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Title: Quantifying NK-Cell Cytotoxicity Potentiated by Chimeric Antigen Receptors and Therapeutic Antibodies Using Live-Cell Imaging and Flow Cytometry

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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**

3. Filming location: Will the filming need to take place in multiple locations? **Yes**

1 floor apart

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: 40

Number of Shots: 58

Interviews

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

INTRODUCTION:

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

- 1.1. **Wilfredo Garcia-Beltran:** Our laboratory studies the role of natural killer cells in health and disease, aiming to harness their potential to improve treatments for solid and blood cancers.
 - 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. **Yuxin Chen:** Existing NK-cell cytotoxicity assays lack standardization across platforms and setups; this protocol provides reproducible live-cell imaging and flow cytometry methods for accurate cytotoxicity 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. **James Townsend:** This protocol enables researchers to quantify NK-cell natural cytotoxicity, evaluate tumor-killing potency of CAR-NK designs, and compare ADCC efficacy of monoclonal antibodies.
 - 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. **Yuxin Chen:** A key consideration while performing this protocol is maintaining accurate, consistent cell counting, pipetting, and plating of NK and target cells to ensure reliable, interpretable results.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **James Townsend:** Future studies can leverage these protocols to explore NK-cell immune evasion, compare tumor susceptibility, and evaluate novel NK-cell-based immunotherapies across cancers.
- 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.

Protocol

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

2. Cell Preparation and Co-Culture Set up for Cytotoxicity Assay

Demonstrator: Yuxin Chen

- 2.1. To begin, prepare 10 milliliters of 2× SYTOX (*Two-X Sytox*) Orange by diluting SYTOX Orange stock solution into NK-92 (*N-K-Ninety-Two*) culture media in a 15-milliliter conical-bottom centrifuge tube [1].
 - 2.1.1. WIDE: Talent adding SYTOX Orange stock solution to NK-92 culture media in a 15-milliliter conical-bottom centrifuge tube. ***Videographer:** Please obtain CU shot as well for this shot.*
- 2.2. Count the target cells using a Luna counter after mixing with trypan blue or use any validated dye exclusion-based cell counting method [1].
 - 2.2.1. Talent counting target cells using an automated cell counter.
- 2.3. Transfer the required amount of target cells into a 15-milliliter conical-bottom centrifuge tube [1]. Centrifuge the tube at 300 *g* for 5 minutes at room temperature [2] and discard the supernatant [3].
 - 2.3.1. Talent transferring the required amount of target cells into a 15-milliliter conical-bottom centrifuge tube.
 - 2.3.2. Talent placing the tube in the centrifuge.
 - 2.3.3. Talent discarding the supernatant from the 15-milliliter conical-bottom centrifuge tube.
- 2.4. Then, resuspend the target cells to a final concentration of 2.5×10^5 (*Two-hundred fifty thousand*) cells per milliliter, in 2× SYTOX Orange media [1].
 - 2.4.1. Talent resuspending the target cells in 2× SYTOX Orange media.
- 2.5. To prepare the effector cell, assess NK-92 cell viability and concentration, and proceed only if viability is greater than 80%, as lower viability may affect assay performance [1].
 - 2.5.1. Shot of the viability result meeting the required threshold.
- 2.6. Count and transfer the required number of NK-92 cells into individual 15-milliliter conical-bottom centrifuge tubes [1].
 - 2.6.1. Talent transfers the required number of NK-92 cells into individual 15-milliliter conical-bottom centrifuge tubes.
- 2.7. After centrifugation, resuspend the cells to a final concentration of 5.0×10^5 (*five*

hundred thousand) cells per milliliter [1].

2.7.1. Talent shows the pellet and resuspends the cells in the tube.

2.8. Prepare effector cell dilutions by adding 240 microliters of effector cells to wells of a 96-well round-bottom plate for the 2 to 1 effector-to-target ratio [1] and add 120 microliters of media to the remaining wells [2].

2.8.1. Talent adding effector cells to the designated wells of the round-bottom 96-well plate.

2.8.2. Talent adding media to the remaining wells

2.9. Perform 2-fold serial dilutions from 2 to 1 to 0.25 to 1 effector-to-target ratios, mixing thoroughly by pipetting at least 5 times and changing tips between transfers [1].

2.9.1. Talent performing serial dilutions across the wells of the round-bottom 96-well plate and mixing the cell suspension.

2.10. Next, obtain the poly-D-lysine-coated flat-bottom 96-well plate [1].

2.10.1. Talent placing the poly-D-lysine-coated flat-bottom 96-well plate on the work surface.

2.11. Immediately before transfer, thoroughly resuspend the target and effector cells without creating bubbles, then add 100 microliters of each cell suspension to the appropriate wells [1]. Then, add 200 microliters of PBS to the surrounding unused wells [2].

2.11.1. Talent resuspending the cells by pipetting up and down before dispensing into the wells and adding the cell suspension to the wells.

2.11.2. Talent adding PBS to the unused plate.

2.12. Centrifuge the plate at 100 g for 2 minutes at room temperature [1].

2.12.1. Talent placing the plate in the centrifuge.

3. Live-Cell Imaging Setup for Cytotoxicity Assay

3.1. Place the plate in the live-cell imaging system [1]. Acquire images every 1 hour for 24 hours at 37 degrees Celsius with 5 percent carbon dioxide [2].

3.1.1. Talent placing the plate into the live-cell imaging system.

3.1.2. SCREEN: JoVE-71377-Shot-3.1.2.mp4: 01:26-01:31.

3.2. Verify the image quality after the first scan and adjust the imaging settings if necessary [1].

3.2.1. SCREEN: JoVE-71377-Shot-3.2.1.mp4: 00:18-00:30

- 3.3. Adjust the spectral unmixing to correct for SYTOX Orange spillover into the green channel. Verify that Orange-positive dead cells are not misidentified as Green-positive cells. [1].

3.3.1. SCREEN: JoVE-71377-Shot-3.3.1.mp4

- 3.4. To configure non-adherent Cell-by-Cell analysis, set the expected object diameter to 15 micrometers to match the approximate cell diameter [1]. Set the threshold sensitivity and texture sensitivity to 5 and the edge sensitivity to 10 to facilitate the separation of cell clusters [2].

3.4.1. SCREEN: 3.4-and-3.5.mp4: 00:13-00:18, 00:34-00:39

3.4.2. SCREEN: 3.4-and-3.5.mp4: 00:39-00:44

- 3.5. Set the accepted object area filter range to 50 to 2000 square micrometers to exclude debris and large objects [1]. Set the eccentricity maximum to 0.95 to exclude non-cellular objects [2].

3.5.1. SCREEN: 3.4-and-3.5.mp4: 00:44-00:54

3.5.2. SCREEN: 3.4-and-3.5.mp4: 00:54-01:00

4. Preparation and Assembly of NK Cell–Mediated ADCC Co-Cultures

Demonstrator: James Townsend

- 4.1. Obtain primary human NK cells cultured for 16 to 24 hours [1]. Transfer 5×10^5 (*five hundred thousand*) target cells into a 15-milliliter conical-bottom centrifuge tube [2].

4.1.1. Talent removes the cell culture plate from the incubator. **NOTE: Use the version with the plate placing tilted in the incubator.**

4.1.2. Talent transferring target cells into a 15-milliliter conical-bottom centrifuge tube.

- 4.2. Wash the cells once with 10 milliliters of PBS [1]. Centrifuge the cells at 300 *g* for 5 minutes at room temperature [1], then remove the supernatant [1].

4.2.1. Talent adding PBS to the target cells for washing.

4.2.2. Talent placing the tube in the centrifuge.

4.2.3. Talent removing the supernatant.

- 4.3. Prepare a 0.5 micromolar working solution of carboxyfluorescein succinimidyl ester or CFSE (*C-F-S-E*) in PBS by diluting 1 microliter of 5 millimolar CFSE stock solution into 10 milliliters of PBS [1].

4.3.1. Talent adding CFSE stock solution into PBS in a 15-milliliter conical-bottom

centrifuge tube.

- 4.4. Resuspend the obtained pellet at approximately 1×10^6 (*one million*) cells per milliliter in the CFSE solution [1].
 - 4.4.1. Talent resuspending the cell pellet in the CFSE solution.
- 4.5. Incubate the cells for 15 minutes at 37 degrees Celsius, wrapped in aluminum foil to protect from light [1].
 - 4.5.1. Talent placing the wrapped centrifuge tube into the incubator.
- 4.6. Then quench the reaction by adding 5 to 10 volumes of culture medium [1] and incubate for 5 minutes at 37 degrees Celsius [2].
 - 4.6.1. Talent adding culture medium to the centrifuge tube to quench the reaction.
 - 4.6.2. Talent placing the centrifuge tube into the incubator.
- 4.7. After centrifuging the cells, resuspend the cell pellet at 2.5×10^5 (*two hundred fifty thousand*) cells per milliliter so that 100 microliters contain 2.5×10^4 (*twenty-five thousand*) cells [1].
 - 4.7.1. Talent resuspending the cells to the required final concentration.
- 4.8. Add 100 microliters per well of target cells to the designated wells of a round-bottom 96-well plate using a multichannel pipette. Plate each condition in triplicate [2].
 - 4.8.1. Talent adding target cells to the designated wells of the round-bottom 96-well plate using a multichannel pipette.
- 4.9. Next, prepare 200 microliters of $4\times$ (*four X*) antibody solution in NK-cell culture medium for each test antibody and matched isotype control [1]. Transfer the antibody solutions to a deep-well plate [1].
 - 4.9.1. Talent preparing antibody solutions in NK-cell culture medium.
 - 4.9.2. Talent transferring antibody solutions into the deep-well plate.
- 4.10. Add 50 microliters of antibody solution to each well containing the target cells and mix thoroughly by pipetting up and down at least 5 times [1].
 - 4.10.1. Talent adding antibody solution to the target-cell wells and mixing by pipetting multiple times.
- 4.11. Add 50 microliters of NK-cell culture medium per well to the no-antibody control wells and mix thoroughly by pipetting [1]. Incubate the plate for 25 to 30 minutes under standard incubation conditions [2].
 - 4.11.1. Talent adding NK-cell culture medium to the no-antibody control wells and mixing by pipetting multiple times.
 - 4.11.2. Talent placing the plate into the incubator.

4.12. Count and obtain 2×10^5 (*two hundred thousand*) NK cells and proceed only if viability is greater than or equal to 75 percent [2].

4.12.1. Talent places the cells in the cell counter with the counter's display in the frame.

4.13. Resuspend the cells to 2.5×10^5 (*two hundred fifty thousand*) cells per milliliter in 800 microliters of primary human NK-cell culture [1]. Add 50 microliters per well of NK cells to the corresponding wells and mix thoroughly by pipetting up and down at least 5 times [1].

4.13.1. Talent resuspending NK cells in primary human NK-cell culture medium.

4.13.2. Talent adding NK cells to the corresponding wells and mixing by pipetting multiple times.

4.14. Next, pipette 50 microliters per well of culture medium into the control wells and mix thoroughly [1].

4.14.1. Talent adding culture medium to the control wells and mixing by pipetting multiple times.

4.15. Carefully inspect the round-bottom 96-well plate to confirm that all wells contain 200 microliters with uniform meniscus levels and no bubbles [1].

4.15.1. Talent visually inspecting the round-bottom 96-well plate for uniform liquid levels and absence of bubbles.

4.16. Centrifuge the plate for 2 minutes at 100 g [1], then incubate it for 16 to 18 hours under standard incubation conditions [1].

4.16.1. Talent placing the plate in the centrifuge.

4.16.2. Talent placing the plate into the incubator.

5. Quantification of Cellular Cytotoxicity by Flow Cytometry

5.1. Centrifuge the plate for 5 minutes at 300 g [1] and discard the supernatant [2].

5.1.1. WIDE: Talent placing the plate in the centrifuge.

5.1.2. Talent discarding the supernatant from the wells of the plate.

5.2. Wash all wells with 200 microliters per well of fluorescence-activated cell sorting or FACS (*Facs*) buffer [1].

5.2.1. Talent adding fluorescence-activated cell sorting buffer to all wells of the plate.

5.3. Prepare a surface antibody master mix in FACS buffer as presented here [1].

5.3.1. TEXT ON A PLAIN BACKGROUND

CD56-BV421 at 1:50 dilution

CD16-BV785 at 1:50 dilution

CD107a-PE-Cy7 at 1:25 dilution

- 5.4. Resuspend the cells in 50 microliters per well of antibody master mix and mix thoroughly by pipetting up and down at least 5 times [1]. Incubate the plate for 15 minutes at 4 degrees Celsius, wrapped in aluminum foil to protect from light [2].
 - 5.4.1. Talent resuspending the cells in antibody master mix and mixing by pipetting multiple times.
 - 5.4.2. Talent placing the wrapped plate into cold storage for incubation.
 - 5.5. Add 150 microliters per well of FACS buffer [1].
 - 5.5.1. Talent adding fluorescence-activated cell sorting buffer to the wells of the plate.
 - 5.6. Following centrifugation, resuspend the cells in 100 microliters per well of 5 micromolar DAPI (*Dapi*) in FACS buffer [1]. Proceed immediately to flow cytometry acquisition without pre-acquisition incubation [2].
 - 5.6.1. Talent resuspending the cells in the 4',6-diamidino-2-phenylindole solution.
 - 5.6.2. Talent transferring the prepared plate for immediate flow cytometry acquisition.
 - 5.7. Perform flow cytometry, using the Cytex Guava instrument [1], and record fluorescence channels for CFSE, BV421 (*B-V-Four-Two-One*), BV785 (*B-V-Seven-Eight-Five*), PE-Cy7 (*P-E-Cy-Seven*), DAPI, and any additional fluorophores [1]. NOTE: The VO is edited for the additional shot
- Added shot: Demonstrator acquiring data using the Cytex Guava instrument (slated as 5.7.1)
- 5.7.2 SCREEN: JoVE-71377-Shot-5.7.2-v3: 00:05-00:29

Results

6. Results

- 6.1. During co-culture, effector cells, live target cells, and dead cells were visualized using real-time, live-cell microscopy [1].
 - 6.1.1. LAB MEDIA: Figure 1B.
- 6.2. CAR2 (*Car-Two*) and CAR3 (*Car-Three*) NK-92 cells exhibited faster killing kinetics than CAR1 (*Car-One*) NK-92 cells, particularly at earlier time points at an equal effector to target ratio [1].
 - 6.2.1. LAB MEDIA: Figure 1C. *Video editor: Highlight the graph labeled “E:T = 1:1” and emphasize the purple and orange curves*
- 6.3. Target-cell killing increased with higher effector to target ratios, including control NK-92 cells, which demonstrated natural cytotoxicity [1].
 - 6.3.1. LAB MEDIA: Figure 1C. *Video editor: Move the graphs from “E:T = 0.25:1” to “E:T = 2:1” and emphasize the green control curve*
- 6.4. CAR2 and CAR3 NK-92 cells demonstrated greater potency at lower effector-to-target ratios at later time points [1], while enhanced potency at intermediate effector-to-target ratios was observed at earlier time points [2].
 - 6.4.1. LAB MEDIA: Figure 1D. *Video editor: Highlight the graph labeled “t = 24 h” and emphasize the purple and orange curves remaining lower than the red curve*
 - 6.4.2. LAB MEDIA: Figure 1D. *Video editor: Highlight the graph labeled “t = 4 h” and emphasize the purple and orange curves dropping below the red curve*
- 6.5. Three parameters, KR50 (*K-R-Fifty*), KT50 (*K-T-Fifty*), and IKI50 (*I-K-I-Fifty*) [1], were defined to quantify CAR NK-92 cell potency, killing kinetics, and overall cytotoxic performance across effector populations [2].
 - 6.5.1. LAB MEDIA: Figure 1E. *Video editor: Highlight the labels “KR50”, “KT50”, and “IKI50” inside the diagram*
 - 6.5.2. LAB MEDIA: Figure 1F.
- 6.6. The addition of daratumumab alone did not reduce target-cell survival, confirming that the antibody had no direct effect on target-cell viability in the absence of effector cells [1].
 - 6.6.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the second bar labeled with daratumumab only, which remains close to 100% survival*
- 6.7. Co-culture with NK cells alone reduced target-cell survival to approximately 70%,

indicating baseline natural cytotoxicity [1].

6.7.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the purple bar corresponding to NK cells alone at approximately 70% survival*

6.8. The addition of daratumumab further reduced cell survival to approximately 37%, consistent with enhanced antibody-mediated cytotoxicity [1]. In contrast, the isotype control condition showed approximately 62% survival, comparable to the NK-only condition [2].

6.8.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the purple bar marked as “37%”*

6.8.2. LAB MEDIA: Figure 2C. *Video editor: Highlight the purple bar marked as “62%”*

6.9. NK-cell degranulation was lowest in NK–cell–target cell co-culture without antibody [1] and highest in the daratumumab-treated condition at approximately 31% [2], while the isotype control showed intermediate degranulation at approximately 19% [3].

6.9.1. LAB MEDIA: Figure 2D. *Video editor: Highlight the left blue bar at approximately 12%*

6.9.2. LAB MEDIA: Figure 2D. *Video editor: Highlight the middle blue bar at approximately 31%*

6.9.3. LAB MEDIA: Figure 2D. *Video editor: Highlight the right blue bar at approximately 19%*

1. SYTOX
Pronunciation link: <https://www.howtopronounce.com/sytox>
IPA: /'sɪtɒks/
Phonetic Spelling: sy·toks
2. Trypan
Pronunciation link: <https://www.merriam-webster.com/dictionary/trypan>
IPA: /'traɪˌpæn/
Phonetic Spelling: try·pan
3. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /'sentrəˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
4. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /ˌsuːpərˈneɪtənt/
Phonetic Spelling: soo·per·nay·tuht
5. Resuspend
Pronunciation link: <https://www.merriam-webster.com/dictionary/resuspend>
IPA: /ˌriːsəˈspend/
Phonetic Spelling: ree·suh·spend
6. Viability
Pronunciation link: <https://www.merriam-webster.com/dictionary/viability>
IPA: /ˌvaɪəˈbɪləti/
Phonetic Spelling: vy·uh·bil·uh·tee
7. Poly-D-lysine
Pronunciation link: <https://www.merriam-webster.com/dictionary/lysine>
IPA: /ˈləɪsiːn/
Phonetic Spelling: pol·ee dee·ly·seen
8. Cytotoxicity
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytotoxic>
IPA: /ˌsaɪtəʊtɪkˈsɪsəti/
Phonetic Spelling: sy·toh·tok·sis·uh·tee
9. Spectral
Pronunciation link: <https://www.merriam-webster.com/dictionary/spectral>
IPA: /'spektrəl/
Phonetic Spelling: spek·truhl
10. Unmixing
Pronunciation link: <https://www.merriam-webster.com/dictionary/unmix>
IPA: /ʌnˈmɪksɪŋ/
Phonetic Spelling: un·mik·sing
11. Adherent
Pronunciation link: <https://www.merriam-webster.com/dictionary/adherent>
IPA: /ədˈhɪrənt/
Phonetic Spelling: uhd·heer·uhnt

12. Micrometers
 Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>
 IPA: /maɪˈkraːmətər/
 Phonetic Spelling: my·krom·uh·ter
13. Eccentricity
 Pronunciation link: <https://www.merriam-webster.com/dictionary/eccentricity>
 IPA: /ˌɛksənˈtrɪsəti/
 Phonetic Spelling: ek·sen·tris·uh·tee
14. Carboxyfluorescein
 Pronunciation link: <https://www.howtopronounce.com/carboxyfluorescein>
 IPA: /kɑːrˌbɒksiˌflʊərəˈsiːn/
 Phonetic Spelling: kar·bok·see·floor·uh·see·een
15. Succinimidyl
 Pronunciation link: <https://www.howtopronounce.com/succinimidyl>
 IPA: /sʌkˌsɪnɪˈmɪdɪl/
 Phonetic Spelling: suk·sin·ih·mid·il
16. Ester
 Pronunciation link: <https://www.merriam-webster.com/dictionary/ester>
 IPA: /ˈɛstər/
 Phonetic Spelling: es·ter
17. CFSE
 Pronunciation link: No confirmed link found
 IPA: /ˌsiː ɛf ɛs ˈiː/
 Phonetic Spelling: see·ef·es·ee
18. Daratumumab
 Pronunciation link: <https://www.howtopronounce.com/daratumumab>
 IPA: /ˌdærəˈtuːmjʊːmæb/
 Phonetic Spelling: da·ruh·too·myoo·mab
19. Isotype
 Pronunciation link: <https://www.merriam-webster.com/medical/isotype>
 IPA: /ˈaɪsəˌtaɪp/
 Phonetic Spelling: eye·suh·type
20. Degranulation
 Pronunciation link: <https://www.merriam-webster.com/medical/degranulation>
 IPA: /diˌɡrænjəˈleɪʃən/
 Phonetic Spelling: dee·gran·yuh·lay·shuhn
21. Flow Cytometry
 Pronunciation link: <https://www.merriam-webster.com/dictionary/cytometry>
 IPA: /floʊ saɪˈtɑːmətri/
 Phonetic Spelling: floh sy·tom·uh·tree
22. Fluorophores
 Pronunciation link: <https://www.merriam-webster.com/medical/fluorophore>
 IPA: /ˈflʊərəˌfɔːr/
 Phonetic Spelling: floor·uh·for

23. DAPI

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

IPA: /'dæpi/

Phonetic Spelling: dap·ee

24. Diamidino

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

IPA: /daɪ,æmɪ'di:nou/

Phonetic Spelling: dye·am·ih·dee·noh

25. Phenylindole

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

IPA: /,fɛnɪl'indoʊl/

Phonetic Spelling: fen·il·in·dohl