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ORIGINAL ARTICLE

Smartphone-based colorimetric ELISA implementation for determination of women's reproductive steroid hormone profiles

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Abstract Biologists frequently collect and analyze biospecimens in naturalistic (i.e., field) conditions to ascertain information regarding the physiological status of their study participants. Generally, field-collected biospecimens need to be stored frozen in the field and then transported frozen to laboratory facilities where traditional biomarker assays, such as enzyme-linked immunosorbent assays (ELISAs), are conducted. As proper storage and transport of frozen specimens is often logistically difficult and expensive, particularly in nonurban field settings, methods that reduce the need for specimen storage and transport would benefit field-research dependent disciplines such as biology, ecology and epidemiology. One limiting factor to running assays in the field is the use of large and expensive equipment to visualize and quantify the assays, such as microplate readers. Here, we describe an implementation of colorimetric ELISA visualization and quantification using two novel and portable imaging instrumentation systems and data processing techniques for the determination of women's reproductive steroid hormone profiles. Using the light absorbance and transmittance properties of the chemical compounds that make up the hormone assay, we were able to estimate unknown hormone concentrations using a smartphone system and a webcam system. These estimates were comparable to those from a standard laboratory multiple reader (smartphone: accuracy = 82.20%, $R^2 > 0.910$; webcam: accuracy = 87.59%, $R^2 > 0.942$). This line of applied research, in the long run, is expected to provide necessary information for examining the extent to which reproductive function varies within and between populations and how it is influenced by psychosocial, energetic and environmental challenges. Our validation of these novel, portable visualization and quantification systems allows for the eventual development of a compact and economical closed system which can be used to quantify biomarker concentrations in remote areas.

Keywords Enzyme-linked immunosorbent assay (ELISA) · Colorimetry · Spectrophotometry · Immunoenzyme techniques · Image processing · Computer-assisted

1 Introduction

Frequent collection and analysis of biospecimens in naturalistic (i.e., field) conditions are typical of longitudinal naturalistic study designs [9]. As traditional hormone assays are conducted in a laboratory setting, field-collected biospecimens need to be frozen and properly stored in the field and then transported frozen to laboratory facilities where specialized equipment is available. In nonurban field sites, freezer space is often scarce and transport of frozen specimens is logistically difficult and expensive. Therefore, methods that reduce the need for specimen storage and transport are important to field-research-dependent disciplines such as biology, ecology and epidemiology.

One option is to perform the assays in the field. This will require portable specialized equipment. For example, enzyme-linked immunosorbent assays (ELISA) generally

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utilize microtiter plate readers to detect changes in color for the *quantification of hormone concentrations*. These plate readers are expensive and require line-electrical power supply to operate in the field. Thus, there is a need for a more cost-effective and portable method to visualize and quantify colorimetric assays. Using a smartphone or a webcam to accomplish the task of quantifying the colorimetric ELISA would be a portable as well as an economical solution which could be deployed in the field. Images of the microtiter plate could be taken using a smartphone or a webcam, and standard *Digital Image Processing techniques* could then be used to analyze the pictures. Digital Image Processing is a form of signal processing where the input is an image (photograph or video frame); the output can be either an image or a set of characteristics or parameters related to the image. Most image processing techniques involve treating the image as a two-dimensional signal and applying standard signal processing techniques to it [5]. These techniques allow for a wider range of algorithms that can be applied to the captured image and extract a considerable amount of information that can be applied for the purpose of analysis. These features offer a new paradigm in image processing applied to biochemical analysis in a portable platform. The image processing techniques can be applied using an application developed exclusively for the smartphone's operating system platform or in a PC/laptop computer application by transferring the pictures. A web-based application could also be deployed for similar purposes for communities where internet access is available.

Here we compare the ability of novel smartphone and webcam systems to accurately quantify women's reproductive hormone levels using ELISA against a standard laboratory microtiter plate reader.

2 Methods

2.1 Ethics

Urine specimen collection in 2000–2001 and analysis were approved by the University of Michigan's Institutional Review Board. Informed consent was obtained from all individual participants included in the study. Secondary analysis of these specimens was approved by Simon Fraser University's Ethics Review Board.

2.2 Archived samples

First morning urine specimens were collected between 2000 and 2001 in the context of the Society, Environment and Reproduction (SER) study [7, 8]. Briefly, urine specimens were collected three times per week from women in two rural Kaqchikel Mayan communities in the southwest highlands of Guatemala who were parous, not using any form of chemical contraception, and at least 6 months from the birth of their last child. Sample was frozen at −10 °C for up to 6 months in the field and then chipped on dry ice from Guatemala to the laboratory at the University of Michigan, where they were archived at −80 °C. In 2010, samples were shipped on dry ice to the Maternal and Child Health laboratory at Simon Fraser University, where they were again stored at −80 °C until analysis for the quantification of reproductive hormone levels.

2.3 Hormone analysis

A competitive ELISA was used to quantify urinary concentrations of pregnanediol glucuronide (PdG), a urinary metabolite of progesterone, a reproductive steroid hormone, in urine samples of unknown concentrations using the protocol described by O'Connor et al. [10]. A negative control comprised of deionized distilled water and a positive control consisting of a pool of urine samples collected from women in the luteal phase of the menstrual cycle were used. All standards, controls and samples were run in duplicate. In this assay, each microtiter well is coated with an anti-PdG monoclonal antibody (clone 220, Quidel). The PdG in each sample or standard competes with horseradish peroxidase—conjugated PdG to bind to the antibodies. Following the addition of substrate to each well, the horseradish peroxidase activity and resulting color intensity are inversely proportional to the concentration of PdG in the sample or standard. The PdG concentration of each sample was quantified by comparing the color intensity of each sample to that of an eight-point standard curve generated using a four-parameter logistic regression. The standard curve ranged in concentration from 19.53 to 2500 ng mL^{-1}.

A Victor X5 2030 multilabel microplate reader (PerkinElmer) was used as a standard microtiter plate reader for quantification of hormone concentration. The absorbance of each well was measured using a test wavelength of 405 nm and reference wavelength of 595 nm. PdG concentrations were estimated from the absorbance of the eight-point standard curve in Workout software (PerkinElmer).

2.4 Experimental smartphone and webcam setup

Concurrently, a series of pictures of the microtiter plate were captured using an iPhone 4S and Logitech c920 webcam. The images taken from the web camera (Logitech c920) are about 8 MP, and the images taken using a smartphone are about 5 MP. As we are dealing with the color of individual pixel or a group of pixels, the white balance and the color reproduction of the camera play an important role compared to the resolution of the image. Higher resolution

gives us more samples in each well leading to a better average. These images were then transferred to a PC for analysis. The process of capturing images using a smartphone and a webcam was performed using an inexpensive setup which consists of the following components.

2.4.1 Light source

In imaging, appropriate light source is essential. The direction of the light will affect how the shape and texture of the subject is recorded. Similarly, the characteristics of the light source have considerable influence on the recorded image and subsequent interpretation of the results. Therefore, it was extremely important that the source of light used for our experiment remained constant, regardless of the time and place where the assay was performed. By using a *well-calibrated, dedicated light source*, we can reduce, if not eliminate, the effect of environmental lighting conditions in the field. For these purposes, the ELastoLite® from Oryon Technologies, an *electroluminescent lamp* that emits light by the direct conversion of electrical energy into light through energized phosphors was used as the source of light spectrum producing uniform light across 5 × 3 inch surface. It was attached to the lid of the microtiter plate.

2.4.2 Enclosure

It was also extremely important to make sure that the images we captured were not adversely affected by the environmental lighting conditions. Hence, we used an enclosure over the entire setup. For the demonstration prototype, this enclosure was built using cardboard, which was lightweight and portable. The inside walls of the enclosure were painted black to avoid any stray reflections of ambient light. This is shown in Fig. 2.

2.4.3 Camera

To capture the images, we used an *iPhone 4S*, which is a common mid-tier or low-end smartphone by today's market standards, and a *Logitech c920* webcam to capture the images. Using two types of portable cameras allowed us to compare the quality of our results and interpret them for configuring the portable analysis systems. Images from both the iPhone and webcam were saved in JPEG lossy compression format.

The microtiter plate was positioned between the light source and the camera in such a way that the camera recorded the light transmitted through the plate. The distance between the electroluminescence sheet and the plate was approximately 1 cm, and the distance between the plate and the camera lens was approximately 30 cm. The optical axis of the camera passed through the center of the electroluminescence panel and the microtiter plate.

2.5 Implementation

As the light passed through the microtiter plate, variation in hormone concentration among the standards, controls and samples altered the amount of light transmitted through each well. Based on the hormone concentration present in

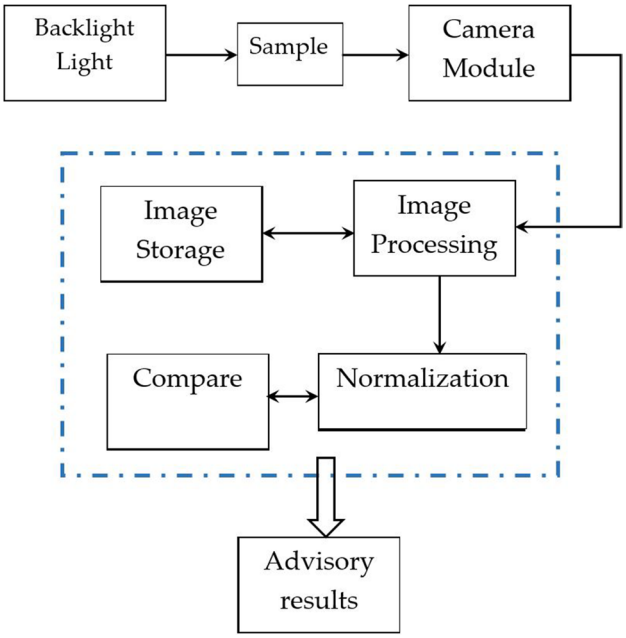

Fig. 1 Flowchart of the imagebased implementation of the colorimetric ELISA

Fig. 2 Imaging prototype which has been using for the initial demonstrations

the sample, the corresponding modulation in the intensity of the color was recorded by the camera. Color intensities of the wells were recorded on the camera as images and were digitally processed in MATLAB® (MathWorks), a fourth-generation programming language developed for implementing the processing of the captured images, using a PC. The MATLAB code was programmed to differentiate color shade variations, which are normally quite difficult to be quantified or observed by the human eye. The code was written such that the RGB values from all of the wells were averaged to *grayscale*, which were then used as the *intensity* values. The wells containing the standards were used as reference points for processing the image. Figure 1 illustrates the process flowchart of our proposed hormone ELISA using smartphone and webcam cameras.

Using the known PdG concentrations of the standards and their intensity values extracted from the smartphone and webcam images of the microtiter plates by the MATLAB program, we were able to plot nonlinear regression curves (i.e., eight-point standard curves) using SigmaPlot 13 (Systat Software Inc.), a scientific data analysis and graphing software. The hormone concentrations of the unknown samples were then interpolated by mapping their MATLAB-derived intensity values onto the standard curves. The total time taken for the analysis part would be a maximum of about a couple of seconds though it depends on the computing power of the system (Fig. 2).

2.6 Statistical analyses

The PdG concentrations quantified by the plate reader were used as a reference for validating the novel smartphone and webcam approaches. Using linear regression models in JMP 12 (SAS Institute), the smartphone- and webcam-based concentration estimates were compared to the concentration results from the Victor™ X5 multilabel plate reader.

3 Results

Figures 3, 4 and 5 show the intensity versus PdG concentration standard curves for the smartphone system, webcam system and the plate reader, respectively. Table 1 shows the predicted PdG concentrations for 10 representative samples of unknown concentration based on the color intensity values extracted from the images captured by the iPhone 4S and the Logitech c920 webcam in comparison with the PdG concentrations based on the absorbance values from the Victor X5 multilabel plate reader. For the purpose of this publication, we have only tabulated estimates from 10 samples (in Table 1). However, the following statistical comparisons of the smartphone, webcam and plate reader systems were based on PdG estimates from 32 samples of unknown concentration.

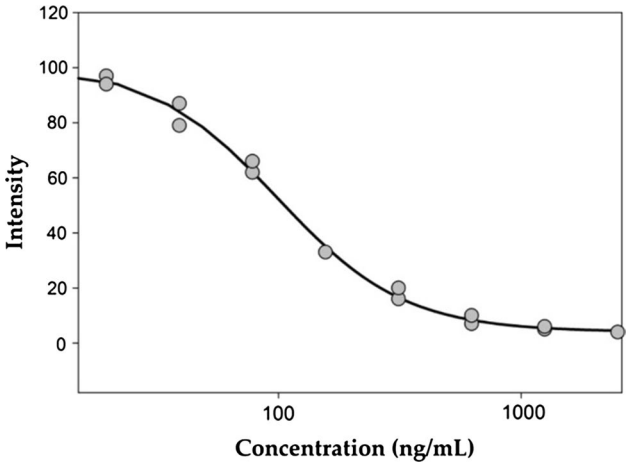

Fig. 3 Intensity versus concentration plot (eight-point standard curve) for the iPhone 4S smartphone

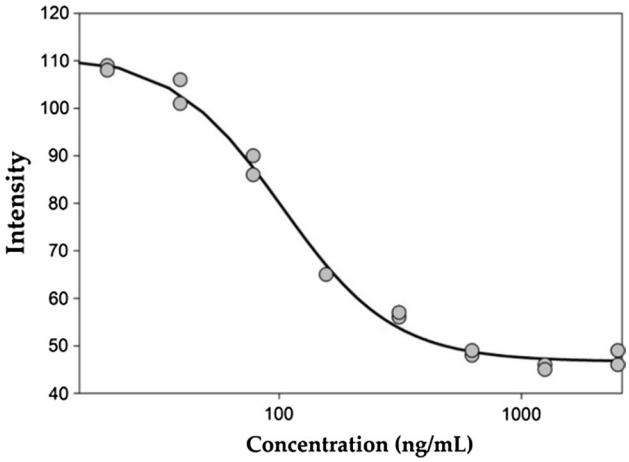

Fig. 4 Intensity versus concentration plot (eight-point standard curve) for the Logitech c920 webcam

The statistical mean accuracy of the iPhone 4S system was estimated to be 82.20%, while that of the Logitech c920 system was 87.59% compared to that of the multiplate reader. The PdG concentration estimates from the smartphone ($R^2 > 0.910$; slope = 0.73; intercept = 25.85) and the webcam systems ($R^2 > 0.942$; slope = 1.19; intercept = −19.48) were significantly positively correlated with those from the Victor multiplate reader ($n = 32$, both $p < 0.0001$) (Fig. 5).

4 Discussion

Our results confirm that the standard curves and estimated unknown concentrations generated by the novel smartphone and webcam imaging and data processing systems

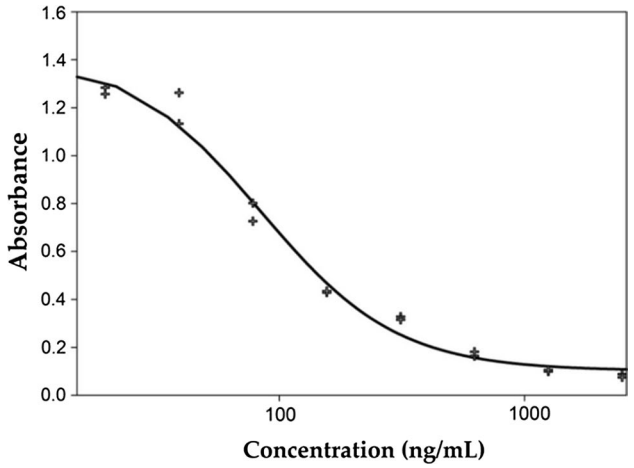

Fig. 5 Absorbance versus concentration plot (eight-point standard curve) for the Victor™ X5 multilabel plate reader

were comparable to a laboratory "golden" standard multiplate reader for the quantification of PdG using a colorimetric ELISA. While lower concentrations of PdG were more comparable among systems, there was a slightly higher variation in the concentration estimates of samples with higher PdG levels (Fig. 6). These higher values fell near the edge of the linear portion of the standard curves in the colorimetric ELISA, where the curves start to flatten out. This suggests that the smartphone and webcam systems were less sensitive at detecting variation among lighter-colored, higher-concentration samples than darker-colored, lower-concentration samples. This issue can easily be fixed by diluting samples so that their concentrations fall on the more linear portion of the standard curve. Sample dilution is a common practice in the laboratory when working with samples with concentrations at the edge of the linear portion of the standard curve [1]. This adjustment would increase both the statistical mean accuracy and the linear correlations of the smartphone and webcam systems compared to the multiplate reader. Thus, our results suggest that large and expensive microplate readers can be replaced with these more economic and widely available portable devices for the quantification of colorimetric ELISAs without compromising measurement accuracy.

These novel systems enable ELISAs to be quantified in the field without having to transport large and expensive microplate readers to remote locations. This would minimize or eliminate the need to freeze and ship specimens back to the laboratory for analyses, and these tasks, which require purchasing and maintaining a freezer and the ability to purchase ample amounts of dry ice to ensure the samples remain frozen during shipping, are often logistically difficult and expensive, particularly in remote field locations. Thus, these novel visualization and quantification systems have the potential to increase researchers' ability to collect and analyze biospecimens in remote field locations, requiring only electricity and refrigeration (for short-term storage of samples and ELISA reagents). The ability to quantify biomarkers while in the field would enable researchers to gauge the current physiological status of their participants, provide faster feedback for experimental manipulations and facilitate the adjustment of study design or planning of follow-up studies in a time-efficient and data-responsive manner.

A single reproductive hormone value per woman is inadequate to determine the woman's reproductive status (e.g., phase of the menstrual cycle). By eliminating the need for expensive microplate readers, these systems decrease the costs of ELISAs, making it more economically feasible for researchers to collect multiple biospecimens per woman. This would allow for the quantification of hormone concentrations within women across consecutive days and the generation of reproductive hormone profiles, which can be used to assess reproductive status and health. In the long run, this line of research is expected to contribute to our understanding of the extent to which reproductive function varies within and between populations and how it is influenced by psychosocial, energetic and environmental challenges.

Similar smartphone-based technologies have been used to acquire images and analyze colorimetric variation in ELISAs for the purpose of quantifying different

Table 1 Comparison of PdG concentrations (ng mL^{-1}) for 10 representative samples estimated using standard laboratory microtiter plate reader (Victor X5), smartphone (iPhone 4S) and webcam (Logitech c920) systems

Unknown	Victor X5 (from workout)	iPhone 4S (from SigmaPlot)	Logitech c920 (from SigmaPlot)
1	203.97	223.54	215.90
2	180.93	146.05	175.62
3	213.49	158.86	195.90
4	140.99	152.24	147.54
5	457.30	485.37	672.67
6	149.17	134.76	162.07
7	212.69	204.17	234.05
8	180.81	165.95	205.39
9	256.85	227.86	234.05
10	292.98	342.45	294.26

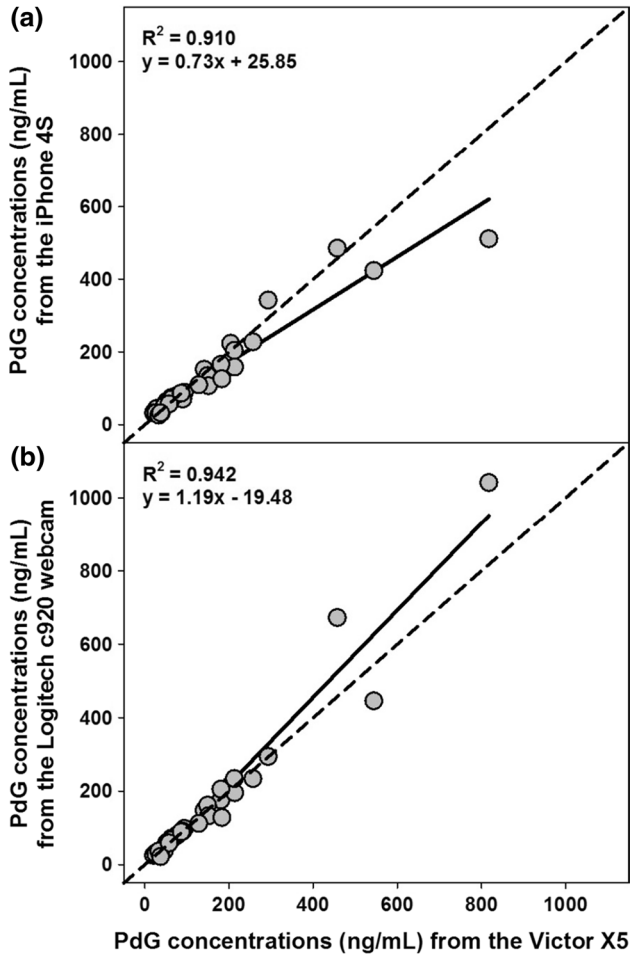

Fig. 6 Linear relationships between the estimated PdG concentrations from the **a** iPhone 4S system and **b** Logitech c920 system and the Victor X5 multiplate reader. PdG concentration estimates from the smartphone and webcam systems were highly positively correlated with those from the microtiter plate reader ($n = 32$, both $p < 0.0001$). *Dashed lines* represent unity

physiological compounds, including proteins and cancer biomarkers [6, 12]. The novelty of our approach remains in providing a economic and portable modeling system adopting the technology to the microtiter plate which has been the laboratory standard tool in analytical research and clinical diagnostic testing laboratories for a couple of decades.

4.1 Future directions

Our future work involves designing an adaptive light illumination system to improve the visualization of colorimetric assay plates, which would change the color temperature and the intensity of the backlight system, thereby assisting the image processing algorithms for better accuracy. We are also developing a software program which would quantify the ELISA using a 4-parameter logistic regression model and employ machine learning and statistical prediction algorithms for higher reliability. The development, validation and integration of novel portable and economical visualization and quantification systems with advances in laboratory technologies for biomarker analyses will allow for the eventual development of compact and economical closed systems that can be used to quantify biomarker concentrations in remote areas or self-sampling kits for point-of-care-based solutions. Furthermore, the incorporation of secure mobile health (mHealth) capabilities into these novel systems would contribute to advances in eHealth (healthcare supported by electronic processes), including facilitating data integration with electronic health records as well as telemonitoring and telemedicine [2–4, 11]. These eHealth solutions are particularly important in remote, rural areas where local access to medical facilities is limited.

5 Conclusions

We have demonstrated smartphone- and webcam-based imaging and data processing systems for the quantification of reproductive hormones using colorimetric ELISAs and have shown that the results obtained from two different portable cameras are comparable to the results obtained using a standard laboratory plate reader. Our demonstration results allow us to propose a portable ELISA visualization and quantification system that can be used in the field to monitor reproductive hormone levels as well as other biomarkers that can be quantified colorimetrically.

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Glossary

ELISA Enzyme-linked immunosorbent assay: laboratory test that uses antibodies and changes in intensity of color or fluorescence to quantify the concentration of the target substance, e.g., hormones

PdG Pregnanediol glucuronide: a urinary metabolite of progesterone

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