

Flow Control-based 3D μ PADs for Organophosphate Pesticide Detection

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Flow control-based paper devices have recently shown great potential for point-of-need analysis, since they allow for the easy operation of multi-step assays by minimizing user operation. In this work, a wax printing method was evaluated as a means to control liquid flow in 3D microfluidic paper-based analytical devices (μ PADs). The resulting flow control-based 3D μ PADs were applied to determine paraoxon-ethyl as a typical organophosphate pesticide model system. The analytical procedure is as simple as applying a 200- μ L sample solution, resulting in reproducible (relative standard deviation of colorimetric signals from 6 independently fabricated devices, 2.63%) colorimetric signals within 1 h of the assay time with the limit of detection (LOD) reaching 25.0 μ g/L. Finally, results obtained for pesticide-spiked water samples analyzed by flow control-based 3D μ PADs showed good agreement with those from a conventional HPLC analysis with UV detection.

Keywords Flow control, 3D μ PADs, biosensor, organophosphates

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Introduction

Although pesticides have brought great economic benefits to the improvement of crop productivity in agriculture, they are also associated with negative impacts on humans.¹ Once being used, they pose potential hazards to human health and the surrounding environment. Pesticide residues can be mostly found in food commodities, surface and ground water, soil, air, non-target vegetation, and living organisms. Thus, a vast variety of health disorders has been linked to pesticides, such as poisoning, reduced liver and immune function, neurological impairment and cardiovascular and respiratory diseases, among others. In efforts to mitigate the negative impacts of pesticides, the detection of their residues plays an important role. Among the analyzed pesticides, organophosphate pesticides, which account for over 38% of totally used pesticides, have been targets for decades due to their main contribution and toxicity.^{2,3} Conventional pesticide analysis mainly relies on UV-HPLC,^{4,5} GC-MS,⁶ fluorescent and chemiluminescent assays.^{7,8} However, these methods require bulky instruments together with fully equipped laboratory infrastructure and well-trained operators. Therefore, it is important to develop fast and accurate screening methods to enable on-site detection of pesticides.

Since the first introduction of microfluidic paper-based analytical devices (μ PADs) by Whitesides' group,⁹ patterned cellulosic paper has attracted significant attention as an excellent substrate for practical analytical assay applications. Their advantageous features including low-cost, readiness for use, safe disposability, simple transport and storage as well as pump-

free sample liquid transportation make μ PADs particularly attractive for point-of-need applications as alternatives to well-established conventional instrumental methods.¹⁰ Not surprisingly, paper-based devices have also been adapted to the colorimetric determination of pesticides without the requirement of using sophisticated instruments. In 2007, No *et al.* first applied an enzyme-based sensing system to paper-based dipsticks consisting of various cellulosic derivatives to determine organophosphate and carbamate pesticides.¹¹ The working principle is based on the inhibition of the acetylcholine esterase (AChE) enzyme activity, depending on the concentration of the target pesticide. Later, Hossain *et al.* successfully developed paper-based dipsticks using sol-gel silica as an entrapment material, which can enhance the enzyme stability and offer an alternative to established lateral flow based methods.^{12,13} Devices were found to be stable for 60 days at 4°C and to achieve a limit of detection (LOD) of 27.5 μ g/L, suitable for paraoxon pesticide analysis. However, these methods still require a user to perform multiple operation steps, like reversing the device at a specific time for signal generation or adding external reagents. In order to overcome these drawbacks, Jahanshahi-Anbuhi *et al.* have developed a paper-based device consisting of two flow channels. One channel with rapid flow enables the interaction of the pesticide in the sample with the AChE enzyme, before the colorimetric substrate indophenyl acetate (IPA) is delivered to the reaction zone through a second and slower flow channel.¹⁴ The pesticide-AChE interaction time leading to target concentration-dependent enzyme inhibition is controlled by a manually operated ON-OFF valve made of paper. Besides the requirement to manually switch a paper flap at a specific moment in the assay, the sample-enzyme interaction time before colorimetric substrate arrival is limited by the length of the "slow" flow channel. In 2015, Sicard *et al.* demonstrated

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the applicability of μ PADs for on-site pesticide analysis by using a smart phone for signal readout.¹⁵ But also that system requires a two-step process to complete the test: the addition of a sample solution and immersing the device in distilled water until the liquid level reaches a defined level. Recently, a very sensitive test strip has been developed by using a similar enzymatic reaction mechanism.¹⁶ A very low LOD (0.01 μ g/L) has been achieved, based on the relatively long pesticide-AChE interaction time (20 min), resulting in a high degree of enzyme inhibition. Once again, users need to add an external reagent, the indoxyl acetate (IDA) colorimetric substrate in this case, which makes this device less favorable for practical applications. In summary, many paper-based devices for colorimetric pesticide analysis have been reported in the literature, but to the best of our knowledge, they do not allow simple and user-friendly assays with sample addition, but generally involve additional operational steps, often at a specific timing, as outlined above. Consequently, there is still room for the development of simpler paper-based devices to overcome this limitation.

A number of researchers have looked at the question of how to control and to manipulate fluid flow in μ PADs, for example, for the purpose of controlling reaction time.¹⁷ Lutz *et al.* have used various concentrations of sucrose deposited inside flow channels as a means to automatically control liquid flow rates and to create time delays.¹⁸ Another approach to precise flow control on μ PADs is to use wax-printing resulting in variations in paper permeability for aqueous solutions.¹⁹ Yet, there are few applications of these flow control-mechanisms for real assays, but most studies used dye solutions for proof-of-concept. Park *et al.* made use of pressed regions in wax-printed channels, where variations in pressing force resulted in modulations of liquid permeability through the pressed region.²⁰ However, one disadvantage of this method is that only delay times of up to 200 s could be achieved, which is insufficient for an enzyme inhibition reaction, as required in the current case.

In order to realize a “walk-away” μ PAD for the automatic analysis of organophosphate pesticides without user intervention. The term of “walk-away” is used in our current work because our devices only need to add sample solution without any additional step, enabling the long incubation time before scanning the images for signal acquisition. For this reason, this work introduces a wax-printed channel integrated into 3D μ PADs as a means for flow control. Liquid flow control has been used to generate a delay for the following assay steps: the inhibition reaction between enzyme and pesticide and the enzymatic reaction between non-inhibited AChE enzyme and the IDA substrate. In addition, all required assay reagents were deposited in individual paper layers, making the addition of external reagents unnecessary. To the best of our knowledge, this is the first report concerning on the integration of wax-printed channels into 3D μ PADs for flow rate control, which can minimize the need for user action.

Experimental

Reagents and chemicals

Acetylcholinesterase (AChE) (electrophorus electricus, Type VI-S), indoxyl acetate (IDA), paraoxon-ethyl, poly-(diallyldimethylammonium chloride) (PDDA) and tris-(hydroxymethyl)aminomethane (Tris) were purchased from Sigma-Aldrich (St. Louis, MO). Ethanol and methanol were purchased from Kanto Chemical Company (Tokyo, Japan). All other reagents, including skim milk and hydrochloric acid

(HCl), were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). CF7 absorbent pads (22 mm $\times$ 50 m) and Whatman grade 4 filter paper were purchased from GE Healthcare Life Science (Buckinghamshire, UK). Hot lamination films (150 μ m thickness, film material: polyethylene terephthalate and polyvinyl alcohol as a thermoplastic adhesive) were obtained from Jointex (Tokyo, Japan). All solutions were prepared in 18.2 M Ω pure water, obtained from a Purelab Flex water system (ELGA, Veolia, UK).

Device fabrication

The design and schematic fabrication procedure for one type of 3D μ PAD for enzyme-based colorimetric detection of organophosphate pesticides (**Device I**) are shown in Fig. 1. **Device I** is composed of two reagent-coated paper wells (1st and 2nd layer), an unmodified paper microfluidic channel for flow control (3rd layer), and an absorbent pad for sample liquid uptake (Fig. 1a). Microfluidic patterns or wells were designed in Microsoft Powerpoint and printed on A4-size paper substrates by a wax printer (ColorQube 8570N or 8580N, Xerox, Norwalk, CT, USA). For fabricating hydrophobic barriers, the wax-printed paper was heated for 2 min at 150°C on a hot plate (Nissin NHS-450ND, Nissinrika, Tokyo, Japan). To enhance their mechanical strength during the assembling step, both sides of each paper layer were coated with lamination films (light blue color in Fig. 1a) on a hot laminator (QHE325, Meiko Shokai, Tokyo, Japan), except for the wax-patterned paper well areas, the top side of the microfluidic channel layer, and the bottom side of the circular area at the end of the microfluidic channel connecting to the absorbent pad. The laminator settings for the substrate thickness and feeding speed were “150 μ m” and “fast mode”, respectively. The wax-patterned paper substrates were then subjected to reagent deposition, as illustrated in Fig. 1b.

To minimize reagent degradation, fresh stock solutions of IDA were prepared immediately prior to preparation of paper devices. IDA working solutions were prepared by diluting a methanolic stock solution to the desired final concentrations with methanol/water (v/v = 50/50). AChE solution (final concentration 100 U/mL) was prepared in Tris-HCl buffer (25 mM, pH = 8.0) and stored in a low-binding plastic tube at -20°C before use. To form the 1st layer of the device, 10 μ L of AChE (100 U/mL) solution was pipetted into the wax-patterned paper wells (ϕ = 0.9 cm), which have been pretreated with 40 μ L of 1% (w/v) aqueous skim milk solution followed by drying overnight at room temperature. Onto the paper wells of the 2nd layer (ϕ = 0.4 cm) pretreated with 0.25% (w/v) aqueous PDDA solution, 10 μ L IDA working solution (40 mM) was pipetted. After cutting into individual strips, reagent-coated paper layers and the microfluidic channel layer were stacked together using double-sided adhesive tape having a circular hole (ϕ = 0.9 cm). Plain filter paper disks (ϕ = 0.9 cm) were used to fill the gaps between each paper layer (Figs. 1a, 2a, and 2b). Finally, a piece of absorbent pad material was attached as a “balance bar” to obtain devices flatly sitting on a surface. Similarly, a second type of 3D μ PAD (**Device II**) for fully reagentless operation was fabricated as outlined in Fig. 2a. In contrast to **Device I**, a blank paper well layer was attached on top of the AChE coated layer to hold in place a first Tris-HCl buffer component coated paper disk (ϕ = 0.9 cm). A second Tris-HCl buffer component coated paper disk is located between the enzyme (2nd layer) and IDA-coated (3rd layer) paper substrate layers. These paper disks serve the purpose of pH buffering function integration (pretreated with 25 μ L of 1 M Tris-HCl buffer, pH = 8.0, followed by drying overnight under ambient condition),

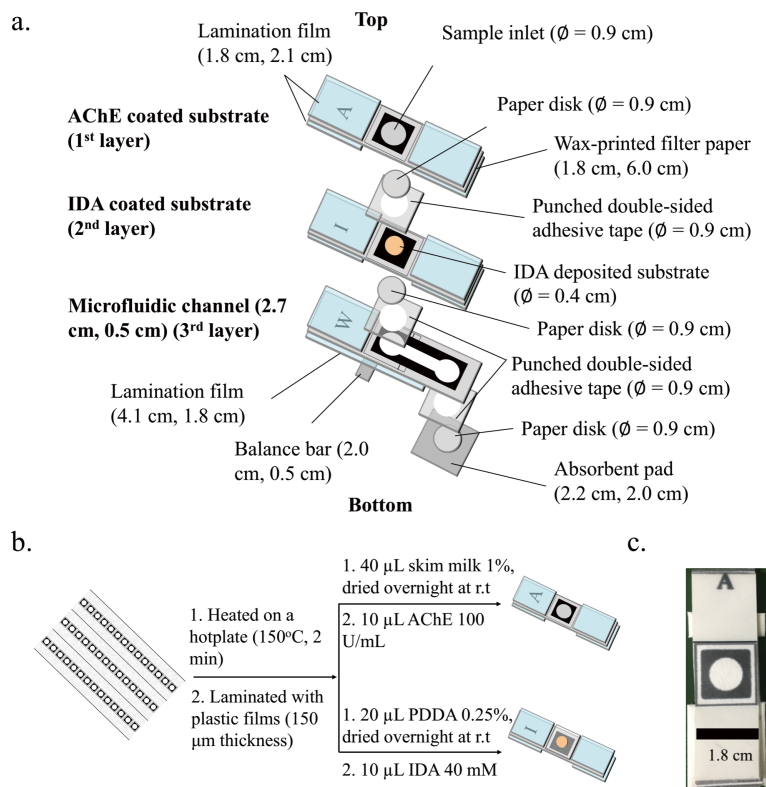


Fig. 1 a) Schematic illustration of a 3D μ PAD (**Device I**) showing the arrangement and layout of individual paper layers, double-sided tape and paper disks. b) Fabrication procedure for AChE and IDA-coated paper substrates (1st and 2nd layer). c) Actual picture of assembled **Device I**.

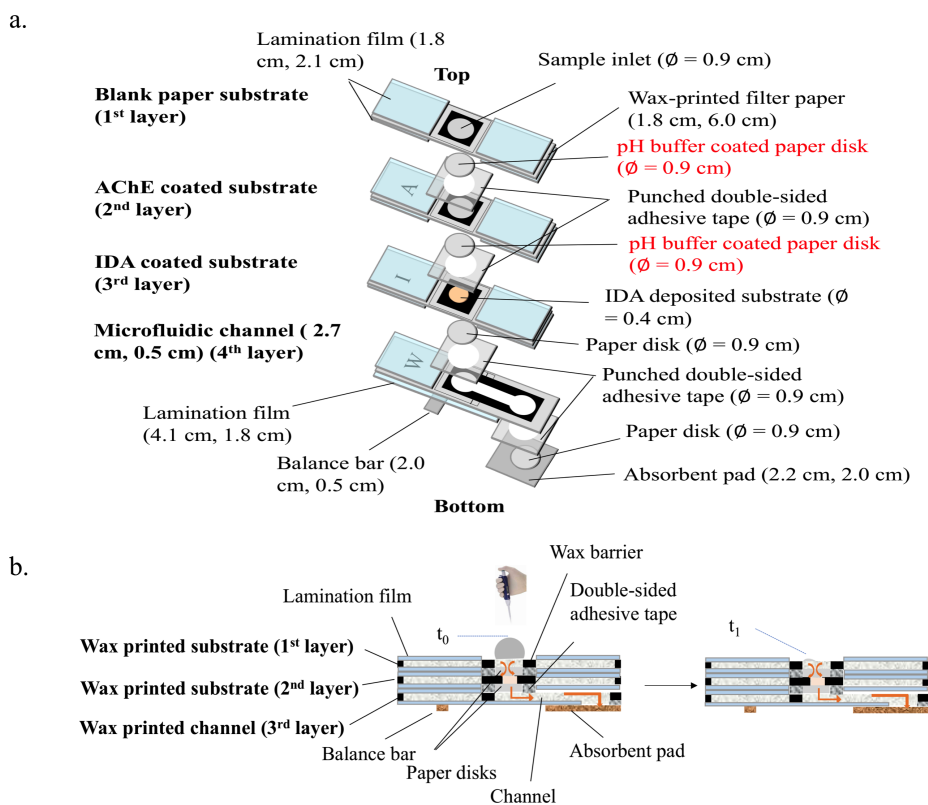


Fig. 2 a) Schematic illustration of a 3D μ PAD with integrated buffer components for reagentless operation (**Device II**). b) Cross-section of a 3D μ PAD (**Device I**) and schematic illustration of the experimental method for evaluating the delay time ($t_1 - t_0$) in 3D μ PADs.

in addition to bridging the gaps between paper layers as mentioned above for the case of **Device I** (Fig. 1a).

Delay time measurement

The dependence of the delay time on the width and length of the wax-patterned microfluidic paper channel (3rd layer of **Device I**) was evaluated, as illustrated in Fig. 2b. After introducing an analyte-free Tris-HCl buffer solution (200 μ L, 50 mM, pH = 8.0), the starting time (t_0) and the time required for transporting the sample liquid (t_1) were recorded, followed by calculating the delay time as $t_1 - t_0$. Here, t_1 is defined as the time when the introduced buffer solution completely disappeared from the surface of the top paper layer of the devices, as visually judged by the naked eyes.

Preparation of standard samples

To minimize any degradation, fresh stock solutions of organophosphate pesticide (paraoxon-ethyl) were prepared prior to analysis. Pesticide standard solutions were prepared in either Tris-HCl (50 mM, pH = 8.0) containing 6% (v/v) ethanol (when working with **Device I**) or in 6% (v/v) aqueous ethanol (when working with **Device II**). All pesticide standard sample solutions were utilized within 3 h after preparation.

Device optimization

For optimizing the 3D devices, the optimal amount of IDA was firstly evaluated by simple paper spot tests (refer to Fig. S1 (Supporting Information) for the detailed experimental procedure). First, 10 μ L of AChE (100 U/mL) diluted with 20 μ L of Tris-HCl (50 mM, pH = 8.0) was applied to the wax-patterned well (ϕ = 0.4 cm) containing 10 μ L of pre-deposited IDA solutions with different concentrations (10 – 60 mM). After a 30-min reaction time, the obtained color spots were scanned from the backside by a CanoScan 9000F Mark II scanner (Canon, Tokyo, Japan) and the green (G) color values of the RGB color coordinates were extracted from the images by ImageJ software (NIH, Bethesda, MD) to quantify the color intensities. Moreover, the dependency of the achieved color intensity on the time of AChE catalyzed hydrolysis of IDA was also evaluated using the identical experimental setup. Color signals were acquired at 5 min intervals.

For evaluating the effect of the channel lengths and widths (3rd layer of **Device I** and 4th layer of **Device II**) on the obtained color intensities, different wax-printed channels with various lengths and widths were fabricated by the wax printer. Then, these channels were integrated into 3D **Device I**. After 200 μ L of an analyte-free Tris-HCl buffer solution (50 mM, pH = 8.0) containing ethanol 6% was introduced into the devices, the delay time was recorded as mentioned above, followed by incubation for an additional 30 min before scanning images for color signal acquisition.

Assay procedure for 3D μ PADs (Devices I and II)

In general, 200 μ L of the respective sample solution was pipetted onto the sample inlet of the 3D μ PADs. After approximately 20 min of liquid transportation and an additional 30 min incubation time, the IDA containing layers (2nd layer of **Device I** or 3rd layer of **Device II**) were detached from the 3D devices and scanned from the top side. ImageJ was used to obtain quantitative color intensities from the scanned images.

Results and Discussion

Working principle and optimization of 3D μ PADs

The working principle for colorimetric detection of organophosphate pesticides is based on the degree of AChE activity inhibition by the target pesticides. Higher pesticide concentrations result in stronger enzyme inhibition, leading to reduced IDA hydrolysis, and hence weaker intensities of the blue colored spots appearing on the paper substrate. Active AChE promotes IDA hydrolysis into a colorless hydroxyl indole, which is subsequently oxidized by atmospheric oxygen to the blue indigo dye (Fig. S2, Supporting Information). To prevent an initial reaction of AChE with IDA before sample introduction, the two reagents were integrated into 3D μ PADs by deposition onto separate paper layers, as shown in Figs. 1a and 2a.

During RGB color analysis, a higher dynamic range was found for the green (G) value compared to the red (R) value (data not shown). Therefore, this parameter has been adapted for quantitative color analysis throughout this work. It should be noted that a weaker blue color gives rise to higher G values. Higher concentrations of the target pesticide are represented by higher G values.

The surface of cellulosic paper is negatively charged due to the presence of ionizable groups (mainly carboxyl groups).^{21,22} It is known that such anionic sites cause strong electrostatic interactions with amino groups of AChE.²³ Therefore, skim milk was used as a blocking agent to limit the electrostatic and hydrophobic interactions between AChE and paper surfaces.²⁴ On the other hand, a positively charged polymer containing quaternary amino groups (PDDA) was applied to the IDA containing layers, serving as immobilization sites for negatively-charged components of AChE (pI = 5.3) at pH = 8.0 and the partially negatively charged form of indigo (pK_1 = 7.97 and pK_2 = 12.7) after the enzymatic reaction between AChE and IDA on the paper surface.^{25,26} PDDA plays an important role as cellulose network-anchored material to electrostatically trap anionic chromogens and enzymes, which prevents their washout into the absorbent pad during the assay. To guarantee a smooth flow of the sample solution, blank paper disks were inserted to fill the gaps between each paper substrate layer.²⁷

For simplicity reasons, the basic colorimetric reaction system (AChE catalyzed hydrolysis of IDA) was optimized with simplified paper spot tests. The concentration and pH value of the Tris-HCl buffer (50 mM, pH = 8.0) were chosen according to a previous report,¹⁶ and IDA working solutions were obtained by dilution with methanol/water (v/v = 50/50) to prevent compromising the hydrophobic wax-printed barriers by excess amounts of methanol. As can be seen from Fig. S3 (Supporting Information), the optimal green intensities were obtained by using 10 μ L of 40 mM IDA for the evaluated IDA concentration range from 10 to 60 mM. The green color intensities decrease from about 101 to 96, reaching a plateau when the IDA concentration is 40 mM. Therefore, the deposition of 10 μ L of 40 mM IDA was chosen for all following experiments as the amount providing strongest color signals in combination with the applied 10 μ L AChE (100 U/mL). Figure S4 (Supporting Information) shows the hydrolysis time-dependent color intensity obtained with 10 μ L of a 40 mM IDA working solution. Although the G values monotonously decreased over time for more than 30 min, the curve started to flatten out, and a 30-min incubation time was chosen in the interest of the total assay time.

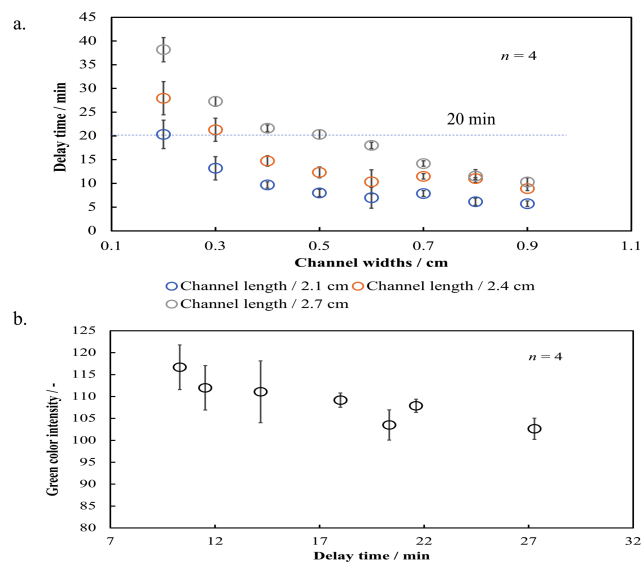


Fig. 3 a) Effect of the channel lengths and widths on the delay time ($t_1 - t_0$). b) Average green color intensity development over time for 3D μ PADs with a 2.7-cm channel length and a 0.5-cm width (**Device I**); error bars represent the standard deviations for measurements obtained with 4 individual single-use devices.

Validation of flow delay in the flow control-based 3D μ PADs

The total time for transportation of the sample liquid in the wax-patterned 3D μ PADs is strongly affected by the geometry of the fluidic channels. In a fundamental study, Hong *et al.* have proven that the retardation of aqueous liquid fluid flow in paperfluidic channels with wax boundaries is caused by a high-contact angle at the boundaries between the solution and the hydrophobic wax barrier.²⁸ Here, fluidic channels fabricated by the wax printing method have been integrated into 3D μ PADs to create a sufficient delay time for the enzyme-based colorimetric determination of organophosphate pesticides, which is required for the enzyme inhibition through interaction with the pesticide. Various combinations of flow channel lengths and widths in the 3rd layer of **Device I** have been evaluated. Figure 3 displays the effect of channel lengths and widths on the observed delay time and the relationship between the delay time and the G color intensities. As a result, the delay time increased with increasing channel length and decreasing channel width (Fig. 3a). The targeted 20 min delay time for incubation was achieved with 3 different designs of 3D μ PADs (channel length $\times$ width: 2.1×0.2 cm, 2.4×0.3 cm, and 2.7×0.5 cm). However, the highest reproducibility (smallest error bars) was experimentally observed for the 2.7×0.5 cm configuration. This is because of patterning the narrower channels (0.2 and 0.3 cm) by wax printing is known to be less reproducible, due to inherently inhomogeneous wax spreading during the hot plate heating step.²⁹ For this reason, we selected the 2.7×0.5 cm configuration to fabricate 3D μ PADs for the enzymatic colorimetric detection of pesticides in this work.

Generally, the obtained G values strongly depend on the delay time. The longer is the delay time, the stronger are the blue color signals and the lower are the G values (Fig. 3b). Since the rate of the flow delay relies on the flow velocity, increasing the channel length or narrowing the channel width suppressed the passive fluidic behavior of the sample liquid moving through the channel. The colorimetric signals reached a stable value and remained constant at around a 20-min delay time.

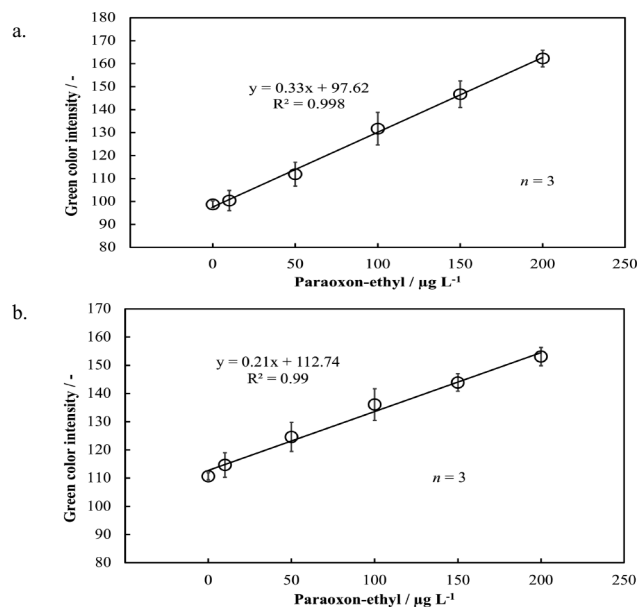


Fig. 4 a) Pesticide response curve obtained with **Device I** type 3D μ PADs shown in Fig. 1a; pesticide standard solutions were prepared in Tris-HCl buffer (50 mM, pH = 8.0) containing 6% (v/v) ethanol. b) Pesticide response curve obtained with reagentless flow control-based 3D μ PADs (**Device II**) in Fig. 2b; pesticide standard solutions were prepared in 6% (v/v) ethanol. Applied sample volume, 200 μL ; incubation time, 30 min; error bars represent standard deviations for measurements with 3 individual single-use devices.

Pesticide detection with flow control-based 3D μ PADs (**Device I**)

To obtain a response curve for organophosphate pesticides using **Device I**, various concentrations of paraoxon-ethyl (from 0 to 200 $\mu\text{g/L}$), prepared in Tris-HCl buffer containing 6% ethanol, were applied to optimized flow control-based 3D μ PADs. The purpose of using the Tris-HCl buffer is to maintain the optimal pH condition for this enzymatic reaction. Ethanol is required to fully dissolve the compound in the standard solutions. In this assay, in accordance with previous optimization experiments, approximately 20 min passed before the entire sample solution was completely adsorbed into the device. The results shown in Fig. 4a indicate a good linear relationship between the paraoxon-ethyl sample concentrations and the G-value intensities with a LOD of 25.0 $\mu\text{g/L}$ estimated according to the 3 method.³⁰

The LOD achieved with the current 3D μ PAD is comparable to that of the bidirectional lateral flow dipsticks reported by Hossain *et al.*¹² Although paper-based pesticide assaying devices with significantly lower LOD (0.01 $\mu\text{g/L}$) have been reported, the total analytical procedure using the 3D μ PAD-based assay is reduced to a single step, requiring only the addition of the sample solution to the device. The 3D μ PADs provide sufficient time not only for pesticide and enzyme interaction, but also for the AChE catalyzed IDA hydrolysis. Hence, the two different reaction steps required for the pesticide assay have been successfully combined into a single device, allowing “walk-away” assays without user intervention.

Additionally, the device-to-device reproducibility of 3D μ PADs (**Device I**) was validated with pesticide containing solutions (100 $\mu\text{g/L}$). Color scans of the IDA containing layers (2nd layer) detached from 6 individually fabricated single-use 3D devices after assay completion are shown in Fig. S5 (Supporting Information). Consequently, the developed 3D

μ PADs showed a reproducible colorimetric response upon exposure to pesticide-containing solutions with a relative standard deviation of 2.63%. Moreover, the storage stability of fully assembled devices at 4°C was found to be at least 35 days, which proves that this device is suitable for on-site analysis (Fig. S6, Supporting Information).

Reagentless flow control-based 3D μ PADs (**Device II**)

In a final effort to minimize the number of operation steps in pesticide analysis, a user-friendly reagentless flow control-based 3D μ PAD (Fig. 2b) was evaluated, which integrates the pH buffering function usually required during sample pretreatment in previously reported studies.^{12,14} For this purpose, Tris-HCl buffer (25 μ L, 1 M, pH 8.0) was pre-deposited in the paper disks (ϕ = 0.9 cm) replacing the blank paper disks used in **Device I**.

The corresponding response curve obtained with **Device II** type 3D μ PADs is shown in Fig. 4b. The LOD for paraoxon-ethyl was estimated as 46.7 μ g/L in this case, which is higher than the value observed for **Device I** type μ PADs where the Tris-HCl buffer was included in the sample solution. In addition to the lower sensitivity, larger signal fluctuations between individually fabricated devices were noted (larger error bars in Fig. 4b compared to Fig. 4a) and the obtained slope of the response curve for **Device II** (0.21) is smaller than that of **Device I** (0.33). The reason for these differences might be the inhomogeneous and slow solubility of the solid Tris-HCl buffer salt components pre-deposited in the paper disks, which results in lower signal intensities in **Device II**.

Table S1 (Supporting Information) summarizes a comparison of the developed flow control-based 3D μ PADs (**Devices I** and **II**) with other previously published paper-based systems for organophosphate pesticide detection regarding the number of assay steps and the LOD. The 3D μ PADs enable single-step assays (only sample application) with suitable LODs (25.0 and 46.7 μ g/L for **Device I** and **II**, respectively), which fulfil the requirement for the detection of maximum allowable pesticide residues in river water or vegetables, set at 50 μ g/L by Japanese regulators, respectively.³¹

Assays of pesticide-spiked river water sample

To demonstrate the potential application of the 3D μ PADs to practical samples, recovery tests using a spiked river water sample were carried out with the two types of flow control-based 3D μ PADs and a conventional UV-HPLC method. The river water sample was collected from Yagami river (Kanagawa, Japan). Pesticides were not detectable in as collected river water both by using the developed 3D μ PADs and HPLC. Therefore, paraoxon-ethyl was spiked into the river water (100 μ g/L) together with ethanol (final conc.: 6% v/v) for **Device II**, or ethanol (final conc.: 6% v/v) and Tris-HCl buffer (final conc.: 50 mM, pH = 8.0) for **Device I**. Table S2 (Supporting Information) summarizes the found pesticide concentrations and the recovery values for each detection method. The result obtained with **Device I** was in good agreement with the conventional UV-HPLC analysis. However, a somewhat unsatisfactory recovery was obtained when using **Device II** (131%). This goes along with the observed better performance of Device I, as mentioned earlier, which is most likely caused by solubility issues of the pre-deposited Tris-HCl buffer components in the case of **Device II**.

Conclusions

Flow control-based 3D μ PADs have been successfully developed and applied for the detection of a typical organophosphate pesticide model. The flow rate of the sample liquid can be controlled by changing the lengths and widths of a paperfluidic channel patterned by printing wax barriers. This system allowed us to achieve a delay time of around 20 min, which provides the time required for sufficient interaction between pesticide, enzyme and IDA. The commonly required two-step assay procedure for pesticide detection with paper-based devices, consisting of enzyme inhibition and enzyme-IDA incubation, has been effectively combined into one complete device requiring solely the application of the sample liquid with no external reagent handling, manual device inversion or switching of paper valves. Using the 3D flow control-based μ PADs (**Device I**), paraoxon-ethyl, a representative pesticide, was detected with a LOD (25.0 μ g/L). Finally, the strategies applied to the development of this type of device will open further opportunities to integrate multi-step assays into μ PADs.

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Supporting Information

Experimental details on spot test experiments, the enzymatic reaction mechanism, results for optimizing the IDA concentration and the hydrolysis time, reproducibility, storage stability, Tris-HCl buffer pH function integration, comparison of the developed flow control-based devices with other previously published articles, results for pesticide-spiked river water sample analysis. This material is available free of charge on the Web at <http://www.jsac.or.jp/analsci/>

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