bio-protocol www.bio-protocol.org/e1974 Vol 6, Iss 20, Oct 20, 2016
DOI:10.21769/BioProtoc.1974

Quantification of Tumor Material Uptake

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[Abstract] Extracellular tumor material including exosomes, microvesicles and apoptotic tumor debris may help cancers invade new organs. Enhancing the removal of extracellular tumor material by immune cells represents a novel immunotherapy approach for preventing cancer metastasis. This protocol quantifies the uptake and removal of extracellular tumor material from circulation and tissues by immune cells. In this assay fluorescent tumor cells are transferred into mice, and then immune cells are quantified by either flow cytometry or imaging cytometry for their uptake of tumor material.

[Background] Recent studies have demonstrated that extracellular tumor material including exosomes, microvesicles and apoptotic tumor debris shed from tumors are important mediators of tumor metastasis, growth and evasion of the immune response (Vader *et al*., 2014; Pucci and Pittet, 2013; Pucci *et al*., 2016). Immune cells have the ability to remove, respond and transport this circulating tumor material (Hanna *et al*., 2015; Pucci *et al*., 2016; Headley *et al*., 2016). This protocol offers a novel approach to quantify tumor material uptake by specific immune cell populations, and may be adapted to test immune targets that regulate tumor material uptake. This protocol may assist in better understanding the immune response to extracellular tumor material, with the hope of eventually developing novel therapies targeting extracellular tumor material in cancer development.

Materials and Reagents

1. 30 gauge insulin syringe (BD, Ultra-Fine™, catalog number: 328431)
2. 15 ml tubes
3. 70 µm cell strainers (Thermo Fisher Scientific, Fisher Scientific, catalog number: 22363548)
4. 15 mm Petri dish
5. 5 ml syringe (BD, Luer-Lok™, catalog number: 309646)
6. 96-well v-bottom plate
7. Lewis lung carcinoma cells expressing red fluorescent protein (LLC-RFP), B16F10 green fluorescent protein (B16F10-GFP) or other fluorescent-tagged tumor cell line (AntiCancer.com, http://www.anticancer.com/Fluorescent_protein_cell_lines_April_2010.pdf)
8. Syngeneic recipient mice. C57BL/6J mice (The Jackson Laboratory, catalog number: 000664) for LLC and B16F10 tumors.
9. TrypLE (Thermo Fisher Scientific, Gibco™, catalog number: 12604013)

Vol 6, Iss 20, Oct 20, 2016
DOI:10.21769/BioProtoc.1974

10. Dulbecco's phosphate-buffered saline (DPBS) (GE Healthcare, Hyclone™, catalog number: SH30013.02)
11. UltraPure™ 0.5 M EDTA, pH 8.0 (Thermo Fisher Scientific, Invitrogen™, catalog number: 15575020)
12. Red blood cell (RBC) lysis buffer (10x) (BioLegend, catalog number: 420301)
13. Fc Block (CD16/32) (BD, Pharmingen™, catalog number: 553141)
14. Fluorochrome conjugated antibodies:
    CD11b-FITC (BioLegend, catalog number: 101206)
    CD115-APC (BioLegend, catalog number: 135510)
    Ly6C-APC-Cy7 (BioLegend, catalog number: 128026)
    CD45-BV421 (BioLegend, catalog number: 103134)
15. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A9418)
16. Live/Dead Fixable Blue (Thermo Fisher Scientific, Molecular Probes™, catalog number: L34962)
17. Sodium azide (Sigma-Aldrich, catalog number: S2002)
18. Flow buffer (FB) (see Recipes)
19. RBC lysis buffer (see Recipes)

Equipment

1. Cell culture flask
2. Centrifuge
3. Multiparameter flow cytometer with optimal excitation and detectors for tumor fluorophore (BD, LSRII or similar) or Amnis imaging cytometer (ImageStream X Mark II)

Software

1. ImageJ software (ImageJ)
2. FlowJo software (version 9.2)
3. Prism software (GraphPad Software)

Procedure

A. Tumor injection

1. Collect LLC-RFP tumor cells at about 70-80% confluency grown in a cell culture flask. Wash with DPBS two times and then gently trypsinize cells for approximately 3-5 min. We find that the greatest amount of reproducibility with tumors that are 70-80% confluent and highly viable (> 95%) when collected for injection.
   Note: If there is any question of tumor cell viability, test for viability by trypan blue staining or other methods after tumor collection.

Vol 6, Iss 20, Oct 20, 2016
DOI:10.21769/BioProtoc.1974

2. Resuspend cells in DPBS and centrifuge for 5 min at 300 *x g*. Repeat DPBS wash.
3. Wash tumor cell with DPBS two times and then gently trypsinize cells. Bring up cells in DPBS and centrifuge for 5 min at 300 *x g*. Repeat DPBS wash.
4. Count cells and bring up at 3 x 10^6 cells/ml.
5. Intravenously inject (in tail vein) 3 x 10^5 Lewis lung carcinoma cells expressing red fluorescent protein (LLC-RFP) or other fluorescent-tagged tumor cell line resuspended in 100 µl of DPBS into the tail vein using a 30 gauge syringe. Recipient mice should be syngeneic for the tumor cell line (C57BL/6J mice for LLC and B16F10 tumors). Optimal internalization of tumor material in circulating myeloid cells is observed between 4 and 72 h after intravenous injection of tumor, but tumor material observer can be observed in lung tissue for at least 3 weeks after injection.

B. Isolation of immune cells
1. Collect samples in 15 ml tubes filled with DPBS (with 2 mM EDTA).
2. Collect 200 µl-1 ml of blood, whole spleen, or whole lung.
3. Centrifuge 300 *x g* for 10 min for blood, and then lyse RBC with 10 ml of RBC buffer for 5 min.
4. Place a 70 µm filter in a 15 mm Petri dish and mechanical dissociate spleen or lung through filter using a 5 ml syringe plunger. Pour cell solution into 15 ml tube and then lyse with 2 ml of RBC buffer for 2-5 min.
5. Wash 1x with 10 ml DPBS and centrifuge 300 *x g* for 10 min (repeat lysis for blood if necessary).
6. Resuspend cells around 1 x 10^7 cells/ml in flow buffer (FB).

C. Staining of immune cells
1. Place ~2 x 10^6 cells/well in a 96-well v-bottom plate.
2. Wash cells in 200 µl in FB, centrifuge for 5 min at 300 *x g*, flick out wash (swing plate over sink to remove liquid).
3. Resuspend cells in 100 µl Fc Block (CD16/32) at a dilution of 1:200 (Make a master mix and add to cells using a multichannel pipettor).
4. Let sit on ice for 5 min.
5. Make a master mix of fluorochrome conjugated antibodies and viability dye to stain immune cell population of interest in FB. Avoid other red channels (PE, PE-Cy7 and Percp5.5) if assaying for LLC-RFP uptake and avoid green channels (FITC/A488/GFP) if assaying for B16F10-GFP. For example, to label myeloid cell populations after LLC-RFP injection we use CD11b-FITC, CD115-APC, Ly6C-APC-Cy7, CD45-BV421, and Live/Dead Blue, but this panel can vary depending on your cell of interest and tumor injected.
6. Wash cells in 200 µl FB, centrifuge for 5 min at 300 *x g*, flick out wash.
7. Resuspend cells in 100 µl of fluorochrome conjugated antibody master mix.
8. Stain compensation controls/beads with single antibodies.
9. Incubate on ice for 30 min.
10. Wash cells 2 x with 200 µl FB, centrifuge for 5 min at 300 *x g*, flick out wash.

Vol 6, Iss 20, Oct 20, 2016
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11. Resuspend in 200 µl of FB
12. Transfer 200 µl to analysis tubes and run samples on the flow cytometer or Amnis imaging cytometer.
13. Gate on live cells, then CD45$^+$ immune cell population of interest, and then RFP$^+$ or GFP$^+$ tumor material found in the immune cell population by flow cytometry (Figure 1) or image tumor material localization using imaging cytometer (Figure 2).

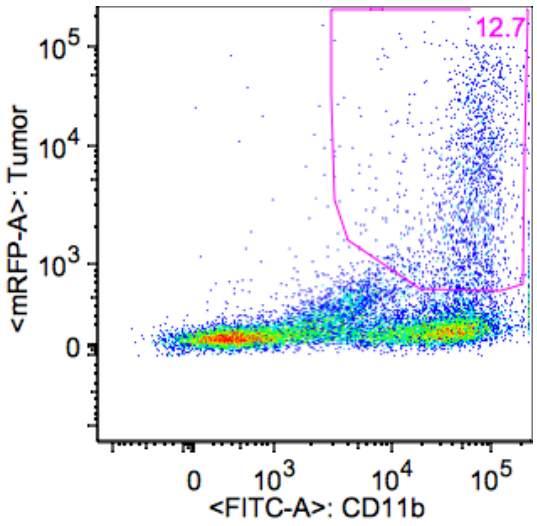

Figure 1. Gating of LLC-RFP$^+$ tumor material in CD11b$^+$ myeloid cells isolated from blood and analyzed by flow cytometry. Cells were gated on Live, CD45$^+$ immune cells before this analysis.

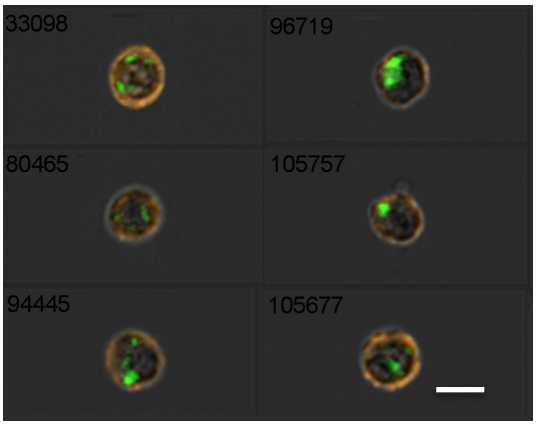

Figure 2. Imaging of B16F10-GFP tumor material uptake (Green) by CD11b$^+$ myeloid cells (Orange) isolated from blood and analyzed by imaging cytometry. Cells were gated on Live, CD45$^+$ immune cells before this analysis. Scale bar in lower right corner is 10 µm.

Vol 6, Iss 20, Oct 20, 2016
DOI:10.21769/BioProtoc.1974

Data analysis

For our flow and imaging cytometry analysis we usually quantify the percent of a cell population with tumor material or the relative amount or size of tumor material in each cell. Calculations of percentages of $CD45^+$ immune cells were based on live cells as determined by forward and side scatter and viability analysis. Average size of tumor material in cells can be measured from imaging cytometry data using ImageJ software. Cell fluorescence was assessed with an LSRII and was analyzed with FlowJo software (version 9.2). Data for all experiments were analyzed with Prism software. Unpaired *t*-tests and two-way analysis of variance were used for comparison of experimental groups. *P* values of less than 0.05 were considered significant. The data appeared to be normally distributed with similar standard deviation and error observed between and within experimental groups.

Notes

1. Always keep the immune cells on ice during collection and staining.
2. Work fast and don't let the samples sit too long before analysis as the internalized tumor material can degrade or lose fluorescence quickly. Analyze sample within 3 h of collection for optimal results.
3. Set detector fairly high on flow cytometer in channel detecting tumor material in order to detect small fragments of tumor engulfed by cells. Though there is no universal range for setting these detectors since cytometers setup and fluorophore brightness varies, detector settings should put tumor material in the upper log scale of the cytometers flow plots without going off scale.
4. For reproducibility, remove any mouse from analysis that was questionably IV injected with tumor as variability in the amount of tumor material injected can greatly affect the amount of tumor uptake measured in immune cells.

Recipes

1. Flow buffer (FB)
   10 g 1.0% BSA
   1 g 0.1% sodium azide
   1 L 1x DPBS
   Store at 4 °C.
2. RBC lysis buffer
   Dilute 10x from stock.

Vol 6, Iss 20, Oct 20, 2016
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Acknowledgments

This work was supported by NIH grants R01 HL118765 and R01 CA202987 (both to C.C.H.), American Heart Association Scientist Development Grant 125SDG12070005 (to R.N.H.), the La Jolla Institute for Allergy and Immunology Board of Directors Fellowship (to R.N.H.).

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