

**RE: Revisions for our JoVE submission JoVE58841-[EMID:3957d6015f7f3e00]**

30th October, 2018

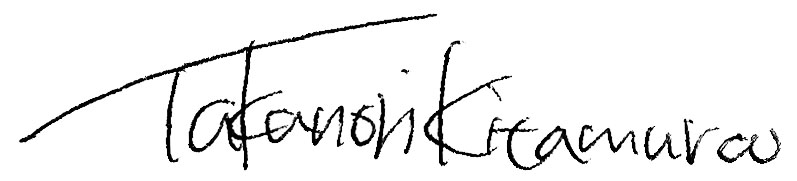
Dear Editor and reviewers,

Thank you so much for your time to review our manuscript. We have submitted the revision of our manuscript "Real time detection of in vitro tumor cell apoptosis induced by CD8+ T cells to study immune suppressive functions of tumor-infiltrating myeloid cells" by Kitamura et al.

We have addressed all comments from the editor and reviewers, and added new figures and extended explanations to the revised manuscript. Please see the following Point-by-point Response (original comments and our responses are written in black and blue respectively).

We hope this paper is now acceptable for publication in the “Journal of Visualized Experiments”

With best regards,



Takanori Kitamura

**Point-by-point Response**

**Editorial comments:**

1) Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

🡪 We have proofread the article and corrected typos and grammatical errors.

2) Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

🡪 We have added this information in the first paragraph of the Protocol.

3) Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:

1.2: Please mention how to confirm that cells are negative for mycoplasma.

1.3-1.5, 4.5: Please specify incubation temperature throughout the protocol.

2.1: Please specify the age and gender of mice. Are the mice anesthetized before injection?

2.3: What volume of PBS is used to wash? How many times?

2.5: Please provide composition of E-DMEM. Refer to Table of Materials if it is purchased.

2.6: How to count the number of live cells?

3.1: Please specify the euthanasia method and mention how to isolate the spleen.

3.2: What is used to grind?

3.5: What is used to adjust the cell concentration?

3.6: Please describe how this is actually done.

3.9: Please add a sub-step that describes the use of flow cytometry.

5.2.1: Please mention the reaction conditions for trypsinization.

5.2.2: How to adjust the density?

🡪 We have added sentences and information to address the above comments.

4) 7, 8 and their sub-steps: Software must have a GUI (graphical user interface) and software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.) to your protocol steps.

🡪 We used the commercial software specifically designed for this microscope system for the analysis, so we do not think that it would be reasonable to describe the analysis steps in such minute detail (i.e steps which refer to elements in the specific GUI for this software). Since, as stated in the manuscript we believe that the principles underlying the analysis can be adapted to other image analysis software, we have elaborated the manuscript to further describe the key parameters necessary for the image analysis which readers can adapt using their preferred software package (Sections 7 and 8). We have also added a link to the online Technical Note for the specific software we used in the Table of Materials, so that readers using the same software can use it as a resource.

5) After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

6) Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

7) Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

🡪 We have highlighted Steps 4-6 in the text, and attached a file showing the relevant sentences (Steps for Video).

8) Line 384: Please remove commercial language (Essen Bioscience).

🡪 We have removed the word “IncuCyte” and “Essen Bioscience”.

9) Figure 2: Please make the scale bars more readable.

🡪 We have substituted a scale bar that is more readable. We have also changed the colors of arrowheads based on Reviewer’s comments.

10) Figures 3-5, supplementary Figure 2: Please change “hr” to “h” for the time unit. Please include a space between the number and the unit of the scale bar in Figure 3.

🡪 We have changed this as suggested.

11) Please upload Figure 3 individually to your Editorial Manager account as a .png, .tiff, .svg, .eps, .pdf, .psd, or .ai file.

🡪 We have uploaded the files as requested.

12) Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique.

🡪 One of the critical steps is distinguishing apoptotic target cells from dying effector cells. In the protocol section, we have mentioned this point and listed controls that should be used (Step 5.7) as well as detailed parameters to set up the masks for accurate detection of apoptotic targets (Steps 7.1 – 8.4). We have also discussed limitations of the current protocol and potential solutions within the Discussion.

13) Table of Equipment and Materials: Please provide lot numbers and RRIDs of antibodies, if available.

🡪 We have added the lot numbers.

14) References: Please do not abbreviate journal titles.

🡪 We have changed this as requested.

**Reviewers' comments:**

**Reviewer #1:**

Manuscript Summary:

This manuscript presents a real-time monitoring method for T-cell mediated tumor cell lysis that can be used to measure the effect of immunosuppressive or immunostimulatory factors, such as immunosuppressive TAMs and MDSCs. The authors provide a very good assessment of existing methods and their shortcomings. This could be a powerful technique with broad applications. Overall, this manuscript has potential, but it needs to address some issues and undergo some revision.

🡪 Thank your for your careful reading and constructive comments.

Major Concerns:

Figure 2 and 3 are a bit confusing to interpret. These are the key figures that the entire paper depends on, but they are not convincing. The movies didn't do much to resolve this. Figure 2: It seems like there's quite a bit of variability in the red expression. It would be helpful to also show the red and green signals without the phase contrast overlay. The one cell that was identified as dead has so many effector cells over it that it is difficult to discern if the cas-3 is fluorescing in the target or effector cells. It's obscured by the size exclusion mask. Maybe zoom in on that better or pick a different field to show. It would be nice to include what the purple and teal masks and the filled arrow are in a figure legend, like the "excluded from the mask" arrow is. This would be extremely helpful for Figure 3 also. I also would like to see more fields shown. Why are so many more effector cells dying than target cells? Is that normal for these types of coculture experiments?

🡪We have changed Figure 2 to show a representative field with higher magnification. We have also added images without phase contrast and legends in the figure.

🡪 It is indeed normal and inevitable that more dead effector cells will be present than target cells in this type of co-culture assay. Since we added a 4-fold higher number of effector cells compared to target cells, green (apoptotic) effector cells were more frequently found in each field. However, this does not present a problem in detecting apoptotic cancer cells as we can exclude the smaller apoptotic effector cells from analysis by use of the size-restriction mask and detect only instances of red/green overlap as apoptotic nuclei of dying cancer cells.

Figure 3: What is going on with the small, rounded target cells in the second row. They look like they are dead or dying but are not fluorescing green. Is this a potential problem for data analysis? Also, the loss of the red signal in your apoptotic cell could complicate your colocalization analysis. It would be helpful if it were more clear that the arrows are showing the same cells in each timepoint. Pick colors other than blue and purple. Maybe yellow for the dead cells? Since we expect colocalization, it would be more intuitive of a color choice. Adding a legend for what the arrows mean would make interpretation of the figure more straightforward.

🡪 The small rounded target cells represent the cancer cells undergoing mitosis, which can be seen in the video. Existence of the mitotic cells does not affect data analysis, as these cells are not undergoing apoptosis (and thus are not showing green fluorescent signal). In order to make this point clear, we have shown the images as a supplementary figure and explained this in the text (second paragraph of Representative Results).

🡪 We have changed the colors of the arrowheads based on the suggestion. We have also modified a legend to explain that arrowheads are tracking the same cells through the different fields/time point.

There are entirely too many mis-numbered and mis-referenced sections, figures and figure legends. This became more frustrating as the paper went on. In many places, more details are warranted. It would be helpful to have more information about the most important reagents (Lenti and cas-3) within the text rather than only relying on the table.

🡪 We have addressed the issues of mis-numbering and referencing in the revised manuscript and have also elaborated upon many details throughout the manuscript. The journal guidelines do not permit us to name the commercial reagents or provide in-depth explanation of their details in the text. We have thus provided the sources and catalogue numbers in Table of Materials.

Minor Concerns:

1) Figure 4 and all the supplementary figures are duplicated.

🡪 This is caused by an unexpected system error. We have fixed the problem.

2) Figure 5: It would be more appropriate to consider the statistical analysis of the data in its entirety rather than pairwise comparisons at each timepoint. Also, what is the statistical test used? Instead of comparing to target only, would it be more appropriate to compare to the E + T to show that you're actually getting statistically significant reduction in killing? The current results showed that statistically significant killing is occurring even with the suppressors.

🡪 Following your suggestion, we have changed the analysis to determine the area under the curve, where the curves depict the apoptotic fraction of the target cell population at each time point. We then used unpaired t-test with Welch’s correction to determine statistical significance.

3) Supplementary Figure 1 Eliminate the contour plots. They don't add anything to the analysis. Your gating for MAMs is wrong. You're clearly including the lower portion of the MDSC population into your gate. Therefore, all of your MAM data is contaminated with MDSCs.

🡪 We have deleted the contour plots.

🡪 M-MDSCs and MAMs are distinguished as CD11bhighLy6Chigh and CD11bhighLy6Clow respectively. The threshold of Ly6C level to distinguish MAMs and M-MDSCs is based on that of resident alveolar macrophages (RMAC). We have mentioned this point in the text (2.4). It has been reported that MAMs and M-MDSCs isolated by this gating strategy have different morphology, distinct gene expression profile, and different mechanisms to suppress CD8+ T cell cytotoxicity (*Frontiers in Immunology* (2018) 8:2004).

4) Supplementary Figure 3: The lack of proliferation in the CD3/28 antibody stimulation condition is troubling.

🡪 We found a significant increase in the proliferation of T cells cultured with CD3/CD28 antibody, this was further increased by a co-culture with MAMs or MDSCs.

5) Reference the methods table somewhere early in the text so that we know it's here. You said you used 70 & 40m cell strainers in the text but your table says 100 and 40m.

🡪 We added the following sentence in the first paragraph of the Protocol; “Information about commercial reagents and equipment are listed in a supplementary Table of Materials.”

🡪 We used 100 and 40 μm strainers, and corrected the typo in the text.

6) Some specific thoughts while reading include, labelled by line number:

51 How long is the cas-3 substrate detectable after a cell has undergone apoptosis?

🡪 It is difficult to determine exactly how long the caspase-3 signal lasts as the cells migrate across the plate bottom and some cells undergo mitosis during the experiment making it difficult to track individual cells without using much shorter timepoints than employed in these experiments. However, based on our time-lapse images, we estimate that green fluorescence from the caspase substrate was detectable for 15 – 20 h after first appearing in a cell undergoing apoptosis.

62 "Progress" seems like an odd word choice.

🡪 We changed the word “progress” to “initiate”.

101 Clarification of "metastasis-associated macrophages" compared to tumor-associated macrophages would be helpful.

🡪 We have added a paragraph explaining MAMs and M-MDSCs (the last paragraph of Introduction).

106 Why are figures 1 & 2 duplicated?

🡪 This is simply an error in uploading the files. We have fixed the problem.

114 Would this approach be appropriate for human cells as well? It seems like it would, but addressing that would help potential adopters of this technique.

🡪 Technically, it is possible to apply our assay to investigate human cells, as activation antibodies against human CD3 and CD28 are commercially available, and a protocol to isolate human TAMs from clinical samples has been established. We have described it in Discussion.

125 Define "low". What is the target cell number or density at lentivirus addition?

🡪 We have changed the sentence as following; “If the proliferation rate of target cells is low (population doubling time greater than 36 h), the number of cells can be increased to 1x104.”

135 How long is necessary to incubate with puromycin at this dose. Also, that dose may not be effective for all cell types.

🡪 We have explained that one should “culture the cells until they are 80-90 % confluent”. We also added the following note: “Concentration of puromycin will be different between target cell types, and should be optimised using un-transfected cells.”

160 Could you include references to support your choice of markers, especially since you're using "MAMs" instead of "TAMs".

🡪 We included references that show gating strategy to detect MAMs (references 10, 11, and 14).

166 E-DMEM needs to be defined here as well.

🡪 We defined the contents of E-DMEM in steps 2.5 and 3.8.

213 This should be moved to 4.4. Why is CSF1 in T cell activation medium? They shouldn't express the receptor.

🡪 We moved it as suggested. CSF1 is not required for T cell activation, but is essential for survival of MAMs and M-MDSCs. We have mentioned this as a note for Step 4.3.

217-219 Number references are wrong.

🡪 Thank you for pointing out this error. We have corrected the typo.

238-252 Stick with scientific notation for cell numbers for consistency.

🡪 Based on the suggestion, we utilized scientific notation throughout the text.

245 I would like to see data on purity. Mouse TAMs release from the plastic with vigorous pipetting.

🡪 We cannot perform a purity check due to the small number of cells. Instead, we have attached some images demonstrating that mouse MAMs are not detached from the well after the collection of co-cultured T cells. We have also shown the representative images showing the morphology of T cells and MAMs cultured in an imaging plate. As shown in the figure, MAMs are obviously larger than T cells and show granules in their cytoplasm. Since we don’t find such a cell type in our time-lapse images, the contamination of MAMs must be negligible.

264 Define "too many". What are approximate cell density limits?

🡪 Our preliminary experiments suggest that an initial effector to target ratio in 8:1 (i.e., total 9x103 cells per well) presents difficulty in distinguishing apoptotic target cells from effector cells in our microscope system due to the increased likelihood of aggregates of apoptotic effector cells overlaying non-apoptotic target cells being falsely detected as co-localization. We have mentioned this point in the note of Step 5.7.

267 "Should" is too gentle. Include a list somewhere of all of the controls that are needed for proper data analysis.

🡪 We have modified this in the note for Step 5.7 as follows: “Wells containing the following four controls are necessary to aid with data analysis: target cells at the density used for the co-culture wells (1x103 cells/well) in medium with and without caspase-3 substrate, effector cells (1x103 cells/well) in medium with and without caspase-3 substrate.”

284 What are the actual fluorophores used?

🡪 We used mKate2 for the nuclear-restricted red fluorescence and a fluorophore with excitation at 488nm for the green fluorogenic activated caspase-3 substrate. We have added this information in the text (Step 6.2), and listed the associated commercial reagents in the Table of Materials.

288 How long is the cas-3 detectable for after a cell undergoes apoptosis? Showing control data on that would be very helpful.

🡪 As mentioned above, it is difficult to determine exactly how long the caspase-3 signal lasts as it is difficult to track individual cells without using much shorter timepoints than those employed in these experiments. As this is a population-based experiment and we calculated the fraction of the target cell population that is apoptotic at any particular time point, this does not present a problem for analysis.

314 It sounds like you're including a significant portion of the effectors in your size exclusion mask. A figure with the nuclear sizes of the target and effectors with where to set the threshold would help others.

🡪 We have updated the methods (Section 7) to list the specific values we used with the specific software employed in these experiments. We have also updated Figure 2 to better depict how the size exclusion mask allows detection of apoptotic target cells.

320 Give more detail on this step and the data analysis in total. Also, your figures clearly show the loss of the red nuclear signal with the gaining of cas-3 signal, so you shouldn't actually get colocalization of signal. How does the analysis address this very significant issue?

🡪 As seen in Figure 2 there is a period of time during which there is co-localization of the 2 signals that is evident when the individual fluorescent channels are examined. We have updated Figure 2 to show the green and red fluorescent channels in isolation, which makes this co-localization clearer.

334 Is dividing or subtracting more appropriate here?

🡪 We have updated our analysis to express the data as the fraction of the population that is apoptotic at each time point. For this analysis procedure, dividing is appropriate particularly as there may be small differences in the number of cells in each well or image and expressing the apoptotic cells as a fraction of the population gives greater accuracy.

367 Figure 3 doesn't really represent that well for MDSCs.

🡪 Given this comment, we have selected another representative field and shown it with higher magnification, which shows that target cells are associated with T cells but do not undergo apoptosis.

391-2 The colors used for the masking aren't ideal.

🡪 We have changed the colors for the mask. We have also replaced the images with those of higher magnification, which we believe makes it clearer how each mask works to produce more accurate analysis.

Page 10 There are a bunch of mis-referenced figure numbers here and you're missing an entire legend.

🡪 We are sorry about the errors. We have scrutinized the manuscript and corrected the errors.

441 This seems counter to what other groups have shown. Do you have any other references to support this conclusion?

🡪 So far, there is no reference reporting the enhancement of CD8+ T cell proliferation by macrophages. However, we have reproducibly found that MAMs enhance antibody-induced proliferation of CD8+ T cells, whereas the expanded CD8+ T cells exert less cytotoxicity. This is a novel finding that emphasizes the importance of the cytotoxicity assay to evaluate the immune suppressive function of macrophages.

453 Did you ever titrate the target cell numbers? That could possibly confirm or refute your conclusion.

🡪 This is a valid point. Since we have not titrated target cell numbers we have removed the line in the manuscript, which speculates on the effect of target cell mitosis on the level of apoptosis observed.

468-9 Can you quantify that and show it? That would be a huge power of this method that you're not really exploiting.

🡪 Technically it is possible to quantify the frequency and duration of effector-to-target interaction utilizing the basis of this assay system. However, it requires the acquisition of higher magnification images with shorter imaging intervals, which is not suitable for the scope of this protocol, i.e., to determine apoptotic fraction of target cell population. We intend to investigate this in future studies into the mechanism underlying suppression.

470 Only that many cells per well.

🡪 We added the word “per well” (the third paragraph in the Discussion).

486 I find it difficult to believe that your microscope signal doesn't have a DAPI channel.

🡪 The microscope that we use was specifically developed for live cell imaging particularly long-term live cell imaging (days or weeks) and the microscope resides in the cell culture incubator (please refer to Table of Materials for manufacturer and specific model). The manufacturer likely does not include a DAPI channel as the excitation for DAPI is in the ultraviolet range of the spectrum and repeated excitation over long-term imaging may cause DNA damage of the experimental cells leading to cell death.

516 What's the difference between this manuscript and reference 6?

🡪 In the reference 6, the apoptotic target cells are detected by flow cytometry or fluorescent microscopy at a fixed time point. In contrast, our assay enables acquisition of time-lapse images and thereby identification of the time of peak apoptosis. As we described in the first paragraph of the Discussion, this is one major advantage of this assay since determination of an optimal time point is important for appropriate comparisons between different conditions. Furthermore, analysis masks utilized in our protocol (target detection mask in red channel, size restricted apoptosis mask in green channel, and their combination, red/green overlap mask) enable more accurate detection of apoptotic target cells.

**Reviewer #2:**

Manuscript Summary:

In manuscript entitled "Real time detection of in vitro tumor cell apoptosis induced by CD8+ T cells to study immune suppressive functions of tumor-infiltrating myeloid cells" Takanori Kitamura and co-authors present in vitro multi-step assay for evaluation of: a) immunosuppressive function of murine MDSCs and TAMs; b) CD8 T cell cytolytic activity against tumor cells. Using real time fluorescence microscopy authors were able visualize the T cell-mediated apoptosis of tumor cells.

This is interesting novel approach to evaluate both immunosuppressive activity of myeloid cells and T cell anti-tumor activity using one multi-step assay. However, several concerns that should be clarified by authors.

Major Concerns:

1) Authors using CD3/CD28 Ab pre-activated CD8 T cells for generation of tumor cell cytolytic (CTL) activity. Since, no antigen has been used for generation of CTLs, authors should mention that this assay is antigen non-specific.

🡪 We have mentioned it in the fifth paragraph of the Discussion.

2) It is not clear, why authors cultured isolated tumor-infiltrating macrophages and m-MDSCs in the presence of recombinant M-CSF. Recombinant M-CSF is potent inductor of macrophage differentiation, which drives differentiation of monocytic MDSCs toward macrophages. As a result, authors evaluated immunosuppressive activity of macrophages only (MAMs and MDSC-derived macrophages).

🡪 CSF-1 (M-CSF) is an essential growth factor for survival of classical monocytes and their derivatives including M-MDSCs and MAMs. We thus need to add recombinant CSF-1 in the culture to maintain the viability of the suppressor cells. The same medium was used for the T cell co-culture with target cancer cells to keep conditions consistent. CSF-1 is found in nano-molar concentrations in all tissues, and is required for monocyte/macrophage viability *in vivo* and thus this is a physiological context for these cells. We have mentioned this point in the note for Step 4.3.

🡪 Using this protocol, we have identified that cytotoxicity of CD8+ T cells cultured with MAMs and M-MDSCs were suppressed via different mechanisms (i.e., CTLA4 and ROS mediated mechanism respectively). It is therefore likely that this assay can evaluate the immunosuppressive activity of both MAMs and their progenitor M-MDSCs.

3) It would be interesting to know whether MAMs (F4/80+/Ly6c-) or tumor-infiltrating m-MDSCs (Ly6c+) are able to kill syngeneic T cells via apoptosis?

🡪 As shown in Supplementary Figure 3, MAMs and MDSCs isolated from the metastatic lung of C57BL/6 mice promote, rather than suppress, the proliferation of CD8+ T cells from syngeneic mice. Furthermore, we did not find a significant reduction in the ratio of live CD8+ T cells cultured with MAMs or MDSCs compared to the cells in mono-culture. It is therefore unlikely that tumor-infiltrating myeloid cells induce apoptosis of T cells in our model.

Minor Concerns:

None

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**Figure for Reviewer#1. The contamination of MAMs in our time-lapse imaging should be negligible.**

**A)** Representative images of wells in the first culture plate before (top row) or after (bottom row) collecting CD8+ T cells. CD8+ T cells were cultured for 4 days without (left column) or with (right column) MAMs, and were collected by gentle pipetting as described in the Protocol. After the collection of T cells, large attached cells (i.e., MAMs) were found in a well of co-culture, which were not found in a well of T cell mono-culture.

**B)** Representative images showing the morphology of CD8+ T cells and MAMs cultured in an imaging plate. CD8+ T cells and MAMs were isolated as described in the Protocol, and seeded into wells of the second culture (imaging) plate. After 18 h, images of the cultured cells were acquired. MAMs are obviously larger than T cells and show granules in their cytoplasm.