

Colorimetric/chemiluminescent vertical flow immunoassay based on multifunctional cobalt–gold bimetallic nanoclusters for Brucella antibody detection and deep learning- assisted semiquantitative analysis

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Abstract

colorimetric/chemiluminescent vertical flow immunoassay (VFI) based on glutathione-protected cobalt–gold bimetallic nanoclusters (GSH@Co–AuNCs) conjugated with staphylococcal protein A as multifunctional catalytic immunoprobes. The probe integrates immunorecognition with peroxidase-like activity, enabling direct colorimetric visualization, nanozyme-amplified colorimetry through 3,3',5,5'-tetramethylbenzidine–H₂O₂ oxidation, and sustained luminol–H₂O₂ chemiluminescence within a common assay architecture. Direct colorimetry remained reliably distinguishable at 0.5 IU mL^{−1}, whereas nanozyme-amplified colorimetry and chemiluminescence both reached 0.2 IU mL^{−1}. The chemiluminescent system additionally exhibited sustained emission for more than 150 min, providing low-background imaging and a broader signal-acquisition window for weak responses. To standardize image interpretation, separate shared-weight dual-branch 18-layer residual network models were developed for nanozyme-amplified colorimetric and chemiluminescent images, yielding internal-test R² values of 0.9647 and 0.9734, respectively. In a preliminary evaluation of 30 clinical serum samples using the serum agglutination test as the reference, nanozyme-amplified colorimetry achieved 95.0% sensitivity, 90.0% specificity, and 93.3% accuracy, while chemiluminescence achieved 95.0%, 100%, and 96.7%, respectively. This work not only demonstrates the integration of multifunctional nanocluster catalysis, sustained chemiluminescence, and regression-based semiquantitative analysis within a single VFI platform, but also provides a general strategy for improving weak-signal discrimination and standardized interpretation in portable immunoassays.

Introduction

Brucellosis is a globally significant zoonotic disease caused by *Brucella* species, with an estimated 2.1 million new human infections annually^[1]. The disease burden is highest in Asia and Africa, where livestock farming and unpasteurized dairy consumption are prevalent. Early diagnosis is challenging because initial symptoms—fever, fatigue, arthralgia, and myalgia—are nonspecific^[2]. Delayed diagnosis can lead to chronic complications including osteoarticular involvement, endocarditis, and neurobrucellosis^[3].

Conventional diagnostic methods include bacterial culture, PCR, Rose Bengal plate test, serum agglutination test (SAT), and ELISA^[4, 5]. Culture is the gold standard but requires weeks and biosafety level 3 facilities. PCR offers high sensitivity but depends on thermal cyclers and trained personnel. Serological tests are simpler but rely on subjective interpretation. These limitations highlight the need for rapid, simple, and reliable point-of-care testing (POCT) in resource-limited settings.

Paper-based vertical flow immunoassay are attractive for POCT due to short assay times, simple operation, and minimal instrumentation. Formats explored for anti-*Brucella* antibody detection include lateral flow assays, SERS-based immunochromatography^[4, 5] and nanozyme-enhanced vertical flow immunoassays (VFIs)^[6, 7]. VFI offers advantages over lateral-flow formats by directing sample transport vertically through the membrane, shortening mass-transfer pathways and facilitating functional

nanomaterial labels^[8]. Recent VFI platforms using Au@Mn₃O₄ and PB@Au nanocomposites have demonstrated multi-signal transduction for Brucella detection^[6, 7]. Deep learning-assisted image analysis has recently been shown to enhance interpretation consistency in paper-based assays, including nanoparticle-amplified vertical flow formats^[9]. However, signal amplification alone does not resolve the challenge of weak or borderline responses. Colorimetric readouts are rapid and intuitive, but reliability near the decision threshold is compromised by membrane background, illumination, and interobserver variability. Chemiluminescence (CL) requires no external excitation and provides lower optical background for weak-signal imaging, and has been successfully integrated with smartphone-based readout for convenient on-site detection^[10, 11]. Integrating colorimetry and CL within a single system offers complementary strengths without separate probes.

Nanozymes provide a versatile platform for integrating catalytic signal amplification with multiple optical readouts in membrane-based immunoassays^[12]. Gold nanoclusters (AuNCs) are attractive due to ultrasmall dimensions and ligand-rich surfaces facilitating bioconjugation and catalytic signal generation^[13]. Incorporation of a second metal can further modulate catalytic behavior^[14]. Glutathione-protected Co–Au bimetallic nanoclusters (GSH@Co–AuNCs) are therefore well suited to such an integrated sensing strategy because they combine efficient H₂O₂ activation with sustained chemiluminescent emission^[15]. Moreover, the GSH-derived surface functionalities provide accessible groups for subsequent conjugation with staphylococcal protein A (SPA), allowing immunorecognition and catalytic signal transduction to be incorporated into a single nanocluster probe.

Our previous studies have demonstrated the feasibility of nanozyme-assisted vertical flow immunoassays for anti-Brucella antibody detection using Au@Mn₃O₄ and PB@Au nanostructures^[6, 7]. These studies established that functional nanomaterials can effectively enhance membrane-associated signals and provide diversified optical readouts for rapid serological analysis. However, improving signal intensity alone does not fully address the interpretation of weak or borderline responses, particularly when the visual contrast between the test region and the membrane background becomes limited. Variations in spot morphology, illumination conditions, image acquisition, and manual delineation may further introduce uncertainty into conventional image-based evaluation. Therefore, in addition to developing sensitive signal-transduction strategies, a more standardized approach for extracting and interpreting assay responses is desirable.

Here, GSH@Co–AuNCs–SPA was used as a multifunctional immunoprobe in a colorimetric/chemiluminescent VFI for anti-Brucella antibody detection. Anti-Brucella antibodies are first captured by lipopolysaccharide (LPS) immobilized at the test region and subsequently recognized by GSH@Co–AuNCs–SPA to form a membrane-bound immunocomplex. The accumulated nanoclusters then generate two complementary catalytic readouts: H₂O₂-mediated oxidation of TMB produces a blue colorimetric signal for rapid visual screening, whereas luminol–H₂O₂ chemiluminescence provides a lower-background imaging readout for weak signals. To reduce subjectivity associated with manual spot delineation and T/C signal measurement, separate regression models were developed for the TMB and

chemiluminescence readouts using a shared-weight C/T dual-branch ResNet-18 architecture^[16]. The TMB model was designed to predict the T/C equivalent spot-diameter ratio, whereas the chemiluminescence model predicted the background-subtracted T/C integrated-intensity ratio. The continuous outputs were subsequently mapped to four predefined semiquantitative response levels. The analytical performance of the platform was evaluated in terms of catalytic behavior, assay sensitivity and specificity, reproducibility, storage stability, and preliminary performance in clinical serum samples. By integrating catalytic signal amplification, complementary optical readouts, and regression-based image interpretation within a common immunorecognition system, this study aims to improve the consistency of weak-signal interpretation while retaining the operational simplicity required for rapid screening.

Experimental

Reagents and materials

HAuCl₄·4H₂O and CoCl₂·6H₂O were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). Glutathione (GSH) was obtained from Beijing Solarbio Science & Technology Co., Ltd. (China). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl), N-hydroxysuccinimide (NHS), bovine serum albumin (BSA), NaCl, Tween-20, EDTA, NaN₃, sucrose, Tris, and staphylococcal protein A (SPA) were purchased from Sigma-Aldrich. H₂O₂ and luminol were obtained from Shanghai Aladdin Biochemical Technology Co., Ltd. (China). Brucella lipopolysaccharide (LPS) and sheep IgG were purchased from Beijing Zhongke Ruijin Technology Co., Ltd. and Hangzhou Yibai Xin Biotechnology Co., Ltd., respectively. VFI housings, absorbent pads, and Millipore Hi-Flow Plus 135 nitrocellulose membranes were obtained from Advanced Microdevices. All aqueous solutions were prepared using deionized water. Clinical serum samples were collected from a brucellosis-endemic region in southern Xinjiang, China.

Synthesis of GSH@Co–AuNCs

GSH@Co–AuNCs were synthesized using a modified previously reported method^[15]. Briefly, 9.87 mL of HAuCl₄·4H₂O solution (24.28 mM) was mixed with 3.00 mL of CoCl₂·6H₂O solution (20 mM). Freshly prepared GSH solution (25 mM, 18.00 mL) was then added under continuous stirring at 500 rpm, resulting in the formation of a pale-yellow precipitate. After 10 min, 2 mL of NaOH solution (1 M) was added dropwise until the precipitate was completely dissolved. The reaction mixture was subsequently heated in an oil bath at 70°C for 24 h. After cooling to room temperature, the product was purified by dialysis against deionized water using a dialysis membrane with a molecular-weight cutoff (MWCO) of 1 kDa.

Preparation of the GSH@Co–AuNCs–SPA signal probe

To prepare the immunosignal probe, the surface carboxyl groups of GSH@Co–AuNCs were activated using EDC/NHS chemistry^[17]. Briefly, 5 mL of GSH@Co–AuNC solution was mixed with 10 μ L of EDC (2 M) and 10 μ L of NHS (4 M) and activated at 4°C for 30 min. SPA solution (1 mg mL⁻¹, 100 μ L) was subsequently added, and the conjugation reaction was allowed to proceed overnight at 4°C. The resulting conjugate was blocked with BSA(1%, w/v) for 1 h and then dialyzed against 10 mM phosphate-buffered saline (PBS, pH 7.4) at 4°C for 24 h using a 1 kDa MWCO dialysis membrane. The purified conjugate was designated GSH@Co–AuNCs–SPA and used as the signal probe in subsequent VFI experiments.

Peroxidase-like activity and steady-state kinetics

The peroxidase-like activity of GSH@Co–AuNCs was evaluated using the TMB–H₂O₂ catalytic system. The total reaction volume was 200 μ L and contained 20 μ L of TMB (2 mM), 20 μ L of H₂O₂ (20 mM), 10 μ L of GSH@Co–AuNCs (0.5 mg mL⁻¹), and acetate–sodium acetate buffer to the final volume. Formation of oxidized TMB (oxTMB) was monitored by measuring the absorbance at 652 nm. The effects of pH (3.0–8.0), temperature (20–80°C), and GSH@Co–AuNC concentration on catalytic activity were systematically evaluated.

Under the selected reaction conditions, steady-state kinetic measurements were performed by maintaining one substrate at a fixed concentration while varying the concentration of the other. The change in absorbance at 652 nm during the initial linear stage of the reaction ($\Delta A_{652} \text{ min}^{-1}$) was recorded. Apparent kinetic parameters were obtained by fitting the experimental data to the Michaelis–Menten equation, with Lineweaver–Burk double-reciprocal plots used as complementary graphical representations. The apparent Michaelis constant (K_m) and maximum reaction velocity (V_{max}) were subsequently determined^[18, 19].

Chemiluminescence performance and kinetics

The chemiluminescence properties of the GSH@Co–AuNCs/luminol–H₂O₂ system were evaluated in black 96-well microplates. The total reaction volume was 200 μ L. Luminol (5 mM) and GSH@Co–AuNCs (0.75 mg mL⁻¹) were first mixed with the corresponding buffer, after which H₂O₂ (2 mM) was added to initiate the reaction. Chemiluminescence signals were continuously recorded for 180 min.

The effects of pH (8.0–13.0), GSH@Co–AuNC concentration (0.05–0.75 mg mL⁻¹), H₂O₂ concentration (0–10 mM), and luminol concentration (0.05–10 mM) on chemiluminescence performance were investigated using single-factor experiments. Because the system exhibited pronounced time-dependent emission behavior, its chemiluminescence performance was evaluated using both the peak intensity and the background-subtracted net area under the curve over the first 10 min (net AUC_{0–10} min).

Evaluation of analytical performance

Anti-Brucella IgG-positive serum with an initial antibody concentration of 1000 IU mL⁻¹ was serially diluted to 100, 20, 10, 5, 2, 1, 0.5, 0.2, and 0.1 IU mL⁻¹. These samples were used to evaluate three signal readouts: direct colorimetry based on the native pale-yellow color of the GSH@Co–AuNCs probe, nanozyme-amplified colorimetry based on GSH@Co–AuNC-catalyzed TMB–H₂O₂ oxidation, and luminol–H₂O₂ chemiluminescence. The lowest reliably detectable concentration was operationally defined as the lowest antibody concentration that could be consistently distinguished from the negative control across replicate measurements. Accordingly, this value represents an experimentally determined signal-discrimination threshold rather than a formal statistical limit of detection.

Analytical specificity was evaluated using Brucella-positive serum together with sera positive for Mycobacterium tuberculosis, Enterococcus spp., Yersinia enterocolitica O:9, and Escherichia coli. Reproducibility was assessed using independently prepared VFI batches, and storage stability was evaluated during storage at 4°C. Unless otherwise specified, measurements were performed in triplicate.

Deep learning-assisted semiquantitative analysis

Separate datasets of 300 TMB and 300 CL images (covering 10 concentration levels) were established and partitioned into training/validation/internal-test sets (80:10:10). Images from the same sample were kept in the same partition to avoid data leakage. Only training images underwent augmentation; validation/test images were ROI-extracted, resized to 224×224, and normalised.

For each modality, a shared-weight dual-branch ResNet-18 architecture^[16] was used: the upper control (C) and lower test (T) regions were processed independently by two weight-sharing branches, and the extracted features were fused for continuous regression. For TMB, the target was the T/C equivalent spot-diameter ratio; for CL, the background-subtracted T/C integrated-intensity ratio. Predicted ratios were mapped to four levels: G0 (negative/very weak), G1 (weak), G2 (moderate), and G3 (strong), using fixed thresholds. Model performance was evaluated using MAE, RMSE, and R²; grading accuracy was assessed via confusion matrices.

Clinical serum samples and reference method

A total of 30 clinical serum samples, comprising 20 serum agglutination test (SAT)-positive and 10 SAT-negative samples, were included in the preliminary clinical evaluation. SAT classification was performed according to the Chinese diagnostic standard for human brucellosis^[20].

For practical binary interpretation, the continuous outputs of the trained regression models were converted into positive or negative results using prespecified decision thresholds established before evaluation of the clinical serum samples. For nanozyme-amplified colorimetric images, a model-predicted T/C equivalent spot-diameter ratio < 0.30 was classified as negative, whereas a ratio ≥ 0.30 was classified as positive. For chemiluminescence images, a model-predicted background-subtracted T/C integrated-intensity ratio < 0.10 was classified as negative, whereas a ratio ≥ 0.10 was classified as

positive. These thresholds corresponded to the predefined G0/G1 boundaries used during model development and were not optimized using the 30-sample clinical cohort. Clinical performance was assessed by comparison with the SAT reference classification.

Results and Discussion

Morphological and structural characterization of GSH@Co–AuNCs

TEM images showed that the primary GSH@Co–AuNCs were predominantly quasi-spherical, with diameters mainly in the range of 5–8 nm. In addition to these primary nanoclusters, larger assemblies of approximately 20–100 nm were observed, indicating aggregation or self-assembly of the ultrasmall building units (Fig. 1a,c). Consistent with the TEM observations, FESEM revealed rough, quasi-spherical structures with characteristic dimensions of approximately 50–80 nm (Fig. 1b), further supporting the presence of assembled nanocluster structures. In contrast, DLS measurements yielded an apparent hydrodynamic diameter of approximately 317 nm with a polydispersity index (PDI) of 0.3–0.5 (Fig. S1). The substantially larger DLS-derived size is attributable to the fact that DLS reflects the hydrodynamic behavior of dispersed particles and is influenced by the GSH ligand shell, the surrounding solvation layer, and contributions from a small fraction of larger aggregates. Therefore, the value of approximately 317 nm should be interpreted as the hydrodynamic diameter of the dispersed assemblies rather than the physical diameter of the individual primary nanoclusters. EDS elemental mapping further showed colocalized Au, Co, S, and N signals in the particle-rich regions, supporting the presence of Co-containing nanoclusters and a GSH-associated surface layer.

X-ray photoelectron spectroscopy (XPS) was used to examine the surface elemental composition and chemical states of GSH@Co–AuNCs before and after SPA conjugation. The survey spectra exhibited signals attributable to Au 4f, Co 2p, S 2p, N 1s, C 1s, and O 1s (Fig. S2). After calibration to the C 1s peak at 284.8 eV, the Au⁰ 4f_{7/2} and Au⁰ 4f_{5/2} components of GSH@Co–AuNCs were located at 84.21 and 87.88 eV, respectively, whereas the corresponding Au(I) components were observed at 85.34 and 89.01 eV. After SPA conjugation, the Au⁰ components shifted to 84.01 and 87.68 eV, while the Au(I) components appeared at 84.96 and 88.63 eV. The coexistence of Au⁰ and Au(I) species is consistent with the surface chemistry of GSH-protected gold nanoclusters, in which Au(I) is commonly associated with Au–S coordination^[15]. The S 2p signals located at approximately 162–164 eV further support the presence of metal–thiolate interactions. Because of the relatively low Co content and limited signal-to-noise ratio of the Co 2p spectrum, the Co-related features, including coordinated species and satellite contributions, were interpreted qualitatively rather than used for quantitative determination of oxidation-state fractions (Fig. 2a–d).

FT-IR spectroscopy provided complementary evidence for GSH coordination and subsequent SPA functionalization. The characteristic S–H stretching band of free GSH at approximately 2521.6 cm⁻¹ disappeared after formation of GSH@Co–AuNCs and remained absent after SPA conjugation,

supporting the involvement of thiol groups in metal coordination. Following SPA conjugation, the absorption bands in the amide-associated region of 1500–1700 cm^{-1} became more pronounced. In parallel, the zeta potential shifted from approximately -20 to -13 mV, indicating a decrease in the net negative surface charge after bioconjugation. These changes are consistent with partial consumption of surface carboxyl groups during EDC/NHS-mediated coupling and the formation of a protein-containing interfacial layer. Taken together, the XPS, FT-IR, and zeta-potential results are consistent with successful SPA immobilization on the GSH@Co–AuNC surface through EDC/NHS-mediated amide coupling between amino groups of SPA and carboxyl groups associated with the GSH ligand shell (Fig. 2e,f).

Peroxidase-Like activity and steady-state kinetics of GSH@Co–AuNCs

In the TMB– H_2O_2 system, an intense blue color accompanied by the characteristic absorbance of oxidized TMB (oxTMB) at 652 nm was observed only when GSH@Co–AuNCs, TMB, and H_2O_2 were simultaneously present (Fig. 3a). This result demonstrates that GSH@Co–AuNCs promote the H_2O_2 -mediated oxidation of TMB and exhibit peroxidase-like catalytic activity. Optimization of the reaction conditions showed relatively high catalytic responses at approximately pH 4.0 and 30°C (Figs. S3 and S4), which were therefore selected for subsequent kinetic measurements. The absorbance at 652 nm increased approximately linearly with increasing GSH@Co–AuNC concentration over the investigated range ($R^2 = 0.996$; Fig. 3d), indicating a concentration-dependent catalytic response. This behavior supports the use of GSH@Co–AuNCs as signal-amplifying probes, because variations in the amount of catalyst retained at the detection region can be translated into corresponding changes in colorimetric output.

Steady-state kinetic measurements revealed clear substrate-dependent behavior toward both H_2O_2 and TMB. When H_2O_2 was varied while the TMB concentration was held constant, the apparent Michaelis constant (K_m) and maximum reaction velocity (V_{max}) were 3.27 mM and $9.25 \times 10^{-7} \text{ M s}^{-1}$, respectively. When TMB was used as the variable substrate, the corresponding apparent K_m and V_{max} values were 0.12 mM and $5.87 \times 10^{-7} \text{ M s}^{-1}$, respectively. The corresponding Lineweaver–Burk plots exhibited good linearity, with R^2 values > 0.99 (Fig. 3b,c,e,f). The relatively low apparent K_m for TMB indicates comparatively strong apparent substrate affinity under the experimental conditions employed. However, apparent kinetic parameters are highly dependent on factors such as pH, temperature, catalyst concentration, and the substrate concentration range used for fitting. Therefore, the K_m value alone should not be taken as evidence that the overall catalytic performance of GSH@Co–AuNCs exceeds that of horseradish peroxidase (HRP)^[18, 21].

The catalytic behavior of the bimetallic nanoclusters may be associated with modulation of the local reaction environment by the Co component. Previous studies on Co–Au nanoclusters have suggested that incorporation of Co can influence interfacial electron transfer and H_2O_2 activation^[15]. Consistent with this interpretation, the hydroxyl-radical trapping experiments described below showed enhanced

·OH generation in the GSH@Co–AuNC/H₂O₂ system under the present experimental conditions. These observations support a mechanism in which facilitated H₂O₂ activation contributes to the peroxidase-like catalytic activity of GSH@Co–AuNCs, although the detailed reaction pathway requires further mechanistic investigation.

3.3. Chemiluminescence performance, kinetics, and optimization of reaction conditions

A pronounced blue chemiluminescent emission in the 425–450 nm region was observed only when GSH@Co–AuNCs, luminol, and H₂O₂ were simultaneously present, whereas omission of any one component resulted in only weak background emission (Fig. 4a). The observed emission band is consistent with the characteristic luminescence of electronically excited 3-aminophthalate generated during luminol oxidation. The maximum chemiluminescence intensity obtained from the spectral scan was approximately 3.5×10^6 a.u., whereas an independent kinetic experiment yielded a background-subtracted peak intensity of approximately 1.68×10^6 a.u. (Fig. 4b). Because these measurements were obtained using different acquisition protocols and data-processing procedures, the absolute signal intensities were not directly compared; instead, the two experiments were used to characterize the spectral and temporal behavior of the chemiluminescent system, respectively.

The GSH@Co–AuNCs/luminol–H₂O₂ system exhibited sustained chemiluminescent emission over an extended period. A faint blue signal remained visually detectable after 150 min under dark conditions (Fig. 4d), while instrumental monitoring showed that approximately 4% of the initial signal remained at 180 min. Direct analysis of the normalized decay curve gave a 50% signal-decay time (t_{50}) of 31.83 min (Fig. 4c). Separately, linear fitting of $\ln(\text{Net CL})$ over the initial 0–60 min interval yielded an apparent first-order decay constant of 0.0179 min^{-1} , corresponding to an apparent fitted half-life of 38.72 min (Fig. S5). The difference between t_{50} and the fitted half-life indicates that the overall decay profile cannot be adequately described by a single-exponential model over the entire measurement period. Instead, the observed kinetics likely reflect multiple time-dependent processes, including an initially rapid reaction phase followed by progressive depletion or redistribution of reactive species and substrates. The extended emission window is advantageous for image-based detection because it reduces the dependence of signal intensity on the exact interval between reagent addition and image acquisition, thereby improving the practical reproducibility of membrane-based chemiluminescence measurements^[22–24].

Hydroxyl-radical (·OH) generation was further investigated using terephthalic acid (TA) as a fluorescent probe. As shown in Fig. S6, the GSH@Co–AuNCs + H₂O₂ group produced a fluorescence intensity of 422.15 ± 3.75 at 425 nm, substantially higher than those of the AuNCs + H₂O₂ group (112.17 ± 1.73), H₂O₂-only group (39.50 ± 0.85), and blank group (34.88 ± 0.90). These results support enhanced ·OH generation in the GSH@Co–AuNC/H₂O₂ system under the experimental conditions employed. Previous studies of Co–Au bimetallic nanoclusters have implicated reactive oxygen species, including ·OH and O₂^{·-}, in related chemiluminescent processes^[15]. However, the present TA-trapping experiment provides

direct evidence only for enhanced $\cdot\text{OH}$ formation; the involvement and relative contributions of other reactive oxygen species remain to be established.

Because the chemiluminescent response was strongly time dependent, reaction conditions were evaluated using both the peak chemiluminescence intensity and the background-subtracted integrated signal over the first 10 min (net AUC_{0-10} min). Both response metrics showed pronounced pH dependence and reached comparatively high values at pH 10.0 (Figs. S7 and S8). This behavior is consistent with the favorable role of alkaline conditions in luminol deprotonation and subsequent oxidative light-emitting reactions reported for nanocluster- and nanozyme-catalyzed luminol- H_2O_2 systems^[11, 15].

The time-dependent chemiluminescence kinetics at varying H_2O_2 and luminol concentrations are shown in Fig. 4e and 4f, respectively. As the H_2O_2 concentration increased from 0 to 2.0 mM, both the peak intensity and the overall kinetic response were progressively enhanced, whereas further increases beyond 2.0 mM led to a marked reduction in emission (Fig. 4e). A similar trend was observed for luminol: increasing the luminol concentration from 0.05 to 5.0 mM resulted in a steady rise in the kinetic curves, with higher concentrations also exhibiting prolonged emission tails (Fig. 4f).

As the concentration of GSH@Co-AuNCs increased from 0.05 to 0.75 mg mL^{-1} , both the peak intensity and net AUC_{0-10} min increased throughout the investigated range (Figs. S9 and S10). Accordingly, 0.75 mg mL^{-1} , the highest concentration evaluated, was selected as the working concentration for subsequent experiments rather than being interpreted as an absolute optimum. Increasing the H_2O_2 concentration enhanced both response metrics up to 2.0 mM, whereas further increases resulted in decreased chemiluminescent responses (Fig. S11). A similar concentration-dependent profile was observed for luminol, with 5.0 mM providing comparatively high peak and integrated signals (Fig. S12). On the basis of these complementary response metrics, pH 10.0, 0.75 mg mL^{-1} GSH@Co-AuNCs, 2.0 mM H_2O_2 , and 5.0 mM luminol were selected as the working conditions for subsequent experiments.

Compared with optimization based solely on peak intensity, the combined use of peak intensity and net AUC_{0-10} min accounts for both the initial emission strength and the cumulative chemiluminescent output over a defined acquisition period. This dual-metric approach is therefore better suited to the evaluation of sustained-emission systems in which signal intensity varies substantially with time. Moreover, the prolonged emission profile provides a broader operational window for image acquisition, helping to reduce variability caused by differences in acquisition timing and thereby supporting more reproducible image-based analysis^[14, 22].

Analytical performance of the VFI

Specificity

To assess the analytical specificity of the GSH@Co-AuNC-based VFI and evaluate potential cross-reactivity, sera positive for *Mycobacterium tuberculosis*, *Enterococcus* spp., *Yersinia enterocolitica* O:9,

and *Escherichia coli* were tested alongside *Brucella*-positive serum. All samples produced a clearly visible pale-yellow signal at the control (C) spot, indicating valid assay performance (Fig. 5). In contrast, a distinct pale-yellow signal at the test (T) spot was observed only for the *Brucella*-positive serum, whereas no discernible T-spot signal was detected for the non-*Brucella* sera examined. These results indicate that the developed VFI enabled specific recognition of anti-*Brucella* antibodies with minimal cross-reactivity within the tested serum panel, supporting its analytical specificity for anti-*Brucella* antibody detection.

Analytical sensitivity and complementarity of the dual-mode readouts

Serial dilutions of anti-*Brucella* IgG-positive serum were used to evaluate the concentration-dependent colorimetric readout, nanozyme-amplified colorimetric readout and chemiluminescence readouts. In the colorimetric readout, the pale-yellow signal at the test (T) region gradually decreased with decreasing antibody concentration. The T spot remained visually distinguishable from the nitrocellulose membrane background at 0.5 IU mL^{-1} , whereas further dilution resulted in signals that could not be consistently differentiated from the negative control. Accordingly, 0.5 IU mL^{-1} was considered the lowest reliably detectable concentration for the intrinsic-color readout under the present experimental conditions.

Following addition of the TMB– H_2O_2 substrate system, membrane-bound GSH@Co–AuNCs catalyzed the formation of blue oxidized TMB, markedly enhancing the contrast of the T region. The amplified colorimetric signal remained reliably distinguishable at 0.2 IU mL^{-1} , representing an improvement over the intrinsic-color readout. At concentrations below 0.2 IU mL^{-1} , the amount of probe retained at the T region was insufficient to generate reproducibly distinguishable color development. These results demonstrate that the peroxidase-like activity of GSH@Co–AuNCs effectively converts probe accumulation into an amplified colorimetric response.

The luminol– H_2O_2 chemiluminescence system produced a concentration-dependent decrease in luminescence intensity with increasing serum dilution. A distinguishable T-region signal was retained at a dilution of 1:5000, corresponding to 0.2 IU mL^{-1} . Thus, under the present experimental conditions, chemiluminescence and TMB colorimetry showed the same lowest reliably detectable concentration. Importantly, the principal advantage of chemiluminescence was not a further reduction in the numerical detection threshold, but its lower optical background and improved contrast for weak membrane-associated signals. In addition, the sustained chemiluminescent emission of the GSH@Co–AuNCs/luminol– H_2O_2 system provided a broader image-acquisition window, which may reduce variability associated with acquisition timing.

Taken together, the two catalytic readouts provide complementary analytical functions. TMB colorimetry enables rapid and visually intuitive screening, whereas chemiluminescence provides a low-background imaging mode that is particularly advantageous for weak or borderline responses. Both signals originate from the same LPS–anti-*Brucella* IgG–GSH@Co–AuNCs–SPA membrane-bound immunocomplex and

therefore do not require separate recognition probes or assay architectures. The values of 0.5 and 0.2 IU mL⁻¹ reported here represent experimentally determined signal-discrimination thresholds rather than formal statistical limits of detection.

Sensitivity of the GSH@Co–AuNC probe and complementarity of the dual-mode readouts

LPS was immobilized at the test (T) spot as the capture component, whereas sheep IgG was immobilized at the control (C) spot. SPA-functionalized GSH@Co–AuNCs served as the signal probe for recognition of captured anti-Brucella antibodies. Serial dilution of anti-Brucella IgG-positive serum from an initial concentration of 1000 IU mL⁻¹ resulted in a progressive decrease in the intrinsic pale-yellow signal of the nanocluster probe. At 0.5 IU mL⁻¹, the T spot remained visually distinguishable from the membrane background, whereas further dilution produced signals that could no longer be reproducibly resolved. Accordingly, 0.5 IU mL⁻¹ was defined as the lowest visually detectable concentration for the intrinsic-color readout. At lower antibody concentrations, the reduced amount of nanoclusters retained at the T spot caused the intrinsic signal to approach the background of the nitrocellulose membrane, thereby limiting reliable naked-eye discrimination of weak-positive samples.

After addition of TMB–H₂O₂, membrane-bound GSH@Co–AuNCs generated an amplified blue colorimetric response. The test-region signal remained reliably distinguishable at 0.2 IU mL⁻¹, indicating improved visual contrast relative to direct colorimetry. Below 0.2 IU mL⁻¹, retained catalyst was insufficient for reproducible color development.

The luminol–H₂O₂ chemiluminescent signal also decreased with serum dilution and remained distinguishable at 0.2 IU mL⁻¹. Thus, chemiluminescence and nanozyme-amplified colorimetry showed the same practical detection threshold, while CL provided lower optical background and a prolonged acquisition window for weak membrane-associated signals.

The TMB and chemiluminescence readouts therefore provide complementary analytical functions. TMB colorimetry offers a rapid and intuitive visual response suitable for field screening, whereas chemiluminescence provides a lower-background imaging mode for weak signals and samples close to the interpretation threshold. Because both catalytic outputs originate from the same LPS–anti-Brucella IgG–GSH@Co–AuNCs–SPA immunocomplex, dual-mode signal acquisition can be achieved without altering the underlying immunorecognition architecture.

Nanozyme-mediated optical amplification has been increasingly explored in membrane-based immunoassays using catalytic colorimetric and chemiluminescent readouts^[25–27]. Relative to our previous Au@Mn₃O₄- and PB@Au-based VFI systems^[6, 7], the present platform does not primarily seek to increase the number of signal modalities. Instead, GSH@Co–AuNCs provide a common catalytic immunoprobe for direct colorimetry, nanozyme-amplified colorimetry, sustained low-background CL, and regression-based image interpretation. Recent instrument-assisted Brucella immunochromatography likewise highlights the need for reliable weak-signal analysis^[28]. Because analytical thresholds across

platforms depend on materials, sample matrices, signal processing, and threshold definitions, direct numerical comparison should be interpreted cautiously.

Reproducibility and stability

Inter-batch reproducibility was evaluated at three representative response levels (low, medium, and high) using three independently prepared batches of VFI test cards. The coefficients of variation (CVs) of the T/C signals were 7.1%, 5.2%, and 3.8% for the low-, medium-, and high-response levels, respectively, with all values remaining below 10%. These results indicate limited inter-batch variation across the tested response range, supporting the reproducibility of the VFI platform under the experimental conditions employed.(Table S1)

Storage stability was evaluated by monitoring the relative assay response of the test cards during storage at 4°C. With the Day 0 response normalized to 100%, the retained signal was 98.1% on Day 7, 92.3% on Day 14, and 85.4% on Day 30. The CVs of replicate measurements at all evaluated time points remained below 5%. Thus, after 30 days of storage at 4°C, the test cards retained 85.4% of their initial response. These findings indicate that the developed VFI maintained relatively stable analytical responses over the 30-day evaluation period, although longer-term studies under different storage conditions are required to establish its shelf-life characteristics.(Table S2)

Deep learning-assisted semiquantitative analysis of VFI images

To reduce variability associated with manual spot delineation and T/C signal measurement, separate regression models were developed for TMB colorimetric and chemiluminescence (CL) images. The datasets contained 300 TMB images and 300 CL images, and the same ROI extraction, resizing, and normalization procedures were applied to both modalities. For each modality, a shared-weight dual-branch ResNet-18 architecture was used. The upper control (C) region and lower test (T) region were processed separately by the two weight-sharing branches, after which the extracted C-spot and T-spot features, together with their difference features, were fused for regression. The TMB model was trained to predict the T/C equivalent spot-diameter ratio, whereas the CL model predicted the background-subtracted T/C integrated-intensity ratio (Fig. 7). The continuous predictions were subsequently mapped to four predefined semiquantitative levels (G0–G3) using fixed thresholds. Therefore, the final grading step represents regression followed by threshold-based stratification rather than direct multiclass classification.

On the independent internal test sets, the TMB model achieved a mean absolute error (MAE) of 0.0404, a root mean square error (RMSE) of 0.0529, and an R^2 of 0.9647 (Fig. 8a, Fig. 8b). Most predicted values were distributed close to the $y = x$ reference line, indicating good agreement between the predicted and manually determined T/C diameter ratios. For the CL model, the corresponding MAE, RMSE, and R^2 values were 0.0667, 0.1010, and 0.9734, respectively (Fig. 8d, Fig. 8e). The predicted CL T/C ratios

likewise showed close agreement with the reference values, indicating that the model captured the major variations in luminescence responses across the test samples.

The two modalities showed somewhat different error profiles. The lower MAE and RMSE obtained for TMB images may be related to the relatively well-defined spot boundaries under standardized bright-field imaging, which provide stable morphological information for diameter-ratio estimation. In comparison, CL images may be more sensitive to variations in local background, exposure, signal diffusion, and dynamic range, resulting in larger absolute prediction errors. The slightly higher R^2 obtained for the CL model does not contradict its higher MAE and RMSE because these metrics describe different aspects of regression performance: MAE and RMSE quantify absolute prediction errors, whereas R^2 reflects the proportion of variation in the reference values explained by the model.

After threshold-based conversion of the continuous predictions, both the TMB and CL models achieved 100.00% grading accuracy on their respective internal test sets. All samples were assigned to the correct G0–G3 level (Fig. 8c, Fig. 8f). This result shows that the regression outputs were sufficiently separated for four-level interpretation under the present test conditions. However, each internal test set contained only 30 images. The observed 100.00% accuracy should therefore be regarded as performance within the current data partition rather than evidence of equivalent performance on independent external samples. In addition, because the G0–G3 levels were derived from predefined thresholds, grading accuracy depends on both regression performance and the selected cutoff values.

Previous studies have shown that convolutional neural networks and smartphone-assisted image analysis can reduce variability associated with manual interpretation and extract quantitative information from immunoassay images^[29, 30]. Machine-learning-assisted dual-mode sensing has also been explored to improve the interpretation of portable optical assays^[31]. In the present study, TMB colorimetry and CL were analyzed separately because the two readouts provide different types of image information. The TMB model mainly relies on relative spot morphology, whereas the CL model estimates the relative luminescence response after background correction. Using the same C/T-based regression strategy for both readouts allows the two VFI modes to be interpreted within a consistent semiquantitative framework without directly mixing heterogeneous optical information at the input stage.

The current dataset nevertheless remains limited. Although 300 images were available for each modality, model performance was evaluated using internal data partitioning only. Models trained on relatively small datasets may show reduced performance when applied to images acquired using different devices, assay batches, exposure settings, or independent sample populations^[32]. Further validation should therefore include independently collected clinical samples, multiple VFI batches, and different imaging conditions. In future external evaluations, Macro-Precision, Macro-Recall, and Macro-F1 should also be reported together with regression metrics to provide a more complete assessment of semiquantitative grading performance.

Overall, the shared-weight dual-branch ResNet-18 models enabled prediction of the T/C spot-diameter ratio from TMB images and the background-subtracted T/C integrated-intensity ratio from CL images. Subsequent threshold-based stratification provided four-level semiquantitative interpretation of both readout modes. This approach reduces the dependence on manual spot measurement and provides a more standardized method for image-based interpretation of dual-mode VFI results.

Preliminary clinical evaluation

Preliminary clinical performance was evaluated using 30 serum samples (20 SAT-positive and 10 SAT-negative). Using the prespecified binary thresholds, nanozyme-amplified colorimetry correctly identified 19/20 SAT-positive and 9/10 SAT-negative samples, corresponding to 95.0% sensitivity, 90.0% specificity, and 93.3% accuracy. Chemiluminescence correctly identified 19/20 SAT-positive and 10/10 SAT-negative samples, corresponding to 95.0% sensitivity, 100% specificity, and 96.7% accuracy. No false-positive result was observed for CL in this cohort, which is consistent with its low-background imaging characteristics (Table S3).

The results show preliminary agreement between the deep learning-assisted VFI and SAT, but the small cohort produces substantial uncertainty in diagnostic-performance estimates. Larger independent multicenter cohorts are needed to validate the prespecified thresholds and assess generalizability.

Conclusions

A GSH@Co–AuNCs–SPA-based colorimetric/chemiluminescent VFI was developed for anti-Brucella antibody detection. The nanocluster immunoprobe supported direct colorimetry and nanozyme-amplified colorimetry, with practical detection thresholds of 0.5 and 0.2 IU mL⁻¹, respectively, while luminol–H₂O₂ chemiluminescence also remained detectable at 0.2 IU mL⁻¹ and provided a sustained low-background imaging window. Shared-weight dual-branch ResNet-18 models enabled continuous T/C regression and predefined semiquantitative stratification. In 30 clinical serum samples, nanozyme-amplified colorimetry achieved 95.0% sensitivity, 90.0% specificity, and 93.3% accuracy, whereas chemiluminescence achieved 95.0%, 100%, and 96.7%, respectively, relative to SAT. These results support the feasibility of integrating catalytic optical readouts with standardized image interpretation in a common VFI platform.

The study is limited by the small, single-centre clinical cohort and lack of independent external validation of the deep-learning models. The platform should be regarded as a promising preliminary approach for rapid screening and semiquantitative analysis; larger multicenter studies with standardised imaging conditions are required to confirm reproducibility and clinical generalisability.

Declarations

Ethics approval and consent to participate

This retrospective study was approved by the Shihezi University Ethics Committee (Approval No. 2022-089). Owing to the retrospective design and anonymised data, the ethics committee waived the requirement for written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors report no conflicts of interest in this work.

AI Disclosure

During the preparation of this work, the graphical abstract and the schematic illustration shown in Fig. 7. were initially generated with the assistance of the image-generation functionality of ChatGPT (OpenAI) and were subsequently manually refined and finalized by the authors. The AI tool was not used to generate, modify, or analyze experimental data or scientific results. The authors reviewed the final graphical abstract and take full responsibility for its content.

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Author Contribution

Hamai Bi: Conceptualization, Data curation, Formal analysis, Writing – original draft; Xiaogang Zhao: Methodology, Investigation, Validation; Mingjie Wei: Data curation; Xia Li: Software, Visualization; Jiahui Wu: Investigation, Resources; Yuankun Wang: Formal analysis, Writing – review & editing; Xindi Zhang : Resources; Halifei Ma: Project administration; Hengxuan Zhou: Methodology, Supervision; Zhihua Xu: Resources, Funding acquisition; Kedong Guo : Investigation, Data curation, Writing – review & editing; Yali Jiang,: Conceptualization, Funding acquisition; Feng Shi: Supervision, Writing – review & editing.

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Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request

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Figures

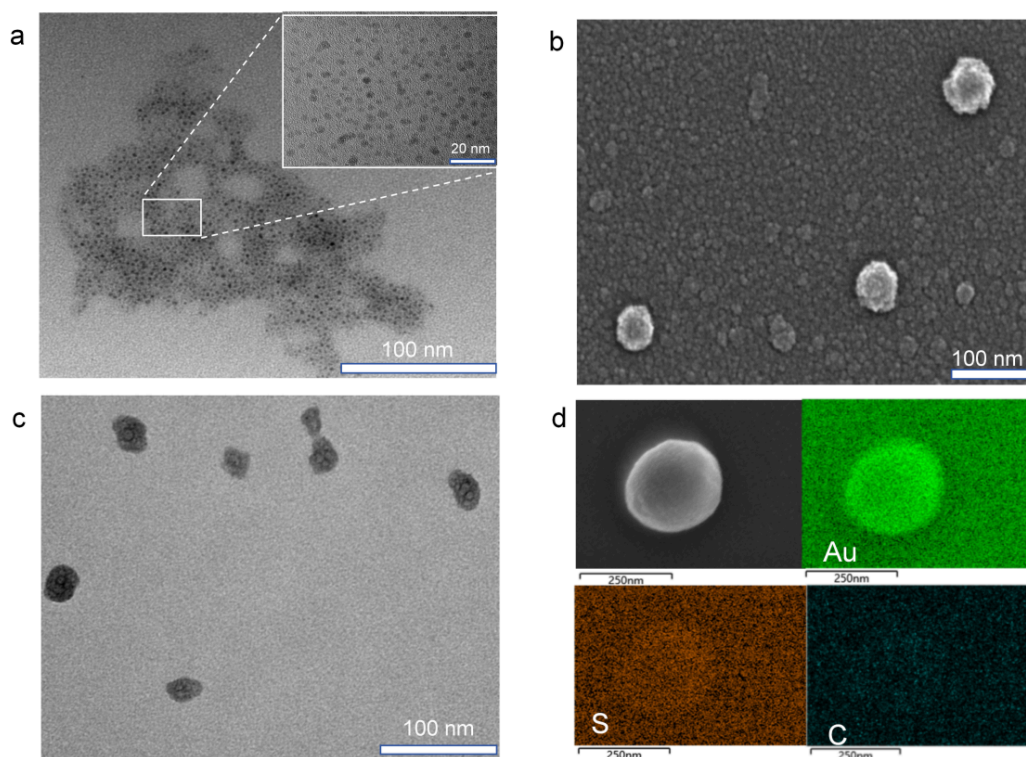


Figure 1

Morphology and elemental composition of GSH@Co-AuNCs. (a) TEM and high-magnification TEM images; (b) FESEM image; (c) Low-magnification TEM image of the nanocluster assembly; (d) Surface distribution maps of Au, Co, S, and N elements.

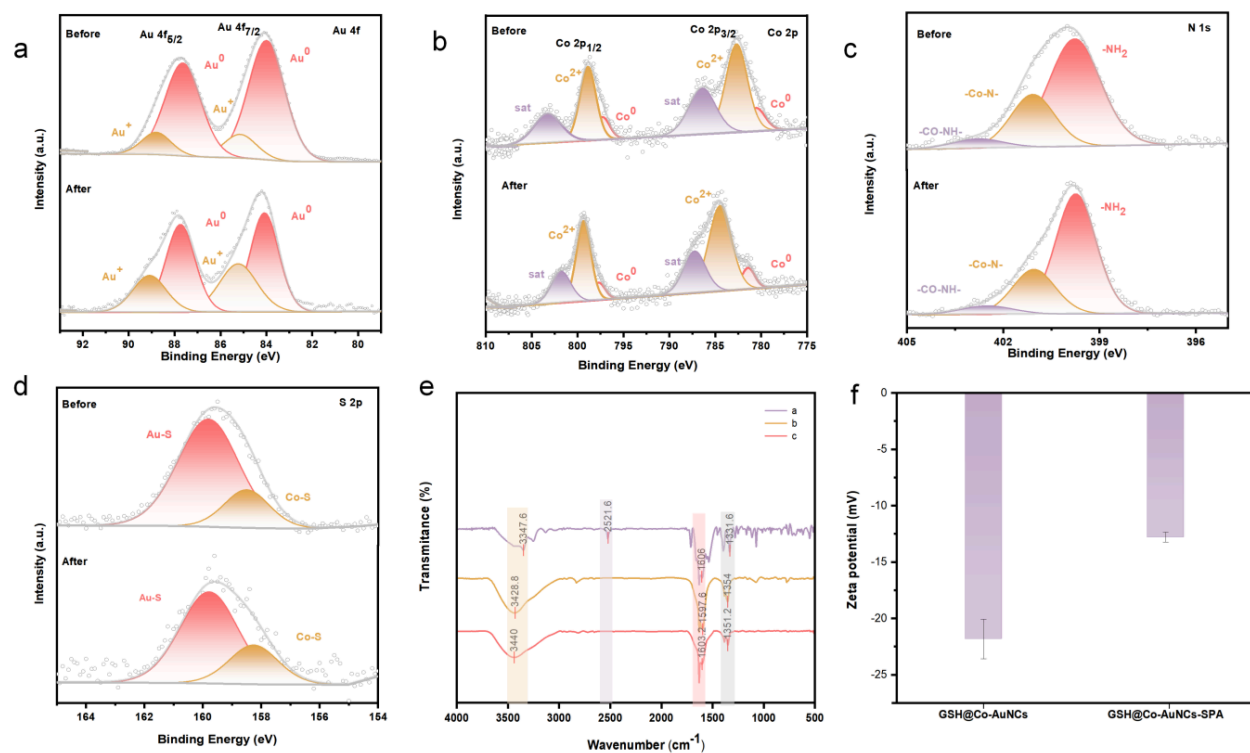


Figure 2

Surface chemical characterization of GSH@Co-AuNCs before and after coupling with SPA. The results include XPS high-resolution spectra (a–d), FT-IR spectra (e), and Zeta potential changes (f).

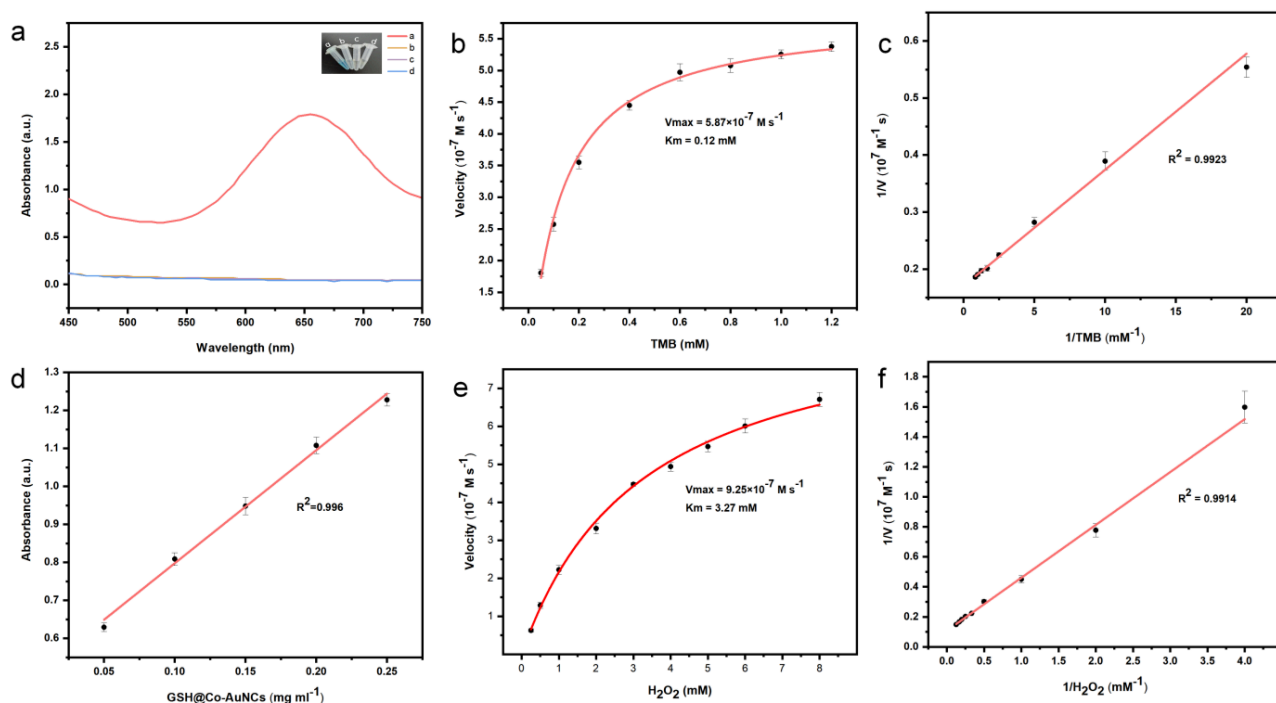


Figure 3

Peroxidase-like activity and steady-state kinetics of GSH@Co-AuNCs nanocomplexes. (a) UV–Vis absorption spectra: a: GSH@Co-AuNCs + TMB + H₂O₂; b: without nanozyme addition; c: without H₂O₂ addition; d: without TMB addition; inset diagrams show corresponding experimental images for each group; (d) With fixed concentrations of H₂O₂ and TMB, the linear relationship between the amount of oxTMB generated and the concentration of GSH@Co-AuNCs nanoparticles was determined; (b–c, e–f) Michaelis–Menten and Lineweaver–Burk fits for GSH@Co-AuNCs; error bars represent the standard deviation (n = 3).

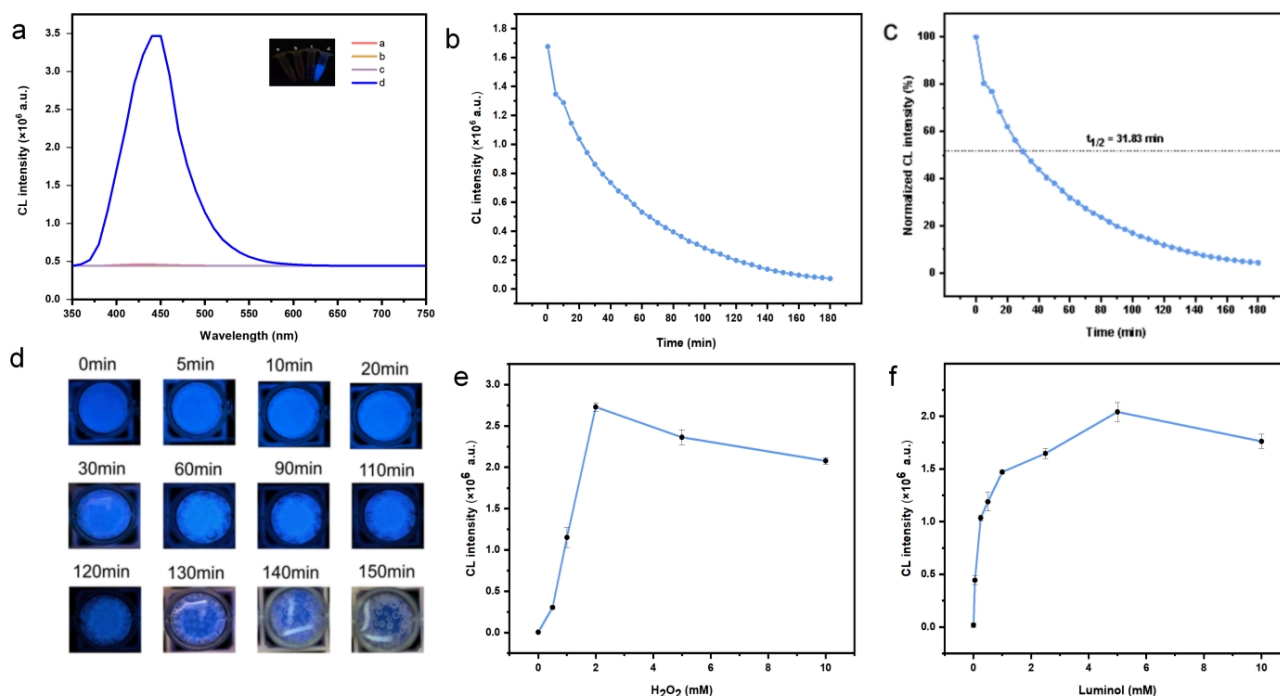


Figure 4

(a) CL emission spectra of the four reaction systems: a: luminol + H_2O_2 ; b: GSH@Co–AuNCs + luminol; c: GSH@Co–AuNCs + H_2O_2 ; d: GSH@Co–AuNCs + H_2O_2 + luminol; **(b)** time-dependent net CL intensity over 0–180 min; **(c)** normalized decay profile and determination of t_{50} ; **(d)** representative CL images at different time points; **(e)** Kinetic curves of chemiluminescence intensity at varying H_2O_2 concentrations with fixed luminol and nanocluster concentrations. **(f)** Kinetic curves at varying luminol concentrations with fixed H_2O_2 and nanocluster concentrations. Data are presented as mean $\pm$ standard deviation (SD) from three independent replicates ($n = 3$).

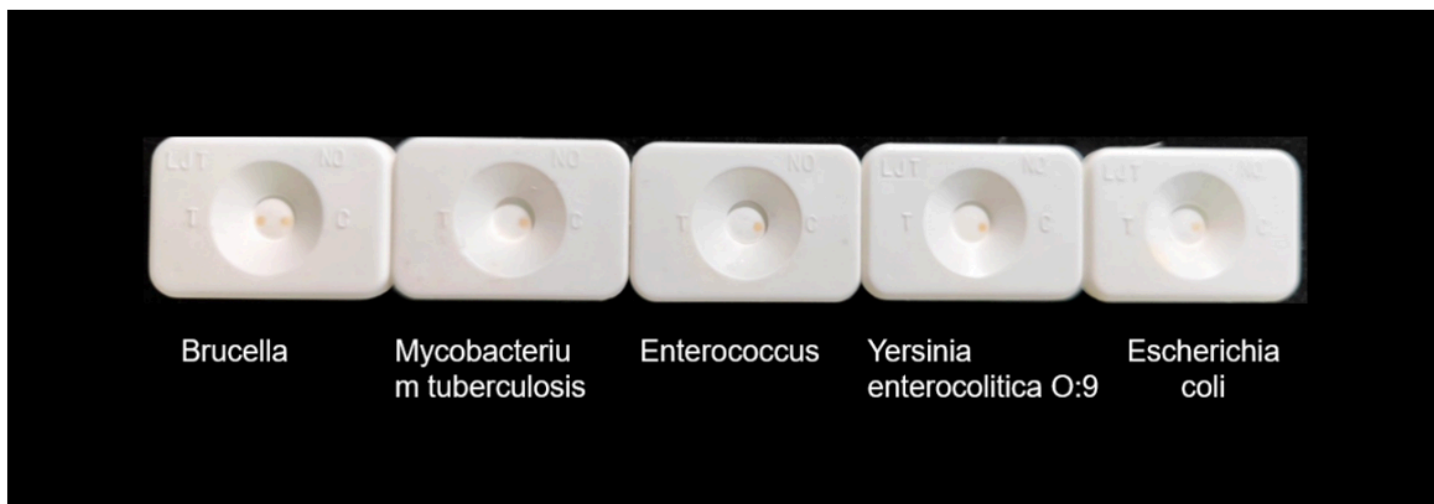


Figure 5

Specificity evaluation of the GSH@Co–AuNCs–SPA VFI assay for Brucella-positive serum samples and potential cross-reactive sera.

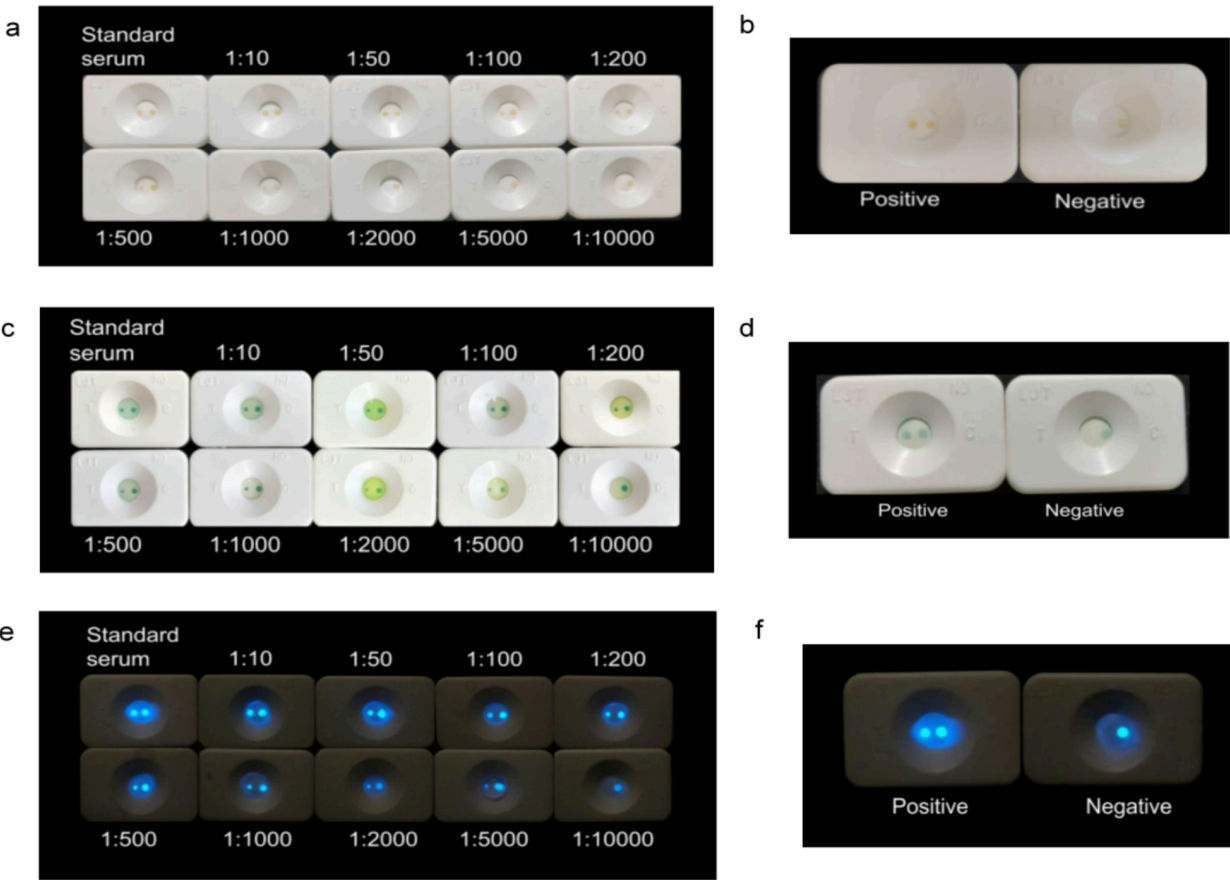


Figure 6

Concentration-dependent responses of the GSH@Co–AuNCs–SPA-based vertical flow immunoassay. (a, b) Direct colorimetric responses based on the native pale-yellow color of GSH@Co–AuNCs at different anti-Brucella antibody concentrations; (c, d) nanozyme-amplified colorimetric responses generated by GSH@Co–AuNC-catalyzed TMB–H₂O₂ oxidation; and (e, f) chemiluminescent responses generated using the luminol–H₂O₂ system. Representative negative and positive assay results are also shown.

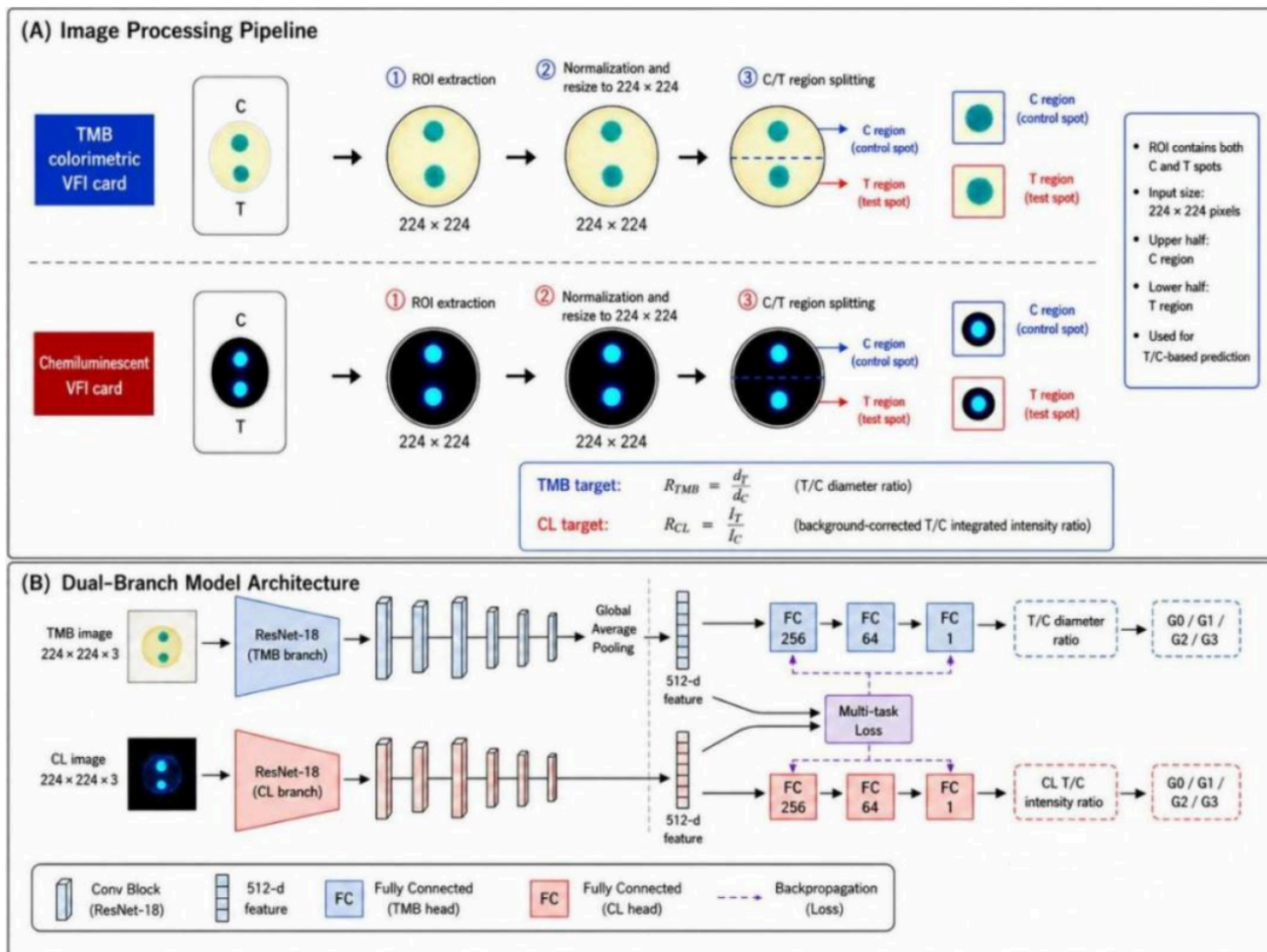


Figure 7

Architecture of the shared-weight dual-branch ResNet-18 regression framework for semiquantitative analysis of nanozyme-amplified colorimetric and chemiluminescent VFI images; This image was created with the assistance of the ChatGPT (OpenAI) image generation tool, and was then manually edited by the author before being finalized.

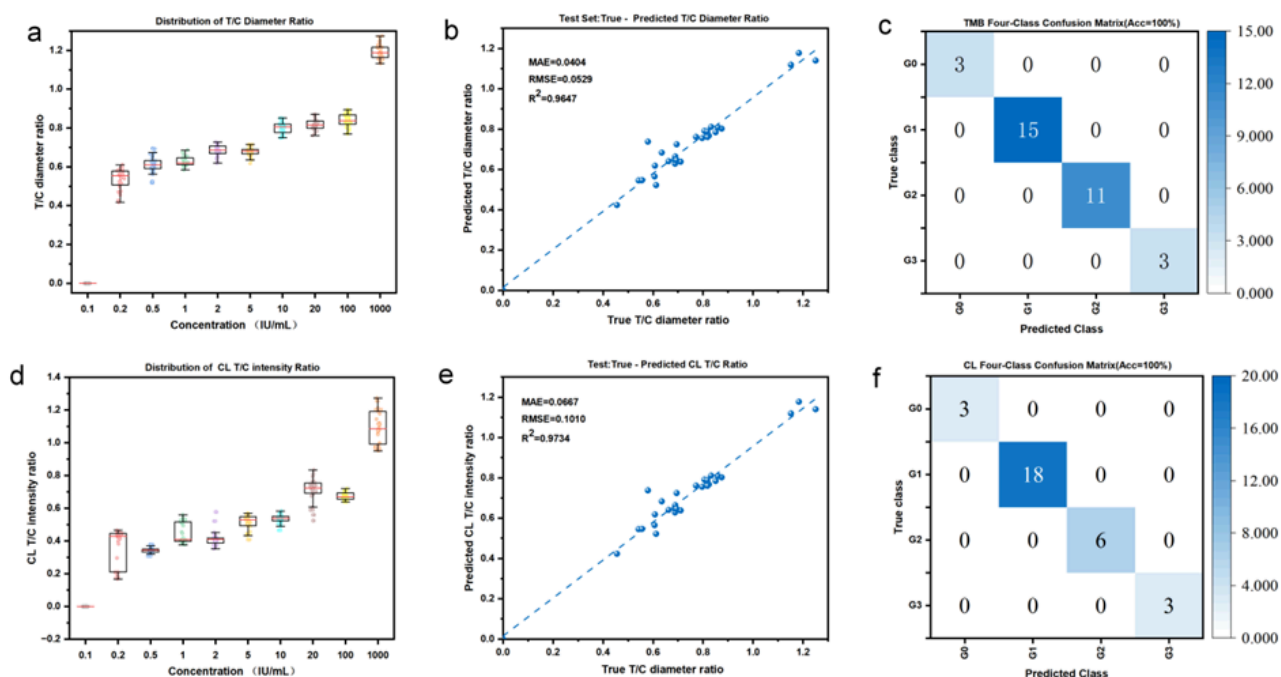


Figure 8

Performance of the shared-weight dual-branch ResNet-18 regression models for semiquantitative analysis of TMB and chemiluminescence images. (a) Distribution of reference T/C equivalent spot-diameter ratios across TMB samples; (b) relationship between reference and predicted T/C diameter ratios in the internal TMB test set; (c) G0–G3 grading confusion matrix for the TMB test set; (d) distribution of reference background-subtracted T/C integrated-intensity ratios across chemiluminescence samples; (e) relationship between reference and predicted T/C integrated-intensity ratios in the internal chemiluminescence test set; and (f) G0–G3 grading confusion matrix for the chemiluminescence test set.

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