

Life Sciences Reporting Summary

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► Experimental design

1. Sample size

Describe how sample size was determined.

Necessary sample sizes (number of analyzed single molecule signals) for TOCCSL experiments were taken from Brameshuber, M. & Schutz, G.J., *Methods Enzymol* 505, 159-186 (2012). The number of analyzed measurements was adjusted to give rise to a standard error of the mean, which supported the drawn conclusions.

2. Data exclusions

Describe any data exclusions.

No data was excluded from the analysis.

3. Replication

Describe whether the experimental findings were reliably reproduced.

All attempts at replication were successful.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.

Allocating samples into experimental groups was not applicable since primary T cells from one mouse strain were used. No specific method for randomization was used.

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

Allocating samples into experimental groups was not applicable and hence blinding was not relevant to this study.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a Confirmed

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- A statement indicating how many times each experiment was replicated
- The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
- A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- Clearly defined error bars

See the web collection on [statistics for biologists](#) for further resources and guidance.

► Software

Policy information about [availability of computer code](#)

7. Software

Describe the software used to analyze the data in this study.

Custom code implemented in Matlab was used to analyze the 2-color TOCCSL and PA/FCS data. ImageJ was used to analyze FRET data. Matlab code for 1-color TOCCSL experiments can be downloaded from GitHub.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* [guidance for providing algorithms and software for publication](#) provides further information on this topic.

► Materials and reagents

Policy information about [availability of materials](#)

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All unique materials used (single chain fragments and fragmented antibodies) are readily available from the authors upon request.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

No commercial antibodies were used.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

No eukaryotic cell lines were used.

b. Describe the method of cell line authentication used.

No eukaryotic cell lines were used.

c. Report whether the cell lines were tested for mycoplasma contamination.

No eukaryotic cell lines were used.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by [ICLAC](#), provide a scientific rationale for their use.

No commonly misidentified cell lines were used.

► Animals and human research participants

Policy information about [studies involving animals](#); when reporting animal research, follow the [ARRIVE guidelines](#)

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Primary T cells were isolated from 5c.c7 TCR transgenic B10.A male and female mice and OT-1 TCR transgenic C57BL6/6 mice (male and female) at age 8-12 weeks.

Policy information about [studies involving human research participants](#)

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

The study did not involve human research participants.