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Combined Single-Cell Measurement of Cytokine mRNA and Protein in Immune Cells V.1

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We use this protocol in our group and it is working.

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Abstract

A key feature of immune cells, such as T cells, is their rapid responsiveness to activation. The response rate of T cells depends on the type of signals they receive, and the signal strength. Studying the underlying mechanisms that define responsiveness, however, is confounded by the fact that immune cells do not uniformly respond to activation. Tools that measure gene products on a single cell level therefore provide additional insights in T cell biology. Here we describe Flow-FISH, a high-throughput assay that allows for the simultaneous measurement of cytokine mRNA and protein levels of the gene(s) of interest by flow cytometry. We present several possible applications of Flow-FISH in human and murine T cells that - with minor adjustments - should also be applicable for other gene products and cell types.

This is a post-peer-review, pre-copyedit version of an article published in Methods in Molecular Biology, and is currently in press.

Attachments

Protocol.pdf 245KB

Figure1.pdf 913KB

Figure2.pdf 941KB

Figure3.pdf 263KB

Guidelines

NOTES

1. The authors have successfully studied the cytokine mRNA and protein levels in T cells isolated from fresh and cryopreserved human PBMCs, and from different mouse organs. Both unsorted bulk single cell solutions, FACS/MACS-isolated T cells and cultured T cells were used.
2. For reproducible results, 3 x 10^5 [human] or 1 x 10^6 [murine] cells per condition are recommended to limit cell loss during this assay. Authors found that cell loss can be up to 30-50% of the input due to e.g. fragility and stickiness of cells to tubes during the procedure. If studying lower cells numbers, irrelevant cells can be spiked in to prevent loss of the cells of interest. When performing Flow-FISH on other cell types, the required cell numbers may have to be reassessed.
3. For both human and murine $CD8^+$T cells, authors use 10 ng/mL PMA and 1 µM ionomycin (both Sigma Aldrich), or 1 µg/mL (human, clone Hit3a) / 2 µg/mL (murine, clone 17.A2, Bioceros) aCD3 and 1 µg/mL aCD28 (human clone CD28.2, murine clone PV-1) . When using peptide (pools), authors use between 1 and 5 µg/mL.
4. The authors prefer activation in 96-well V-bottom plate (human and murine) or Lo-Bind Eppendorf tubes (human), but if required, 96-well F- or U-bottom plates are also suitable.
5. Not all fluorophores are compatible with the buffers used in this protocol. For instance, some tandem dyes may disintegrate. **Table 1**depicts the fluorophores that the authors tested.
6. Human $CD8^+$ T cells were stained with Near-IR viability dye (BD Biosciences, 1:800), aCD4-AF700 (BD Biosciences clone RPA-T4, 1:200), and aCD8-BV605 (Biolegend clone RPA-T8, 1:200). When studying different T cell populations, this panel can be expanded with other surface markers as previously reported [29].
7. Murine $CD8^+$ T cells were stained with Near-IR viability dye (1:800), aCD8a-AF488 (Biolegend clone 53-6.7, 1:200), and aCD44-PE-Cy7 (Biolegend clone IM7, 1:300).
8. Labeling T cells with APC- and/or PE-labeled MHC class I tetramers is compatible with the Flow-FISH protocol and could be used to study antigen-specific T cells.
9. The authors recommend using an RNAse inhibitor without requirements for DTT, as DTT can affect the FISH-staining.
10. Due to the cell permeabilization, RNAs are exposed to RNAses from the outside. Therefore, supplementing buffers and reagents with RNAse inhibitors and the use of RNAse free tips is essential from this step onwards.
11. Human T cells were stained with aIFN-g-eF450 (eBioscience clone 4S.B3), aTNF-a-BV785 (Biolegend clone MAb11), and aIL-2-AF488 (Biolegend clone MQ1-17H12). All antibody dilutions: 1:100.
12. Murine T cells were stained with aIFN-g-PE or PE-Cy7 (clone XMG1.2), aTNF-a-PE (clone MP6-XT22) and aIL-2-PE or PE-Cy7 (clone JES6-5H4; all eBioscience). All antibody dilutions: 1:200.
13. Best results were achieved when measuring activation-induced gene products. This also facilitates the probe set-up and probeset validation.
14. The authors used Quasar 570 or 670-labeled probes (Biosearchtech) to simultaneously measure mRNA and protein levels of more than one gene product [29]. Probes can also be custom-labeled [35], which allows for the expansion of the fluorophore panel and hence the number of RNAs that can be simultaneously measured.
15. A minimum of 25 unique probes per target is recommended to achieve optimal signal-to-noise ratio [36]. Therefore, smFISH is limited to RNA molecules with a length of at least 550 nucleotides.
16. Online tools can be used for probe design, e.g. **https://www.biosearchtech.com**. Authors verified probe specificity with BlastN (NCBI), and eliminated all probes that showed >90% homology in the same species with a second target, in

particular when this gene was highly expressed in the cells of interest. To determine gene expression in immune cells, authors used ImmGen (**https://www.immgen.org**).

17. The authors determined the optimal probe concentration in a titration assay (see **Figure 3A)**. For both human and murine IFN-g probes, a final probe concentration of 15 nM was used. When using different cells, the optimal probe concentration needs to be retritrated, as fluorescence intensity is dependent on cell size and granularity.
18. To determine background staining by the RNA probes, a competition assay with unlabeled cold probes can be performed (see **Figure 3B**).
19. Fluorescence intensity and background fluorescence can be assessed with a non-relevant probeset labeled with an identical fluorophore. A non-specific probeset targeting a gene that is not expressed in the cell type of interest, or that is from a different species, should be used (see **Figure 3C**).
20. Authors used FISH probes at a final concentration of 15 nM. However, the optimal concentration should be determined upon a titration assay (see **Note 17**). Note that the concentration will have to be adjusted to the number of samples and cells used.
21. Using a swing-out centrifuge significantly increases the yield of cells after centrifugation.

Materials

T cell activation

1. (Isolated) human/murine T cells. (*see* **Notes 1** and **2**).
2. Iscove's Modified Dulbecco's medium (IMDM, supplemented with 8% heat-inactivated Fetal Calf Serum (FCS), 2mM L-glutamine, 20 U/mL penicillin G sodium, 20 µg/mL streptomycin sulfate, and for murine, T cells 15 µM 2-mercaptoethanol.
3. Stimuli (i.e. aCD3 + aCD28, 12-O-Tetradecanoylphorbol-13-acetate (PMA) + Ionomycin, target cells expressing the antigen or loaded with antigen, or soluble antigen [GP_{33}, NP_{396}, or $OVA_{257-264}$], or peptide pools, *see* **Note 3**).
4. Monensin (Thermo Fisher Scientific, cat. no. 00-4505-51).
5. Polystyrene 96-well F-bottom (Nunc, 249570) or V-bottom (Costar, 3595) plates or 1.5 mL Lo-Bind Eppendorf tubes (Sigma Aldrich, cat. no. Z666548) (*see* **Note 4**).

Extra- and intracellular staining with antibodies and FISH probes

1. FACS buffer: 1x Phosphate buffered saline (PBS) supplemented with 1% FCS and 2 mM EDTA. Store at 4º C.
2. Cell surface antibodies for separating T cell populations (*see* **Notes 5 - 8**).
3. Fixable live/dead dye (i.e. Near-IR Dead Cell Stain Kit, Thermo Fisher Scientific, cat. no. L10119).
4. Fixation/Permeabilization Solution kit (BD Biosciences, cat. no. 554714).
5. RNAse A/B/C inhibitor (New England Biolabs, cat. no. M0314) (*see***Note 9**).
6. Perm/Wash solution: Dilute BD Perm/Wash™ buffer 1:10 in RNAse-free water. Store at 4º C. The buffer can be used for subsequent experiments. Before use, take the necessary amount and add 4 IU/mL RNAse inhibitor (*see* **Note 10**). Store in fridge until use.
7. Intracellular protein antibodies (*see* **Notes 5, 11**, and **12**).
8. FISH probes (custom design, *see* **Notes 13 - 20**).
9. 20x SSC buffer: RNAse free water, 3M sodium chloride and 0.3 M sodium citrate. Store at 4 ºC. The buffer can be used for subsequent experiments. If the buffer contains crystals, prepare fresh.
10. Flow-FISH wash buffer: 30 mL RNAse-free water, 5 mL de-ionized formamide (Sigma Aldrich, cat. no. 47671) and 5 mL 20x SSC. Before use, add 40 IU RNAse inhibitor/mL (*see* **Note 9**). Prepare fresh for each experiment for optimal results.
11. Flow-FISH hybridization buffer: Combine 8 mL RNAse-free water, 1 mL de-ionized formamide, 1 mL 20x SSC and 1 g dextran sodium sulfate salts (Sigma Aldrich, cat. no. 42867) Of note, dextran sodium sulfate salts will require thorough mixing or vortexing to dissolve. Can be stored at 4º C and used in sequential experiments. Before use, take the necessary amount and add 40 IU RNAse inhibitor/mL (*see* **Note 10**).
12. 1.5 mL Lo-Bind Eppendorf tubes.
13. 96-well V-bottom plates.
14. 5 mL polystyrene round-bottom tubes (Corning, 352058).
15. RNAse free pipette tips (*see***Note 10**).
16. Swing-out centrifuge (*see* **Note 21**).
17. Plate centrifuge.
18. Thermocycler.

Before start

The PDF file attached to this protocol contains notes (as mentioned in parentheses) to clarify some steps. Please refer to the PDF protocol before starting the experiment.

This is a post-peer-review, pre-copyedit version of an article published in Methods in Molecular Biology, and is currently in press.

1. Stimulation and extracellular staining of human and murine CD8+ T cells

1 Activate 3 x 105 [human] or 1 x 106 cells [murine] in 200 μL medium containing the stimulus of choice and 2 μM monensin in a sterile 96-well plate according to standard operating procedure or as described in [29, 31]. Include a sample without the stimulus as control. Cover the plate with a lid and culture cells for the desired amount of time in a humidified incubator at 37 °C + 5% CO2 (see Notes 1 - 4).

2 Remove plate from incubator. Depending on the preference of the experimenter, samples can be transferred into tubes now or at step 3.2.3 [human]/3.3.6 [murine]. Otherwise, perform surface antibody staining in the stimulation plate. In case plate-bound antibodies were used for activation, transfer cells to tubes or to a 96-well V-bottom plate. Spin for 3 min at 650 x g, maximum acceleration and brake, at 4 °C. Of note, all subsequent centrifugation steps are all performed with maximum acceleration and brake. Remove supernatant.

3 Add desired surface antibodies in 30 μL FACS-buffer and resuspend by pipetting. Incubate for 20 min at 4 °C in the dark (see Notes 5 - 7).

4 Add 150 μL FACS-buffer and spin for 3 min at 650 x g at 4 °C. Remove supernatant.

5 Add 50 μL Perm/FIX solution. Resuspend the cell pellet either by briefly vortexing or pipetting and incubate for 20-30 min at 4 °C in the dark (see Note 10).

5.1 The protocol for human and murine T cells diverge here. The original protocol developed for human T cells [29] required slight alterations to preserve the protein staining of cytokines in murine T cells [31].

2. Intracellular mRNA and protein staining of human CD8+ T cells

6 During fixation, prepare the hybridization mix by denaturing FISH probes in 20 μL RNAse-free water. Heat to 65 °C for 5 min in a thermocycler (see Notes 13 - 20).

7 After cooling down to room temperature, add the intracellular antibodies to the denatured FISH probes and add 4 IU/mL RNAse inhibitor. Incubate at RT in the dark for 5 min to allow the neutralization of RNAses (see Notes 5 and 11).

8 Prepare hybridization solution by diluting the denatured FISH probes and intracellular antibodies from step 7 in RNAse inhibitor pre-treated hybridization buffer. Adjust for the amount of samples and the final concentration of probes to be added to the sample, as determined by a probe titration assay (see Note 17). I.e., for 5 samples, add 80 μL hybridization buffer to the 20 μL containing 1 μL of probes from step 3.2.1.

9 After fixation, add 150 µL RNAse inhibitor pre-treated Perm/Wash solution to the cells and transfer to 1.5 mL Lo-bind Eppendorf tubes. Add 1mL RNAse inhibitor pre-treated Flow-FISH wash buffer and spin cells for 5 min at 570 x g at room temperature in a swing-out centrifuge (see Note 21).

10 Remove supernatant while leaving a small “dead” volume of ± 50 µL, as to not disturb the cell pellet and thus limit cell loss. Add 50 µL of hybridization mix containing the FISH probes and the intracellular antibodies as prepared in step 3.2.3. Vortex 2 sec to resuspend, or scratch the bottom of the tube on a rack.

11 Incubate overnight at 37 ºC + 5% CO2 in a humidified incubator.

12 The following day, wash cells by adding 1 mL RNAse inhibitor pre-treated Flow-FISH wash buffer and spin cells for 10 min at 570 x g at room temperature in a swing-out centrifuge.

13 Remove supernatant while leaving ±100 µl, as to not disturb the cell pellet. Resuspend the cell pellet in the residual volume and transfer to a 5 mL polystyrene round-bottom tube and measure mRNA and protein levels on a suitable flow cytometer.

3.2 Intracellular mRNA and protein staining of murine CD8+ T cells

14 During fixation, pipette the required amount of intracellular antibodies in RNAse-free tubes (see Notes 5 and 12). De-activate RNAses present in the antibodies by adding 4 IU/mL RNAse inhibitor to intracellular antibodies and incubate at 4 ºC for 10 min. Denature FISH probes in 20 µL RNAse-free water by heating to 65 ºC for 5 min in a thermocycler (see Notes 13 - 20).

15 After fixation, add 150 µL RNAse inhibitor pre-treated Perm/Wash solution to the cells and spin for 3 min at 650 x g, at 4 ºC. Remove supernatant.

16 Perform intracellular staining by adding desired intracellular antibodies prepared in step 14 in 30 µL RNAse inhibitor pre-treated Perm/Wash solution and resuspend by pipetting. Incubate for 20 min at 4 ºC in the dark.

17 Wash cells with 150 µL RNAse inhibitor pre-treated Perm/Wash solution and spin cells for 3 min at 650 x g at 4 ºC. Remove supernatant.

18 Wash cells twice with 200 µL RNAse inhibitor pre-treated Flow-FISH wash buffer. Between washes, spin cells for 3 min at 650 x g, at 4 ºC. Remove supernatant.

19 Prepare hybridization solution by diluting the denatured FISH probes from step 14 in RNAse inhibitor pre-treated hybridization buffer. Adjust for the amount of samples and the final concentration of probes to be added to the sample, as determined by a probe titration assay

(see Note 17). I.e., for 5 samples, add 80 µL hybridization buffer to the 20 µL containing 1 µL of probes from step 3.3.1.

20 Resuspend cells in 50 µL RNAse inhibitor pre-treated Flow-FISH wash buffer and transfer to 1.5 mL Lo-Bind Eppendorf tube. Add 50 µL of hybridization buffer supplemented with FISH probes as prepared in step 3.3.6.

21 Incubate overnight at 37 ºC + 5% CO2 in a humidified incubator.

22 Prior to acquisition, wash cells by adding 1 mL RNAse inhibitor pre-treated Flow-FISH wash buffer and spin cells for 10 min at 570 x g at room temperature in a swing-out centrifuge (see Note 21). Remove supernatant while leaving a small “dead” volume of ±100 µL, as to not disturb the cell pellet.

23 Resuspend the cell pellet by pipetting in the residual volume and transfer to a 5 mL polystyrene round-bottom tube. Measure samples/cells on a suitable flow cytometer.