *Foxp3* gene.
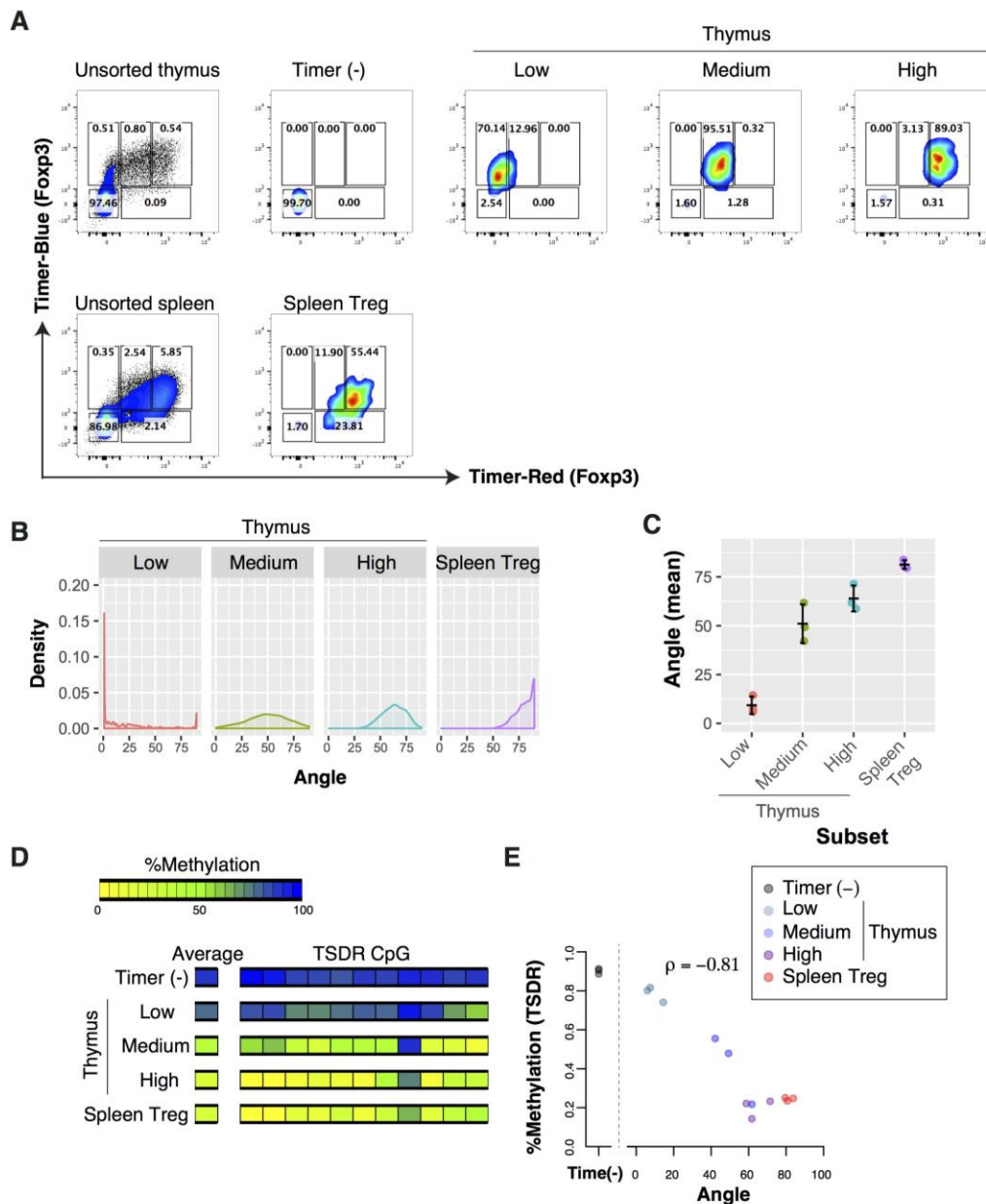
Thymic cells, or splenic Treg, were sorted according to increasing Tocky-time (i.e. Timer-Angle) in Blue+ cells. Displayed are (**A**) flow cytometry plots of Blue vs. Red fluorescence, (**B**) Timer Angle density plots, or (**C**) Mean Timer Angle in the sorted T-cell populations, n=3. DNA was extracted for methylation analysis, and thymic samples were compared to Timer (-) and splenic Treg samples (**D**) Heatmap showing average TSDR methylation rates of flow sorted T-cell subsets. (**E**) Average TSDR methylation rates plotted

against mean Timer Angle in sorted T-cell subsets sorted, n = 3 mice for each subset. Spearman's correlation coefficient is shown. Data were combined from two experiments.

Supplementary Material

Title: An engineered cellular Timer for analysing the time domain of cellular differentiation in vivo

Authors: David Bending[1], Paz Prieto Martín[1], Alina Paduraru[1], Erik Marzaganov[1], Marie Laviron[1], Satsuki Kitano[2], Hitoshi Miyachi[2], Tessa Crompton[3], and Masahiro Ono[1*]

This file contains 4 supplementary figures and 1 supplementary table
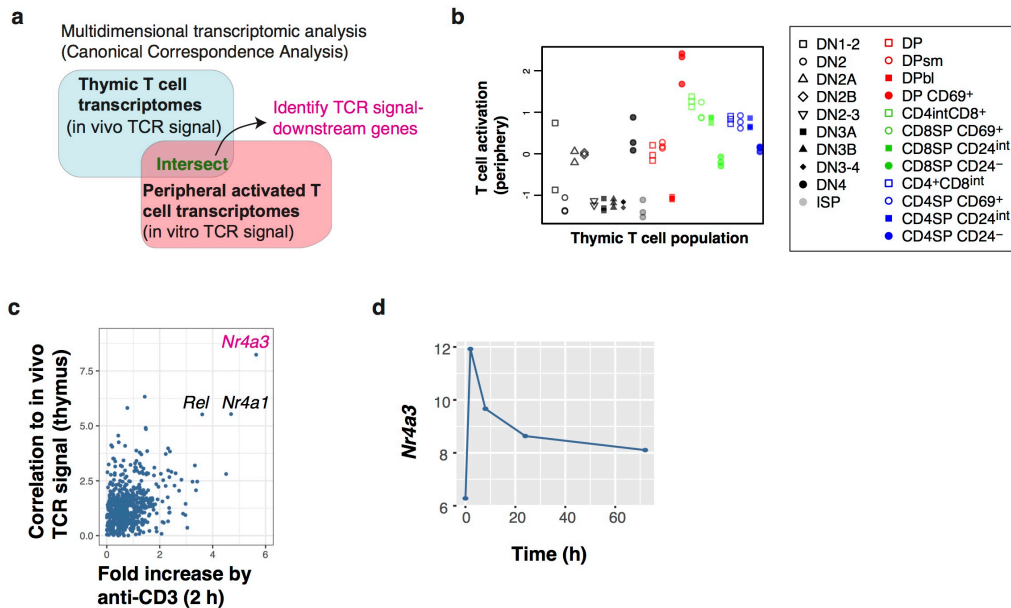
Supplementary Fig. 1: CCA identifies *Nr4a3* as a downstream target of TCR signalling

 (**a**) Schematic showing rationale for identifying genes that are downstream of TCR signalling. Cross-dataset analysis of the transcriptomes of thymic T cell populations (in vivo signal, GSE15907) and those of TCR-signalled peripheral CD4+ T cells data (in vitro signal, GSE48210). CCA was performed to identify the candidate genes used by both thymic and peripheral T cells. (**b**) Thymic T cell population data was analysed by CCA using activated and resting T cells from GSE48210 as the explanatory variable. The output of CCA is composed of (1) cell sample score, (2) gene score, and (3) biplot value for explanatory variable, and thus allowing the cross-level analysis of cells, genes, and biological process. Thus, using the axis that is correlated with T cell activation, T cell activation scores of thymic T cell populations were specified and visualised. (**c**) Scatter plot showing the fold change increase by anti-CD3 stimulation and the correlation to the thymic T cell populations that received TCR signals. Cross-dataset analysis was performed by CCA of the transcriptome data from thymic T cell populations (*in vivo* signal, GSE15907) and peripheral CD4+ T cells that received *in vitro* anti-CD3 stimulation (*in vitro* signal, GSE48210), in order to identify candidate genes upregulated by TCR signals in both thymic and peripheral T cells. (**d**) Time course analysis of *Nr4a3* transcripts upon anti-CD3 stimulation (from GSE48210).
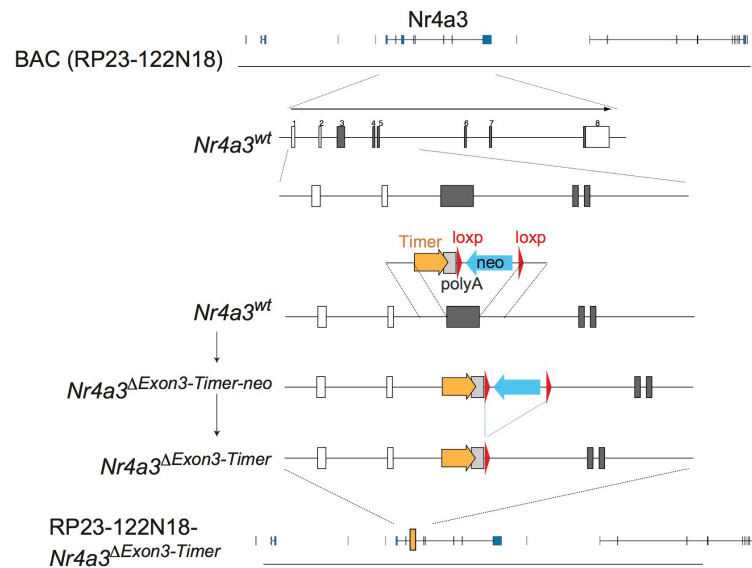
**Supplementary Fig. 2: DNA construct design for *Nr4a3*-Tocky**

The BAC fragment RP23-122N18 was modified using the BAC recombineering approach. Briefly, the first coding exon of the *Nr4a3* gene was replaced by the Timer gene using a targeting vector containing 5' and 3' homology arms, the *Timer* gene, and the neomycin resistant gene (neo). The neomycin gene was deleted by inducible Cre expression in BAC-carrying bacteria, and the modified BAC (RP23-122N18-*Nr4a3*$^{\Delta Exon3\text{-}Timer}$) was amplified and purified for pronuclear injection.
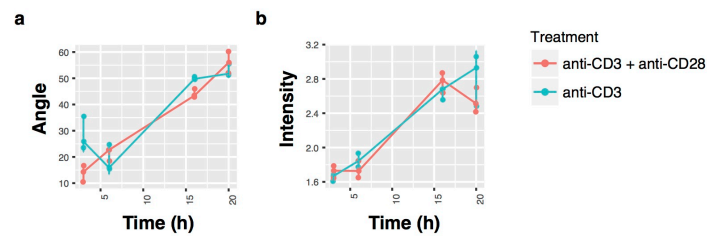
Supplementary Fig. 3: Anti CD28 stimulation does not alter *Nr4a3*-Tocky dynamics

Nr4a3-Tocky T-cells were stimulated on either anti-CD3 or anti-CD3 and anti-CD28-coated plates for the indicated time points and Timer fluorescence was analysed and transformed into (**a**) Timer-Angle and (**b**) Timer–Intensity data. Bars represent mean ± SD.

Tocky (Timer of Cell Kinetics and Activity) technology
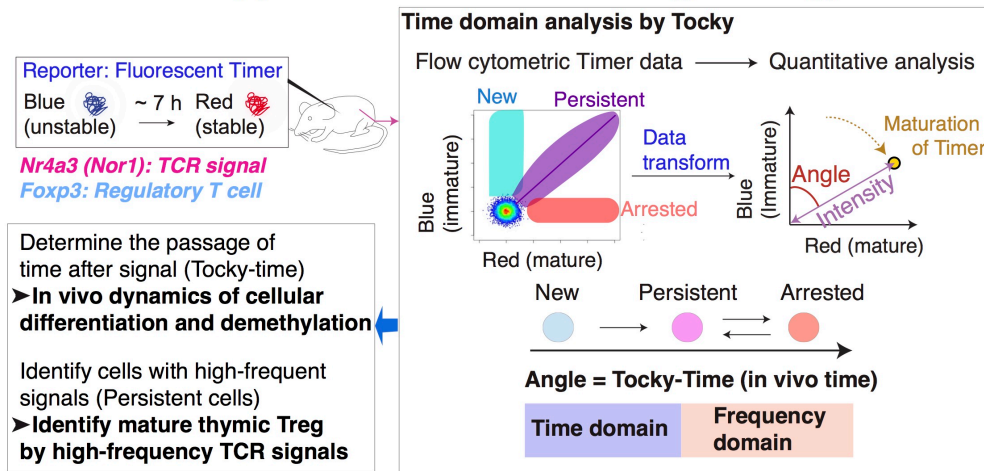
Supplementary Fig. 4: Summary of Tocky technology

Tocky system harnesses the power of Fluorescent Timer protein in combination with a computational algorithm to define the Time and frequency domains of cellular differentiation (Tocky-Time) using flow cytometry. This permits the determination of the relative passage of time following signalling cues, as well as identifying cells that are receiving continuous or very frequent differentiation signals.

Supplementary Table 1. Differences between Tocky, Fate-mapper, and GFP/FP reporters.

	GFP reporter (and other conventional FP reporters)	Fate-mapping reporter (e.g. Cre: Rosa-FP double transgenic)	Tocky
Time domain analysis	No. Do not distinguish new expressors from pre-existing expressors	NA	Reveal the time frame of early differentiation following key signalling
Frequency domain analysis	No, due to long half-life of reporter proteins	No information	Identification of cells with different frequencies of transcriptional activity and finding unique functional states (e.g. antigen-specific T cells)