

# Single-Molecule Detection on a Protein-Array Assay Platform for the Exposure of a Tuberculosis Antigen

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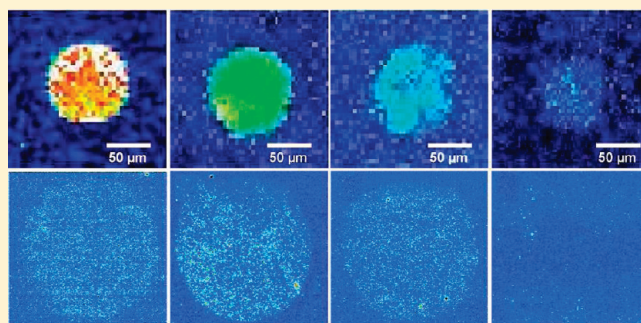
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**ABSTRACT:** Based on a single-molecule sensitive fluorescence-linked immunosorbent assay, an analytical platform for the detection of lipoarabinomannan (LAM), a lipopolysaccharide marker of tuberculosis, was established that is about 3 orders of magnitude more sensitive than comparable current ELISA assays. No amplification step was required. Also, no particular sample preparation had to be done. Since individual binding events are detected, true quantification was possible simply by counting individual signals. Utilizing a total internal reflection configuration, unprocessed biological samples (human urine and plasma) to which LAM was added could be analyzed without the requirement of sample purification or washing steps during analysis. Samples containing about 600 antigen molecules per microliter produced a distinct signal. The methodology developed can be employed for any set of target molecules for which appropriate antibodies exist.

**KEYWORDS:** Antibody detection, single-molecule sensitivity, quantification, tuberculosis



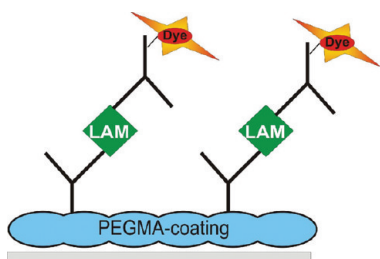
Tuberculosis (TB) is the most relevant bacterial infection on a global basis and a re-emerging infectious disease in western countries. The WHO estimates that the disease kills 1.8 million people every year, and 9.4 million new cases were registered in 2008. In total, about 2 billion people are infected worldwide.<sup>1,2</sup> Untreated active TB results in a mortality rate of about 68% compared to approximately 5% with treatment.<sup>1</sup> Consequently, reliable case finding of active disease is of utmost importance for disease control. However, current methods of clinical diagnosis often lack sensitivity. Bright field microscopy is still considered the method of choice in most endemic settings. Although very specific, it only provides sensitivities between 30% and 60%. Other assays, such as PCR or solid culture, are elaborate and time-consuming. Interferon- $\gamma$  assays are mostly useless for the diagnosis of active TB in high endemicity due to high numbers of infected individuals and related false positive rates. Therefore, more sensitive and reliable diagnostic platforms and biomarkers are required.

Antigen biomarkers that are specific for *Mycobacterium tuberculosis* and detectable in body fluids (e.g., urine) are an attractive option for the development of new diagnostic assays.<sup>3,4</sup> One promising new lead as a TB biomarker is lipoarabinomannan (LAM), a 17.5 kDa major cell-wall lipopolysaccharide specific to the genus *Mycobacterium*. LAM is also present in some fungi

species; however, LAM of different mycobacteria and fungi differs in its terminal arabinose residues. Therefore, antibodies can be raised that exhibit high specificity for each particular mycobacterial strain.<sup>5</sup> LAM is released both from metabolically active or degrading bacterial cells.<sup>3,5,6</sup> Once in the bloodstream, it is filtered out by the kidneys and can therefore be detected in urine. State of the art ELISA assays for the detection of tuberculosis antigens have shown sensitivities of around 1 ng/mL, equal to a concentration of about  $10^{-11}$  M.<sup>3-5</sup> Higher sensitivities are prerequisite to better diagnostics. Actual analyte concentrations vary from nanomolar to attomolar in a complex milieu like serum or urine, in which there is also an excess of nontarget proteins, typically leading to high background reading and undefined cross-reactions that are limiting detection accuracy.<sup>7</sup> This lack of sensitivity demands new approaches. During the past few years, protein microarrays have been developed for multiple applications.<sup>8-10</sup> Most protein microarrays used for diagnostic or analytic purposes, however, represent a miniaturization of the ELISA concept and face similar limitations of sensitivity and handling. However, also the currently most sensitive protein arrays reach their limit of detection

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**Figure 1.** Schematic presentation of the FLISA assay. A LAM-specific antibody was attached to a PEGMA-coated surface. After binding of LAM, a second, fluorescently labeled antibody indicates the presence of the antigen.

in the picomolar to femtomolar range,<sup>11,12</sup> similar to that achievable with “best in class” ELISA methods.

Our approach to improve LAM detection was based on a fluorescence-linked immunosorbent assay (FLISA) combined with a single-molecule detection mode. In this system, the requirement of two independent binding events assures specificity. Direct labeling of the detection antibody is preferable to enzyme-linked amplification for its technical simplicity. Detection of individual molecules enables both high sensitivity and high accuracy of the assay and permits true quantification by means of directly counting bound molecules. Also other detection approaches with single-molecule sensitivity exist, such as flow cytometry,<sup>13</sup> atomic-force spectroscopy using cantilevers or optical tweezers, and advanced ELISA based formats.<sup>14</sup> Due to a highly complex design, however, these methods are not easily applicable in field use or routine diagnostics. In this study, two unique LAM-specific antibodies were used. One antibody was spotted as capture reagent and covalently attached to a coated glass slide. After sample incubation, bound LAM was made visible with a second, dye-labeled antibody, and the fluorescence signal was recorded (Figure 1). With this simple assay format, even unprocessed samples could be analyzed with ultrahigh sensitivity. In addition, the ability of counting the molecules bound to the target eliminates the need for standard curves.

## MATERIALS AND METHODS

### Chemicals

All chemicals were from Sigma Aldrich (Steinheim, Germany) unless stated otherwise. *N,N*-Dimethylformamide (DMF) was obtained in peptide grade quality from Biosolve BV (Valkenswaard, The Netherlands) and dried over molecular sieves (0.4 nm; Roth, Karlsruhe, Germany). Fmoc-ss-alanine (>99%) was purchased from Iris Biotech (Marktredwitz, Germany). Nitrogen from AirLiquide (Düsseldorf, Germany) was used as inert gas for all reactions that are sensitive to oxygen and humidity. For chemical treatment of the glass slides (Menzel Gläser, Braunschweig, Germany), a custom-built Teflon synthesis box for up to 30 glass slides (format 24 × 50 mm) was used. Monoclonal capture (AK24) and detection (AK29) antibodies were provided by FIND (Geneva, Switzerland). Antibody affinities are in the low nanomolar range, as determined by Biocore SERS. FIND will provide more detailed information to interested parties upon request. All plasma and urine samples were collected from healthy donors, whose written informed consent was obtained.

### Surface Coating and Activation

The PEGMA surfaces were produced according to well-established protocols.<sup>15</sup> For activation, 10 PEGMA-coated

surfaces were equilibrated at room temperature and soaked in 30 mL of dry demethylformamide (DMF) for 30 min. Subsequently, the DMF was removed, and 100 mL of activation solution (100 mL of dry DMF, 1 g of *N,N'*-disuccinimidyl carbonate (DSC), and 1.5 mL of *N,N*-diisopropylethylamine) was added. After 60 min, the surfaces were washed three times with 100 mL of dry DMF for 3 min. Then, they were dried and stored under nitrogen at 4 °C until spotting of capture molecules.

### Capture Antibody Coupling

The capture antibody (2 μM AK24) was coupled to the surface via solid pin contact printing in 0.2 M sodium carbonate buffer, pH 8.4. The humidity during the whole spotting process was kept at 80%. After the spotting process, the slides were washed three times with 265 mM NaCl, 5.4 mM KCl, 20 mM sodium phosphate, 4 mM potassium phosphate (2 × PBS), pH 7.2, dried, and stored at −80 °C until used for sample incubations.

### Surface Blocking

Prior to sample incubation, a deactivation of the PEGMA surfaces was performed. Slides were incubated in 0.2 M sodium carbonate buffer, pH 8.4, containing 9% aminoethanol for 10 min. Then, the deactivation solution was removed, and the surfaces were washed two times with water and dried in a stream of nitrogen.

### Labeling of the Detection Antibody

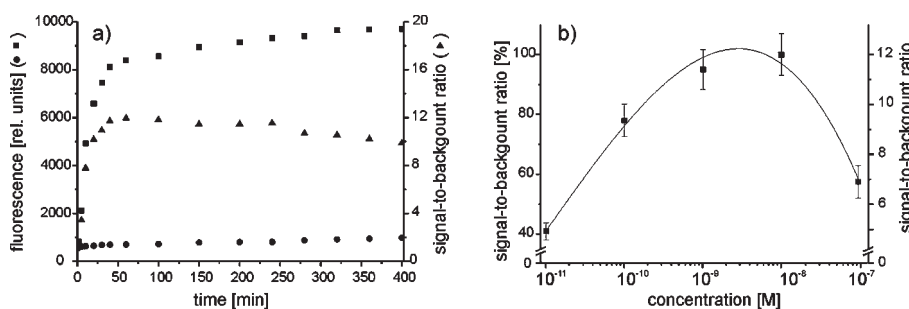
The fluorescent dye ATTO633 of ATTO-TEC (Siegen, Germany) was dissolved in dry and amine-free DMF for a 2.5 mM stock solution. The concentration was checked by measurement of the UV absorption at 633 nm. Ten microliters of the antibody (AK29) stock solution ( $1.4 \times 10^{-5}$  M = 2 mg/mL) was diluted in 20 μL of 0.2 M sodium carbonate buffer, pH 8.4. Subsequently, 0.3 μL of the dye stock solution was added. The reaction mixture was left at room temperature for 2.5 h. The labeled antibody was purified using a gel chromatography column (Illustra NAP-5 column; GE Healthcare, Munich, Germany) resulting in a 150 nM stock solution of labeled antibody, which was stored in aliquots at 4 °C.

### Incubation

The incubation of the arrayed antibodies with samples and labeled detection antibody was done in secure seals (Invitrogen, Karlsruhe, Germany). Urine samples were used without any processing but for the spiking-in of particular concentrations of LAM. Plasma had to be diluted 1:50 in PBS, since the viscosity of the undiluted material was too high to be used in the incubation seals. For calibration experiments, also LAM in PBS buffer was used. Prior to incubation, the array surface was wetted with 2 × PBS for 30 min. Then, the LAM-containing sample was added. After 10 min, labeled detection antibody was added to a final concentration of  $10^{-9}$  M. Incubation in a total volume of 20 μL took place for 40 min, if detection occurred on standard fluorescence scanners. Subsequently, the slides were washed with 2 × PBS, pH 7.2, and water and dried with nitrogen. In single-molecule detection experiments, the incubation time was 15 min, followed by direct measurement without any washing.

### Standard Scanner Detection

Slides were kept in the dark until read-out. Scanning was at 633 nm and 5 μm resolution using a ScanArray 5000 (Perkin-Elmer, Waltham, USA). For data comparison, the scanner photomultiplier tube gain and laser power was kept constant in



**Figure 2.** Effect of incubation duration. (a) The mean fluorescence signal intensities of 36 spots (■) and the related background signals (●) are shown, which were obtained on PEGMA-coated slides. Spots were produced with  $2 \times 10^{-6}$  M antibody. The arrays were incubated with  $10^{-13}$  M LAM for different periods. Subsequently, the labeled detection antibody was applied at a concentration of  $10^{-9}$  M. The autofluorescence of the surface (about 1,800 units) was subtracted from all measurements. The triangles (▲) represent the signal-to-background ratio. (b) Signal-to-background ratios are presented that were detected with different concentrations of detection antibody using samples of  $10^{-13}$  M LAM.

all measurements. The fluorescence signals were converted into numerical values by the built-in software ScanArray Express.

### Single-Molecule Detection

Read-out was performed on a scanner with single-molecule sensitivity.<sup>17</sup> As fluorescence excitation source, a 647 nm Ar<sup>+</sup> laser (Coherent Innova, Dieburg, Germany) was used at an illumination intensity of  $P = 0.1$  kW/cm<sup>2</sup>. To achieve a homogeneous excitation profile and an adjustable spot size, the shape of the laser beams were controlled using two cylindrical telescopes. The cell samples were illuminated in total internal reflection configuration using a 100 $\times$  oil immersion objective ( $\alpha$ -Fluar, NA = 1.45, Zeiss, Germany). The fluorescence signal was imaged on a back-illuminated CCD camera (Princeton Instruments, Trenton, USA; chip size 1340  $\times$  100 pixels and pixel size 20  $\mu$ m). The arrays were mounted on a scanning stage Scan IM 120  $\times$  100 (Märzhäuser, Bonn, Germany) with 20 nm minimum step size. Scanning was at full speed (116 ms/pix) and resolution (200 nm/pix). The changes in the distance between objective and sample surface were corrected with a Focus Hold System.<sup>18</sup> With this scanning configuration, the average intensity of a single binding event of a labeled antibody was 1332 ( $\pm$  449) counts/pix. Although the microarrays were quite homogeneous, spot diameters varied up to 10%. Therefore, the numbers were standardized to a typical spot area of 6360  $\mu$ m<sup>2</sup>.

## RESULTS

Bright fluorescence signals and a low background are prerequisite to the detection of individual molecules. Background may be caused by a variety of factors, such as unspecific binding events or autofluorescence of the materials used. To minimize unspecific adsorption, the microarray glass surfaces were coated with poly(ethylene glycol)methacrylate (PEGMA). This PEGMA film was originally designed for peptide array supports in proteome research.<sup>15</sup> As shown by X-ray photoelectron spectroscopy, unspecific adsorption of proteins did not occur in incubations with BSA, lysozyme, fibrinogen,  $\gamma$ -globulin, and human serum. Moreover, the PEGMA coating is superior to commonly applied blocking agents, since the latter contain proteins that produce unspecific background themselves,<sup>16</sup> which is especially critical for single-molecule detection. Concomitant to repelling unspecific protein binding, the PEGMA coating provided a high and variable specific loading capacity of up to 40 nmol/cm<sup>2</sup>. The actual antibody density on the surface and the array printing conditions were other critical factors.

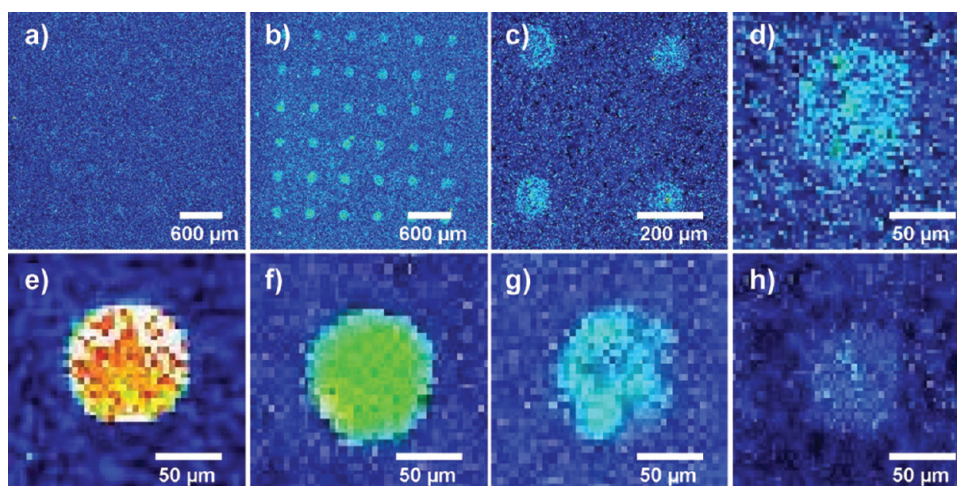
Printing worked best at a humidity of 80% with a sodium carbonate buffer and an antibody concentration of  $2 \times 10^{-6}$  M.

Beside fabrication parameters, also the sample incubation conditions affected performance. Usually, ELISA assays require a relatively long incubation time in order to generate detectable signals. To investigate the influence of incubation time on signal and background intensities, microarrays were incubated with LAM for different time intervals prior to the addition of the labeled detection antibody. After washing, detection was performed in a standard low-resolution fluorescence scanner. A LAM concentration of  $10^{-13}$  M was chosen. This low concentration prevented saturation of the capture antibodies. Due to fast binding kinetics, 50% and 80% of the maximum fluorescence signal were achieved after 10 and 40 min, respectively (Figure 2a). The background signal increased very rapidly during the first minute but rose at a much slower rate subsequently. Incubation times of about 60 min resulted in the highest signal-to-background ratio.

The concentration of the detection antibody was another important parameter that influenced signal intensities (Figure 2b). Antibody concentrations ranging from  $10^{-7}$  to  $10^{-11}$  M (which equals 14 to 1.4 ng/mL) were investigated. The highest signal-to-background ratio of 11.7 ( $\pm$  0.4) was achieved with an antibody concentration of  $10^{-8}$  M (1.4  $\mu$ g/mL). Higher concentrations caused a significant increase in fluorescence background, resulting in dramatically lower signal-to-background ratios. For reasons of optimal resource utilization, the concentration of the detection antibody was set to  $10^{-9}$  M (140 ng/mL). At this concentration, the signal-to-background ratio is only 4% below its maximum but the amount of antibody needed is reduced by 90%. Even at a concentration of  $10^{-10}$  M (14 ng/mL), a signal-to-background ratio of 9.4 (80% of optimum) was achieved.

### Antigen Detection Using a Conventional Fluorescence Scanner

Spotting the PEGMA-coated surface with the capture antibody according to the protocol described in the Methods section resulted in homogeneous grids (Figure 3). Spot diameter was 100 ( $\pm$  7)  $\mu$ m. For proof of principle experiments, microarrays were incubated with unprocessed human plasma (diluted 1:50) that was spiked with LAM to a final concentration of  $10^{-13}$  M. After adding  $10^{-9}$  M of the fluorescently labeled detection antibody, 40 min of incubation, and a washing step, the fluorescence was recorded by a commercial standard scanner system with 5  $\mu$ m resolution. The fluorescence signal (Figure 3b–e) was



**Figure 3.** Fluorescence signals measured with a standard fluorescence scanner. Detection was done after 40 min of incubation in the presence of  $10^{-9}$  M fluorescence-labeled detection antibody. (a) Signal resulting from an incubation of unprocessed human plasma (diluted 1:50) containing no LAM and (b) spiked with  $10^{-13}$  M LAM. In panels c and d, images of the same grid are shown at higher resolution. In the lower row of panels, one typical spot is shown as recorded for a urine sample spiked with LAM with concentrations of  $10^{-11}$  M (e),  $10^{-12}$  M (f),  $10^{-13}$  M (g), and  $10^{-14}$  M (h), respectively.

**Table 1. Comparison of Detection Sensitivities<sup>a</sup>**

sample type	LAM concn [M]	overall fluorescence signal intensity		single-molecule detection	
		fluorescence signal/spot [rel units]	unspecific binding [rel units]	no. of molecules per spot	unspecific binding; no. of molecules
plasma	$10^{-12}$	$14,280 \pm 1,232$	$800 \pm 39$	ND	ND
	$10^{-13}$	$4,632 \pm 468$	$730 \pm 37$	$1,040 \pm 160$	$240 \pm 24$
	$10^{-14}$	$1,721 \pm 218$	$726 \pm 38$	$480 \pm 80$	$232 \pm 16$
urine	$10^{-12}$	$21,730 \pm 1,731$	$710 \pm 41$	ND	ND
	$10^{-13}$	$8,113 \pm 747$	$640 \pm 42$	$2,400 \pm 320$	$320 \pm 32$
	$10^{-14}$	$2,241 \pm 376$	$630 \pm 38$	$1,070 \pm 300$	$240 \pm 24$
PBS buffer	$10^{-11}$	ND	ND	$24,700 \pm 1,763$	$532 \pm 62$
	$10^{-12}$	$24,319 \pm 1,587$	$700 \pm 32$	$9,000 \pm 860$	$474 \pm 43$
	$10^{-13}$	$9,237 \pm 1,145$	$600 \pm 31$	$3,600 \pm 184$	$280 \pm 12$
	$10^{-14}$	$2,975 \pm 374$	$610 \pm 33$	$1,360 \pm 96$	$160 \pm 8$
	$10^{-15}$	$824 \pm 255$	$605 \pm 32$	$800 \pm 160$	$240 \pm 8$
control	0	$912 \pm 146$	$480 \pm 17$	$230 \pm 24$	$230 \pm 24$

<sup>a</sup> Relative fluorescence signals and single molecule counts are shown that were recorded in the presence of  $10^{-9}$  M detection antibody for different body fluids spiked with various LAM concentrations. The unspecific binding was analyzed on spot-sized areas located between the actual antibody spots. The control contained PBS buffer and detection antibody but no LAM. For each signal intensity value, the average of 36 measurements is shown. Next to it, the results from the single-molecule detection modus are presented. The number of molecules per spot was averaged over 6 spots. ND = not determined.

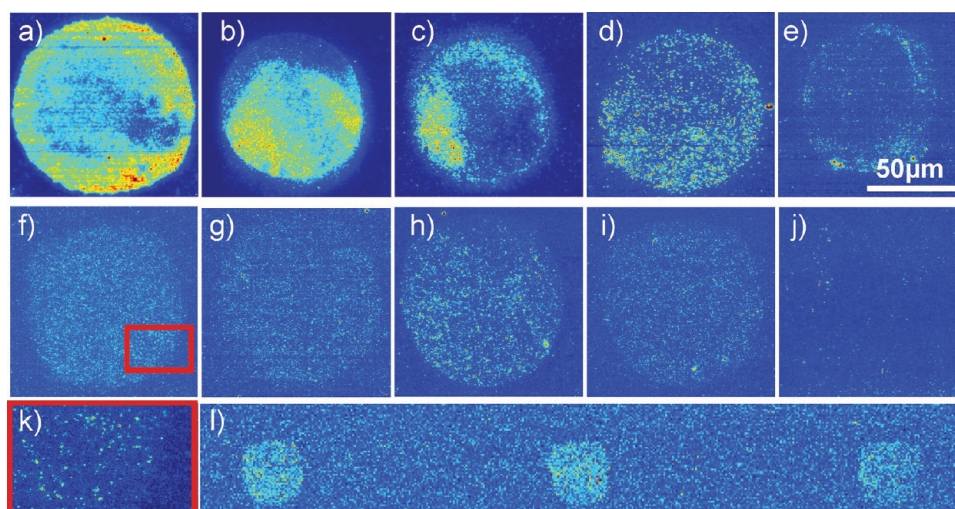
approximately 10 times higher than that of the negative control generated by the same process but without LAM (Figure 3a). However, the signal-to-background ratio was only about 6.5. The unspecific binding of the aggregate of LAM and detection antibody is probably higher than that of the detection antibody alone, resulting in a higher background when LAM is present.

Table 1 summarizes all measurements of buffer, unprocessed urine, and plasma samples (diluted 1:50) spiked with different concentrations of LAM. An antigen concentration of  $10^{-13}$  M could be clearly identified in all media investigated. In unprocessed human plasma, however, the signal intensity dropped by 30–40% compared to urine samples or buffer. Furthermore, the background signal increased slightly. Therefore, we found for plasma samples signal-to-background ratios of 18 ( $c(\text{LAM}) = 10^{-12}$  M) and 6 ( $c(\text{LAM}) = 10^{-13}$  M). Under the same conditions, signal-to-background ratios of 30 and 12 were obtained for urine samples, and 35 and 15 for antigen alone,

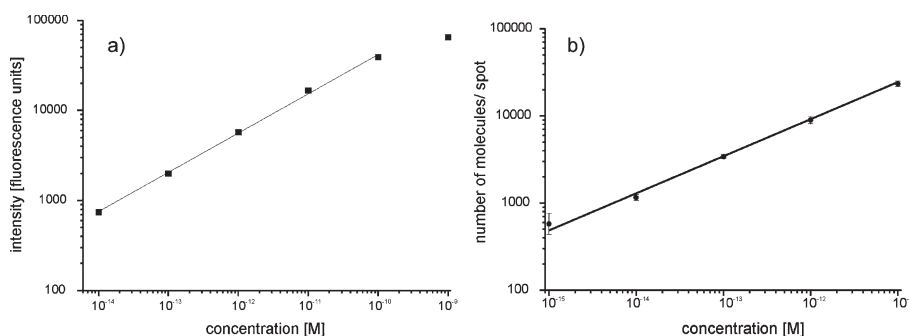
respectively. The detection limit of  $10^{-14}$  M LAM corresponds to approximately 120,000 molecules in a 20  $\mu\text{L}$  sample. All experiments showed high reproducibility. The fluorescence intensities of all experiments vary by about 7–10% both across a single array and between different arrays.

#### LAM Detection Using a Single Molecule Sensitive Read-Out

For more sensitive and quantitative measurements, analyses were performed on a scanner with single-molecule sensitivity.<sup>17–20</sup> The samples were illuminated in a total internal reflection configuration. Such a read-out allows for easy discrimination between fluorescence arising from individual fluorescent molecules and background caused by light scattering at the surface. Therefore, few bound molecules are sufficient to generate a positive signal, although the average fluorescence does not increase significantly (Figure 4). At the applied scanning parameters, about 1,300 photons were detected per dye molecule of a



**Figure 4.** Single-molecule detection. The fluorescence signal of a typical array spot is shown as recorded by a single-molecule sensitive CCD camera after 15 min of incubation of samples containing  $10^{-9}$  M labeled detection antibody. In the top row, images are shown after incubation of PBS buffer spiked with purified LAM at concentrations of  $10^{-11}$  M (a),  $10^{-12}$  M (b),  $10^{-13}$  M (c),  $10^{-14}$  M (d), and  $10^{-15}$  M (e). Below, results are shown that were obtained with plasma (f and g) as well as urine (h and i) samples spiked with  $10^{-13}$  M and  $10^{-14}$  M LAM, respectively. A magnified part of panel f is shown in panel k. As a control (j), a plasma sample containing no LAM but dye-labeled detection antibody was used. In panel l, we show a software-binned area covering multiple spots, demonstrating the homogeneity of the arrays; a typical array scanned at the condition of panel f is shown.



**Figure 5.** Linearity of the assays. PBS buffer was spiked with different LAM concentrations, and the fluorescence was recorded with a standard scanner (a) and a single-molecule sensitive CCD camera (b). All values are corrected by the negative control (sample without LAM) showing 900 fluorescence units or 230 molecules/spot, respectively.

detection antibody. The background scattering in the same area resulted in only 200–350 counts. Signal arising from the surface could therefore be identified easily. On the other hand, impurities and defect structures on the surface often show brighter fluorescence and can lead to signals that are similar to individual bound antibodies.

In the following, background is defined as the number of signals (molecules) generated by adsorbed detection antibodies or impurities in areas between the spots that are of the same size as the spots. After incubation with LAM in PBS buffer but without fluorescent antibodies, less than 10 molecules per spot-sized area were found. In another control experiment, a sample without LAM but containing fluorescently labeled detection antibody in a 1 nM ( $10^{-9}$  M) concentration was performed (Figure 4j). The surface was imaged after 15 min incubation without any washing. In contrast to many other surface-based assay formats, washing steps are not needed because the total internal reflection excitation does only excite molecules located near the surface (<100 nm distance). Due to nonspecific adsorption of detection antibodies, we found between 200 and 250 fluorescent molecules per spot-sized area. This background

signal was found in the actual antibody spots as well as the area in between the spots, demonstrating that the binding procedure of the capture antibodies does not affect the surface properties significantly.

The detection limit of purified LAM in PBS buffer was at least 10 times better compared to the result with a standard scanner (Figure 4; Table 1). Even a 20  $\mu$ L sample with a LAM concentration of  $10^{-15}$  M, corresponding to about 12,000 molecules, showed distinct signals (Figure 4e). On average, there were about 800 molecules per spot, which is three times the number found between the spots. The recovery rates were 0.2%, 0.7%, 2.7%, 10.2%, 31%, respectively, for LAM concentrations of  $10^{-11}$  M down to  $10^{-15}$  M. In urine (undiluted) or plasma (1:50 dilutions) spiked with LAM, the detection limit was  $10^{-14}$  M (Figure 4g and i). In line with the results with a standard fluorescence scanner, the number of bound molecules per spot decreases by approximately 30% with urine and 70% with plasma. However, the urine or plasma samples did not produce higher background in terms of molecules per spot-sized area. Indeed, the light scattering on the surface was also increased, but this signal was eliminated by just counting molecules that exhibited a

fluorescence intensity above a threshold that is typical for single molecules. This data shows that urine and plasma contain only negligible amounts of fluorescent impurities that stick to the surface and thus enhance the background.

Figure 5 shows the signal intensity plotted versus LAM concentration. The data shown were obtained by spiking different amounts of LAM into PBS buffer; urine or plasma samples produced comparable results (not shown). The linear slope in the double logarithmic plot is in line with a pseudo-first-order binding kinetics of the LAM/detection antibody complex to the capture antibody.<sup>21</sup> The data point of the LAM concentration  $10^{-9}$  M does not match the linear fit. Probably, the binding kinetics of detection antibody and LAM cannot be neglected, because both binding partners have similar concentrations, whereas for all other measurements the detection antibody is in excess.

## DISCUSSION

Progress in suppressing unspecific protein binding enhances immunosorbent assays to a degree that sensitivity becomes limited by the detection technique and the binding kinetics of the analyte. As a proof of principle, we set up an assay for the tuberculosis associated antigen LAM, achieving a detection limit of  $10^{-14}$  M with standard fluorescence scanners. This is already about 3 orders of magnitude more sensitive than candidate ELISA assays suggested for tuberculosis diagnostics.<sup>3</sup> Besides a significant increase in sensitivity, our approach has other advantages, too. First, total turnaround time is short; the whole assay procedure takes about 20 min when using the single-molecule sensitive approach, including sample preparation, incubation, and fluorescence read-out. Second, it is cost-effective as a result of the small antibody amounts needed for both capture and detection. Third, different capture antibodies could be used simultaneously, which would enable the detection of different analytes or isoforms within one sample at a time in a multiplex detection mode.

To achieve even better sensitivity and for enabling quantification, a sensitive CCD camera system was applied that permits the detection of individual fluorescent molecules. This technique improved sensitivity by at least another order of magnitude, detecting a few hundred target molecules on relatively large spot areas of 100  $\mu\text{m}$  diameter. In addition, truly quantitative measurements across several orders of magnitude are made possible by this assay configuration, since molecules can be counted individually. Because of excitation via total internal reflection, only molecules near the surface are illuminated. Consequently, no time-consuming washing steps are needed in contrast to most other surface-based assay systems. The system therefore allows real-time monitoring of the analyte binding event. Lastly, also unprocessed clinical samples can be studied since the background generated from light scattering can be separated from the specific signals.

The described technique is widely applicable to the development of new assays in medical diagnostics and research. For analyzing the binding of complex, directly fluorescence-labeled protein mixtures to large sets of arrayed antibodies,<sup>22</sup> for example, this technique is highly relevant and important as a result of its sensitivity, ability to quantify, and analysis of unprocessed samples. With regard to tuberculosis, the method provides a sensitivity benchmark for the development of LAM antigen immunoassays for TB case detection, a much desired

achievement for rapid TB diagnostics. With regard to medical application, the approach reduces assay complexity because of fewer sample preparation steps and thus results in a quicker analysis. Also, the risk of contamination is reduced, and fewer reagents are consumed. Although single-molecule detection equipment is not yet state-of-the-art in diagnostics, such hardware is becoming more and more a routine tool. However, even without, the process could replace all current ELISA-based formats used for clinical applications without causing any major changes to the overall setting.

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