Dr. Bajaj, Review Editor of the Journal of Visualized Experiments,

Thank you for your input to our article “Analysis of Natural Killer cell metabolism” JoVE61466R1. Here we provide a detailed reply to the editorial comments:

1. **Line 58:**

   Editor comment: “Most of the details which do not directly discuss the representative result obtained by using the protocol above can be moved to the introduction. Figures talking about the introduction can be referenced in the introduction section. However please refer figures in order.

   Also claims in this section need citations.”

   Reply: This text has been moved from Results to the Introduction and citations have been added (references 7 and 8)

2. **Line 132:**

   Editor comment: “Citation?”

   Reply: We have rephrased the sentence “Here we show that this is a valid method for the study of metabolic changes in resting and IL-15 activated human NK cells”

3. **Line 134:**

   Editor comment: “Citation?”

   Reply: Reference 17 has been added.

4. **Line 143:**

   Editor comment: “Please highlight complete sentences throughout.”

   Reply: This has been done.

5. **Line 227:**

   Editor comment: “The pellet above was resuspended in something?”

Reply: The pellet is resuspended in NK separation Buffer and now it has been clearly stated.
"2.2.1. Count the cells from 2.1.6 and resuspend them in NK Separation Buffer (1x 10^8 PBMCs/mL).

2.2.2. Take 10 ml of the cell resuspension (10^9 PBMCs) and place them into a 50 mL tube."

6. **Line 258:**

   Editor comment: "Do you remove the medium before resuspending?"

   Reply: The medium is removed by centrifugation. It is stated in the text now "2.3.1. Take 0.25 x 10^6 cells per sample from step 2.2.8, remove the medium by centrifuging at 800 *x g* for 5 min at room temperature and resuspend the cell pellet in 500 µL of PBS."

7. **Line 264:**

   Editor comment: "How?"

   Reply: It is explained now "Wash 2x by resuspending in 5 mL PBS and centrifuging at 800 *x g* for 5 min, at room temperature."

8. **Line 276:**

   Editor comment: "From which step? 2.2.8?"

   Reply: Step 2.2.8. was added to the text "Resuspend 0.75 x 10^6 cells from step 2.2.8 in 100 µL of IMDM containing 10% HS in a well of a 96 well-plate (round bottom)."

9. **Line 297:**

   Editor comment: "Please reword as it matches with previously published literature."

   Reply: The sentence has been rephrased "Open the sensor cartridge package and separate the sensor cartridge from the utility plate. Add 200 µl of calibrant solution in each well of the utility plate and put back the sensor cartridge onto the plate, validating that the sensors are completely submerged in the solution."

10. **Line 326:**

    Editor comment: "Please reword as it matches with previously published literature."

Reply: The step has been rephrased “4.1.2. Pipet 25 µl of the cell adhesive solution to each well of the assay plate and incubate at room temperature for 20 minutes. After that, remove the solution and wash 2x with 200 µl of sterile water per well. Let the wells dry by keeping the plate open for 15 minutes inside a cell culture hood.”

11. Line 335:

Editor comment: “Please bring out cohesivity in the protocol. Cells from which step? Volume and number of cells used in this assay etc.”

Reply: This information has been added

Editor comment: “What is the appropriate assay medium?”

Reply: This information has been added

Editor comment: “Please instead include your preferred cell concentration. What is volume of the medium?”

Reply: This information has been added

“4.2.1. Centrifuge cells from step 2.2.8 at 200 *x g* for 5 min at room temperature. Remove supernatants and wash cells in warmed mitochondrial stress test medium (if a mitochondrial stress test is being performed) or glycolysis stress test medium (if a glycolysis stress test is being performed). Pellet cells again and resuspend to the preferred cell concentration in the same medium (resuspension volume will depend on the cell concentration chosen; since each well will contain 180 µL of the cell suspension, prepare 0.26 x 10^6, 0.52 x 10^6, 1.04 x 10^6, 2.08 x 10^6, 4.17 x 10^6 and 8.33 x 10^6 cells/mL cell suspensions for 0.047 x 10^6, 0.094 x 10^6, 0.187 x 10^6, 0.375 x 10^6, 0.75 x 10^6 and 1. 5 x 10^6 cells per well respectively).”

12. Line 351:

Editor comment: “Wasn’t this already prepared in the reagent preparation section? So redundant here.”

Reply: In the reagent preparation section we describe how to prepare the stock solutions. This refers to working 10x solutions. This is clarified in the text now. Line 182 “1.2.3. Prepare stock solutions for reagents: Oligomycin (ATP synthase inhibitor), 10 mM stock solution in DMSO; 2,4-dinitrophenol (DNP, uncoupler), 1 M stock solution in DMSO; antimycin A (complex III inhibitor), 10 mM stock solution in DMSO; rotenone (complex I inhibitor), 10 mM stock solution in DMSO. Make 30 µL aliquots of all reagents and store at -20 °C. “

13. Line 359:

Editor comment: “wasn’t this done initially?”

Reply: In the reagent preparation section we describe how to prepare the stock solutions. This refers to working 10x solutions. This is clarified in the text now “**4.3 Preparation of 10x working solutions to load into sensor cartridge**.”

14. Line 364:

Editor comment: “Is the working solutions of the compounds prepared before?”

Reply: To prepare this 10x working solutions, use the stock solutions from 1.2.3.

“4.3.1. For the mitochondrial stress test, prepare 2.5 mL each of 10 µM oligomycin, 1 mM DNP, and a mixture of 10 µM rotenone and 10 µM antimycin A, in mitochondrial stress assay medium (use the stock solutions from step 1.2.3). Final concentrations in the well after injection will be 1 µM oligomycin, 0.1 mM DNP and 1 µM antimycin A/rotenone.

4.3.2. For the glycolysis stress test, prepare 2.5 mL of a mixture of 10 µM rotenone and 10 µM antimycin A in glycolysis stress assay medium (use the stock solutions from step 1.2.3). Dissolve glucose in glycolysis stress test medium for a 100 mM solution and 2-deoxy-glucose (2-DG) in glycolysis stress test medium for a 500 mM solution. Final concentrations in the well after injection will be 10 mM glucose, 1 µM antimycin A/rotenone and 50 mM 2-DG.”

15. Line 375:

Editor comment: “Please check this and bring out clarity? Solutions from 4.3.2 have different concentrations.” “How do you identify appropriate cartridge? Please spell out the details. Do you have different port for different assay? Do you load each compound in different ports? “

Reply: Hydrated cartridge comes from step 3.2. Loading of the ports is shown in table 2. “4.3.3. Warm 10x solutions to 37 °C, check pH and readjust to 7.4 if required. Load compounds prepared in step 4.3.1. (for a mitochondrial stress test) or 4.3.2 (for a glycolysis stress test) into ports A, B and C of the hydrated sensor cartridge (from step 3.2) using a multichannel micropipettor and the port-loading guides provided with the cartridge, as shown in **Table 2**.”

16. Line 384:

Editor comment: “After loading with loading solution? “

Reply: After loading ports A, B, and C with compounds. “4 Incubate the loaded sensor cartridge at 37 °C in a CO_2-free incubator while setting up the program.”

17. Line 389:

Editor comment: “Please include an example of condition used in your experiment?“

Reply: This information has been added to the text
“4.4.1. Open the extracellular flux analyzer software, and using the *Group Definitions* and *Plate Map* tabs indicate groups of wells that have similar conditions (for example, wells with the same number of cells, or wells with either resting cells or IL-15-stimulated cells). Also, indicate background correction wells (by default A1, A12, H1, and H12 will be set, but additional wells can be used) and empty wells.”

18. Line 400:

Editor comment: “Few steps are missing here, what happens after you put the assay plate? When and how do you inject the compounds in the plate? Which port has what compounf etc. For how long is this done?“

Reply: The run is fully automated. This is indicated now.
“4.4.3 Begin the program using the *Run Assay* tab. Place the sensor cartridge (hydrated and loaded with 10x compounds) and utility plate onto the tray when prompted. After the calibration step (15 – 20 min), replace the calibrant plate for the assay plate (without lid) with attached cells. After this, the run is fully automated (the machine will perform measurements and injections).

NOTE: It is possible to perform a mitochondrial stress test and a glycolytic stress test in the same plate, as long as the specific compounds are loaded into the proper ports (oligomycin, DNP and antimycin/rotenone in ports A, B and C respectively of the wells where a mitochondrial stress test is performed; glucose, antimycin/rotenone and 2-DG in ports A, B and C respectively of the wells where a glycolysis stress test is performed), the same volumes for injections are used in each series of ports and wells for each test are properly identified using the *Group Definitions* and *Plate Map* tabs of the software.

4.4.4. After the completion of the run, retrieve the data and analyze them using the software.”

19. Line 434:

Editor comment: “Which reagent?”

Reply: The cell proliferation assay reagent
“5.1.4. Add 200 µL of the cell proliferation assay reagent to each well.”

20. Line 440:

Editor comment: “Please reword this part as it matches with the previously published literature. Other option is to just include citation instead.”

Reply: This part has been reworded

“**5.2. Protein content determination**

5.2.1. Carefully, aspirate completely the assay medium from each well without touching the cells and freeze the cells at -20^{o}C for at least 1 hour. Alternatively, the cells can be kept frozen for longer times (up to 1 week) until the analysis is performed.

5.2.2. Add 50 µL of radioimmunoprecipitation assay (RIPA) lysis medium supplemented with 1x protease inhibitors (from a 100x stock solution) to each well. Place the plate on a shaker for 5 min at room temperature, and then incubate the plate on ice for 30 min for a complete cell lysis.

5.2.3. Centrifuge the plate for 5 min at 200 *x g* at room temperature. This step will pellet cellular debris to prevent interference with the protein measurement.

5.2.4. Measure protein concentration by bicinchoninic acid (BCA) assay according to manufacturer's recommendations.”

21. Line 571:

Editor comment: “Please label the x-axis.”

Reply: The x-axis in Figure 5 has been labeled as “Time(min)”.