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## CONFINED CHEMILUMINESCENCE DETECTION OF NANOMOLAR LEVELS OF H<sub>2</sub>O<sub>2</sub> IN A PAPER-PLASTIC DISPOSABLE MICROFLUIDIC DEVICE USING A SMARTPHONE

*Elise Lebiga, Renny Edwin Fernandez, Ali Beskok*

### **Abstract:**

We report the design and characterization of a disposable light shielded paper-plastic microfluidic device that can detect nanomolar levels of H<sub>2</sub>O<sub>2</sub> using a smartphone camera and a light sealed accessory. Chemiluminescent reaction of H<sub>2</sub>O<sub>2</sub> with Bis (2,4,6-trichlorophenyl) oxalate in presence of rubrene and imidazole is confined to a paper reaction site where the individual reagents are delivered via plastic microfluidic channels. The net photon emission from the chemiluminescent reactions, detected by a smartphone, is correlated with H<sub>2</sub>O<sub>2</sub> concentrations. With a total reagent volume of 25 μL, the sensor system was able to detect H<sub>2</sub>O<sub>2</sub> concentrations as low as 250 nM. The smartphone based chemiluminescent sensing system has great potential as a point of care diagnostic tool for monitoring nanomolar levels of H<sub>2</sub>O<sub>2</sub> in biological samples.

### **Introduction**

Chemiluminescent assay is steadily getting accepted as a highly sensitive and reliable methodology that can lead to the development of affordable transportable, and disposable lab-on-a-chip type devices [1]. Chemiluminescent sensors are most favored in clinical applications where nanomolar levels of analytes must be detected with minimal skilled labor. Due to their sensitivity and easy instrumental setup, chemiluminescence (CL) assays have been integrated to lab-on-a-chip devices [2]. Earlier versions of miniaturized CL glucose biosensors [3] were operated using an external peristaltic pump to mix reagents with human serum within a 25x45x5 mm device. Zhao et al., 2009 integrated microchip electrophoresis with chemiluminescence detection (MCE-CL) to determine intracellular content of GSH in human red blood cells, which proved to be 100 times more sensitive than the laser fluorescence detection (LIF) techniques. Incorporation of chemiluminescence to paper-microfluidics has led to the development of various immunoassay platforms [5-9]. Laminated paper-based analytical devices (LPADs), produced using techniques similar to the fabrication of identification cards, have recently led to the development of many microfluidic sensors [10, 11]. Chemiluminescent assays have been successfully incorporated onto LPADs for simultaneous detection of biomolecules [12].

After the advent of smartphones empowered with high resolution cameras, researchers started to explore the possibility of cellphones as sensors in point of care applications [13]. The mean pixel value in photographs of the colorimetric reactions has been readily correlated to the detection of pH changes in sweat and saliva [14]. Human blood and sweat samples have been analyzed for C-reactive proteins (as low as 0.07 ng mL<sup>-1</sup>) using a smartphone-based colorimetric reader [15]. A direct industrial application was demonstrated recently when a smartphone-based microfluidics assay was used to differentiate 10 different kinds of red wines [16]. Electro-generated chemiluminescence reactions (ECL) conducted on paper microfluidic substrates have been reported to detect tris(2,2'-bipyridyl)ruthenium(II) (Ru(bpy)<sub>3</sub><sup>2+</sup>) concentrations as low as 250 μM with smartphone camera detection [17]. Very recently, a chemiluminescent (CL)-lateral flow immunoassay was conducted using a smartphone to quantitatively detect salivary cortisol (as low as 0.3 ng/mL) in the clinically accepted range [18]. Unlike electro-generated chemiluminescence

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3 reactions (ECL), where light emission can be restricted to the diffusion layer of an electrode  
4 area, a chemiluminescent reaction (CL) lacks spatial and temporal control over the reaction and  
5 commences as soon as the reagents come in contact with the analyte. However, incorporation of  
6 electrodes into a disposable device makes it complicated and expensive. Most of the recently  
7 developed devices for conducting chemiluminescence reactions on lab-on-chip environments are  
8 based on paper, PMMA, or PDMS substrates (ESI, Table S1). Naturally, reactions on paper-  
9 based devices are more prone to signal spread due to its porous nature, and can adversely affect  
10 the credibility of the chemiluminescent assay. Regardless of its high merit in sensitivity, a point-  
11 of-care device can only be reliable if it is devoid of any unaccountable operational errors. As per  
12 the guidelines by WHO, a point of care device has to be affordable, sensitive, specific, user-  
13 friendly, rapid, and robust to be considered as ASSURED [19]. Hence, a chemiluminescent  
14 reaction that is not confined within a small reaction area will have inferior sensitivity due to  
15 signal loss. To investigate the extent of signal loss, we fabricated an all-paper device that is  
16 conceptually analogous to most of the paper-microfluidic devices that have been used for  
17 chemiluminescence and colorimetric detection (ESI S1). We noticed that the chemiluminescent  
18 reaction has spread across the channels from the point of reagent introduction due to the capillary  
19 force offered by the highly porous paper substrate. The reagents quickly diffused away from the  
20 reagent delivery point, spreading the signal along its travel with a ~20% loss in net photon  
21 emission.

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26 The objective of the current research is to confine a CL reaction to a paper reaction site by  
27 disallowing the reagents to prematurely react inside the delivery channel and preventing any  
28 signal loss. We report a smartphone-based sensing system designed for accurate detection of  
29 chemiluminescence using a disposable paper-plastic microfluidic device. The paper-plastic  
30 microfluidic device is housed inside a custom-made smartphone accessory that also acts as a  
31 light shield for improved detection limits in the absence of background light. The  
32 chemiluminescent reaction of H<sub>2</sub>O<sub>2</sub> with Bis (2,4,6-trichlorophenyl) oxalate in the presence of  
33 rubrene and imidazole was confined to a paper reaction site while the reagents are delivered  
34 through the plastic microfluidic channels. The smartphone accessory ensures a light sealed  
35 environment for video recording of the reaction. The net photon emission of the reaction from  
36 the paper-plastic device was found to have a proportional correlation with the H<sub>2</sub>O<sub>2</sub> concentration  
37 from 100 μM up to 250 nM.

### 38 39 40 41 **Design and fabrication**

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44 The disposable microfluidic device comprises of multiple layers of paper and plastic channels  
45 stacked and sealed using thermal lamination. A cutout of the channel design is constructed with a  
46 precision die cutter (SilverBullet™) onto a plastic film. The specifications for the proposed  
47 design are as follows: three inlets measuring 1.5 mm diameter lead to three 10 mm long  
48 channels. The three plastic microfluidic channels connect to a paper reaction site that is 6 mm in  
49 diameter (Fig. 1a, 1b). The device comprises of two 200 μm thick plastic sheets securing the 1.5  
50 mil (~38 μm) thick lamination film inside. Layers 2-4 are thermally laminated on a hot plate at  
51 120°C for two minutes and were allowed to cool for five minutes before the top most layer (L1)  
52 with inlets was laminated. The bottommost layer (L5) is made up of black vinyl adhesive in  
53 order to avoid the interference of stray light during a reaction. The whole device measures 3 cm  
54 x 2 cm. A thicker paper (Whatman® qualitative filter paper, Grade 1) was found to give slightly  
55 better chemiluminescent signals than the current choice of (Kimwipe®). However, it required a  
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higher temperature for thermal assembly, which was too high for the plastic components to withstand without deformation. The 3D printed smartphone accessory allowed a light sealed video recording using iPhone (**Fig. 1c**, **Fig. 1d**). Each reaction was recorded for over a period of 60 seconds. Digital images of the snap shots were converted to 8-bit grayscale and the mean pixel value at the reaction site of the device was analyzed using ImageJ, image analysis software (National Institutes of Health). The microfluidic device was inserted into the accessory through a groove at a distance of 48 mm from the smartphone camera. First, 5  $\mu\text{L}$  of rubrene was added followed by equal volumes of imidazole and TCPO and allowed to settle for 1 minute. Finally, 5  $\mu\text{L}$   $\text{H}_2\text{O}_2$  is injected and smartphone video record begins. Each reagent is dissolved in 9:1 mixture of ethyl acetate and acetonitrile. The order of the addition of reagents was found to be crucial in reducing the background signal. Experiments were done for 1  $\mu\text{M}$ , 10  $\mu\text{M}$ , and 100  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  with 10 mM TCPO: Additional experiments were conducted with 15 mM TCPO with 250 nM and 750 nM  $\text{H}_2\text{O}_2$  concentrations.

## RESULTS AND DISCUSSION

During a chemiluminescent reaction, an excited molecule instantly loses its electronic energy in the form of a photon. The intensity of emitted photon can be expressed as in **Equation 1**.

$$I_{CL} = \Phi_{CL} \frac{dC}{dt} \quad (1)$$

$I_{CL}$  is dependent upon both the rate of chemical reaction,  $dC/dt$ , and chemiluminescent quantum yield,  $\Phi_{CL}$ . CL intensity as a function of time follows an exponential decay pattern as observed by many researchers. Various analytical methods have been used by researchers to describe the behavior of CL reaction. In an ideal homogeneous system where the free radicals are not constrained, the decay of CL emission demonstrate simple second-order kinetics [20]. The chemiluminescent reaction is inherently dynamic where the intensity of the emitted light changes as a function of time, reaction area of confinement, and liquid volume. Another way was to consider the chemiluminescence signal at the same time point for different conditions [21]. Hence, net photon emission from a chemiluminescent reaction conducted in a known volume can be quantified by integrating the decaying emission intensities over a period of time. **Equation 2** expresses the mathematical representation for the area under the curve (AUC), in which an average value of two subsequent light intensities ( $m$ ) are taken and multiplied by the time elapsed between points. Characterization of a chemiluminescent reaction using AUC reaction takes into account the light intensities at regular time intervals as well as the decay rate associated with each reaction.

$$AUC_G = \sum_{i=1}^{n-1} \frac{(m_{(i+1)} + m_i) \cdot t_i}{2} \quad (2)$$

By taking the sum of all of these points along the graph we obtained a numerical value that reflects both the peak intensity and the decay rate of the signal over time.

### 2.2 Chemiluminescent reaction on paper reaction site

The current design was found to successfully confine the reaction to the paper reaction site without premature reactivity or signal loss. The paper-plastic microfluidic device consists of a paper reaction site with a defined area where the reagents and the analyte can be sequentially delivered through plastic microfluidic channels using a micropipette. The chemiluminescence

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3 reactions in confined reaction sites were observed to proportionally change with  $\text{H}_2\text{O}_2$   
4 concentrations. After a series of optimization experiments, a protocol was developed for  
5 conducting chemiluminescent reactions with minimal reagent volumes (ESI, S3). Very evident  
6 differences were observed in the amount of photons generated for various concentrations of  
7  $\text{H}_2\text{O}_2$  as noticed from the reaction snapshots (Fig. 2). At high concentrations, visual observation  
8 of the light emission at various instances was very revealing of the ongoing reaction, which  
9 attained a peak intensity almost instantly and died down after a period of time. The rate of light  
10 emission from the reaction followed an exponentially decaying curve for intensity values plotted  
11 against time for  $\text{H}_2\text{O}_2$  concentrations. To evaluate the sensitivity of the proposed method, the  
12 chemiluminescent reactions were recorded for  $\text{H}_2\text{O}_2$  concentrations from 100  $\mu\text{M}$  to 1  $\mu\text{M}$ . Some  
13 of the previous studies have related analyte concentrations to the peak intensity of a  
14 chemiluminescent reaction [12, 22]. However the analyte concentration not only affects the peak  
15 intensity but it also affects the rate of decay of the signal [23]. Area under the curve (AUC) of a  
16 chemiluminescent intensity time plot appropriately represents the net photon emission, taking  
17 into consideration the light emission from the whole reaction and not just the maxima.  
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22 Experiments were conducted on the disposable microfluidic devices housed in the light sealed  
23 smartphone accessory. The device was inserted into the groove of the smartphone accessory  
24 (Fig. 1c) and the reagents were flown into the paper reaction site through the plastic microfluidic  
25 channels. The smartphone accessory allows the phone to be rested in an inclined position (Fig.  
26 1d) for operation. All added reagents were added sequentially. First, 5  $\mu\text{L}$  rubrene was pipetted  
27 in the inlet and is allowed to flow into the confined reaction site. Imidazole was then added and  
28 allowed to settle followed by TCPO addition. The reaction was recorded with the smartphone  
29 camera from start to finish just before the introduction of  $\text{H}_2\text{O}_2$ , 60 seconds post TCPO addition.  
30 Fig. 2 shows the snap shots of chemiluminescent reactions conducted in the proposed paper  
31 plastic device at varying  $\text{H}_2\text{O}_2$  concentrations for 10 mM TCPO recorded in an iPhone<sup>TM</sup> 5S. The  
32 light intensity-time decay data were taken immediately after mixing the reagents. We noticed  
33 that the onset of light emission was almost instantaneous and died down in less than 60 seconds.  
34 The variation in light emission during chemiluminescent reactions for 100, 10, 1  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  are  
35 featured in Fig. 3. It can be seen that the intensities were quite different from each other at every  
36 instant of the reaction. The light emission was found to completely decay within 40, 20, 10  
37 seconds for 100, 10 and 1  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$ , respectively. The peak intensities were also found to be  
38 proportional to  $\text{H}_2\text{O}_2$  concentrations as much as the rate of decay. A perceivable difference in the  
39 net photon emission can be noticed between concentrations, although at lower concentrations the  
40 reaction is extremely fast and feeble.  $\text{H}_2\text{O}_2$  concentrations below 1  $\mu\text{M}$  did not produce  
41 perceivable signals that could be recorded using the smartphone camera.  
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### 46 2.3 Sensitivity assessment studies

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48 The effect of varying concentrations of Bis (2,4,6-trichlorophenyl) oxalate (TCPO) on  
49 chemiluminescence was evaluated by conducting experiments with 5, 10 and 15 mM TCPO  
50 where rubrene and imidazole concentrations are held constant although it has been reported that  
51 imidazole might have an effect on the intensity of the chemiluminescent signal [24]. The  
52 variation of chemiluminescence intensity was noted be a function of  $\text{H}_2\text{O}_2$ , although the intensity  
53 values are also dependent on TCPO levels. For the same  $\text{H}_2\text{O}_2$  concentrations, the  
54 chemiluminescent intensities were considerably lower when TCPO concentrations were reduced  
55 to half. The  $\text{H}_2\text{O}_2$  concentrations below 10  $\mu\text{M}$  did not record perceivable signals when 5 mM  
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3 TCPO were used. Higher intensity levels were observed for 10 mM TCPO concentrations than 5  
4 mM. However, reactions with H<sub>2</sub>O<sub>2</sub> concentrations below 1 μM did not yield chemiluminescent  
5 signals even with 10 mM TCPO. However, experiments done with 15mM TCPO were able to  
6 detect H<sub>2</sub>O<sub>2</sub> below 1 μM (250-750 nM). **Fig. 4** depicts the chemiluminescent reactions of 250  
7 and 750 nM. We can observe a distinct difference in the net photon emission between 250 nM  
8 and 750 nM. The reaction was extremely fast and the entire light emission was completed in less  
9 than 10 seconds. It was observed that light emission patterns followed the same trend as that of  
10 reactions conducted in confined reaction sites. The AUC values for 100, 10, and 1 μM H<sub>2</sub>O<sub>2</sub>  
11 values were found to be around 316.4 ± 7, 197.9 ±19, and 36.7 ± 9.8, respectively. The  
12 experiments performed with 15 mM TCPO with 750 and 250 nM H<sub>2</sub>O<sub>2</sub> yielded 78.3 ± 8.3 and  
13 37.8 ± 6.7, respectively.  
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18 It has been reported that Lab-on-a-chip devices made on paper proved to produce reproducible  
19 results even after weeks of storage [12]. Performance of our sensor under storage conditions was  
20 evaluated by a set of experiments by immobilizing the reagents on the reaction site for 24 hours.  
21 Paper reaction sites immobilized with imidazole were found to produce chemiluminescent  
22 signals after 24 hours of storage at 4 °C. However, TCPO- and rubrene-immobilized surfaces did  
23 not yield any light even at 100 μM H<sub>2</sub>O<sub>2</sub>, confirming their instability under immobilized  
24 conditions. More detailed studies on immobilized paper surfaces are in progress.  
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## 27 Conclusion

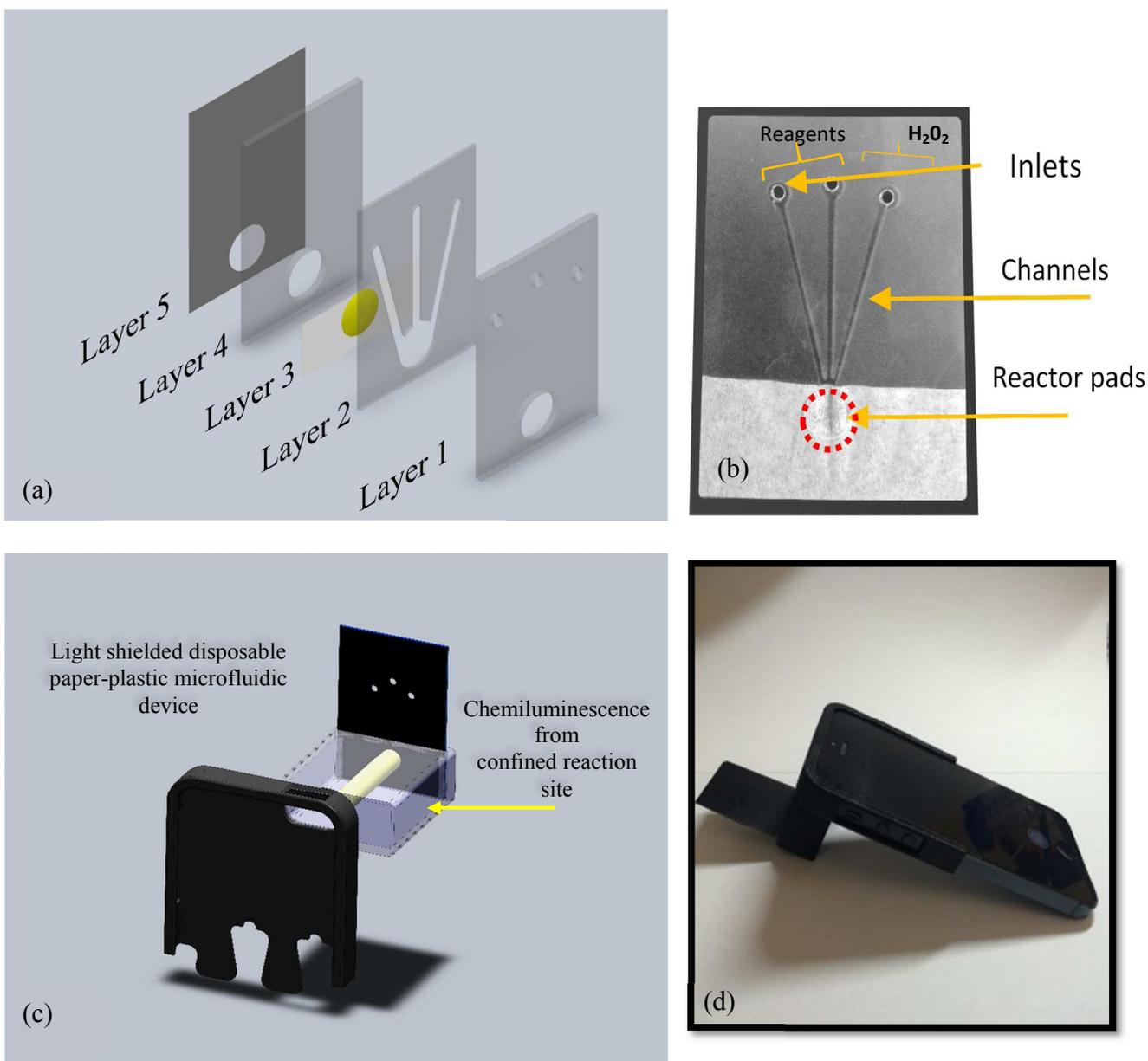
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29 We have developed a disposable microfluidic sensor for reliable detection of  
30 chemiluminescence. The photon emission arising from the interaction of rubrene with  
31 intermediates derived from hydrogen peroxide and Bis (2,4,6-trichlorophenyl) oxalate(TCPO) in  
32 presence of imidazole was detected with the paper-plastic microfluidic device and a smartphone  
33 accessory. The exponentially decaying CL reaction was successfully recorded using a  
34 smartphone camera and the AUC representation of the reaction showed a proportional variation  
35 for nanomolar levels of H<sub>2</sub>O<sub>2</sub> concentrations. The proposed sensing system was found to be  
36 sensitive for H<sub>2</sub>O<sub>2</sub> concentrations as low as 250 nM.  
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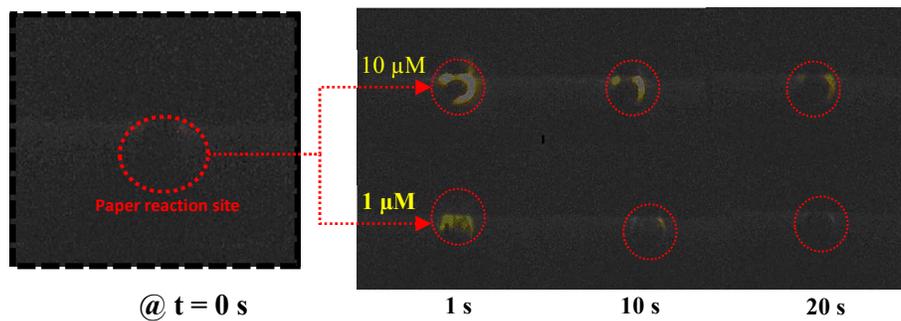
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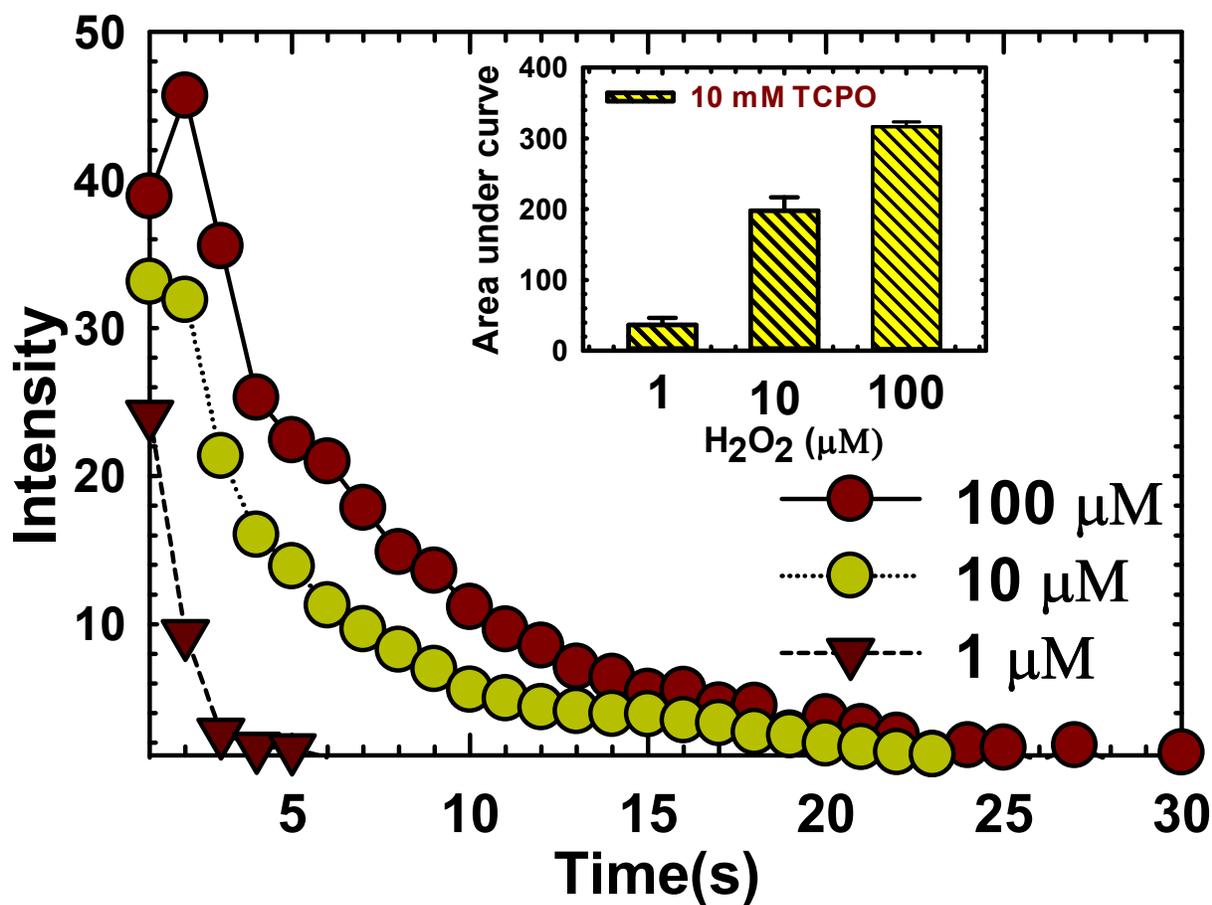


**Fig. 1:** Custom designed smartphone sensor system module for chemiluminescent detection.

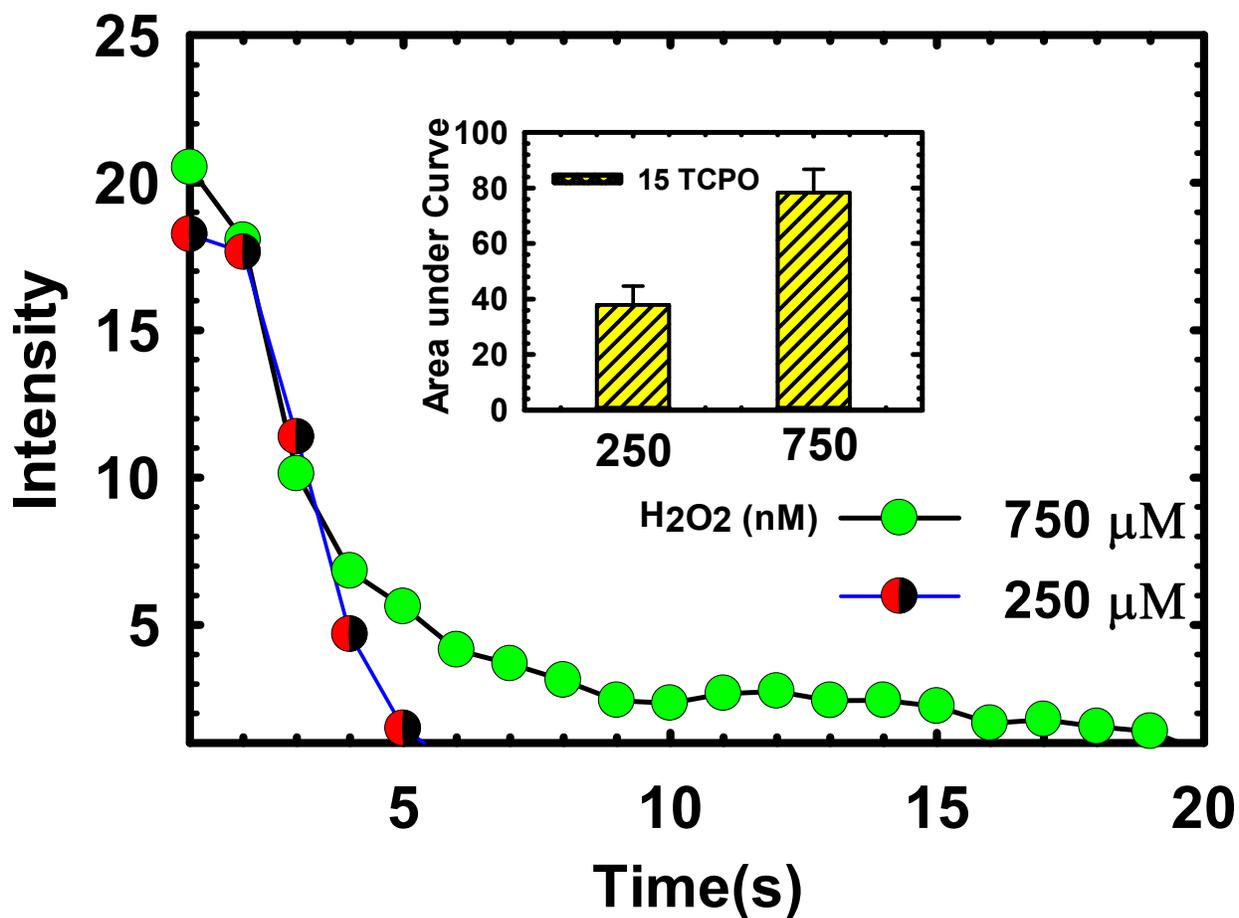
(a) Layer 1: Thermal lamination film with fluid inlets, Layer 2: Thermal lamination film with channel design, Layer 3: Paper substrate, Layer 4: Thermal lamination film with outlet, Layer 5: Vinyl backing for light-sealing. (b) shows the custom-designed paper-plastic disposable microfluidic device, (c): Design of the smartphone accessory for video capture of chemiluminescent reaction, (d): The disposable microfluidic device with smartphone accessory.



**Fig. 2:** Snap shots of chemiluminescent reactions conducted in the proposed paper plastic device at varying  $\text{H}_2\text{O}_2$  concentrations ( $10$  and  $1 \mu\text{M}$ ) for  $10 \text{ mM}$  TCPO recorded in an iPhone<sup>TM</sup> 5s.



**Fig. 3:** Variation of the chemiluminescent response as a function of time as recorded by a smartphone using the paper-plastic microfluidic device for 100, 10 and 1  $\mu\text{M}$  of  $\text{H}_2\text{O}_2$  for 10mM TCPO. Inset shows the corresponding area under curve (AUC) values.



**Fig. 4:** Variation of the chemiluminescent response as a function of time as recorded by a smartphone using the proposed paper-plastic microfluidic device for 750 and 250 nM of H<sub>2</sub>O<sub>2</sub> for 15mM TCPO. Inset shows the corresponding area under curve (AUC) values.