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Complete List of Authors:	Vu, Binh; University of Houston, Chemical Engineering Brosamer, Kristen; University of Houston, Biomedical Engineering McDaniel, Naiyah; Prairie View A&M University, Department of Chemistry Kourentzi, Katerina; University of Houston, Department of Chemical and Biomolecular Engineering Willson, Richard; University of Houston, Department of Chemical Engineering Fernando, Harshica; Prairie View A&M University

COMMUNICATION

"Glow ELISA": Sensitive Immunoassay with Minimal Equipment and Stable Reagents

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Binh V. Vu [a], Kristen Brosamer [b], Naiyah McDaniel[c], Katerina Kourentzi [a], Richard C. Willson [a,b,d,e], and Harshica Fernando[c]

Glow enzyme-linked immunosorbent assay (Glow ELISA) uses inexpensive and shelf-stable glow stick reagents to chemically excite fluorescent reporters, obviating the need for excitation light sources, filters, and complex optics. It achieves excellent limits of detection while offering portability and equipment cost comparable to lateral flow immunoassays.

Introduction

Enzyme-Linked Immunosorbent Assay (ELISA) is widely used for detecting and quantifying target analytes in biological and clinical samples. Advancements in assay chemistry, microfluidics, and automation have been implemented in an effort to simplify the ELISA workflow[1–5] and several commercially available ELISAs are now based on a 90-minute, 3-step, single-wash protocol (e.g. SimpleStep ELISA from Abcam or the Quantikine ELISA from R&D Systems). ELISA readout, however, still relies on complex and expensive plate readers that measure either absorbance or fluorescence. These are common in centralized laboratories or large hospitals but often cost-prohibitive for smaller laboratories or other resource-limited settings. Moreover, the operation of these sophisticated instruments often requires specialized training, limiting their accessibility and practicality.

[a.] *William A. Brookshire Department of Chemical and Biomolecular Engineering, University of Houston, Houston, Texas, USA*
[b.] *Department of Biomedical Engineering, University of Houston, Houston, Texas, USA*
[c.] *Department of Chemistry, Prairie View A&M University, Prairie View, Texas, USA*
[d.] *Tecnológico de Monterrey, Institute for Obesity Research, Monterrey, Nuevo Leon, Mexico.*
[e.] *Tecnológico de Monterrey, Escuela de Medicina y Ciencias de la Salud, Monterrey, Nuevo Leon, Mexico.*
E-mail: bvvu@uh.edu, willson@uh.edu, hufernando@pvamu.edu

The widespread availability of smartphones with high-resolution cameras and substantial computing power has paved the way for their utilization in scientific research and medical diagnostics opening new possibilities for simplified ELISA readout towards the democratization of highly-sensitive immunoassays.[6] These approaches simplify the ELISA readout by replacing the plate reader with a smartphone.[7–10] However, they require the addition of an external light source to excite the reporters or to minimize the effect of the variability of ambient light.

Here we present a simple, smartphone-readable "Glow" ELISA technology based on the shelf-stable oxalate reagents (originally bis(2,4,6-trichlorophenyl) oxalate, TCPO[11]) used in glowsticks (and more recently by our group in lateral flow assays[12]) that does not require an external light source. In glowsticks TCPO reacts with H_2O_2 to produce the high-energy intermediate 1,2-dioxetanedione, which chemi-excites any of several fluors to produce light of different colours. Oxalate chemi-excitation reaction is among the most efficient light-producing reactions (QY 5–50%[13] depending on fluorophore). In this first demonstration of Glow ELISA, fluorescent resorufin is produced by horseradish peroxidase (HRP)-catalysed oxidation of a fluorogenic substrate, Amplex Red (10-acetyl-3,7-dihydroxyphenoxazine). Chemi-excitation of resorufin produces a bright glow that is readily captured by a smartphone camera (Figure 1). This approach has wide applicability, as Amplex Red can report the activity of diverse enzymes producing H_2O_2 and Glow chemistry can excite a broad range of fluors, supporting colour multiplexing.

Human chorionic gonadotropin (hCG), the most widely used pregnancy marker and immunoassay model system, was used for this initial demonstration of Glow ELISA. We also found that Glow ELISA imaged with an unmodified smartphone has comparable sensitivity to a fluorescent ELISA read by a

conventional plate reader for the detection of HIV-1 p24 protein in human serum.

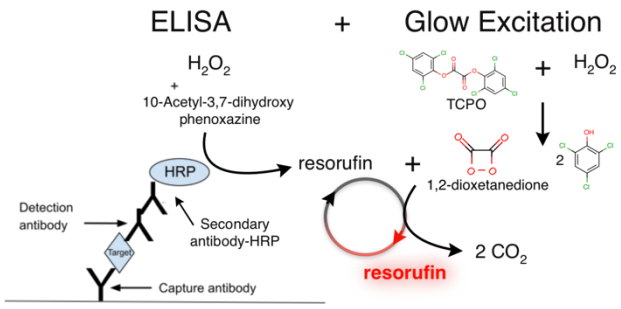

Figure 1. Glow ELISA. Glow ELISA has the same format and workflow as traditional ELISA except for the signal generation and readout. The Amplex Red reagent (a fluorogenic ELISA substrate), in the presence of HRP enzymes, reacts with hydrogen peroxide and is converted to the fluorescent dye resorufin. TCPO reacts with hydrogen peroxide to generate the energy-rich intermediate 1,2-dioxetanedione, which chemically excites nearby resorufin molecules to emit visible light.

Materials and methods

Materials

Bovine serum albumin (BSA) (Cat# A9418), human chorionic gonadotropin (hCG) (Cat# CG10), Tween-20 (Cat# P1379), hydrogen peroxide 30% (Cat# 216763-500ML), butyl benzoate (Cat# 293296), tert-butanol (Cat# 471712), Acetone (Cat# 270725-1L), and Bis(2,4,6-trichlorophenyl) Oxalate (Cat# O3629) were purchased from Millipore-Sigma. White 96-Well Immuno Plates (Cat# 436110), 1-Step TMB ELISA Substrate Solutions (Cat# 34028), and Amplex UltraRed Reagent (Cat# A36006) were purchased from Thermo Fisher Scientific. Transparent 96-Well Polystyrene Medisorp Plates (Nunc Cat# 467320) and Black 96-Well Polystyrene Plates (Corning Cat# 3991) were purchased from Fisher Scientific. Mouse anti-β hCG antibody (clone 2; Cat#CGBCG-0402) and Goat polyclonal anti-hCG-α antibodies (Cat# ABACG-0500) were purchased from Arista Biologicals. Peroxidase AffiniPure Donkey anti-Goat IgG (H+L) (Cat# 705-035-003) was purchased from Jackson Immuno Research. HIV-1 p24 SimpleStep ELISA Kit (Cat# ab218268) was purchased from Abcam. Phosphate buffered saline (1X PBS) tablets were purchased from Takara. Serum from anonymous healthy donors was obtained from Gulf Coast Regional Blood Center, Houston, Texas, and stored at −20 °C until used.

Methods

Detection of hCG with Glow ELISA

Medisorp polystyrene microplate wells were coated with 100 µL of 5 µg/mL monoclonal mouse anti-β hCG antibody in PBS and incubated overnight. The wells were then blocked with 300 µL of 3% BSA in PBS for 1 hr at 37°C. After blocking, a 2-fold dilution series of hCG diluted either in 1% BSA in PBS or in 25% human serum (pre-diluted in PBS) was added to the wells. PBS-1% BSA was added to blank wells to serve as negative control. The plate was incubated for 1 hr at 37°C. The wells were then decanted and washed 3 times with PBS-0.1% Tween 20 using a Tecan Hydroflex plate washer. Next, goat polyclonal anti-α hCG antibody diluted in PBS-1% BSA (100 µL of 500 ng/mL) was added to the wells and incubated for 1 hr at 37°C. After incubation, the wells were decanted, and the plate was washed 3 times with PBS-0.1% Tween-20. Next, secondary anti-goat antibody-HRP conjugate was added to the wells (100 µL of 1:3000 Ab in PBS-1% BSA), and the wells were washed 3 times. The ELISA signal was then developed and read using three different methods: TMB absorbance, optically-excited Amplex Red fluorescence, and Amplex Red + Glow chemi-excitation. The colorimetric signal was developed using TMB (100 µL), incubated for 5 min, then stopped using 2M H_2SO_4. The absorbance (OD450) was measured using a Tecan Infinite M200 Pro. The fluorescent signal was developed using freshly prepared Amplex Red substrate (50 µL/well), incubated for 10 min, then the samples were transferred to black polystyrene plates. The optically-excited fluorescent signal was measured (Excitation 530 nm/Emission 590 nm) using Tecan plate reader. After reading of optically-excited fluorescence, samples were transferred to white polypropylene plates (25 µL/well) and Glow substrate (100 µL/well) was added to develop the Glow signal. Glow reagent was freshly prepared (for each well: 25 µL of 30 mM of TCPO in butyl benzoate, 25 µL of 3% H_2O_2 in t-butanol, and 50 µL of acetone), added to each well, then immediately imaged inside a dark box (Figure 2) using the built-in Samsung Note 8 camera in Pro mode with an exposure time of 10 s (max), white balance set to 5500 K, and ISO set to 800 (max). The limit of detection (LoD) values were determined as the minimum tested analyte concentration with signal above the mean of the blank plus three standard deviations of the blank (µ + 3σ; denoted by the horizontal dashed lines on the graphs).

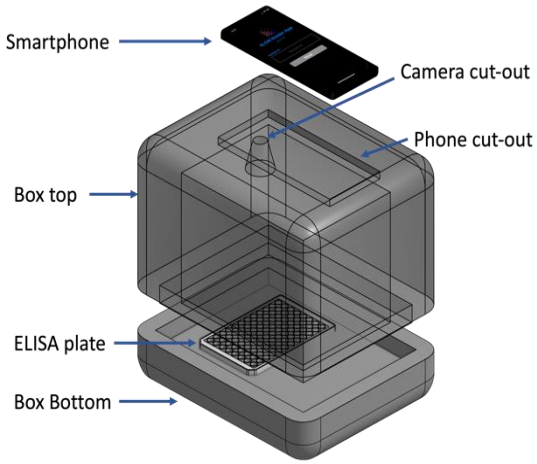

Figure 2. Dark box for smartphone-based readout. The Styrofoam box was opacified with aluminium foil wrapped on the outside and had a shallow cut-out for the smartphone and a thru-hole for the camera. The internal surface is preferably covered with light absorbing material to minimize reflection. However, the dark box used in this work was not treated since the reflection was not significant, as shown in Figure SI1.

Analysis of Smartphone Images

Glow ELISA signal intensities were extracted from the smartphone image's red channel using the NIH ImageJ[14] particle analysis tool. Colour channels were isolated by navigating to "Image," "Color," and "Split Channels." Only the red channel was selected. To separate the signal from the circular wells from the background signal, the threshold was adjusted with "Adjust" and "Threshold" in the "Image" menu. Threshold levels were adjusted until the wells were correctly segmented. The "Auto" button can help obtain an initial threshold; if necessary the threshold can then be manually fine-tuned. Next, the "Analyze Particles" tool from the "Analyze" menu was applied. In the particle analysis dialog box, the minimum and maximum particle size were specified based on the expected sizes of the circular wells. The "Display Results" option was enabled, and the "Results" window provides measurements for each identified well, including the area and the integrated sum of pixel values of the red channel as a measure of the well's signal intensity. The average intensity of each well was calculated by dividing the integrated intensity by its area.

Comparison of sensitivity between different readout methods

Detection antibody-HRP conjugate included in the HIV-1 p24 ELISA kit was serially diluted and added to the wells in triplicate. TMB and Amplex Red were used as HRP substrates. TMB reactions were stopped after 10 minutes of incubation, and the absorbance was read at 450 nm. Amplex Red reactions were read with fluorescence, absorbance, and Glow after 10 minutes. Sensitivity was determined as previously described at a dilution level below 1:81,000, equivalent to 4 mU/ml.

Detection of HIV-1 p24 in Serum with Glow ELISA

A commercially available colorimetric ELISA (ab218268 HIV-1 p24 SimpleStep ELISA® Kit) for the detection of HIV-1 p24 in serum was used according to the manufacturer's protocol. The 96 well plate-format strips included with the kit were supplied ready to use. All reagents and the working standards were prepared as directed in the protocol. All materials and prepared reagents were equilibrated to room temperature before use. The test samples were prepared by first spiking p24 protein into 50% serum (prediluted 1:1 with PBS) to prepare the highest p24 concentration sample. Subsequent samples were prepared by serial dilutions of the first sample with 50% serum to generate samples with p24 concentrations ranging from 150 to 5 pg/mL. 50% serum without p24 served as the negative control. 50 µL of samples in triplicate were added to appropriate wells followed by 50 µL of Antibody Cocktail. The plate was sealed and incubated for 1 hour at room temperature on an orbital shaker (Cat# 7744-20220, Bellco Glass, Inc) at 400 rpm. After incubation, the plate was washed thrice with 350 µL 1X Wash Buffer PT (supplied in the kit) using a Tecan Hydroflex plate washer. After the last wash, the plate was inverted and blotted against clean paper towels to remove excess liquid. The ELISA signal was developed using (50 µL/well) Amplex Red instead of the colorimetric TMB supplied in the kit. The Amplex Red substrate was prepared fresh (10 mM substrate in anhydrous DMSO) according to the Manufacturer's instructions. The absorbance and fluorescence were measured with the plate reader as described previously. After measurement of optically-excited fluorescence, Glow substrate was added to the wells

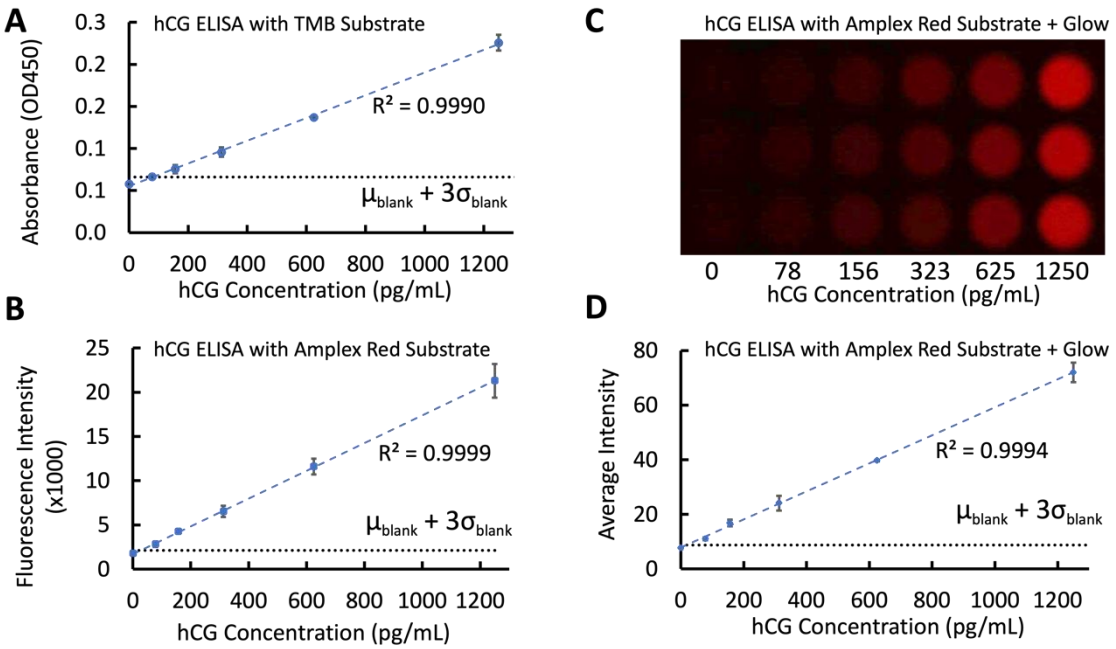

Figure 3. Comparison of three different methods of reading out hCG ELISA. (A) hCG ELISA was developed with TMB as substrate with 5-minute incubation and stopped with 2M H_2SO_4. The absorbance of the samples was read at 450 nm with the plate reader. (B) hCG ELISA was developed with Amplex Red with 10-minute incubation. The optically-excited fluorescence (Excitation 530 nm/Emission 590 nm) was read with the plate reader. (C) the developed substrate in (B) was transferred to the white polypropylene plate for Glow chemical excitation and a long-exposure (8 s) image of the plate wells was immediately taken. (D) Glow intensity signal was extracted from the image (C) using ImageJ. The dashed horizontal lines mark the mean of the blank plus three times the standard deviation of the blank ($\mu_{blank} + 3\sigma_{blank}$) for determining the limit of detection (LoD).

to develop a Glow signal. Glow reagent was mixed fresh (for each well: 50 µL of 30 mM of TCPO in butyl benzoate, 50 µL of 3% H_2O_2 in t-butanol, and 100 µL of acetone) and added to each well, then immediately imaged with a Samsung Note 8 as described above for hCG.

Results and discussion

Amplex Red is highly sensitive and ThermoFisher Scientific's best fluorogenic substrate for horseradish peroxidase. Upon reaction with H_2O_2 the substrate is converted to red-fluorescent resorufin. Resorufin also can be read by absorbance due to its high extinction coefficient (58,000 $cm^{-1}M^{-1}$)[15]. When chemi-excited with the Glow (peroxyoxalate) chemistry, resorufin emits a bright red (585 nm) light readily imaged in a smartphone camera's red channel.[16]

Detection of hCG with Glow ELISA

As an initial proof of concept, we detected hCG in buffer using three different readout methods: absorbance, optically-excited fluorescence, and Glow chemically-excited fluorescence. By employing Amplex Red substrate followed by chemi-excitation with peroxyoxalate chemistry, we achieved a very low limit of detection of 78 pg/mL, generating a vivid red light while keeping the background noise to a minimum (Figure 3). Determining the optimal incubation time of the Amplex Red substrate prior to chemi-excitation proved to be crucial, as the introduction of the glow reagent halts the generation of resorufin. We determined that the ideal time to introduce the Glow reagent is 5 to 10 minutes after incubation or when the well with highest concentration of the standard curve has a faint pink colour; in any case detection of p24 in serum done repetitively (or any standardized assay) would have a fixed incubation time. To compare Glow with standard readout techniques, we used TMB substrate with the absorbance measured at 450 nm and Amplex Red substrate with the fluorescence reading (Excitation 530 nm/Emission 590 nm) using a plate reader. Subsequently, we added the Glow reagent to the developed Amplex Red and captured images using a smartphone camera with a Styrofoam dark box. All three readout methods yielded excellent linearity in the 78-1250 pg/mL range. Both optical and chemically-excited fluorescence gave LoD of 78 pg/mL, well below that obtained with absorbance detection (156 pg/mL). To test the applicability of Glow ELISA to complex clinical samples, we demonstrated the detection of hCG in 25% serum and at lower hCG concentrations (16 pg/mL to 500 pg/mL), with a LOD of 63 pg/mL and excellent linearity (R^2 = 0.98) (Figure 4).

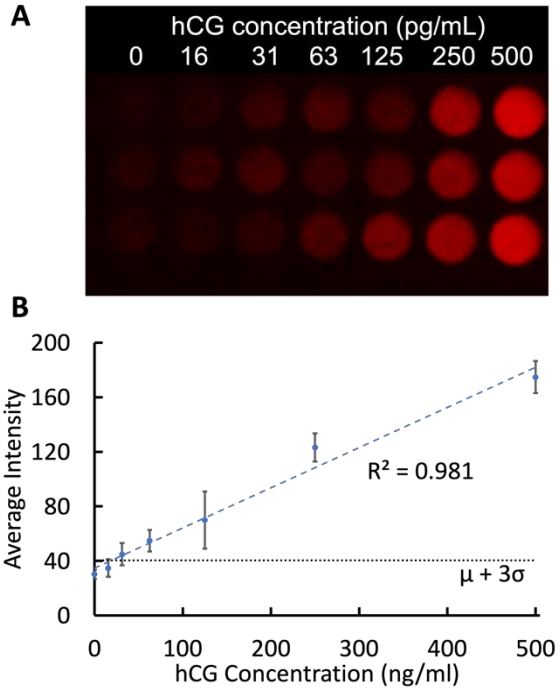

Figure 4. Glow ELISA detection of hCG in 25% serum. Dilution series of 16 - 500 pg/mL of hCG in 25% serum in triplicate detected with Glow ELISA. (A) Smartphone image of plate wells taken immediately after Glow excitation. (B) Graph of average Glow intensities extracted from Glow images in (A) by ImageJ analysis.

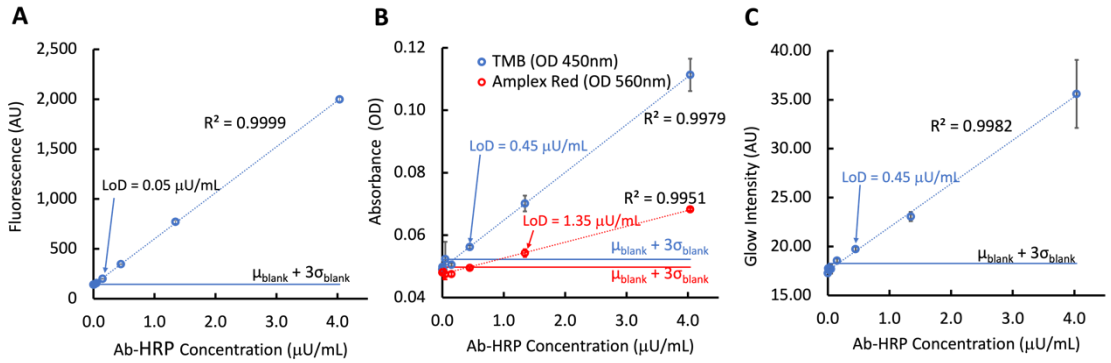

Figure 5. Comparison of the detection of antibody -HRP (Ab-HRP) conjugate with optically-excited fluorescence, absorbance (TMB and Amplex Red), and Glow excitation. A dilution series of Ab-HRP (0.01 – 4 µU/mL) in PBS (n=3) was detected with either TMB or Amplex Red substrates with 10 minutes incubation. (A) Fluorescent intensity readout using a plate reader (Excitation 530 nm/Emission 590 nm) and (B) Absorbance readout of TMB and Amplex Red substrates at 450 nm and 560 nm, respectively. (C) Glow intensity extracted from smartphone Glow image analysed with ImageJ. The mean of the blank plus three times the standard deviation of the blank ($\mu_{blank} + 3\sigma_{blank}$) was used to determine the limits of detection (LoD).

Comparison of sensitivity between different readout methods

To eliminate variability from multiple steps in ELISA, known amounts of antibody-HRP conjugate were serially diluted and added to the wells (n=3). The enzyme activity of the antibody-

HRP conjugate was assessed by referencing the standard curve generated by diluting the HRP supplied in the Amplex Red kit (10 U/mL stock concentration). TMB and Amplex Red were used as HRP substrates. After 10 minutes of incubation, the TMB reactions were stopped, and the absorbance was read at 450 nm. Amplex Red reactions were read out with fluorescence, absorbance, and Glow after 10 minutes of incubation. All the readout methods showed excellent linearity for the Ab-HRP range of 0.01 – 4 μU/mL (Figure 5). Fluorescent intensity readout produced the lowest LoD, 0.05 μU/mL, while both TMB absorbance and Glow readout methods gave the same LoD of 0.45 μU/mL. Amplex Red absorbance was the least sensitive with LoD of 1.35 μU/mL.

Detection of HIV-1 p24 in Serum with Glow ELISA

In order to demonstrate the application of the chemi-excited Glow ELISA in a clinically relevant scenario, we used it to read out a commercial ELISA for HIV-1 p24 protein in human serum. fluorogenic reporter enzyme substrate with chemical excitation of fluorescence. This innovative technique generates a bright visible light signal which can be captured using a smartphone and a basic dark box made of cardboard or Styrofoam. Glowsticks provide proof-of-concept for packaging and portable use of Glow reagents. Glow ELISA surpasses the sensitivity of absorbance ELISA and matches the performance of optically-excited fluorescence in both hCG and HIV-1 p24 model assays. We envision integrating a smartphone app[17–19] that enables automated signal analysis, further streamlining the process. This approach eliminates the need for intricate and costly plate-reading equipment often unavailable in resource-limited settings. The Amplex Red reagent with Glow chemi-excitation is applicable to a wide range of peroxide-producing enzymatic reactions.

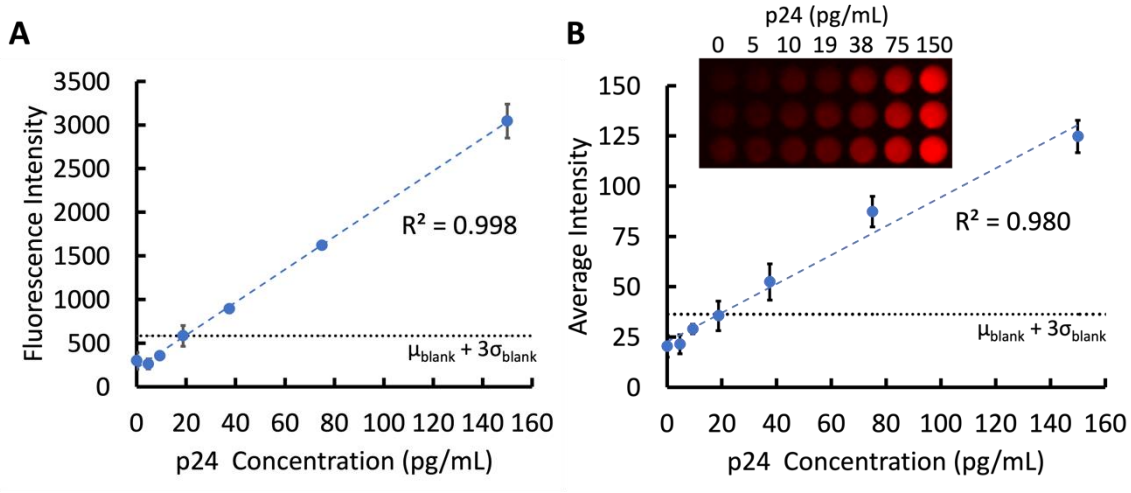

Figure 6. Comparison of the detection of HIV-1 p24 in 50% serum with optically-excited fluorescent and Glow ELISA. A dilution series of p24 (5 - 150 pg/mL) in 50% serum (n=3) was detected with (A) Fluorescent intensity using a plate reader (Excitation 530 nm/Emission 590 nm) and (B) Glow intensity extracted from smartphone Glow image (inset) analysed with ImageJ.

The p24 concentration in the test samples ranged from 5 to 150 pg/mL in 50% serum; negative controls were 50% serum devoid of p24. Both optically-excited fluorescence and Glow chemi-excited ELISA gave an LoD of 37.5 pg/mL, as illustrated in Figure 5, and each gave good linearity within the 5-150 pg/mL range (Figure 6). The average coefficient of variation (%CV) for the Glow ELISA was 15%, and that with optically-excited fluorescence was 13%. The Glow ELISA has a slight lower R^2 linearity, probably due to automatic image processing by the phone firmware (e.g., JPEG compression, gamma correction). This would not matter for many yes/no pathogen diagnostics, but RAW format image capture could improve the linearity of Glow ELISA. A smartphone app could both capture RAW images and automate image analysis (as we have previously developed for other light-based reporters[17]).

Conclusions

Inspired by glowsticks (extremely bright, shelf-stable for years, temperature-tolerant, and routinely used even by young children) we have developed a Glow ELISA by combining

Author Contributions

B.V.: Conceptualization, Methodology, Investigation, Validation, Visualization, Funding Acquisition, Writing—original draft, review and editing; K.B.: Conceptualization, Methodology, Investigation, Validation, Writing—review and editing; N.M.: Conceptualization, Methodology, Investigation, Validation; K.K.: Methodology, Resources, Visualization, Funding Acquisition, Writing—review and editing; R.C.W.: Conceptualization, Methodology, Supervision, Visualization, Funding Acquisition, Writing—review and editing; H.F.: Conceptualization, Methodology, Resources, Supervision, Funding Acquisition, Writing—review and editing.

Conflicts of interest

B.V.V. and R.C.W. are named inventors of IP which could relate to the subject of this paper. All other authors declare no competing interests.

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