

## Electronic Supplementary Information

### Plasmonic Gold Nanoparticles as Multifaceted Probe for Tissue Imaging

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## I. Chemicals and Materials

Optimal cutting temperature (OCT) compound used for cryosectioning was purchased from Sakura Finetek USA (CA, USA). Glass slides (24×30 mm, 0.13–0.16 mm thick) and the adhesive pen for frozen tissue sections attachment (Fro-Tissuer) were purchased from Ted Pella (Redding, USA). Piranha solution for glass cleaning was prepared by sulfuric acid (A.R. grade, 98%) purchased from RCI Labscan (Bangkok, Thailand) and 30% hydrogen peroxide (Ph Eur. Grade) purchased from VWR Chemicals (Radnor, PA, USA). Formaldehyde (for molecular biology, 36.5-38% in H<sub>2</sub>O) and glycine (ACS Reagent, ≥98.5%) were purchased from Sigma Aldrich (St. Louis, MO, USA) for pre-fixation and washing. Phosphate buffered saline (PBS) buffer solution was prepared using the Ultrapure, ACS Reagent grade Na<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, NaCl, KCl purchased from Affymetrix (Santa Clara, CA, USA). Tween-20 (Affymetrix) was mixed with PBS for the preparation of PBST (0.5% Tween 20) buffer solution. Bovine serum albumin (BSA) (≥ 98%) purchased from Sigma Aldrich and normal goat serum (NGS) purchased from Abcam (Cambridge, MA, USA) were used for blocking.

Anti-megalin antibody (1mg/mL, mouse monoclonal) and anti-podocin antibody (1mg/mL, rabbit monoclonal) used as the primary antibodies were purchased from Abcam. Anti-mouse conjugated 1.4nm AuNPs, anti-rabbit conjugated 1.4nm AuNPs, Au enhancement kit (GoldEnhance™ LM), and Ag enhancement kit (HQ Silver) were purchased from Nanoprobes (NY, USA). Anti-mouse conjugated 18nm AuNPs was purchased from Jackson ImmunoResearch Laboratories (PA, USA). Anti-mouse IgG and anti-rabbit IgG (2mg/mL, goat polyclonal) purchased from Abcam and N-hydroxysuccinimide AuNPs (NHS-AuNPs, 20nm) purchased from Cytodiagnostics (Ontario, Canada) were used for the preparation of anti-mouse conjugated 20nm AuNPs and anti-rabbit conjugated 20nm AuNPs. Anti-mouse IgG conjugated Alexa Fluor® 488 (2mg/mL, goat polyclonal, preadsorbed) and Anti-rabbit IgG conjugated Alexa Fluor® 647 (2mg/mL, goat polyclonal, preadsorbed) were purchased from Abcam.

For other chemicals and solvents, sodium thiosulfate was purchase from Sigma Aldrich. Absolute ethanol (ACS, Reag. Ph Eur. Grade) and HPLC grade water were purchased from Scharlau (Barcelona, Spain).

## II. Experimental Procedures

### **Chromogenic, Electron Microscopic Analysis, and Imaging Mass Spectrometric Analysis**

***Tissue Preparation.*** Experiments involving the uses of animals and tissue collections were performed in compliance of Animals (Control of Experiments) Ordinance of Hong Kong and approved by the Committee on the Use of Live Animals for Teaching and Research, The University of Hong Kong. A male Sprague Dawley rat kidney was embedded in optimal cutting temperature (OCT) compound. The kidney was cut into section slices (thickness: 12  $\mu\text{m}$ ) with a cryostat (FE/FSE, Thermo Fisher Scientific, Inc. Waltham, MA, USA) at chamber temperature  $-20^{\circ}\text{C}$ . Using an adhesive pen, the kidney tissue sections were adhered onto Piranha cleaned glass slides. The kidney tissue section was fixed with 4% formaldehyde for 8 minutes then quenched with 50mM glycine. After washing with PBS, the tissue section was blocked using 10% normal goat serum (NGS) in 1% BSA-PBST.

***Antibody Incubation.*** For primary antibody incubation, the tissue was either incubated with anti-megalin antibody (mouse monoclonal, 1 : 200) or anti-podocin antibody (rabbit monoclonal, 1 : 200) in 1% BSA-PBST at  $4^{\circ}\text{C}$  for overnight to maximize the antigen labeling. After washing with PBST and 1% BSA-PBST to remove excess primary antibodies, the tissue was incubated with corresponding secondary antibody-conjugated AuNPs, either anti-mouse conjugated AuNPs (1.4nm AuNP, 1 : 100, Nanoprobes) or anti-rabbit conjugated AuNPs (1.4nm AuNP, 1 : 100, Nanoprobes) in 1% NGS in 1% BSA-PBST at room temperature for 1 hour. The labeled tissue was then washed with 1% BSA-PBST, PBST, and then PBS. For the preparation of control rat kidney tissue section, the primary antibody solution was replaced by pure 1% BSA-PBST during primary antibody incubation. Then, anti-mouse conjugated AuNPs (1.4nm AuNP, 1 : 100, Nanoprobes) was used as secondary antibody.

***On-Tissue Au Enhancement.*** On-tissue Au enhancement was performed using GoldEnhance<sup>TM</sup> LM (Nanoprobes) to enlarge the size of AuNPs. Following the protocol suggested by Nanoprobes, the Au enhancement solution was prepared and applied onto the kidney tissue sections. After 9 minutes, the enhancement reaction was quenched with 1% sodium thiosulfate. After washing with PBST, PBS, and water, the tissue was dehydrated using 30%, 70%, 80%, 95%, and 100% ethanol, sequentially.

### **Plasmon-Enhanced Fluorescence Analysis**

***Preparation of anti-mouse and anti-rabbit conjugated AuNPs.*** Anti-mouse IgG (goat polyclonal) and anti-rabbit IgG (goat polyclonal) were conjugated to NHS-AuNPs (20nm) purchased from Cytodiagnosics. Following the suggested protocol, 40 $\mu\text{L}$  of 1mg/mL antibodies were diluted to 90 $\mu\text{L}$  using the provided reaction buffer solution. The diluted antibody was then added to lyophilized NHS-AuNPs for conjugation and reacted for 3 hours at room temperature. 10 $\mu\text{L}$  quencher solution (Tris buffer) was added to quench any unreacted NHS moieties. To remove unreacted proteins and the quenching reagents, antibody-conjugated AuNPs were centrifuged at 5500g and washed with 1% BSA-PBST. The antibody-conjugated AuNPs were resuspended in 1% BSA-PBST and stored at  $4^{\circ}\text{C}$ .

***Antibody Incubation.*** For primary antibody incubation, the tissue was simultaneously incubated with anti-megalin antibody (mouse monoclonal, 1:200) and anti-podocin antibody (rabbit monoclonal, 1:200) in 1% BSA-PBST at  $4^{\circ}\text{C}$  for overnight to maximize the antigen labeling. After washing with PBST and 1% BSA-PBST to remove excess primary antibodies, the tissue was incubated with a mixture of 4 secondary antibody-conjugated AuNPs and fluorophores, including

- (1) anti-mouse conjugated AuNPs (20nm AuNP) with dilution factor of 1 : 120 – 1200, corresponding to  $10.9\text{E}+10 - 1.1\text{E}+10$  AuNPs/mL,
- (2) anti-rabbit conjugated AuNPs (20nm AuNP) with dilution factor of 1 : 120 – 1200, corresponding to  $10.9\text{E}+10 - 1.1\text{E}+10$  AuNPs/mL,
- (3) anti-mouse IgG conjugated Alexa Fluor® 488 (1 : 1000)
- (4) anti-rabbit IgG conjugated Alexa Fluor® 647 (1 : 1000)

in 1% NGS in 1% BSA-PBST at room temperature for 1 hour. The labeled tissue was then washed with 1% BSA-PBST, PBST, PBS, and water. The tissue was then dehydrated using 30%, 70%, 80%, 95%, and 100% ethanol, sequentially.

### **Duplex Imaging Mass Spectrometric Analysis**

***Antibody Incubation for Labeling Podocin.*** For primary antibody incubation, the tissue was simultaneously incubated with anti-megalin antibody (mouse monoclonal, 1 : 200) and anti-podocin antibody (rabbit monoclonal, 1 : 200) in 1% BSA-PBST at 4°C for overnight to maximize the antigen labeling. After washing with PBST and 1% BSA-PBST to remove excess primary antibodies, the tissue was incubated with anti-rabbit conjugated AuNPs (1.4nm AuNP, 1 : 100, Nanoprobes) in 1% NGS in 1% BSA-PBST at room temperature for 1 hour for the recognition of anti-podocin antibody. The labeled tissue was then washed with 1% BSA-PBST, PBST, and then PBS.

***On-Tissue Ag Enhancement.*** On-tissue Ag enhancement was then performed using HQ Silver (Nanoprobes) to convert the 1.4nm AuNP seeds to large AgNPs. Following the protocol suggested by Nanoprobes, the Ag enhancement solution was prepared and applied onto the kidney tissue sections. After 7 minutes, the enhancement reaction was quenched with 1% sodium thiosulfate and the tissue was then washed thoroughly with PBST and 1% BSA-PBST.

***Labeling of Megalin after Ag Enhancement.*** For the recognition of anti-megalin antibody, the tissue was incubated with anti-mouse conjugated AuNPs (18nm AuNP, 1 : 10, Jackson ImmunoResearch) in 1% NGS in 1% BSA-PBST at room temperature for 1 hour. The labeled tissue was then washed with 1% BSA-PBST, PBST, PBS, and water. The tissue was dehydrated using 30%, 70%, 80%, 95%, and 100% ethanol, sequentially.

### **Data Acquisition and Data Processing**

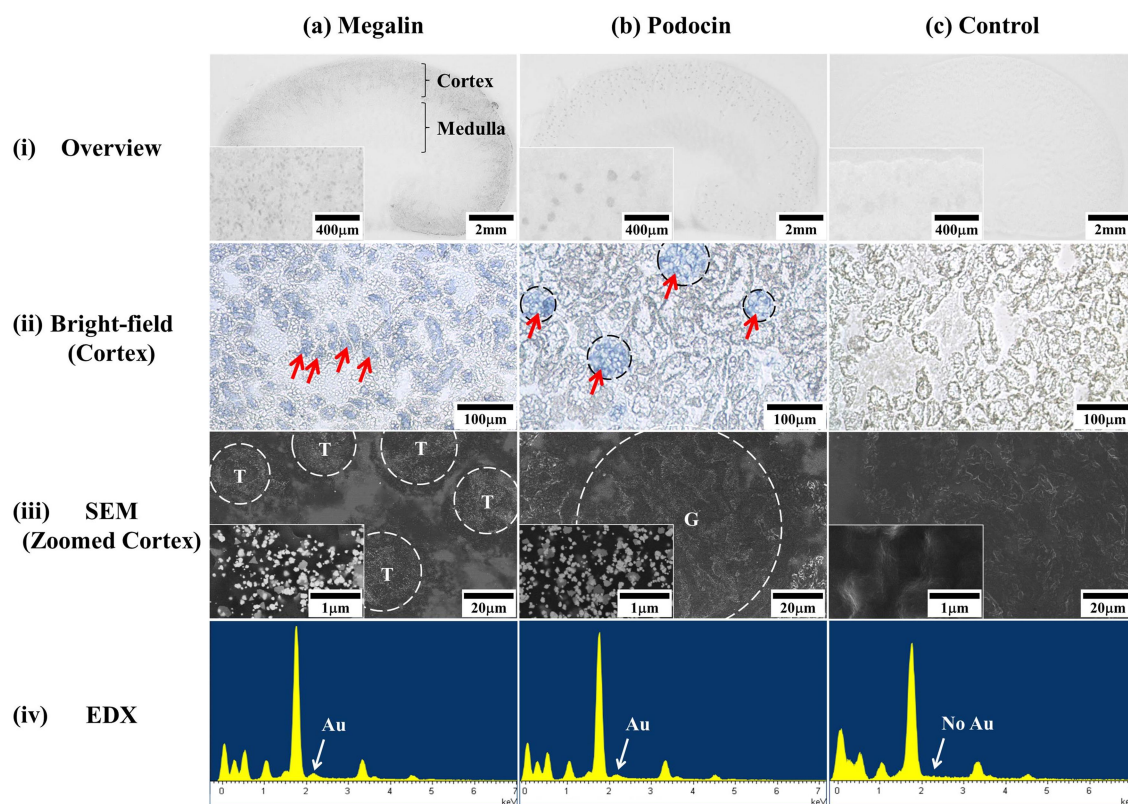
***Optical Microscopy Measurements.*** The photographs of the kidney tissue sections were recorded using a digital camera with a macro lens (EOS 550D with EF 100 mm f/2.8 Macro USM, Canon, Japan). All the bright field optical micrographs of tissue sections were recorded using an inverted light microscope (Eclipse TE2000-U; Nikon, Japan) for locating the proximal convoluted tubules and glomeruli on the cortex region of the kidney tissue sections.

***Electron Microscopy Measurements.*** All the scanning electron microscopic (SEM) measurements were performed with Hitachi S-4800 FEG (Hitachi High-Technologies, Japan). The tissues were examined at operating voltage 5kV. For improving the electrical conductivity, a thin layer of carbon was coated on the tissue surface using a coater (SCD 005; Bal-Tec AG, Liechtenstein) before analysis.

**Fluorescence Image Measurements and Data Processing.** All the fluorescence micrographs were recorded using confocal laser scanning microscope (Carl Zeiss LSM710 NLO, Zeiss, NY, USA) with an objective EC Plan-Neofluar 10x (0.3 Ph1 M27). The instrument was controlled via the software ZEN 2010 (Zeiss, NY, USA). The frame scan mode was used with a frame size of  $1024 \times 1024$ , bidirectional scanning with a scanning speed of 7, line average of 4, and 16-bit depth. The pinhole size was adjusted to 1 Airy unit. For a fair comparison of fluorescence intensities, the gain (master), the digital offset, and the digital gain were kept at 800, 0, and 1.0 respectively for all the measurements. The fluorescence of Alexa Fluor 488 was captured using 488 nm excitation by Argon laser with 493–630 nm detection, while the fluorescence of Alexa Fluor 647 was captured using 633 nm excitation by HeNe laser with 638–755 nm detection. The fluorescence intensity of both Alexa Fluor 488 and Alexa Fluor 647 were extracted using the software ZEN 2010.

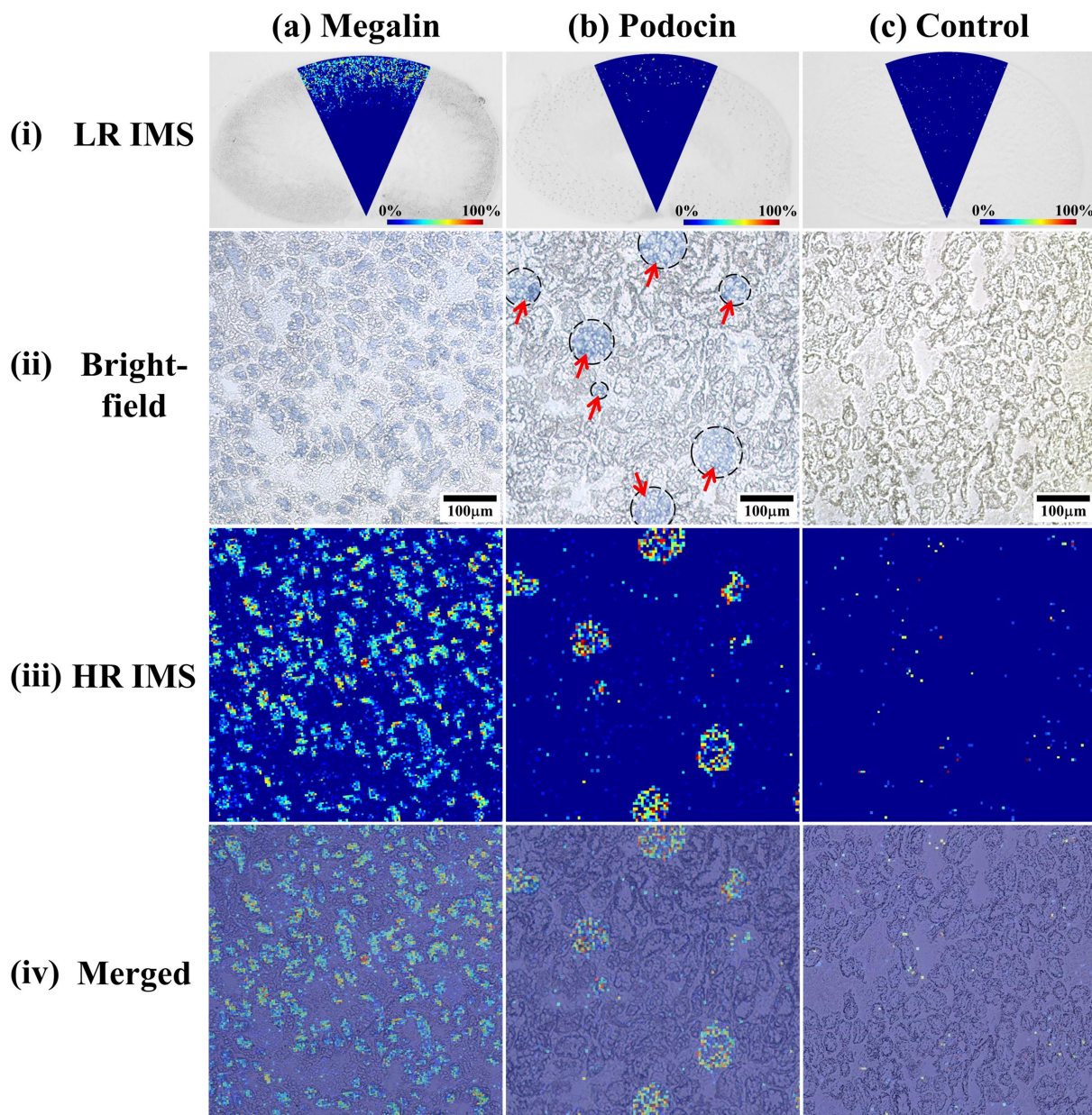
**Mass Spectrometric Measurements and Data Processing.** All the mass spectrometric measurements were performed with UltrafleXtreme MALDI-TOF/TOF mass spectrometer equipped with a 355nm solid state smartbeam Nd:YAG laser (Bruker, Bremen, Germany). The tissue section slides were mounted onto the standard stainless steel MALDI plate using electrically conductive double-sided tape (9713 XYZ-Axis, 3M, St. Paul, MN, USA). Reflectron mode was adopted to acquire high quality IMS images. The instrumental parameters used are as follows: ion source 1 at 20.3kV; ion source 2 at 16.7kV; lens voltage at 7.92kV; reflector 1 at 21.1kV; reflector 2 at 10.65kV; delay time at 10ns; laser shot number at each raster position, 10; laser shot frequency, 50Hz; acquired mass range,  $m/z$  5 – 700; raster width, 50 $\mu$ m for low resolution imaging and 5 $\mu$ m for high resolution imaging. The vacuum pressure was kept at around  $10^{-6}$  –  $10^{-7}$  mbar in the source and  $10^{-7}$  –  $10^{-8}$  mbar in the analyzer. The instrument was controlled via the Bruker Daltonics flexControl 3.4 software (Bruker). Imaging mass spectrometry data were recorded with the aid of using flexImaging 4.1 software (Bruker). In the duplex analysis, the reporter gold ion at  $m/z$  197 was used for tracing megalin and silver ion at  $m/z$  107 was used for tracing podocin. The IMS images were constructed using SCiLS Lab 2016a 4.0.0 software (SCiLS Lab, Bremen, Germany).

### III. Supplementary Figures



**Fig. S1** Images showing the (a) megalin labeled, (b) podocin labeled and (c) control (without labeling), rat kidney tissue sections prepared using antibody-conjugated AuNPs with on-tissue Au enhancement. (i) The overview photographic images showing both megalin and podocin are located in the cortex region. (ii) The bright-field optical micrographs of the cortex region showing that megalin located at the proximal convoluted tubules and the podocin at the glomeruli were stained blue (indicated by red arrows). (iii) Scanning electron micrographs of the cortex region. The tubules are labeled as T and the glomerulus is labeled as G. Enhanced AuNPs are shown in the insets. The enlarged SEM figures were shown in Fig. S4. (iv) Energy-dispersive X-ray spectroscopy (EDX) showing the presence of Au in (a) megalin labeled and (b) podocin labeled rat kidney tissue sections but absent in (c) control rat kidney tissue section.





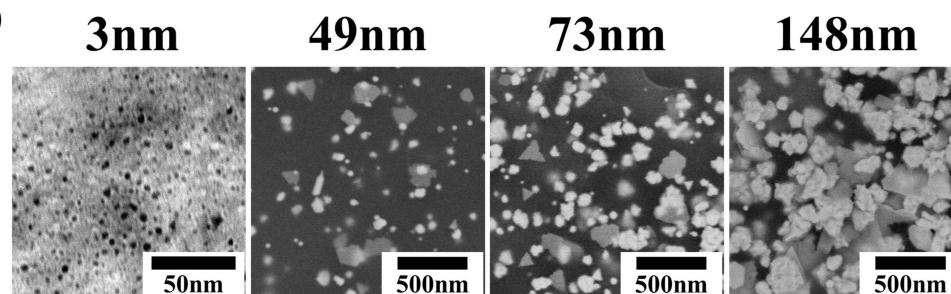
**Fig. S2** IMS results showing (a) megalin labeled, (b) podocin labeled and (c) control (without labeling), rat kidney tissue sections prepared using antibody-conjugated AuNPs with on-tissue Au enhancement. By (i) low resolution IMS, antigens are observed at the cortex region which is coherent with the results from the overview photographic images. Both of the (ii) bright-field optical micrographs at the cortex region and the (iii) high resolution IMS (HR-IMS) images of the same area have good coherence. (iv) Merged images are prepared by overlapping the bright-field image and the HR IMS image for easy correlation of the histological features.

(a)

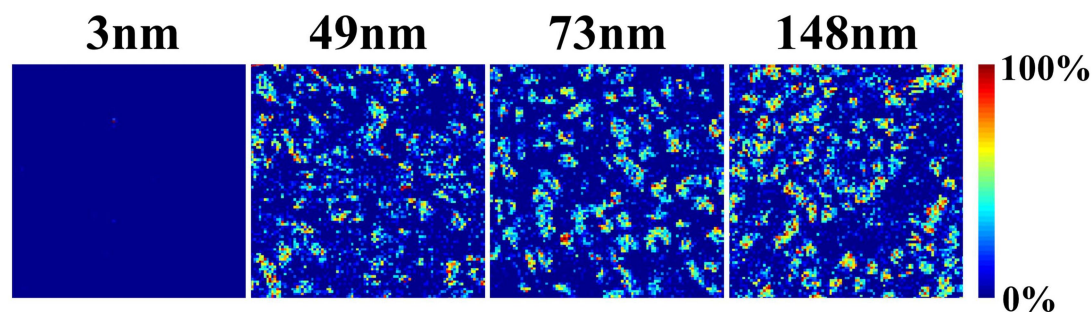
(i)

Source of AuNPs	Size Measured Using SEM/ TEM
1.4nm (Purchased from Nanoprobes)	$3 \pm 2$ nm (TEM)
1.4nm with 4 minutes Au Enhancement	$49 \pm 20$ nm (SEM)
1.4nm with 9 minutes Au Enhancement	$73 \pm 41$ nm (SEM)
1.4nm with 18 minutes Au Enhancement	$148 \pm 62$ nm (SEM)

(ii)

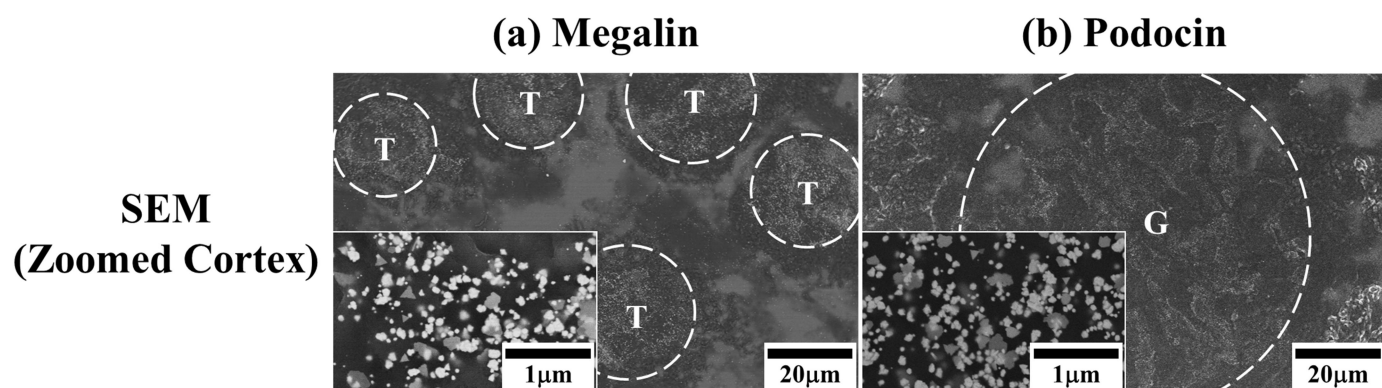


(b)

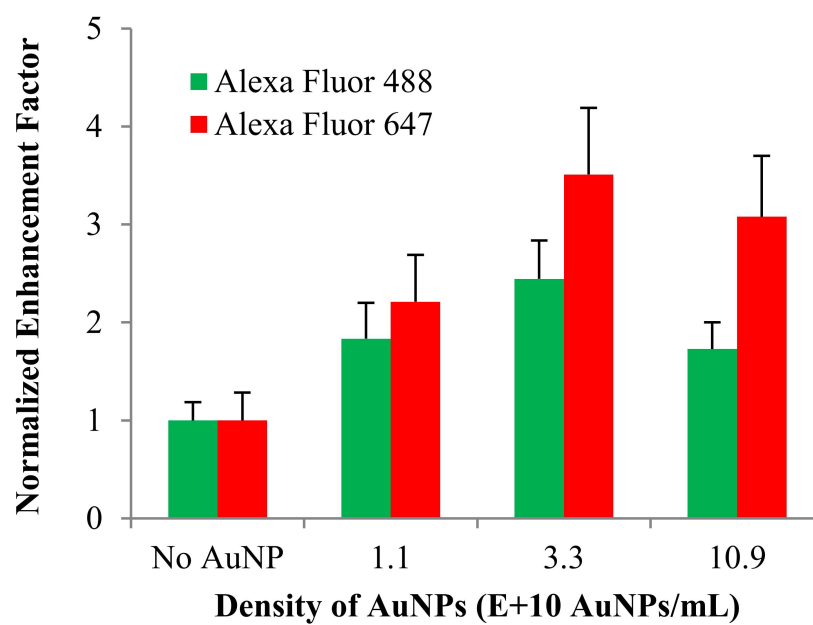


**Fig. S3** (a-i) The size distribution of the AuNPs after 0 – 18 mins gold enhancement and (a-ii) the corresponding TEM and SEM micrographs. The anti-mouse conjugated 1.4nm AuNPs brought from Nanoprobes was characterized to be 3nm. After 4 minutes, 9 minutes, and 18 minutes gold enhancement, the AuNPs were growth to 49nm, 73nm, and 148nm respectively. (b) High resolution IMS images were obtained using a wide range of AuNP sizes from 3nm to 148nm, corresponding to 0 – 18 minutes Au enhancement.





**Fig. S4** Enlarged scanning electron micrographs of the cortex region showing (a) megalin located at the proximal convoluted tubules and (b) podocin at the glomeruli. The tubules are labeled as T and the glomerulus is labeled as G. Enhanced AuNPs are shown in the insets.



**Fig. S5** Bar chart showing the normalized enhancement factor of Alexa Fluor 488 (green) and Alexa Fluor 647 (red) by different concentration of antibody-conjugated AuNPs.