FISH-Flow, a protocol for the concurrent detection of mRNA and protein in single cells using fluorescence *in situ* hybridization and flow cytometry

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We describe a flow-cytometry-based protocol for intracellular mRNA measurements in nonadherent mammalian cells using fluorescence *in situ* hybridization (FISH) probes. The method, which we call FISH-Flow, allows for high-throughput multiparametric measurements of gene expression, a task that was not feasible with earlier, microscopy-based approaches. The FISH-Flow protocol involves cell fixation, permeabilization and hybridization with a set of fluorescently labeled oligonucleotide probes. In this protocol, surface and intracellular protein markers can also be stained with fluorescently labeled antibodies for simultaneous protein and mRNA measurement. Moreover, a semiautomated, single-tube version of the protocol can be performed with a commercially available cell-wash device that reduces cell loss, operator time and interoperator variability. It takes ~30 h to perform this protocol. An example of FISH-Flow measurements of cytokine mRNA induction by *ex vivo* stimulation of primed T cells with specific antigens is described.

INTRODUCTION

Development of the FISH-Flow protocol

Analysis of gene expression in whole tissues using real-time RT-PCR, DNA microarrays and RNA sequencing provides insight into the state of cells in tissues under normal and pathological conditions. However, gene expression may vary among cells of the same type and in the same tissue because of cell-cycle, epigenetic and stochastic differences[1]. Gene expression variability among cells can be explored by single-cell analyses.

Single-molecule fluorescence *in situ* hybridization (sm-FISH) has been the method of choice for single-cell gene expression analysis for >15 years. sm-FISH was initially developed by Singer and colleagues[2]. We modified their procedure to render it particularly simple and reliable[3]. In our protocol, ~50 oligonucleotide probes, each coupled with a fluorochrome, bind to the same mRNA molecule (**Fig. 1a**). Deposition of multiple fluorescent moieties renders each single mRNA molecule detectable by high-magnification fluorescence microscopy as an intensely fluorescent, diffraction-limited spot. Spots are enumerated to quantify target mRNA abundance[3–5]. This method has been widely used in laboratories around the world (>650 citations on Google Scholar as of 2016) to unravel multiple, diverse features of single-cell gene expression. The power of microscopy-based sm-FISH lies in its ability to allow determination of integer counts of target mRNAs per cell and to provide information about the spatial distribution of RNAs[6]. However, microscopy-based mRNA quantification is typically applied to small numbers of cells attached to a glass surface (usually ~100 cells) and is labor-intensive. The small sample size limits the ability to assess cell population behaviors and precludes identification of rare cell subsets displaying particular expression patterns. To overcome the limitations of the microscopy-based platform, we adapted sm-FISH to flow cytometry[7,8].

Unlike microscopy, flow cytometry, which has long been the platform of choice for single-cell fluorescence measurements, allows for high-throughput, multiparametric analyses[9]. Therefore, it is used to analyze complex cellular phenotypes for many cells at the same time. Because of the unique properties of flow cytometry, our flow-cytometry-based embodiment of sm-FISH, which we call FISH-Flow[7,8], constitutes an important advancement over microscopy-based sm-FISH for the study of single-cell gene expression. With its ability to analyze thousands of cells simultaneously, FISH-Flow can be used to distinguish cells producing particular mRNAs (as few as ten molecules of mRNA per cell[7]) from cells that do not. An example of parallel microscopy-based sm-FISH and FISH-Flow analysis is shown in **Figure 1b**,**c**. FISH-Flow will be particularly useful for the study of single-cell gene expression in rare subpopulations, such as circulating cancer cells, and for the study of antigen-specific cellular responses.

In the present protocol, we highlight key modifications of the sm-FISH procedure that were necessary to perform FISH on cells in suspension (microscopy is typically performed with cells adhering to a glass surface), reduce background fluorescence and detect cellular mRNA by flow cytometry. We also describe a semi-automated FISH-Flow protocol (**Box 1**) using the BD FACS Lyse Wash Assistant (LWA; BD Biosciences), which is a cell-wash device developed for automation and standardization of sample preparation for flow cytometry. The semiautomated protocol can process up to 40 samples at the same time. It reduces labor, processing time, cell loss and variability among samples and operators. In addition, we describe protocols for the simultaneous detection of mRNA molecules and protein markers (located on the cell surface and/or intracellularly) in the same cells.

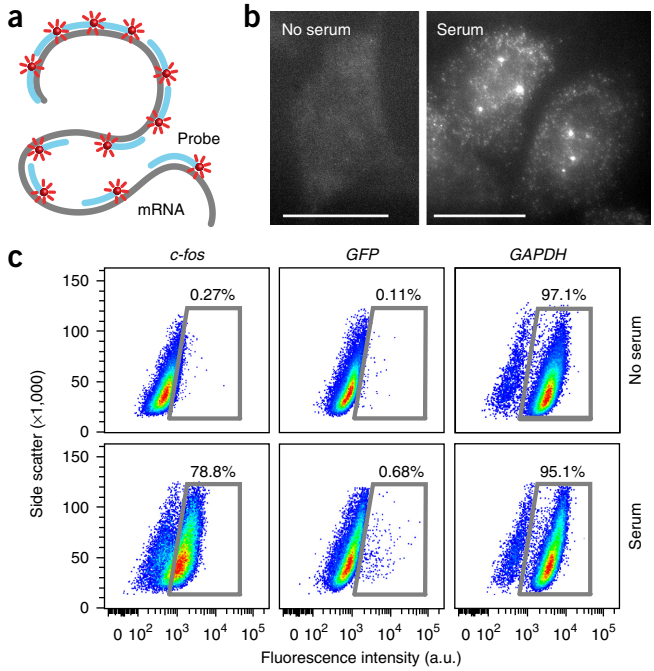

Figure 1 | Comparison of mRNA expression analysis by microscopy and FISH-Flow. (**a**) Schematic representation of sm-FISH probes tiled along the length of an mRNA target. Binding of multiple fluorescently labeled probes to each mRNA molecule results in fluorescent signals that are bright enough to enable detection by flow cytometry and by fluorescence microscopy. (**b**) Representative microscopy images of HeLa cells after sm-FISH using *c-fos* probes. Cells were cultured on 0.17-mm-thick glass coverslips coated with gelatin and subjected to serum treatment as described in the Results section, hybridized with *c-fos* probes and imaged according to the previously described adherent cell protocol[3]. The presented images are merged optical slices. Scale bars, 5 µm. Images were acquired using a Zeiss Axiovert 200 M microscope equipped with a 100× objective. (**c**) Scatter plots of HeLa cells after hybridization with probes against *c-fos* mRNA, *GFP* mRNA (a negative control) and *GAPDH* mRNA (a positive control). Cells were either left untreated or treated with serum, detached, resuspended and processed according to the PROCEDURE. Frequencies of cells positive for *c-fos*, *GFP* (negative control) and *GAPDH* mRNA (positive control) are reported above each gate. Gates were set separately, based on no-serum controls in each case. Cy5 fluorescence was recorded using a 640-nm laser and a 670/30 BP filter. A total of 50,000 events per sample were analyzed.

Applications

The FISH-Flow protocol can be used for a wide range of applications. For example, it can be applied to multiple types of cells in suspension. Cells that are not cultured in suspension can be obtained from liquid tissues, detached from Petri dish surfaces or suspended by trituration of solid tissues. Moreover, a large variety of RNA targets, including viral RNAs, are detectable within cells. In the present protocol, we report detection of mRNAs for cytokines in peripheral blood mononuclear cells (PBMCs); *c-fos* and *GAPDH* mRNAs in HeLa cells; and the interferon-gamma-regulated GTPase, IRGA6, present in macrophage cell lines. Although not shown here, *Irga6* mRNA is also readily detected in primary murine macrophages. Other laboratories have reported using an RNA flow cytometry protocol to detect HIV RNA in infected PBMCs[10]. As sm-FISH probe sets are designed from the knowledge of the mRNA nucleotide sequence, any known gene transcript can be targeted. In addition, the concurrent detection of mRNA and protein in the same cell, which is discussed below, should create new possibilities for multiplex analyses by flow cytometry.

Comparison with other methods

Advantages of flow-cytometry-based single-cell mRNA measurements, relative to microscopy-based measurements, include much higher numbers of cells analyzed (at least three to four orders of magnitude), the ability to perform multiparametric phenotyping and decreased labor. A distinction between the two techniques is the type of information they provide: microscopy provides integer counts of mRNA per cell, whereas flow cytometry provides integrated fluorescence intensity measurement for each cell. Although a limitation of FISH-Flow is the loss of the intracellular spatial resolution afforded by fluorescence microscopy, the possibility exists that protocols that incorporate imaging through the use of flow cytometers such as ImageStream (Amnis, Millipore) could be developed.

FISH-Flow also offers several advantages over conventional, antibody-based flow cytometry. First, it allows concurrent detection of mRNA and cell surface markers, thereby adding a new dimension (gene expression) to the cell phenotyping obtained with conventional flow cytometry. Second, as mRNA is produced before protein and typically has a much shorter half-life, the use of mRNA (rather than protein) as an analyte allows analysis of faster and more dynamic gene expression responses to stimuli. In conventional methods, protein that may already be present in the cell before stimulation must be taken into account, and studies of temporal progression of the response are confounded by protein that can be detected for many hours (typically, up to 24 h) after the initial response to stimulus[7]. Third, RNA flow cytometry circumvents the need for protein-secretion inhibitors that are used for detection of secreted proteins, such as cytokines, by intracellular staining and flow cytometry. Avoiding protein-secretion inhibitors provides a more accurate representation of cell state, particularly when it is defined by the presence of surface markers that are exposed only in response to stimulus (for example, T-cell activation markers). Fourth, RNA flow cytometry can assay a much broader set of targets than antibody-based flow cytometry for the following reasons: (i) abundance of both coding and noncoding RNAs can be assessed; (ii) designing nucleic acid probes is more straightforward than obtaining suitable antibody probes for the analysis of protein targets; and (iii) nucleic acid probes can be readily designed to bind only under desired conditions. Finally, assay standardization amenable to automation can be much more easily achieved with RNA than with protein analytes.

Several RNA FISH methodologies exist. In addition to the FISH-Flow protocol discussed here, an RNA flow cytometry method that uses branched DNA amplification technology has been reported[11]. However, this technology adds multiple hybridization and wash steps to the assay, which makes it less suited to automation. Furthermore, we found that background signals in unstimulated cells are so high that a rare population of activated T cells cannot be detected. In addition to fixed-cell assays, several methods exist for mRNA detection in live cells. These procedures use either molecular beacons or SmartFlare probes and have been used for the detection of abundant mRNA targets in live cells by flow cytometry[6,12,13]. As their use has not been extensive, it is currently difficult to comment on their advantages and limitations *vis-à-vis* the FISH-Flow protocols.

Limitations

One of the limitations of the FISH-Flow method is that fewer fluorochromes have been tested for nucleic acid probe conjugation than for antibody labeling. A second limitation is that the requirement for ~50 (at least 30) 17- to 20-nt-long probes per set limits the selection of target mRNAs to those that are at least 500-nt long. Third, as mRNAs are typically present in lower copy numbers than the proteins they encode, the RNA-based assay

Box 1 | LWA semiautomated protocol

▲ **CRITICAL** Warm all the LWA tanks containing buffers to RT before use. Warm complete RPMI in a 37 °C water bath.

Cell culture and stimulation ● TIMING 7 h

1. Proceed as in Steps 1 and 2 of the PROCEDURE to determine the number of samples, and thaw and wash the PBMCs. Label 12 × 75-mm polystyrene round-bottom tubes according to the number of the samples (**Table 1**).
2. Resuspend the cells in complete RPMI at the determined cell concentration, and prepare unstimulated and stimulated cells as described in Step 3 of the PROCEDURE. Transfer the cell suspension to the 12 × 75-mm polystyrene round-bottom tubes labeled in step 1 of this box and incubate at 37 °C in a 5% CO_2 humidified atmosphere for 6 h. ▲ **CRITICAL STEP** Do not exceed 1 ml of volume for each tube. Leave the cap loose to allow gas exchange during the incubation.
3. During the incubation, transfer the required amount (5 µl per sample) of FcX to a 1.7-ml microcentrifuge tube. Store it at 4 °C for up to 10–12 h. Prepare master mixes for fluorescently labeled antibodies and for FMO controls as described in Step 4 of the PROCEDURE, using **Table 1** as a reference. Add the appropriate amount of antibody to 50 µl of Brilliant Stain Buffer for each sample. If multiple BV or BUV dyes are not used together, the antibodies can be mixed in 50 µl of PBS-FISH buffer instead of Brilliant Stain Buffer. Store the antibody master mixes at 4 °C for up to 10–12 h in the dark.
4. Turn on the LWA. Open the reagent door and connect the PBS-FISH, HWB and Fix tanks to the instrument. Check the water level in the spindle-cleanse tank and the liquid level in the waste tank. Position the three-way stopcock in order to use the PBS-FISH tank as the cell wash tank.

Antibody staining ● TIMING 45 min

5. After stimulation, uncap the tubes from step 2 of this box (do not discard the caps) and transfer each tube to the LWA carousel rack, starting from position 1. Select the PBS-FISH wash protocol and press the 'Run' button.

? TROUBLESHOOTING

6. Remove the carousel, add 5 µl of FcX to each tube, mix well and incubate for 5 min, at RT. Add the appropriate amounts of antibody and FMO mixes to the corresponding tubes, resuspend them well and incubate the tubes for 25 min at 4 °C in the dark. Leave the tubes in the carousel during the incubation at 4 °C. ▲ **CRITICAL STEP** Dispense the FcX and antibody mixtures directly into the cell suspension. Avoid dispensing the mixtures onto the tube walls.

? TROUBLESHOOTING

Fixation and permeabilization ● TIMING 1.5 h

7. After the incubation, place the carousel back into the LWA, select the PBS-FISH wash protocol and press the 'Run' button.
8. Lift the carousel, then place it back into the LWA, select the PFA-PBS-FISH wash protocol and press the 'Run' button.
9. After the wash, lift the carousel, then place it back into the LWA. Open the reagent door and adjust the three-way stopcock to select the external HWB tank as the cell wash tank. Select the permeabilization–HWB wash protocol and press the 'Run' button.

▲ **CRITICAL STEP** If the stopcock is not properly turned, the LWA will fail to dispense the HWB.

? TROUBLESHOOTING

Hybridization ● TIMING overnight, 14–16 h

10. Thaw the HB 20% dx and the probe's working concentration vials (5 ng μl^{-1}) according to the number of samples. Prepare a master mix by dispensing 2 µl (10 ng) of fluorochrome-conjugated probes in 98 µl of HB for each sample (final concentration of 0.1 ng μl^{-1}). Mix well by vortexing. Remove the tubes from the carousel. Dispense 100 µl of the probe mix into each tube and thoroughly resuspend the cell pellet by pipetting. Cap each tube tightly, briefly vortex and incubate overnight, at 37 °C in the dark. ▲ **CRITICAL STEP** HB 20% dx is highly viscous. Vortex the probe mix several times to obtain a homogeneous solution. If bubbles are present, briefly spin the tube for a few seconds in a microcentrifuge. ▲ **CRITICAL STEP** Slowly pipette the mix directly into the cell suspension and pipette until you obtain a clear homogeneous solution. ▲ **CRITICAL STEP** Protect the probe vials from direct light exposure. ▲ **CRITICAL STEP** Prolonged hybridization at 37 °C (>16 h) can lead to fluorochrome tandem dye degradation and associated loss of signal.

■ **PAUSE POINT** Incubate the samples overnight at 37 °C.

? TROUBLESHOOTING

Flow cytometry acquisition and data analysis ● TIMING (1–3 h)

11. After overnight incubation, briefly vortex each tube, remove its cap and place it back into the carousel. Insert the carousel into the LWA. Ascertain that the external HWB tank is connected, select the Post-Hybridization wash protocol and press the 'Run' button.
12. Remove the carousel and acquire the data at the flow cytometer per the individual laboratory's protocol.
13. Analyze the data with flow cytometry data analysis software.

? TROUBLESHOOTING

TABLE 1 | Example of a five-color experimental setup to measure the expression of *IFNG* mRNA in PPD-stimulated PBMCs isolated from an LTBI+ donor.

Tube no.	Stimulation	Condition	Surface marker antibodies				mRNA probes
1	—	Unstimulated	CD3	CD4	CD8	CD56	*IFNG* Cy5
2	PPD	Stimulated	CD3	CD4	CD8	CD56	*IFNG* Cy5
3	PPD	FMO 1	—	CD4	CD8	CD56	*IFNG* Cy5
4	PPD	FMO 2	CD3	—	CD8	CD56	*IFNG* Cy5
5	PPD	FMO 3	CD3	CD4	—	CD56	*IFNG* Cy5
6	PPD	FMO 4	CD3	CD4	CD8	—	*IFNG* Cy5
7	PPD	FMO 5	CD3	CD4	CD8	CD56	—
8	PMA/ionomycin	Positive control	CD3	CD4	CD8	CD56	*IFNG* Cy5

yields signals having lower intensity than those associated with protein detection. Moreover, as with all fluorescence-based methods, the cell types that can be analyzed by this method may be limited to those cells that exhibit low-level autofluorescence in the fluorescence channels that are used for the analysis.

Experimental design

The protocol is divided into the following sections: (Steps 1–4) preparation of samples, (Steps 5–8) antibody staining of cell surface markers, (Steps 9–15) fixation and permeabilization, (Step 16) probe hybridization and (Steps 17 and 18) data acquisition by flow cytometry. Two versions of the protocol are described—a manual protocol and a semiautomated protocol in which the LWA cell-wash device mentioned above is used (**Box 1**). In addition, two optional steps are described for the manual protocol: (i) a viability dye staining for dead cell discrimination (performed before fixation and permeabilization as part of Step 5) and (ii) antibody staining for intracellular proteins (performed after permeabilization, Steps 12–14).

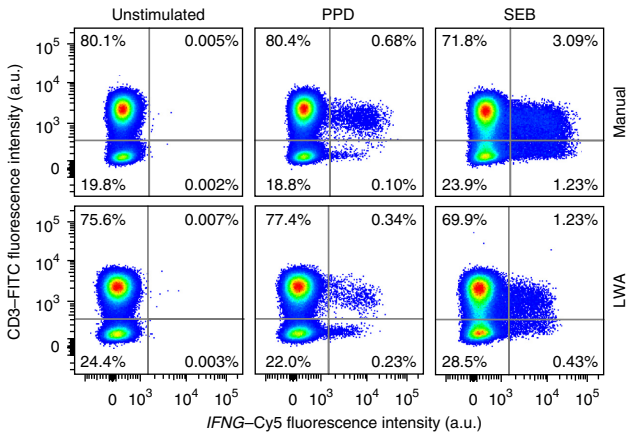

Figure 2 | Analysis of *IFNG* mRNA expression in T cells using manual and LWA semiautomated FISH-Flow protocols. PBMCs from an LTBI+ donor were either not stimulated or were stimulated with purified protein derivative (PPD) or staphylococcal enterotoxin B (SEB) for 6 h. Top row: cells were incubated in a 24-well plate and processed according to the PROCEDURE. Bottom row: cells were incubated in polystyrene FACS tubes and processed according to the LWA semiautomated protocol (**Box 1**). Frequencies of cells expressing *IFNG* mRNA in CD3+ and CD3− cells are reported. Gates were set based on FMO controls and unstimulated samples. Cy5 fluorescence was recorded using a 640-nm laser and a 670/30 BP filter. A total of 100,000 events per sample were analyzed.

The FISH-Flow protocols are described using an assay in which memory T cells from asymptomatic individuals infected with *Mycobacterium tuberculosis* (LTBI+) are identified by *M. tuberculosis*-antigen-specific stimulation of PBMCs *ex vivo*[7,8]. This example is suited to protocol demonstration, because *ex vivo* antigen stimulation results in activation of a small number of T cells that produce mRNAs encoding cytokines such as IFNG and tumor necrosis factor-α. These serve as targets of sm-FISH probes. The ability to detect a small number of cells producing a target mRNA demonstrates the specificity and the power of the assay.

In **Figures 1–5**, we also illustrate key features of the protocol by (i) comparison of the FISH-Flow assay with the sm-FISH assay using suspended versus adherent human HeLa cells, in which the use of positive and negative controls is also described (**Fig. 1**); (ii) analysis of *IFNG* mRNA expression in activated T cells (**Fig. 2**); (iii) concurrent analysis of *Irga6* mRNA and protein in the mouse macrophage cell line RAW264.7 (**Fig. 3**); (iv) adaptation of the cell-wash instrument to a semiautomated FISH-Flow protocol (**Fig. 4**); and (v) five-color, multiplex analysis of cell surface markers and *IFNG* mRNA (**Fig. 5**).

Preparation of samples.

The protocol should be initiated with at least 1×10^6 cells per condition to obtain at least 200,000 events analyzed by flow cytometry. Conditions should include (i) the test sample; (ii) positive control(s), such as a constitutively expressed gene or cell treatments that induce strong gene expression; and (iii) negative control(s), such as unstimulated samples, or hybridization with probes against a foreign RNA not present in the cell. When antibody-based staining of surface markers is included, staining with isotype control antibodies must be used to assess nonspecific binding. In addition, when performing multiparametric analysis, it is important to include also 'fluorescence minus one' (FMO) controls, which allow for the precise identification of cells that exhibit fluorescence above background levels[14]. **Table 1** shows the sample and control setup for the example application described below.

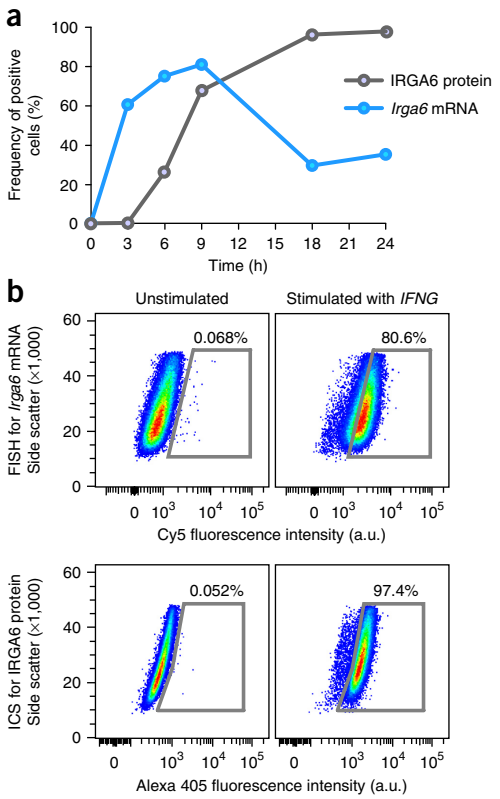

Figure 3 | Flow cytometric analysis of *Irga6* mRNA and IRGA6 protein in mouse macrophage cell line RAW264.7. Cells were stimulated with 100 U ml^{-1} of IFN-γ or left unstimulated and collected at the indicated time points. Samples were processed according to the PROCEDURE. Cy5 fluorescence was recorded using a 640-nm laser and a 670/30 BP filter, whereas Alexa 405 fluorescence was recorded using a 405-nm laser and a 450/50 BP filter. A total of 100,000 events per sample were analyzed. (**a**) Kinetics of *Irga6* mRNA and IRGA6 protein induction by IFNG-γ stimulation of RAW264.7 cells. (**b**) Scatter plots showing peak production of *Irga6* mRNA (after 9 h of stimulation, upper panels) and IRGA6 protein (after 24 h of stimulation, lower panels). The frequencies of cells positive for *Irga6* mRNA and IRGA6 protein are reported above each gate. Gates were set based on unstimulated samples.

The experiments should be performed with viable, well-preserved cells (cell viability ≥90%). Damaged or aged cells are more susceptible to physical damage and cell lysis, leading to the formation of aggregates and cell debris, as well as increases in background fluorescence signals and the rate of false-positive signals[15]. If adherent cells are used, cells should be detached and resuspended before performing the assay. An example of the FISH-Flow assay, starting with adherent cells, is presented in **Figure 1c**.

(Optional) viability dye staining.
Dead cells often exhibit autofluorescence or give rise to false-positive results by binding nonspecifically to antibodies used for surface marker staining. Therefore, it is normal practice in flow cytometry to characterize the sample, at least in initial assays, in terms of relative proportions of live and dead cells. This result is routinely achieved by using viability dyes—e.g., LIVE/DEAD Fixable Dead Cell Stains (Molecular Probes/Thermo Fisher Scientific).

The LIVE/DEAD staining procedure is straightforward. Before surface antibody staining (Step 5), cells are resuspended in 100 µl of 1× PBS containing the dye and incubated for 15 min at room temperature (RT, 20–25 °C). At the end of the incubation, the cells are washed twice with PBS–FISH buffer. It is important to use PBS (rather than PBS-FISH buffer) in the LIVE/DEAD staining step, as the amine-reactive dye used for staining is not compatible with protein-containing buffers, such as the PBS-FISH buffer. The optimal amount of amine-reactive dye must be determined by preliminary titration experiments[16]. We suggest inclusion of heat-killed cells (5 min at 60 °C) as a positive control, and an unstained control to discriminate autofluorescent cells from dead cells during data analysis, as reported by Perfetto *et al.*[16].

Antibody staining of cell surface proteins.
After stimulation, cells can be stained with fluorochrome-labeled antibodies against surface protein markers to identify cell populations of interest. The optimal concentration of antibody is determined in preliminary titration experiments. The FISH-Flow assay is compatible with most commercial fluorochromes; good results are obtained with the bright protein-based fluorochromes phycoerythrin (PE) and allophycocyanin (APC), and their conjugates with organic dyes, such as CF594, Cy5 and Cy7.

Tandem dyes such as Brilliant Violet (BV) and Brilliant Ultraviolet (BUV), which are designed to yield particularly strong signals with excitation by violet and UV lasers, are also compatible. However, we found that the fluorescence of these dyes decreases after overnight hybridization at 37 °C. Moreover, the assay is not compatible with PerCP and PerCP-Cy5.5 dyes, which degrade during the hybridization and washing steps. Qdot nanocrystals have not been tested. When using multiple BV or BUV dyes, the buffer provided by the manufacturer should be used (e.g., Brilliant Stain Buffer (BD Biosciences)) to prepare the antibody mixture, as it is designed to avoid aggregation of polymeric dyes.

Fixation and permeabilization.
Cell fixation is performed in 4% (vol/vol) paraformaldehyde (PFA) for 30 min at RT after surface antibody staining. It is important to avoid overfixation of the samples, which results in enhanced autofluorescence. The traditional microscopy-based sm-FISH protocols use 70% (vol/vol) alcohol for permeabilization[3]. Although this method works well, we recommend 0.2% (vol/vol) Tween 20 instead, as it greatly increases compatibility of the protocol with the fluorochromes used for surface marker staining (see above; when we compared permeabilization with Triton X-100, saponin and Tween 20, we obtained the highest values of fluorescence intensity and positive cell frequencies with Tween 20). As permeabilization can reduce cell size, adjusting forward-scatter area (FSC-A) and side-scatter area (SSC-A) scaling is recommended for visualizing the population of interest during flow cytometry data acquisition.

(Optional) antibody staining of intracellular proteins.
Use of fluorochrome-conjugated antibodies for staining of intracellular proteins requires a minor modification of the permeabilization step, as is indicated in Steps 12–14. We observed that adaptation of intracellular staining to the FISH-Flow assay results in a 30–40% reduction of the intracellular staining signal relative to that obtained with commercial kits. Therefore, parameters of the intracellular staining protocols, such as amount of antibody and incubation time, should be reoptimized when used in the

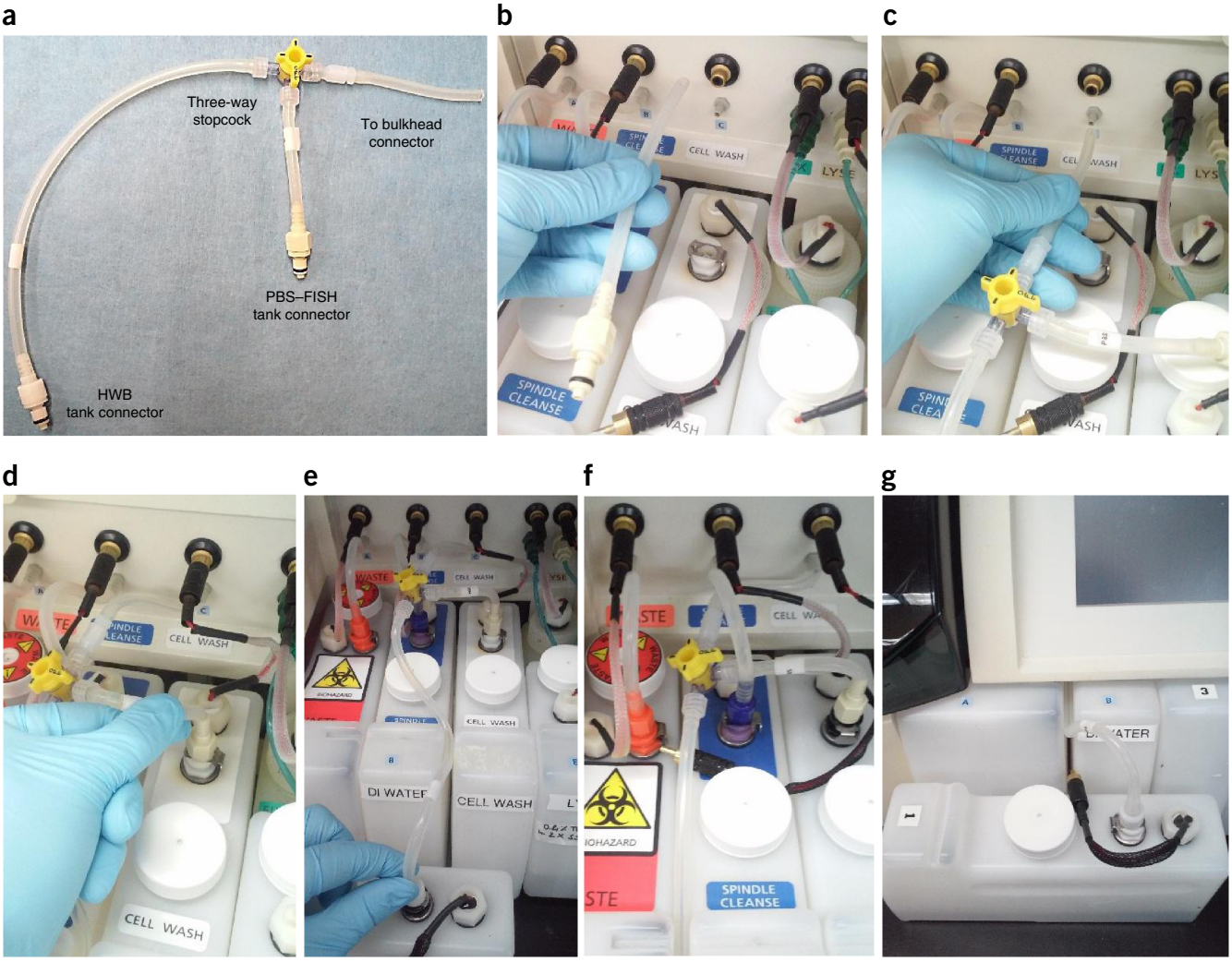

Figure 4 | Installation of the external tank in the LWA instrument. (**a**) Tubes with a three-way Luer-lock stopcock for connecting the instrument with the external tank. (**b**) Removal of the tube from the LWA wash tank and the barb connection on the instrument bulkhead. (**c**) Attachment of the bulkhead connector tube to the bulkhead cell wash connector, (**d**) Connection of the shorter tube to the internal cell wash tank (containing PBS-FISH buffer). (**e**) Connection of the longer tube to the external cell wash tank (HWB). (**f**,**g**) Positioning of (**f**) the stopcock and (**g**) the tubing to avoid tube tightening or pinching. The level sensor in panels **b** and **c** was removed to illustrate the barb connection on the instrument bulkhead.

context of the FISH-Flow assay. The application of intracellular protein measurements with FISH-Flow is shown in **Figure 3**.

Nucleic acid hybridization.

Although hybridization reactions are performed under excess probe conditions, it is important to optimize probe concentrations in order to maximize signal-to-background ratios. To optimize the amount of probe, multiple probe concentrations should be initially tested in small increments above and below 0.1 ng μl^{-1} probe (this concentration is optimal in our protocol), and separation between the positive and negative cell populations should be assessed by flow cytometry. As a minimum of 6–8 h is required for the oligonucleotide probes to bind the target mRNA, we perform hybridization with sm-FISH probes overnight (14–16 h) at 37 °C for convenience. Longer hybridization times should be avoided with cells stained with fluorochrome-conjugated antibodies to minimize fluorochrome degradation. At the end of the entire procedure, samples can be analyzed immediately or stored at 4 °C in hybridization wash buffer (HWB) for up to 24 h. We note that hybridization conditions, such as reaction volume, concentration of formamide and dextran sulfate in the hybridization buffer (HB) and the type of plastic of the reaction tubes, are critical. Thus, tubes and volumes should be used as described in the protocol.

An important difference between the HWBs used in the adherent cell protocols described earlier and the present cell suspension protocol is that the latter includes 0.2 mg ml^{-1} BSA. Without BSA, cells tend to stick to the walls of the tubes, resulting in cell loss during cell washes.

Data acquisition by flow cytometry.

After hybridization, samples are washed twice in HWB to remove unbound probes and are resuspended in HWB for flow cytometry data acquisition. The samples must be maintained in HWB during acquisition. If the cell suspension is too concentrated for data acquisition, the sample should be diluted in HWB (dilution of the 2× saline-sodium citrate in HWB can cause probe dissociation from the target mRNA). With the semiautomated

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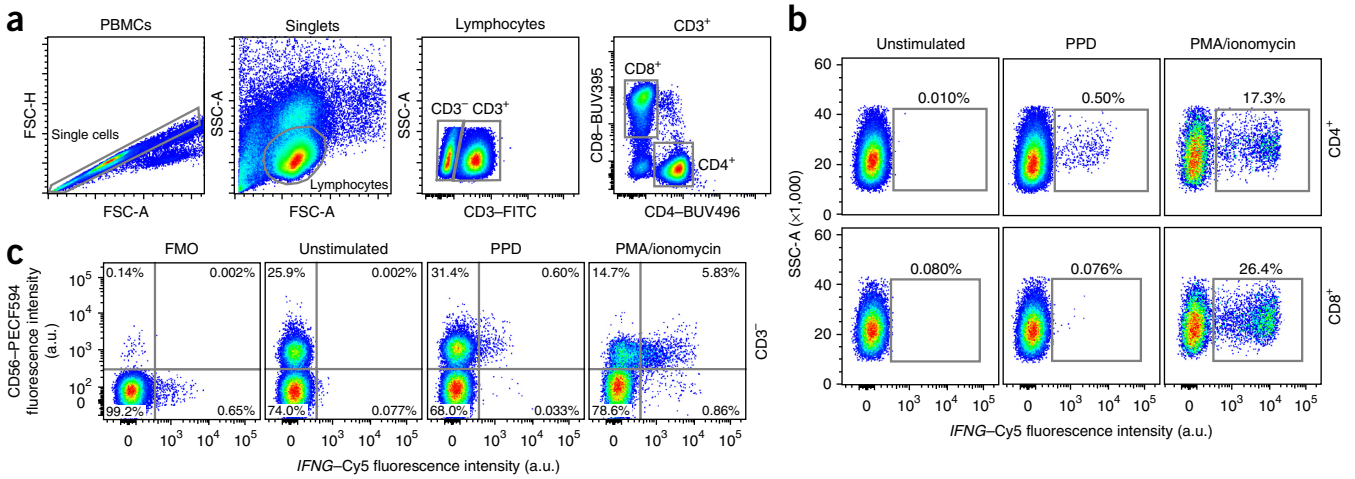

Figure 5 | Multiplex five-color analysis of *IFNG* mRNA expression in PBMC subsets. PBMCs from an LTBI+ donor were left unstimulated, or were stimulated with PPD for 6 h or with PMA/ionomycin for 2 h, and then were processed using the LWA semiautomated protocol (**Box 1**). In addition to hybridizing the cells with Cy5-labeled probes against *IFNG* mRNA, they were also stained for CD3, CD4, CD8 and CD56 surface markers using antibodies labeled with the indicated dyes. (**a**) Gating strategy. Gates on each cell population are indicated by gray boundaries. From left to right: single-cell gating includes single cells and excludes aggregates using FSC-A and FSC-H; lymphocytes are gated using FSC-A and SSC-A; $CD3^-$ and $CD3^+$ cells are gated using SSC-A and the FITC channel; and $CD4^+$ and $CD8^+$ cells are gated using the BUV395 and BUV496 channels on the $CD3^+$ population. Gates for CD3, CD4 and CD8 were set using FMO controls. (**b**) Frequencies of $IFNG^+CD4^+$ and $IFNG^+CD8^+$ cells under unstimulated conditions, under PPD stimulation and under PMA/ionomycin stimulation. A lower total number of cells were acquired for the PMA/ionomycin stimulation relative to the other two conditions, because that stimulation leads to significant cell loss. Gates were set based on the unstimulated samples. (**c**) Relative frequencies of *IFNG* mRNA and CD56 expression in $CD3^-$ cells. Gates were set based on FMO control and unstimulated samples. An example of the FMO control for staining without anti-CD56 antibody is shown in the panel (left). The following lasers and BP filters were used for each fluorochrome: Cy5, 640-nm laser and 670/30 BP filter; FITC, 488-nm laser and 530/30 BP filter; BUV395 and BUV496, 355-nm laser and 379/28 and 513/30 BP filters, respectively; PE-CF594, 561-nm laser and 610/20 BP filter. A total of 200,000 events per sample were analyzed. PMA, phorbol 12-myristate 13-acetate.

protocol, the LWA carousel can be directly transferred to a flow cytometer equipped with a BD FACS Loader for automatic sample acquisition.

For flow cytometer calibration, data acquisition, and minimization of day-to-day variability in performance, follow the recommendations of Perfetto and colleagues[17]. With multicolor experiments, compensation for spectral overlap must be performed by including single-color controls (cells or polystyrene beads). To obtain these controls, cells are hybridized with only the probe, whereas polystyrene beads are labeled with antibodies conjugated with the same fluorochrome used with the oligonucleotide probes. Combining of beads and cells is not recommended: it can lead to incorrect compensation calculations, as cells and beads have different levels of autofluorescence. It is important that the fluorescence signal obtained with the compensation control be equal to or brighter than the fluorescence signals of the samples analyzed.

Optimal laser and filter sets for different dyes are as follows: 488-nm laser and 530/30 BP filter—Alexa 488 and FITC; 561-nm laser and 610/20 BP filter—Alexa 594 and PE-CF594; 640-nm laser and 670/30 BP filter—Cy5; 355-nm laser and 379/28 BP filter—BUV395; and 355-nm laser and 513/30 filter—BUV496. As mentioned above, FSC-A and SSC-A require scaling to properly visualize the population of interest. Moreover, the forward-scatter height (FSC-H) parameter should be included to allow for single-cell gating during data analysis (**Fig. 5a**). The number of events to acquire is best determined by taking into account (i) the number of cells present in the population of interest and (ii) the frequencies of positive cells expressing the target mRNA (relative to control samples)[18].

Data analysis.

Flow cytometry data analysis can be performed using software such as FlowJo or FACS Diva. An example of data analysis for a five-color assay using a sequential gating strategy is described in **Figure 5**. First, doublets and cell aggregates are excluded by using FSC-A and FSC-H parameters, thus allowing gating on single cells (singlets). Second, the population of interest (lymphocytes, in this example) can be selected among the singlets by using FSC-A and SSC-A parameters (**Fig. 5a**). Third, lymphocyte subpopulations such as $CD3^+$, $CD3^-$, $CD4^+$ and $CD8^+$ can be identified using fluorescence channels compatible with the fluorochromes used for surface marker staining. Fourth, FMO controls are required to accurately set the gates (an example is shown in **Fig. 5c**). Fifth, a threshold is set by gating on control samples such as unstimulated cells (**Figs. 3b** and **5b**,**c**) or untreated cells (**Fig. 1c**). As a result, the number of cells expressing the target mRNA above an empirical threshold can be determined. Within this scheme, additional quality-control procedures will include supplementary negative and positive controls, such as those indicated above in the 'Preparation of samples' section, to minimize the occurrence of false-positive and false-negative results and to verify the position of the gates (**Fig. 1c**).

Manual and semiautomated versions of the FISH-Flow protocol.

The manual protocol begins with *ex vivo* stimulation of PBMCs in 24-well plates, followed by transfer of cells to microcentrifuge tubes, in which hybridization and wash steps are performed. Washes are carried out by centrifugation followed by removal of the supernatant fluid. Finally, the cells are transferred to polystyrene

FACS tubes for data acquisition with the flow cytometer. In its semiautomated version (**Box 1**), the FISH-Flow assay is performed in an LWA instrument, which is modified to include an additional external cell wash tank (**Fig. 4**). In the semiautomated protocol, cells are dispensed and maintained in the same polystyrene FACS tubes throughout the procedure, from antigen stimulation through data acquisition, by flow cytometry. No cell transfer occurs during the procedure. The two versions of the protocol yield similar results in terms of fraction of cells that are classified as expressing the target mRNA, even though the samples processed in the LWA show somewhat decreased fluorescence intensity of mRNA-positive cells relative to the manual procedure (**Fig. 2**). The decrease in signal intensity is due to a combination of three factors: (i) vessel plastic (polystyrene for FACS tubes versus polypropylene for microcentrifuge tubes), (ii) final concentration of dextran sulfate in the HB (5% versus 10%) and (iii) final volume of hybridization reaction (~400 µl versus 50 µl) in the semiautomated relative to the manual protocol. The advantages of the semiautomated assay are parallel processing of multiple samples, increased cell recovery, reduced operator time and reduced operator-to-operator variability. Thus, the semiautomated assay would be particularly useful in settings such as clinical laboratories, in which the number of samples to be processed is large and the number of available cells is limited.

Both the manual and the semiautomated protocols are typically performed in 2 d. On day 1, cells are stimulated, stained with surface antibodies, fixed, permeabilized and hybridized overnight with sm-FISH probes. On day 2, samples are washed and analyzed by flow cytometry, followed by data analysis.

MATERIALS

REAGENTS

▲ **CRITICAL** Some of the materials and reagents listed here are specific to the PBMC protocol. Specific reagents within some classes, such as antibodies, cell culture medium and cell stimulation agents, will be particular to different applications.

Cell culture reagents

- Fresh or cryopreserved PBMCs from consenting healthy human donors
  ! **CAUTION** Experiments must be carried out in class II biological safety cabinets while wearing personal protective equipment. Biohazardous waste must be disposed of per institutional protocols.
- RPMI Tissue Culture Medium (1640 1× without L-glutamine; Corning, cat. no. 15-040-CV)
- L-glutamine (200-mM solution; Corning, cat. no. 25-005-CI)
- Penicillin–streptomycin solution (50×; Corning, cat. no. 30-001-CI)
- FBS, heat-inactivated (Seradigm, cat. no. 1500-500)
- Phorbol 12-myristate 13-acetate (PMA; Sigma, cat. no. P8139) **!** **CAUTION** PMA causes skin irritation and is a carcinogen; handle the compound wearing personal protective equipment.
- Ionomycin calcium salt (Enzo Life Sciences, cat. no. ALX-450-007)
  ! **CAUTION** Ionomycin calcium salt is highly toxic. Avoid skin contact and inhalation. Handle the compound while wearing personal protective equipment.
- Tuberculin purified protein derivative (batch RT 50, 1 mg ml^{-1}; Staten Serum Institute, cat. no. 2391)
- *Staphylococcus* enterotoxin B (EMD Millipore, cat. no. 324798) **!** **CAUTION** *Staphylococcus* enterotoxin B is toxic. Avoid inhalation, ingestion or contact with skin, eyes or mucous membranes. Handle it while wearing personal protective equipment.

Antibodies

- Co-stimulatory antibodies CD28/CD49d (BD Biosciences, cat. no. 347690)
- Human TruStain FcX (Fc receptor blocking solution; (BioLegend, cat. no. 422302)

Fluorescently labeled antibodies and flow cytometry reagents

▲ **CRITICAL** In the examples described, we have used the following antibodies (all from BD Biosciences), but other antibodies/reagents can be used.

- CD3 FITC clone HIT3a (cat. no. 555339), excitation maximum = 490 nm, emission maximum = 525 nm
- CD4 BUV496 clone SK3 (cat. no. 564651), excitation maximum = 355 nm, emission maximum = 496 nm
- CD8 BUV395 clone RPA-T8 (cat. no. 563795), excitation maximum = 348 nm, emission maximum = 395 nm
- CD56 PE-CF594 clone B159 (cat. no. 562289), excitation maximum = 496 and 564 nm, emission maximum = 612 nm
- Brilliant Stain Buffer (BD Biosciences, cat. no. 563794)
- BD CompBeads compensation beads (BD Biosciences, cat no. 552843)

FISH-Flow reagents

- Custom amino-labeled or fluorochrome-labeled sm-FISH probe sets (LGC Biosearch)
- Milli-Q water (EMD Millipore, Milli-Q Integral water purification system)
- Biopak filter for Milli-Q purification system (EMD Millipore, cat. no. CDUFBI001)
- Nuclease-free water (Thermo Fisher Scientific, cat. no. AM9939)
- 10× PBS (RNase-free; Thermo Fisher Scientific, cat. no. AM9625)
- 32% (vol/vol) Paraformaldehyde solution (electron microscopy grade; Fisher Scientific/Electron Microscopy Sciences, cat. no. 50-980-495)
  ! **CAUTION** Paraformaldehyde is highly toxic. It is a strong irritant and has caustic effects on skin, eyes and mucous membranes. There is limited evidence of carcinogenic effects. Handle it in a well-ventilated workspace while wearing the recommended personal protective equipment.
- Deionized formamide (Thermo Fisher Scientific, cat. no. AM9342)
  ! **CAUTION** Formamide may cause eye, skin and respiratory tract irritation. It contains one or more substances that are classified as carcinogenic, mutagenic and/or reprotoxic. Handle it under a fume hood while wearing the recommended personal protective equipment.
- Tween 20 (Sigma, cat. no. P2287)
- 20× Saline-sodium citrate (Thermo Fisher Scientific, cat. no. AM9763)
- Dextran sulfate sodium salt from *Leuconostoc* spp. (Sigma, cat. no. D8906)
- tRNA (ribonucleic acid transfer from baker's yeast (*Saccharomyces cerevisiae*; Sigma, cat. no. R8759)
- UltraPure BSA (RNase-free BSA, 50 mg ml^{-1}; Thermo Fisher Scientific, cat. no. AM2618)
- BSA (fraction V, heat shock; Roche, cat. no. 03116999001)
- Ethanol (Fisher Scientific, cat. no. S25309B) **!** **CAUTION** Ethanol is highly flammable

EQUIPMENT

- 1.7-ml Polypropylene microcentrifuge tubes (Corning-Axygen, cat. no. MCT-175-C)
- 24-well Polystyrene plates (Greiner Bio-One/Cellstar, cat. no. 662160)
- 5-ml Polystyrene round-bottom tubes (12×75-mm, FACS tubes; BD-Falcon, cat. no. 352054)
- 50-ml RNase/DNAse-free conical polypropylene centrifuge tubes (Fischer Scientific, cat. no. 05-539-13)
- Swinging-bucket centrifuge with microcentrifuge tube adaptor (e.g., Eppendorf, model no. 5810R or equivalent)
- In-house vacuum line or vacuum pump
- Liquid aspirator setup (Guardian Suction Canister System; Cardinal Health, cat. no. 65651-230)
- Vortex mixer (Fischer Scientific, cat. no. 02215365)
- Incubator (37 °C, 5% CO_2)
- Water bath (37 °C)
- Laminar flow hood
- Syringe filters (0.22 and 0.45 µm; Fischer Scientific, cat. nos. 09-719A and 09-719B)
- Syringe (60 ml, with Luer Lock; Fischer Scientific, cat. no. NC0896720)
- BD FACS Lyse Wash Assistant (LWA; (BD Biosciences, cat. no. 337146)
- Additional LWA wash tanks (BD Biosciences, cat. no. 33635107)

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- Additional LWA wash tank tubing assembly (Silastic laboratory tubing with 0.125-inch interior-diameter, 1/8-inch hose barb coupling insert and three-way Luer-lock stopcock (CPC, cat. no. PMC2202)
- Software such as FlowJo v10.2 (https://www.flowjo.com/solutions/flowjo/downloads) or BD FACSDiva (https://www.bdbiosciences.com/us/instruments/research/software/flow-cytometry-acquisition/bd-facsdiva-software/m/111112/overview) for flow cytometry data analysis
- Flow cytometer (BD Biosciences, model no. BD Fortessa X20 or BD LSRII)

REAGENT SETUP

Probe design, labeling and storage For each target mRNA, we design 48 oligonucleotide probes using the Stellaris Probe Designer program available at http://www.singlemoleculefish.com. Even though 20–30 probes can give excellent signals with microscopy[19], we advise designing at least 40 probes per target mRNA in FISH-Flow experiments. The Stellaris probe designer program screens against probes that can bind to genomic sequences other than the intended target. Two control probe sets should be included—one against a gene that is expected to be absent from the cells (to serve as the negative control; we use *GFP* mRNA), and a constitutively expressed mRNA as the positive control (we use *GAPDH*) (**Fig. 1c**). The negative control set is needed to evaluate nonspecific binding of probes and to set the gate for flow cytometry analysis.

Some probes bind nonspecifically within cells. In microscopic analysis, this background is visible as diffuse fluorescence that does not affect image analysis, because the analysis algorithms specifically recognize spots within the diffuse background (**Fig. 1b**). However, in flow cytometry this background signal is recorded as integrated fluorescence; identifying cells that express target mRNAs requires that they are more fluorescent than those that do not express the target mRNA. In the case of PBMCs, we found that cells that express about ten molecules of target mRNA can be distinguished from the cells that do not express any[7]. However, some probe sets produce unacceptably high background signals. In those cases, we have found that high background stems from just one or several probes among the probe set. These probes (that presumably bind to nontarget nucleic acids) can be identified and removed by testing combinatorial subpools. An example is shown in **Table 2**, in which an initial set of 48 *IFNG* mRNA probes was reduced to 40 probes.

In the FISH-Flow assay, the best results are obtained with probes conjugated with the Cy5 dye. Multiplex assays can be performed with probes labeled with Cy5, Alexa 488 and Alexa 594 dyes. However, given the low fluorescence intensity of Alexa 488 and 594 dyes and the relatively high autofluorescence detected at those emission wavelengths, these fluorochromes should be used only for detection of highly transcribed genes. Thus, it is advisable to first evaluate the expression of each new gene of interest using Cy5-labeled probes and to then consider the possibility of alternative fluorochromes.

We obtain probe sets (10-nmol scale synthesis) with a 3′-amino modification from LGC Biosearch Technologies and then conjugate them with the desired amino-reactive fluorescent dye. Alternatively, probes can be purchased prelabeled from LGC Biosearch Technologies. The dye conjugation and subsequent HPLC purification procedures have been described[5,19]. The stock solutions of the purified probe sets (~100 µM in water) can be stored at −80 °C for several years. Multiple aliquots at lower working concentrations (5–25 ng µl^{-1}) should be stored to prevent repeated freeze–thaw cycles. Nucleotide sequences of probes against *IFNG* mRNA, which serve as our example target in this protocol, are presented in **Table 2**.

Complete RPMI Supplement RPMI 1640 medium with 2 mM L-glutamine, 1× penicillin–streptomycin solution and 10% (vol/vol) FBS. Store the medium at 4 °C for up to 3 months.

PBS-FISH buffer PBS-FISH buffer contains 1× PBS and 0.2 mg ml^{-1} RNase-free BSA. Prepare the buffer by diluting 5 ml of 10× PBS and 200 µl of 50 mg ml^{-1} RNase-free BSA to a final volume of 50 ml with nuclease-free water. Store the buffer at 4 °C for up to 3 months. ▲ **CRITICAL** All buffers should be prepared using DNase- and RNase-free reagents, unless otherwise stated. ▲ **CRITICAL** Use autoclaved graduated cylinders for preparation of the buffers. Rinse the cylinder with 70% (vol/vol) ethanol and wash it with nuclease-free water to reduce nuclease contamination. ! **CAUTION** Ethanol is highly flammable.

4% (vol/vol) PFA 4% (vol/vol) PFA is 4% (vol/vol) PFA in 1× PBS. Prepare the solution fresh according to the number of samples (0.4 ml per sample). Multiply the number of samples by 0.4 ml to determine the final volume. Dilute the 32% (vol/vol) PFA stock solution and 10× PBS with water to obtain a final concentration of 4% (vol/vol) PFA in 1× PBS. ▲ **CRITICAL** Prepare fresh PFA solution on the day of the experiment. Avoid direct exposure to light. ! **CAUTION** Paraformaldehyde is highly toxic, an irritant and has caustic effects on skin, eyes and mucous membranes. Handle it while wearing the recommended personal protective equipment.

10% (vol/vol) Tween 20 in 1× PBS Add 5 ml of Tween 20 and 5 ml of 10× PBS to 40 ml of nuclease-free water in a 50-ml polypropylene tube. Store the mixture at RT for up to 6 months. ▲ **CRITICAL** Tween 20 is very viscous. Pipette slowly and rinse the pipette at least five to six times before discarding.

Permeabilization buffer Permeabilization buffer is 0.2% (vol/vol) Tween 20 in 1× PBS. Prepare permeabilization buffer by adding 1 ml of the 10% (vol/vol) Tween 20 solution to 49 ml of 1× PBS. Store the solution at RT for up to 6 months.

Intracellular staining buffer Intracellular staining (ICS) buffer consists of 0.5% (vol/vol) Tween 20 in PBS-FISH buffer. Prepare the ICS buffer by adding 2.5 ml of the 10% (vol/vol) Tween 20 solution to 47.5 ml of PBS-FISH buffer. Store it at 4 °C for up to 3 months.

Hybridization wash buffer HWB contains 2× saline-sodium citrate, 10% (vol/vol) formamide and 0.2 mg ml^{-1} RNase-free BSA in nuclease-free water. Prepare HWB by adding 5 ml of formamide to 25 ml of nuclease-free water; mix the solution well and add 5 ml of 20× saline-sodium citrate and 200 µl of 50 mg ml^{-1} RNase-free BSA; bring the solution to a final volume of 50 ml with nuclease-free water. Store it at 4 °C for up to 3 months. ! **CAUTION** Formamide is toxic and may cause eye, skin and respiratory tract irritation. Handle it while wearing personal protective equipment. ▲ **CRITICAL** Warm formamide to RT before preparing the buffer.

Hybridization buffer 10% (wt/vol) dextran sulfate (HB 10% dx) HB 10% dx contains 10% (wt/vol) dextran sulfate, 2× saline-sodium citrate, 10% (vol/vol) formamide, 1 mg ml^{-1} tRNA and 0.2 mg ml^{-1} BSA in nuclease-free water. Prepare HB 10% dx by resuspending 5 g of dextran sulfate and 50 mg

TABLE 2 | List of *IFNG* mRNA probe sequences.

1	ATCAGAACAATGTGCTGCAC	21	CCCCATATAAATAATGTTAA
2	TCTAATAGCTGATCTTCAGA	22	CTTATTTGATTGATGAGTCT
3	CCAAAGGACTTAACTGATCT	23	CATTACACAAAAGTTGCTAT
4	TCTTGTATCAAGCTGATCAG	24	GACAGTCACAGGATATAGGA
5	GCCAAAGAAGTTGAAATCAG	25	CAGAAAACAAAGGATTAAGT
6	TCATCGTTTCCGAGAGAATT	26	CACATAGCCTTGCCTAATTA
7	GAGCTGAAAAGCCAAGATAT	27	CCCTGAGATAAAGCCTTGTA
8	CAAGAGAACCCAAAACGATG	28	TTAGGTTGGCTGCCTAGTTG
9	TATGGGTCCTGGCAGTAACA	29	AAACACACAACCCATGGGAT
10	AAGGTTTTCTGCTTCTTTTA	30	GTTCATTGTATCATCAAGTG
11	GACCTGCATTAAAATATTTC	31	CTGGATAGTATCACTTCACT
12	CCATTATCCGCTACATCTGA	32	CATATTTTCAAACCGGCAGT
13	TTAGTCAGCTTTTCGAAGTC	33	AAGCACTGGCTCAGATTGCA
14	GACATTCAAGTCAGTTACCG	34	AGTTCTGTCTGACATGCCAT
15	GTTCATGTATTGCTTTGCGT	35	TCAGGGTCACCTGACACATT
16	AGTTCAGCCATCACTTGGAT	36	CTCCTGAGATGCTATGTTTT
17	TTCGCTTCCCTGTTTTAGCT	37	TTGGAAGCACCAGGCATGAA
18	CGAAACAGCATCTGACTCCT	38	CAGTCACAGTTGTCAACAAT
19	ATTACTGGGATGCTCTTCGA	39	TGAGTTACTTTCCATTTGGG
20	CAAATATTGCAGGCAGGACA	40	GTGAACTTACACTTTATTCA

of tRNA in 30 ml of nuclease-free water. Dissolve the reagents by mixing with a vortexer or in a tube rotator. Add 5 ml of 20× saline-sodium citrate, 200 µl of 50 mg ml^{-1} RNase-free BSA and 5 ml of formamide and bring the solution to a final volume of 50 ml with nuclease-free water. Mix well in a tube rotator until the solution becomes homogeneous, and filter it using a 0.4-µm filter unit. Store it in aliquots (1 ml per tube) at −20 °C for up to 6 months. **! CAUTION** Formamide is toxic and it may cause eye, skin and respiratory tract irritation. Handle it while wearing personal protective equipment. **▲ CRITICAL** Warm formamide to RT before preparing the buffer.

BSA 100 mg ml^{-1} stock solution for LWA protocol Add 5 g of BSA to 30 ml of nuclease-free water. Mix the solution in a tube rotator until it is completely solubilized. Add 5 ml of 10× PBS and bring the solution to a final volume of 50 ml with nuclease-free water. Mix it well, filter first with a 0.4-µm filter unit and then with a 0.22-µm filter unit. Store it at 4 °C for up to 6 months.

PBS-FISH buffer for LWA protocol PBS-FISH buffer contains 1× PBS and 0.2 mg ml^{-1} BSA. Add 100 ml of 10× PBS and 2 ml of 100 mg ml^{-1} BSA stock solution to 898 ml of nuclease-free water. Transfer the solution to the LWA cell wash tank. Store the tank at 4 °C when not in use (for up to 3 months).

8% (vol/vol) PFA for LWA 8% (vol/vol) PFA is 8% (vol/vol) PFA in 1× PBS. Add 50 ml of 32% (vol/vol) PFA and 20 ml of 10× PBS to 130 ml of nuclease-free water. Mix the solution well and transfer the solution to the LWA Fix tank. Store the tank at 4 °C protected from light exposure when not in use (for up to 1 month). **! CAUTION** Paraformaldehyde is highly toxic, causes irritation and has caustic effects on skin, eyes and mucous membranes. Handle it while wearing the recommended personal protective equipment.

10% (vol/vol) Tween 20-2× saline-sodium citrate for LWA protocol Add 5 ml of Tween 20 and 5 ml of 20× saline-sodium citrate to 40 ml of nuclease-free water in a 50-ml polypropylene tube. Store the solution at RT for up to 6 months. **▲ CRITICAL** Tween 20 is very viscous. Pipette slowly and rinse the pipette at least five to six times before discarding.

Permeabilization buffer for LWA protocol Permeabilization buffer contains 0.4% (vol/vol) Tween 20 and 2× saline-sodium citrate in nuclease-free water. Add 20 ml of 10% (vol/vol) Tween 20 and 50 ml of 20× saline-sodium citrate to 430 ml of nuclease-free water. Mix the solution well and transfer it to the LWA lyse tank. Store it at RT for up to 6 months.

HWB for LWA protocol HWB contains 2× saline-sodium citrate, 10% (vol/vol) formamide and 0.2 mg ml^{-1} BSA in nuclease-free water. Add 50 ml of formamide, 50 ml of 20× saline-sodium citrate and 1 ml of 100 mg ml^{-1} BSA stock solution to 399 ml of nuclease-free water. Mix the solution well and transfer it to the LWA external cell wash tank. Store it at 4 °C for up to 3 months. **! CAUTION** Formamide is toxic and may cause eye, skin and respiratory tract irritation. Handle it while wearing personal protective equipment. **▲ CRITICAL** Warm formamide to RT before preparing the buffer.

HB 20% (wt/vol) dextran sulfate (HB 20% dx) for LWA protocol HB 20% dx contains 20% (wt/vol) dextran sulfate, 2× saline-sodium citrate, 10% (vol/vol) formamide, 2 mg ml^{-1} tRNA and 0.2 mg ml^{-1} nuclease-free BSA in nuclease-free water. Add 10 g of dextran sulfate and 100 mg of tRNA to 30 ml of nuclease-free water. Incubate the solution under agitation at 37 °C until it is completely solubilized. Add 5 ml of 2 × saline-sodium citrate, 5 ml of formamide and 200 µl of RNase-free BSA; mix the solution well and adjust to a final volume of 50 ml with nuclease-free water. Incubate the solution in a tube rotator until it becomes homogeneous and store it in aliquots (1 ml/tube) at −20 °C for up to 6 months. **! CAUTION** Formamide is toxic and may cause eye, skin and respiratory tract irritation. Handle it while wearing personal protective equipment. **▲ CRITICAL** The buffer is very viscous; mix it well and pipette slowly. Warm formamide to RT before preparing the buffer.

EQUIPMENT SETUP

LWA instrument setup Install the LWA according to the instrument user's guide. Fill the spindle-cleanse tank with Milli-Q water. If Milli-Q water is not available, fill the tank with filtered (0.22-µm filter), autoclaved dH_2O. Open the reagent door and install the tanks containing the FISH-Flow reagents, prepared as described in the Reagent Setup. Install the extra tank tubing assembly and the three-way stopcock (**Fig. 4a**) by disconnecting the fluid connector (on the tank) and by pulling the tube from the instrument bulkhead connector (**Fig. 4b**). Connect the extra tank tubing assembly via the bulkhead connector tube to the cell wash connector in the bulkhead (**Fig. 4c**), attach the shorter tube tank connector to the internal cell wash tank (PBS-FISH tank) (**Fig. 4d**) and attach the longer tube tank connector to the external tank (HWB tank) (**Fig. 4e**). Position the tubes and the stopcock in order to avoid tube tightening (**Fig. 4f**). Collocate the external tank tube between the waste and spindle-cleanse tanks and close the reagent door (**Fig. 4g**). **▲ CRITICAL** Monitor the fluid level of the external tank. The tank is not connected to a float sensor, and the instrument will not issue a warning if the fluid levels are low.

▲ CRITICAL Before filling the tanks with reagents, rinse with 70% (vol/vol) ethanol and incubate them for 30 min at RT. Discard the ethanol and rinse the tanks two to three times with autoclaved dH_2O (we use Milli-Q water filtered with a 0.22-µm Biopak polisher filter).

LWA protocol customization Follow the steps reported in the LWA user's manual to create a customized protocol. Use the parameters reported in **Table 3** to create and set up each FISH-Flow protocol. Protocols can be arranged in the instrument's main screen by using the 'assign buttons' feature.

Flow cytometer We have used BD Fortessa X20, BD LSRII and BD FACS Aria II flow cytometers for FISH-Flow experiments. Different flow cytometers must be tested and evaluated by the investigator. In multicolor experiments, use polystyrene compensation beads for single-color compensation controls. Beads can be used according to the manufacturer's instructions; however, use HWB to resuspend the beads in order to have the same acquisition conditions as the cell samples. For mRNA probes, Cy5- or Alexa-labeled commercial antibodies (such as isotype control antibodies) can be used as compensation control. Use an APC filter set (band-pass = 670/30) for Cy5-labeled probes and an FITC filter set (band-pass = 530/30) for Alexa 488. To obtain brighter signals from Alexa 594-labeled probes, use a flow cytometer equipped with a 561-nm laser and a 610/20 band-pass filter set.

PROCEDURE

▲ CRITICAL Warm all the buffers to RT before use. Warm complete RPMI in a 37 °C water bath.

▲ CRITICAL Perform all the centrifugation steps using a swinging-bucket rotor. Substantial cell loss will occur if samples are processed in centrifuges with fixed-angle rotors.

Cell culture and stimulation ● TIMING 7 h

1| Determine the number of total cells required for each experiment. Use 1–5 × 10^6 cells for each condition.

2| Thaw frozen PBMC vials in a 37 °C water bath, and slowly pipette the contents of the vial into warm complete RPMI (10 ml of medium for each milliliter of frozen cells) in a 50-ml centrifuge tube. Centrifuge the tube at 400*g* for 5 min at RT. Remove the supernatant fluid by aspiration, wash the cells by mixing with 10 ml of complete RPMI and centrifuge at 400*g* for 5 min at RT.

3| Aspirate the supernatant and resuspend the cells in complete RPMI at a cell concentration of 1–5 × 10^6 cells ml^{-1} and add 1 µl ml^{-1} of CD28/CD49d costimulatory antibodies (from a 0.1-mg ml^{-1} stock solution). Transfer 1 ml of these cells to a well of a 24-well plate for the unstimulated control. To the remainder of the cells, add 10 µg ml^{-1} PPD, mix well, transfer

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TABLE 3 | LWA protocol settings for FISH-Flow assay.

Protocol name	Step type	Step name	Variable	Value
PBS-FISH wash	Wash	PBS-FISH wash	Precipitation *g* force	461*g*
			Precipitation time	10 s
			Wash *g* force	361*g*
			Wash volume	6,400 µl
PFA-PBS-FISH wash	(i) Dispense	8% PFA	Reagent selection	Fix tank
			Dispense volume	300 µl
			Perform mix	Yes
			Max. Mix delay	1 s
			Defer until before next pause	No
	(ii) Incubate		Incubation time	30 min
			Intermittent mix	2.5 min
	(iii) Wash	PBS-FISH wash	Precipitation *g* force	461*g*
			Precipitation time	10 s
			Wash *g* force	361*g*
			Wash volume	6,400 µl
Permeabilization-HWB wash	(i) Dispense	0.4% Tween 20	Reagent selection	Lyse tank
			Dispense volume	350 µl
			Perform mix	Yes
			Max. mix delay	1 s
			Defer until before next pause	No
	(ii) Incubate		Incubation time	30 min
			Intermittent mix	10 min
	(iii) Wash	HWB wash	Precipitation *g* force	461*g*
			Precipitation time	10 s
			Wash *g* force	461*g*
			Wash volume	6,400 µl
Post-hybridization wash	(i) Dispense	0.4% Tween 20	Reagent selection	Lyse tank
			Dispense volume	400 µl
			Perform mix	Yes
			Max. Mix delay	1 s
			Defer until before next pause	No
	(ii) Wash	HWB wash	Precipitation *g* force	500*g*
			Precipitation time	30 s
			Wash *g* force	500*g*
			Wash volume	6,400 µl

to a 24-well plate and incubate for 6 h. For the positive control, add PMA and ionomycin at the final concentrations of 25 ng ml^{-1} and 0.5 µM, respectively, for the last 2 h of incubation. Incubate the plate at 37 °C in a 5% CO_2 humidified atmosphere.

4| During cell incubation, prepare the FcX and antibody master mixes as follows. For the former, combine 5 µl of FcX and 45 µl of PBS-FISH buffer per sample. Prepare a master mix for the complete set of fluorescently labeled antibodies and for each FMO control, using **Table 1** as a reference. Add the appropriate amount of anti-target antibodies to 50 µl of Brilliant Stain Buffer for each sample. If multiple BV or BUV dyes are not used together, antibodies can be mixed in 50 µl of PBS-FISH buffer instead of Brilliant Stain Buffer. Store the antibody master mixes at 4 °C in the dark for up to 10–12 h. Label 1.7-ml centrifuge tubes using the sample numbers reported in **Table 1**.

Antibody staining ● TIMING 1 h

5| After stimulation, remove the plate from the incubator, resuspend the cells by pipetting, transfer the cell suspension to the labeled 1.7-ml microcentrifuge tubes from Step 4 and centrifuge at 400*g* for 5 min at RT. Remove the supernatant fluid

by aspiration with a tip connected to the vacuum line, resuspend the pellet in the residual volume, add 1 ml of PBS-FISH buffer and centrifuge at 400*g* for 5 min at RT.
▲ **CRITICAL STEP** Note that if the operator chooses to perform LIVE/DEAD staining, cell pellets should be resuspended in 1× PBS (not PBS-FISH buffer), incubated with the appropriate amount of dye (see Experimental Design) and washed in PBS-FISH buffer before Step 6, as described in the 'Viability dye staining' section of the Experimental Design).
▲ **CRITICAL STEP** Beginning at this Step, briefly resuspend the pellet by gently flicking the bottom of the tube after each centrifugation. This practice will strongly reduce the formation of cell aggregates.
▲ **CRITICAL STEP** For Steps 5–15, when aspirating the supernatant fluid, use the 100-µl mark on the wall of the tube as a reference point to stop aspirating.
? TROUBLESHOOTING

6| Aspirate the supernatant, resuspend the cell pellet in the residual volume and add 50 µl/sample of FcX master mix from Step 4. Incubate the mixture for 5 min at RT. Add the appropriate volume of complete antibody mixture or FMO mixture from Step 4 to each sample, and incubate for 25 min at 4 °C in the dark.
▲ **CRITICAL STEP** Dispense the FcX and antibody mixtures directly into the cell suspension. Avoid dispensing the mixtures onto the tube walls.
▲ **CRITICAL STEP** From this Step onward, protect the tubes from direct light exposure to avoid fluorochrome degradation.
? TROUBLESHOOTING

7| Prepare fresh 4% (vol/vol) PFA in 1× PBS while the samples are incubating at 4 °C.

8| Add 0.5 ml of PBS-FISH buffer to each tube and centrifuge the tubes at 400*g* for 5 min at RT. Aspirate the supernatant, resuspend the cells, repeat the wash by adding 0.5 ml of PBS-FISH buffer and centrifuge the tubes at 400*g* for 5 min at RT.

Fixation and permeabilization ● TIMING 2 h
9| Aspirate the supernatant and resuspend the cell pellet in the residual volume by flicking the tube. Add 0.4 ml of 4% (vol/vol) PFA to each tube from Step 7, mix well and check for the presence of cell aggregates. If visible aggregates are present, resuspend by pipetting until the cell suspension is homogeneous. Incubate the samples for 30 min at RT in the dark.
▲ **CRITICAL STEP** It is important to have a homogeneous cell suspension during PFA fixation. Cell aggregates cannot be dissociated after fixation.

10| Add 0.5 ml of PBS-FISH buffer to each tube and centrifuge the tubes at 650*g* for 5 min at RT. Aspirate the supernatant, resuspend the cells in the residual volume, repeat the wash by adding 0.5 ml of PBS-FISH buffer and centrifuge the tubes at 650*g* for 5 min at RT.

11| Aspirate the supernatant and resuspend the cell pellet in the residual volume by flicking the tube. At this step, intracellular staining of proteins can be introduced, which is described in Steps 12–14. If no intracellular staining is performed, add 0.4 ml of permeabilization buffer to each tube, mix well, incubate for 30 min at RT in the dark and proceed directly to Step 15.

12| Prepare antibody and FMO control master mixes by adding the appropriate amounts of antibodies to 100 µl of ICS buffer for each sample.

13| Resuspend the cells in 400 µl of ICS buffer, incubate for 5 min at RT and centrifuge at 650*g* for 5 min at RT.

14| Aspirate the supernatant, resuspend the pellet in the residual volume and add 100 µl of the intracellular antibody or FMO control mix. Incubate for 30 min at 4 °C (time and temperature can be adjusted according to experimental conditions).

15| Add 0.5 ml of HWB to each tube and centrifuge at 650*g* for 5 min at RT. Aspirate the supernatant, resuspend the cells, repeat the wash by adding 0.5 ml of HWB and centrifuge at 650*g* for 5 min at RT.
? TROUBLESHOOTING

Hybridization ● TIMING overnight, 14–16 h
16| Aspirate the supernatant, leaving ~50 µl of residual volume (a low residual volume is required to avoid excessive dilution of the HB). Resuspend the pellet by gently flicking the tube. Thaw an aliquot of HB 10% dx along with vials of the probe's working concentration (5 ng μl^{-1}) according to the number of samples. Prepare a master mix by dispensing 1 µl (5 ng) of

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fluorochrome-conjugated probes in 49 µl of HB 10% dx for each sample (final concentration = 0.1 ng μl^{-1}). Mix well by vortexing. Dispense 50 µl of the mixture to each tube and thoroughly resuspend the cell pellet by pipetting, avoiding bubbles. Incubate the tubes overnight, at 37 °C in the dark.

▲ **CRITICAL STEP** Protect the probe vials from direct light exposure. Prolonged hybridization at 37 °C (>16 h) can lead to fluorochrome tandem dye degradation with associated signal loss.

? TROUBLESHOOTING

■ **PAUSE POINT** Samples should be incubated overnight at 37 °C.

Flow cytometry acquisition and data analysis ● TIMING (1–3 h)

17| Add 500 µl of HWB to each tube, mix well and centrifuge at 650*g* for 5 min at RT. Aspirate the supernatant, leaving ~100 µl of residual volume, then resuspend the cells in the residual volume, repeat the wash by adding 0.5 ml of HWB and centrifuge at 650*g* for 5 min at RT. Aspirate the supernatant, resuspend the cells and add 300 µl of HWB to each tube. Transfer the cell suspension to polystyrene round-bottom tubes and acquire the data in the flow cytometer per the individual laboratory's protocol.

18| Analyze the data with flow cytometry data analysis software.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice for the manual and semiautomated protocols can be found in **Tables 4** and **5**.

TABLE 4 | Troubleshooting for the PROCEDURE (manual protocol).

Step	Problem	Possible reason	Solution
5	Cell aggregation/lysis after stimulation	Unhealthy cells, low viability, incorrect thawing procedure	Improve cell culture conditions. Determine viability and cell recovery after thawing. We recommend starting with ≥90% viable cells
		Tube vortexing	Do not vortex. Resuspend the cells by gently flicking the tube
	Decreased/loss of fluorescence intensity of LIVE/DEAD fixable dye	Used PBS-FISH for viability cell staining	Use 1× PBS instead of PBS-FISH during viability cell staining
6	Heterogeneous antibody staining among samples	Antibody mix was dispensed on the wall of the tube	Dispense the antibody mix directly into the cell suspension
		Poor mixing after antibody addition	Mix well by pipetting or by flicking the tube
15	No visible pellet; low cell yield	Low initial cell concentration	Start the assay with a minimum of 1×10^6 cells per condition
		High cell loss due to high aspiration rate of the vacuum aspirator	Decrease the vacuum pressure. Add small tips (e.g., 10–200 µl) to the aspirator pipette to reduce aspiration
		Wrong centrifuge was used	Use only centrifuges with swinging-bucket rotors
		Poor sample handling	Gently resuspend the cell pellet by flicking the tubes
16	Poor separation of the antibody-surface-stained cells from the negative population	Degradation of the fluorochrome	Choose another fluorochrome. Start with nontandem dyes, as they are more stable than tandem dyes. Avoid prolonged hybridization time (>16 h)
		Incorrect amount of antibody	Titrate the antibody to determine optimal concentration
18	High background in probe's emission channels	High cell autofluorescence	Check the cell autofluorescence using an unstained control sample without labeled nucleic acid probes or antibodies
		Nonspecific binding of the probes	Use an irrelevant gene control probe (e.g., *GFP* or prokaryotic genes)
		Insufficient washing	Increase the washing volumes
		Cells treated with reagents/compounds exhibiting fluorescent properties	Avoid treating the cells with fluorescent compounds/reagents
	No/weak probe signal	Target gene poorly expressed. mRNA copy number below the assay sensitivity (<10 copies)	Confirm gene expression with an alternative method (e.g., RT-PCR)
			Use a highly expressed gene as a positive control
	Abnormal FSC-A and SSC-A values; large amounts of debris	Low cell viability; rough handling	Start with a good percentage of viable cells (cell viability ≥90%). Avoid cell damage by mechanical stress (e.g., vortexing)

TABLE 5 | Troubleshooting for LWA semiautomated protocol (**Box 1**).

Step	Problem	Possible reason	Solution
Box 1, step 5	Cell aggregation/ lysis after stimulation	Unhealthy cells; low viability; incorrect thawing procedure	Improve cell culture conditions. Determine viability and cell recovery after thawing. We recommend starting with ≥90% viable cells
		Tube vortexing	Do not vortex. Resuspend the cells by gently flicking the tube
	Lysed cells in each tube	Wrong cell wash tank (HWB) was selected	Turn the three-way stopcock in order to select the PBS-FISH tank
	No wash or cells partially washed (presence of medium in tubes)	Wrong positioning of the three-way stopcock; cell wash tank not connected properly; pinched tubes	Check for misalignment of the three-way stopcock and for cell wash tube pinches or tightening. Perform a wash test with a tube containing 1 ml of RPMI
Box 1, step 6	Heterogeneous antibody staining among samples	Antibody mix dispensed on the wall of the tube	Dispense the antibody mix directly into the cell suspension
		Poor mixing after antibody addition	Mix well by pipetting or by flicking the tube
Box 1, step 9	No probe signal	External HWB cell wash tank was not connected after the PFA-PBS-FISH protocol	Turn the three-way stopcock to select the HWB external tank as the cell wash tank
Box 1, step 10	Samples exhibit variable basal fluorescence (variable fluorescence intensity of the negative cell population)	Probe master mix was not mixed properly	Prepare a homogeneous probe solution. Vortex the probe master mix
		Probe mix was dispensed onto the walls of the tube. Poor sample mixing after probe mix addition	Dispense the probe mix into the cell suspension and briefly vortex each tube to resuspend thoroughly
Box 1, step 13	Poor separation of the antibody-surface-stained cells from the negative population	Degradation of the fluorochrome	Choose another fluorochrome. Start with nontandem dyes, as they are more stable than tandem dyes. Avoid prolonged hybridization time (>16 h)
		Wrong amount of antibody was used	Titrate the antibody to determine optimal concentration
	High background in probe emission channels	High cell autofluorescence	Check the cell autofluorescence using an unstained control sample without labeled nucleic acid probes or antibodies
		Nonspecific binding of the probes	Use an irrelevant gene control probe (e.g., *GFP* or prokaryotic genes)
		Cells were treated with reagents/compounds exhibiting fluorescent properties	Avoid treating the cells with fluorescent compounds/reagents
	No/weak probe signals	Target gene was poorly expressed; mRNA copy number was below the assay sensitivity (<10 copies)	Confirm gene expression with an alternative method (e.g., RT-PCR)
			Use a highly expressed gene as a positive control
	Abnormal FSC-A and SSC-A values; large amounts of debris	Low cell viability; rough handling	Start with highly viable cells (cell viability ≥90%). Avoid cell damage by mechanical stress (e.g., vortexing)

● TIMING

Steps 1–4, cell culture and stimulation: 7 h
Steps 5–8, antibody staining: 1 h
Steps 9–15, fixation and permeabilization: 2 h
Step 16, hybridization: overnight 14–16 h
Steps 17 and 18, flow cytometry acquisition and data analysis: 1–3 h
Box 1, Steps 1–4, cell culture and stimulation: 7 h
Box 1, Steps 5 and 6, antibody staining: 45 min
Box 1, Steps 7–9, fixation and permeabilization: 1.5 h
Box 1, Step 10, hybridization: overnight 14–16 h
Box 1, Steps 11–13, flow cytometry acquisition and data analysis: 1–3 h

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ANTICIPATED RESULTS

Initial experiments were performed to determine the applicability of FISH-Flow to the detection of signals seen by fluorescence microscopy and to establish the specificity of the method. In these experiments, HeLa cells were subjected to serum starvation for 48 h and left either untreated or treated with serum for 30 min as described[20]. Fluorescent spots associated with *c-fos* gene induction in serum-starved HeLa cells were detected by fluorescence microscopy (**Fig. 1b**). When the experiment was performed using FISH-Flow, it was found that almost 80% of the serum-treated cells expressed detectable amounts of *c-fos* mRNA, whereas only ~0.3% of untreated cells expressed *c-fos* mRNA (**Fig. 1c**). By contrast, the proportion of positive cells was very similar in treated and untreated cells when hybridization was performed with probes against an irrelevant gene (*GFP*) (~0.7% and 0.1%, respectively) (negative control) or a constitutively expressed gene (*GAPDH*) (97% and 95%, respectively) (positive control) (**Fig. 1c**). Thus, FISH-Flow detects induction of gene expression in a specific manner.

To evaluate the FISH-Flow protocol in T-cell activation assays, we stimulated PBMCs from an LTBI$^+$ donor with the mycobacterial antigen tuberculin PPD. As a positive activation control, we used staphylococcal enterotoxin B (SEB), which is a super antigen and, as such, does not require immunological memory. In these experiments, we co-stained the cells with an anti-CD3 antibody that serves as a T-cell marker. These assays were performed using both the manual and the semiautomated protocols. Typical results are shown in **Figure 2**. The probes against *IFNG* mRNA stained a significant fraction of T cells in stimulated cells, whereas only very few unstimulated cells were found in the gate of positive cells. Even though the semiautomated protocol resulted in a lower number of IFNG$^+$CD3$^+$ cells (35–50% fewer cells) as compared to manual assessment, the two protocols yielded qualitatively similar results. Thus, both the manual and the semiautomated versions of the protocol are apt to reveal the induction of cytokine gene expression in antigen-specific T cells after *ex vivo* exposure to antigen.

We applied the FISH-Flow to a second example of cell stimulation, which involved a different cell type. We took advantage of the notion that stimulation of the mouse macrophage cell line RAW264.7 with IFN-γ induces production of IRGA6. We applied FISH-Flow and intracellular staining to IFN-γ-stimulated RAW264.7 cells and unstimulated controls to concurrently detect induction of *Irga6* mRNA and IRGA6 protein in the same cells. In one example, we performed a time-course experiment to monitor the kinetics of accumulation of *Irga6* mRNA and IRGA6 protein in the same cells. We observed that levels of *Irga6* mRNA decline after 9 h, whereas levels of IRGA6 protein continue to rise for 24 h (**Fig. 3**). This time-course example illustrates how multiplex analysis of both mRNAs and proteins may provide integrated information on multiple aspects of the cellular response to a stimulus.

To demonstrate multiplex detection of surface phenotypic markers and intracellular mRNA, we stained cells with four surface markers that identify different PBMC subsets. After antigen-specific (PPD) stimulation or nonspecific global stimulation (PMA/ionomycin) of PBMCs isolated from an LTBI$^+$ donor, we used the semiautomated protocol to process the samples. In this example, we performed antibody staining against the surface markers CD3, CD4, CD8 and CD56 (a marker of natural killer cells[21]), followed by hybridization with *IFNG* mRNA probes. The scattering properties of the cells were used to distinguish single cells from cell clusters and lymphocytes from all other peripheral blood cells (**Fig. 5a**), whereas the cell surface markers allowed for identification of T cells, NK cells, and CD4$^+$ and CD8$^+$ T-cell subsets (**Fig. 5a**). In agreement with a previous report[22], the analysis showed prevalent induction of *IFNG* mRNA in CD4$^+$ T cells (**Fig. 5b**) and in CD56$^+$ cells among the CD3$^-$ population (**Fig. 5c**). Treatment with PMA/ionomycin resulted in a strong *IFNG* induction in CD4$^+$, CD8$^+$ and CD56$^+$ cells (**Fig. 5c**), as expected from global stimulation. This example illustrates the powerful analytical possibilities that result from combining antibody staining and mRNA detection in FISH-Flow.

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AUTHOR CONTRIBUTIONS R.A., M.L.G. and S.T. conceived and designed the experiments. R.A. and S.T. performed the experiments. R.A., M.L.G. and S.T. analyzed the data. Y.B. and R.P. contributed to the initial FISH-Flow protocol. F.R., K.L. and P.V. contributed to the initial FISH-Flow experiments. D.M. and J.S. developed the modified LWA instrument and contributed to semiautomated protocol design and data analysis. Y.Z. and G.S.Y. designed and provided cells for the concurrent FISH-Flow/ICS time-course experiments. S.T. developed the RNA FISH probes. A.A.L. obtained blood samples from patients. M.L.G. supervised the study. R.A., M.L.G. and S.T. wrote the manuscript.

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