

## Phagocytosis Assay to Measure Uptake of Necroptotic Cancer Cells by BMDCs

Tania Løve Aaes<sup>1,2</sup>, Dmitri V. Krysko<sup>1,2,3</sup>, and Peter Vandenabeele<sup>1,2,4,\*</sup>

<sup>1</sup>Molecular Signaling and Cell Death Unit, VIB Inflammation Research Center, Ghent, Belgium; <sup>2</sup>Department of Biomedical Molecular Biology, Cancer Research Institute Ghent (CRIG), Ghent University, Ghent, Belgium; <sup>3</sup>Department of Basic Medical Sciences, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium; <sup>4</sup>Methusalem Program, Ghent University, Ghent, Belgium  
\*For correspondence: [peter.vandenabeele@irc.vib-ugent.be](mailto:peter.vandenabeele@irc.vib-ugent.be)

**[Abstract]** This protocol is a flow cytometry-based method to measure the phagocytosis efficiency of necroptotic target cells by bone marrow-derived dendritic cells (BMDCs) *in vitro* (Aaes *et al.*, 2016). The method is a slightly modified and updated version of the protocols used in previously published papers (Krysko *et al.*, 2006; Brouckaert *et al.*, 2004). In brief, the target cells are labeled with a CellTracker™ dye before they are induced to undergo cell death. After a co-culture period of 2 h with BMDCs, the cells are immunostained with a dendritic cell marker and dead cell marker, and the phagocytic efficiency is quantified using a flow cytometer. This protocol can readily be used for target cells undergoing cell death modalities other than necroptosis.

**[Background]** Studying the phagocytic uptake of necroptotic cells by BMDCs *in vitro*, is a preliminary step in the examination of immunogenic cell death models (Obeid *et al.*, 2007). Efficient uptake will allow the phagocyte to cross-present antigens to leukocytes and thereby create an immune reaction towards the dead target cells. In this protocol we make use of a CellTracker™ dye. This type of dye may be toxic in certain concentrations, which may vary depending on which cell type is used. Thus, we recommend users to first find the optimal concentration of the dye for the target cell in use. Optimally, the CellTracker™ dye itself should not induce any cell death, but should label the target cells so that they become easily separable from the CD11c-positive BMDCs.

### Materials and Reagents

1. Delta treated 10 cm Petri dishes (Thermo Fisher Scientific, Nunc™, catalog number: 153066)
2. 15 ml polystyrene centrifuge tubes (Corning, Falcon®, catalog number: 352095)
3. 6-well suspension plates (SARSTEDT, catalog number: 83.3920.500)
4. 96 V well, 2.0 ml polypropylene plate (Greiner Bio One, MASTERBLOCK®, catalog number: 780285)
5. 5 ml round-bottom polystyrene tubes (Corning, Falcon®, catalog number: 352054)
6. Bone marrow-derived dendritic cells (BMDCs) isolated from BALB/c WT mice (see Procedure)
7. CT26 Necroptosis-inducible cells (DD\_RIPK3) (Aaes *et al.*, 2016)
8. Dulbecco's modified Eagle medium (DMEM), high glucose (Thermo Fisher Scientific, Gibco™,

- catalog number: 41965062)
9. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10270106)
  10. Sodium pyruvate (Sigma-Aldrich, catalog number: S8636)
  11. L-glutamine (Lonza, catalog number: BE17-605F)
  12. Roswell park memorial institute (RPMI) 1640 medium (Thermo Fisher Scientific, Gibco™, catalog number: 52400025)
  13. 2-mercaptoethanol (50 mM) (Thermo Fisher Scientific, Gibco™, catalog number: 31350010)
  14. Murine GM-CSF (VIB Protein Service Facility, UGent-VIB Inflammation Research Center)
  15. ACK lysing buffer (Lonza, catalog number: 10-548E)
  16. Dulbecco's phosphate-buffered saline (DPBS) (Thermo Fisher Scientific, Gibco™, catalog number: 14190094)
  17. CellTracker™ green CMFDA dye (Thermo Fisher Scientific, Molecular Probes™, catalog number: C7025)
  18. Doxycycline (Sigma-Aldrich, catalog number: D9891)
  19. B/B homodimerizer (Takara Bio, catalog number: 635059)
  20. Purified rat anti-mouse CD16/CD32 (Mouse BD Fc Block™) (BD, Pharmingen™, catalog number: 553142)
  21. APC hamster anti-mouse CD11c (BD, Pharmingen™, catalog number: 550261)
  22. SYTOX® blue nucleic acid stain (Thermo Fisher Scientific, Molecular Probes™, catalog number: S11348)
  23. BD FACSuite CS&T Research Beads Kit (BD, FACSuite™, catalog number: 650621)
  24. DMEM CT26 culture medium (see Recipes)
  25. RPMI BMDC/Co-culture medium (see Recipes)
  26. FACS buffer (see Recipes)
  27. Staining solution (see Recipes)

## **Equipment**

1. Table top centrifuge
2. BD FACSVe™ flow cytometer (BD, model: BD FACSVe™)

## **Software**

1. BD FACSuite™ software (BD)
2. FlowJo software version 10.0.8 or newer (FlowJo)

## **Procedure**

Bone marrow cells were isolated by crushing the femurs and tibias of 7-week-old BALB/c WT mice. Red blood cells were lysed with ACK lysing buffer, and the cells were differentiated into dendritic cells for eight days using RPMI culture medium – 400,000 cells per 2 ml per 6-well. On day three, 2 ml of fresh culture medium was added, and on day six the medium was replaced with fresh culture medium.

### **A. Cell death induction in target, CT26 DD\_RIPK3, cells**

1. Seed  $1 \times 10^6$  CT26 DD\_RIPK3 cells in 10 ml DMEM culture medium per 10 cm Petri dish.
2. Wait 5 h for the cells to attach.
3. Wash the Petri dishes with serum-free DMEM.
4. Add 10 ml of serum-free DMEM + 1  $\mu$ M CellTracker™ green CMFDA to each plate.
5. Incubate for 30 min at 37 °C.
6. Remove the medium, and wash the plate with RPMI culture medium.
7. Add 10 ml RPMI culture medium to each Petri dish, incubate for 2 h at 37 °C.
8. Induce cell death by adding 1  $\mu$ g/ml doxycycline + 10 nM B/B homodimerizer.
9. Incubate for another 18 h at 37 °C.

### **B. Co-culture with BMDCs**

1. Collect the BMDCs and CT26 target cells into separate 15 ml Falcon® tubes
  - a. Live CT26 cells are collected by adding EDTA (2 ml per 75 cm<sup>2</sup>), incubating at 37 °C for 2 min, then collecting in RPMI culture medium (8 ml per 75 cm<sup>2</sup>). Centrifuge at 300 x g for 5 min.
  - b. Dead CT26 cells are collected by pipetting only the cells in solution. Centrifuge at 400 x g for 5 min.
2. Count the cells and resuspend them in RPMI culture medium.
3. Seed the cells in 6-well suspension plates.
4. Seed 400,000 BMDCs + 400,000 CT26 cells (ratio 1:1) in a total volume of 4 ml per well.
5. Incubate for 2 h at 37 °C.
6. Include wells with cells for single stain and compensation controls:
  - a. CT26 cells Non-stained target cell control
  - b. CT26 cells – cell death-induced SYTOX® blue single stain control
  - c. CT26 cells CellTracker™ green single stain control
  - d. BMDCs Non-stained BMDC control
  - e. BMDCs CD11c-APC single stain control

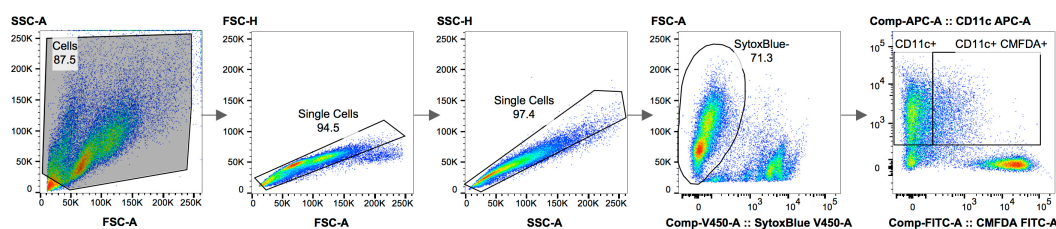
### **C. Immunostaining**

1. Transfer the cells to a deep 96-well plate.
2. Spin down at 400 x g for 5 min at 4 °C.
3. Discard the supernatant by flicking the plate, and resuspend the pellet in 200  $\mu$ l FACS buffer.

4. Spin down at 400 x g for 5 min at 4 °C.
5. Resuspend the samples in 200 µl of staining solution (or in single stain solution) with a final antibody concentration (APC-CD11c) of 0.1 µg per 8 x 10<sup>5</sup> cells.
6. Incubate for 30 min at 4 °C covered from light.
7. Wash the samples and spin down at 400 x g for 5 min at 4 °C.
8. Resuspend in FACS buffer + 1.25 µM SYTOX<sup>®</sup> blue (or in FACS buffer only for the single stained samples).
9. Transfer to 5 ml Falcon<sup>®</sup> round-bottom tubes.
10. Keep the samples on ice (for maximum 1 h) until acquisition on the flow cytometer.
11. On the flow cytometer use FACSuite<sup>™</sup> to acquire minimum 50-100,000 events per sample.

#### D. Gating strategy in FlowJo

True uptake of CMFDA-labeled dead cell material by BMDCs is determined using a gating strategy that allows analysis of only single cells and is determined as CD11c<sup>+</sup> CMFDA<sup>+</sup> double-positive cells expressed as a percentage of all CD11c<sup>+</sup> cells. See Figure 1 and Table 1 for the gating strategy and compensation matrix used in FlowJo.



**Figure 1. Gating strategy used for the analysis in FlowJo.** Figure 1 shows the gating strategy used, when analyzing the results in FlowJo. Using the forward and side scatter area (FSC-A vs. SSC-A), the debris is excluded from the analysis. In the following two steps, doublet cells are removed, by drawing a gate around the single cells, which roughly gather along the diagonal of FSC-A vs. FSC-H, followed by SSC-A vs. SSC-H. A Live/Dead staining, in this case SYTOX<sup>®</sup> blue, is used to eliminate dead cells. In the final panel, the live, SYTOX<sup>®</sup> blue-negative, cells can now be analyzed for phagocytosis by plotting Cell Tracker Green CMFDA (target cells) vs. CD11c-APC (BMDCs).

**Table 1. Compensation matrix generated in FlowJo for the analysis of the phagocytic BMDCs**

Compensation matrix	FITC-A::CMFDA	APC-A::CD11c APC-A	V450-A::SytoxBlue V450-A
FITC-A :: CMFDA FITC-A	100	0.0618	0.0835
APC-A :: CD11cAPC-A	0.4078	100	0.1801
V450-A :: SytoxBlue V450-A	1.7078	0.68	100

## **Data analysis**

1. Software
  - a. BD FACSuite™ software (BD)
  - b. FlowJo software version 10.0.8 or newer (FlowJo)
2. The experiment should be repeated a minimum of three times, each time including technical triplicates of each sample. The statistical difference between BMDC uptake of live cells versus that of necroptotic cells can be analyzed with a Mann-Whitney non-parametric *t*-test.
3. The phagocytic BMDCs (see gating strategy in the legend of Figure 1) are calculated as the percentage of double-positive CMFDA<sup>+</sup> CD11c<sup>+</sup> cells (BMDCs that have engulfed target cells) out of all CD11c<sup>+</sup> cells (the total BMDC population).

## **Notes**

To verify the efficiency of the cell death induction in the target cells, it is advised to run a cell death analysis on the flow cytometer in parallel with the phagocytosis analysis. Cell death analysis should include the nucleic acid staining combined with an Annexin V fluorescent probe (Aaes *et al.*, 2016). Tet-On induction for 18 h with doxycycline results in > 85% cell death.

## **Recipes**

1. DMEM CT26 culture medium  
*Note: Store at 4 °C; heat to 37 °C before use.*  
DMEM  
10% FBS  
1.3% Na-pyruvate  
1.4 mM L-glutamine
2. RPMI BMDC/Co-culture medium  
*Note: Store at 4 °C; heat to 37 °C before use.*  
RPMI  
5% FBS  
1.3% Na-pyruvate  
1.4 mM L-glutamine  
50 μM 2-Me  
20 ng/ml mGM-CSF
3. FACS buffer  
*Note: Store at 4 °C.*  
DPBS  
0.5% FBS

4. Staining solution

*Note: Prepare just before use, keep on ice.*

FACS buffer

Fc Block™ diluted 1/250

APC Hamster anti-mouse CD11c diluted 1/350

### **Acknowledgments**

Peter Vandenabeele's research group is part of the Cancer Research Institute Ghent (CRIG). Funding is supported by Interuniversity Attraction Poles (IAP 7/32), Research Foundation Flanders (FWO), Methusalem grants, Ghent University grants and the Belgian Foundation Against Cancer. The authors declare no conflicts of interest.

### **References**

1. Aaes, T. L., Kaczmarek, A., Delvaeye, T., De Craene, B., De Koker, S., Heyndrickx, L., Delrue, I., Taminau, J., Wiernicki, B., De Groote, P., Garg, A. D., Leybaert, L., Grooten, J., Bertrand, M. J., Agostinis, P., Berx, G., Declercq, W., Vandenabeele, P. and Krysko, D. V. (2016). [Vaccination with necroptotic cancer cells induces efficient anti-tumor immunity](#). *Cell Rep* 15(2): 274-287.
2. Brouckaert, G., Kalai, M., Krysko, D. V., Saelens, X., Vercammen, D., Ndlovu, M. N., Haegeman, G., D'Herde, K. and Vandenabeele, P. (2004). [Phagocytosis of necrotic cells by macrophages is phosphatidylserine dependent and does not induce inflammatory cytokine production](#). *Mol Biol Cell* 15(3): 1089-1100.
3. Krysko, D. V., Denecker, G., Festjens, N., Gabriels, S., Parthoens, E., D'Herde, K. and Vandenabeele, P. (2006). [Macrophages use different internalization mechanisms to clear apoptotic and necrotic cells](#). *Cell Death Differ* 13(12): 2011-2022.
4. Obeid, M., Tesniere, A., Ghiringhelli, F., Fimia, G. M., Apetoh, L., Perfettini, J. L., Castedo, M., Mignot, G., Panaretakis, T., Casares, N., Metivier, D., Larochette, N., van Endert, P., Ciccosanti, F., Piacentini, M., Zitvogel, L. and Kroemer, G. (2007). [Calreticulin exposure dictates the immunogenicity of cancer cell death](#). *Nat Med* 13(1): 54-61.