Colloids and Surfaces B: Biointerfaces 80 (2010) 251–255

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Short communication

Detecting interferon-gamma release from human CD4 T-cells using surface plasmon resonance

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ARTICLE INFO

Article history:
Received 7 May 2010
Received in revised form 8 June 2010
Accepted 17 June 2010
Available online 25 June 2010

Keywords:
CD4 T-cells
Antibody–antigen interactions
Immunoassay
SPR
Cytokine biosensors

ABSTRACT

Cytokine secretion by leukocytes is an important indicator of immune response to pathogens and therefore has significant implications in disease diagnostics. Given heterogeneity of leukocyte subsets and the ability of multiple cell subsets to secrete the same cytokines, connecting cytokine production to a specific leukocyte subset is a distinct challenge. In the present paper we describe a strategy combining antibody (Ab)-based affinity cell separation and surface plasmon resonance (SPR) for capturing human CD4 T-cells and for label-free detection of cell-secreted interferon (IFN)-γ – an important inflammatory cytokine. Human blood was introduced into a flow chamber modified with anti-CD4 Abs resulting in capture of $CD4^+$ T-cells. After mitogenic activation of cells inside the flow chamber, culture medium was routed onto an SPR chip modified with monoclonal IFN-γ Abs. SPR signal observed in this experiment correlated with cytokine production by T-cells. The strategy of combining SPR detection with cell purification may be used in the future for label-free, sensitive detection of multiple cytokines or proteins secreted by the desired cell subset.

1. Introduction

White blood cells or leukocytes are central players in innate and adaptive immune responses ranging from wound healing to infectious diseases. Leukocytes in peripheral blood are comprised of multiple cell subsets that are distinguished based on cell/nucleus morphology, surface marker expression and cytokine production [1]. CD4 T-cells, also known as T-helper (Th) cells, represent an important leukocyte subset responsible for mounting complex adaptive immune responses. The death of CD4 T-cells during HIV leads to compromised immunity associated with AIDS [2]. Cytokine secretion and signaling is central in T-cell-mediated activation, proliferation and differentiation of other cells during an immune response [3,4]. The types and the levels of secreted cytokines correlate with the ability of CD4 T-cells to mount an immune response to a given pathogen, and therefore contain disease diagnostic information.

Cytokine detection is traditionally carried out using sandwich immunoassay or ELISA. While robust and sensitive, these traditional approaches require multiple washing steps, are time-consuming and do not allow real-time detection. Limitations of traditional immunoassay approaches have spurred the development of label-free biosensors where cytokine molecules are detected using impedimetric [5,6] or surface plasmon resonance (SPR)-based sensors [7]. SPR is especially well suited for determining surface binding events and has been used extensively for monitoring antigen–antibody interactions in general [8] and for cytokine detection in particular [9–11]. The use of SPR for detecting secreted cellular products has been infrequent and largely limited to bioreactor monitoring [12,13].

Our goal in this study was to employ SPR for blood cell diagnosis by detecting cytokine release from CD4 T-lymphocytes—a leukocyte subset commonly monitored in infectious disease diagnosis and management. A significant challenge in profiling cytokine secretion in a heterogeneous cell suspension such as blood is the fact that the same cytokines can be produced by several leukocyte types. For example, IFN-γ is commonly secreted by CD4 T-cells but may also be produced by CD8 T-cells, macrophages and neutrophils. To address this challenge, our laboratory has been developing sur-

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doi:10.1016/j.colsurfb.2010.06.015

faces micropatterned with antibody (Ab) molecules for capturing leukocytes in the immediate vicinity of cytokine immunosensors [14,15].

The present paper combined Ab-based capture of T-cells from blood with SPR analysis to detect IFN-γ release from human primary CD4 T-cells. A flow chamber modified with CD4 Abs was used to capture pure CD4 T-cells from human blood upstream of an SPR instrument. IFN-γ released by the captured cells was then detected by routing effluent from the fluidic chamber onto an SPR chip modified with IFN-γ-sensing Ab layer. Coupling a miniature cell purification device to an SPR sensor may open new opportunities for blood diagnostics and may be used in the future for label-free, multiplexed detection of cytokine or other protein molecules released by a desired cell subset.

2. Materials and methods

2.1. Materials

Silane adhesion promoter, 3-acryloxypropyl trichlorosilane, was purchased from Gelest, Inc. (Morrisville, PA), 10× phosphate-buffered saline (PBS) without calcium and magnesium, paraformaldehyde (PFA), surfactant TWEEN® 20, Na_4EDTA, $KHCO_3$, NH_4Cl, poly(ethylene glycol) diacrylate (PEG-DA) (MW575), anhydrous toluene (99.9%), bovine serum albumin (BSA) were purchased from Sigma–Aldrich (Saint Louis, MO), and neutravidin from Pierce, Rockford, IL. Monoclonal purified mouse anti-human CD4 Abs (13B8.2) from Beckman-Coulter (Fullerton, CA), and biotinylated goat anti-human IFN-γ and IL2 Abs from R&D Systems (Minneapolis, MN) used for capturing T-lymphocytes and cytokines. Anti-human CD3ε-FITC (UCHT1) and anti-CD4-PE (L120) were used for immunostaining of surface-bound cells and were purchased from BD Pharmingen. Human recombinant IFN-γ and IL-2 were from R&D Systems (Minneapolis, MN). T-cell activation reagents: phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma–Aldrich. Cell culture medium RPMI 1640: with L-glutamine was purchased from VWR. Medium was supplemented with fetal bovine serum (FBS) and penicillin/streptomycin purchased from Invitrogen. Glass slides (25 mm × 75 mm × 1 mm) were obtained from VWR (West Chester, PA).

2.2. Preparation of cell capture surfaces and blood samples

Standard glass slides (3 in. × 1 in.) were silanized and coated with poly(ethylene glycol) (PEG) hydrogel according to the protocols described by us previously [16]. Anti-CD4 antibody solution was printed onto hydrogel-coated glass slides using handheld microarrayer (MicroCaster, Schleicher & Schuell, Keene, NH) to create 56 by 12 array of 500 μm diameter Ab spots. Ab printing solution consisted of mouse anti-CD4 adjusted to concentration of 0.2 mg/ml in 1× PBS. To ensure uniform spreading of droplet contents upon printing, 19 μl of dissolved Ab molecules were mixed with 1 μl of 0.1% TWEEN 20 surfactant.

In T-cell capture experiments, 3 ml of blood was collected from adult human subjects through venipuncture under sterile conditions with informed consent and approval of the Institutional Review Board of University of California, Davis (protocol number 200311635-6). Blood was then depleted of erythrocytes according to previously published protocols [16,17]. Leukocytes were collected and resuspended in 1× PBS at concentration of ~10^6 cells/ml prior to cell capture experiments.

2.3. Capture of T-cells in an antibody-modified flow chamber

In order to capture T-cells, a 3 in. × 1 in. glass slide with imprinted Ab spots was placed into a custom-made parallel-plate flow chamber. The flow chamber, described by us in greater detail previously [16], consisted of two polycarbonate plates (50.8 mm × 127 mm × 6.35 mm (width × length × height)) that could accommodate a standard glass and could be secured with screws. A 100 μm thick gasket was used to create a headspace between the upper plate and a glass slide, defining the channel height. The effective dimensions of the fluid conduit were 25.4 mm × 76.2 mm × 0.100 mm (width × length × height). Both plates had a glass window allowing for *in situ* observation of leukocyte capture. Tubing was inserted into the chamber through inlet and outlet holes and was connected to a syringe pump (PHD22/2000, Harvard Apparatus, Holliston, MA) to create pressure-driven flow in the chamber.

Blood depleted of red blood cells (RBCs) as described in the previous section was introduced into the flow chamber at flow rate of 200 μl/min corresponding to shear stress of 0.8 dyn cm^{-2}. Flow rate to shear stress conversion was made using an equation for infinitely wide parallel-plate flow chamber [17]. This flow rate was maintained for ~10 min to capture T-cells on Ab spots. In order to wash away non-specific blood cells, flow rate was increased to 500 μl/min (2 dyn/cm^2 shear stress). Removal of the non-specific cells was monitored by brightfield microscopy and was determined to be complete after 10–15 min. Approximately 250 cells were bound on each 500 μm diameter Ab spot and the total number of cells captured on a 56 × 12 array of spots was ~100,000.

Captured blood cells were identified as CD4 T-cells using immunofluorescent staining. In these experiments, anti-CD3-FITC (green) and -CD4-PE (red) Abs were diluted 1/10 in 1× PBS containing 0.01% (w/v) BSA and were injected into flow chamber containing captured T-cells. Cells were incubated with labeling Abs for 45–60 min in the dark at room temperature.

2.4. Preparation of SPR chips for IFN-γ sensing

Spreeta sensors (Texas Instruments) were used in SPR sensing experiments. Prior to assembly of the biorecognition layer, Spreeta sensor was cleaned by immersion in Piranha solution (1 part H_2O_2 and 3 parts H_2SO_4) for 5 min. Subsequently, the gold surface was exposed to neutravidin solution (0.01% by wt. in 1× PBS) until saturation of SPR signal was observed (~20–30 min). In the next step, SPR chip was exposed to 1.5 μg/ml solution of biotinalyted anti-IFN-γ Ab dissolved in 1× PBS. In the final step, SPR chip surface was exposed to 2 μg/ml biotin solution to block unreacted neutravidin sites on the surface.

The SPR sensor was characterized by injections of known concentrations of recombinant human IFN-γ. In these experiments, one channel of the dual-channel SPR chip was modified with anti-IFN-γ Abs as described above while the other channel was modified with neutravidin followed by biotin blocking. This second channel was used as a reference to assess non-specific binding of recombinant IFN-γ in the absence of recognition Ab layer. Different concentrations ranging from 0.05 to 2.0 μg/ml were prepared by dissolving IFN-γ in 1× PBS and were injected at flow rate of 0.1 ml/min into a flow cell of an SPR instrument. Signal saturation was typically achieved after 20 min. Specificity of IFN-γ detection was established by challenging Ab-modified SPR sensor to a high concentration (1 μg/ml) of non-specific cytokine IL-2.

2.5. Detection of T-cell-secreted IFN-γ using SPR

To detect endogenous IFN-γ, CD4 T-cells were captured in the flow chamber as discussed in the preceding section. IFN-γ-sensing SPR chips were prepared as described above. The flow cell with T-cells was then placed into a custom-made environmental chamber operating at 37 °C and connected so that solution bathing the cells in the flow chamber was passed through an SPR instrument

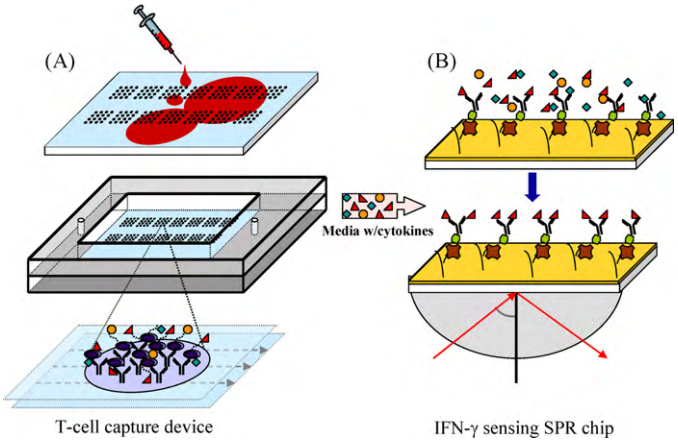

Fig. 1. Schematic of the proposed cytokine detection strategy. (A) Glass slides imprinted with anti-CD4 Abs spots are enclosed inside a flow chamber and exposed to RBC-depleted human blood. (B) After affinity selection, CD4 T-cells are activated *in situ* to secrete cytokines. Media bathing T-cells is then sent into an SPR chip containing Ab layer for detection of IFN-γ.

and then returned to the flow chamber. This solution consisted of RPMI1640 phenol red-free cell culture media with 10% FBS. In order to induce T-cells to secrete cytokines, culture media was supplemented with mitogens, PMA (50 ng/ml) and ionomycin (1 μg/ml). In these experiments, both channels of an SPR chip were modified with anti-IFN-γ Abs. One channel received culture media bathing the cells while the other channel was challenged with fresh media that did not come in contact with cells. This was done in order to distinguish SPR signals due to serum-containing media without IFN-γ from signals due to media with IFN-γ produced by T-cells.

3. Results and discussion

In this paper we describe a strategy of combining an Ab-modified cell capture flow chamber with an SPR instrument (see Fig. 1) to detect IFN-γ release from human CD4 T-cells. The strategy of capturing the desired cell subset upstream of an SPR sensor may be used in the future for multiplexed, label-free detection of cytokines for diagnostics or monitoring of blood based on leukocyte function.

3.1. Capturing CD4 T-cells in an Ab-modified flow chamber

Detection of cytokines in blood serum is performed regularly using immunoassays. However, blood is a heterogeneous cell suspension comprised of many different cells types and serum analysis does not reveal which cell type was responsible for cytokine production. To resolve this difficulty, we purified the desired leukocyte type (CD4 T-cells) upstream of the cytokine-sensing SPR instrument. The RBC-depleted human blood was pumped across an array of 500 μm diameter anti-CD4 Ab spots printed on a hydrogel-coated glass slide. In a series of previous publications, we demonstrated that a non-fouling PEG hydrogel coating combined with optimal flow conditions allows to localize leukocyte binding on Ab domains and to minimize non-specific blood cell attachment elsewhere on the surface [14,16,17]. Fig. 2A shows an array of 500 μm diameter spots after passing RBC-lysed blood through a flow chamber. As can be seen from this image, cells are captured on the spot with little or no cell adhesion occurring on the surrounding hydrogel coating. Immunofluorescent staining of the cell arrays with anti-CD3-FITC (green) and anti-CD4-PE (red) was used to determine cell phenotype. Representative images shown in Fig. 2B and C demonstrate that leukocytes bound to anti-CD4 Abs

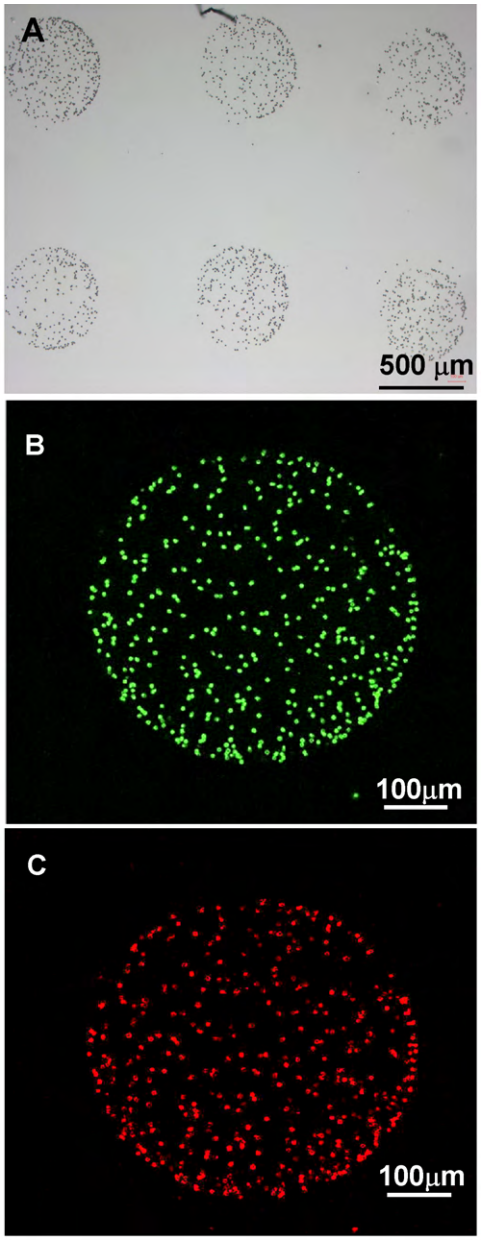

Fig. 2. Capture of human T-cells on printed anti-CD4 Ab arrays. (A) Brightfield images of cells captured on 500 μm diameter Ab spots. (B and C) Representative immunofluorescence staining results showing that cells captured on Ab spots were stained with CD3-FITC (green) and CD4-PE (red). The $CD3^+CD4^+$ staining identifies captured leukocytes as CD4 T-cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

spots where $CD3^+CD4^+$ identifying them as CD4 T-cells. Prevalence of CD4 T-cells on anti-CD4 Ab spots seen in Fig. 2 is consistent with our previous reports of high purity (>95%) capture of CD4 and CD8 T-cells on Ab-modified surfaces [17].

3.2. Characterization of SPR sensor for IFN-γ detection

Prior to detecting cytokine release from cells, we sought to characterize SPR sensor performance using recombinant IFN-γ. SPR sensing surface was prepared by physical adsorption of neutravidin, followed by attachment of biotinylated anti-IFN-γ Abs and finally saturating the surface with biotin molecules to block unreacted neutravidin sites. Fig. 3A shows responses of this SPR sensor to varying concentrations of IFN-γ. The SPR signal was

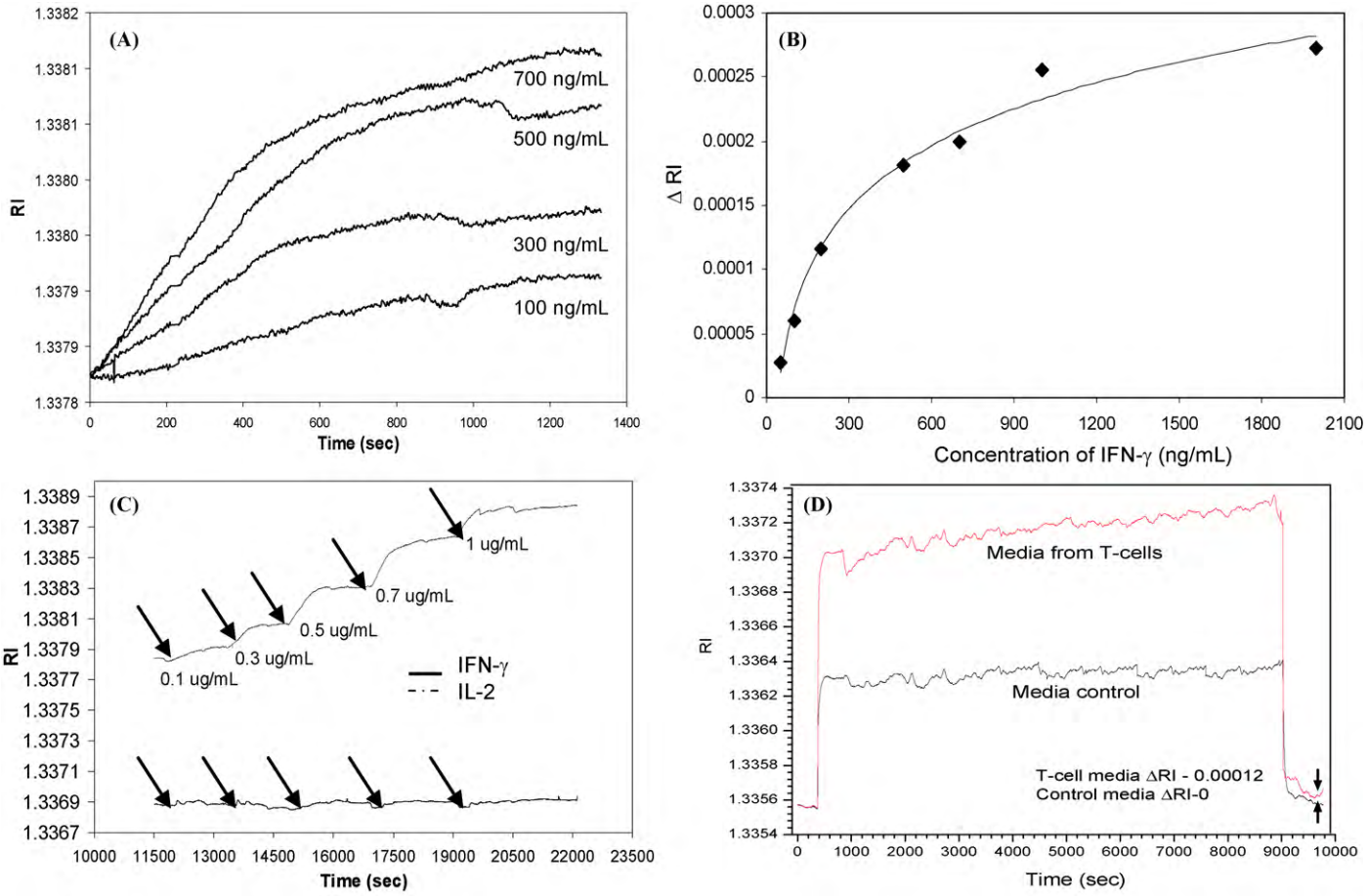

Fig. 3. (A) SPR curves for binding of different IFN-γ concentrations. (B) Calibration curve of SPR signal vs. cytokine concentration. (C) Specificity of cytokine detection. Solid curve shows binding of different IFN-γ concentrations on anti-IFN-γ chip surface. Dashed curve shows lack of binding of IL-2 (non-specific cytokine) on anti-IFN-γ chip. (D) Detection of T-cell-secreted IFN-γ using SPR. Working channel (red curve) was exposed to media that came from T-cell capture fluidic device, while the reference channel was exposed to media that did not come in contact with cells. A detectable signal was observed in the working channel after ~2.5 h of cell stimulation with mitogen. Signal corresponded to ~200 ng/ml of IFN-γ. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

normalized to the response of the reference channel that did not contain sensing Ab layer but was exposed to analyte. As seen from binding curves in Fig. 3A and a calibration curve in Fig. 3B, our SPR sensor provided detectable signals in response to 0.05 μg/ml IFN-γ and was saturated at 1 μg/ml of IFN-γ. Importantly, challenging IFN-γ sensing SPR chip with a high concentration of a non-specific cytokine, IL-2, did not result in detectable signal (Fig. 3C). This pointed to specificity of SPR sensor towards IFN-γ.

3.3. Detection of T-cell-secreted IFN-γ using SPR sensor

A central question explored in this study was how to tie cytokine release to a specific cell subset in a complex blood sample? Previously, we proposed co-localizing cell- and cytokine-capture Abs on micropatterned surfaces in order to capture desired cells next to cytokine immunosensors [14,15]. However, in these past studies, cytokine release was detected by sandwich immunoassay—an approach requiring labeling and multiple washing steps. In the present study, Ab-modified flow chamber was employed for capturing CD4 T-cells but instead of sandwich immunoassay an SPR chip was used for label-free sensing of cytokine production. A representative SPR sensogram demonstrating detection of IFN-γ is described in Fig. 3D. In this experiment, T-cells were captured in the flow chamber and mitogenically activated to induce cytokine production. It should be noted that mitogenic activation broadly activates T-cells to secrete several types of cytokines, including IFN-γ. The media bathing the cells was circulated through an SPR chip modified with anti-IFN-γ Ab layer. One channel of the dual-channel SPR chip was exposed to media with T-cell-secreted cytokines whereas the other channel was challenged with control media that did not come in contact with cells. Significantly, circulation of T-cell media through the working channel of an SPR instrument for ~2.5 h resulted in detectable SPR signal ΔRI – 0.00012 (see Fig. 3D). Using calibration curve in Fig. 3B this SPR signal was converted into IFN-γ concentration of ~200 ng/ml. The reference SPR channel exposed to media that did not come in contact with cells had no detectable signal. The results of Fig. 3D demonstrate that it is possible to detect endogenous, T-cell-secreted IFN-γ using SPR instrument.

In a separate set of experiments, T-cells were incubated in culture media without mitogens and this media was then routed onto an SPR chip. No signal was observed in this scenario (data not shown) confirming that IFN-γ detection in our SPR sensor was a direct consequence of T-cell producing/releasing cytokines in response to mitogenic stimulation.

4. Conclusion

The present paper sought to combine cell purification and SPR sensing for detection of IFN-γ release from CD4 T-cells. Anti-CD4

Ab-containing flow chamber was used to capture pure CD4 cells from RBC-depleted human blood. This flow chamber was then connected to an SPR instrument so as to circulate cell culture media and bring it into contact with an SPR chip containing IFN-γ Ab layer. Capturing CD4 T-cells upstream of an SPR sensor allowed label-free detection and quantification of secreted IFN-γ molecules. Our paper addresses a significant challenge of determining molecules secreted by a specific cell subset that is present in a heterogeneous sample such as blood. Pure CD4 T-cells were captured in ∼10 min and could then be connected to cytokine-sensing SPR instrument for label-free detection of secreted cytokines. A high concentration of IFN-γ was detected after only 2 h mitogenic stimulation which is much shorter then overnight activation used in traditional cytokine secretion assays. Importantly, given the emergence of SPR instruments capable of multiplexing, the strategy described here may be easily extended to parallel, real-time and label-free detection of multiple cytokines or other proteins secreted by the desired cell type.

Acknowledgements

The authors would like to thank Profs. Louie and Marcu for use of their microscopes. GS and MK were supported in part by the fellowship from the National Center for Biotechnology, Republic of Kazakhstan. This study was also supported in part by the NSF EFRI grant (#0937997) awarded to AR. ALS acknowledges working at the National Science Foundation during the time this study was carried out. The views expressed in this article are those of the authors and do not reflect the views of the National Science Foundation.

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