

Submission ID #: 71979

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Project Page Link: <https://review.jove.com/account/file-uploader?src=21953643>

Title: TCR Signal Strength and Duration in Primary Mouse CD8⁺ T Cell Activation and Differentiation: A Standardized In Vitro Approach

Authors and Affiliations:

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NOTE: The affiliations are edited during post-shoot according to text review page. But the article is not published yet, so the author names/affiliations might change

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**

If **Yes**, we will need you to record using screen recording software.

We recommend using the screen capture program [OBS](#). JoVE's tutorial for using OBS Studio is provided at this link: <https://review.jove.com/v/5848/screen-capture-instructions-for-authors?status=a7854k>

As these files are necessary for finalizing your script, please upload all screen-captured video files to your project page as soon as possible.

3. Filming location: Will the filming need to take place in multiple locations? **No**

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 18

Number of Shots: 53

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

Authors: The statements are edited for length and clarity. Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**.

INTRODUCTION:

~~REQUIRED:~~ Summarize your research focus and the main questions it aims to answer.

- 1.1. **Jiayi Ouyang:** Our research focuses on understanding how graded TCR stimulation regulates CD8⁺ T cell activation, proliferation, and functional differentiation in vitro.

- 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Jiayi Ouyang:** Existing methods typically use a single saturating anti-CD3 concentration, whereas our protocol uses graded concentrations to systematically study the effects of TCR signal strength.

- 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.3. **Jiayi Ouyang:** The most important considerations are ensuring a precise anti-CD3 gradient coating, high cell purity, and a consistent cell seeding density.

- 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.4. **Jiayi Ouyang:** This protocol also provides a platform for optimizing T cell conditioning for adoptive immunotherapy and in vitro priming assays.

- 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Animal Care and Use Committee of Third Military Medical University.

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

***Author:** For better continuity and flow of the protocol, selected unhighlighted portions (Narration 3.1 and 4.2) have also been included alongside the highlighted steps.*

2. CellTrace Violet labeling for Proliferation Tracking

Demonstrator: Jiayi Ouyang

- 2.1. To begin with, obtain the anti-CD3 (*C-D-three*) vial and place it on ice [1]. Prepare the anti-CD3 dilutions in sterile PBS [2-TXT] and add 50 microliters of each anti-CD3 solution to the wells of a flat-bottom 96-well plate [3].
 - 2.1.1. WIDE: Talent bringing the anti-CD3 and placing them on ice
 - 2.1.2. Talent preparing anti-CD3 dilutions using sterile PBS **TXT: Anti-CD3 concentrations- 0.1, 0.5, 1, 5, and 10 µg/mL**
 - 2.1.3. CU: Talent adding 50 µL of anti-CD3 solution to the wells of 96-well plate
- 2.2. Seal the plate with sealing film [1] and incubate it overnight at 4 degrees Celsius [2]. Prepare a fresh 10 micromolar CellTrace Violet or CTV, (*CTV*) working solution by vortexing the 5 millimolar CTV stock solution [3] and diluting it with sterile PBS [4].
 - 2.2.1. Talent sealing the plate with sealing film
 - 2.2.2. Talent placing the plate at 4°C
 - 2.2.3. Talent vortexing 5mM CTV stock solution
 - 2.2.4. Talent diluting the stock CTV solution with sterile PBS
- 2.3. Resuspend naive CD8⁺ (*C-D-eight positive*) T cells isolated from mouse spleens in pre-chilled PBS [1-TXT]. Rapidly add an equal volume of the 10 micromolar CTV working solution to the cell suspension [2], then mix thoroughly by pipetting to obtain a final CTV concentration of 5 micromolar [3].
 - 2.3.1. Talent resuspending the cells in the PBS **TXT: Cell Density: 8×10^7 cells/mL**
 - 2.3.2. CU: Talent adding an equal volume 10 µM of CTV solutions to the cell suspension
 - 2.3.3. Talent mixing the solution by pipetting up and down

- 2.4. Incubate the cells at room temperature in the dark for 20 minutes [1], gently inverting the tubes every 5 minutes to ensure thorough mixing [2]. Add 5 volumes of R10 (*R-ten*) culture medium [3] and incubate the cells on ice for 5 minutes to remove any free dye [4].
- 2.4.1. Talent placing the cells in the dark
 - 2.4.2. CU: Talent gently inverting the tube every 5 minutes
 - 2.4.3. Talent adding 5 volumes of culture medium
 - 2.4.4. Talent placing the cells on ice for 5 minutes
- 2.5. Centrifuge the cells at 500 *g* for 5 minutes [1], then resuspend the pellet in freshly pre-warmed complete R10 medium supplemented with anti-CD28 (*C-D-twenty-eight*) and interleukin-2 [2-TXT]. Remove the overnight pre-coated 96-well plate from 4 °C storage [3]. Aspirate the coating solution [4] and wash each well twice with 200 microliters of sterile PBS before adding the cells [5].
- 2.5.1. Talent placing the cells in the centrifuge and closing the lid
 - 2.5.2. Talent resuspending the pellet in R10 medium **TXT: Anti-CD28: 1 µg/mL; IL-2: 10 ng/mL.**
 - 2.5.3. Talent removing the plates from 4°C storage
 - 2.5.4. Talent aspirating the coating solution
 - 2.5.5. Talent washing the wells with 200 µL of the sterile PBS
- 2.6. Add 250 microliters of the cell suspension, containing 6×10^5 cells, to each well of the pre-coated 96-well plate [1]. Incubate the cells at 37 degrees Celsius with 5% Carbon dioxide for up to 48 hours [2].
- 2.6.1. Talent adding 250 µL of the cell suspension to the wells of the pre-coated 96-well plate
 - 2.6.2. Talent placing the plate at 37 °C with 5% Carbon dioxide

3. Intracellular Cytokine Blocking

- 3.1. Resuspend the remaining naïve CD8⁺ T cells in R10 medium supplemented with 1 microgram per milliliter anti-CD28 and 10 nanograms per milliliter interleukin-2 [1-TXT]. Add 250 microliters of the cell suspension, containing 6×10^5 cells, to each well of the pre-coated 96-well plate [2]. Culture the cells at 37 degrees Celsius with 5% carbon dioxide for up to 48 hours [3].

- 3.1.1. Talent resuspending the cells in the R10 medium **TXT: Cell Density: 2.4×10^6 cells/mL**
- 3.1.2. Talent adding 250 μ L of the cell suspension to the wells of the pre-coated 96-well plate
- 3.1.3. Talent placing the plate at 37°C
- 3.2. Five hours before harvest, add 100 microliters of fresh R10 medium supplemented with PMA, ionomycin, and protein transport inhibitors to each well **[1-TXT] [2]**. Gently mix the cells **[3]** and return the plate to 37 degrees Celsius to block cytokine secretion and allow intracellular cytokine accumulation **[4]**. At 24 and 48 hours, transfer the cells to a 96-well round-bottom plate **[5]**.
 - 3.2.1. Talent adding the 100 μ L of fresh medium **TXT: PMA- Phorbol 12-myristate 13-acetate**
 - 3.2.2. **TEXT ON A PLAIN BACKGROUND:**
PMA- 50 ng/mL
Ionomycin- 1 μ g/mL
Protein transport inhibitor Monensin-1:1000
Brefeldin A-based protein transport inhibitor- 1:1000 *Video editor: This is ratio!*
 - 3.2.3. Talent gently mixing the cells
 - 3.2.4. Talent placing the plate at 37°C
 - 3.2.5. Talent transferring the cells to a 96-well round-bottom plate
- 3.3. Centrifuge the cells at 500 *g* for 1 minute **[1]** and resuspend the pellet in 200 microliters of flow cytometry staining buffer, or FACS (*Facs*), buffer **[2]**. Centrifuge again at 500 *g* for 1 minute **[3]**, aspirate the supernatant **[4]**, and wash the cells twice with 200 microliters of FACS buffer **[5]**.
 - 3.3.1. Talent placing the plate in the centrifuge and closing the lid
 - 3.3.2. Talent resuspending the pellet in 200 μ L of FACS after discarding the supernatant
 - 3.3.3. Talent placing the plate in the centrifuge and closing the lid
 - 3.3.4. Talent aspirating the supernatant after centrifugation.
 - 3.3.5. Talent washing the cells with 200 μ L of staining buffer
- 3.4. For surface marker staining, add 50 microliters of an antibody cocktail containing anti-CD44 (*C-D-forty-four*), anti-CD8 (*C-D-eight*), and a fixable viability dye to the cells **[1-**

TXT]. Incubate for 30 minutes on ice in the dark **[2]**. Wash the cells three times with 200 microliters of FACS buffer to remove unbound antibodies **[3]**. Add 200 microliters of fixation and permeabilization solution **[4]** and incubate the cells for 30 minutes on ice in the dark **[5]**.

3.4.1. CU: Talent adding 50 µL of antibody cocktail to the cells **TXT: Antibody Dilution-1:400 In FACS buffer** *Video editor: This is ratio!*

3.4.2. Talent placing the plate on the ice and keeping it in the dark

3.4.3. Talent washing the cells with 200 µL of FACS

3.4.4. Talent adding 200 µL of fixation and permeabilization solution

3.4.5. Talent placing the plate on the ice and keeping it in the dark

3.5. Centrifuge the cells at 500 *g* for 1 minute **[1]** and discard the supernatant into hazardous chemical waste **[2]**. Then, wash the cells three times with 1× (*one-x*) permeabilization wash buffer **[3]**.

3.5.1. Talent placing the plate in the centrifuge and closing the lid

3.5.2. Talent discarding the supernatant into hazardous chemical waste

3.5.3. Talent washing the cells with permeabilization wash buffer

3.6. For intracellular cytokine staining, add 50 microliters of an antibody cocktail containing anti- tumor necrosis factor-alpha and anti-Granzyme B to the cells **[1-TXT]**. Then, incubate for 30 minutes on ice in the dark **[2]**. Wash the cells three times with 1× permeabilization wash buffer to remove unbound antibodies **[3]**, followed by a final wash with FACS buffer **[4]**. Resuspend the cell pellet in 200 microliters of FACS buffer **[5]**.

3.6.1. CU: Talent adding 50 µL of antibody cocktail to the cells **TXT: Antibody Dilution 1:100 in 1x permeabilization wash buffer** *Video editor: This is ratio!*

3.6.2. Talent placing the plate on the ice and keeping it in the dark

3.6.3. Talent washing the cells with permeabilization wash buffer

3.6.4. Talent washing the cells with FACS buffer

3.6.5. Talent resuspending the cell pellet in 200 µL of FACS buffer

4. Flow Cytometric Analysis

4.1. Acquire each sample on a flow cytometer **[1]**. Exclude debris using Forward-scatter versus side-scatter gating **[2]** and doublets using singlet gating **[3]**, collect at least 1×10^4 CD8⁺ T cells per sample **[4]**.

- 4.1.1. Talent loading the samples onto the flow cytometer
- 4.1.2. SCREEN: #71979_screenshot_1: 00:09 -00:17
- 4.1.3. SCREEN: #71979_screenshot_1: 00:19 -00:26
- 4.1.4. SCREEN:#71979_screenshot_1: 00:28 -00:41

Authors: The following step is included for better connection of the protocol to the result, although it was not highlighted.

- 4.2. For the proliferation samples, calculate the proportion of cells that have undergone one or more divisions [1]. For the intracellular cytokine samples, obtain the mean fluorescence intensity [2].

- 4.2.1. SCREEN: #71979_screenshot_2: 00:02 -00:12
- 4.2.2. SCREEN: #71979_screenshot_3: 00:16 -00:18 *Video editor: When VO says, “the intracellular cytokine samples” emphasize the first two rows in the FlowJo Tables popup window and if possible, slow down the video to cover the narration.*

Author: The highlighted protocol specifies analysis of the percent positivity of each marker. Although results for both percent positivity and mean fluorescence intensity (MFI) are provided, the MFI results are presented in the script. Therefore, the statement has been revised accordingly, and its correctness has been confirmed by the author.

Results

5. Results

5.1. CTV dilution histograms show the proliferative response of CD8⁺ T cells to increasing anti-CD3 stimulation over time [1]. At 6 hours, minimal proliferation was observed across all stimulation conditions, with proliferating fractions ranging from 0.2% [2] to 4.7% [3].

5.1.1. LAB MEDIA: Figure 2

5.1.2. LAB MEDIA: Figure 2 *Video Editor: Please highlight the peak in the first graph in the left panel*

5.1.3. LAB MEDIA: Figure 2 *Video Editor: Please highlight the peak in the fifth graph in the left panel*

5.2. By 36 hours, proliferation was minimal at 0.1 microgram per milliliter anti-CD3 [1] but increased at higher concentrations, accompanied by distinct CTV dilution peaks at 5 [2] and 10 microgram per milliliter [3]. By 48 hours, proliferation rose markedly in a concentration-dependent manner [4], reaching 89.5% at 10 µg/mL, with additional CTV dilution peaks indicating successive rounds of cell division [5].

5.2.1. LAB MEDIA: Figure 2 *Video Editor: Please highlight the peak in the first graph in the middle panel*

5.2.2. LAB MEDIA: Figure 2 *Video Editor: Please highlight all the peaks in the fourth graph in the middle panel*

5.2.3. LAB MEDIA: Figure 2 *Video Editor: Please highlight all the peaks in the fifth graph in the middle panel*

5.2.4. LAB MEDIA: Figure 2 *Video Editor: Please sequentially highlight peaks in all graphs except the fifth graph in the right panel*

5.2.5. LAB MEDIA: Figure 2 *Please highlight peaks in the fifth graph in the right panel*

5.3. Expression of the effector molecule Granzyme B increased with increasing anti-CD3 concentration at 24 hours, reaching its highest level at 10 micrograms per milliliter, indicative of early effector differentiation [1]. By 48 hours, however, this trend reversed, with the highest stimulation doses showing a marked reduction in Granzyme B expression [2].

- 5.3.1. LAB MEDIA: Figure 5C *Video Editor: Please show the increase by drawing a line from the blue bar to the black bar in the left graph corresponding to 24 hours*
- 5.3.2. LAB MEDIA: Figure 5C *Video Editor: Please highlight the black bar in the left graph corresponding to 48 hours, along with the 10 µg/mL (black) legend in the side of the graph*

- 5.4. Expression of the effector molecule Tumor necrosis factor- α peaked under strong stimulation of 10 micrograms per milliliter at 24 hours [1]. By 48 hours, higher anti-CD3 concentrations led to a significant decline in the expression, indicating reduced production of effector molecules [2].
 - 5.4.1. LAB MEDIA: Figure 5C *Video Editor: Please highlight the black bar in the right graph corresponding to 24 hours, along with the 10 µg/mL (black) legend in the side of the graph*
 - 5.4.2. LAB MEDIA: Figure 5C *Video Editor: Please highlight the black bar in the right graph corresponding to 48 hours, along with the 10 µg/mL (black) legend in the side of the graph*

1. Micromolar
Pronunciation link: <https://www.merriam-webster.com/dictionary/micromolar>
IPA: /ˌmaɪkroʊˈmoʊlər/
Phonetic Spelling: my·kroh·moh·ler
2. Millimolar
Pronunciation link: <https://www.merriam-webster.com/dictionary/millimolar>
IPA: /ˌmɪləˈmoʊlər/
Phonetic Spelling: mil·uh·moh·ler
3. CellTrace Violet
Pronunciation link: No confirmed link found
IPA: /ˈsɛlˌtreɪs ˈvaɪələt/
Phonetic Spelling: sel·trays vy·uh·luht
4. Naïve
Pronunciation link: <https://www.merriam-webster.com/dictionary/naive>
IPA: /naɪˈiːv/
Phonetic Spelling: ny·EEV
5. Interleukin-2
Pronunciation link: <https://www.merriam-webster.com/dictionary/interleukin>
IPA: /ˌɪntərˈluːkɪn tuː/
Phonetic Spelling: in·ter·LOO·kin two

6. Intracellular
Pronunciation link: <https://www.merriam-webster.com/dictionary/intracellular>
IPA: /,ɪntrəˈsɛljələr/, /,ɪntrəˈsɛljələr/
Phonetic Spelling: in·truh·SEL·yuh·ler; in·trah·SEL·yuh·ler
7. Cytokine
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytokine>
IPA: /ˈsaɪtəˌkaɪn/
Phonetic Spelling: SYE·tuh·kine
8. PMA
Pronunciation link: No confirmed link found
IPA: /,piːˌɛmˈeɪ/
Phonetic Spelling: pee·em·AY
9. Ionomycin
Pronunciation link: No confirmed link found
IPA: /aɪˌɑːnəˈmaɪsɪn/
Phonetic Spelling: eye·on·uh·MY·sin
10. Phorbol
Pronunciation link: <https://www.merriam-webster.com/medical/phorbol>
IPA: /ˈfɔːrˌbɔːl/, /ˈfɔːrˌboʊl/
Phonetic Spelling: FOR·bawl; FOR·bohl
11. Myristate
Pronunciation link: <https://www.merriam-webster.com/medical/myristate>
IPA: /mɪˈrɪsˌteɪt/
Phonetic Spelling: mih·RIS·tayt
12. Acetate
Pronunciation link: <https://www.merriam-webster.com/dictionary/acetate>
IPA: /ˈæsəˌteɪt/
Phonetic Spelling: ASS·uh·tayt
13. Phorbol 12-myristate 13-acetate
Pronunciation link: No confirmed link found
IPA: /ˈfɔːrˌbɔːl twelv mɪˈrɪsˌteɪt ˌθɜːrˈtiːn ˈæsəˌteɪt/
Phonetic Spelling: FOR·bawl twelve mih·RIS·tayt thur·TEEN ASS·uh·tayt
14. Monensin
Pronunciation link: <https://www.merriam-webster.com/medical/monensin>
IPA: /moʊˈnɛnsən/
Phonetic Spelling: moh·NEN·suhn
15. Brefeldin
Pronunciation link: No confirmed link found
IPA: /brəˈfɛldɪn/
Phonetic Spelling: bruh·FEL·din
16. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsentrəˌfjuːdʒ/
Phonetic Spelling: SEN·truh·fyooj

17. Centrifugation
 Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifugation>
 IPA: /ˌsentrəˈfjuːˈɡeɪʃən/
 Phonetic Spelling: sen·truh·fyoo·GAY·shuhn
18. Supernatant
 Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
 IPA: /ˌsuːpərˈneɪtənt/
 Phonetic Spelling: soo·per·NAY·tuhnt
19. Flow cytometry
 Pronunciation link: No confirmed link found
 IPA: /floʊ saɪˈtɑːmətri/
 Phonetic Spelling: floh sy·TOM·uh·tree
20. FACS
 Pronunciation link: No confirmed link found
 IPA: /fæks/
 Phonetic Spelling: faks
21. Permeabilization
 Pronunciation link: No confirmed link found
 IPA: /ˌpɜrmiəbələˈzeɪʃən/
 Phonetic Spelling: per·mee·uh·buh·luh·ZAY·shuhn
22. Granzyme B
 Pronunciation link: No confirmed link found
 IPA: /ˈgrænzəɪm biː/
 Phonetic Spelling: GRAN·zyme bee
23. Tumor necrosis factor-alpha
 Pronunciation link: No confirmed link found
 IPA: /ˈtuːməɹ nəˈkroʊsɪs ˈfæktər ˈælfə/
 Phonetic Spelling: TOO·mer nuh·KROH·sis FAK·ter AL·fuh
24. Cytometric
 Pronunciation link: No confirmed link found
 IPA: /ˌsaɪtəˈmetrɪk/
 Phonetic Spelling: sy·tuh·MET·rik
25. Doublets
 Pronunciation link: <https://www.merriam-webster.com/dictionary/doublet>
 IPA: /ˈdʌbləts/
 Phonetic Spelling: DUB·luhts
26. Singlet
 Pronunciation link: <https://www.merriam-webster.com/dictionary/singlet>
 IPA: /ˈsɪŋɡlət/
 Phonetic Spelling: SING·gluht
27. Fluorescence
 Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescence>
 IPA: /flʊˈresəns/, /flɔːˈresəns/
 Phonetic Spelling: floo·RES·uhns; flaw·RES·uhns

28. Proliferation

Pronunciation link: <https://www.merriam-webster.com/dictionary/proliferation>

IPA: /prəˌlɪfəˈreɪʃən/

Phonetic Spelling: pruh·lif·uh·RAY·shuhn

29. Proliferative

Pronunciation link: <https://www.merriam-webster.com/medical/proliferative>

IPA: /prəˈlɪfəˈreɪtɪv/

Phonetic Spelling: pruh·LIF·uh·ray·tiv

30. Histograms

Pronunciation link: <https://www.merriam-webster.com/dictionary/histogram>

IPA: /ˈhɪstəˌɡræmz/

Phonetic Spelling: HIS·tuh·gramz

31. Effector

Pronunciation link: <https://www.merriam-webster.com/dictionary/effector>

IPA: /ɪˈfektər/

Phonetic Spelling: ih·FEK·ter

32. Differentiation

Pronunciation link: <https://www.merriam-webster.com/dictionary/differentiation>

IPA: /ˌdɪfəˈrenʃiˈeɪʃən/

Phonetic Spelling: dif·uh·ren·shee·AY·shuhn