Supporting Information

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A Nanostructured Microfluidic Immunoassay Platform for Highly Sensitive Infectious Pathogen Detection

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Chemical Reagents

SU-8 10 photoresist and developer were purchased from MicroChem (MicroChem Corp., USA). Poly(dimethylsiloxane) (PDMS, Sylgard\textsuperscript{®} 184) and curing agent were obtained from Dow Corning Corp (Dow Corning Silicones Co., Ltd, USA). Ammonium hydroxide (28%), 3-glycidoxypropyltrimethoxysilane (GPTMS), trimethylsilyl chloride and zinc nitrate hexahydrate were purchased from Alfa Aesar. EZ-Link\textsuperscript{®} sulfo-NHS-LC-Biotin was purchased from the Thermo Fisher Scientific. Ethanolamine, potassium permanganate (KMnO\textsubscript{4}) and bovine serum albumin (BSA), NAPTM-5 Columns Sephadex\textsuperscript{TM} G-25 DNA Grade and the Amicon Ultra centrifugal filters (3.5 kD) were purchased from Sigma-Aldrich. DyLight\textsuperscript{®} conjugated goat
anti-mouse IgG was purchased from the Thermo Scientific Company. Cy*3-streptavidin (Cy3-SA) was obtained from the Invitrogen. The methoxy polyethylene glycol thiol (mPEG-SH, MW, 2000) was purchased from the Laysan Bio, Inc. Monoclonal antibody to H5 hemagglutinin (H5 mAb) and to H7 hemagglutinin (H7 mAb) produced in mouse, H1N1 virus, H3N2 virus, H5N2 AIV, H7N2 AIV, newcastle disease virus (NCDV) and infectious bursal disease virus (IBDV) were obtained from the Wiley Lab/Avian Virology (Penn State University). All the other chemical reagents were obtained from VWR (VWR, USA) and used without further purification.

S1. Design and Fabrication of the ZnO-NIM

The design of the microfluidic chip is shown in Figure S1. There are six star-like branched channels as inlets for the sample loading and one main outlet in the center. The outlet is connected to a syringe pump. The upper layer of the microfluidic chip is the fluid flow channel with a herringbone structure on the top, while the bottom is the ZnO nanorod slide.

Figure S1 The mask of a six star-like branch microchannels (A) and the herringbone structure design.

The two layer herringbone structure is fabricated as previous reports.\textsuperscript{[1, 2]} Briefly, the negative SU-8 10 photoresist was spin-coated on a clean silicon wafer at 1000 rpm for 30 s to obtain a 20 μm thick photoresist master. After exposure with UV through a mask for 15 s and
baked at 65 ºC for 1 min, 95 ºC for 2 min respectively, a second layer of 20 μm SU-8 10 photoresist was spin-coated onto the first layers. After the second exposure was under UV with a herringbone structure on the mask for another 15 s, the silicon wafer was backed at 65 ºC for 3 min, 95 ºC for 5 min. After developing in the solution, the photoresist mold was exposed to the trimethylsilyl chloride vapor for 10 min in order to facilitate the mold release. The PDMS microchannel was fabricated by the standard soft lithography method. The PDMS prepolymer (part A: part B = 10:1 w/w) was well mixed together and poured onto the photoresist master and then baked in an oven at 75 ºC for 4 h. After that, the solid PDMS was peeled off from the master and punched with a blunt needle for the inlets and outlets. The stamp-stick room-temperature method was used to bind the PDMS slab with the glass slide with the ZnO surfaces together. Briefly, the PDMS prepolymer (part A: part B = 5:1 w/w) was well mixed together and degassed in a vacuum. After that, a spin-coated method was used to produce a thin layer of the PDMS film on a clean silicon wafer with the speed of 6,800 rpm/min. The PDMS slab with the herringbone structure was put onto the wet PDMS film and picked it up immediately from the wet PDMS film. Then the PDMS slab with some wet PDMS was carefully put onto the glass slide with the ZnO nanorods and let the process allow to ~12 h. The bonded ZnO-NIM could be used for the pathogen detection. Figure S2 gives the image of microfluidic chip (Figure S2A), the herringbone structure and the roughness ZnO nanorod surface (between the fluid channels) (Figure S2B).
Figure S2 Image of a microfluidic chip (A) and the herringbone structure on the microfluidic chip (B).

S2. Large-scale Growth of ZnO Nanorods on the Glass Slides

Standard 75×25 or 75×50 mm glass slides were washed with ultra-pure water and activated with 100 mL of 5 mM freshly prepared KMnO₄ solution for 30 min at room temperature. After washed with ultra-pure water for three times, the activated glass slides were vertically immersed into 125 mL of the ZnO nanorods growth medium, which included 0.1 M of Zn(NO₃)₂, 4% (v/v) of NH₃·H₂O (28%) and 10% (v/v) of ethanolamine (98%). The glass dish containing the glass slides and the ZnO nanorods growth medium was placed on a hotplate with a controlled temperature at ~ 75°C. The ZnO nanorods growth process was allowed to about 1 h without any agitation. After that, the glass slides with ZnO nanorods were washed with ultra-pure water and dried under N₂ flow.

S3. SEM Image of ZnO Nanorods Fabricated on Glass Slides
Figure S3 SEM image of the ZnO nanorods taken at 45 °C angle.

S4. Study of Fluorescence Intensity Enhancement by ZnO Nanorods

We use Cy3-SA and fluorescent beads to study the fluorescence enhanced by the ZnO nanostructure glass surface compared to the flat bare glass. First, a small PDMS reservoir was fabricated on a ZnO nanostructured glass slide or bare glass slide. Then, 100 nm Fluoro-Max dyed green fluorescent particles (1.05 mg/mL, Thermal Scientific) or 2.5 μL of Cy3-SA (1 μg/mL) was added into the PDMS reservoir. After the solution was dried, the reservoirs were observed under a reflective fluorescence microscope (Olympus IX71), which was the same way we imaged the on-chip sandwich ELISA in ZnO-NIMs and conventional sandwich ELISA in multiwell plate. The results were shown in Figure S4. The fluorescence intensity of the fluorescence bead samples on the flat bare glasses and ZnO nanostructured surfaces are 1153.3 ±
126.7 and 1984 ± 745.7 (A.U.), respectively. Meanwhile, the fluorescence intensity of Cy3-SA dye on the flat bare glass and ZnO nanostructured surfaces were 4990 ± 432.0 and 6456 ± 591.2 (A.U.) respectively. Therefore, the fluorescence enhancement by the ZnO nanorods is calculated to be ~ 1.3-1.7 fold. It should be noticed that for this experiment we kept the same total number of fluorophores, thus purposely eliminated the effect of presumably larger effective area therefore more potential binding sites on the ZnO nanorod modified surface. The effect is purely optical. Also to mimic the sandwich ELISA on the device surface, we allowed the samples to dry so that the fluorophores are on the surface.

![Bar Chart](image)

**Figure S4** The fluorescence intensity enhancement effect by the ZnO nanorods (n = 3). A, Fluorescent beads on the glass slides; B, Fluorescent beads on the ZnO nanorods surfaces with the same concentration of the beads on A; C, Cy3-SA on the glass slides; D, Cy3-SA on the ZnO nanorods surfaces with the same concentration of the dye on C.

**S5. FR-IR Characterization of GMPTS Modified ZnO Slides**
Figure S5 FT-IR characterization of the ZnO nanorods on the glass after the GMPTS modification.

S6. Conventional Sandwich ELISA Detection of the H5N2 AIV

For the conventional sandwich ELISA experiments, 100 μL of 4 μg/mL H5 mAb was added into the 96 well plate (Greinier Bio-One) and incubated overnight at 4 °C. After that, the plate was washed with PBS plus 0.05% tween 20 (PBST). Then, 200 μL of the 1% BSA in the PBS was added into each well and incubated for 1.5 h to block the nonspecific adsorption on the plate surface. 100 μL of H5N2 AIV samples of different concentrations were added into the mAb-modified wells and incubated for another 1 h. The antibody was exactly the same mAb for H5N2 AIV as we used for the sandwich ELISA on ZnO-NIM. After washing with PBST, 100 μL of 10 μg/mL H5 mAb-biotin was added into each well and incubated for 1 h. At last, 100 μL of 10 μg/mL Cy3-SA was added into the wells and incubated for 45 min. After washing with PBST,
the fluorescence signal was recorded. The detection limit of this conventional ELISA assay for H5N2 AIV is determined as $\sim 8 \times 10^4$ EID$_{50}$/mL.

![Graph showing fluorescence intensity versus H5N2 AIV concentration](image)

**Figure S6** The fluorescence intensity versus the H5N2 AIV concentration in the conventional sandwich ELISA assay performed in multiwell plates.

**S7. Simultaneous Detection of Multiplexed Pathogens**

Two subtypes of AIV, H5N2 and H7N2, are simultaneously detected in a single ZnO-NIM. The H5 mAb and H7 mAb were loaded into ZnO-NIM from different branched channels. It should be noted that, the mAbs were continuously introduced into the ZnO-NIM from the corresponding branched channels at a very low flow rate of 3 μL/h in order to inhibit the undesired mixing of the two mAbs in the same branched channel. After the blocking and washing process, the H5N2 AIV, H7N2 AIV and the negative control samples were introduced into the ZnO-NIM from different branched channels sequentially. The concentration of the H5N2 AIV is $3.6 \times 10^6$ EID$_{50}$/mL, while the virus titer of the H7N2 AIV sample is 1:512 determined by the hemagglutination test. After washing, the mixture of the biotin labeled
detection H5 mAb-biotin and H7-mAb-biotin was loaded into the ZnO-NIM and reacted with the captured H5N2 AIV and H7N2 AIV in the different branched microchannels respectively. Then Cy3-SA was loaded and reacted with the H5-mAb-biotin and H7-mAb-biotin. The fluorescent images from different branched channels were recorded.

S8. Release of the Captured Virus by Dissolving the ZnO Nanorods under the Acidic pH Condition

To release captured H5N2 AIV in the ZnO-NIM, the device was perfused with slightly acidic PBS buffer whose pH was adjusted to 6.0. The process began with adding 50 μl pH adjusted PBS to each of the six inlet wells. The flow rate from the center outlet was 15 μl/min. The ZnO release by PBS took ~20 min. Then the device was rinsed three times. 24 μl PBS was added to each of the six inlet wells each time, and the flow rate from the center outlet was 60 μl/min.

After that, the solution was collected for the real-time reverse transcriptase PCR (rRT-PCR) assay to identify the virus. Meanwhile the microchannels were observed under microscope for the comparison of the surface morphology before and after virus release. As the negative control for the rRT-PCR, ZnO was dissolved with 1×PBS buffer (pH 6.0) from a ZnO-NIM with no virus captured.

The rRT-PCR for H5N2 AIV Matrix gene was conducted in a 25 μL reaction system by using one-step RT-PCR kit (QIAGEN, Valencia, CA) as described previously.[5] Briefly, the reaction mixture consisted of 5 μL of 5× reaction buffer, 1 μL of each primer (10 pmol/μL) and probe (5 pmol/μL), 0.8 μL enzyme mixture, 1 μL of dNTP mixture (10 mM each dNTP), 0.5 μL RNase inhibitor (40 U/μL) (New England Biolabs. Inc., MA, USA), 2 μL of RNA template and 13.7 μL of RNase-free water. The one-step rRT-PCR was performed with the 7300 Real-time
PCR System (Applied Biosystems, Foster City, CA, USA) and the thermal cycling profile for the rRT-PCR proceeded as follows: 50 °C for 30 min and 94 °C for 15 min followed by 45 cycles of denaturation at 95 °C for 10 s and annealing and elongation at 60 °C for 60 s. Fluorescence signals for each sample were collected at the end of each annealing and elongation step. The cycle threshold (Ct) values for each sample and standard curve were analyzed by the SDS software program (version 1.4).

S9. Comparison with Other Immunosensors for the Virus Detection in Literatures

References
Table S1 Summary of results reported on several immunosensors for the detection of the AIVs

| Detection method                                      | Analyte   | Detection limit | Detection range     | Detection time | Complex samples | Reference |
|--------------------------------------------------------|-----------|-----------------|---------------------|----------------|-----------------|-----------|
| Electrochemical immunoassay                            | H1N1      | 1 pg/mL         | $10^{-2}$-$10^{3}$ ng/mL | 4 h            | Not mention     | 6         |
| Colorimetric magnetomterialso sensor                   | H9N2      | 17.5 pg/mL      | 20 pg/mL-1ng/mL     | 1.5 h          | Chicken heart, dung and serum | 7         |
| Magnetic silica nanoparticles based resonance light scattering | AIV<sup>(a)</sup> | 0.15 ng/mL | 0.5-50 ng/mL | 80 min | Chicken serum | 8         |
| Electrochemical immunoassay                            | Avian leukosis viruses | 180 TCID<sub>50</sub>/mL<sup>(b)</sup> | $10^{2.22}$-$10^{5.58}$ TCID<sub>50</sub>/mL | Not given | Not mention | 9         |
| Antigen-competitive ELISA                              | IBV<sup>(c)</sup> | $10^{5.4}$ EID<sub>50</sub>/mL<sup>(d)</sup> | $10^{5.3}$-$10^{6.9}$ EID<sub>50</sub>/mL | Not given | Not mention | 10        |
| Surface plasmon resonance immunoassay                   | H6 AIV    | 5.14-10<sup>8</sup> EID<sub>50</sub>/0.1 mL | Not given | Not given | Not mention | 11        |
| Impedance measurement with RBC amplification           | H5N1      | $10^{5}$ EID<sub>50</sub>/mL | >$10^{6.8}$ EID<sub>50</sub>/mL | ~2 h           | Not mention    | 12        |
| Immunomagnetic microfluidic chip with RT-PCR amplification | H1N1      | ~10<sup>2</sup> TCID<sub>50</sub> | Not given | 3.5 h | Throat Swab Samples | 13        |
| Droplet Digital Loop-Mediated Isothermal Amplification | H5N1      | 10 copies/µL   | $10^{5}$-$10^{8}$ copies/µL | Not given | Allantoic fluid | 14        |
| Microfluidic immunomagnetic Fluorescence                | H9N2      | 3.7×10<sup>4</sup> copies/µL | 3.0-10<sup>5.1</sup>-10<sup>7</sup> copies/µL | 55 min | Chicken dung, liver, and lung | 15        |
| AIV Ag Test Kit                                        | H5 AIV<sup>(g)</sup> | $10^{5.5}$ EID<sub>50</sub>/mL or 1 HAU<sup>(f)</sup> | >$10^{5.5}$ EID<sub>50</sub>/mL or 1 HAU | ~30 min | Egg layer | 16        |
| ZnO-NIM chips Without amplification                    | H5N2      | $10^{5.56}$ EID<sub>50</sub>/mL | $10^{5.56}$-$10^{6.56}$ EID<sub>50</sub>/mL | 1.5 h | Allantoic fluid | This work |

(a) AIV: avian influenza virus; (b) TCID<sub>50</sub>: 50% tissue culture infective dose; (c) IBV: infectious bursal virus; (d) EID<sub>50</sub>:50% embryo infective dose; (e) Avian influenza type A subtype H5 virus (H5N1-1559); (f) HAU: Hemagglutinating unit

References

[1] A. D. Stroock, S. K. Dertinger, A. Ajdari, I. Mezic, H. A. Stone and G. M. Whitesides, *Science*, 2002, 295, 647-651.

[2] S. L. Stott, C. H. Hsu, D. I. Tsukrov, M. Yu, D. T. Miyamoto, B. A. Waltman, S. M. Rothenberg, A. M. Shah, M. E. Smas, G. K. Korir, F. P. Floyd, Jr., A. J. Gilman, J. B. Lord, D. Winokur, S. Springer, D. Irimia, S. Nagrath, L. V. Sequist, R. J. Lee, K. J. Isselbacher, S. Maheswaran, D. A. Haber and M. Toner, *Proc. Natl. Acad. Sci.*, 2010, 107, 18392-18397.

[3] D. C. Duffy, J. C. McDonald, O. J. Schueller and G. M. Whitesides, *Anal. Chem.*, 1998, 70, 4974-4984.
[4] S. Satyanarayana, R. N. Karnik and A. Majumdar, *J. Microelectromech. Syst.*, 2005, **14**, 392-399.

[5] E. Spackman, D. A. Senne, T. J. Myers, L. L. Bulaga, L. P. Garber, M. L. Perdue, K. Lohman, L. T. Daum and D. L. Suarez., *J. Clin. Microbiol.* 2002, **40**, 3256-3260.

[6] Y. Li, M. Hong, Y. Lin, Q. Bin, Z. Lin, Z. Cai and G. Chen, *Chem. Commun.* 2012, **48**, 6562-6564.

[7] C. H. Zhou, J. Y. Zhao, D. W. Pang and Z. L. Zhang, *Anal. Chem.*, 2014, **86**, 2752-2759.

[8] X. Zou, H. Huang, Y. Gao and X. Su, *Analyst*, 2012, **137**, 648-53.

[9] K. Shang, J. Zhu, X. Meng, Z. Cheng and S. Ai, *Biosens. Bioelectron.*, 2012, **37**, 107-111.

[10] R. V. Bronzoni, A. A. Pinto and H. J. Montassier, *Avian Pathol.*, 2001, **30**, 67-71.

[11] X. Zhao, Y. C. Tsao, F. J. Lee, W. H. Tsai, C. H. Wang, T. L. Chuang, M. S. Wu and C. W. Lin, *J. Virol. Methods*, 2016, **233**, 15-22.

[12] J. Lum, R. Wang, K. Lassiter, B. Srinivasan, D. Abi-Ghanem, L. Berghman, B. Hargis, S. Tung, H. Lu and Y. Li, *Biosens. Bioelectron.*, 2012, **38**, 67-73.

[13] B. S. Ferguson, S. F. Buchsbaum, T. T. Wu, K. Hsieh, Y. Xiao, R. Sun and H. T. Soh, *J. Am. Chem. Soc.*, 2011, **133**, 9129-9135.

[14] Y. Hu, P. Xu, J. Luo, H. He and W. Du, *Anal. Chem.* 2017, **89**, 745-750.

[15] R. Q. Zhang, S. L. Liu, W. Zhao, W. P. Zhang, X. Yu, Y. Li, A. J. Li, D. W. Pang and Z. L. Zhang, *Anal. Chem.*, 2013, **85**, 2645-2651.

[16] ANIGEN Rapid H5 AIV Ag Test Kit: http://www.gentaurpromo.com/animal_desease/files/AIVH5Brochure.pdf. (Commerical available)