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Protocol for T-cell Adhesion Strength on Tumor Cells under Flow Conditions

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[Abstract] This method allows evaluating the relative adhesion strength between T lymphocytes and specific adherent target cells using a shear force in flow chambers. It is based on the measure of the resistance of conjugates formed between T cells and adherent tumor cells to shear stress in a microfluidic system. For this purpose, T cells, stained with a CellTracker probe, are added into flow channels containing a monolayer of adherent target cells and their progressive detachment under a constant shear stress is then recorded using a fluorescent microscope.

Material and Reagents

1. Adherent tumor cells [such as non-small cell lung carcinoma (NSCLC) cell lines)
2. Specific T-cell clones (generated either from autologous tumor-infiltrating T lymphocytes (TIL) or peripheral blood lymphocytes (PBL))
3. RPMI 1640 (Life Technologies, Gibco®, catalog number: 61870044)
4. DMEM-F12 (Life Technologies, Gibco®, catalog number: 31331093)
5. UltroserG (Pall, catalog number: 15950-017)
6. Fetal Bovin Serum (Life Technologies, Gibco®, catalog number: 10270-106)
7. Human serum AB (Institut de Biotechnologies Jacques Boy)
8. Penicillin and streptomycin (Life Technologies, Gibco®, catalog number: 15140122)
9. Sodium Pyruvate (Life Technologies, Gibco®, catalog number 11360029)
10. IL-2
11. 10x PBS (Life Technologies, Gibco®, catalog number: 70011-036)
12. CellTracker probe (CellTracker™ Green CMFDA) (Life Technologies, Invitrogen™, catalog number: C2925)
13. Complete DMEM: tumor cell culture medium (LC medium) (see Recipes)
14. RPMI-based T-cell complete medium (see Recipes)

Equipment

1. Microscope Zeiss LSM-510 (ZEISS) with a heated incubation chamber and CO_2 supply

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2. Micro-Slides VI, ibiTreat (ibidi GmbH, catalog number: 80606), two silicon tubes (1.6 mm of inner diameter) with a plastic clip, two Elbow Luer connectors (ibidi GmbH, catalog number: 80646)
3. Syringe pump (high flow rate > 50 ml/min)
4. 60 ml syringe (Becton, Dickinson and Company, catalog number: 300866)
5. Humidified incubator at 37 °C with 5% CO_2
6. A recipient for waste flow buffer (Erlenmeyer)
7. Centrifuge (Beckman Coulter, model: GS-6R)

Procedure

A. Adherent tumor cell preparation

1. Seed adherent tumor cells into IBIDI channels by adding 60 µl of tumor cell suspension in LC medium. Micro-slides VI, ibiTreat characteristics are the following:
   Number of channels: 6
   Minimal volume per channel: 30 µl
   Channel length: 17 mm
   Channel width: 3.8 mm
   Channel height: 0.4 mm
   Growth area: 0.6 cm^2 per channel
   Tumor cell concentration may vary according to the cell type (for instance: 1.6 x 10^6 cells/ml for NSCLC cell lines described in Reference 1). Cells should be at 90-95% of confluence the day of experiment.
2. Incubate the IBIDI slide in a humidified incubator at 37 °C for at least 2 h for cell attachment.
3. Fill gently the reservoirs with another 60 µl of LC medium. Avoid pipetting directly into the channels not to detach the cells.
4. Incubate overnight at 37 °C, 5% CO_2.
   Note: In case of tumor cell treatment (example siRNA transfection), cells should be plated two days before using the same experimental conditions. Medium may need to be changed every 24 h. Be sure that the cells are all alive and just reaching 90-95% confluence the day of experiment.

B. T-cell preparation

1. Wash T cells with PBS 1x by centrifugation at 350 *x g* for 5 min.
2. Stain cells with CellTracker Green (CMFDA) according to the manufacturer's protocol. Briefly, resuspend cells at 2 x 10^6/ml in PBS and add one volume of CellTracker Green (CMFDA 2x) diluted in PBS (final concentration 1 µM). Incubate for 15 min at 37 °C.

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3. Wash T cells twice with RPMI-based complete medium by centrifugation at 350 *x g*, 5 min.
4. Resuspend T cells in T-cell medium, at final concentration 2 x 10^6 cells/ml, in 24 flat bottom well plates.
5. Incubate T cells overnight in humidified incubator at 37 °C with 5% CO_2.

C. T-cell adhesion strength under flow conditions

1. The following day, warm the thermostatic chamber of the microscope at 37 °C and 5% CO_2 (Figure 1A).

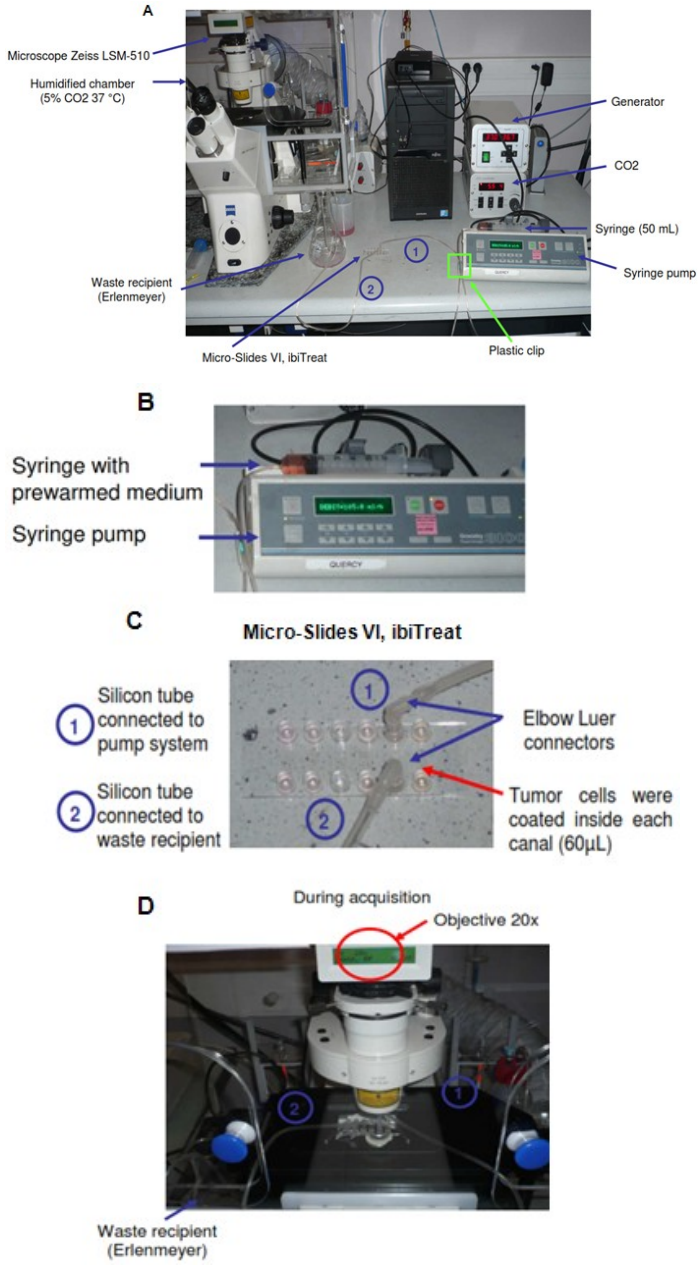

Figure 1. Flow system. A. Whole flow system; B. Pump system; C. Details of Micro-Slides IBIDI connections; D. During acquisition, the slide is fixed under the microscope and connected to the pump system.

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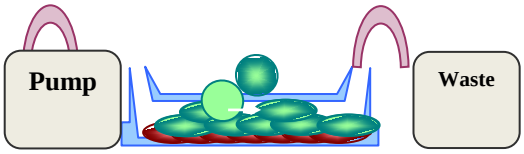
2. Equilibrate RPMI-based complete medium (500 ml) inside the incubator at 37 °C and 5% CO_2.
3. Prepare syringe pump (Figure 1B).
4. Connect the tube carrying a plastic clip (position closed) to the syringe and fill the syringe with prewarmed medium. Put the Elbow Luer connector and prime the tube (Figure 1A).
5. Put the IBIDI slide (Figure 1C) under the microscope objective (20x) (Figure 1D). Be sure that the reservoirs are completely full. If not, add some medium.
6. Connect the tube to one extremity of the IBIDI channel making sure there are no air bubbles remaining inside. This step is critical, because bubbles increase the risk of tumor cell detachment, influence the flow rate and can even stop the flow.
7. Use the second tube to connect the opposite extremity of the channel with a bottle collecting wastes (Figure 2).



Red: adherent tumor cell layer
Green: T cells
Blue: LC medium
White arrow: direction of the flow

Figure 2. T-cell adhesion under flow conditions. Stained T lymphocytes were incubated for 15 min on a monolayer of autologous tumor cells previously seeded into IBIDI channels. The IBIDI slide is then connected by silicon tubes, in one side to a pump (with a syringe filled with prewarmed medium) and in the other side to the waste recipient.

8. Release the clip on the tube connecting the syringe to the IBIDI slide (Figure 1A).

Note: Test the system to validate the maximal flow rate that doesn't detach tumor cells. This rate will be applied to determine the T-cell adhesion strength on tumor cells.

9. Wash T cells with RPMI by centrifugation at 350 *x g* for 5 min.
10. Resuspend T cells in RPMI medium at final concentration 2 x 10^6 cells/ml.
11. Replace the medium filling the channels with 50 µl of T cells suspension. Be careful to not detach tumor cells or introduce bubbles inside channels.
12. Incubate 15 min at 37 °C.
13. After incubation, prepare the flow system using the same conditions described for the test assay.
14. Add 50 ml pre-warmed medium inside the syringe.
15. Start the acquisition just before the flow (Figure 1D).

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16. Acquire images every 2 s for 60 s. It is expected that T cells adhere more firmly to tumor cells that express adherence molecules (such as ligands for integrins expressed by T cells) than tumor cells that do not express these molecules. (Figure 3)

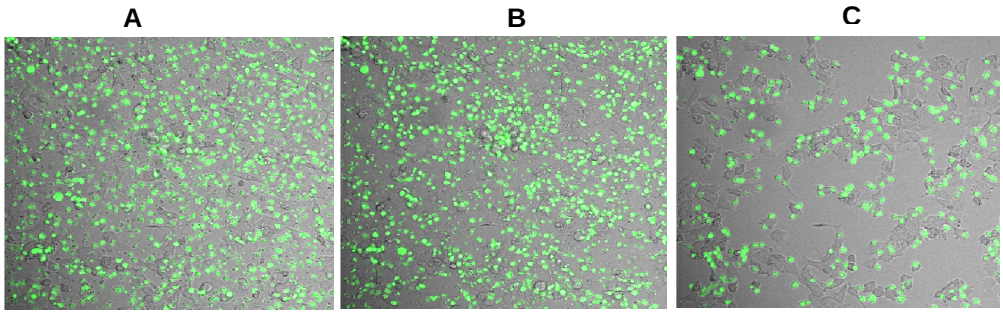

Figure 3. Representative images acquired at different time lapses during T cell adhesion protocol. A. 0 sec; B. 250 sec; C. 640 sec at the flow rate of 100 ml/h.

Recipes

1. Complete DMEM: tumor cell culture medium (LC)
   DMEM-F12 supplemented with
   10% decomplemented Fetal Bovine Serum
   1% UltroserG
   1% Penicillin and streptomycin
   1% Sodium pyruvate
2. RPMI-based T-cell complete medium
   RPMI 1640 complemented with
   10% Human serum AB
   1% penicillin and streptomycin
   1% of Sodium pyruvate
   IL-2 (100 U/ml)

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