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A quantitative, label-free visual interference colour assay platform for protein targeting and binding assays



Ana Lopez-Campistrous¹, Hillary Sweet^{2,3}, Ciaran Terry², Craig Garen⁴, Yu Wan⁵, Robert E. Burrell², Kyle Moxham², Matthew Nickel^{2,5}, Michael Serpe⁶, Michael Joyce⁷, Lorne Tyrrell⁷ & Todd P. W. McMullen^{1,5}✉

The vast array of immunoassay technologies used to assess protein interactions is costly or platform-specific. We present a label-free visual interference colour assay (VICA) that quantifies peptide and protein interactions by creating an iridescent surface allowing direct visualisation without spectrophotometric optics or microfluidics. A nanoporous aluminium oxide surface is tuned to match the refractive indices of the overlying protein layers to generate visual interference colours. To functionalise the surface, we created an affinity-capture system using a protein A-carboxyglutamic (GLA) construct that orients antibodies to enhance the signal. Using off-the-shelf antibodies, the platform can isolate analytes in buffer, whole blood, or serum. This surface generates a discernible colour change at concentrations as low as 50 femtomoles/mm² and can monitor oligomer formation in sequential steps on the same slide. VICA provides comparable kinetic parameters to biolayer interferometry and traditional immunoassays while also allowing characterisation of proteins in large macromolecular complexes.

Thousands of different commercial immunoassays using a variety of reporter mechanisms (e.g., colorimetric, fluorometric or chemiluminescent) share common requirements for spectrophotometric reader infrastructure^{1–3}. Miniaturised examples exist, but these are often designed for a single analyte or require dedicated cartridges, thus requiring a substantial investment for a small number of tests^{4,5}. Antibody specificity is central to the accuracy of these assays, while the reporter moiety and spectrophotometric features define assay fidelity and range, which are directly proportional to the investment in infrastructure^{6–9}. Proteins and reagents are often fixed for different platforms. Consequently, deployment is often limited by barriers in cost, reagent supply, and access to stable infrastructure⁷.

The ability to detect protein–protein or protein–antibody interactions without complex reporter chemistry or spectrophotometric technology presents substantial theoretical advantages in experimental design and cost. Some of the earliest visual colorimetric tools were first reported by Langmuir and Shaefer¹⁰, creating interference colours through changes in the optical path length of light with protein binding on a partially reflective layer. Unfortunately, subsequent attempts to create a measurement device were limited due to poor colour contrast, complex device synthesis, and modest

sensitivity^{10–13}. The silicon and silane-based chemical methods used to immobilise proteins and antibodies to the surface are complex, have variable efficiencies, and have limited applications with different protein or antibody moieties^{13–16}. The variable sensitivity of the devices was a barrier to adoption, and the fixed refractive index of silicon dioxide also limits its experimental utility^{12,17}. A solution to the sensitivity problem was to create a porous aluminium layer on tantalum that minimised light scatter and differences in refractive index^{17,18}. However, protein deformation and degradation on catalytic aluminium surfaces, poor immobilisation of proteins and loss of their biological activity have limited the commercialisation of these devices^{19–21}.

To remove the need for spectrophotometric optical processing, we created an iridescent surface capable of generating visible colours with nanometre changes in optical path length. This label-free visual interference colour assay (VICA) platform for the detection and quantification of protein–protein or protein–peptide interactions uses a porous anodised aluminium layer on tantalum that minimises light scatter by matching the refractive indices of the aluminium oxide and adsorbed protein layer(s). Film thickness,

¹Departments of Surgery and Oncology, University of Alberta, Edmonton, AB, Canada. ²Department of Biomedical Engineering, University of Alberta, Edmonton, AB, Canada. ³SN Biomedical Inc, Edmonton, AB, Canada. ⁴Department of Physics, University of Alberta, Edmonton, AB, Canada. ⁵Pavonis DiagnosticsTM, Edmonton, AB, Canada. ⁶Department of Chemistry, University of Alberta, Alberta, AB, Canada. ⁷Department of Medical Microbiology and Immunology, University of Alberta, Alberta, AB, Canada. ✉e-mail: tpm1@ualberta.ca

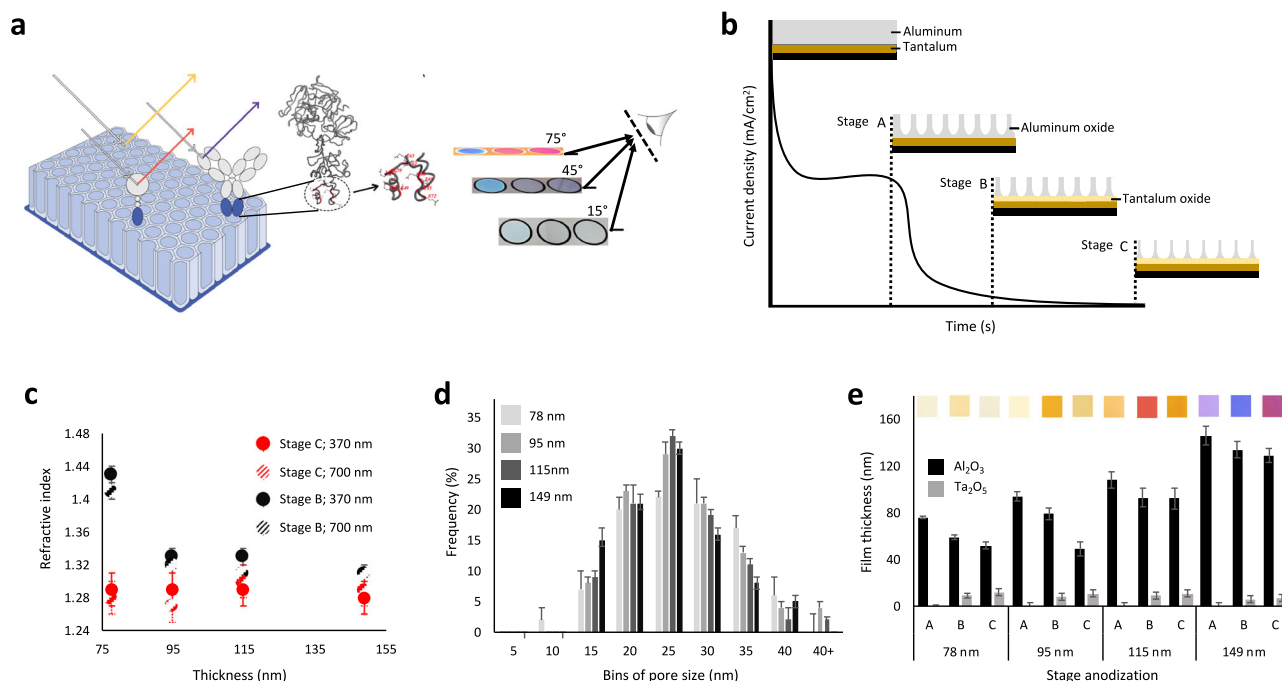


Fig. 1 | Visual interference colour development using anodised aluminium oxide for protein detection. **a** Representation of a thin film device with interference colours and reflections. A change in film thickness by adsorption of a protein monolayer will be observed as a change in colour due to the interference of the reflected wavelengths. The 3D structure of prothrombin was generated using I-TASSER. The 10 Gla residues (red) are located at sites 49, 50, 57, 59, 62, 63, 68, 69, 72 and 75 on the full-length protein. Set up for visualising colour changes generated on thin films. The optimal viewing angle is 15° from the surface (75° from normal) through a polarising filter. The best viewing was completed with a white light background. **b** Current density-time plot during the anodization of aluminium-tantalum thin films in 0.4 M phosphoric acid under potentiostatic conditions (8 V). The critical stages are labelled. Pore and Al_2O_3 formation began immediately upon

starting at a current of 8 V in 0.4 M stirred phosphoric acid (Stage A). Stage B begins with the formation of the tantalum oxide (Ta_2O_5) layer, and Stage C represents the complete etching process after a period of low current density and changes in pore configuration. **c** Optical constants (η) in the xy plane for devices with different initial aluminium thicknesses after aluminium oxide (stage B) and tantalum oxide (stage C) pore formation at wavelengths of 370 and 700 nm. The extinction coefficients were zero. **d** Histograms of the raw pore diameter after complete anodization for each of the initial aluminium thin film thicknesses with error bars representing standard deviation (SD). **e** The thicknesses of the aluminium oxide and tantalum oxide layers at different stages, as determined by ellipsometry with error bars representing SD ($n = 4$), were compared to the corresponding observed interference colours.

porosity, and refractive index can be tuned for individual proteins to further improve precision and accuracy. We address the challenges of a biologically hostile aluminium oxide surface by creating an interfacial layer to protect against potential oxidation using a novel immobilisation methodology defined by a high-density carboxylic acid domain. Modelled from the carboxyglutamic acid (GLA) domain of prothrombin, the carboxylic acid-rich domain allows us to orient proteins on the aluminium oxide surface where it acts as a stable base layer for the immobilisation of proteins. Our approach provides a monolithic surface that avoids the typical passive immunoassay problems of random orientation, poor adsorption, or low surface density, where typically no more than 10% of antibody epitopes are available for binding^{22,23}. VICA output is a predictable and precise colour sequence that can be observed by the eye or camera, with a simple polarisation filter to improve contrast. Chemical crosslinking, as well as GLA-protein A and GLA-Fc antibody constructs, demonstrated the utility of the designer GLA domains to bind and orient capture proteins on the thin film surface. This platform can be used for quantitative assays or the rapid assessment of protein-protein complexes of up to four proteins in buffer, serum, or whole blood. The kinetics of potential molecular associations can be assessed without the complex spectrophotometric infrastructure of ELISA, bilayer interferometry (BLI), or surface plasmon resonance (SPR). We anticipate that VICA can provide a novel platform to reveal and quantify a spectrum of biomolecules and their biomolecular interactors, including metabolites in discovery or clinical studies.

Results

Generating visual interference colours using anodised aluminium oxide for protein detection

We hypothesised that we could use interference colours to precisely visualise nanomolar concentrations of analytes on an anodised alumina surface without a spectrophotometer (Fig. 1a). To immobilise and orient proteins on the anodised aluminium oxide surface, we used a negatively charged protein construct modelled on the GLA domain of prothrombin^{24,25}. Prothrombin is a vitamin K-dependent protein with a highly compact negatively charged domain with ten GLA residues concentrated in 8.3 nm^3 as illustrated in Fig. 1a using I-TASSER to render the 3D structure^{26–28}. Anodization to configure pores in the aluminium oxide allowed us to generate the appropriate phase shifts for the first- or second-order interference colours with a suitable contrast that can be observed without a spectrophotometer (Supplementary Fig. 1). Matching the refractive indices of the aluminium oxide surface and protein enabled the detection of changes in the path length of light reflected at the air/protein and aluminium/tantalum interfaces while minimising noise from other reflections. The matching of the refractive indices can be achieved by tailoring the porosity and pore size of the anodic aluminium oxide through modulation of the anodization conditions, such as electrical potential, electrolyte composition, time and temperature. The final colours, observed at a 15° angle through a polarising filter, are a consequence of the changes in the optical path length that are directly proportional to the amount and size of the protein immobilised on the surface.

To define the characteristics of the aluminium oxide layer that generate appropriate phase shifts and colour changes without noise from other

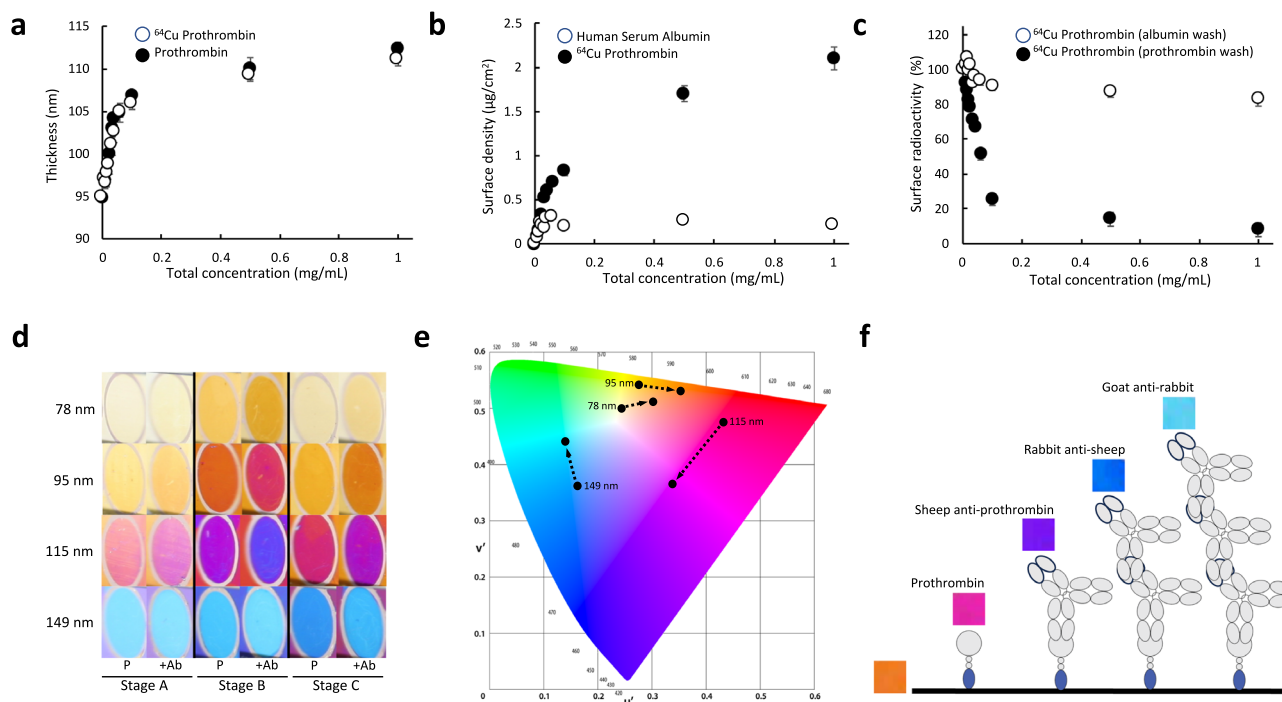


Fig. 2 | Stability of the GLA-mediated aluminium oxide surface-protein interaction and precision of RGB coordinate measurements. **a** The thickness profiles derived by ellipsometry for aluminium oxide (95 nm) with radiolabelled and native prothrombin applied to the surface for 15 minutes, shown as a function of prothrombin concentration ($n = 4$), SD represented by error bars. **b** Effect of varying prothrombin concentration on the resulting surface density ($n = 4$) with error bars representing SD. The same experiments were completed with human serum albumin. **c** Radiolabelled exchange experiments were completed with prothrombin and human serum albumin, with the labelled prothrombin placed on the surface, followed by incubation with unlabelled prothrombin and albumin for 120 min at various concentrations ($n = 4$), SD represented by error bars. **d** Interference colours of the aluminium oxide surface after protein

exposure. The initial aluminium thicknesses are shown on the left of the images, and the stages of oxidation and the protein solution exposure are shown at the bottom of the image. The solutions consisted of prothrombin (P) and prothrombin + prothrombin antibody (+Ab). **e** Corresponding plot of colours for Stage C demonstrating the colour shift with the addition of antibody to prothrombin on a CIE 1976 chromaticity diagram. The CIE coordinates of the x- and y-axes indicate the specific colours that can be perceived by the human eye. **f** Sequential protein stacking experiment. Prothrombin (250 $\mu\text{g/mL}$) was added to the surface, followed by incubation with a prothrombin sheep IgG antibody (100 $\mu\text{g/mL}$), a rabbit anti-sheep IgG (100 $\mu\text{g/mL}$) and a goat anti-rabbit IgG (100 $\mu\text{g/mL}$). Representative images of the observed interference colours are shown.

reflections, we set up an experimental matrix in which the optical path length was modulated, starting with four different initial sputtered aluminium thicknesses: 78 ± 2.2 , 95 ± 1.9 , 115 ± 2.8 and 149 ± 3.1 nm. We used anodization to modulate the refractive index of these thin aluminium films through the introduction of pores. The formation of the aluminium and tantalum oxide layers was determined by current density as a function of time (Fig. 1b). Representative SEM images at the three stages of anodization are shown in Supplementary Fig. 2, and mean pore diameters measured by SEM as a function of anodization are shown in Supplementary Fig. 3. The impact of pore formation on the refractive index of the alumina layer at different thicknesses was determined by constructing an ellipsometry model. Ellipsometry at wavelengths of 370 to 700 nm was used to calculate the refractive indices when the samples were anodized to stages B and C, as shown in Fig. 1c. The refractive index varied with the initial film thickness in stage B. By stage C, the refractive indices for all aluminium oxide layers converged to create a consistent interface for protein immobilisation. The refractive index matching process minimises the loss of signal due to refracted light and reflections between the protein and aluminium oxide layers. For these films, more than 90% of the pores were between 15 and 35 nm in diameter (Fig. 1d), minimising protein complex formation within the pores. The presence of protein in the pores would increase the effective refractive index of the aluminium oxide layer, ultimately decreasing visualisation due to light scattering from a mismatch of the protein and aluminium oxide layer refractive indices. Ellipsometry was also used to determine how the anodised aluminium oxide and tantalum film thickness changed throughout the anodization process (Fig. 1e). The tantalum oxide layer was formed by stage B of anodization and was uniform in thickness at 12 nm for

all different starting aluminium thicknesses as it impedes current flow and stops further electrochemical oxidation. The interference colours generated by thin films of varying initial thickness, as anodization progressed, were also visualised through a polarising film and are shown at the top of Fig. 1e for each stage and thickness. With changes in the optical path length, we demonstrated that there is a range of colours available for visualisation with changes in the alumina thickness. It is also clear that the creation of a tantalum oxide layer, in combination with changes in thickness, will alter the observed interference colours. We observed that nanometre changes in thickness generated significant changes in colour that could potentially be used to fine-tune the aluminium oxide surface to optimise analyte detection based on protein size or concentration.

Simple RGB coordinate analysis to quantify protein–protein interactions

With an iridescent aluminium oxide surface responding to nanometre changes in optical path length through colour change, we then examined the strength and durability of GLA tag-mediated immobilisation on the thin film surface. Using prothrombin as an example of a GLA-linked protein, ellipsometry quantified changes in the optical path length of the thin film upon the binding of both radiolabelled and native prothrombin to examine exchange on the surface, as well as changes when competing with high levels of albumin. We used radiolabelled ^{64}Cu -prothrombin, human albumin and prothrombin mixtures to monitor surface density changes. As a control, unlabelled and ^{64}Cu -labelled prothrombin demonstrated an equal affinity for the aluminium oxide surface (Fig. 2a). We observed a stronger preference of the surface for prothrombin than for albumin (Fig. 2b), and

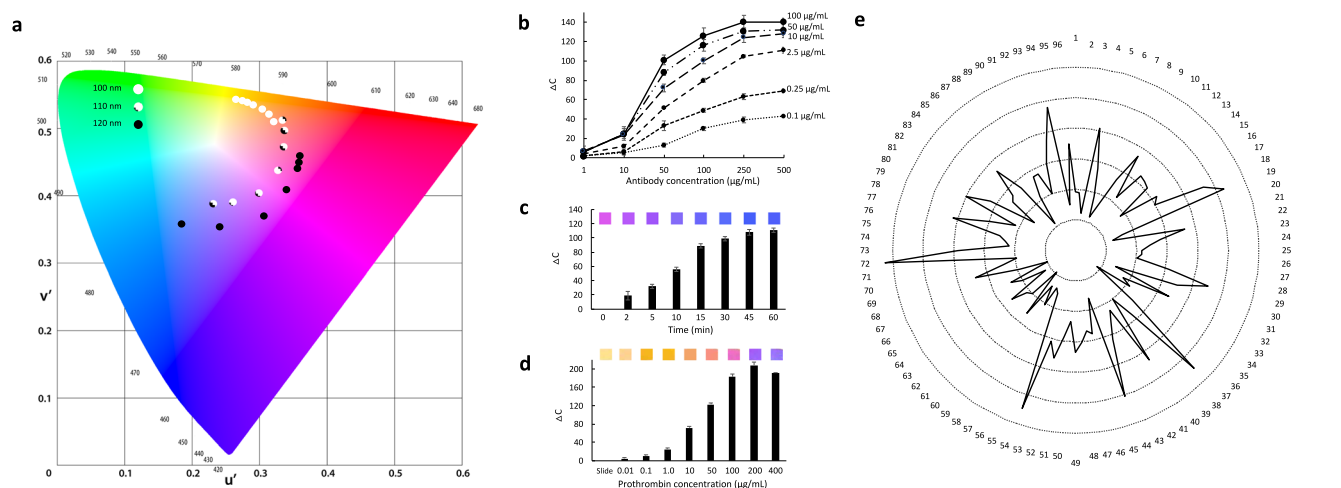


Fig. 3 | Tuning the aluminium oxide thickness to examine changes in the dynamic range of colour changes for the applied prothrombin antibody. **a** Prothrombin antibody was added to prothrombin (100 $\mu\text{g/mL}$) on aluminium oxide surfaces of three different thicknesses to generate the colours plotted on the CIE chart ($n = 5$), the standard deviation falls within the symbols. **b** The stepwise increase in the amount of prothrombin on the surface was used to identify signal saturation with increasing concentrations of antibody added. As the level of prothrombin increased between 0.1 and 50 $\mu\text{g/mL}$, an increase in signal intensity was detected, but at prothrombin concentrations beyond this level, we approached the signal intensity maximum, indicating the thin film surface binding capacity of the antibody, consistent with the results of the radiolabelling studies ($n = 3$; error bars represent SD). **c** Aluminium oxide surface (110 nm) tunability and range, as defined by changes in

exposure time, are represented graphically. Results were quantified using Euclidean geometry (ΔC) as outlined in Extended Fig. 5. For fixed concentrations of prothrombin (50 $\mu\text{g/mL}$) and prothrombin antibody (50 $\mu\text{g/mL}$), increased exposure time allowed for increasing changes in colour through 45 min, beyond which further colour change was minimal at these concentrations. Measurements in triplicate with error bars representing SD. **d** The observed colour changes with the application of increasing concentrations of prothrombin to a fixed concentration (100 $\mu\text{g/mL}$) of prothrombin antibody on the surface for 30 min. Measurements in triplicate with error bars representing SD. **e** Radial representation of the Euclidean geometry (ΔC) changes (in increments of 20 starting at -20 at centre) measured antibody–antigen binding using the FORMoscreen system of buffers.

incubation with unlabelled prothrombin induced concentration-dependent exchange with the surface protein. However, human serum albumin did not displace substantial amounts of prothrombin even when the slides were washed for 30 min (Fig. 2c). The stability of the GLA-mediated aluminium oxide interaction allows for prolonged measurements with minimal impact on the integrity of the protein/alumina interface.

The changes in observed colours with protein binding using our VICA system were explored under different aluminium oxide thicknesses and stages of anodization, as outlined in Fig. 2d. As predicted, different initial thicknesses provided a range of observable colours upon antibody binding to prothrombin when added in sequence to the aluminium oxide surface. Changes in pore structure during different stages of anodization and the presence or absence of the tantalum oxide layer all manifested as changes in colour. At the lower (78 nm) and upper (148 nm) extremes of initial aluminium thickness, the changes in observable colour with prothrombin or prothrombin plus antibody binding are less discernible than those observed between 95 and 115 nm. As measured by ellipsometry, the resulting attenuation of light in this thickness range spans the green wavelengths, giving the complementary colours from red to blue. As a simple and precise tool for the quantification of changes, we utilised red, green, and blue (RGB) coordinates as well as the CIE 1976 colour chart for visualisation. Using the RGB coordinates allowed the rapid quantification of differences in colour that can be stored and compared using Euclidean geometry, which is routinely used for colorimetric analyses. Defined as ΔC , the distance between two points is based on the RGB coordinates, and colours can be converted to the CIE LUV colour space to visually represent colour changes in two dimensions, as plotted on the CIE chart (Fig. 2e and Supplementary Fig. 4a)^{29–31}. Using 110 nm slides in our model system, we utilised the RGB geometric quantification system for a multistep experimental pathway example. We used the prothrombin substrate and sequentially layered antibodies with varying species reactivity to quantify the change in interference colour on the same slide (Fig. 2f). The sequentially added proteins progressively altered the optical path length (see Fig. 1) and caused changes in the observed colour. The addition of inappropriate competing species of

IgG antibodies did not significantly abrogate binding, as demonstrated by the small variations in the observed colours, quantified by ΔC (Supplementary Fig. 4b). In our studies of this surface with bound prothrombin, we observed minimal binding of non-targeted proteins in both simple and complex biological solutions, including human serum, which was consistent with previous studies demonstrating the low affinity of antibodies and most other proteins for porous alumina²⁰ (Supplementary Fig. 4b). Moreover, the precision of the device and measured RGB coordinates with prothrombin or anti-prothrombin binding is high, with a coefficient of variation of less than 3% within a given slide and 6% between slides based on 21 separate measurements in human serum. (Supplementary Fig. 5).

Quantitative measurements of protein–protein binding and screening antibody formulations

To demonstrate the simplicity and range of this platform using a simple camera, we examined the quantitative performance characteristics of this visual interference colour immunoassay using prothrombin as a model system. First, we demonstrated the concept of tuning using the prothrombin–prothrombin antibody binding model. The observed changes in RGB coordinates are proportional to the initial aluminium oxide surface thickness, whereby the colour range, plotted in Fig. 3a, was documented for three different thicknesses. This feature can be exploited to alter the signal-to-noise ratio. For example, we determined the signal intensity and limit of detection for each initial aluminium oxide thickness using spiked samples of prothrombin antibody in pooled human serum (Supplementary Fig. 6). Based on the calculated surface imprint of prothrombin at $\sim 12 \text{ nm}^2$, a 7 mm diameter circle with a surface area of $\sim 38 \text{ mm}^2$ should approach saturation at approximately 79 fmol/mm^2 . This was consistent with the signal generated from the addition of prothrombin antibodies to increasing levels of bound prothrombin, with colour signal saturation above $10 \mu\text{g/mL}$, 68 fmol/mm^2 (Fig. 3b). Furthermore, the ability to quantify these colour shifts was preserved under different lighting intensities between 250 and 4200 lux (Supplementary Fig. 7). We also determined that the observed changes in interference colours can be predicted as a time-dependent (Fig. 3c) and

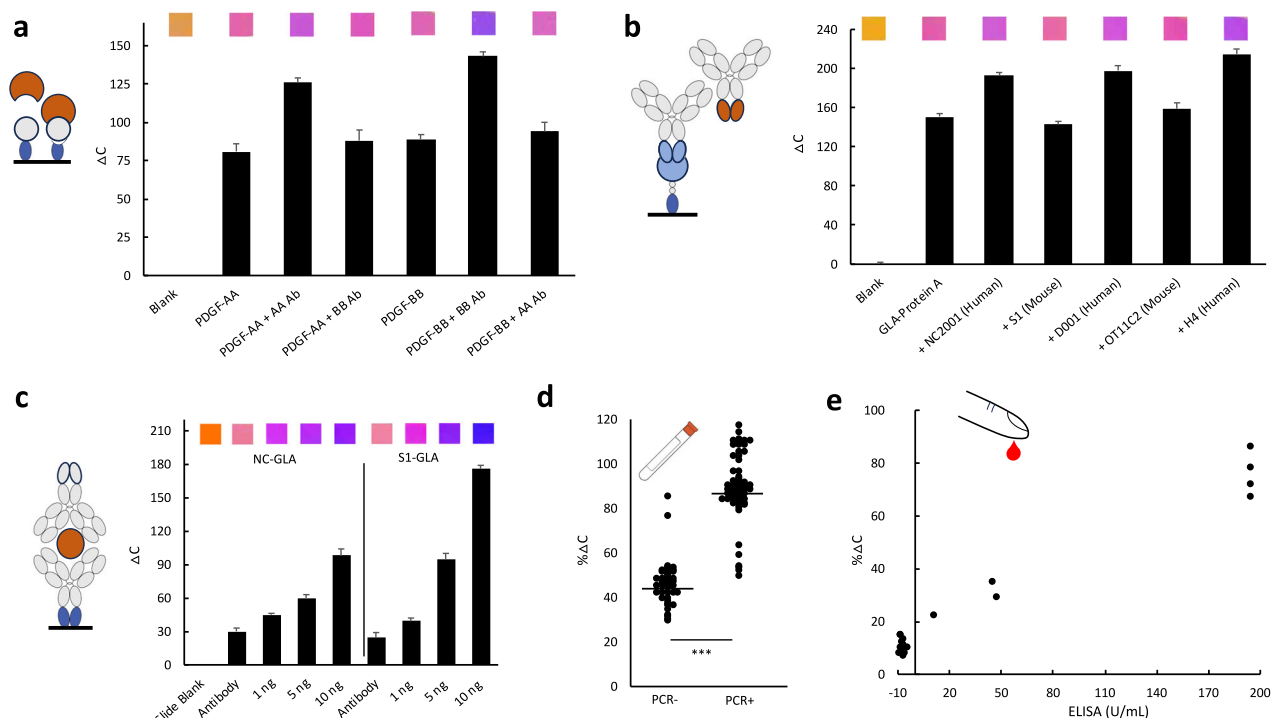


Fig. 4 | Practical applications of the aluminium oxide surface in the measurement of protein interactions. **a** Utility of aluminium oxide surface (110 nm) in the assessment of antibody specificity to identify cross-reactivity and binding efficacy for antigen-antibody pairs as qualified using Euclidean geometry (ΔC) ($n = 3$) with error bars representing SD. PDGF-AA and PDGF-BB crosslinked to prothrombin were subsequently exposed to their respective antibodies. Antibodies directly applied to the prothrombin surface do not induce a significant colour change. **b** The binding of a novel GLA-protein A construct to different human and mouse IgG antibodies. Using a recombinant hybrid protein containing the GLA sequence of prothrombin and protein A, we created an antibody pulldown system. Protein A alone does not bind the thin film surface, but the GLA-protein A construct produces a strong and reproducible colour shift when applied to the surface. Using this construct in solution with different IgG species (mouse and human), we performed a pulldown experiment, which revealed

specific binding of protein A to human IgG antibodies but not to mouse IgG clones ($n = 3$), error bars represent SD. We confirmed the specificity of human IgG binding for this construct using native gels. (data not shown). **c** Sandwich assay using novel GLA-Fc antibodies for SARS-CoV-2 (100 $\mu\text{g}/\text{mL}$) to capture the SARS nucleocapsid or S1 antigens spiked at varying levels in naïve human serum and applied to the surface for 15 min, washed, and then incubated with a commercial unmodified corresponding antibody ($n = 3$) with error bars representing SD. **d** Serum isolates tested for antibodies to the SARS-CoV-2 nucleocapsid defined by exposure using PCR and serology over a three-month period of sample acquisition (negative $n = 35$; positive $n = 48$; $p < 0.0001$, unpaired t -test). **e** Fresh capillary blood quantification of SARS-CoV-2 nucleocapsid antibody in fresh capillary blood specimens correlated with antibody levels detected by ELISA.

concentration-dependent (Fig. 3d) effect of the applied analyte. This permits the user to image and quantify protein-protein (such as antigen-antibody) interactions in complex solutions to define the components and kinetics of molecular associations. To demonstrate the utility of the rapid assay for measuring antibody-antigen interactions, we used the FORMOScreen system, designed for the optimisation of antibody formulations, to assess antibody binding in our model system. We observed significant changes in the avidity of antibody binding with variations in pH, detergent, and ionic strength, as quantified by ΔC in the radar chart in Fig. 3e.

To assess the utility of this approach following long-term storage, we prepared thin film slides with prothrombin and stored them at room temperature in a dark environment. We measured changes in colour upon the binding of primary anti-prothrombin antibodies followed by a secondary IgG antibody. The results indicated the ability to quantify binding up to 180 days after the slides were first prepared, and the platform works well at low temperatures (4 °C) as well (Supplementary Fig. 8).

Direct and sandwich assays for proteins in buffers and biological solutions

To fully explore the potential applications of the VICA with direct and sandwich immunoassays on human or cell culture specimens without a spectrophotometer, we demonstrated the use of three different methods to immobilise proteins on the surface. First, platelet-derived growth factor ligands (PDGF-AA and PDGF-BB)

were crosslinked to prothrombin using the common glutaraldehyde method to affix the proteins to the VICA surface. In crossover binding assessments, anti-PDGF-AA or anti-PDGF-BB antibodies were added to the spots to demonstrate clear and precise shifts in colour only when appropriate antibody-antigen pairs were allowed to interact (Fig. 4a). No colour shift was observed when bound PDGF-AA was incubated with anti-PDGF-BB antibodies or when antibodies were incubated with the prothrombin-coated surface in the absence of ligand. We also evaluated binding in anisotropic environments (mixture of proteins in solution) by assessing the interaction of three different PDGFRA antibodies to their antigen. The opportunity to bind PDGFRA in a dynamic environment using a two-step procedure demonstrated affinity differences between polyclonal (Ab#2-Thermo Scientific Cat. PA5-32545, and Ab#1-Millipore Cat. 07-276) and monoclonal (Ab#3-Cell Signaling, Cat. 5241) antibodies (Supplementary Fig. 9). This also demonstrates how our platform can also be utilised for protein binding on the surface as well as for anisotropic binding in solution. Second, we engineered a novel affinity-capture protein A-GLA construct based on GLA and an intervening Kringle domain that allows the oriented immobilisation of protein A to the anodised aluminium surface (Supplementary Fig. 10). Demonstrating the isotype specificity of protein A, interference colour changes are only observed when the protein A-GLA construct specifically binds human IgG antibodies

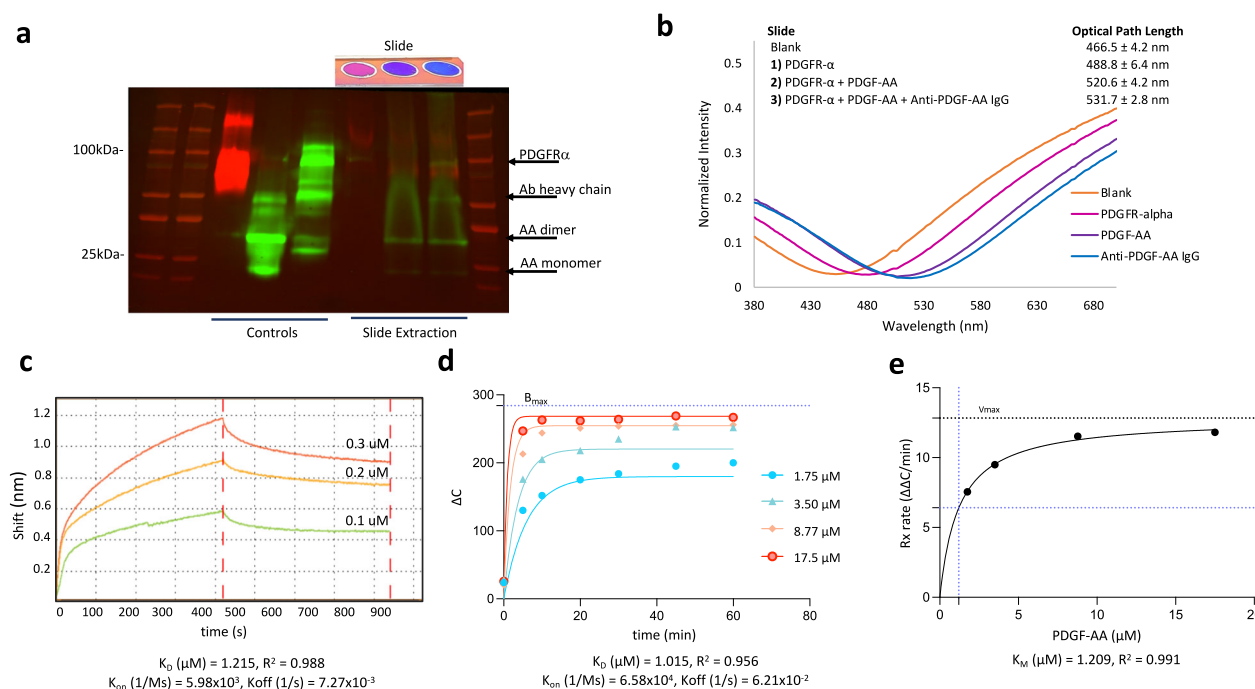


Fig. 5 | Validation of PDGFRA and PDGF-AA binding using VICA, BLI and Western blot. **a** Proteins bound to anodised alumina surface were eluted with a bicarbonate buffer solution and their presence was documented by western blot. Lanes 1–3 size controls for PDGFRA, PDGF-AA and anti-PDGF-AA Ab; lanes 3–6 protein complexes eluted from the surface. **b** Ellipsometry was used to determine slide thickness as the protein layers were added to the slide to demonstrate protein–

protein interactions. **c** The K_D of PDGF-AA binding to PDGFRA was determined by BLI using local fitting and a 1:1 binding model. **d** The K_D of PDGF-AA binding to PDGFRA was determined using VICA and association kinetics to calculate saturation binding curves at multiple ligand concentrations (non-linear least squares fitness model, 95% confidence). **e** The K_M of PDGF-AA binding to PDGFRA was determined using VICA (non-linear least square fitness model, 95% confidence).

but not mouse IgG (Fig. 4b). These results indicate that capture of a large variety of antigens may be possible using VICA with commercially available antibodies. Third, we engineered two novel anti-SARS-CoV-2 antibodies whose Fc region was fused to the GLA domain of prothrombin. GLA-Fc antibody constructs were designed and produced for both the nucleocapsid and the S1 portion of the spike protein of SARS-CoV-2. These constructs enabled the monolithic orientation of the antibodies directly on the thin film surface. These antibodies were used as capture agents for either the spike or nucleocapsid proteins of SARS-CoV-2, which then allowed detection antibodies to create a sandwich immunoassay. Increasing interference colour differences were observed with increasing doses of either the SARS-CoV-2 spike or nucleocapsid proteins (Fig. 4c). This response was not observed when either the capture antibody or the antigen was omitted from the assay (data not shown). Finally, using a direct assay design with SARS-CoV-2 antigens immobilised via a GLA domain, we examined the levels of anti-SARS-CoV-2 nucleocapsid antibodies in human serum (Fig. 4d) and fresh whole blood (Fig. 4e). When serum samples were examined, 2 out of 42 samples that were negative by PCR had higher interference colour changes, and 5 out of 62 of the PCR-positive samples had low interference colour changes (sensitivity 92%, specificity 95%) (Fig. 4d). When an anti-SARS-CoV-2 nucleocapsid-specific ELISA (EDITM, San Diego, USA) was performed, antibody levels determined by ELISA correlated (r^2 = 0.93) to those determined using VICA in fresh blood by capillary sampling (Fig. 4e). Similar results were obtained using VICA with SARS-CoV-2 spike protein compared to quantitative ELISA (ProLab, Toronto, CAN) (Supplementary Fig. 11). Overall, the stability of the platform in complex biological mixtures and the ability to stack potential reporter moieties allow for signal amplification without the need for specialised optic readers or electronics. In quantitative assessments, the affinity-capture monolithic

orientation provided optimal optical properties while avoiding the need for polymers or other functional group chemistries that exhibit variable reaction efficiencies.

Comparison of PDGFRA and ligand binding parameters using VICA, BLI, and Western Blots

To assess VICA platform fidelity, we examined western blots of isolates from prepared slides and completed a kinetic analysis of comparable BLI experiments to confirm the precision of our platform as defined by changes in optical path length and observed colours. First, we eluted the proteins from the anodised alumina surface and analysed the composition by western blot (Fig. 5a) to document the sequential changes in protein deposition corresponding to the changing colour observed on the slide. Secondly, we completed direct measurements of changes in thickness with immobilisation of proteins using ellipsometry on the same slides. We observed precise and reproducible changes in optical path length (Fig. 5b), consistent with the sequential addition of different proteins, individually, or as a complex (SEM less than 1.5%). In a separate set of experiments, we completed kinetics measurements using VICA and biolayer interferometry (BLI) for comparison. Association kinetics with multiple ligand concentrations was used to determine the dissociation constant (K_D) of PDGF-AA binding to PDGFRA using BLI and VICA (Fig. 5c, d respectively). We also used VICA on an enzyme kinetics model to extrapolate the maximum velocity of the interaction (Fig. 5e) and calculate the Michaelis-Menten constant (K_M). The comparable values obtained from both VICA and BLI techniques indicated that VICA possesses similar capabilities to BLI in terms of kinetics measurements. Additional time course and time and equilibrium experimental designs are possible with VICA, given the ability to complete sequential experiments on a slide for K_{on} or K_{off} determinations over hours, days, or longer if needed. Lastly, we demonstrated

signal amplification of up to two orders of magnitude by using secondary biotinylated antibodies that allowed conjugation to streptavidin-conjugated nanoparticles (Supplementary Fig. 12a). Western blots also quantitatively correlated with proteins captured on a slide with visualisation to under 1 µg/ml (Supplementary Fig. 12b).

Discussion

We created a platform with an iridescent surface capable of measuring individual proteins or complexes as low as 50 femtomoles of protein/mm², suited for the study of peptide- or protein-protein interactions in their native conformation. We utilise the electrostatic properties of aluminium oxide surfaces and their ability to act as both Lewis acid and base to immobilise structures on the surface with a negatively charged amino acid construct to the surface, the GLA domain of prothrombin³². The benefit of an affinity-capture mode of immobilisation, in contrast to passive surface immunoassays, is that a monolithic orientation avoids random orientation of proteins that may result in steric hindrance and inefficient capture of the moiety of interest^{33,34}. It also avoids the need for polymers or other functional group chemistries that exhibit variable reaction efficiencies. The stability of the GLA-mediated aluminium oxide interaction allows for prolonged measurements with minimal impact on the integrity of the protein/alumina interface. This not only permits experimentation in complex biological solutions, but also allows for sequential experimentation and multiple steps, which addresses challenges in other techniques that are limited by a single reaction and single measurement. We believe that this is an excellent adjunct to the current standard binding assays where variable temperatures and times are needed to determine binding affinities.

The sensitivity can be varied significantly depending on the surface saturation. The limit of detection is determined by the number of molecules on the surface needed to sufficiently increase the optical path length so that a phase shift can be visibly observed. This means that volume and kinetics are the primary rate-limiting determinants. In this study, we used small (<20 µl) volumes, but increasing the volume of a solution of a given concentration can increase the sensitivity up to 1000 times. Minimising noise by altering thin film thickness also permits increased sensitivity by matching the refractive indices of the aluminium oxide surface and protein, eliminating undesirable scattering of light at the protein/alumina interface. The manufacture of the thin film substrate is highly reproducible because the tantalum oxide layer serves as a high-fidelity marker of anodization completion, impeding current flow to stop further electrochemical oxidation. For the purposes of this study, we created a custom enclosure system that blocks external light where colour patterns can be adjusted. Designed and presented with the goal of simplicity using a simple camera and a polarising filter, our current analysis does not preclude the feasibility of more detailed analysis of the spectral output that we believe does contain information regarding protein concentration, binding, and configuration. We also note that human visual perception consists of a much higher density of green receptors; therefore, eyes are most sensitive to discern the contrasting colours in this range. Future work would examine how the alumina structure could be optimised for visual human perception through the complex interplay between brightness, hue, and chroma^{33,35}.

Combining the robust aluminium oxide with an affinity-capture system allows for long-time course experiments and equilibrium experiments, given the ability to reuse slides to complete K_{on} or K_{off} determinations over hours or days if needed. Moreover, with a single construct or crosslinking reaction, off-the-shelf antibodies or proteins can be used to create qualitative or quantitative assays that can work in buffers, as well as complex solutions

including cell culture, serum, or whole blood. Our experimental surface permits binding in the anisotropic environment of solution, or ordered in two dimensions with the pull-down of specific antibodies to the surface. Overall, the platform has a broad range of applications, from multiplex screening for interactome discovery research, to affinity measurements, to validation of antibodies in different detergents in complex solutions for preclinical validation or clinical diagnostic tests. This platform is a fraction of the cost of spectrophotometric methods and the platform can be used in remote or low-resource settings.

Methods

Thin film deposition

Unprocessed silicon wafer substrates 100 mm in diameter (University Wafer, South Boston, MA) were cleaned in piranha solution consisting of a 1:3 ratio of hydrogen peroxide (H₂O₂ 30%) (J.T. Baker, Center Valley, PA) and sulfuric acid (H₂SO₄ 96%) (J.T. Baker, Center Valley, PA). The wafers were dried completely using nitrogen gas in a spin rinse dryer and coated using planar magnetron sputtering (Kurt J. Lesker Company, Jefferson Hills, PA). The purity targets were 99.95% for tantalum and 99.9995% for aluminium (Kurt J. Lesker Company). During aluminium deposition, the power density was maintained at 6.6 W/cm² with an argon pressure between 7 and 10 mTorr and a substrate rotation of 20 rpm. The deposition of optically thick tantalum (>50 nm) was followed by sputtering of aluminium at depths varying from 70–140 nm.

Thin film oxidation

The wafers were cleaved into pieces of varying sizes, no larger than 6.5 cm in the greatest dimension, and one-step anodization was carried out at 8 V under potentiostatic conditions for varying durations to reach the desired stages of thin film formation³⁶. Preliminary tests were required to determine the range of thicknesses capable of producing visible colour shifts in the first-order interference range with adsorbed monolayers of protein; the current density (mA/cm²) was used to determine the stage of oxidation for sample selection, as outlined in Nickel et al.³⁷. The anode (sputtered layers to be oxidised) and cathode (aluminium foil) were kept parallel to one another with 4 cm of separation. The electrolyte used for anodization consisted of 300 mL of 0.4 M phosphoric acid (H₃PO₄) at room temperature (21 ± 2 °C). The anodization current density changes followed a precise pattern during initial pore formation and at the beginning of anodised aluminium (Al₂O₃) generation, and the current density decreased as (Ta₂O₅) formation began. All anodization runs were capped at a period of 900 s. All experiments were carried out with a magnetic stir bar stirring the electrolyte solution at 300 rpm.

FE SEM

Field emission scanning electron microscopy (FE SEM, Zeiss Sigma) images of the anodised aluminium oxide slides were taken using a through-the-lens detector. An accelerating voltage of 3 or 4 keV was used with apertures of 15 µm. All FE SEM images were collected under ultrahigh vacuum conditions (<1 × 10⁻⁸ Torr) on prepared samples with a pixel dwell time of 64 µs and a probe current of 35 pA. Device samples for analysis were cleaved following anodization and mounted on microscope stubs. Images of the surface layers, which were taken prior to the introduction of protein complexes, were evaluated to determine the average pore diameter and area fraction. ImageJ NIH open-source software (<https://imagej.nih.gov/ij/>) was used to conduct this test by first setting the scale and adjusting the threshold³⁸. Average pore diameters were extracted from pores measured along three horizontal and three vertical lines from representative regions of the slide. The mean, standard deviation, minimum, and maximum values were obtained.

Ellipsometry

A variable angle spectroscopic ellipsometer (VASE) was used to analyse the optical constants and thicknesses of the thin film device layers²⁵. Models were built using a bottom-up approach on WVase32® software (J. A. Woollam Co., Inc., Lincoln, NE). The base layer used in the model was 0.5 mm of silicon and subsequent layers of 200 nm tantalum, followed by a thin tantalum pentoxide (Ta₂O₅) layer and porous aluminium oxide (Al₂O₃) thin films formed during the anodization process. For all samples, Ψ and Δ values were recorded for wavelengths in the visible wavelength range from 300 to 700 nm at 10 nm increments, typically at angles of 55° to 75° from normal, at 10° intervals. Various modes of fitting were used to accurately represent the properties of each layer. A Lorentz oscillator layer was used for modelling the tantalum metal, and the Cauchy layers were used for modelling the tantalum and alumina. No improvements were demonstrated when using the effective medium approximation (EMA) to model surface roughness, and scans were conducted to confirm that depolarisation was negligible during the examination of the porous alumina layer.

Reagents, proteins, and antibodies

Glutaraldehyde (50% wt/v) and L-lysine were purchased from Sigma Aldrich (Darmstadt, Germany). Human prothrombin (APP006B) and an anti-prothrombin antibody (CL20110A) were purchased from Aniera Diagnostica (West Chester, OH, USA). Rabbit anti-sheep IgG (172-1017 secondary Ab) was obtained from Bio-Rad (Hercules, CA, USA). Gibco PDGF-AA (PHG0035), Gibco PDGF-BB (PHG0046), phosphoric acid (H₃PO₄) and goat anti-rabbit IgG (31210 secondary Ab) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibodies against PDGF-AA (sc-128) and PDGF-BB (sc-127) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-PDGFRA antibodies were from Thermo Fisher Scientific (Waltham, MA, USA), Cat. # PA5-32545, Millipore (Temecula, CA, USA), Cat. # 07-276 and Cell Signaling Technology (Danvers, MA, USA), Cat. # 5241. SARS-CoV-2 S1 chimeric antibody (HC2001), SARS-CoV-2 nucleocapsid chimeric antibody (HC2003), and SARS-CoV-2 recombinant S1 and nucleocapsid proteins were obtained from GenScript (Piscataway, NJ, USA). Biotinylated rabbit anti-sheep IgG (ab6746) was obtained from Abcam Limited (Cambridge, UK) and streptavidin conjugated 40 nm gold nanospheres (AUIR40) were from nanoComposix Inc. (San Diego, CA, USA). GLA-modified protein A was obtained from Cambridge Protein Works (Cambridge, UK). FORMOScreen formulations were obtained from Jena Bioscience (Jena, Germany). EDI™ COVID-19 Nucleocapsid IgG Quantitative ELISA Kit was obtained from Epitope Diagnostics, Inc. (San Diego, CA, USA), and the Prolisa™ Quantitative Anti-SARS-CoV-2 IgG EIA was from Pro-Lab Diagnostics (Richmond Hill, ON, Canada).

Patient specimens

Ethics approval was obtained through the University of Alberta Health Research Ethics Board ID Pro00018758. A total of 104 human serum specimens were obtained from Alberta Precision Laboratories; 62 samples were collected between 3- and 10-weeks post-PCR-confirmed COVID-19 infection, and 42 samples were collected from serology-confirmed negative individuals.

Adsorption of biologics and linking of proteins to the anodised aluminium oxide surface

Lyophilised purified human prothrombin was reconstituted in 20 mM Tris-HCl, 0.1 M NaCl buffer at pH 7.4 and 18 µL of protein solution at varying concentrations was applied within 7 mm diameter spots and incubated for defined durations under 100% relative humidity and room temperature. The solution was then removed with a pipette, and the slide was rinsed thoroughly with deionized water. Antibody complexation on the surface was completed by exposing the specific device surface containing adsorbed

prothrombin to varying concentrations of antibody. For protein crosslinking to prothrombin, a fresh glutaraldehyde solution, 5% v/v in 25 mM phosphate buffer, pH 7.0, was deposited onto the prothrombin-coated circles for one hour at room temperature and then rinsed with PBS (phosphate-buffered saline). Then, the protein of interest was added to the prothrombin spot for two hours at room temperature. Secondary antibodies for protein layer stacking were sequentially incubated for 30 min at room temperature. To stop protein binding events to the surface, once the incubation times were completed the protein samples were removed by pipetting and the slides were thoroughly rinsed with deionized water, visualised and photographed.

Prothrombin radiolabelling

The 2-S-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA) (Macrocyclics Inc., Plano, TX) chelator was purified by RP-HPLC (H₂O, 0.2% trifluoroacetic acid (TFA)/acetonitrile (CH₃CN)), yielding p-SCN-Bn-NOTA-3TFA. The prothrombin concentration was determined by using a Beckman Coulter DU 730 (Beckman Coulter, Brea, CA) to obtain A280 measurements with a percent solution extinction coefficient of $\epsilon_{1\%} = 13.8 \text{ (g/100 mL)}^{-1} \text{ cm}^{-1}$ for prothrombin. Sodium bicarbonate (NaHCO₃), TBS (Tris-buffered saline), and sodium acetate (NaOAc) buffers were pre-treated with Chelex 100 resin and freshly prepared using trace metal-grade salts. Prothrombin was conjugated to the macrocyclic chelator NOTA via isothiocyanate bioconjugation with the lysine residues of the protein. The prothrombin solution (in TBS, 100 µL, 10 mg/mL) (ACOA, Aniera, West Chester, OH) was subjected to spin filtration to achieve buffer exchange into NaHCO₃ (0.15 M, pH 8.8). The resulting solution (500 µL, 852 µg, 1.7 mg/mL) was reacted overnight with p-SCN-Bn-NOTA-3TFA (0.112 mg, 141 nmol, 12 eq.) in a NaHCO₃ buffer solution at 37 °C and 300 rpm. The NOTA-prothrombin conjugate solution was purified from reactants using a size exclusion column while achieving buffer exchange into TBS (pH 7.4). The remainder was subjected to spin filtration using Amicon Ultra 0.5 mL 50k spin filters (Millipore Ltd., Eto-bicoke, ON) in NaOAc (0.25 M, pH 5.5) for ⁶⁴Cu labelling. The solution was aliquoted (100 µg in 61.4 µL of buffer) and stored at −78 °C. Prothrombin labelling was performed with ⁶⁴Cu (Mallinckrodt Institute of Radiology, St. Louis, MO). For this purpose, 126–163.8 MBq [⁶⁴Cu]Cl₂ in 20 µL of NaOAc buffer was added to the NOTA-prothrombin solution (100 µg, in 61.4 µL of NaOAc). The reaction mixture was shaken for 1 h at 30 °C at 550 rpm. Next, 10 µL of 50 mM EDTA was added. After 10 min, incorporation was assessed using TLC (SiO₂, 50 mM EDTA, R_f(Protein) = 0, R_f(⁶⁴Cu-EDTA) = 1). The solution was purified using size exclusion chromatography while achieving buffer exchange into TBS, pH 7.4. The fraction containing the highest amount of radioactivity was used.

Colour detection and statistical analysis

Protein immobilisation on the anodised aluminium oxide surface was observed by the eye with a polarising film, which eliminates p-polarised light off the device surface. To generate the strongest colour contrast by matching s-polarised light reflection intensities off the alumina and underlying surfaces, the light reflection was viewed at an incidence angle of 75°. The experimental setup simply required polarising film in a camera with the angle of assessment of the slide at 75° from normal (see Fig. 1). The red, green, and blue (RGB) coordinate system was used to quantitatively define visible colours. To obtain the coordinates, images were captured under the same conditions and uploaded to a colour coordinate output system (imagecolourpicker.com), and the three coordinates over five consistent positions for each sample spot or the blank slide were averaged. The quantification and comparison measurements were defined by Euclidean geometry with independent subtraction of the R, G, and B components to determine the difference, ΔC . OriginLab (OriginLab Corporation) was used to convert RGB coordinates to the CIE 1976 colour space. GraphPad (GraphPad Software, Inc.) was utilised for the analysis of kinetic results; the mean values are provided in the graphs, and standard deviations are denoted

by error bars. Measures of significance were determined by Student *t*-tests. *P* values less than 0.01 were considered significant.

Data availability

The data supporting the findings of this study are available within the paper and its Supplementary Information files. There are no restrictions on the availability of materials or information. Raw data files are available from the corresponding author upon reasonable request.

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Author contributions

T.P.W.M., A.L.-C. and R.E.B. conceived and planned the experiments. A.L.-C., H.S. and M.N. carried out the experiments. C.T., C.G. and K.M.

contributed to sample preparation. Y.W. and M.S. contributed to the interpretation of the results. M.J. and L.T. contributed material. T.P.W.M. took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Competing interests

TPWM is founder of Pavonis Diagnostics Inc. REB is author of US patent No. US12061195B2, in the name of Pavonis Diagnostics Inc, related to the method of immobilization on aluminium oxide surfaces. All other authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Todd P. W. McMullen.

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