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A Homogeneous Immunoassay Method for Detecting Interferon-Gamma in Patients with Latent Tuberculosis Infection

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IFN-γ release assays (IGRAs) have been developed as viable alternative diagnostic tools for detecting latent tuberculosis infection (LTBI). A customized homogeneous sandwich luminescent oxygen channeling immunoassay (LOCI) was used to quantify IFN-γ levels in IGRAs. Samples were collected from healthy volunteers (n = 40) who were T-Spot-negative and T-Spot-positive patients (n = 32) at rest. Then the amount of IFN-γ in the supernatant of IGRAs was measured by LOCI. The results demonstrated a low background, and high sensitivity, specificity, accuracy, and reproducibility, and a short assay time (only 30 min) with LOCI for IFN-γ. The recovery range was 81.63–102.06%, the coefficients of variation were below 5%, and the limit of detection was 19.0 mIU/ml. Excellent agreement between LOCI IFN-γ and the T-SPOT.*TB* test was obtained (97.2% agreement, κ = 0.94). The LOCI IFN-γ concentrations were significantly higher in T-Spot-positive patients than in the healthy group ($p < 0.001$). Moreover, as observed for the comparative LOCI IFN-γ assay, IFN-γ concentrations were related to the numbers of T-SPOT.*TB* spots. We have established an in vitro blood test for LTBI diagnosis, defined as LOCI IFN-γ. A high level of agreement between the LOCI IFN-γ method and T-SPOT.*TB* assay was observed in clinical studies that showed the LOCI IFN-γ method could determine LTBI. This study shows acceptable performance characteristics of the LOCI IFN-γ assay to diagnose LTBI.

Keywords: LOCI technology, interferon-gamma quantitative detection, interferon-gamma release assay, latent tuberculosis infection

Introduction

Tuberculosis (TB), which ranks as the second leading infectious disease-related cause of death, remains a major global health challenge, accounting for 1.5 million deaths and 9 million new reported cases worldwide in 2013 [11, 23]. Approximately one-third of the global population have latent tuberculosis infection (LTBI) [1, 17, 22] and 5–20% of infected persons will develop active disease within 2 to 5 years [22]. Accurate diagnosis and treatment of LTBI patients can substantially reduce their risk of developing active disease, and would probably prevent further *Mycobacterium tuberculosis* (Mtb) transmission [4, 18, 21]. However, assessments of the testing accuracy of TB infection remains a challenge in the field of tuberculosis control because of the lack of a gold standard for confirming a LTBI diagnosis. Currently, all existing tests, including the tuberculin skin test (TST) and interferon-gamma (IFN-γ) release assays (IGRAs), are indirect approaches for detecting a cellular immune response to Mtb.

For the past century, the TST has been the only screening tool available for detecting LTBI. The TST has several known limitations [18], and so IGRAs have been developed as viable alternative diagnostic tools owing to their improved sensitivity and specificity [6, 9]. Indeed, it was recommended by the US Centers for Disease Control and

Prevention in 2005 that the IGRAs should replace the TST [14]. There are two commercially available ex vivo IFN-γ assays: the QuantiFERON-TB Gold In-Tube (QFT) assay (Cellestis/Qiagen, Australia) and the T-SPOT.*TB* assay (Oxford Immunotec, UK), which are the most accepted IGRAs and have been approved by the US Food and Drug Administration, Health Canada, and the CE (Conformité Européenne, which designated it for use in Europe). QFT measures IFN-γ released by Mtb-specific T cells after incubation with whole blood stimulated in vitro with Mtb-specific antigens, such as the 6 kDa early secreted antigenic target (ESAT-6) and culture filtrate protein 10 kDa (CFP-10). The other assay, T-SPOT.*TB*, is a semi-quantitative experiment based on the detection of the number of Mtb-specific T cells, in which each spot represents one IFN-γ-producing T cell that had been stimulated with Mtb-specific antigen. IGRAs are likely to be a useful method for identifying individuals with progressive infections who are likely to progress to active disease [5, 8, 15]. Studies have shown that the T-SPOT.*TB* assay appears to be more sensitive than QFT [12, 19]. However, the use of existing IGRA-based commercial tools is time-consuming and laborious. Additionally, the results of T-SPOT.*TB* assays must be evaluated by an experienced operator to quantify the spot number on T-SPOT.*TB* plates. Furthermore, QFT and T-SPOT.*TB* assays have a higher material cost, require a well-equipped laboratory and repeated washing steps, and are not suitable for automation. Therefore, it was essential to develop a quantitative IFN-γ system that does not rely on experienced doctors and that integrates the advantages of high sensitivity, cost effectiveness, and ease of use in the clinical laboratory for resource-limited settings.

In this study, we use a luminescent oxygen channeling immunoassay (LOCI) for detecting IFN-γ secreted by memory T cells after stimulation with Mtb-specific antigen. LOCI is a bead-based sandwich immunoassay, which is based on the proximity (less than 200 nm) of donor and acceptor beads when analyte molecules are captured, resulting in the emission of light by chemiluminescence. LOCI has been widely utilized to study aspects of cell signaling, biomarker quantification, and many aspects of drug discovery, principally high-throughput screening [2, 3, 7, 10, 16]. This new diagnostic test aimed to combine T-SPOT.*TB* and QFT, including the separation of peripheral blood mononuclear cells (PMBCs) from whole blood and the quantification of IFN-γ concentrations. LOCI is a homogeneous immunoassay without washing steps, shows fast reaction kinetics, and has been established to be highly sensitive, highly reproducibile, easy to operate, and well suited for automated high-throughput applications. Based on the LOCI IFN-γ test we propose here, the relationship between the number of T-SPOT.*TB* spots in the test and IFN-γ concentrations from the same individual were carefully compared.

Materials and Methods

Materials

Two different IFN-γ antibodies were used in this study; the capture antibody was clone 350B 10G6 with the concentration of 1 mg/ml (Invitrogen AHC4432; CA, USA), and the detection antibody was biotin-conjugated clone 67F 12A8 with the concentration of 0.5 mg/ml (Invitrogen AHC4539). The IFN-γ Standards was purchased from the National Institute for Biological Standards and Control (NIBSC code: 82/587; UK). Serum-free RPMI medium 1640 and AIM-V medium were purchased from Gibco. The automatic blood cell counter was obtianed from Sysmex (Japan).

In the LOCI assay, streptavidin-coated sensibeads and chemibeads coated with an anti-tag monoclonal antibody are linked by the peptide to generate a LOCI signal. Beads were obtained from Beyond Biotech (China), and contained unconjugated Eu-acceptor beads and streptavidin-coated sensibeads. The high-throughput homogeneous luminescence immunoassay instrument (Shanghai Questbitech Company, China) was used to carry out the LOCI test. The 96-well ELISA plate used in PBMC cultures were from Jet Biofil. ESAT-6, CFP-10, and phytohemagglutinin (PHA), as well as the T-SPOT.*TB* kit were purchased from Oxford Immunotec (UK). The mass concentration of PHA was 0.0015%. The mass concentrations of ESAT-6 and CFP-10 were both 0.03%.

Statistical Analysis

SPSS 13.0 (SPSS Inc., IL, USA) was used for the statistical analysis of experimental data. The normality of LOCI IFN-γ levels was assessed using the nonparametric Kolmogorov–Smirnov test. A paired *t* test was performed to compare the differences in matrix interference study. Receiver operator characteristic (ROC) curve analysis was used to set cut-off values for the LOCI IFN-γ method. Because of the non-normally distributed data, the Wilcoxon test was used to compare differences between controls and T-spot-positive groups. Mann–Whitney U tests were used to compare differences between T-spot-positive groups. Agreement between the T-SPOT.*TB* assay and LOCI IFN-γ test results was assessed using the kappa (κ) statistic. The κ values were considered as follows: ≤0.4 was poor, >0.75 was excellent, and values in between were considered to show fair to good agreement. All results were expressed as means ± standard deviation (SD). A *p*-value <0.05 was considered to indicate a statistically significant difference.

Study Population Characteristics

When newly diagnosed active TB was ruled out, clinical blood samples ($n = 72$) obtained at the Shanghai Chest Hospital were

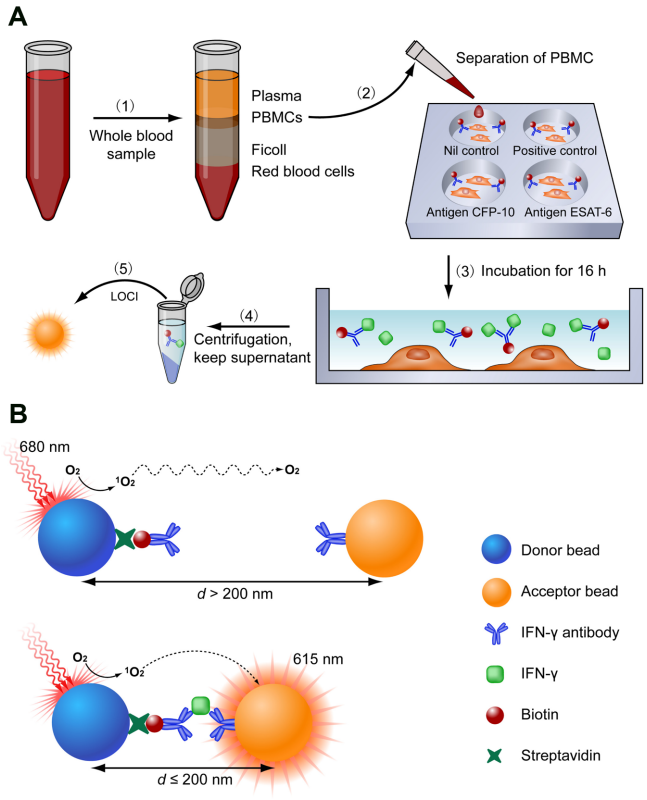

Fig. 1. (**A**) A diagram of the IFN-γ detection protocol using LOCI technology for diagnosing LTBI and (**B**) the principles of LOCI for detecting IFN-γ.

incorporated into this study. The study was approved by the Hospital Ethics Committee of Shanghai Chest Hospital. The mean age of the 32 T-spot-positive patients was 60.6 years (SD, 10.9; range, 27–76 years; 59.4% male, 40.6% female). The mean age of the 40 T-Spot-negative individuals was 56.7 years (SD, 13.1; range, 24–79 years; 60% male, 40% female).

Sample Assay Procedure

For each patient, 5 ml venous blood samples were collected in sterile vacutainers with heparin within 4 h. A schematic diagram of the proposed method is shown in Fig. 1. (1) Separated PMBCs: Peripheral blood mononuclear cells, including T cells, were separated from whole blood samples by standard Ficoll-HyPaque density-gradient centrifugation at 1,000 ×*g* for 22 min at room temperature, followed by washing twice with serum-free RPMI 1640 medium. (2) Seeded PBMCs: PBMCs were resuspended in AIM-V medium and counted using an automatic blood cell counter (Sysmex, Japan), allowing all PBMCs numbers to be adjusted to 2.5 million cells per 1 ml AIM-V medium. A total of four wells on 96-microwell plates were seeded at a concentration of 2.5×10^5 PBMCs (100 µl) per well. (3) Stimulation and incubation: 50 µl AIM-V medium and 50 µl PHA solution were added to the nil and positive control wells, respectively. Additionally, 50 µl of one of two different peptides encoding Mtb-specific antigens, ESAT-6 and CFP-10, was added separately to two other wells. Next, 0.75 µl of biotin-conjugated IFN-γ antibody (0.5 mg/ml) was added to each well. The microwell plates were incubated at 37°C with 5% CO_2 for 16–20 h. If a patient had Mtb infection, T cells in the PBMCs would recognize Mtb-specific antigens and secrete IFN-γ, in which case the biotin-conjugated detection antibody would capture IFN-γ and form IFN-γ–antibody–biotin complexes. (4) Supernatant collection: PBMCs were removed by centrifugation and the supernatant, including IFN-γ–antibody–biotin complexes, were harvested and tested for the presence of IFN-γ produced in response to peptide antigens. (5) LOCI detection: The concentration of IFN-γ in supernatants was measured by LOCI. Results for each sample were reported relative to a standard curve that was prepared by testing dilutions of the cytokine standard. The IFN-γ signal for TB antigen and PHA were corrected by subtracting the background level derived from the respective nil control.

Samples from each individual were also detected using a T-SPOT.*TB* kit and analyzed according to the manufacturer's instructions, and were defined as positive or negative based on the manufacturer's recommended criteria. Spots were counted using a magnifying glass.

LOCI Detection Protocol

Preparation of acceptor beads. Mouse monoclonal anti-IFN-γ antibody was bound to acceptor beads. First, acceptor beads with a size of 200 nm with active aldehyde groups on the surface were separated by centrifugation to remove the supernatant, and then were dispersed in 0.1 M phosphate buffer (pH 7.0) by sonication. Second, 0.1 mg of 350B 10G6 antibody was dialyzed in 50 mM 2-(*N*-morpholino) ethanesulfonic acid buffer (pH 6.0) to obtain an antibody solution. Then, the antibody solution was added to 5 mg of acceptor beads with 10 µl of sodium cyanotrihydridoborate ($NaCNBH_3$, 25 mg/ml) and 12.5 µl of 1% Tween-20, which then was incubated at 37°C for 48 h. After this incubation, any unbound antibody was removed by centrifugation. Immunoluminescent acceptor beads were resuspended at 10 mg/ml and stored at 4°C.

LOCI immunoassay. The LOCI immunoassay was performed in 96-well plates (Coring Life Sciences, Corning, NY, USA) containing 50 µl of each sample that included IFN-γ–antibody–biotin complexes, 25 µl of acceptor beads coupled to anti-IFN-γ antibody, and 25 µl of immunoluminescent reagent (Tris buffer, 10 mmol/l, pH 8.0). Then, plates were placed in the high-throughput homogeneous luminescence immunoassay instrument. The reaction mixture was incubated at 37°C for 15 min. Then, 175 µl (60 µg/ml) of LOCI Common Reagent, which contains donor beads conjugated to streptavidin, was added by an automatic pipettor and plates were incubated at 37°C in the dark for another 10 min, after which the relative light unit (RLU) was read on the LOCI HT instrument with a laser excitation at 680 nm and emission at 610 nm. Samples were analyzed in triplicates in plates. The total detection time for the LOCI immunoassay was 30 min.

Assay Performance Characteristics of IFN-γ Measurements by LOCI

Standard curve and detection limit of IFN-γ by LOCI. The IFN-γ standard solution was prepared by diluting IFN-γ (3,000 IU/ml, stored at –20°C) in AIM-V medium. The double-ratio dilution method based on AIM-V was employed to generate standard concentrations of 8,000, 4,000, 2,000, 1,000, 500, 250, 125, and 0 mIU/ml. Each concentration was analyzed in triplicates by LOCI. Standard solutions were used immediately after they were prepared.

To determine the assay detection limit for the LOCI IFN-γ assay, 20 zero calibrators were run to calculate the mean ± 2SD of the zero calibration. The concentration of the zero calibration ± 2SD was set as the detection limit using the EP17-A procedure.

The recovery rate of IFN-γ by LOCI. To determine the recovery rate of LOCI for IFN-γ measurements, we obtained sample 1 and sample 2 by mixing the supernatant collection of clinical nil control and positive control wells detected by T-SPOT.*TB* assay. Sample 1 and sample 2 contained 74.45 and 6,619.18 mIU/ml IFN-γ respectively via the LOCI proposed in this work. Then samples at 11 different concentration levels of IFN-γ were obtained by mixing with samples 1 and 2 at different ratios, as shown in Table 1. All the 11 samples were measured by LOCI.

The mean recovery rate was used to calculate the ratio between the measured and theoretical values.

Stability. Control samples were prepared by "spiking" IFN-γ in the AIM-V medium for calibration. The stability of the control samples at 4°C was tested at 0, 4, 8, and 24 h, and the IFN-γ concentrations were 4,127 and 2,073 mIU/ml, respectively.

Intra- and inter-assay measurements of precision. A total of three samples that contained low, medium, and high levels of IFN-γ from the sample pool were used to evaluate the precision of the immunoassay. To assess intra-assay variation, 20 duplicates of each sample were run in the same assay on the LOCI HT instrument. The intra-assay precision was calculated based on the variation among the 20 measurements of IFN-γ concentrations. To determine the inter-assay variation, the mean, SD, and coefficient of variation (CV) of each sample in 20 independent tests were each calculated in duplicates on each sample each day.

Matrix interference study. To evaluate matrix effects, the IFN-γ calibration curves obtained using AIM-V medium were compared with the calibration curve that was obtained using PBS buffer.

The volume of each IFN-γ sample could dramatically influence the assessments of assay performance. Here, sample volumes of 25 and 50 µl were used to estimate this effect. Comparisons of LOCI signals were performed using a paired *t* test.

Result and Discussion

Calibration Curve and the Detection Limit of IFN-γ by LOCI

As shown in Fig. 2, a linearized standard curve (y = 5539.6x + 1649.6) of the LOCI IFN-γ assay was generated using the LOCI method. The standard curve showed an intensity that ranged from 1,000 to 50,000 counts per second, which indicated concentrations of 0 to 8 IU/ml IFN-γ. The correlation coefficient (R^2) was 0.999, which represented excellent linear quantification. The detection limit of the LOCI IFN-γ assay was 19.0 mIU/ml, which was based on 20 blank samples that were calculated from the mean of the zero calibrator ± 2SD. The LOCI assay was very sensitive, with a detection limit of 19.0 mIU/ml, which was one order of magnitude lower than the cutoff value of the QFT method (350 mIU/ml), allowing for the measurement of very small concentrations of IFN-γ. These

Table 1. The recovery rate of IFN-γ by LOCI.

Sample No.	Sample 1[a] added volume (ml)	Sample 2[b] added volume (ml)	Theoretical values (mIU/ml)	Measured values (mIU/ml)	Recovery (%)
1	0.20	0.00	74.45	74.45	100.00
2	0.18	0.02	728.92	595.02	81.63
3	0.16	0.04	1,383.39	1,224.89	88.54
4	0.14	0.06	2,037.87	1,856.58	91.10
5	0.12	0.08	2,692.34	2,598.39	96.51
6	0.10	0.10	3,346.82	3,313.12	98.99
7	0.08	0.12	4,001.29	3,865.66	96.61
8	0.06	0.14	4,655.76	4,709.48	101.15
9	0.04	0.16	5,310.24	5,339.06	100.54
10	0.02	0.18	5,964.71	6,087.64	102.06
11	0.00	0.20	6,619.18	6,619.18	100.00

[a]Sample 1: 74.45 mIU/ml IFN-γ.

[b]Sample 2: 6619.18 mIU/ml IFN-γ.

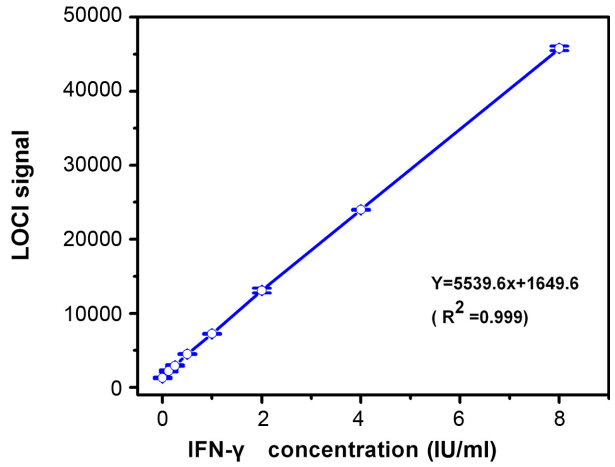

Fig. 2. A calibration curve for detecting IFN-γ by LOCI.
A total of eight standard IFN-γ points based on a 2-fold serial dilution that ranged from 0 to 8 IU/ml were used to generate the standard curve. Error bars indicate standard deviations (n = 3).

results indicate that LOCI for IFN-γ was feasible and efficient.

Recovery Rate and Stability of IFN-γ by LOCI

The recovery rate of LOCI IFN-γ is shown in Table 1. The recovery rate was defined as the (measured concentration) / (theoretical concentration) × 100%. The recovery ranged from 81.63% to 102.06%, and the average recovery was 96.10%. These results showed a good recovery and indicated that the accuracy of the LOCI IFN-γ method that we developed was acceptable.

To evaluate the influence of sample testing time, we tested the storage stability of IFN-γ after stimulation with Mtb-specific antigens. Control samples were prepared by "spiking" IFN-γ in AIM-V medium, similarly to the calibration samples. The stability of the control samples at 4ºC was tested at 0, 4, 8, and 24 h by LOCI, and the IFN-γ concentrations were 4,127 and 2,073 mIU/ml, respectively. The LOCI signal showed that the change in IFN-γ activity of the control samples was less than 10% in 24 h. This result indicated that samples could be measured within 24 h after PBMC stimulation.

Precision of the LOCI System

As shown in Table 2, the intra- and inter-assay coefficients of variation were 4.41% and 4.92%, respectively. These findings indicate that the reproducibility and precision of the proposed method were excellent, and were significantly lower than those for previously reported quantitative cytokine detection methods based on ELISA (which were ~15%) . Moreover, the CV of the zero IFN-γ calibration was only 4.68%, which was much lower than for the commercial ELISA kit (>25%), indicating that the LOCI method was highly sensitive (see Fig. 2). The reason for this is that the LOCI is a no-wash, fast reaction kinetic, and homogeneous detection method that eliminates the need for multiple washing, staining, and blocking steps that are required for ELISA or ELISpot assays, and that often result in analyte dilution, human contamination, and unpredictable signal variation. The precision profile showed that LOCI showed greater accuracy, reproducibility, and precision compared with standard ELISA methods. The detection limit was sufficient for measuring clinically relevant IFN-γ levels, even in immunocompromised populations, such as HIV-infected patients or children [13, 20].

Matrix Interference Studies

To evaluate the matrix effect, we compared the calibration curves performed in AIM-V medium versus PBS buffer. A comparison of the LOCI signal indicated no significant difference ($p < 0.05$) between the different matrices when the sample volume was 25 or 50 μl. The signal-to-noise ratio at a concentration of 8 IU/ml in a standard sample increased from 23.5 to 34.2 when the total sample volume was increased from 25 to 50 μl (shown in Fig. 3), because the amount of secreted IFN-γ increased when the sample volume was increased. To improve detection sensitivity, a 50 μl IFN-γ sample was selected for use in subsequent experiments.

Associations Between IFN-γ Levels Detected Using LOCI and T-SPOT.*TB* Results for all 72 Samples

In our process of designing the IFN-γ LOCI method, PBMCs were stimulated by Mtb-specific antigens for 16 h

Table 2. Intra- and inter-assay measurements of precision.

Quality control (n = 20)	Average measured value (IU/ml)		CV (%)	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
Low	0.34 ± 0.015	0.34 ± 0.016	4.41	4.70
Middle	1.02 ± 0.037	1.02 ± 0.045	3.62	4.41
High	6.91 ± 0.23	6.91 ± 0.34	3.32	4.92

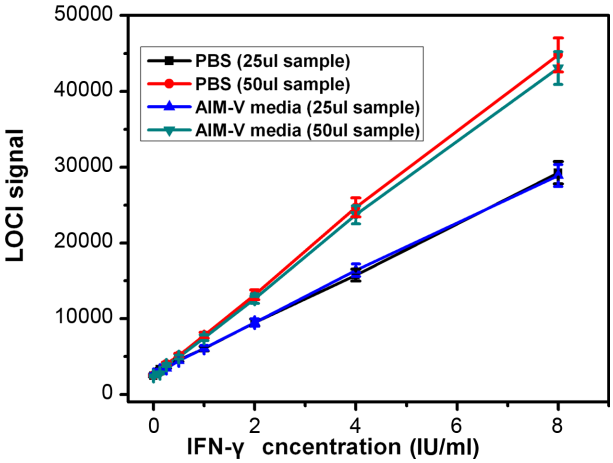

Fig. 3. Calibration curves for different matrixes and sample volumes obtained by LOCI.

Each point represents the mean value of triplicate measurements.

without capture antibody (biotinylated IFN-γ monoclonal antibody), and the agreement between the IFN-γ LOCI and T-SPOT.*TB* results was extremely poor (κ < 0.3). Most notably, in weakly positive individuals (6 ≤ spots of T-SPOT.*TB* < 10), the IFN-γ levels showed almost no difference compared with T-SPOT.*TB*-negative individuals. If the capture antibody was added during the process of Mtb-specific antigen stimulation, excellent agreement was observed between IFN-γ LOCI and T-SPOT.*TB*. Based on this finding, we suspect that IFN-γ was unstable in the incubation environment. Furthermore, we suspect that IFN-γ captured in the form of IFN-γ–antibody complexes was much more stable than IFN-γ alone. To further explore this possibility, we measured several IFN-γ samples of a known concentration (0, 125, 1,000, and 8,000 mIU/ml) by LOCI and incubated them for 16 to 20 h at 37°C with 5% CO_2. We found that the activity of IFN-γ was reduced by almost 30% prior to incubation. This finding demonstrated that detecting IFN-γ–antibody complexes after secretion from T cells was a more robust diagnostic strategy.

To determine the optimal cutoff values yielding higher sensitivity and specificity of measurements of IFN-γ levels by LOCI, 32 T-spot-positive patient and 40 control volunteer samples were used to generate ROC curves. At IFN-γ concentrations of 31.08 mIU/ml, Youden's index peak value with good sensitivity and specificity was 0.94 and 1.0, respectively. Based on a cutoff of 31.08 mIU/ml, we compared the LOCI IFN-γ method with the T-SPOT.*TB* results, as shown in Table 3. There was excellent agreement between our proposed method and the T-SPOT.*TB* test results (97.2% agreement, κ = 0.94). This finding demonstrated

Table 3. Agreement between the LOCI IFN-γ method and T-SPOT.*TB* results.

LOCI	T-Spot		
	Positive	Negative	Total
Positive	30	0	30
Negative	2	40	42
Total	32	40	72

that LOCI IFN-γ could be suitable for the clinical diagnosis of LTBI. As shown in Fig. 4A, the LOCI IFN-γ concentrations were significantly higher in T-spot-positive patients than in the healthy group of patients ($p < 0.001$). The median IFN-γ concentration of healthy participants was 0 mIU/ml (interquartile range [IQR], 0–7.94), and the 95th percentile was 22.15 mIU/ml. The median IFN-γ concentration of T-spot-positive patients was 92.72 mIU/ml (IQR, 22.82–265.04), and the 95th percentile was 997.07 mIU/ml. Moreover, as observed in the comparative LOCI IFN-γ assay, IFN-γ concentrations showed a robust correlation with the number of T-spot dots (Fig. 4B). We grouped all samples into one of three groups based on the spot number obtained using the T-SPOT.*TB* test, termed weak positive (6 ≤ spots < 10), positive (10 ≤ spots < 30), and strong positive (spots ≥ 30). The IFN-γ concentrations were significantly higher in the strong positive group compared with the positive group ($p < 0.05$). However, we detected no significant difference between the weak positive and positive groups ($p < 0.05$).

Comparison Between the Present LOCI IFN-γ Assay and a Conventional Test

In contrast to conventional TST, a major advantage for the LOCI IFN-γ assay is that it was not affected by nontuberculous *Mycobacterium* infection and *Mycobacterium bovis* bacillus Calmette-Guérin vaccination. The concentrations of IFN-γ detected by LOCI showed low background, and high sensitivity, accuracy, and reproducibility. More importantly, this new method required a detection time of only 30 min without a multi-step wash procedure. These advantages make LOCI easy to automate, potentially allowing high-throughput clinical applications, and it could be used in a microfluidic platform and for point-of-care testing equipment.

The K value for the agreement between the T-SPOT.*TB* and LOCI IFN-γ methods (using a 31.08 mIU/ml cut-off) in all 72 subjects was 0.94. The overall agreement was 97.2% (70/72), with a positive correlation of the T-SPOT.*TB* and LOCI IFN-γ methods of 93.8% (30/32), and negative correlation of 100% (40/40).

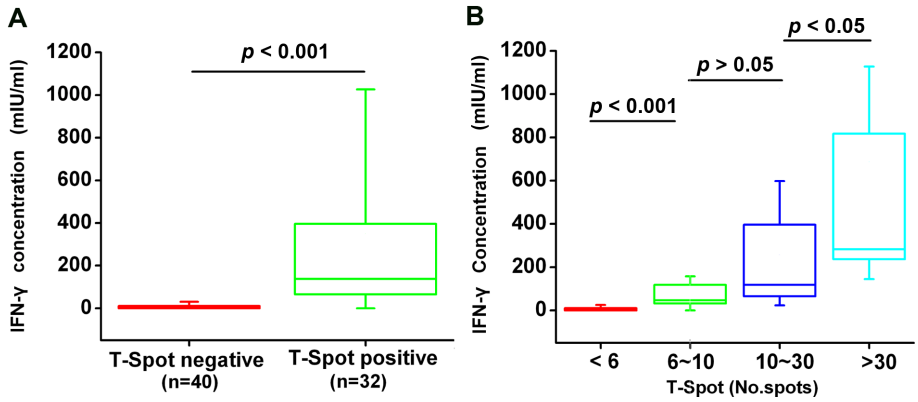

Fig. 4. A comparison of IFN-γ levels measured by LOCI and T-SPOT.*TB* results for all 72 samples.
(**A**) IFN-γ levels in T-spot-positive patients (n = 32) and healthy participants (n = 40). The median IFN-γ concentrations in T-spot-negative controls and T-spot-positive patients were 0 and 92.72 mIU/ml, respectively. (**B**) The correlation between the results obtained using the LOCI IFN-γ method and spot counts for the T-SPOT.*TB* test for all 72 samples.

In this present study, we established and applied a homogeneous assay for the detection of IFN-γ secreted by memory T cells after stimulation with Mtb-specific antigen for the diagnosis of LTBI. To the best of our knowledge, this represents the first time that IFN-γ has been measured using a LOCI detection method. There were a few highlights in this study: (i) The present assay can quantitatively detect IFN-γ in the range of 19–8,000 mIU/ml, with a very low LOD of 19 mIU/ml (equal to 1.9 pg/ml IFN-γ). With extremely low CVs, the reproducibility of the proposed method was excellent and showed the potential to be highly sensitive. (ii) The homogeneous method has remarkably fast reaction kinetics, so the assay time was short. Measurements could be obtained in a total assay time of 30 min, whereas 2.5–4 h is required for a conventional ELISA. (iii) Clinical studies showed that the LOCI IFN-γ method is highly correlated with the T-SPOT.*TB* assay, so it could potentially be used for the clinical detection of LTBI.

Based on all of these advantages, this immunoassay can be considered for use as an analytical tool with high-throughput potential for the clinical diagnosis of LTBI. Larger studies will be needed to improve the diagnostic power (sensitivity and specificity) of this LOCI IFN-γ method compared with the traditional T-SPOT.*TB* assay.

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