

# Profiling T Cell Activation Using Single-Molecule Fluorescence In Situ Hybridization and Flow Cytometry

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Flow cytometric characterization of Ag-specific T cells typically relies on detection of protein analytes. Shifting the analysis to detection of RNA would provide several significant advantages, which we illustrate by developing a new host immunity-based platform for detection of infections. Cytokine mRNAs synthesized in response to ex vivo stimulation with pathogen-specific Ags are detected in T cells with single-molecule fluorescence in situ hybridization followed by flow cytometry. Background from pre-existing in vivo analytes is lower for RNAs than for proteins, allowing greater sensitivity for detection of low-frequency cells. Moreover, mRNA analysis reveals kinetic differences in cytokine expression that are not apparent at the protein level but provide novel insights into gene expression programs expected to define different T cell subsets. The utility of probing immunological memory of infections is demonstrated by detecting T cells that recognize mycobacterial and viral Ags in donors exposed to the respective pathogens. *The Journal of Immunology*, 2015, 194: 000–000.

To diagnose an infection, the presence of the pathogen is usually detected by culturing the pathogen from host body fluids or by in vitro amplification of the pathogen's nucleic acids. Alternatively, either Ab- or T cell-mediated immune responses elicited in the host are probed. The functional states of effector and memory T cell subsets are often defined by the pattern of expression of cytokines IL-2, IFN- $\gamma$ , and TNF- $\alpha$ , and their phenotypic state is defined by their surface markers such as CD4 and CD8 (1, 2). It has been proposed that the medically significant goal of distinguishing between different stages of some asymptomatic infections, for example, between a stable latent *Mycobacterium tuberculosis* infection and infection that has begun progression to active disease, will require comprehensive characterization of multiple surface markers and secreted cytokines in single T cells responding to Ags (1–3).

In the interrogation of T cell-mediated immunity by commonly used methods, PBMCs are stimulated with pathogen-derived peptides ex vivo and the responses of Ag-specific T cells that result in cytokine production are measured by ELISA or intracellular immunofluorescence staining (1, 4). However, the slow turnover of these protein analytes limits the utility of ex vivo assays in two ways. Sensitivity is compromised due to remnants of

protein from prior in vivo activation. Additionally, the persistence of proteins prevents kinetic analysis of changes in gene expression in response to stimulation by Ag as a means to distinguish among T cell subsets and identify single-cell signatures.

To overcome these drawbacks, we focused on the detection of mRNAs rather than proteins produced during T cell activation. As targets for analysis, mRNAs are preferable because they are relatively short-lived (consequently, RNA produced in response to previous in vivo activation is degraded by the time the ex vivo stimulation is performed). Moreover, in contrast to proteins, which require empirical production of useful Abs, probes can be generated systematically for any mRNA from the knowledge of its sequence. Another advantage is the ability to develop standardized detection protocols rather than methods that must be varied depending on the localization of a protein analyte or properties of the detection reagent. This benefit is particularly significant because treatments to retain secreted proteins are avoided because mRNAs are always retained intracellularly. We have previously developed single-molecule fluorescence in situ hybridization (sm-FISH) for extremely sensitive detection of mRNAs in cells. In this technique, mRNAs are detected by hybridization with ~50 short oligonucleotide probes, which renders each mRNA molecule sufficiently bright to be visualized in fluorescence microscopy (5). However, microscopy is poorly suited to detection of rare cells that are activated by Ag.

In this study, we present a new platform for characterizing activated T cells by combining sm-FISH with flow cytometry. This method yields improved sensitivity compared with detection of protein analytes and allows measurements of response kinetics. We also demonstrate multiplex detection of RNA and protein analytes for functional characterization of cells at the RNA level and their phenotypic characterization at the protein level. Pathogen-specific responses are readily detected for active tuberculosis and for T cell memory of common viral infections and anti-tetanus vaccination.

## Materials and Methods

### Study subjects

Tuberculosis (TB) patients and latent TB infection (LTBI)-negative participants were enrolled from the Lattimore Practice Clinic in Newark and the Middlesex County TB Clinic (Rutgers Biomedical and Health

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Abbreviations used in this article: FISH, fluorescence in situ hybridization; LTBI, latent tuberculosis infection; SEB, *Staphylococcus aureus* enterotoxin B; sm-FISH, single-molecule fluorescence in situ hybridization; TB, tuberculosis; TMR, teramethylrhodamine.

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Sciences, New Jersey Medical School) under a protocol approved by the New Jersey Medical School Institutional Review Board. Consented participants (20–65 y old) were eligible when they were under clinical investigation for active pulmonary TB or when screened for latent TB infection by tuberculin skin test. Their infection status was confirmed by microbiological and radiological procedures as well as by QuantiFERON-TB Gold (Cellestis, Valencia, CA) testing.

#### Isolation of PBMCs, cell culture, and stimulation assays

Buffy coats (New York Blood Center, Long Island City, NY, and New Jersey Blood Center, East Orange, NJ) or heparinized fresh blood was used to separate PBMCs on Ficoll (Sigma-Aldrich, St. Louis, MO). All experiments were repeated with PBMCs derived from at least three different individuals unless indicated otherwise.

In stimulation assays, PBMCs were cultured in RPMI 1640 (Mediatech, Manassas, VA) supplemented with 10% FBS, glutamine, penicillin, and streptomycin (culture medium) at a density of  $5 \times 10^6$  to  $8 \times 10^6$  cells/ml. For global stimulation, cells were cultured with 25 ng/ml PMA (Sigma-Aldrich) and 0.5  $\mu$ M ionomycin (free acid) (Alexis Biochemicals, San Diego, CA) for the indicated times at 37°C and 5% CO<sub>2</sub>.

For Ag-specific peptide stimulations, we employed an optimized stimulation regimen described previously (6). PBMCs were incubated on ice with anti-CD28 mAb 9.3 (Bio X Cell, West Lebanon, NH) at 10  $\mu$ g/ml for 0.5 h. CD28 mAb-treated PBMCs were seeded into six-well plates in culture medium supplemented with IL-1 $\beta$  (20 ng/ml), IL-6 (10 ng/ml), and IFN- $\alpha$  (0.2 U/ml) (PeproTech, Rocky Hill, NJ) for stimulation with pooled mixtures of peptides from PM-CEF-E and PM-CEFT-MHC-II (JPT Peptide Technologies, Berlin, Germany) at 2  $\mu$ g/ml for 2 h. The peptides comprise Ag from influenza virus, EBV, human CMV, and tetanus toxoid (*Clostridium tetani*). Alternatively, cells were stimulated for 2 h with *Staphylococcus aureus* enterotoxin B (SEB; American Type Culture Collection, Manassas, VA) at 1.6  $\mu$ g/ml.

PBMCs obtained from TB and LTBI-negative donors were similarly preincubated with anti-CD28 mAb and then stimulated in culture medium supplemented with cytokines and with a mixture of overlapping peptides derived from *M. tuberculosis* proteins ESAT6 and CFP10 (Oxford Immunotec, Oxford, U.K.) at 8.25  $\mu$ g/ml each for 2 h.

#### Synthesis of sm-FISH probes

We designed 48–50 probes for each target mRNA using a probe design program (available at: <http://www.singlemoleculefish.com>). Oligonucleotides were obtained with 3'-amino modifications from BioSearch Technologies (Novato, CA). They were pooled, coupled with tetramethylrhodamine (TMR) or Cy5 fluorophores, and the labeled products were purified using previously described procedures (5).

#### FISH

Stimulated PBMCs were collected in microcentrifuge tubes, washed with FBS/PBS buffer (0.5% FBS, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM KH<sub>2</sub>PO<sub>4</sub>, 137 mM NaCl, 27 mM KCl [pH 7.4]), and fixed in 4% formaldehyde/PBS buffer at 4°C for 16 h. Fixed cells were washed again with FBS/PBS buffer and then permeabilized in 70% ethanol at 4°C for 2–4 h. Permeabilized cells were equilibrated in 10% formamide/2 $\times$  SSC (Ambion/Life Technologies, Grand Island, NY) hybridization wash buffer containing 0.5% FBS and 2 mM vanadyl-ribonucleoside complex (New England BioLabs, Ipswich, MA) supplemented with 10% culture medium mentioned above. Cells were then resuspended in a 50- $\mu$ l volume of hybridization buffer (10% formamide, 10% dextran sulfate, 2 mM vanadyl-ribonucleoside complex, 0.02% RNase-free BSA,  $\mu$ g/ $\mu$ l *Escherichia coli* tRNA) containing Cy5-labeled mRNA probes and incubated overnight at 37°C. The probe concentration ranged from 3 to 20 ng/reaction. The cells were then washed by incubating twice for 30 min at room temperature in 400  $\mu$ l hybridization wash buffer.

#### Flow cytometry

After hybridization and wash, PBMCs were immediately analyzed by flow cytometry in the same buffer. Single-color flow cytometry was performed on a BD LSR II flow cytometer (FITC detection using 488-nm laser and 530/30-nm emission filter, and Cy5 detection using 633-nm laser and 660/20-nm emission filter). Two-color cytometry and cell sorting was performed on BD FACSAria II flow cytometer (FITC detection using 488-nm laser and 530/30-nm emission filter, TMR detection using 561-nm laser and 582/15-nm emission filter, and Cy5 detection using 640-nm laser and 670/30-nm emission filter). Lymphocytes were gated first on size and granularity and then for the relevant fluorochrome. Data analysis was performed with either FACSDiva (Becton Dickinson, San Jose, CA) or FlowJo (Tree Star,

Ashland, OR) software. Sorted cells were centrifuged for subsequent image analysis. In some experiments, as indicated in the text, cells were immunostained for cell surface markers or intracellular cytokines prior to hybridization with RNA probes. Proteins were detected with specific allophycocyanin-conjugated mAbs (eBioscience, San Diego, CA) according to manufacturer protocols.

#### Imaging and mRNA quantification

After hybridization or sorting by flow cytometry, cells were centrifuged and resuspended in 5  $\mu$ l freshly prepared deoxygenated mounting medium designed to prevent photobleaching (5). For microscopy, the cells were sandwiched between a coverslip-bottom dish and an agar pad prepared using the deoxygenated mounting medium. Imaging and counting of mRNA spots were performed using equipment and a custom image processing program as previously described (5). In cells with very high expression, it is difficult to resolve individual molecules present in clusters. In these cases, the number of molecules was determined by dividing the overall intensity of the cell by the unit intensity of single molecules.

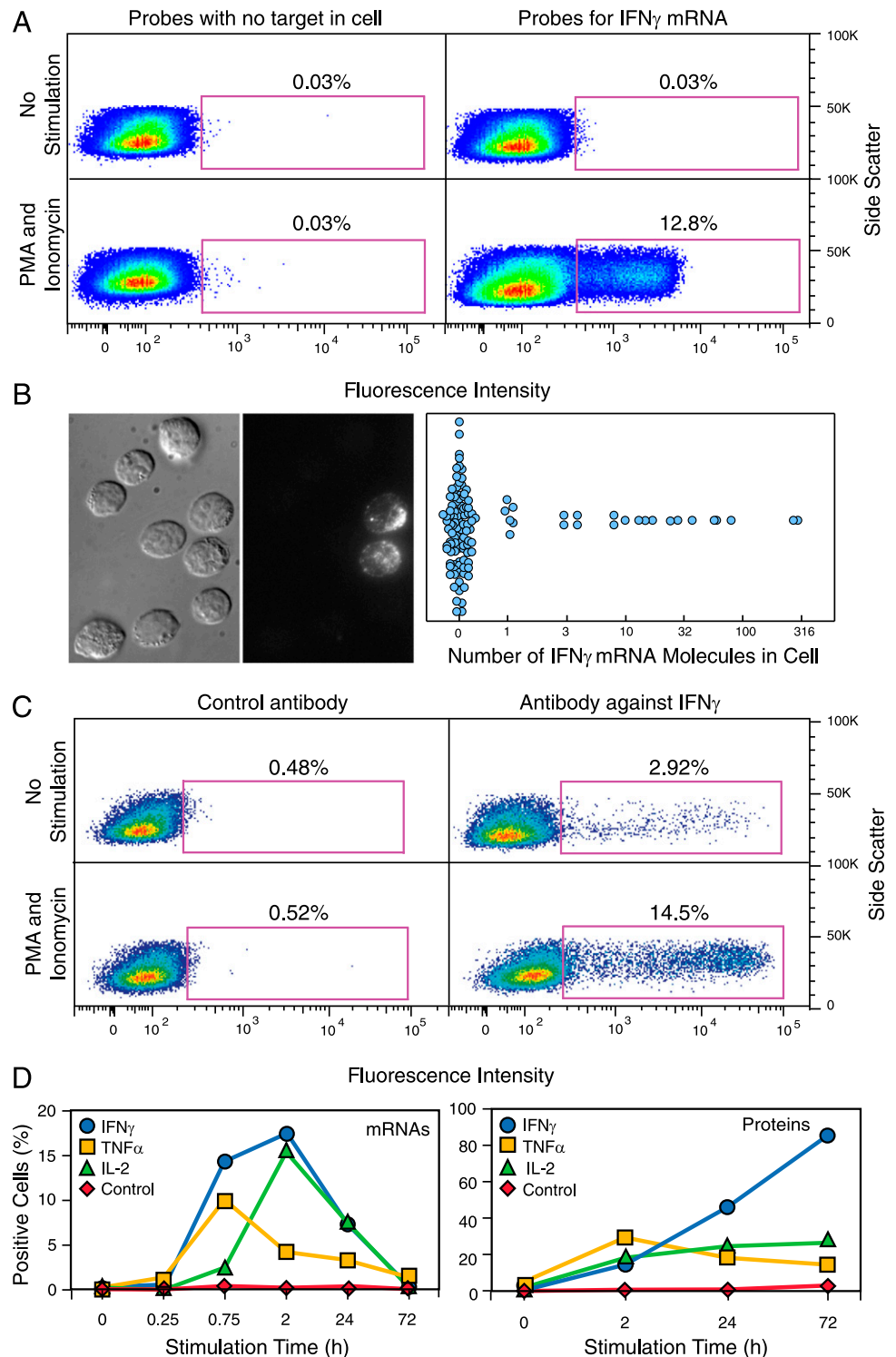
## Results

Pathogen-specific T cells are a very small fraction of PBMCs in blood. It would be challenging to identify them using microscopy-based measurements previously used with sm-FISH, so we sought to determine whether detection would be amenable to flow cytometry. To explore this possibility, we first stimulated PBMCs with a mixture of PMA and ionomycin (which induce the protein kinase C pathway and a downstream gene expression cascade in a high proportion of PBMCs), then fixed and permeabilized cells prior to probing with a set of sm-FISH probes specific to IFN- $\gamma$  mRNA and labeled with Cy5. Flow cytometric analysis indicated that 12.8% of the cells were specifically labeled and the rest remained relatively unstained (Fig. 1A, lower right panel). Negative controls (unstimulated cells hybridized with IFN- $\gamma$  probes or stimulated cells hybridized to a probe set with no cellular target) showed only 0.03% of stained cells.

Applications of sm-FISH that are based on microscopic analysis (5) achieve single-molecule sensitivity by distinguishing spot-like signals characteristic of the target mRNA molecules from diffuse background fluorescence generated by nonspecifically bound probes. This distinction is not attainable in flow cytometry because only the integrated fluorescence from each cell is recorded. To estimate the effective detection sensitivity of this method, we performed fluorescence microscopy on stimulated cells and determined the number of mRNA molecules present in individual cells. Quantitative analysis of the results show that the brightest 12.8% of cells expressed four or more IFN- $\gamma$  transcripts whereas the other cells had less or none (Fig. 1B). The close correspondence between the data from microscopy and flow cytometry indicates that the sensitivity of flow cytometric detection is  $\sim$ 5 mRNA molecules. To provide additional support for this limit, we sorted cells into different bins and then determined the average number of mRNA molecules per cell by microscopy. This analysis revealed similar sensitivity of detection (5–10 molecules) and showed that the number of expressed mRNAs per cell in each bin is proportional to the average fluorescence intensity of the bin (Supplemental Fig. 1).

We next determined how the population of activated T cells expressing IFN- $\gamma$  transcripts compares to the cell population identified by measuring the corresponding protein analyte. After stimulating cells in the presence of brefeldin A, which prevents protein secretion, intracellular IFN- $\gamma$  was stained with a specific fluorophore-conjugated mAb. As shown in Fig. 1C, 14.5% of the cells express IFN- $\gamma$  protein, a population very similar to that detected by mRNA staining (Fig. 1A). However, 2.92% of unstimulated cells were expressing IFN- $\gamma$  protein, whereas only 0.03% of cells were expressing IFN- $\gamma$  transcripts. This dramatic difference indicates that IFN- $\gamma$  proteins persist in cells from prior in vivo stimulations,

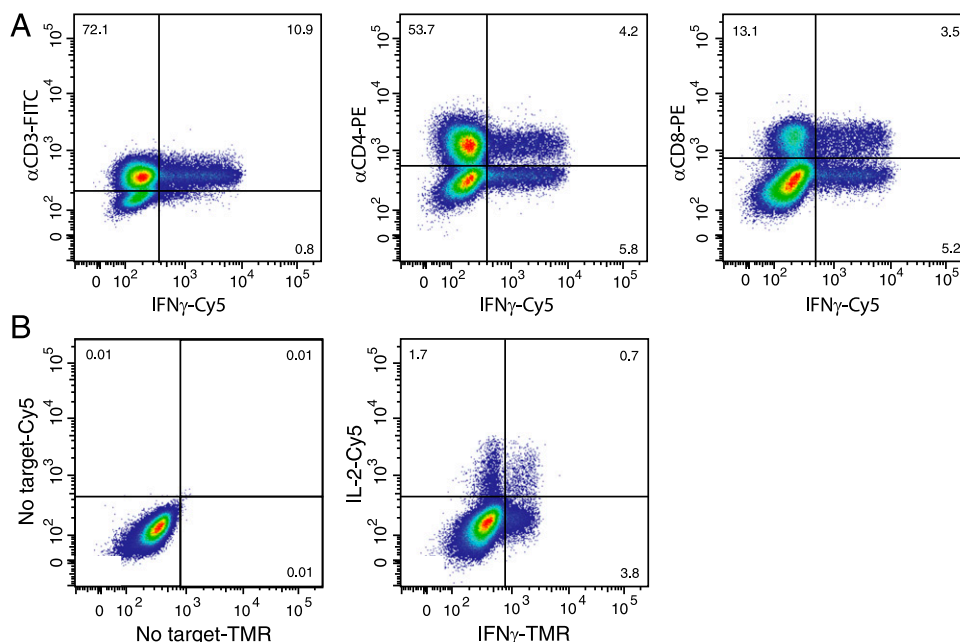
**FIGURE 1.** Flow cytometry–based detection of IFN- $\gamma$  mRNA and protein analytes in activated T cells. Ficoll-isolated PBMCs were stimulated with PMA and ionomycin. **(A)** Following a 2-h stimulation, cells were fixed, permeabilized, and hybridized to a Cy5-labeled probe set specific for IFN- $\gamma$  mRNA (*right panels*). The control probe set (*left panels*) targets GFP mRNA, which is not expressed in the cells and is used to gate on positive signal. **(B)** Cells from the same sample used in the flow cytometry readout shown in *lower right panel* of **(A)** were analyzed by microscopy using an  $100\times$  oil objective, and the number of IFN- $\gamma$  mRNA molecules in each cell was determined. In the *left and middle panels*, diffraction interference contrast image and fluorescence image of the same cells, respectively, are shown. In the *right panel*, the number of IFN- $\gamma$  mRNA molecules found in 124 cells that were analyzed is shown. **(C)** Cells stimulated in the presence of brefeldin A were analyzed for IFN- $\gamma$  protein production. Cells were fixed, permeabilized, and immunostained with either anti-IFN- $\gamma$  or isotype control allophycocyanin-conjugated mAb. **(D)** PBMCs from a different donor were stimulated for the indicated times and then examined for induced cytokine expression. In the *left panel*, the percentages of cells that express IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 mRNAs detected by Cy5-labeled probes and flow cytometry are shown. In the *right panel*, the percentages of cells in the same cultures expressing IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 proteins identified by intracellular staining and flow cytometry are shown. Time axis is not to scale.



whereas the encoding mRNAs are more rapidly degraded. Therefore, we detect only the mRNA synthesized *de novo* in response to *in vitro* stimulation, which indicates that mRNA-based assays have higher signal-to-background ratios than do conventional immunofluorescence-based ones. Moreover, the detection of IFN- $\gamma$  mRNA by itself identifies activated cells.

It has been reported that individual cells secrete different cytokines with discrete kinetics (7). To explore whether the signature of these kinetic differences can be traced to different times of induction of cytokine mRNAs in single cells, we monitored the levels of mRNAs encoding IL-2, IFN- $\gamma$ , and TNF- $\alpha$  as

a function of time after PMA/ionomycin stimulation. We found that although the level of each mRNA first increases and then declines in the population, the time of the rise and fall is different for each species (Fig. 1D, Supplemental Fig. 2). We also explored the expression of several other mRNAs that are induced upon T cell activation and found that they too exhibit distinct kinetics (data not shown). These observations provide an additional temporal parameter for defining functional subpopulations of activated T cells based on the ability to observe the decline as well as the increase in mRNA levels over relatively short time periods.

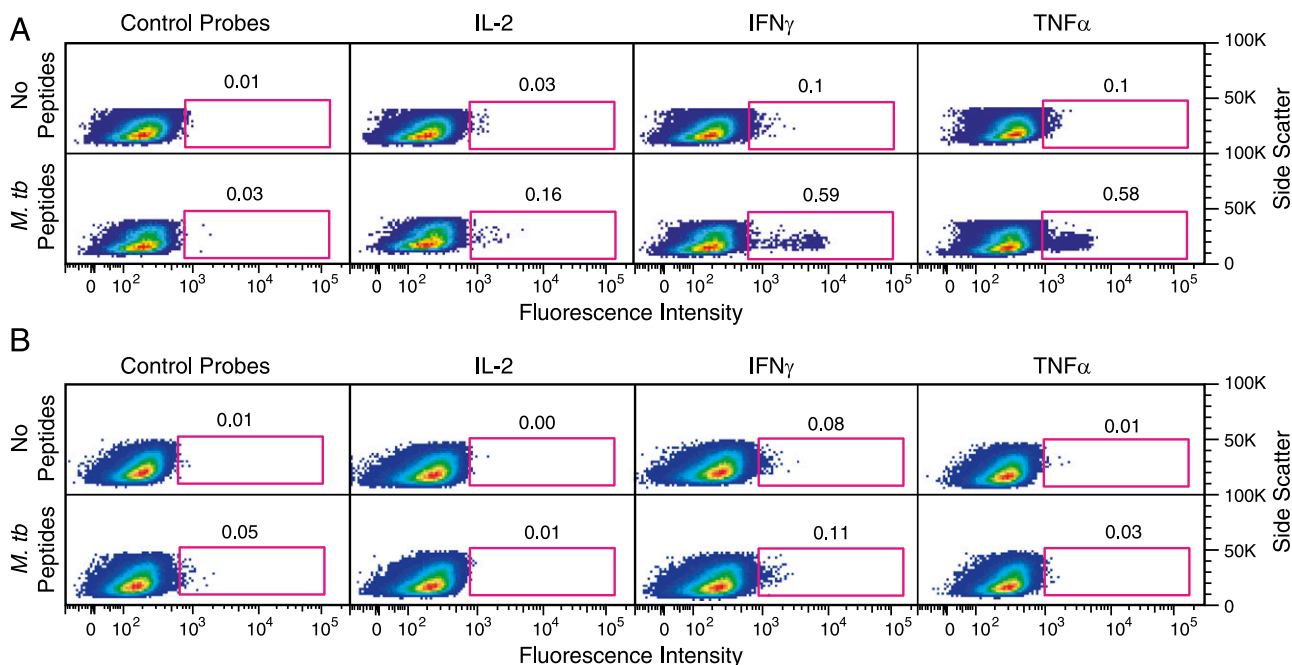


**FIGURE 2.** Multiplex profiling of T cells for surface markers and cytokine expression. PBMCs were stimulated with PMA and ionomycin for 2 h. **(A)** Activated T cells were double stained for surface markers CD3, CD4, or CD8 (FITC- or PE-conjugated mAb) and IFN- $\gamma$  mRNA (Cy5-labeled mRNA probe). **(B)** Activated T cells were double stained for IL-2 and IFN- $\gamma$  mRNAs with TMR- and Cy5-labeled mRNA probes. Frequencies of cells in the indicated quadrants are shown for one of two experiments that were performed.

Furthermore, the changing ratios between mRNAs and proteins over time may hold additional information about the signatures of responses to stimulation.

To demonstrate simultaneous detection of protein and mRNA markers, we stained PMA/ionomycin-stimulated cells for markers of T cell subsets, CD3, CD4, or CD8 with specific Abs, while also probing for IFN- $\gamma$  mRNA by sm-FISH. These bichromatic profiles identify the proportions of cells in each subset that respond

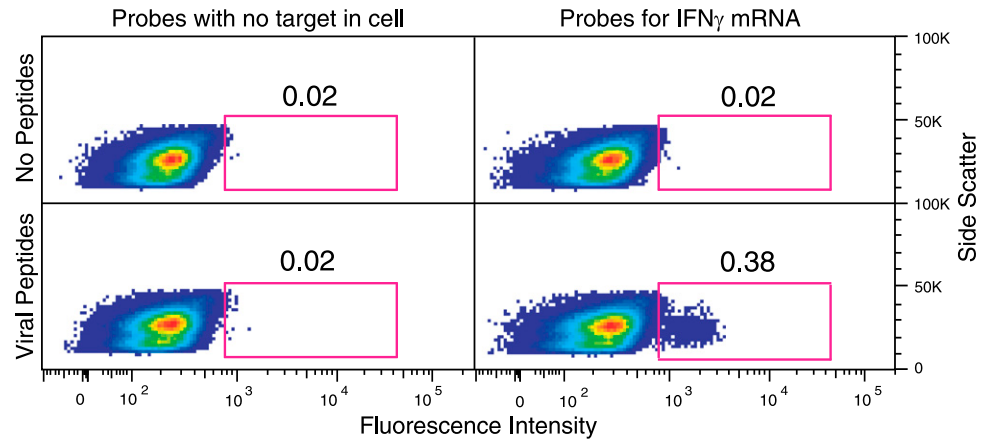
to the stimulation by expressing IFN- $\gamma$  mRNA (Fig. 2A). Moreover, the capability of this method to detect the expression of multiple cytokines was shown by two-color labeling of stimulated cells with mRNA probes conjugated to two distinguishable fluorophores. Three stimulated cellular populations, each expressing either one or both of the IL-2 and IFN- $\gamma$  mRNAs labeled with Cy5 or TMR, were clearly resolved (Fig. 2B, right panel). Because a large variety of fluorophores spanning the entire spectrum are



**FIGURE 3.** Detection of *M. tuberculosis*-specific T cells by mRNA-based flow cytometric analysis in peripheral blood. PBMCs obtained from a TB patient **(A)** or from an individual without LTBI **(B)** were cultured for 2 h in the presence of IL-1 $\beta$ , IL-6, and IFN- $\alpha$  alone (upper panel row) or with anti-CD28 Ab and a mixture of peptides derived from ESAT6 and CFP10 proteins (lower panel row). Frequencies (percentages) of positive cells are shown.



**FIGURE 4.** Detection of IFN- $\gamma$  mRNA expression in Ag-specific T cells. PBMCs obtained from healthy donors were stimulated for 2 h in medium without (*upper row*) or with a mixture of peptides (*lower row*) derived from common viruses and tetanus toxoid. Cells were probed with IFN- $\gamma$  mRNA-specific, Cy5-labeled probes and analyzed by flow cytometry. The frequencies of Ag-specific T cells expressing IFN- $\gamma$  mRNA molecules are shown.



available for labeling Abs and sm-FISH probes, comprehensive typing of polyfunctional T cells based on multiple activation markers including mRNAs is now possible.

We next applied the assay for detection of *M. tuberculosis*-specific T cells in PBMCs of active pulmonary TB patients. A brief, 2-h stimulation with a peptide mix derived from ESAT6 and CFP10 proteins, which is used in a clinical assay of TB infection (T-SPOT.TB, Oxford Immunotec), showed that Ag-specific T cells from an infected donor respond with a 6-fold increase in the synthesis of mRNAs encoding IL-2, IFN- $\gamma$ , and TNF- $\alpha$  (Fig. 3A). Because the pathogen-specific T cells are very few, only a small fraction of the peptide-stimulated PBMCs expressed this activation profile (0.16–0.59%) as compared with a global PMA/ionomycin stimulus (10–16%) (Fig. 3A, Supplemental Fig. 2). Importantly, tuberculin skin test-negative donors showed no increase in cytokine mRNA synthesis upon stimulation with the same Ag (Fig. 3B).

We tested the early response to SEB, a superantigen that forms a linkage between MHC class II and TCR molecules, to examine T cell activation that is Ag-dependent yet memory-independent. This T cell activation procedure is expected to be relatively insensitive to immunological exposures in individuals and thus allows us to validate the reproducibility of flow cytometric measurements of cytokine mRNA expression in activated T cells among different donors and for different donations from the same donor. For one donor who was tested twice 4 wk apart, small but consistently detectable fractions of cells were found to express the three cytokines mRNAs upon stimulation with SEB for 2 h (Supplemental Fig. 3, Supplemental Table I). We similarly tested two more donors, each at two different times, and found that the number of activated cells was similar (Supplemental Table I). Thus, flow cytometric measurements of cytokine mRNA expression reproducibly detects the activated cells. Fold variations with SEB stimulation between serial donations were less than those with TB-derived peptide stimulation in healthy donors compared with TB-infected donors (compare Fig. 3 with Supplemental Table I).

To show detection of T cells retaining the memory of common viral infections and vaccinations, we stimulated PBMCs obtained from a healthy donor with a mixture of peptides derived from human CMV, influenza virus, EBV, and tetanus toxoid. Because the immunological memory of these pathogens and vaccinations is highly prevalent in the United States population (8), a response to one or more of the Ag is likely in most donors. This peptide mixture induced expression of IFN- $\gamma$  in 0.38% of the cells for an individual, with only 0.02% being positive with control probes (Fig. 4). For six individuals the average percentages of positive cells with IFN- $\gamma$  probes were  $0.26 \pm 0.07$  with Ag stimulation and  $0.07 \pm 0.06$  without Ag stimulation ( $p = 0.001$ ). Alternatively, the

percentages of positive cells with the control probes were  $0.02 \pm 0.01$  with Ag stimulation and also  $0.02 \pm 0.01$  without Ag stimulation ( $p = 0.45$ ). This study indicates that T cells specific to the set of viral Ag can be detected by our assay.

## Discussion

In the present study, we show a highly sensitive method to detect T cell activation in single cells soon after they are stimulated. Using sm-FISH and flow cytometry, which we call FISH-Flow, we are able to detect as few as 5–10 copies of an RNA target per cell. The rapid turnover of RNA compared with protein greatly increases the ability to detect induction in response to ex vivo stimulation, as a lower level of prior expression exists for RNA than for proteins. For a memory-dependent stimulus with TB-derived peptides, the assay shows clearly detectable Ag-specific responses in exposed donors compared to unexposed ones. Additionally, we show that a memory-independent stimulus, SEB, also induces reproducible responses.

Although in the present report we focus on mRNAs encoding cytokines, Ag-driven expression of other RNAs can similarly be studied. However, because posttranscriptional modulation, translational control, and other determinants of gene product abundance can sometimes alter the correspondence between mRNA and the proteins that they encode, the relationship between the two should be established on a case-by-case basis as we do in the present study. Multiplex mRNA profiling in combination with surface markers of T cell subsets as a function of time after stimulation creates a repertoire of analytical possibilities that will be diverse enough to distinguish between different stages of infections, responses to therapy, and efficacy of immunization (9–11).

The extension of sm-FISH from microscopy to flow cytometry enables reliable monitoring of changes in gene expression in much larger cell populations compared with what was possible by microscopy, permitting more accurate single cell-based studies in many disciplines. Detection of RNAs produced by intracellular pathogens (12) and their hosts simultaneously also creates powerful new analytical possibilities of host–pathogen interaction studies.

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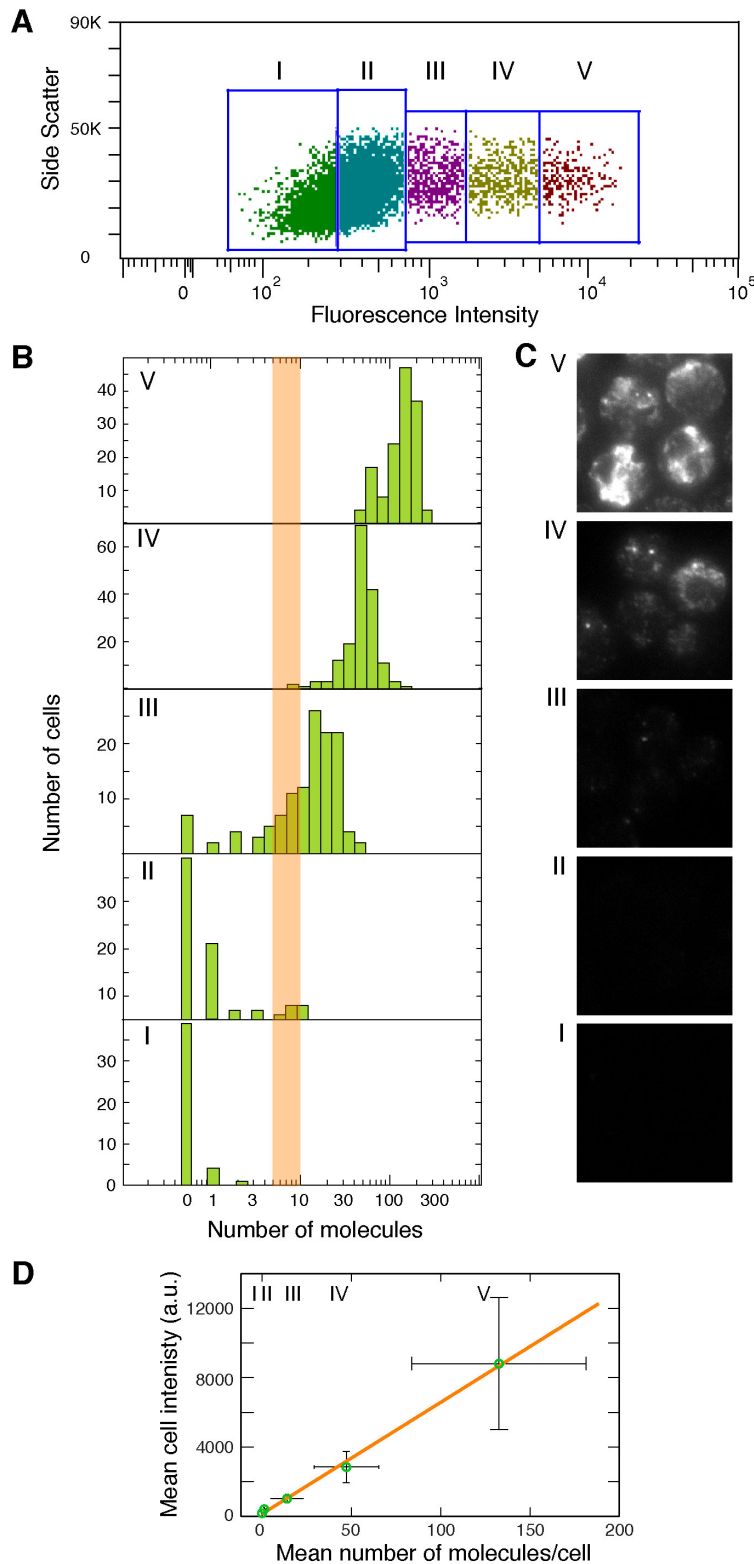
## Disclosures

Rutgers University receives royalties from the sale of prelabeled sm-FISH probes by Biosearch Technologies Inc., which markets them as Stellaris probes. A fraction of these proceeds is distributed to Sanjay Tyagi's lab-

oratory for research and to him personally. These proceeds do not influence the conclusions of this research.

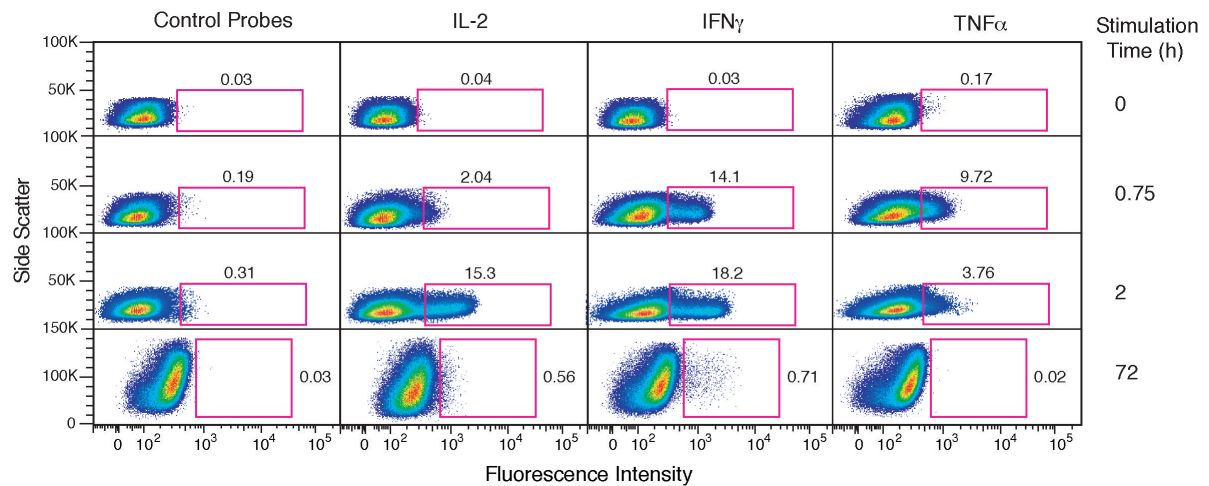
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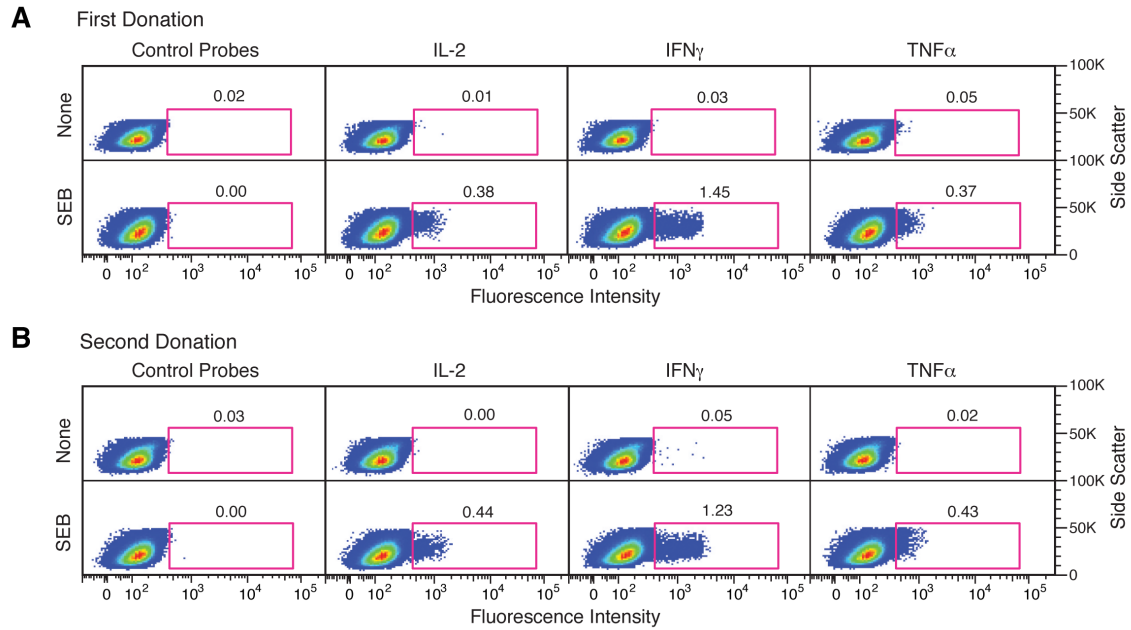
### Supplementary Fig. 1.

Determination of the sensitivity of mRNA detection in flow cytometry. **(A)** PBMC stimulated with PMA and ionomycin for 2 h were hybridized to IFN- $\gamma$  mRNA-specific probes and analyzed by flow cytometry. IFN- $\gamma$ -expressing cells were sorted into five different bins based on their intensity. The right edge of bin II was obtained from the right edge of unstimulated control cell population and served as the boundary between cells that don't express IFN- $\gamma$  mRNA and those that express it. **(B)** Collected cells, 65 to 170 in each bin, were analyzed by microscopy and the observed mRNA distributions in each bin are plotted. The sensitivity of detection lies within the indicated orange stripe because a line drawn within the stripe assigns fewest cells into the wrong category (i.e., cells that lie to the left of the boundary in bin III or to the right of the boundary in bin II). For example, if one considers the detection limit to be 10 molecules/cell, from a total of 609 cells analyzed for all the bins, 553 (91%) are assigned in correct category, whereas, 46 cells are assigned in incorrect categories. **(C)** Images of representative cells in each bin. **(D)** The mean flow cytometric intensity of cells is linearly proportional to the mean number of mRNAs per cell in each bin ( $R=0.998$ ).



**Supplementary Fig. 2.** Kinetics of IL-2 and TNF- $\alpha$  mRNAs expression following stimulation with PMA and ionomycin. All the probes were labeled with Cy5. The gate for each time point and percentage of positive cells was determined from control probes shown on the left. A proportion of cells expressing TNF- $\alpha$  transcripts (upper right panel) reflect pre-existing in vivo activation in this donor. The altered distribution of cells in 3 day culture occurs due changes in size and granularity of cells.





**Supplementary Fig. 3.** Detection of cytokine mRNA expression in T cells from the same donor on two separate occasions. PBMC obtained from a healthy donor with 4 week interval were stored frozen and assayed at the same time. PBMC were stimulated for 2 hours in culture medium without or with SEB (Materials and Methods). Cells were probed with control or cytokine mRNA-specific, Cy5-labeled probes and then analyzed by flow cytometry. The frequencies of the activated T cells expressing IL-2, IFN- $\gamma$ , and TNF- $\alpha$  mRNAs are shown. **(A)** PBMC obtained at time zero were stimulated in medium without (upper row) or with SEB (lower row). **(B)** PBMC obtained after 4 weeks from the same donor were stimulated with in medium without (upper row) or with SEB (lower row).

**Supplementary Table 1.**

Reproducibility of the PBMC responses to stimulation by SEB.

Probes	Donor	1		2		3	
	Donation	1st	2nd	1st	2nd	1st	2nd
Control	- SEB	0.02	0.03	0.02	0.00	0.02	0.02
	+ SEB	0.00	0.00	0.00	0.00	0.01	0.00
IL-2	- SEB	0.01	0.00	0.02	0.00	0.00	0.00
	+ SEB	0.38	0.44	0.11	0.16	0.18	0.25
IFN $\gamma$	- SEB	0.03	0.05	0.03	0.01	0.01	0.00
	+ SEB	1.45	1.23	0.36	0.62	0.63	1.51
TNF $\alpha$	- SEB	0.05	0.02	0.03	0.02	0.01	0.03
	+ SEB	0.37	0.43	0.18	0.25	0.20	0.22

Numbers indicate the percent of cells expressing cytokine mRNAs.