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Title: Bead-Based Multiplex Flow Cytometry Assay for Functional Profiling of CAR T Cells

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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, all done**
- 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: 20

Number of Shots: 41

Interviews

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

Authors: Answers are slightly edited in accordance with journal guidelines. 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. **Christina Pitzka or César Evaristo:** Our research focuses on CAR T-cell functional characterization and has developed a flow cytometry-compatible multiplex assay for profiling effector molecule secretion.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Christina Pitzka or César Evaristo:** This procedure can be applied to T and CAR T-cell research for functional characterization, mechanistic investigations, QC testing, and potency assessment.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Christina Pitzka or César Evaristo:** This protocol enables quantification of cytokines, chemokines, and cytotoxic mediators that reflect CAR T-cell activation and effector function.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

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

- 1.4. **Christina Pitzka or César Evaristo:** The most important considerations in this protocol are co-culture duration, effector-to-target ratio, sample dilution, and maintaining quantifiable analyte levels.

- 1.4.1. 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?~~

- 1.5. **Christina Pitzka or César Evaristo:** Following this procedure, supernatant profiling can be paired with flow cytometric CAR T-cell phenotyping or cytotoxicity assays.
- 1.5.1. 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 Ethics Committee of the Ärztekammer Nordrhein at Miltenyi Biotec, Bergisch Gladbach

Protocol

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

2. Cytokine Sample Preparation and Multiplex Bead Assay Setup

Demonstrators: Christina Pitzka or Anna Foerster-Marniok

2.1. To begin, prepare cell cultures of engineered human chimeric antigen receptor T or CAR T (*Car-T*) cells with target cells either expressing or lacking the cognate antigen, at an effector-to-target cell ratio of 2.5 to 1 [1].

2.1.1. Talent arranging a culture plate for each time point and adding antigen-expressing target cells to the labeled wells. ***Videographer:** Please obtain CU shot as well for this shot.*

Videographer's NOTE: 2.1.1. 2x Close Up; 1x Long shot

Videographer's NOTE: 2.2.1. plates are already marked in 2.1.1.

2.2. Define 1 or multiple time points for supernatant collection to analyze secreted cytokines or monitor cytokine kinetics over a defined time frame [1-TXT].

2.2.1. Talent marking multiple collection time points, including 4, 8, 16, 24, and 48 hours. **TXT: Defined time frame: 4, 8, 16, 24, and 48 hours**

2.3. Reconstitute the analyte standard by adding 200 microliters of buffer or cell culture media to the pellet and mix gently [1]. Prepare 6 reagent tubes [2] and perform a 1 to 5 dilution series of the reconstituted standard down to 3.2 picograms per milliliter or 0.6 picograms per milliliter [3]. Prepare a blank sample [4].

2.3.1. Talent adding 200 microliters of buffer or cell culture media to the analyte standard pellet and mixing it. **Videographer's NOTE:** 2.3.1. 2 versions - one with pan, one without

2.3.2. Talent arranging 6 labeled reagent tubes on the bench.

2.3.3. Talent performing a serial dilution transfer between reagent tubes.

2.3.4. Shot of blank sample tube.

2.4. At defined hours post-co-culture, centrifuge the co-culture at 300 g for 5 minutes at 21 degrees Celsius [1]. Use the supernatants directly or dilute them to fit the standard curve range [2].

2.4.1. Talent placing plates into the centrifuge.

2.4.2. Talent diluting the supernatant in a fresh plate to match the standard curve range.

3. Assay Workflow

- 3.1. Pre-wet MACSPlex (*Max-plex*) Filter Plate with 200 microliters of MACSPlex Buffer per well [1] and use a vacuum manifold or centrifuge to aspirate until the wells are drained [2].
 - 3.1.1. Talent pipetting 200 microliters of MACSPlex Buffer into the wells of the MACSPlex filter plate.
 - 3.1.2. Talent placing the MACSPlex Filter Plate on a vacuum manifold or in a centrifuge to aspirate the buffer.
- 3.2. Vortex the capture bead mix to thoroughly resuspend the beads [1]. Add 50 microliters of the standard or sample [2] and 20 microliters of pre-diluted beads to the wells of a 96-well filter plate [3].
 - 3.2.1. Talent vortexing the capture bead mix tube.
 - 3.2.2. Talent pipetting 50 microliters of standard or sample into the wells of the filter plate.
 - 3.2.3. Talent adding 20 microliters of pre-diluted beads to the wells of the filter plate.
- 3.3. Incubate the plate for 2 hours at room temperature with shaking at 450 rpm in the dark [1]. Wash the wells twice with 200 microliters of buffer [2], using a vacuum manifold [3].
 - 3.3.1. Talent placing the 96-well plate on a shaker for incubation in the dark.
 - 3.3.2. Talent dispensing buffer into the wells of the MACSPlex Filter Plate.
Videographer's NOTE: 3.3.2. and 3.3.3. together in one shot (name of file: 3.3.2.)
 - 3.3.3. Talent placing the plate on a vacuum manifold to remove the wash buffer.
- 3.4. Add 100 microliters of 1 to 50 pre-diluted detection reagent 1 to the wells [1]. Incubate for 1 hour at room temperature with shaking at 450 rpm in the dark [2]. Wash the wells twice [3].
 - 3.4.1. Talent adding pre-diluted detection reagent 1 to the wells of the plate.
 - 3.4.2. Talent placing the plate on a shaker for incubation in the dark. **Videographer's NOTE: 3.4.2. reuse of 3.3.1**
 - 3.4.3. *Reuse 3.3.2 and 3.3.3.*
- 3.5. Add 100 microliters of 1 to 50 pre-diluted detection reagent 2 to the wells [1]. Incubate for 30 minutes at room temperature with shaking at 450 rpm in the dark [2]. Wash the wells once [3].

- 3.5.1. Talent adding pre-diluted detection reagent 2 to the wells of the plate.
- 3.5.2. Talent placing the plate on a shaker for incubation in the dark. **Videographer's NOTE: 3.5.2. reuse of 3.3.1**
- 3.5.3. *Reuse 3.3.2 and 3.3.3.*

- ~~3.6. For acquisition, resuspend the samples in 200 microliters of buffer [1].~~
- ~~3.6.1. Talent pipetting buffer into the wells and resuspending the samples thoroughly.~~ **Videographer's NOTE: 3.6.1. „resuspending“ - this step is not included in the essay protocol (note of Anna Foerster-Marniok**

4. Data Acquisition and Cytokine Analysis

- 4.1. Use the 2D barcode on the capture beads vial to load the acquisition settings [2] into the MACSQuant (*Max-Quant*) flow cytometer [1].
 - 4.1.2.,. Talent positioning the capture bead vial in front of the 2D barcode reader.
NOTE: Moved 4.1.2 before 4.1.1
 - 4.1.1. SCREEN: 70344_screenshot_70344_screenshot_1 00:00-00:03.
- 4.2. After logging in to the software, select **MACSPlex Filter Plate** as the sample rack [1]. Click the **Barcode** button on the toolbar to activate the 2D code reader [2].
 - 4.2.1. SCREEN: 70344_screenshot_2 00:00-00:10.
 - 4.2.2. SCREEN: 70344_screenshot_3 00:01-00:08.
- 4.3. Present the vial to the reader with the barcode facing the blinking light at a distance of 0.5 to 2.5 centimeters [1].
 - 4.3.1. Talent positioning the vial in front of the barcode reader with the code aligned toward the blinking light.
- 4.4. In the **Autolabel** tab, assign the capture beads as MPX human Cytotoxic T and Natural Killer Cell and select **NoLabel (No-label)** [1]. Define the group positions for the serial dilutions of the cytotoxic T and natural killer cell standard [2]. In the **Settings** tab, select **Express Mode**, set **Type** to **Analysis**, and set **Mode** to **MACSPlex Standard (Max-plex-standard)**, then apply gentle mixing [3].
 - 4.4.1. SCREEN: 70344_screenshot_4 00:00-00:13.
 - 4.4.2. SCREEN: 70344_screenshot_5 00:00-00:14.
 - 4.4.3. SCREEN: 70344_screenshot_6 00:04-00:18.
- 4.5. Assign the unknown samples similarly and define their positions [1]. Optionally, group the samples and enter sample identifiers and descriptions [2]. In the **Settings** tab, select **Express Mode** again, set **Type** to **Analysis**, and set **Mode** to **MACSPlex Sample** [3].

- 4.5.1. SCREEN: 70344_screenshot_7 00:00-00:03.
- 4.5.2. SCREEN: 70344_screenshot_8 00:00-00:15.
- 4.5.3. SCREEN: 70344_screenshot_9 00:00-00:13

- 4.6. In the **Experiment** table, verify that the rack definitions are correct [1]. Ensure proper liquid levels in the filter plate wells [2] and start the measurement [3].
 - 4.6.1. SCREEN: 70344_screenshot_10 00:03-00:08 AND 00:12-00:17.
 - 4.6.2. Shot of the filter plate wells showing appropriate liquid levels.
 - 4.6.3. SCREEN: 70344_screenshot_11 00:02-00:08

- 4.7. Using the phycoerythrin or PE (*P-E*), fluorescein isothiocyanate or FITC (*Fit-C*), and allophycocyanin or APC (*A-P-C*) channels, acquire data on a flow cytometer equipped with blue and red lasers [1]. Confirm that the **Express Mode** templates are being used for automated gating and data analysis in the MACSQuant (*Max-Quant*) flow cytometer [2].
 - 4.7.1. Talent loading samples onto a flow cytometer and starting the acquisition run.
 - 4.7.2. SCREEN: 70344_screenshot_11 00:08-00:11 and 00:35-00:39.

- 4.8. Alternatively, upload FSC (*F-S-C*) or MQD (*M-Q-D*) data files to the web-based Inspector (*in-SPEK-tor*) tool for automated quantitative cytokine measurement and export-ready results [1].
 - 4.8.1. SCREEN: 70344_screenshot_12 00:05-00:17 and 00:21-00:30.

- 4.9. Verify proper separation of the 12 different capture bead populations in the FITC versus PE channel [1].
 - 4.9.1. SCREEN: 70344_screenshot_13 00:09-00:16.

- 4.10. Apply the standard curve to calculate cytokine concentrations [1]. Finally, export and visualize the results [2].
 - 4.10.1. SCREEN: 70344_screenshot_13 00:17-00:2.
 - 4.10.2. SCREEN: 70344_screenshot_14 00:15-00:23.

Results

5. Results

- 5.1. Using multiplex bead encoding, distinct bead clusters representing individual analytes were clearly resolved in the biaxial plot [1].
 - 3.1.1. LAB MEDIA: Figure 2.
- 5.2. Following co-culture with tumor target cells, CAR T cells secreted high levels of Granzyme B [1], interferon gamma or IFN-g (*Interferon-gamma*) [2], tumor necrosis factor alpha or TNF-a (*T-N-F-Alpha*) [3], and granulocyte-macrophage colony-stimulating factor or GM-CSF (*G-M-C-S-F*), indicating potent activation and cytotoxic effector function [4].
 - 5.2.1. LAB MEDIA: Figure 3. *Video editor: Emphasize the row Granzyme B 49950.2 pg/mL on the right side table and the corresponding tall bar above Granzyme B in the left graph.*
 - 5.2.2. LAB MEDIA: Figure 3. *Video editor: Emphasize the row IFN-g 48367.8 pg/mL on the right side table and the corresponding tall bar above IFN-g in the left graph.*
 - 5.2.3. LAB MEDIA: Figure 3. *Video editor: Emphasize the row TNF-a 7055.2 pg/mL on the right side table and the corresponding tall bar above TNF-a in the left graph.*
 - 5.2.4. LAB MEDIA: Figure 3. *Video editor: Emphasize the row GM-CSF 6653.5 pg/mL on the right side table and the corresponding tall bar above GM-CSF in the left graph.*
- 5.3. Interleukin-2 or IL-2 (*I-L-two*) levels exceeded the upper detection limit, consistent with strong proliferative signaling [1].
 - 5.3.1. LAB MEDIA: Figure 3. *Video editor: Emphasize the row IL-2 >10000.0 pg/mL shown in red text in the right table and the corresponding high bar above IL-2 in the left graph.*
- 5.4. Cytokines associated with regulatory or alternative helper T-cell responses, including IL-6 [1], IL-10 [2], IL-17A [3], IL-21 [4], and monocyte chemoattractant protein 1 or MCP-1 (*M-C-P-one*), were present at low or near-undetectable levels [5].
 - 5.4.1. LAB MEDIA: Figure 3. *Video editor: Emphasize the short bar above "IL-6" in the left graph and the row "IL-6 38.0 pg/mL" in the right table.*
 - 5.4.2. LAB MEDIA: Figure 3. *Video editor: Emphasize the very short bar above "IL-10" in the left graph and the row "IL-10 <3.2 pg/mL" in the right table.*
 - 5.4.3. LAB MEDIA: Figure 3. *Video editor: Emphasize the short bar above "IL-17A" in the left graph and the row "IL-17A 24.0 pg/mL" in the right table.*

- 5.4.4. LAB MEDIA: Figure 3. *Video editor: Emphasize the short bar above “IL-21” in the left graph and the row “IL-21 <16.0 pg/mL” in the right table.*
- 5.4.5. LAB MEDIA: Figure 3. *Video editor: Emphasize the nearly flat bar above “MCP-1” in the left graph and the row “MCP-1 0.8 pg/mL” in the right table.*

- 5.5. CAR T cells exhibited rapid and robust cytokine release upon recognition of antigen-positive targets, with strong upregulation of Granzyme B [1], INF-g [2], TNF-a [3], GM-CSF [4], and IL-2 within 8–24 hours, reaching peak levels by 48 hours [5].
 - 5.5.1. LAB MEDIA: Figure 4. *Video editor: Highlight the blue Antigen⁺ line in the “Granzyme B” graph rising sharply between 8 and 48 hours.*
 - 5.5.2. LAB MEDIA: Figure 4. *Video editor: Highlight the blue Antigen⁺ line in the “IFNg” graph reaching high values by 24–48 hours.*
 - 5.5.3. LAB MEDIA: Figure 4. *Video editor: Highlight the blue Antigen⁺ line in the “TNF-a” graph, increasing steeply and remaining high at 48 hours.*
 - 5.5.4. LAB MEDIA: Figure 4. *Video editor: Highlight the blue Antigen⁺ line in the “GM-CSF” graph rising progressively toward 48 hours.*
 - 5.5.5. LAB MEDIA: Figure 4. *Video editor: Highlight the blue Antigen⁺ line in the “IL-2” graph reaching the upper range by 24–48 hours.*

- 5.6. Moderate increases in IL-6 [1], IL-17A [2], and IL-21 were also observed [3].
 - 5.6.1. LAB MEDIA: Figure 4. *Video editor: Highlight the blue Antigen⁺ line in the “IL-6” graph showing a moderate increase over time.*
 - 5.6.2. LAB MEDIA: Figure 4. *Video editor: Highlight the blue Antigen⁺ line in the “IL-17A” graph gradually increasing toward 48 hours.*
 - 5.6.3. LAB MEDIA: Figure 4. *Video editor: Highlight the spike in the blue Antigen⁺ line in the “IL-21” graph around 16 hours.*

- 5.7. IL-4 [1], IL-10 [2], MCP 1 [3], and Perforin remained low or modestly elevated [4].
 - 5.7.1. LAB MEDIA: Figure 4. *Video editor: Highlight the near-flat lines in the “IL-4” graph.*
 - 5.7.2. LAB MEDIA: Figure 4. *Video editor: Highlight the blue Antigen⁺ line in the “IL-10” graph.*
 - 5.7.3. LAB MEDIA: Figure 4. *Video editor: Highlight the nearly flat lines in the “MCP-1” graph.*
 - 5.7.4. LAB MEDIA: Figure 4. *Video editor: Highlight the blue Antigen⁺ line in the “Perforin” graph.*

- 5.8. Co-cultures with antigen-negative targets showed minimal cytokine induction across all analytes [1].
 - 5.8.1. LAB MEDIA: Figure 4. *Video editor: Emphasize the red Antigen⁻ lines remaining close to baseline across all 12 cytokine graphs.*

1. Cytokine

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

IPA: /ˈsaɪtəˌkaɪn/

Phonetic Spelling: sy·tuh·kyne

2. Multiplex

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

IPA: /ˈmʌltiˌpleks/

Phonetic Spelling: muhl·tee·pleks

3. Chimeric

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

IPA: /kaɪˈmerɪk/

Phonetic Spelling: ky·MER·ik

4. Antigen

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

IPA: /ˈæntəjən/

Phonetic Spelling: an·tuh·juhn

5. Reconstitute

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

IPA: /ˌriːˈkɑːnstəˌtuːt/

Phonetic Spelling: ree·KON·stuh·toot

6. Analyte

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

IPA: /'ænə,lait/

Phonetic Spelling: an·uh·lyte

7. Microliter

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

IPA: /'maɪkroʊ,lɪ:tər/

Phonetic Spelling: my·kroh·lee·ter

8. Pellet

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

IPA: /'pelət/

Phonetic Spelling: pel·uht

9. Picogram

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

IPA: /'pi:kɒ,græm/

Phonetic Spelling: pee·koh·gram

10. Centrifuge

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

IPA: /'sentərəˌfjuːdʒ/

Phonetic Spelling: sen·truh·fyooj

11. Supernatant

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

IPA: /ˌsuːpərˈneɪtənt/

Phonetic Spelling: soo·per·NAY·tuhnt

12. Vortex

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

IPA: /ˈvɔːrˌtɛks/

Phonetic Spelling: vor·teks

13. Resuspend

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

IPA: /ˌriːsəˈspend/

Phonetic Spelling: ree·suh·SPEND

14. Phycoerythrin

Pronunciation link: <https://www.howtopronounce.com/phycoerythrin>

IPA: /ˌfaɪkoʊˈrɪθrɪn/

Phonetic Spelling: fy·koh·ih·RITH·rin

15. Fluorescein

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

IPA: /flɔːˈresiːn/

Phonetic Spelling: flohr·ESS·ee·in

16. Isothiocyanate

Pronunciation link: <https://www.howtopronounce.com/isothiocyanate>

IPA: /ˌaɪsoʊˌθaɪoʊˈsaɪəˌneɪt/

Phonetic Spelling: eye·soh·thy·oh·SYE·uh·nayt

17. Allophycocyanin

Pronunciation link: <https://www.howtopronounce.com/allophycocyanin>

IPA: /əˌloʊˌfaɪkoʊˈsaɪənɪn/

Phonetic Spelling: uh·loh·fy·koh·SYE·uh·nin

18. Cytometer

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

IPA: /saɪˈtɑːmətər/

Phonetic Spelling: sy·TOM·uh·ter

19. Biaxial

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

IPA: /baɪˈæksɪəl/

Phonetic Spelling: by·AK·see·uhl

20. Analytes

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

IPA: /'ænəˌlaɪts/

Phonetic Spelling: an-uh·lytes

21. Granzyme

Pronunciation link: <https://www.howtopronounce.com/granzyme>

IPA: /'grænzaim/

Phonetic Spelling: gran·zyme

22. Interferon

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

IPA: /ˌɪntərˈfɪrɔːn/

Phonetic Spelling: in·ter·FEER·on

23. Granulocyte

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

IPA: /'grænjələˌsaɪt/

Phonetic Spelling: gran·yuh·luh·syte

24. Macrophage

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

IPA: /'mækroʊˌfeɪdʒ/

Phonetic Spelling: mak·roh·fayj

25. Proliferative

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

IPA: /prəˈlɪfərətɪv/

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

26. Chemoattractant

Pronunciation link: <https://www.howtopronounce.com/chemoattractant>

IPA: /ˌkiːmoʊəˈtræktənt/

Phonetic Spelling: kee·moh·uh·TRAK·tuhnt

27. Cytotoxic

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

IPA: /ˌsaɪtəʊˈtɔːksɪk/

Phonetic Spelling: sy·toh·TOK·sik

28. Perforin

Pronunciation link: <https://www.howtopronounce.com/perforin>

IPA: /pərˈfɔːrɪn/

Phonetic Spelling: per·FOR·in