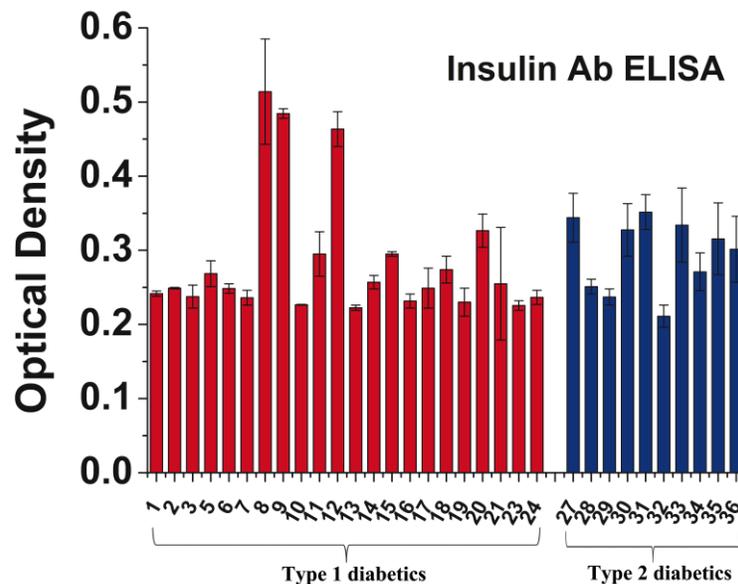
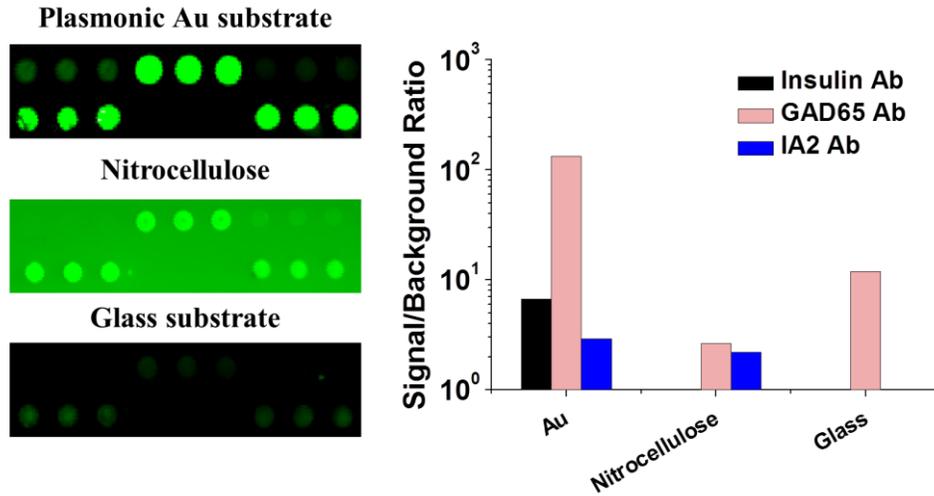


A Plasmonic-plasmonic Chip-chip for Biomarker biomarker Discovery discovery and Diagnosis diagnosis of Typetype-1 Ddiabetes

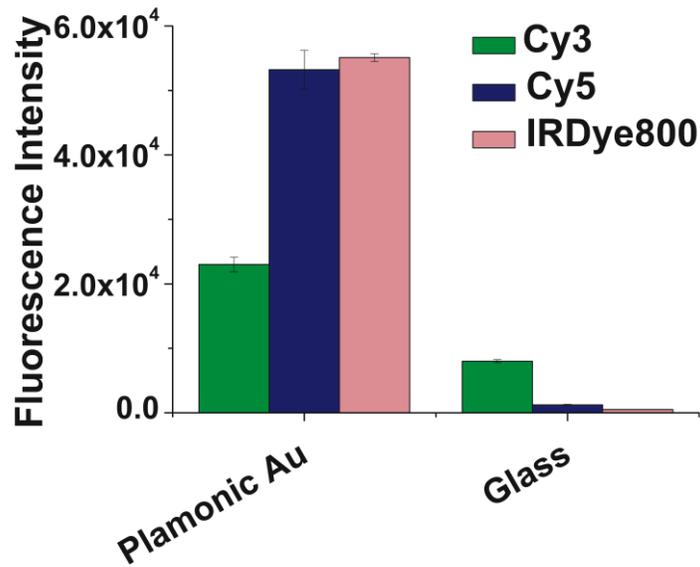
Bo Zhang, Rajiv B- Kumar, Hongjie Dai & Brian J- Feldman



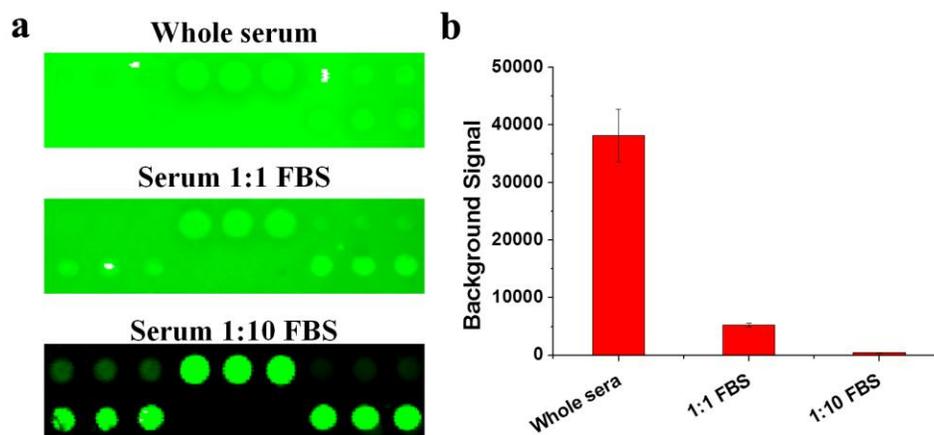
Supplemental Figure 1. ELISA assay for insulin autoantibody quantification. Serum samples from 22 T1D and 10 T2D patients were tested for insulin autoantibody against recombinant human insulin by direct ELISA on a 96-well microtiter plate. Serum was diluted 1:10 and 100 μ L were applied to each well. Rabbit-anti-human-IgG conjugated with HRP was used as the detection antibody, and following addition of tetramethylbenzidine, and subsequently 2M H_2SO_4 to stop the substrate reaction, optical density (450 nm) was quantified with a plate reader. No consistent difference was detectable between the two groups using this platform.



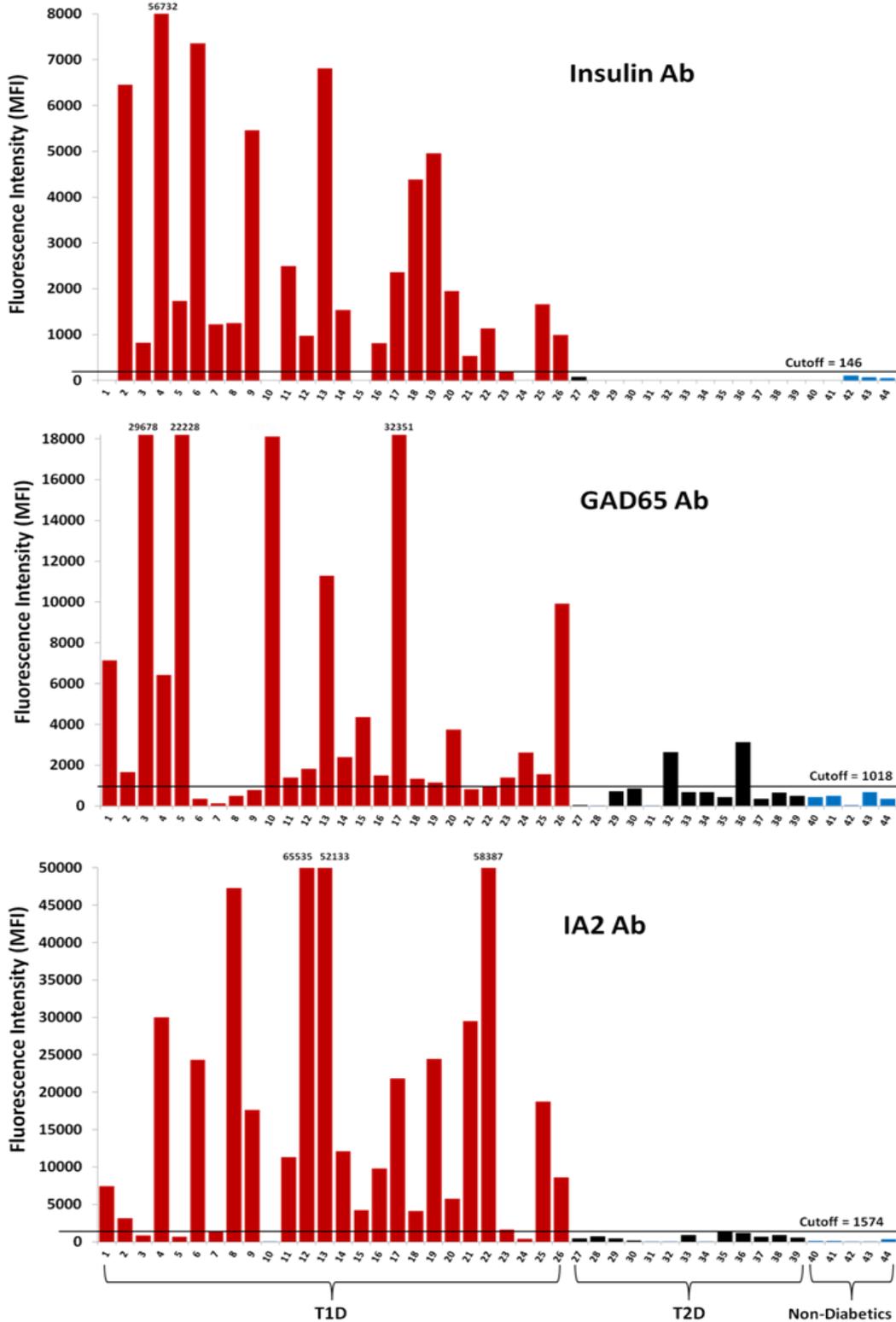
Supplemental Figure 2. Islet antigen microarray performance on plasmonic chip, nitrocellulose substrate and glass substrate. Fluorescence mapping results (left) and signal/background ratios (right) obtained with an example patient's serum sample on the three different substrates, showing that insulin autoantibodies were only detectable on the plasmonic platform. Also, the NIR fluorescence signal-to-noise ratios for the detection of GAD65 and IA2 autoantibodies were far superior on the gold plasmonic platform compared to glass and nitrocellulose.



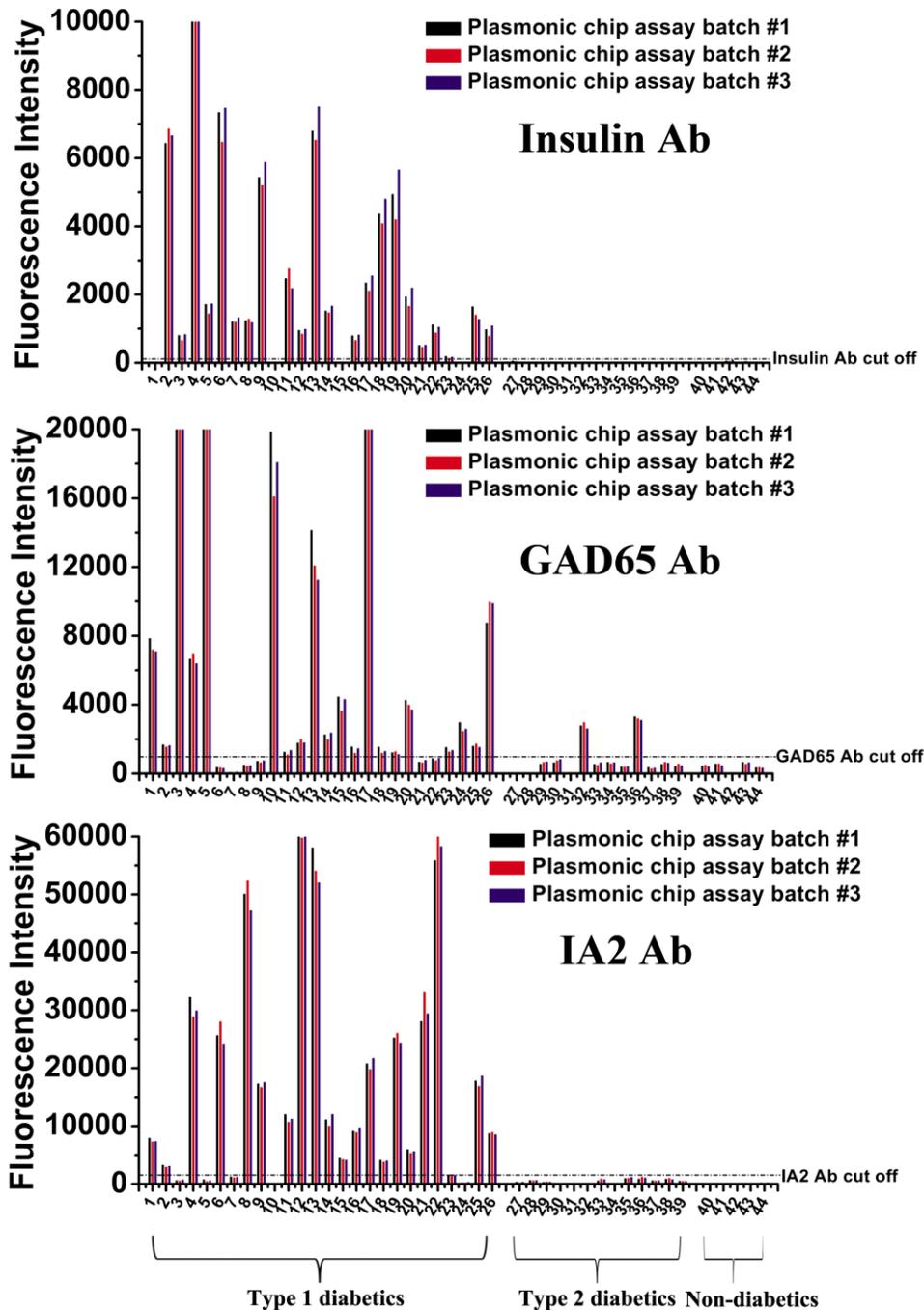
Supplemental Figure 3. Fluorescence enhancement of Cy3, Cy5 and IRDye800 on plasmonic gold substrates. Mean fluorescence intensity of Cy3/Cy5/IRDye800 for monolayer of avidin conjugated Cy3/Cy5/IRDye800 on plasmonic gold (Au) compared to glass substrate. Fluorescence was measured with GenePix4000B for Cy3 and Cy5 fluorescence and a Li-Cor Odyssey scanner for the IRDye800 fluorescence. A ~3, ~50 and ~100 fold enhancement was observed for Cy3, Cy5 and IRDye800 respectively on the plasmonic gold compared to glass substrate.



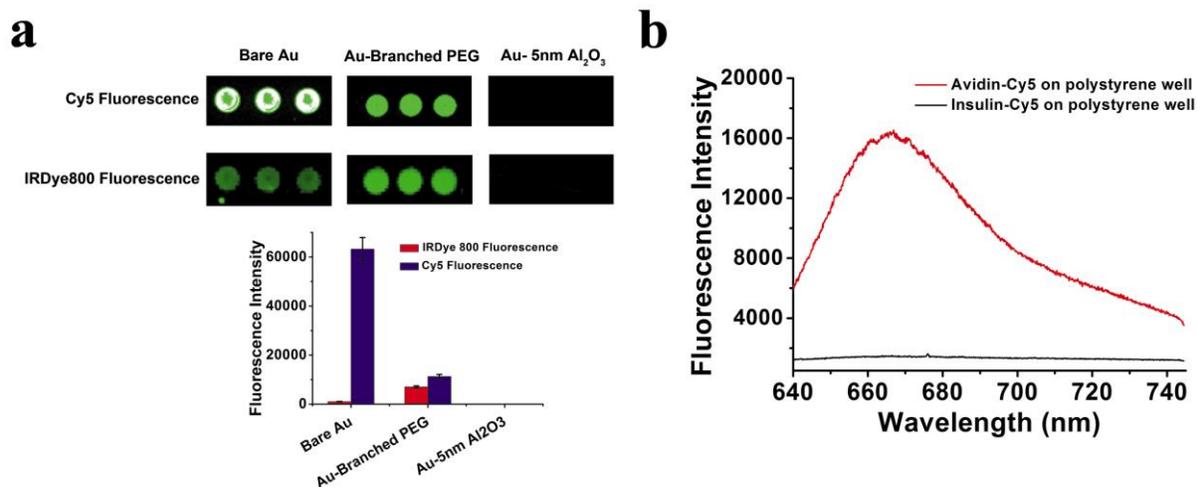
Supplemental Figure 4. Optimization of human serum incubation condition. Fluorescence images (a) and background signal plot (b) demonstrate that, compared to whole serum incubation or 1:1 dilution in FBS, 1:10 dilution of human serum in whole FBS improves signal/background ratio of the microarray by vastly decreasing background caused by non-specific binding (NSB).



Supplemental Figure 5. Quantification of plasmonic gold signal for three autoantibodies for every patient. Diabetes autoantibody titers were measured and compared in 26 patients with T1D (red bars), 13 patients with T2D (black bars), and 5 non-diabetic volunteers (blue bars).



Supplemental Figure 6. Reproducibility of the plasmonic gold chip platform for detecting islet autoantibodies. Islet autoantibodies were measured in every diabetic patient in three independent experiments (black/red/blue bars for each patient) using three different chips made by the identical reaction steps and conditions. The quantified IRDye800 signal for each islet antigen was highly reproducible across the independent experiments.



Supplemental Figure 7. Immobilization of insulin on gold plasmonic substrates treated with different surface chemistry and polystyrene 96 well plate. a) Top panel: Cy5 fluorescence images and bottom panel: IR800 signal quantification for detecting insulin autoantibodies in the serum of a T1D patient. Cy5-labeled insulin was spotted onto plasmonic gold substrate (through microarray printing, see online method) with different surface modifications as indicated (bare, branched-PEG coating and aluminum oxide coating respectively) followed by incubation with serum from T1D patient known to have insulin autoantibody and subsequent detection with IRDye800 labeled anti human IgG secondary antibody. The fluorescence intensity of Cy5 reflects the amount of insulin immobilized on the plasmonic substrate while the fluorescence intensity of IRDye800 reflects the amount of insulin autoantibody captured on the insulin spot. While more insulin was immobilized on bare Au compared to on Au-Branched PEG, fewer autoantibodies were detected. This suggests that insulin proteins on bare gold are less well recognized by autoantibodies than insulin on a PEG cushion layer on gold because of changes in the protein tertiary structure. No insulin is immobilized on the Au – 5nm Al₂O₃ surface after incubation in human serum and secondary antibody solution, suggesting weak binding of insulin to aluminum oxide resulting in loss of protein by solution rinsing. **b)** Cy5 fluorescence on a typical polystyrene 96 well plate after incubation with Cy5 labeled avidin and Cy5 labeled insulin solutions respectively. The avidin-Cy5 binds to the well but the insulin-Cy5 does not, indicating that insulin immobilization in ELISA wells does not work by standard adsorption process. This could contribute to difficulties in detecting insulin autoantibodies by standard ELISA approaches.

Substrate	Sensitivity	Specificity
Plasmonic chip	84.6%	100%
Nitrocellulose	57.7%	100%
Glass	38.4%	100%

Supplemental Table 1. Sensitivity and specificity result for insulin autoantibody detection of T1D on the three substrates.

	RIA	Plasmonic Gold Chip
Time to complete assay	1-2 days for each autoantibody (Insulin, GAD65, or IA2)	Less than 2 hours for all three autoantibody (Insulin, GAD65 and IA2)
Able to multiplex	No	Yes
Sample size	> 1 ml	2 μ l
Reagent requirements	Iodine-125 labeled insulin, Iodine-125 labeled GAD65 and Iodine-125 labeled IA2 (25 μ L each) Anti-human antibody (100 μ L, 1.5 μ g/ml)	Insulin, GAD65 and IA2 (30 nL each, 0.1mg/ml) IRDye800 labeled anti human antibody (20 μ L, 0.15 μ g/ml)
Labor	Handling solutions containing radioactive materials, washing steps, centrifuge steps	Solutions with no biohazard, applying solution to microarray surface, washing step
Biosafety	Radioactive materials	No biohazard
Major Equipment	Gamma scintillation counter calibrated for I ¹²⁵ Temperature control (4C)	Scanner
Sensitivity (T1D vs T2D Cohort)	100%	100%
Specificity (T1D vs T2D Cohort)	85%	85%

Supplemental Table 2. Comparison of RIA and Gold Plasmonic platforms.

	T1D, n = 26	T2D, n=13	Non-Diabetic, n=5
Gender (M or F)	14 M, 12 F	8 M, 5 F	3M, 2F
Age (y)	9.7 ± 3.8 (1.9-19.5)	14.1 ± 2.4 (10.2-18.2)	8.0 ± 3.3 (3.9-13.2)
Body Mass Index (kg/m ²)	18.0 ± 4.6 (12.0-29.9)	31.6 ± 9.2 (18.7-46.7)	19.1 ± 2.6 (15.3-22.1)
Serum Bicarbonate level (mEq/L)	16.0 ± 7.2 (<5-30)	24.5 ± 5.2 (12-32)	N.D.
Hemoglobin A1c (%)	11.3 ± 2.2 (6.7- >15)	10.0 ± 2.9 (6.6- >15)	N.D.

Serum Bicarbonate <5 meq/L = 5 meq/L; Hemoglobin A1c >15% = 15%

Values reported as sample mean ± S.D. (absolute range); N.D. = not done

Supplemental Table 3. Comparison of the T1D, T2D and non-diabetic subject groups.

Supplemental Methods

Materials

Superfrost Plus glass slides were purchased from Fisher Scientific and rinsed with acetone, isopropanol (IPA) and methanol prior to use. Two-pad and Sixteen-pad Whatman FAST nitrocellulose slides, chloroauric acid trihydrate, hydroxylamine HCl, sodium borohydride, cysteamine, mercaptohexadecanoic acid, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) were purchased from Sigma–Aldrich. Ammonium hydroxide (30% ammonia) and Hyclone fetal bovine serum were purchased from Fisher Chemicals. IR800cw-NHS ester was purchased from Licor Biosciences. 6-armed poly(ethylene glycol)–amine was purchased from SunBio. Fetal bovine serum was purchased from Invitrogen.

Recombinant human insulin was purchased from Lilly, GAD65 and IA2 (ICA512) antigens were purchased from Kronus and tetanus toxoid antigen was purchased from Santa Cruz Biotech. Goat anti-human IgG antibody and human IgG were purchased from Vector Lab.

Human serum

Institutional Review Board approval for this study was obtained from Stanford University. Once parental consent and subject assent (when appropriate) were obtained, 2.5 mL of blood was drawn in a red-top venipuncture tube (silicon coated glass) and 2.5 mL in a lavender-top venipuncture tube (liquid K₂EDTA in glass). Samples were stored at 4°C until processing. For processing, the 2.5 mL of whole blood collected in the lavender-top tube was divided into 125 µL aliquots in cryovials and stored at -80°C. The 2.5 mL collected in the red-top tube was centrifuged at 300 rpm for 15 min. The serum supernatant was then divided into 75 µL aliquots in cryovials and stored at -80°C. Samples were subsequently thawed and assessed in parallel.

Clinical diagnosis and monitoring of T2D patients

All patients were monitored regularly for at least six months post diagnosis. Ten of the 13 patients categorized as T2D were transitioned completely off of insulin for over one month without developing ketosis or DKA and were able to maintain good blood glucose control with diet and exercise or diet, exercise and metformin. The other three patients had prolonged periods of insulin omission without ketosis and all three of these patients tested negative for autoantibodies on both platforms. One of these three patients tested positive for a novel mutation in the HNF4a gene (that has not been reported to be associated with MODY).

ELISA assay procedure

96-well microtiter plates (BD Falcon) were coated with 100 µg recombinant human insulin (Lilly). The antigen was incubated at 4°C overnight followed by washing with PBST and then blocked overnight at 4°C with 5% non-fat milk. 100 µL of 1:10 diluted serum for each subject was dispensed into the wells, and incubated overnight at 4°C. Samples were incubated with a 1:5000 dilution of rabbit-anti-human-IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology) for 2 hrs and then washed several times. Following plate washing, 50 µL of 3,3',5,5'-tetramethylbenzidine (Sigma) was added to each well for 30 min, and then an equal

volume of 2M H₂SO₄ (Sigma) was added to stop the substrate reaction. The optical density (450 nm) of each well was quantified using a plate reader (BioTek Synergy H1).

Preparation of evaporated gold film on glass slides

Evaporated gold films were deposited via e-beam evaporation on an Innotec ES26C E-Beam Evaporator. The vacuum pressure of the system prior to deposition was 5e-7 Torr. The e-beam parameters were 10 kV at ~.1 A which resulted in a deposition rate of roughly 2 Å/s.

Preparation of plasmonic gold film on glass slides

Glass slides were immersed in 3 mM HAuCl₄ followed by adding ammonium hydroxide at 20 μL ammonium hydroxide per mL of HAuCl₄ solution with rapid shaking for 1 minute. The slide was washed twice with deionized (DI) water to remove unbound gold ions and immersed into 1 mM NaBH₄ to reduce gold clusters on the glass slide to gold nanoparticle seeds. After further washing twice with water, the slides were incubated in a solution of HAuCl₄ and hydroxylamine at a 1:1 ratio and shaken for 5 min, followed by a 10 min incubation to complete the growth step. After washing with water twice and drying, the slide was checked for plasmon resonance by using a Cary 300UV–Vis–NIR absorbance spectrometer after correcting for background absorbance from the glass substrate. Scanning electron micrographs were acquired on an FEI XL30 Sirion SEM with FEG source at 5 kV acceleration voltage.

Construction of multilayer surface chemistry on gold film

Gold slides were immersed into 10 mM mercaptohexadecanoic acid in ethanol overnight at room temperature. After rinsing with ethanol and drying, the carboxylic group functionalized gold slide was immersed in a solution of 20 μM 6-arm poly (ethylene glycol)-amine (*M_n* ~10,000 Da) and 20 mM each of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and NHS in DMF. After rinsing the gold slide with dimethylformamide (DMF), ethanol and drying, the substrate was immersed in 10 mM succinic anhydride DMF solution with triethylamine at 1 μL per mL. This step transforms the free amine groups on the poly(ethylene glycol) chain into carboxylic groups. Following another washing step with DMF and ethanol, the slide was incubated in 20 mM each of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and NHS in DMF to activate the carboxylic group.

Microarray printing and microarray sandwich assay procedure

The NHS-activated gold slides above (or nitrocellulose slides, glass slides, NHS-activated evaporated gold slides) were loaded into a microarray printing robot (Bio-rad VersArray Chipwriter) where 3 μM antigens in PBS were printed using solid pins at 25 °C and 60% humidity, resulting in microarray feature diameters of ~400 μm. The slides were dried in a desiccator and then blocked in phosphate buffered saline–Tween 20 (PBST) solution containing 5% fetal bovine serum. Human serum was diluted into FBS solution (1:10), and 20 μL of each solution was applied to each set of spots, and incubated (shaking) for 1 hr at room temperature, followed by washing twice with PBST and once with PBS. The array was then incubated in 1 nM IR800 conjugated goat anti-human IgG in FBS for 20 min (shaking) at room temperature in the dark. Chips were washed twice with PBST and once with PBS, followed by immersion in DI

water and subsequent drying with compressed air. The total processing time to test the human samples for autoantibodies using the plasmonic gold chips was less than 2 hrs.

Fluorescence measurement and analysis

A Licor Odyssey scanner was used to scan the IRdye800–secondary antibody labeled microarrays on different substrates using the 800 nm channel with the gain set to 6.0 as defined by the system, and the resolution set to 42 μm . Microarray fluorescence images were analyzed by Genepix 6.1, and the spot features were automatically identified by the program. The fluorescence intensity of each spot was background corrected, and the average of mean pixel intensity values for features printed in duplicates. Note that the background signal was typically on the order of 10, while the spot signals were in the range of 10-100,000 for various autoantibody concentrations.

Calibration curves for insulin Ab, GAD Ab and IA2 Ab

For generating the calibration curves of each autoantibody, a serial standard samples with known amounts of autoantibody (Kronus) was applied to different microarrays on plasmonic chips, following the same procedure as described for human serum. Fluorescence signal vs. autoantibody concentration was plotted and was used for calibrating autoantibody concentrations in patient serum samples.

Statistical analysis

We measured the mean fluorescence intensity (MFI) for each subject against insulin, GAD65 and IA2 (ICA512) islet antigens on the plasmonic gold platform, and directly compared these results to the known corresponding RIA data. Positive thresholds for the Au platform were determined by MFI measurements of control samples (obtained from the Kronus RIA kits) corresponding with the commercial Esoterix RIA positive threshold. We compared positive and negative MFI signals on our platform against RIA results to determine sensitivity and specificity for each diabetes autoantibody by receiver-operating characteristic (ROC) plots. Using subsequent clinical diagnosis determined from clinical progression and insulin needs at six months after disease presentation, we assessed each subject's diabetes autoantibody profile against their clinical diagnosis to compare the sensitivity and specificity of our platform against RIA as a diabetes diagnostic tool. We used a positive signal in any of the three autoantibodies as diagnostic of T1D. As the autoantibody profile included three MFI values in non-normal distribution, we conducted quadratic discriminate analysis of the subject data.