Paper-based diagnostic devices

Spencer A. Schultz[1], *Isabelle C. Noxon*[1], *Tyler A. Sisley*[1] *and Andres W. Martinez*[1]

2.1 Introduction

Paper, broadly defined as any porous hydrophilic membrane, has served as a platform for diagnostic assays for millennia. One of the earliest recorded examples of a paper-based assay comes from *c*. 77 A.D. when Pliny the Elder, a Roman naturalist, described a qualitative test for detecting the presence of adulterants in verdigris using papyrus impregnated with an extract of gallnuts [1,2]. Starting with simple qualitative colorimetric assays and moving all the way to the current field of paperfluidics, paper offers several unique advantages as a platform for conducting a wide variety of diagnostic assays as well as for collecting, storing and transporting samples. This chapter will provide an overview of existing diagnostic devices made primarily out of paper and then focus on paper-based microfluidic devices, the next generation of paper-based diagnostic devices that promises to extend the use of paper as a material for fabricating diagnostic devices well into the future.

Paper is defined formally as a flexible sheet made from pressed cellulosic fibers (Figure 2.1) [3]. The process of making paper was developed in China during the Han Dynasty (206 B.C. to 220 A.D.) [4]. Written records credit Tsai Lun, an official in the Han Court, with developing the modern form of paper as well as the papermaking process in 105 A.D. [5]. Although the equipment used for making paper has evolved over time, the essential steps of the papermaking process have remained unchanged: first, a dilute suspension of cellulosic fibers is prepared in water; then, the fibers are deposited onto a screen forming a mat; and, finally, the mat of fibers is pressed and dried to produce a sheet of paper [5]. The cellulosic fibers used to make paper can come from many sources, but most diagnostic devices are made with paper produced from cotton fibers made primarily of cellulose (Figure 2.2(a)) [4]. Many other types of porous hydrophilic membranes have been developed over time and have been incorporated into diagnostic devices [3]. Among these, porous membranes made from nitrocellulose are used most commonly because of their high protein-binding affinity

[1]Department of Chemistry and Biochemistry, California Polytechnic State University, San Luis Obispo, CA, USA

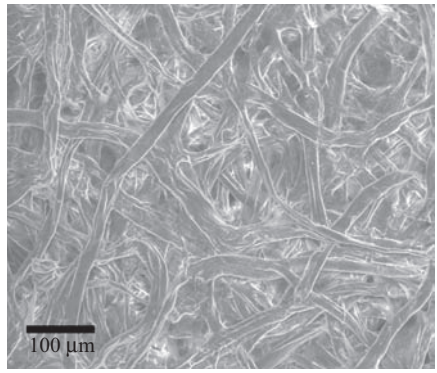

Figure 2.1 SEM micrograph (400×) of the surface of Whatman No. 1 chromatography paper

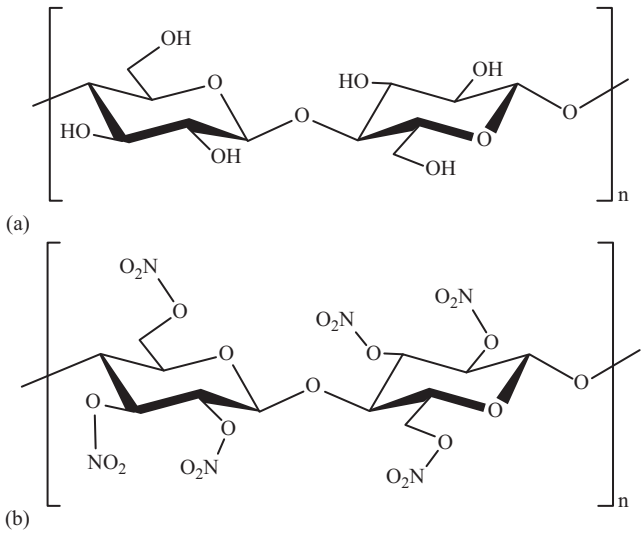

Figure 2.2 Chemical structures of cellulose (a) and nitrocellulose (b)

(Figure 2.2(b)) [3]. An enormous diversity of papers with varying chemical composition, thickness, and pore size are available, and each has unique characteristics that could be harnessed for specific applications in paper-based diagnostic devices.

Paper has many inherent characteristics that make it attractive as a platform for diagnostic assays. Paper is inexpensive and widely available, so scientists across the globe and throughout history have been able to work with paper and develop tests that are compatible with paper [2,4,6]. Paper is typically white and provides an excellent background for assays that produce a colored product or a change in color. Another attractive characteristic of paper is that it has a large surface-to-volume ratio, so a small volume of fluid (∼1 µL) can typically produce a spot on paper that is large enough (∼0.5 cm in diameter) to be seen with the naked eye. The

large surface-to-volume ratio of paper also allows for reagents and samples to be dried and stored on paper-based devices. Paper can even be modified chemically in order to covalently bond reagents to the fibers [7]. Paper can also serve as the stationary phase for chromatographic separations of analytes [8]. Paper is disposable—one simple method for disposing paper-based tests is through incineration. Paper wicks fluid by capillary action, so fluids will move through a paper-based device without any external sources of power. And finally, as mentioned previously, paper can be produced from a wide variety of fibers and with a wide variety of characteristics (e.g., pore size, wicking speed, protein binding ability), which can be harnessed for specific applications and can even be combined in a single device to enable particular capabilities.

2.2 Current paper-based diagnostic devices

One of the best-known examples of a paper-based test is litmus paper, which is used to determine whether a solution is acidic or basic [7]. Scientific reports dating back to the nineteenth century describe the use of litmus paper for analyzing samples, and it is still sold commercially and used routinely by professional scientists and students [6,9]. In addition to litmus paper, a huge variety of other colorimetric tests have been developed to detect the presence of analytes on paper, but most of these tests have not been developed into commercial products [2,10–12]. Currently, there are two primary forms of commercially available paper-based diagnostic devices: dipstick assays and lateral-flow assays (LFAs) [3]. A third form of paper-based devices known as paper-based arrays are used primarily to collect, store, and transport samples but are also used in academic research to conduct assays.

2.2.1 Dipstick devices

Dipstick assays are one of the first examples of paper-based diagnostic devices for clinical use [4]. Dipstick devices comprise one or more small test pads of paper that have been impregnated with reagents for specific colorimetric assays and that are adhered to a plastic strip, which serves as a handle for the device (Figure 2.3). Dipstick assays are performed by quickly immersing the dipstick into the sample to be tested and then observing the colors that are produced on the test pads. The colors produced on the test pads can often be compared to a color-coded chart provided with the dipstick in order to make a semiquantitative determination of the concentration of the analyte in the sample [3].

The first pH dipstick test strips were patented and commercialized in the 1920s [4]. These devices can be purchased to determine the pH of a solution within a given range with varying amounts of sensitivity, both of which are determined by the number of test pads on the dipsticks as well as the specific indicators used on the test pads [13]. The first dipstick assays for clinical use were developed in the 1950s and were introduced commercially in the 1960s [3]. These initial dipstick assays were developed to quantify the concentration of glucose in urine by harnessing enzymatically catalyzed reactions involving glucose oxidase and horseradish peroxidase [14]. Urinalysis dipstick tests now include as many as ten

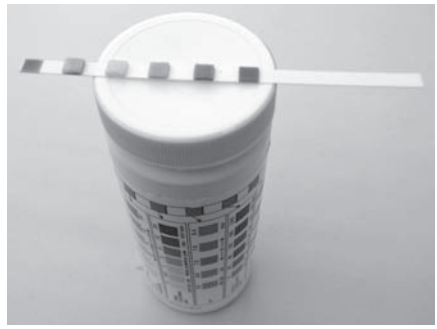

Figure 2.3 Dipstick device for analysis of water [18]

test pads to detect or analyze glucose, bilirubin, ketones, nitrite, urobilinogen, protein, blood, leukocytes, pH, and specific gravity [3,15,16]. These tests allow for the diagnosis of a variety of conditions and diseases including kidney disease, urinary tract infections, carbohydrate metabolism disorders, and liver disease [4,15,16]. For more sophisticated urinalysis tests, the dipsticks can be analyzed by a detector that analyzes the color of the test pads in order to improve the accuracy and precision of the tests [3,17]. Another common application for dipstick tests is for the analysis of water samples. Dipstick devices for testing drinking water and swimming pools can be used to quickly determine pH, total hardness, total chlorine, total bromine, free chlorine, total alkalinity, and cyanuric acid levels [18].

The main advantages of dipstick devices are that they are inexpensive, easy to use and provide immediate results. Dipstick tests are also simple to design and manufacture. The disadvantages of dipstick assays are that they are typically qualitative or semiquantitative, they often have limited sensitivity and selectivity, and they can only be developed for analytes that can react to produce a color change or be linked to a reaction that produces a color change [6]. Dipstick assays often also require a relatively large volume of sample in order to wet the test pads. For these reasons, dipstick devices are most practical for testing relatively simple samples, such as urine and water, that are abundant and contain relatively high concentrations of analytes, such as glucose or chlorine. In order to overcome some of the limitations with dipstick assays, LFAs were developed to expand the range of analytes that could be detected as well as the sensitivity and selectivity of the assays.

2.2.2 Lateral-flow devices

As the name suggests, lateral-flow devices rely on the capillary wicking of fluids across a porous membrane in order to detect an analyte [19]. Lateral-flow devices are made typically of several different types of paper that are treated with different reagents and held in contact with each other to create a single fluidic path that wicks a sample from a sample port to an absorbent pad, which serves as a waste-collection zone (Figure 2.4) [20]. In a typical LFA, a few drops of sample are introduced to the sample port, and the sample then wicks across a conjugate pad in

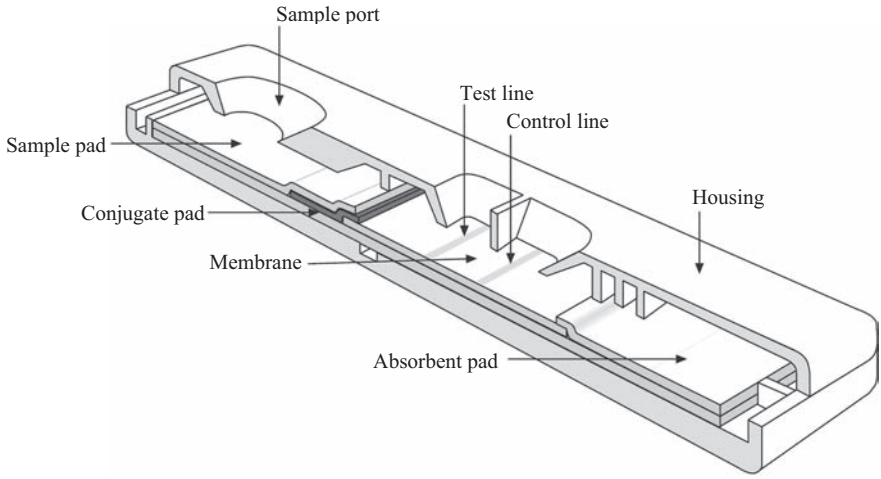

Figure 2.4 Schematic diagram of a lateral-flow device. Reproduced by permission of EMD Millipore Corporation. 2002, 2008 EMD Millipore Corporation

which it interacts with a labeling reagent—typically an antibody bound to a colored particle—that binds specifically to the analyte. The labeled analyte then wicks across a membrane containing a capture reagent—typically a second antibody—that is immobilized on the device in a narrow test line and that captures selectively the labeled analyte. This process generates a colored line or band on the device when the analyte is present. All the other components of the sample as well as any excess labeling reagent continue to wick along the device into the waste zone. Most commercial devices also include a control line containing a second capture reagent on the membrane that binds only to the labeling reagent and serves to confirm that the capture reagents are working properly [3].

LFAs are widely used in laboratories, hospitals, and in the home where they can detect conditions such as pregnancy, disease, drug use, contamination, infection, and organ malfunction [3,21–23]. The first LFAs were developed in the late 1960s and have become a user-friendly, robust, affordable, point-of-care platform for diagnostics [19]. The first commercial LFAs were developed to detect human chorionic gonadotropin (hCG); a high level of this biomolecule is a positive indicator for pregnancy [24]. This test was fast (1–3 min), simple to use even for untrained users, required little user input, used small sample sizes, and was subsequently made commercial by 1988 [24]. The hCG test is now used globally to detect pregnancy and can be purchased for less than US$ 1 [25].

The three main facets of LFAs are the device or platform, sample preparation, and the immunochemical reaction. The LFA platform is composed of a sample pad, a fiberglass conjugate pad for storage of the labeled reagent, a membrane that is typically nitrocellulose, an absorbent waste pad, and often a small plastic holder that encases the assembled device (Figure 2.4) [19]. Although nitrocellulose is

brittle and flammable, it is the membrane used in most LFAs because it is porous, wicks fluids by capillary action, and its surface has a high affinity for binding proteins [4,23]. This binding characteristic is essential for the detection of analytes, as LFAs rely on selective binding and retention of the labeled analyte. Fabrication of commercial LFAs begins with treatment of the different components. The sample pad is pretreated, the fiberglass conjugate pad is loaded with the labeled reagent, and the membrane is printed with test and control lines of capture reagents and then blocked for nonspecific adsorption [20]. Each component is dried, and the device is assembled, cut into strips, and placed into cartridges for packaging [26].

The hCG urine test does not require much sample preparation, but there are many other LFAs that detect biological components from blood plasma or serum that do require treatment. Often sample preparation includes collection of blood, treatment with anticoagulants, separation of blood cells from plasma or serum, and dilution of the sample [19]. These steps are important, but are cumbersome and time consuming—especially at the point of care in which expensive equipment for sample treatment is difficult to obtain or not available [27].

The immunochemical reaction is arguably the most important component of LFAs. There are two main types: immunoassays that rely on antibody–antigen interactions, and nucleic acid-based assays that rely on nucleic acid aptamers and target molecule binding or nucleic acid hybridization [19]. These immunochemical reactions rely heavily on the sensitive and specific interactions that consequently determine the sensitivity and selectivity of the assay itself [19]. The component of the immunochemical reaction that allows for visual detection is the colorimetric probe, which is conjugated to an antibody, protein, or DNA sequence to make the labeling reagent. The probes are most commonly latex beads, gold nanoparticles, fluorescent tags, quantum dots, and enzymes. The type of colorimetric probe is determined by several factors including the type of sample, the analyte, pH, and device storage conditions. Immunochemical reagents can be synthesized against a myriad of proteins and biomolecules. The heterogeneity of immunochemical combinations is part of what makes LFAs such a widely studied and versatile platform [19].

The main advantages of LFAs, as with most other paper-based devices, are that they are inexpensive and easy to use—the user typically only has to apply a few drops of sample to the device and then read the resulting colored lines, unless more sophisticated sample preparation is required [3]. What sets LFAs apart from other paper-based devices, like dipsticks, is that by harnessing the wicking capabilities of paper, LFAs perform what is effectively a chromatographic separation of the analyte from the other components of the sample as well as a concentration of the analyte along the test line. Both these actions lead to highly sensitive and specific assays. Some more sophisticated LFAs include a second step in which an amplification reagent is added to the device in order to enhance the color of the test line and further improve the sensitivity of the device [3]. Another advantage of LFAs is that as they rely on immunochemical reactions to detect analytes, the same basic device design can be used to detect a huge range of analytes by simply changing the labeling reagent and the capture reagent [19]. On their own, LFAs are typically qualitative and only provide yes/no results, but more recent LFAs are compatible

with electronic readers that analyze the intensity of color that develops at the test line or rely on fluorescent labels in order to make quantitative determinations [3].

2.2.2.1 Vertical-flow devices

Vertical-flow devices rely on the same analyte-detection principles as lateral-flow devices and can be used to conduct the same types of assays, but, instead of lining up the different components of the device horizontally as in LFAs, the various components are stacked vertically. The advantage of stacking the layers vertically is that the path length for the sample to wick through the device is significantly reduced; however, these assays typically require multiple steps by the user in order to be performed correctly [3].

2.2.3 Paper-based arrays

Paper-based arrays are used primarily for the collection, storage, and transportation of samples. Paper-based arrays typically comprise a card marked with multiple circular zones that can be filled with liquid samples. The zones are often pretreated with reagents to improve the stability of the analytes or to disinfect the samples. Samples, such as blood, can be spotted on the array and dried, and then the array can be shipped to a central laboratory where the samples can be eluted from the array and analyzed [28]. This process allows for large numbers of samples to be collected and stored in a convenient format that does not require refrigeration [29]. The sample collection can also be performed in the field with minimal instrumentation [29].

One common application for paper-based arrays is in dried blood spot (DBS) testing [28]. This technique was developed in the early 1900s but gained widespread use in the 1960s and 1970s when Robert Guthrie used DBS to develop a technique for the systematic screening of newborns for metabolic diseases [30]. Now known as the Guthrie test, newborns around the world are tested for a series of metabolic disorders and diseases by collecting their blood from a heal prick on a DBS card [28]. In fact, DBS can be used to detect a wide range of analytes and diseases including HIV, dengue, hepatitis, and small molecule drugs [28,29,31].

An alternative application of paper-based arrays is for the synthesis of libraries of short peptides or small molecules via a technique known as spot synthesis [32–35]. Once prepared, these arrays can be used to conduct a wide range of assays including binding assays, enzyme assays, and studies with living organisms [36].

2.3 Paper-based microfluidic devices

In 2007, the Whitesides group at Harvard University published an article describing the first example of what would become known as paper-based microfluidic devices or microfluidic paper-based analytical devices (microPADs) (Figure 2.5) [37]. Having contributed a substantial body of work to the field of conventional microfluidic devices, including the development of soft lithography [38,39], George

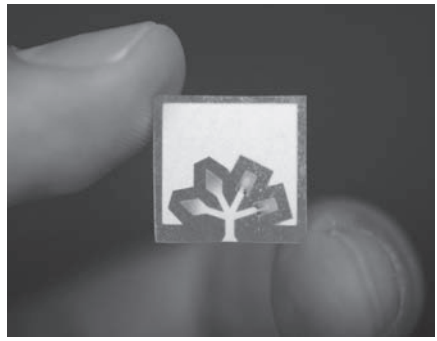

Figure 2.5 A paper-based microfluidic device for detecting glucose (left zones) and protein (right zones)

Whitesides was looking to develop a new class of devices that married the advantages of lateral-flow and dipstick devices, such as capillary wicking and low cost, with the advantages of conventional microfluidic devices, such as low volumes of sample, multiplexing, and controlled sequential steps. The ultimate goal of the Whitesides group was to develop a new class of ultra-low-cost point-of-care diagnostic devices that could be deployed to resource-limit settings and improve access to healthcare to populations around the globe.

Paper was a natural choice as a material for fabricating this new class of devices for all the same reasons mentioned in the introduction. The main innovation described in the initial publication from the Whitesides group was the development of a method to pattern paper into a network of hydrophilic channels and test zones bounded by hydrophobic barriers [37,40]. The channels could wick samples and distribute them into the test zones where assays would take place. Although examples of patterning paper using waxes had been described previously [2,41], what set the work from the Whitesides group apart was the focus on the development of a new class of low cost, very simple to use portable diagnostic devices [37,42]. This work catalyzed a surge of interest in the development of paper-based assays and resulted in the establishment of the new field of paperfluidics [3,6,12].

2.3.1 Fabrication of paper-based microfluidic devices

Fabrication of paper-based microfluidic devices involves the patterning of a piece of paper using hydrophobic inks to define hydrophilic channels and test zones bounded by hydrophobic barriers [12]. Several methods for patterning paper have been developed since 2007, each with their own set of advantages and disadvantages [6,12]. Although the methods are very diverse in terms of equipment and inks, they can be classified broadly into two categories: indirect and direct patterning. In indirect patterning, the first step of the process involves coating the entire piece of paper with a hydrophobic ink or reagent such as a wax, a polymer, or a resin. In the second step, the hydrophobic reagent is etched away selectively to

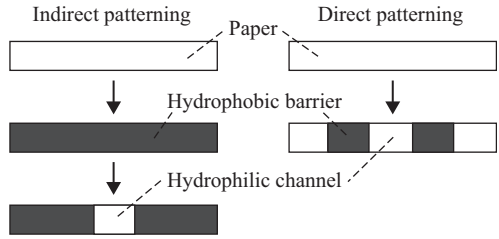

Figure 2.6 Cross-sectional schematic diagram of indirect and direct patterning of paper

produce hydrophilic channels. In direct patterning, the hydrophobic ink is printed directly on the paper in the desired pattern (Figure 2.6).

The first published method for fabricating paper-based microfluidic devices was an example of indirect patterning and was an adapted form of photolithography [37]. In this approach, the paper is first impregnated with SU-8 photoresist, a negative photoresist, and dried. The paper is then exposed to UV light through a photomask in order to cross-link the exposed portions of the photoresist. Finally, the unexposed portions of the photoresist are dissolved and removed from the paper using a solvent such as acetone to yield the desired pattern of channels and test zones in the paper [37,40,42]. Other photopolymers, in addition to SU-8, have also shown to be useful for patterning paper via indirect patterning [3].

Indirect patterning was also achieved using an inkjet printer [43]. The process involves first coating a piece of paper with polystyrene to make it hydrophobic. The polystyrene is then selectively removed from specific areas of the paper by printing toluene onto the paper using an inkjet printer in the desired pattern. The toluene washes the polystyrene out of the paper and returns the washed paper to its original hydrophilic state.

A third example of indirect patterning using plasma treatment involves impregnating a sheet of paper with alkyl ketene dimer, a reagent used commonly for sizing paper to make it more hydrophobic [44]. The reagent is then selectively etched away by placing the paper between two metal stencils and treating it in a vacuum plasma reactor. The areas of the paper that are exposed to the plasma, through the metal stencils, are made hydrophilic and the areas of paper that are protected by the stencil remain hydrophobic.

In direct patterning, hydrophobic agents are applied onto the paper in the desired pattern. Hydrophobic agents are applied typically through conventional printing technologies, such as inkjet printing, screen printing, flexographic printing, and stamping, but could even be applied by hand. Each technique has its own set of advantages and disadvantages in terms of cost, resolution, throughput, and technical difficulty. One of the most popular methods of direct patterning is wax printing [45–47]. In wax printing, a solid-ink printer is used to print wax directly on the surface of paper in any desired pattern. The printed paper is then heated to reflow the wax and create hydrophobic barriers within the paper. The entire process can be

completed in less than 5 min for an entire sheet of paper, and features a small as 1 mm can be patterned reliably [46]. The equipment required for wax printing, namely the solid-ink printer and the heater (e.g., oven, hotplate, thermal laminator, heat gun, and iron) can be purchased for less than US$ 1,000, and the process does not require a clean room or a fume hood, so wax printing can be performed by users with limited resources or limited experience with fabrication [12]. Another advantage of wax printing is that there is virtually no production-cost penalty for making small batches of devices at a time, which makes it well suited for laboratory-scale prototyping.

In comparing indirect and direct patterning methods, indirect patterning methods are generally more time consuming as they involve at least two steps, but often several more. Indirect patterning methods also consume larger quantities of reagents and solvents as the entire piece of paper is treated and made hydrophobic. Direct patterning methods are generally faster, less expensive, and less technically challenging than indirect methods. One advantage of indirect patterning, however, is that it tends to allow for higher resolution patterns to be produced. Specifically in the case of photolithography, the resolution of the pattern is controlled by the resolution of the exposure to UV light, so smaller features can be produced using this method compared to wax printing [12]. In one instance, direct and indirect methods of patterning were combined to pattern paper with Teflon in order to generate microPADs that were compatible with organic solvents [48].

A third method of preparing microPADs is via shaping or cutting. In this approach the desired network of channels and test zones is cut out of a sheet of paper, either manually or using a laser cutter or cutting plotter [49,50]. This method is simple to perform and does not require any reagents. The devices fabricated via cutting usually have to be encased in plastic to protect the paper, provide structural support to the paper, and facilitate manipulation of the devices [12].

Once the patterning process is complete, devices can be further modified to add additional capabilities. One common post-patterning step is to encase devices in plastic using either tape, thermal lamination sheets or toner [49,51,52]. Another option is to add additional features to the device such as electrodes. Electrodes are typically printed onto the devices after they are patterned using conductive inks [53–57]. Devices can also be modified to control the wicking in the device either by incorporating valves [58], adding reagents or shunts that slow down wicking [59,60], or adding additional layers of paper or plastic to speed up wicking [52,57,61]. Finally, multiple layers of patterned paper can be stacked on top of each other to produce three-dimensional (3D) microPADs (Figure 2.7) [62–65]. The layers of paper in the 3D device can either be bonded to each other permanently using permanent adhesives or tapes, or they can be held together temporarily using a manifold or removable adhesives or tapes.

To complete the fabrication process, reagents for the assay must be applied to the device. Typically reagents are deposited onto microPADs from solution, and the solutions are then allowed to dry leaving the reagent behind on the device. Reagents can be deposited manually using a pipet or capillary tube, or can be

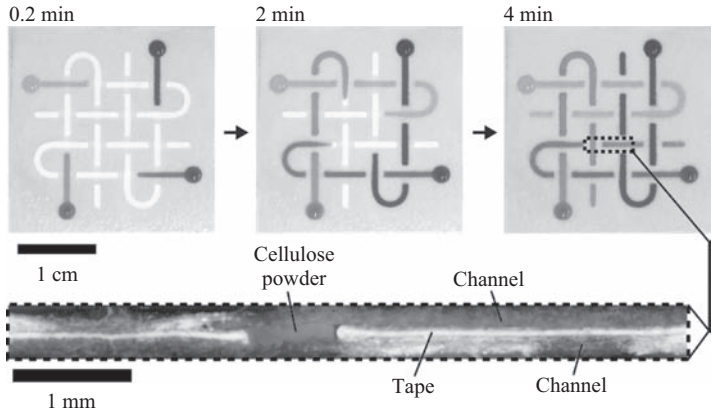

Figure 2.7 Top view and cross-sectional view of a three-dimensional microPAD assembled from two layers of patterned paper and a layer of double-sided tape. Reproduced by permission of The National Academy of Sciences from Reference 62. 2008 National Academy of Sciences, USA

deposited using an inkjet printer or liquid-dispensing robot. More recently, a method for solid deposition of reagents using custom-made pencils was described, which led to improved shelf life of the reagents on the devices [66].

2.3.2 Applications of paper-based microfluidic devices

Initial research on paper-based microfluidic devices focused primarily on the development of methods of fabricating devices. The first devices consisted generally of a sample inlet that directed the sample into one more channels and then into multiple test zones in which reagents for colorimetric assays were deposited (Figure 2.5). These initial devices required small volumes of fluid (≤ 20 μL) and could perform multiple assays simultaneously from a single sample-addition step [37,42]. As work with paper-devices expanded, new applications and unique assays were developed, many of which were enabled by the unique capabilities of microPADs. This section will highlight four areas of applications for microPADs that show promise for future diagnostic devices.

Colorimetric assays, assays that result in a color change in response to the presence of analyte, remain one of the most common types of assays conducted on microPADs [3]. A wide range of colorimetric assays have developed for microPADs including indicator-based assays, enzymatic assays, sorbent-based assays, as well as combinations as these approaches such as enzyme-linked immunosorbent assays (ELISAs) [3]. These assays have been shown to be useful for detecting a wide range of analytes including ions, small molecules, proteins, and DNA. Concurrent with the development of colorimetric assays on microPADs, several methods for quantifying the resulting colorimetric signal on the devices were

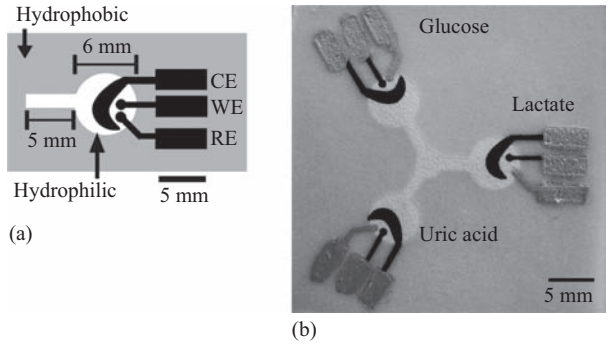

Figure 2.8 Schematic of an electrochemical paper-based microfluidic device including three electrodes (a), and image of an actual device for detecting glucose, lactate, and uric acid (b). Adapted with permission from Reference 53. 2009 American Chemical Society

developed. One promising approach is the use of digital image colorimetry, in which a digital image of the results of a colorimetric assay is obtained, and then the pixel intensity of the color is measured [67,68]. The images could be obtained using a scanner, a digital camera or, most interestingly in the context of portable point-of-care diagnostic assays, a camera phone [42]. The results can be calibrated through either external calibration or standard addition and can typically produce results with relative errors and relative standard deviations within ten percent [68].

As an alternative to colorimetric assays, paper-based assays that rely on electrochemical detection offer many advantages, especially when it comes to performing quantitative tests [53,55,56]. Electrochemical paper-based assays have been demonstrated for a wide range of analytes with high accuracy and precision, and with the ability to detect analytes at femtomolar concentrations [6,69,70]. The electrodes for electrochemical assays are typically printed directly on the microPADs (Figure 2.8), and an external reader is used typically to perform the assay [6].

A third type of paper-based assays that eliminate the need for sophisticated external readers are chronometric assays, in which the signal from the assay is the time that it takes the sample to wick across a channel in a device (Figure 2.9) [64,71–74]. These assays rely on the unique properties of microPADs to wick fluids along channels in paper as well as the ability to pattern reagents in multiple layers of paper and then assemble these layers into a single 3D device. In these devices, a reagent that creates and impermeable or semi permeable barrier is added to a channel in the device. The presence of the analyte triggers a chemical reaction that results in the degradation of the barrier. So, when the analyte is present, the sample wicks across the channel more quickly than when the analyte is absent. Chronometric assays stand out for their simplicity, sensitivity, and low limits of detection, which rival traditional ELISA [74].

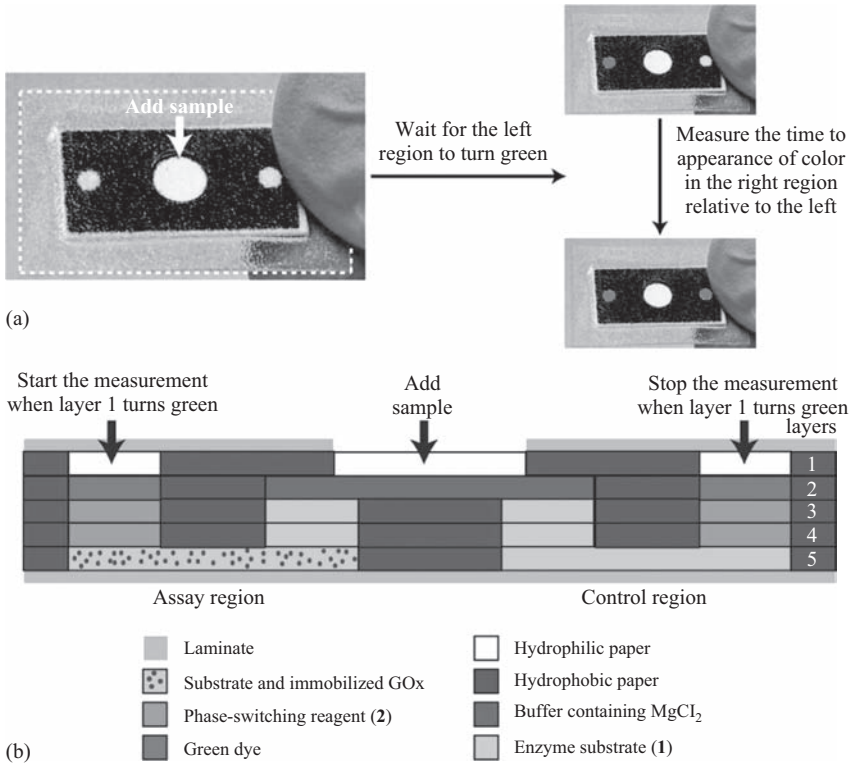

Figure 2.9 A chronometric paper-based assay (a) and a cross-sectional schematic of the layers and components making up the device (b). Adapted with permission from Reference 74. 2013 American Chemical Society

In addition to developing specific types of quantitative assays, the paperfluidics community has devoted considerable effort toward developing devices capable of performing multiple sequential steps in order to automatically perform multistep assays, such as ELISAs or enzyme-inhibition assays. For example, a device that performs a typical lateral-flow immunoassay and then automatically adds an amplification reagent to enhance the intensity of the test line on the device and improve the limit of detection of the assay was developed by designing a device with multiple fluid inlets leading to a single-test zone (Figure 2.10) [50,75,76]. Alternatively, a similar type of assay was achieved by carefully designing a series of channels that would wick fluid from a single sample inlet and deliver it to a test zone at different times [77]. The development of fluidic diodes provided a third option for achieving automated multistep assays on microPADs [78,79]. Finally, paper-based enzyme-inhibition tests that require incubation of an enzyme with a sample followed by introduction of the enzyme substrate were automated by using erodible polymeric bridges, which allowed for the introduction of single-use timed shut-off valves into microPADs [58].

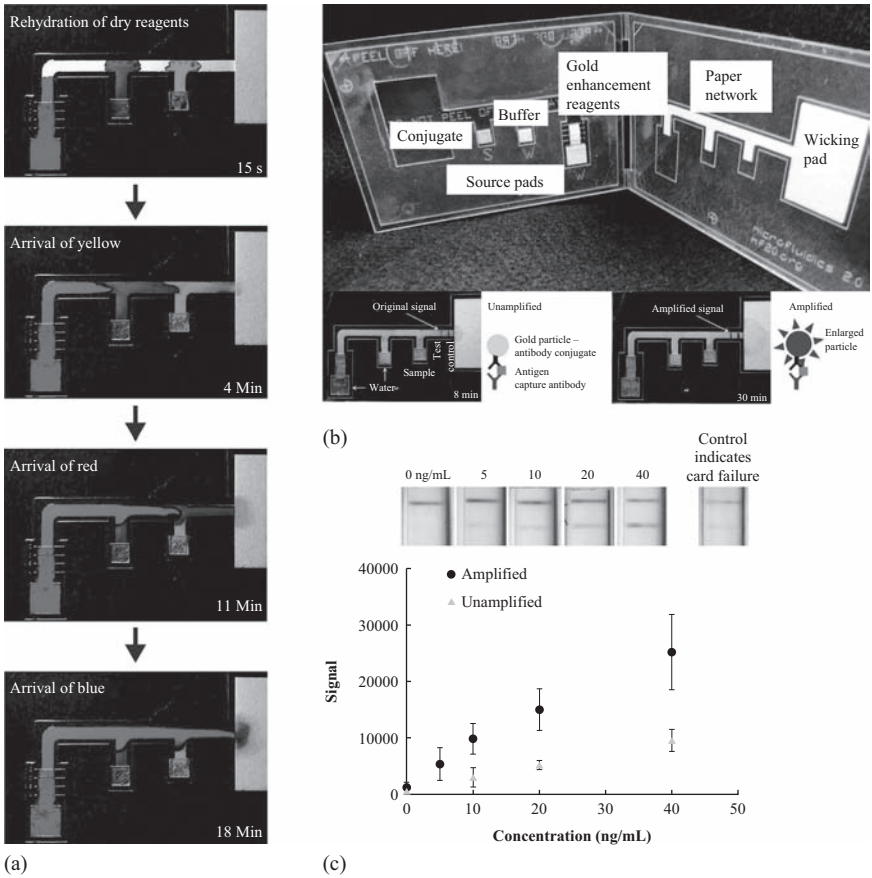

Figure 2.10 Multistep paper-based assay. (a) Images of the device performing multiple sequential delivery of reagents using dyes as sample reagents. (b) Image of actual device for detecting Malaria. (c) Results of the test showing the improved signal obtained using the multistep device (amplified). Adapted with permission from Reference 76. 2012 American Chemical Society

2.4 Conclusions

Paper offers several advantages as a platform for conducting simple, point-of-care diagnostic assays. Current, commercially available paper-based diagnostic devices, such as dipstick assays and LFAs, will continue to be used widely due to their low cost and simplicity. A new generation of paper-based devices is currently being developed and promises to extend those applications of paper for point-of-care diagnostics.

The concept of making diagnostic devices out of paper is being adopted by a growing community of researchers. MicroPADs were first developed as diagnostic devices for use in developing countries but are now being developed to detect a wide range of analytes and could be used to monitor air, soil, and water quality; they could be used as diagnostic devices for animals and plants; they could be used in home healthcare to diagnose disease or monitor drug levels; and they could be used by the military and first-responders to assess a person's health status or detect toxins, biohazards, or explosives. MicroPADs also have many potential applications in basic research.

One of the great aspects of paper-based microfluidic devices is that there is a very low barrier to entry, both in terms of the cost of equipment and the technical expertise that is required to fabricate devices. A pair of scissors and a paper towel is all that is really needed to make simple paper-based devices. So, much like open-source software, we look forward to seeing contributions to this field from all kinds of scientists from all over the world and seeing where we can go with this simple yet-powerful technology.

References

[1] Pliny, B.J., Riley H.T. *The Natural History of Pliny*. 1857. Vol VI. pp. 140–1; 235–8; 362–4.

[2] Yagoda H. 'Applications of confined spot tests in analytical chemistry: Preliminary paper'. *Industrial Engineering Chemistry and Analytical Edition*. 1937;**9**(2):79–82.

[3] Yetisen A.K., Akram M.S., Lowe C.R. 'Paper-based microfluidic point-of-care diagnostic devices'. *Lab on a Chip*. 2013;**13**(12):2210–51.

[4] Hu J., Wang S., Wang L., *et al*. 'Advances in paper-based point-of-care diagnostics'. *Biosensors and Bioelectronics*. 2014;**54**:85–97.

[5] Hunter D. *Papermaking: The History and Technique of an Ancient Craft*. New York, NY: Dover Publications Inc.; 1978.

[6] Cate D.M., Adkins J.A., Mettakoonpitak J., Henry C.S. 'Recent developments in paper-based micro fluidic devices'. *Analytical Chemistry*. 2015;**87**:19–41.

[7] Pelton R. 'Bioactive paper provides a low-cost platform for diagnostics'. *TrAC – Trends in Analytical Chemistry*. 2009;**28**(8):925–42.

[8] Block R.J., Durrum E.L., Zweig G. *A Manual of Paper Chromatography and Paper Electrophoresis*. New York: Academic Press; 1955.

[9] Davy J. 'On gaseous compound of organic oxide and chlorine'. *Philosophical Transactions of the Royal Society of London*. 1812;**102**:144–51.

[10] Feigl F., Anger V., Koslow R.H. 'Spot tests in inorganic analysis'. *Journal of The Electrochemical Society*. 1973;**120**:261C.

[11] Hoffman J.H., Weaver J.W. 'Qualitative spot tests', in *Analytical Methods for a Textile Laboratory*. Research Triangle Park, NC: American Association of Textile Chemists and Colorists; 1984. pp. 156–206.

[12] Martinez A.W., Phillips S.T., Whitesides G.M., Carrilho E. 'Diagnostics for the developing world: Microfluidic paper-based analytical devices'. *Analytical Chemistry*. 2010;**82**(1):3–10.
[13] 'EMD Millipore pH Indicator Strips' [Online]. Available: http://www.emdmillipore.com/Web-US-Site/en_CA/-/USD/ViewParametricSearch-SimpleOfferSearch?SearchTerm=*&SingleResultDisplay=SFProductSearch&SearchContextCategoryUUIDs=UJWb.qB.jtAAAAE_9A93.Lxj. [Accessed: 30-Jun-2016].
[14] Free A.H., Adams E.C., Kercher M.L., Free H.M., Cook M.H. 'Simple specific test for urine glucose'. *Clinical Chemistry*. 1957;**3**(3):163–8.
[15] Boyd J., Barratt J. 'Interpretation and management of abnormal dipstick urinalysis'. *Medicine (Baltimore)*. 2011;**39**:(6):312–16.
[16] Roberts J.R. 'Urine dipstick testing: Everything you need to know urinalysis: A comprehensive review'. *Emergency Medicine News*. 2007;**29**:24–7.
[17] Peele J.D., Gadsden R.H., Crews R. 'Semi-automated vs. visual reading of urinalysis dipsticks'. *Clinical Chemistry*. 1977;**23**(12):2242–6.
[18] 'AquaChek Pool & Spa Test Strips' [Online]. Available: http://www.aquachek.com/pool-spa-owners/aquachek-select-7-in-1/. [Accessed: 15-Jun-2016].
[19] Bahadir E.B., Sezgintürk M.K. 'Lateral flow assays: Principles, designs and labels'. *TrAC Trends in Analytical Chemistry*. 2016;**82**:286–306.
[20] EMD Millipore. *Rapid Lateral Flow Test Strips*. Billerica, MA: Millipore Corporation; 2008.
[21] Chan C.P.Y., Sum K.W., Cheung, K.Y., *et al*. 'Development of a quantitative lateral-flow assay for rapid detection of fatty acid-binding protein'. *Journal of Immunological Methods*. 2003;**79**:91–100.
[22] Posthuma-Trumpie G.A., Korf J., Van Amerongen A. 'Lateral flow (immuno) assay: Its strengths, weaknesses, opportunities and threats. A literature survey'. *Analytical and Bioanalytical Chemistry*. 2009;**393**(2):569–82.
[23] Sajid M., Kawde A.N., Daud M. 'Designs, formats and applications of lateral flow assay: A literature review'. *Journal of Saudi Chemical Society*. 2015;**19**(6):689–705.
[24] Wild D. *The Immunoassay Handbook*, 4th ed. Amsterdam: Elsevier Science; 2013.
[25] 'Clinical Guard One Step HCG Pregnancy Test Strip' [Online]. Available: https://www.clinicalguard.com/one-step-hcg-pregnancy-test-strip-p-704.html. [Accessed: 28-Jun-2016].
[26] Sharma S., Zapatero-Rodriguez J., Estrela P., O'Kennedy R. 'Point-of-care diagnostics in low resource settings: Present status and future role of microfluidics'. *Biosensors*. 2015;**5**(3):577–601.
[27] Chin C.D., Linder V., Sia S.K. 'Lab-on-a-chip devices for global health: Past studies and future opportunities'. *Lab on a Chip*. 2007;**7**(1):41–57.
[28] Lehmann S., Delaby C., Vialaret J., Ducos J., Hirtz C. 'Current and future use of "dried blood spot" analyses in clinical chemistry'. *Clinical Chemistry and Laboratory Medicine*. 2013;**51**(10):1897–909.

[29] Meesters R.J., Hooff G.P. 'State-of-the-art dried blood spot analysis: An overview of recent advances and future trends'. *Bioanalysis*. 2013;**5** (17):2187–208.
[30] Guthrie R., Susi A. 'A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants'. *Pediatrics*. 1963;**32**: 338–43.
[31] Stevens R., Pass K., Fuller S., *et al*. 'Blood spot screening and confirmatory tests for syphilis antibody'. *Journal of Clinical Microbiology*. 1992;**30** (9):2353–8.
[32] Frank R. 'Spot-synthesis: An easy technique for the positionally addressable, parallel chemical synthesis on a membrane support'. *Tetrahedron*. 1992;**48** (42):9217–32.
[33] Frank R. 'The SPOT-synthesis technique: Synthetic peptide arrays on membrane supports – Principles and applications'. *Journal of Immunological Methods*. 2002;**267**(1):13–26.
[34] Blackwell H.E. 'Hitting the SPOT: Small-molecule macroarrays advance combinatorial synthesis'. *Current Opinion in Chemical Biology*. 2006;**10** (3):203–12.
[35] Kramer A., Reineke U., Dong L., *et al*. 'Spot synthesis: Observations and optimizations'. *Journal of Peptide Research*. 1999;**54**(4):319–27.
[36] Hilpert K., Winkler D.F.H., Hancock R.E.W. 'Peptide arrays on cellulose support: SPOT synthesis, a time and cost efficient method for synthesis of large numbers of peptides in a parallel and addressable fashion'. *Nature Protocols*. 2007;**2**(6):1333–49.
[37] Martinez A.W., Phillips S.T., Butte M.J., Whitesides G.M. 'Patterned paper as a platform for inexpensive, low-volume, portable bioassays'. *Angewandte Chemie International Edition*. 2007;**46**:1318–20.
[38] Xia Y., Whitesides G.M. 'Soft lithography'. *Annual Review of Materials Science*. 1998;**28**(1):153–84.
[39] Xia Y., Whitesides G.M. 'Soft lithography'. *Angewandte Chemie International Edition*. 1998;**37**(5):550–75.
[40] Martinez A.W., Phillips S.T., Wiley B.J., Gupta M., Whitesides G.M. 'FLASH: A rapid method for prototyping paper-based microfluidic devices.' *Lab on a Chip*. 2008;**8**(12):2146–50.
[41] Muller R.H., Clegg D.L. 'Automatic paper chromatography'. *Analytical Chemistry*. 1949;**21**:1123–5.
[42] Martinez A.W., Phillips S.T., Carrilho E., Thomas S.W., Sindi H., Whitesides G.M. 'Simple telemedicine for developing regions: Camera phones and paper-based microfluidic devices for real-time, off-site diagnosis'. *Analytical Chemistry*. 2008;**80**(10):3699–707.
[43] Abe K., Suzuki K., Citterio D. 'Inkjet-printed microfluidic multianalyte chemical sensing paper'. *Analytical Chemistry*. 2008;**80**(18):6928–34.
[44] Li X., Tian J., Nguyen T., Shen W. 'Paper-based microfluidic devices by plasma treatment'. *Analytical Chemistry*. 2008;**80**(23):9131–4.

[45] Lu R., Shi W., Jiang L., Qin J., Lin B. 'Rapid prototyping of paper-based microfluidics with wax for low-cost, portable bioassay'. *Electrophoresis*. 2009;**30**(9):1497–500.
[46] Carrilho E., Martinez A.W., Whitesides G.M. 'Understanding wax printing: A simple micropatterning process for paper-based microfluidics'. *Analytical Chemistry*. 2009;**81**(16):7091–5.
[47] Lu Y., Shi W., Qin J., Lin B. 'Fabrication and characterization of paper-based microfluidics prepared in nitrocellulose membrane by Wax printing'. *Analytical Chemistry*. 2010;**82**(1):329–35.
[48] Deiss F., Matochko W.L., Govindasamy N., Lin E.Y., Derda R. 'Flow-through synthesis on teflon-patterned paper to produce peptide arrays for cell-based assays'. *Angewandte Chemie International Edition*. 2014;**53**(25):6374–7.
[49] Fenton E.M., Mascarenas M.R., Lopez G.P., Sibbett S.S. 'Multiplex lateral-flow test strips fabricated by two-dimensional shaping'. *ACS Applied Materials and Interfaces*. 2009;**1**(1):124–9.
[50] Fu E., Ramsey S.A., Kauffman P., Lutz B., Yager P. 'Transport in two-dimensional paper networks'. *Microfluidics and Nanofluidics*. 2011;**10** (1):29–35.
[51] Schilling K.M., Lepore A.L., Kurian J.A., Martinez A.W. 'Fully enclosed microfluidic paper-based analytical devices'. *Analytical Chemistry*. 2012;**84** (3):1579–85.
[52] Camplisson C.K., Schilling K.M., Pedrotti W.L., Stone H.A., Martinez A.W. 'Two-ply channels for faster wicking in paper-based microfluidic devices'. *Lab on a Chip*. 2015;**15**(23):4461–6.
[53] Dungchai W., Chailapakul O., Henry C.S. 'Electrochemical detection for paper-based microfluidics'. *Analytical Chemistry*. 2009;**81**(14):5821–6.
[54] Nie Z., Nijhuis C.A., Gong J., *et al*. 'Electrochemical sensing in paper-based microfluidic devices'. *Lab on a Chip*. 2010;**10**(4):477–83.
[55] Nie Z., Deiss F., Liu X., Akbulut O., Whitesides G.M. 'Integration of paper-based microfluidic devices with commercial electrochemical readers'. *Lab on a Chip*. 2010;**10**(22):3163–9.
[56] Adkins J., Boehle K., Henry C. 'Electrochemical paper-based microfluidic devices'. *Electrophoresis*. 2015;**36**(16):1811–24.
[57] Renault C., Anderson M.J., Crooks R.M. 'Electrochemistry in hollow-channel paper analytical devices'. *Journal of the American Chemical Society*. 2014;**136**(12):4616–23.
[58] Jahanshahi-Anbuhi S., Henry A., Leung V., *et al*. 'Paper-based microfluidics with an erodible polymeric bridge giving controlled release and timed flow shutoff'. *Lab on a Chip*, 2013;**14**(1):229–36.
[59] Lutz B., Liang T., Fu E., Ramachandran S., Kauffman P., Yager P. 'Dissolvable fluidic time delays for programming multi-step assays in instrument-free paper diagnostics'. *Lab on a Chip*. 2013;**13**(14):2840–7.
[60] Toley B.J., McKenzie B., Liang T., Buser J.R., Yager P., Fu E. 'Tunable-delay shunts for paper micro fluidic devices'. *Analytical Chemistry*. 2013;**85** (23):11545–52.

[61] Jahanshahi-Anbuhi S., Chavan P., Sicard C., *et al*. 'Creating fast flow channels in paper fluidic devices to control timing of sequential reactions'. *Lab on a Chip*. 2012;**12**:5079–85.

[62] Martinez A.W., Phillips S.T., Whitesides G.M. 'Three-dimensional microfluidic devices fabricated in layered paper and tape'. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;**105**(50):19606–11.

[63] Liu H., Crooks R.M. 'Three-dimensional paper microfluidic devices assembled using the principles of origami'. *Journal of the American Chemical Society*. 2011;**133**(44):17564–6.

[64] Lewis G.G., DiTucci M.J., Baker M.S., Phillips S.T. 'High throughput method for prototyping three-dimensional, paper-based microfluidic devices'. *Lab on a Chip*. 2012;**12**(15):2630.

[65] Schilling K.M., Jauregui D., Martinez A.W. 'Paper and toner three-dimensional fluidic devices: Programming fluid flow to improve point-of-care diagnostics'. *Lab on a Chip*. 2013;**13**:628–31.

[66] Mitchell H.T., Noxon I.C., Chaplan C.A., *et al*. 'Reagent pencils: A new technique for solvent-free deposition of reagents onto paper-based microfluidic devices'. *Lab on a Chip*. 2015;**15**:2213–20.

[67] Byrne L., Barker J., Pennarun-Thomas G., Diamond D., Edwards S. 'Digital imaging as a detector for generic analytical measurements'. *TrAC – Trends in Analytical Chemistry*. 2000;**19**:517–22.

[68] Chaplan C.A., Mitchell H.T., Martinez A.W. 'Paper-based standard addition assays'. *Analytical Methods*, 2014;**6**(5):1296.

[69] Cunningham J.C., Kogan M.R., Tsai Y.J., Luo L., Richards I., Crooks R.M. 'Paper-based sensor for electrochemical detection of silver nanoparticle labels by galvanic exchange'. *ACS Sensors*. 2015;**1**(1):acssensors.5b00051.

[70] Scida K., Cunningham J.C., Renault C., Richards I., Crooks R.M. 'Simple, sensitive, and quantitative electrochemical detection method for paper analytical devices'. *Analytical Chemistry*. 2014;**86**(13):6501–7.

[71] Lewis G.G., Ditucci M.J., Phillips S.T. 'Quantifying analytes in paper-based microfluidic devices without using external electronic readers'. *Angewandte Chemie International Edition*. 2012;**51**(51):12707–10.

[72] Lewis G.G., Phillips S.T. 'Quantitative point-of-care (POC) assays using measurements of time as the readout: A new type of readout for mHealth'. in *Mobile Health Technologies: Methods and Protocols*. New York, NY: Humana Press; 2015. pp. 213–229.

[73] Lewis G.G., Robbins J.S., Phillips S.T. 'A prototype point-of-use assay for measuring heavy metal contamination in water using time as a quantitative readout'. *Chemical Communications*. 2014;**50**(40):5352–4.

[74] Lewis G.G., Robbins J.S., Phillips S.T. 'Point-of-care assay platform for quantifying active enzymes to femtomolar levels using measurements of time as the readout'. *Analytical Chemistry*. 2013;**85**(21):10432–9.

[75] Fu E., Liang T., Ramachandran S., Lutz B., Yager P. 'Two-dimensional paper network format for amplified lateral flow assays'. In *Proceedings of*

the 15th International Conference on Miniaturized Systems for Chemistry and Life Sciences.. 2011;1891–3.

[76] Fu E., Liang T., Spicar-Mihalic P., Houghtaling J., Ramachandran S., Yager P. 'Two-dimensional paper network format that enables simple multistep assays for use in low-resource settings in the context of malaria antigen detection'. *Analytical Chemistry*. 2012;**84**(10):4574–9.

[77] Apilux A., Ukita Y., Chikae M., Chailapakul O., Takamura Y. 'Development of automated paper-based devices for sequential multistep sandwich enzyme-linked immunosorbent assays using inkjet printing'. *Lab on a Chip*. 2013;**13**(1):126–35.

[78] Chen H., Cogswell J., Anagnostopoulos C., Faghri M. 'A fluidic diode, valves, and a sequential-loading circuit fabricated on layered paper'. *Lab on a Chip*. 2012;**12**(16):2909.

[79] Gerbers R., Foellscher W., Chen H., Anagnostopoulos C., Faghri M. 'A new paper-based platform technology for point-of-care diagnostics'. *Lab on a Chip*. 2014;**14**:4042–9.