Fabrication and Demonstration of Ultra-Sensitive and Fast Fluorescence Immunoassay Using Novel Nanoplasmonic Sensor inside Microfluidic Channels

Ruoming Peng, Chao Wang, Liangcheng Zhou, Qi Zhang, Weihua Zhang and Stephen Y. Chou*
NanoStructure Laboratory, Princeton University, Princeton, NJ08544

Abstract

We report fabrication and performances of a new immunoassay device, which integrates a novel nanoplasmonic sensor array inside a microfluidic channel. The new assay has demonstrated 10^6 fold detection limit enhancement of a model direct Protein A immunoassay over glass reference (from 2nM to 850 aM, i.e. 120 ng/ml to 50 fg/ml) and 6 fold of incubation time reduction (2 hours to 20 min) compared to conventional 96-well plate immunoassay.

Keywords

Microfluidics, microchannel, nanochannel, nanoscale, plasmonics, fluorescence, fluorescence enhancement, immunoassay, limit of detection, incubation time, nanofabrication, nanoimprint, nanostructures, and Protein A.

Introduction

One major challenge in fluorescence immunoassay (FIA) is to drastically increase its sensitivity while significantly reduce incubation time [1]. Nanoplasmonic structures can enhance the fluorescence signal [2], hence improving immunoassay sensitivity [3]. Microfluidic channels have been widely explored for Lab-on-chip, due to the advantage of much less incubation time and efficient deliver and use of analyte [4]. It is desirable to combine nanoplasmonic sensor and microfluidic technology together to build an ultra-sensitive and fast immunoassay plate, however nanofabrication is challenging.

Here, we report fabrication and performances of a new immunoassay platform that integrates novel nanoplasmonic sensor into microfluidic channels and was fabricated by nanoimprint [5]. The new nanoplasmonic sensor is a 3D nanostructure, termed "disk coupled dots on pillar nanoantenna-array" (D2PA), which offers fluorescence enhancements much higher than previous approaches [6, 7]. The new assay platform has demonstrated 10^6 fold enhancement in the limit of detection (LoD) in a model direct Protein A immunoassay over glass reference (from 2nM to 850 aM, i.e. 120 ng/ml to 50 fg/ml) and 6 fold reduction in incubation time (2 hours to 20 min) compared to conventional 96-well plate immunoassay.

Assay Structure and Fabrication

The assay has D2PA sensors in microfluidic channel (Fig. 1a). The D2PA sensor consists of a dielectric nanopillar array (200 nm pitch, 70nm diameter and 56 nm height) with an Au nanodisks on top of each pillar, an Au backplane on the foot, and random nanodots (5 nm to 15 nm) on the pillar sidewalls (Fig. 1b). All metal components are self-aligned with each other and have nanogaps between them.

The fabrication of the microfluidic assay was done by three layer technology, where each layer are fabricated separately and then assembled. The three layers are: bottom D2PA sensor channel layer, middle PDMS inlet and outlet layer, and top thin glass cover layer (Fig. 1b). To fabricate D2PA, Cr dots arrays were first patterned through nanoimprint. Nanopillars were then created through reactive ion etching (RIE) in photolithography defined regions. Then six shallow micro-channels (5μm deep, 400μm wide) and square reservoirs (5 μm deep, 500μm wide) were fabricated by photolithography followed with RIE. Finally, 40nm Au was evaporated on selected sensor area. Before sealing, the device was immersed in Dithiobis Succinimidyl Undecanoate (DSU) in 1, 4 dioxane solution to coat a self-assembly monolayer (SAM) as capture agent for the immunoassay.

The PDMS inlet-and-outlet layer was fabricated through spinning and imprint [5]. A PDMS film of 8 μm thick was first spin-coated onto a thin glass coverslip and then imprinted with a Si master mold. After imprinting, the PDMS was cured and then peeled off from the Si mold. Finally, the bottom layer with D2PA was aligned and bond to the PDMS/thin glass cover (Fig. 1d-g).

For comparison, we also fabricated reference devices: (a) the microfluidic devices the same as D2PA microfluidic assay except no Au; for checking nanoplasmonic effects; and (b) D2PA plate on in 96-well plate (e.g. large fluid volume and no microfluidic channels) for checking microfluidic channel effects.

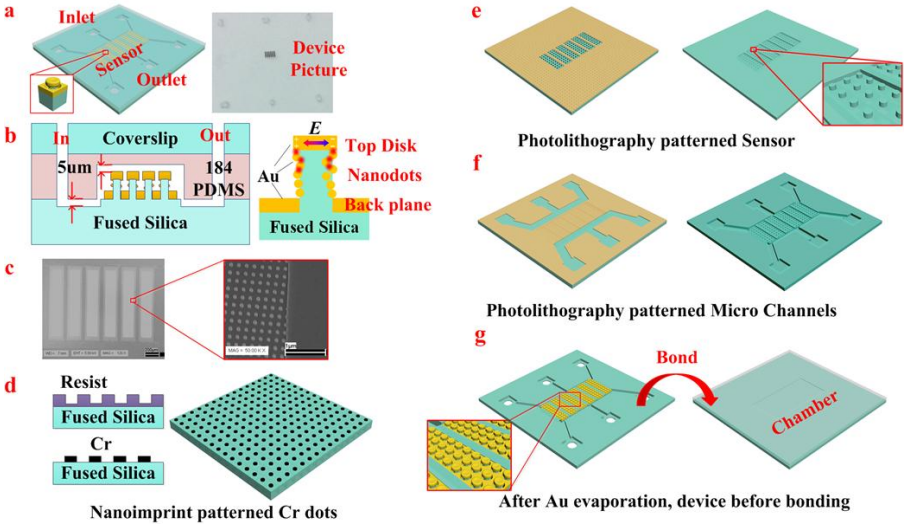

Figure 1. Device structure and fabrication process. (a-b) Device architecture; (c) SEM image of Cr dots array fabricated by nanoimprint; (d-e) bottom-sensor and channel layer fabrication; (f) middle PDMS inlet-and-outlet layer; (g) Au evaporation and bonding of all three layers.

Experiment

The immunoassay we used to test the detection limit and incubation time of our new microfluidic assay is a direct fluorescence assay that detects fluorescence labeled (IRDye800CW) Protein A using the DSU monolayer. In the test, the labeled Protein A in PBS buffer solutions with concentrations from 1 fM to 100 nM (from 60 fg/ml to 6 mg/ml) of volume of 100μL were separately injected into channels (one concentration per assay) using a flow rate of 5μL/min. Protein A molecules were captured on the DSU SAM through the ester-amine reaction, the unbounded molecules were flushed out using 100 μL washer ((PBS+0.5% TWEEN-20) with a flow rate of 10 μL/min.

The assay in conventional 96-well plate reference was performed in a standard way: 100μL labeled Protein A solutions were first added into separate wells and let it incubate for 2 hours. Then, each well was washed three times with washing solution. To read the immunoassay, fluorescence signal was collected through an inverted microscope equipped with an EM-CCD (Fig.2a) and averaged over an area of 100 μm by 100 μm. For each concentration, 5 replicates were measured to obtain the standard deviation.

Results

We observed significant LoD enhancements and incubation time reduction in microchannel D2PA over the references. In fact, six orders of magnitude enhancement in detection sensitivity (LoD) was achieved on our new plasmonic device. Figure 2b shows the fluorescence intensity versus the Protein A concentration of the microchannel D2PA assay and the two references. Using the standard five-parameter logistic regression model, the microchannel D2PA assay demonstrated a limit LoD of 850 aM (50 fg/ml), which is about 10^6 fold better than the identical assay on the reference sample without Au coating (LoD =2 nM). And the reference of D2PA in 96-well plate assay gave a LoD of 1fM, similar to that of microchannel D2PA assay.

The improvement of limit of detection can be ascribed to two reasons: (1) the giant fluorescence enhancement of D2PA [8]; (2) the proper adhesive and spacer layer DSU, which captured Protein A and balanced the plasmonic enhancement and quenching effect of metallic structure.

Table 1 Limit of detection comparison.

Sample	*D2PA in channel*	*D2PA in 96-well plate*	*Nanopillars in channel without Au coating*
Limit of detection	*850aM*	*1fM*	*2nM*
Improvement (fold)	*2.4×10^6*	*10^6*	*1*

We also observed that the incubation time in the microchannel D2PA assay is 6-fold shorter than D2PA in 96-well plate (from 2 hours to 20 min). The fast incubation is due to drastically reduce of average diffusion distance between the molecules to the capture layer which is D2PA surface.

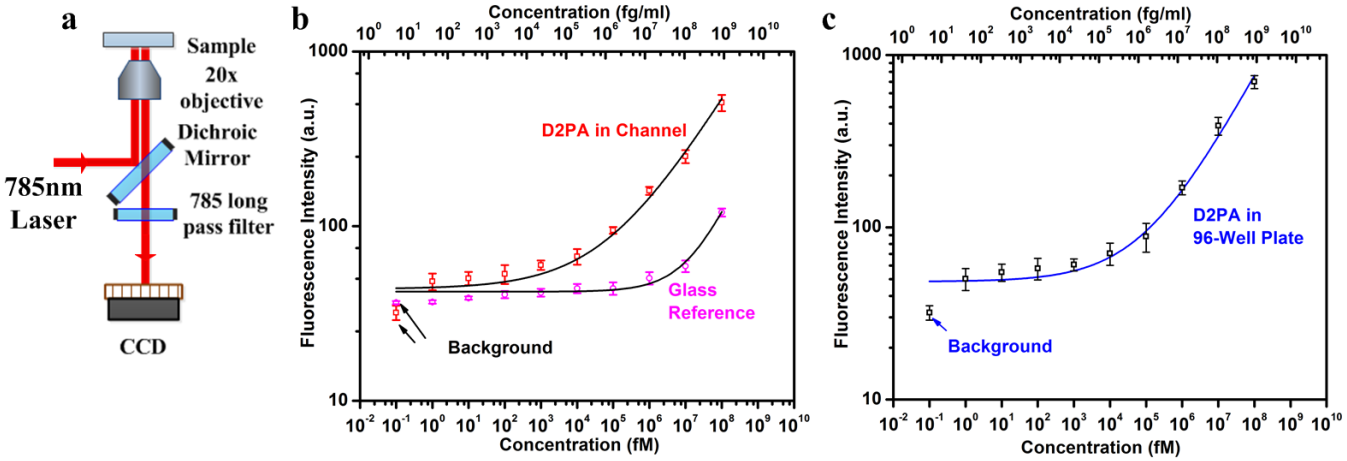

Figure 2. (a) Optical setup of the model immunoassay experiment. The laser beam scan area is 100 μm by 100 μm; (b)-(c) Fluorescence Intensity versus Concentration. The five parameters logistic regression model shows a detection limit of 850 aM for the D2PA in channel device; 2nM for the glass reference and 1fM for the D2PA in 96-well plate assay.

In summary, we report the fabrication and performances of a new immunoassay microfluidic device integrated with a novel plasmonic sensor plate. 10^6 fold enhancement of sensitivity and a 6-fold reduction of incubation time were achieved on a fluorescence-based direct immunoassay.

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* Contact author: Chou@princeton.edu