Video Article

Fabrication of Three-dimensional Paper-based Microfluidic Devices for Immunoassays

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Abstract

Paper wicks fluids autonomously due to capillary action. By patterning paper with hydrophobic barriers, the transport of fluids can be controlled and directed within a layer of paper. Moreover, stacking multiple layers of patterned paper creates sophisticated three-dimensional microfluidic networks that can support the development of analytical and bioanalytical assays. Paper-based microfluidic devices are inexpensive, portable, easy to use, and require no external equipment to operate. As a result, they hold great promise as a platform for point-of-care diagnostics. In order to properly evaluate the utility and analytical performance of paper-based devices, suitable methods must be developed to ensure their manufacture is reproducible and at a scale that is appropriate for laboratory settings. In this manuscript, a method to fabricate a general device architecture that can be used for paper-based immunoassays is described. We use a form of additive manufacturing (multi-layer lamination) to prepare devices that comprise multiple layers of patterned paper and patterned adhesive. In addition to demonstrating the proper use of these three-dimensional paper-based microfluidic devices with an immunoassay for human chorionic gonadotropin (hCG), errors in the manufacturing process that may result in device failures are discussed. We expect this approach to manufacturing paper-based devices will find broad utility in the development of analytical applications designed specifically for limited-resource settings.

Video Link

The video component of this article can be found at https://www.jove.com/video/55287/

Introduction

Paper is widely available in a range of formulations or grades, can be functionalized to tune its properties, and can transport fluids autonomously by capillary action or wicking. If paper is patterned with a hydrophobic substance (*e.g.*, photoresist[1] or wax[2]), the wicking of fluids can be controlled spatially within a layer of paper. For example, an applied aqueous sample can be directed into a number of different zones to react with chemical and biochemical reagents stored within the paper. These paper-based microfluidic devices have been demonstrated to be a useful platform for the development of portable and inexpensive analytical assays[3,4,5,6,7]. Applications of paper-based microfluidic devices include point-of-care diagnostics[8], monitoring of environmental contaminants[9], detection of counterfeit pharmaceuticals[10], and delocalized healthcare (or "telemedicine") in limited-resource settings[11].

Multiple layers of patterned paper can be assembled into an integrated device where hydrophilic zones from neighboring layers (*i.e.*, above or below) connect to form continuous fluidic networks whose inlets and outlets may be coupled or left independent.[12] Each layer can comprise a unique pattern, which enables the spatial separation of reagents and multiple assays to be performed on a single device. The resulting three-dimensional microfluidic device is not only capable of wicking fluids to enable analytical assays (*e.g.*, liver function tests[13] and electrochemical detection of small molecules[14]), but it can also support a number of sophisticated functions (*e.g.*, valves[15] and simple machines[16]) common to traditional microfluidic approaches. Importantly, because paper wicks fluids by capillary action, these devices can be operated with minimal effort from the user.

Since reagents can be stored within the three-dimensional architecture of a paper-based device, complex protocols can be reduced to a single addition of aqueous sample to a device. Recently, we introduced a general three-dimensional device architecture that can be used for the development of paper-based immunoassays using the wax-printing technique to create patterned layers.[17,18] These studies focused on how aspects related to the design of the device-number of stacked layers used, composition of the layers, and the pattern of the three-dimensional microfluidic network-controlled the overall performance of the immunoassay. Ultimately, we were able to use these design rules to facilitate the rapid development of a multiplexed immunoassay[19]. In this manuscript, a previously developed immunoassay for human chorionic gonadotropin (hCG; pregnancy hormone)[17] is used as an example to illustrate the strategies that we have developed for the assembly and manufacture of three-dimensional paper-based immunoassays. Accordingly, we focus on the assembly and operation of a device rather than the development of an assay.

In a sandwich immunoassay, which is the format used to detect hCG, a capture antibody specific to one subunit of the hormone is coated onto a solid substrate, which is then blocked to limit the non-specific adsorption of a sample or any subsequent reagent. This substrate is most often a polystyrene microwell plate (*e.g.*, for an enzyme-linked immunosorbent assay or ELISA). The sample is then added to a well and allowed to incubate for a period of time. After rigorous washing, an antibody specific to the other subunit of hCG is added and allowed to incubate. This detection antibody may be conjugated to a colloidal particle, enzyme, or fluorophore in order to produce a measurable signal. The well is again washed prior to interpreting the results of an assay (*e.g.*, using a plate reader). While commercial kits rely on this time-consuming multistep process, all of these steps can be performed rapidly in paper-based microfluidic devices with minimal intervention to the user.

The device used for the hCG immunoassay comprises six active layers, which are, from top to bottom, used for sample addition, conjugate storage, incubation, capture, wash, and blot (**Figure 1**). The sample addition layer is made from qualitative filter paper. It facilitates the introduction of a liquid sample and protects the reagents in the conjugate layer from contamination from the environment or accidental contact by the user. The conjugate layer (qualitative filter paper) holds the color-producing reagent (*e.g.*, colloidal gold-labeled antibody) for the immunoassay. The incubation layer (qualitative filter paper) allows the sample to travel laterally within the plane of the paper to promote binding of the analyte with reagents before reaching the next layer, the capture layer. The capture layer (nylon membrane) contains ligands specific for the analyte adsorbed to the material. After the assay is completed, this layer is revealed to enable visualization of the completed immunocomplex. The wash layer (qualitative filter paper) draws excess fluids including free conjugate reagents away from the face of the capture layer into the blot layer (thick chromatography paper). The six-layer device is held together by five layers of patterned, double-sided adhesive: four layers of permanent adhesive maintain the integrity of the assembled device and one layer of removable adhesive facilitates peeling of the device to inspect the results of the immunoassay on the capture layer.

For the purpose of this manuscript, we use only negative and positive control samples of hCG (0 mIU/mL and 81 mIU/mL, respectively) to provide representative results of a paper-based immunoassay, which permits a dedicated discussion of the relationship between fabrication methods and the performance of a device. In addition to demonstrating how to manufacture devices successfully, we highlight several manufacturing errors that could lead to the failure of a device or irreproducible assay results. The protocol and discussion detailed in this manuscript will provide researchers with valuable insight into how paper-based immunoassays are designed and fabricated. While we focus our demonstration on immunoassays, we anticipate that the guidelines presented herein will be broadly useful for the manufacture of three-dimensional paper-based microfluidic devices.

Protocol

1. Preparation of Paper-based Microfluidic Device Layers

1. Prepare patterns for layers of paper, nylon, and adhesive using a graphic design software program.[6] Each layer may have a different pattern.
   NOTE: The pattern may include alignment holes that are not required for a functional paper-based immunoassay, but assist with the reproducible manufacture of three-dimensional devices. Placement of these holes will differ if devices are assembled individually, in strips, or as full sheets. The software program used to design patterns may vary based on the choice of patterning technique (*e.g.*, photolithography, wax printing, or cutting).[6]
2. Spray the work area with a solution of 70% (v/v) ethanol and water. Wipe the work area with a clean paper towel.

2. Preparation of Paper Layers: Sample Addition, Conjugate Storage, Incubation, and Wash Layers

1. Prepare layers of qualitative filter paper using a large tabletop paper cutter. Cut a stock sheet of paper into a standard paper size to facilitate patterning using a solid ink (wax) printer. For example, a single 460 x 570 mm^2 sheet can make 4 sheets of US Letter paper (8.5 x 11 $inches^2$). Handle paper with clean gloves at all times to minimize contamination.
2. Load a cut sheet of chromatography paper into the printer tray. Print previously designed layers (see **Figure 1**).
   NOTE: A pattern can be printed directly onto this sheet using the automatic feed. Only one sheet of paper should be printed at a time to avoid paper jams. For all layers, use the "Enhanced" print settings.

3. Preparation of Nylon Membrane Layer: Capture Layer

1. Cut the stock roll of nylon membrane into sheets (7.5 x 10 $inches^2$) using a tabletop paper cutter. Take great care in handling the nylon membrane to maintain its integrity and protect against ripping. Store any unused material in a desiccator cabinet, as nylon membranes are moisture sensitive.
   NOTE: Cut sheets are narrower than US Letter paper. Because nylon membranes are thin and fragile, they cannot be processed by the printer directly and require support. Details are discussed below.
2. Using a wax printer, print a capture layer pattern onto a piece of copy paper and tape it to a light box to serve as a guide for the positioning of the nylon membrane. The light box aids the alignment of multiple layers.
3. Place a clean sheet of copy paper onto the previously printed sheet of copy paper. Tape the clean sheet of paper to the light box, but do not tape the two sheets together.
4. Place a cut sheet of nylon membrane onto the clean piece of copy paper. Make sure that membrane covers the printed area of the bottom layer of copy paper. Tape all four sides of the nylon membrane to the clean sheet of copy paper.
   NOTE: Make sure that the nylon membrane is flat and smooth so that there are no problems with printing (*e.g.*, paper jams or uneven printing of wax). Wax may be printed on the tape where the nylon membrane is attached to the copy paper. If this occurs, areas where nylon is incompletely patterned due to tape coverage should be discarded. For future preparations, larger pieces of nylon membrane can be used to avoid this printing error.

5. Load a sheet of nylon membrane (supported by the copy paper affixed to it) into the manual feed printer tray. Print only one sheet of nylon membrane at a time.
   NOTE: There are no preparation steps required for the blot layer, as it is not patterned.

4. Creating Hydrophobic Barriers in the Printed Layer

1. Tape the printed layers onto an acrylic frame for even heating above and below the layer when placed in a gravity convection oven. Keep the nylon membrane taped to the support sheet of copy paper until after the wax is melted and hydrophobic barriers are formed.
   NOTE: The acrylic frame is a custom-made, laser cut piece of 1/2" thick acrylic plastic. Two frame sizes depending on the number of devices being fabricated were used. The outer border of the smaller frame measures 11 5/8" x 2 3/4", and the inner hole of the frame measures 10 3/8" x 1 3/4". The outer border of the larger frame measures 11 5/8" x 8 7/8", and the inner hole of the frame measures 10 1/4" x 7 7/8". The open, inner space allows for even melting of wax through the entire thickness of the paper.
2. Place the layers in the oven at 150 °C for 30 sec until the wax melts into the thickness of the paper. Confirm that the wax has permeated the thickness of the paper by turning it over and checking for imperfections in the design.
   NOTE: Forced air ovens or hot plates may also be used to melt the solid wax ink. Melting times or temperatures may vary depending on the heating method.
3. Remove the paper and nylon membrane from the acrylic frame. Also, remove nylon membrane from support sheet of copy paper.

5. Preparation of Adhesive Layers

1. Pattern double-sided sheets of adhesive films using a robotic knife plotter, using design files previously prepared (step 1.1). Protect any exposed adhesive surface using a sheet of wax liner.
   NOTE: The double-sided adhesive should be patterned with holes that allow the sample to flow through layers as a continuous fluidic pathway. The wax liner is easily removed from the adhesive, and serves to protect it from contamination and tearing during cutting. A laser cutter or die press may also be used to pattern layers of adhesive films.

6. Backing of Device Layers with Adhesive

1. Spray the light box with a solution of 70% (v/v) ethanol and water. Wipe with a clean paper towel.
2. Tape a patterned layer of paper or nylon membrane that needs to be backed with adhesive onto the light box with the printed side down.
3. Peel one side of protective liner from the patterned sheet of adhesive and affix it to the layer of paper or nylon membrane. Use the light box to ensure proper alignment of patterns. Press together. Place the partially assembled device into a protective slip.
   NOTE: The protective slip is a folded piece of lamination film backing that protects the devices from contamination or damage by ensuring that they do not contact the laminator rollers.
4. Pass the resulting two-layer assembly through an automated laminator to completely press the adhesive and paper together, removing any pockets of air from the adjoined layers.
   NOTE: Air pockets between the layers of the device can interfere with device integrity and wicking reproducibility by causing leaks.

7. Treatment of Conjugate Layer with Reagents for Immunoassays Prior to Device Assembly

1. Tape conjugate layer onto an acrylic frame such that the hydrophilic zone to be treated is suspended and not in contact with the frame.
2. Add 2.5 µl of 100 mg/ml bovine serum albumin (BSA) in 1x phosphate buffered saline (PBS) to the hydrophilic zone on the conjugate layer. Allow it to dry at room temperature for 2 min and then at 65 °C for 5 min.
   NOTE: This volume is just enough to wet the zone of the paper. The BSA solution helps to prevent aggregation of the colloidal nanoparticles during the drying process, which will facilitate the release of the nanoparticles when the paper and reagents are rehydrated by the sample.
3. Add 5 µl of 5 O.D. colloidal gold nanoparticle conjugated to anti-β-hCG antibody, and repeat the drying process.
   NOTE: The units of concentration of colloidal gold nanoparticles are often expressed as optical density (O.D.) as measured by absorbance at λ = 540 nm. No treatment is required for the wick pad before device assembly in Section 10.

8. Treatment of Lateral Channel with Reagent for Immunoassays Prior to Device Assembly

1. Tape lateral channel layer onto an acrylic frame such that the hydrophilic zone to be treated is suspended and not in contact with the frame.
2. Add 10 µl of blocking agent (5 mg/ml non-fat milk and 0.1% (v/v) Tween 20 in 1x PBS) to treat the lateral channel. Repeat the same drying process (2 min at room temperature and then at 65 °C for 5 min) as the conjugate layer.

9. Treatment of Capture Layer with Reagents for Immunoassays Prior to Device Assembly

1. Tape capture layer onto an acrylic frame such that the hydrophilic zone to be treated is suspended and not in contact with the frame.
2. Treat the capture layer with 5 µL of 1 mg/ml anti-α-hCG antibody and then allow the sample to dry at room temperature for 2 min followed by 8 min at 65 °C.
3. Add 2 µL of blocking agent (5 mg/ml non-fat milk and 0.1% (v/v) Tween 20 in 1x PBS). Repeat the drying process for the capture layer.
   NOTE: This amount is appropriate to coat the papers without occluding the pores of the nylon membrane, which can happen when too much blocking agent is used.

10. Assembly of Three-dimensional Paper-based Microfluidic Devices

1. Tape the wash layer to the light box (printed side facing upwards). If alignment holes are used, remove them from subsequent layers using a handheld hole-punch tool.
2. Remove the protective film on the back of the capture layer to expose the adhesive. Align the capture layer above the wash layer using the alignment holes as a guide. Press the two layers together. Avoid touching hydrophilic zones to minimize contamination or damage to the device. Tweezers may be used to assist assembly.
3. Remove the protective film on the back of the incubation layer to expose the adhesive. Align the incubation layer above the capture layer and press them together. Continue adding layers in this manner until all active layers are assembled.
4. Place the partially assembled device into a protective slip and firmly affix the layers together using a laminator.
5. Remove the protective film on the back of the wash layer and affix the blot layer to the bottom of the device. Repeat lamination step 10.4 to complete the assembly of the three-dimensional paper-based microfluidic device. Cut desired number of devices from strips or sheets of fully-assembled devices using scissors.
   NOTE: Full sheets of devices, strips of devices, or single devices may be prepared using a similar approach.

11. Performing a Paper-based Immunoassay

1. Add 20 µl of a sample to the hydrophilic zone on top of the device (*i.e.*, the sample layer).
2. Wait for the sample to wick completely into the device, then add 15 µl of wash buffer (0.05% v/v Tween 20 in 1x phosphate buffered saline). After the first aliquot of wash buffer has wicked completely into the device, add a second 15 µl aliquot of wash buffer.
   NOTE: The wash buffer has completely wicked into the device when the liquid droplet has disappeared, showing no meniscus on the surface of the paper. The assay is complete when the second aliquot of wash buffer has completely entered the device.
3. **To reveal the results of the assay, peel away the three top layers of the device using tweezers to expose the capture layer.**
   1. Interpret the results of the assay qualitatively by observing the presence or absence of color. Alternatively, image the readout layer using a desktop scanner and use image processing software or algorithms to quantify results and characterize the distributions of intensity within a detection zone.[20]

Representative Results

Obtaining reproducible assay performances in three-dimensional paper-based microfluidic devices relies on a fabrication method that ensures consistency among devices. Towards this goal, we have identified a number of manufacturing processes and material considerations, and discuss them here in the context of demonstrating a paper-based immunoassay. We use a wax printing method to form hydrophobic barriers within paper-based microfluidic devices (**Figure 2A**).[2] This method is ideal because it relies only on widely available office equipment, requires minimal procedural steps to complete, and does not require the use of chemicals (*e.g.*, photoresists) that might interfere with protein adsorption or alter the wettability of paper fibers. Further, wax printing produces fluidic pathways with reproducible dimensions, which is critical for assays with repeatable performances and duration times. After the hydrophobic barriers are formed, adhesive sheets are applied to layers to facilitate assembly of three-dimensional devices (**Figure 2B**). Any reagents required for the immunoassay can be applied after the adhesive film is added to the back of a layer (**Figure 2C**). This procedure is useful for fabrication processes in an academic laboratory because many devices can be prepared in parallel. The assembly process for an immunoassay device is completed after all layers of the device are stacked and laminated together (**Figure 2D**). We add sample to begin the assay. In this example, we use a urine control set for pregnancy tests, which contains negative and positive samples of hCG in buffer, as samples to demonstrate the operation of our devices and the reproducibility of assays performed using them. Two aliquots of wash buffer are then added sequentially. Once the final aliquot of wash buffer has completely entered the device, the assay is considered complete. The top three layers are then peeled away to reveal the capture layer (**Figure 3A**). This step irreversibly damages the device ensuring that it cannot be used again. The completion of a paper-based immunoassay results in a qualitative color readout that can indicate a negative or positive output upon visual inspection. The objectivity of these results is apparent in uncorrected images acquired using a flatbed scanner (**Figure 3B**).

Failed experiments can often highlight certain procedural steps whose importance may be otherwise imperceptible when the analysis of an experiment is focused on successful results. We demonstrate three errors in the manufacture and assembly of three-dimensional paper-based microfluidic devices that result in failures of the immunoassay: (i) Occasionally, device failures are not apparent until after an assay is completed. For example, a misalignment between layers comprising the incubation channel and capture zone can cause the development of an irregular pattern in the positive signal, which may result in the misinterpretation of the qualitative signal by a user (**Figure 4A**). (ii) If the wax is not printed in a sufficient amount or not allowed to melt completely through the full thickness of the paper, then the integrity of the resulting hydrophobic barriers may be compromised. Incomplete formation of these barriers will cause a loss of control over wicking and lead to leaks within the device. For example, instead of confining flow to a channel within a layer, a semi-permeable wax barrier will allow fluid to wick elsewhere in the plane of the paper. Without defined channels, the sample is unlikely to reach the capture or wash layers. The user will perceive this kind of error as a greatly shortened assay duration time. We demonstrate this device failure by applying a solution of red food coloring to a layer whose wax pattern was not allowed to melt for the full 30 sec (**Figure 4B**). An immunoassay using such a layer was "completed" in 6 min, which is clearly different than the expected duration of 15 min. (iii) Assays that take longer than expected to complete may indicate a malfunction in the fabrication of a device. For example, improperly cut adhesive or occluded pores due to the application of an excessive amount of reagents (*e.g.*, blocking agents or colloidal gold) could prohibit a sample or wash buffer from entering the device (**Figure 4C**).

Overall, our manufacturing protocol is useful to fabricate numerous paper-based microfluidic devices in parallel on a scale that is useful for an academic laboratory. We demonstrate the performance of the hCG paper-based immunoassay prepared using this method by performing 70 assays in parallel: 35 negative replicates and 35 positive replicates. For the purposes of this demonstration, we prepared a set of layers with the designs of our device, affixed the layers of paper with adhesive, and then cut the sheets into rows of devices. Each sheet was cut into 7 rows, which contained ten devices. This facilitated the arrangement of the layers onto the smaller acrylic frames where the layers are taped and then treated with reagents needed to perform the assays. This method of device preparation is suggested in a note in the protocol. Following the treatment of layers, the devices were assembled in strips of ten and then laminated. After the final device fabrication steps were completed, the devices remained in the strips of ten and sample was added to each device. We observed a 0% failure rate for devices fabricated using our protocol. We used an open-source image processing software[20] to quantify the results of these assays. While a number of methods are available to analyze the intensity distribution in circular spots (*e.g.*, radial or linear distributions)[21] we measure the mean intensity from the green channel of an RGB image of the device using the entire detection spot as a region of interest.[17,18,19] We then normalize the measurements of both positive and negative assays by subtracting the raw negative data (**Figure 3B**). We determined the coefficient of variation for each data set to be 1% for assays performed using negative samples and 3% for assays performed using positive samples.

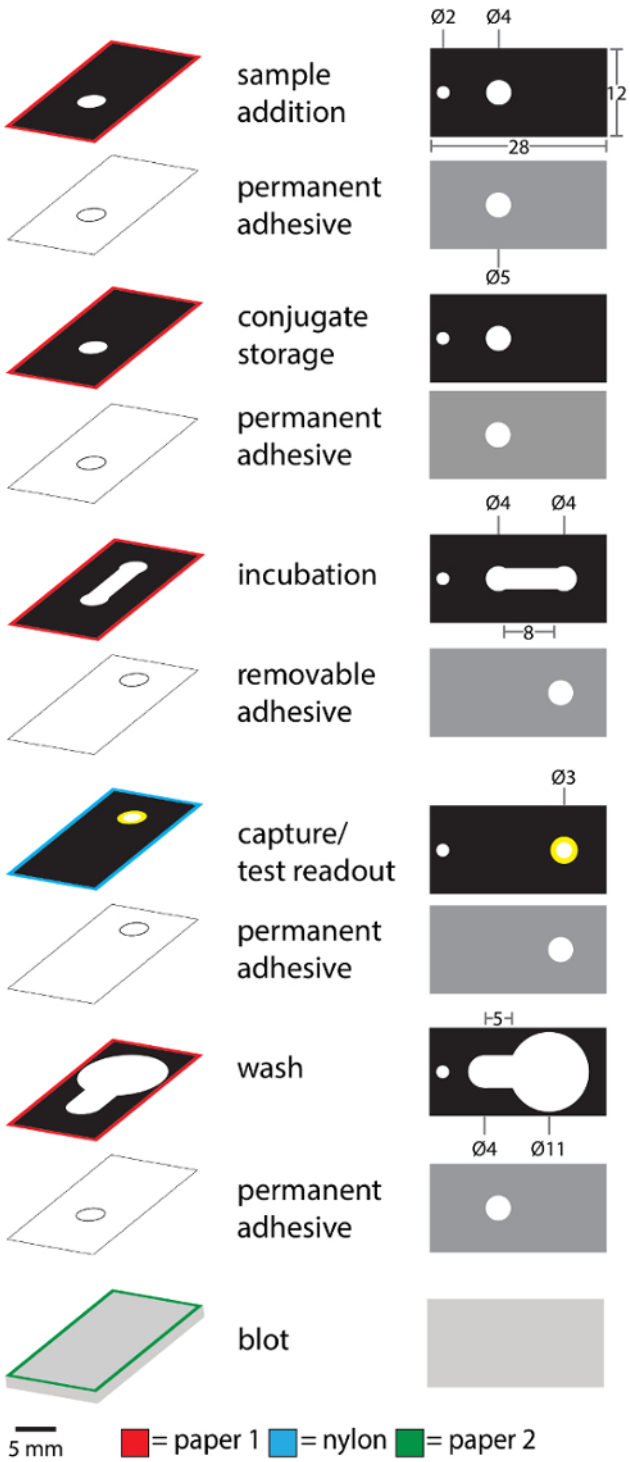

Figure 1: Schematic of three-dimensional paper-based device. This illustration shows the hydrophobic and hydrophilic regions that define the fluidic pathway within the device, as well as the patterned layers of permanent and removable adhesive that hold layers together. Each layer is labeled by the function it performs in the assay. The red, blue, or green outline on each layer indicates the material used to fabricate that specific layer (red: chromatography paper, blue: nylon membrane, green: thick chromatography paper). Dimensions are given for each zone within the device in mm. Please click here to view a larger version of this figure.

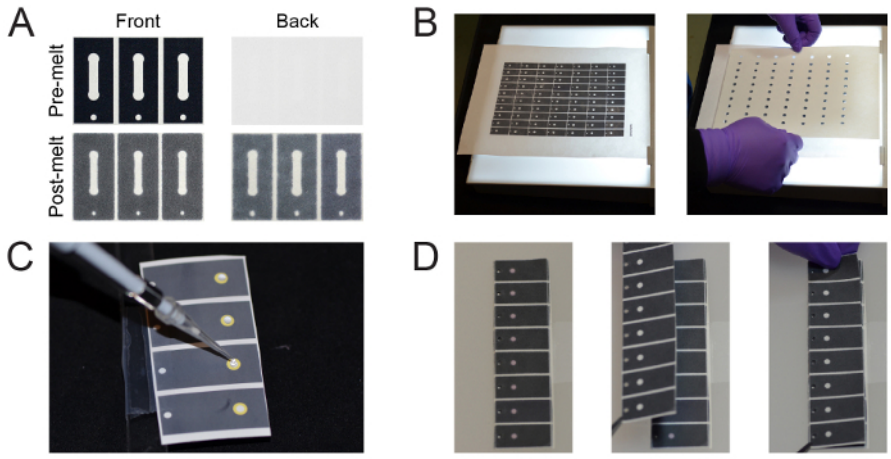

Figure 2: Procedure used to fabricate immunoassays from three-dimensional paper-based microfluidic devices. (**A**) Images of the front and back of a sheet of chromatography paper patterned using wax printing before and after heating. (**B**) A sheet of chromatography paper backed with a film of patterned adhesive. (**C**) Treatments applied to the hydrophilic zones of a strip of patterned nylon membrane. (**D**) Assembly of strips of a multilayer device using a light box and alignment holes as a guide. Please click here to view a larger version of this figure.

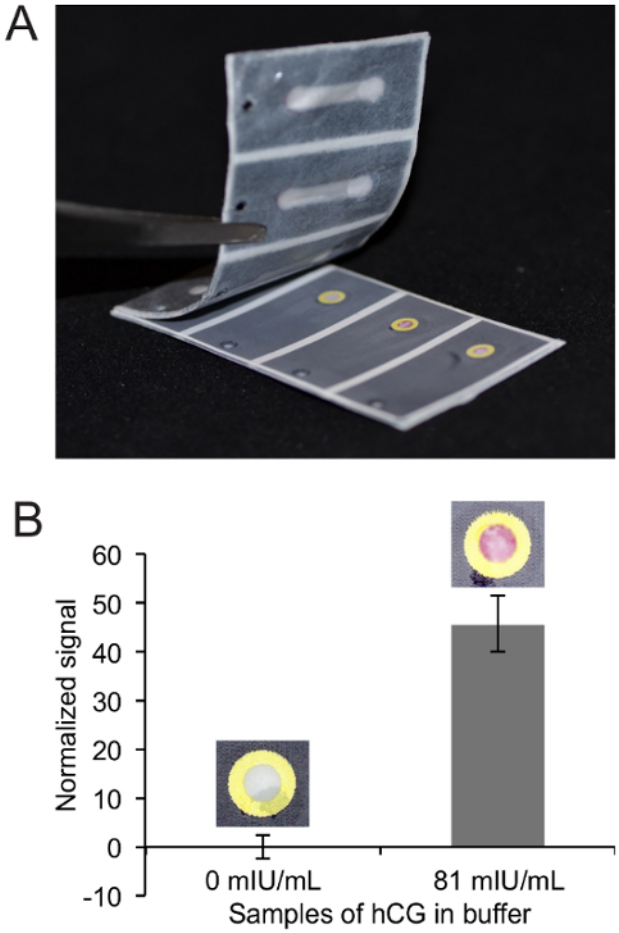

Figure 3: Interpreting results of a paper-based immunoassay. (**A**) The top three layers of the paper-based device are peeled back to expose the capture layer and interpret the results of the assay. (**B**) Graphical representation of the performance of a paper-based immunoassay for hCG. The results depicted are the averages of 70 replicates performed simultaneously where 35 replicates each are used for positive and negative samples of hCG. Error bars represent the standard deviation of the data set. Uncorrected, representative images depicting positive (red color) and negative (white color) results from an hCG immunoassay are shown above their respective data. Please click here to view a larger version of this figure.

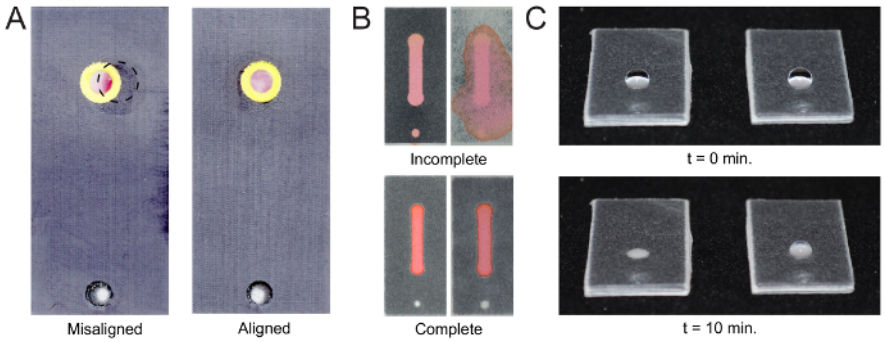

Figure 4: Examples of manufacturing errors. (**A**) Due to misalignment of the lateral channel above the capture layer, the positive signal is concentrated in a small area of the readout zone. A "wet" circular region (dashed outline) can be observed to the right of the readout zone resulting from contact between the misaligned lateral channel with the capture layer (left). Image of a positive readout on the capture layer of a properly aligned device (right). (**B**) Incomplete melting of wax throughout the thickness of a layer can lead to leaks within the device. Food coloring has been added to the solution to assist with visualization of sample in layers with incomplete or fully-formed hydrophobic barriers. (**C**) Improperly cut adhesive can block the fluidic network between layers of paper, which stops the flow of a sample. Please click here to view a larger version of this figure.

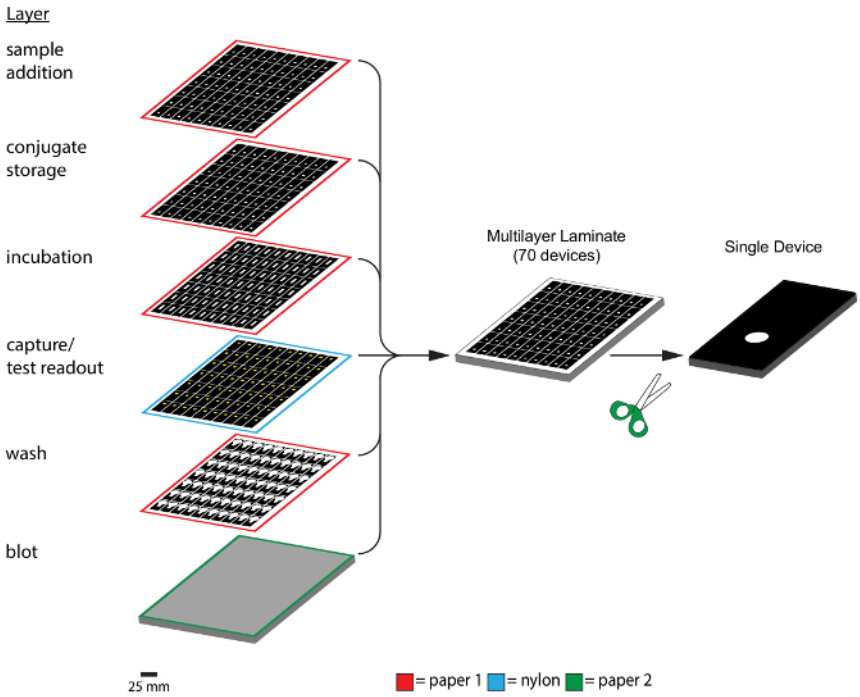

Figure 5: Manufacturing three-dimensional paper-based microfluidic devices. The schematic depicts the assembly and lamination of multiple layers of patterned paper into completed three-dimensional devices. In this example, 70 devices can be made simultaneously. The layers of adhesive and alignment holes have been removed from the schematic for simplification purposes. After assembly, individual devices can be removed and used in assays. The red, blue, and green outlines on each layer indicate the material used to fabricate that specific layer (red: chromatography paper, blue: nylon membrane, green: thick chromatography paper). The Scale bar = 25 mm except for the separate device (right), which has dimensions of 12 x 28 mm^2. Please click here to view a larger version of this figure.

Discussion

Identifying a reproducible manufacturing strategy is an essential component of assay development.[22] We use a sequential, layer-by-layer approach to manufacture three-dimensional paper-based microfluidic devices. In contrast to those methods that apply folding or origami techniques to produce multilayer devices from a single sheet of paper[23,24] additive manufacturing offers a number of advantages: (i) Multiple materials can be incorporated into a single device architecture without modification to methods for the printing, alignment, or assembly of layers. (ii) Patterned adhesive films can be integrated into the assembly process. These films affix adjoining layers, and, based on the strength of the adhesive, can be reversible to enable peeling and evaluation of internal layers. Moreover, adhesives provide structural integrity to the three-dimensional device, which precludes the need for binder[25] clips or machined enclosures.[23] (iii) Individual sheets of US Letter chromatography paper can accommodate an array of replicates, which can greatly improve the throughput of laboratory-scale manufacturing (**Figure 5**). This is particularly beneficial when evaluating numerous experimental conditions that require technical replicates. By this approach, 70 three-

dimensional paper-based devices can be prepared simultaneously. (iv) Similar multilayer lamination approaches are used for the high-volume manufacture of numerous commercial products in healthcare (*e.g.*, wound care dressings and transdermal patches), which consequently lowers the production barrier to translating these three-dimensional paper-based microfluidic devices.

In addition to facilitating peeling and assembly, the choice of adhesive is also critical to the design of the three-dimensional fluidic network. An adhesive film can serve as an additional barrier between layers of paper, which can enable masking of hydrophilic zones on adjacent layers. In practice, the use of thin layers of adhesive is desirable. If the adhesive is too thick (*e.g.*, many double-sided tapes), then the gap formed between layers of paper will be too large to facilitate wicking and must be filled with a hydrophilic substance (*e.g.*, cellulose powder) to regain function.[12] While this additional step adds complexity to manufacture and the substance used may interfere with some assays, these gaps become a useful feature for the production of controllable, fluidic push-down valves.[15] Other forms of adhesive have been used in the manufacture of three-dimensional paper-based microfluidic devices. Adhesive sprays offer a simple method to affix layers to each other.[26] Using this method, the adhesive material is applied uniformly onto both the hydrophobic and hydrophilic area of the paper. An advantage to this method is that additional equipment (*e.g.*, knife plotter or laser cutter) is not needed to design the pattern for the adhesive layer. However, the conditions for the uniform application of the adhesive spray must be determined experimentally for each type of material used. The topography of the material may affect the adhesive-material interface and longer spray times may be needed for rougher surfaces. In addition, spraying adhesive onto the hydrophilic zones of the fluidic pathway may result in impaired wicking by altering the wettability of the paper. Alternatively, the use of stencils[27] or screen printing[8] may be used to pattern adhesive directly onto layers of patterned paper.

Two major considerations for the development of three-dimensional paper-based microfluidic devices are the choice of materials and the design of the fluidic network. (i) We select materials and combinations of materials based on wicking rate, wet strength, thickness, and protein-binding capacity. Wicking rate can influence the duration of an assay and the amount of time reagents have to react or bind within a layer. Different grades of paper are characterized by wicking rates based on, for example, the treatment of the paper, its porosity, and its thickness. It is possible to use multiple layers of paper to increase the effective wicking rate of a device.[28] A good wet strength is desirable for applications that require handling (*e.g.*, peeling an immunoassay) after the device has been saturated with a sample. Materials that are too thick or that cannot be passed through commercial printer due to fragility will require an alternative method to produce patterned channels (*e.g.*, photolithography). However, in contrast, thicker materials are ideal for blot layers (or sinks) to draw fluids through the device. Many grades of nylon membranes are available commercially, which may differ in their ability to bind proteins irreversibly to the capture zone. Material substitutions (*e.g.*, nitrocellulose instead of a nylon membrane) can also influence binding capacity, which may affect the sensitivity of the assay. (ii) The use of symmetry in the design of fluidic networks ensures that the unique channels patterned into three-dimensional devices behave identically (*e.g.*, filled simultaneously), which is critical for multiplexed assays.[19] Symmetry further simplifies layer design, assists with layer alignment when assembling full sheets of devices, and can minimize waste. Modifications to the device design can influence the performance of the assay. For example, increasing the length of the lateral channel in the incubation layer will affect the duration of the assay, because the fluid will wick a proportionately longer distance before reaching the outlet.[17] In applications that rely on the binding of a target biomolecule, a longer assay time may be advantageous because it can increase the fraction of bound, labeled species prior to immobilization on the capture layer.

In conclusion, we have presented a method to fabricate three-dimensional paper-based microfluidic devices that support the development of immunoassays. This method, which uses a type of additive manufacturing to produce multilayer devices, facilitates the production of devices at a scale that is suitable for laboratory research. The protocol described in this manuscript is specific for paper-based immunoassay devices; however, we expect the procedures related to the assembly of these immunoassays-wax printing, patterning adhesive, aligning layers, and lamination-will be readily extendable to numerous three-dimensional paper-based microfluidic device architectures. An understanding of fabrication methodology can lead to the development of new point-of-care assays with a broad range of applications in healthcare, environment, and agriculture.

Disclosures

The authors have nothing to disclose.

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References

1. 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 Chip.* **8** (12), 2146-2150, (2008).
2. Carrilho, E., Martinez, A.W., & Whitesides, G.M. Understanding wax printing: a simple micropatterning process for paper-based microfluidic devices. *Anal. Chem.* **81** (16), 7091-7095, (2009).
3. Martinez, A.W., Phillips, S.T., Butte, M.J., & Whitesides, G.M. Patterned paper as a platform for inexpensive, low-volume, portable bioassays. *Angew. Chem. Int. Ed.* **46** (8), 1318-1320 (2007).
4. Martinez, A.W., Phillips, S.T., & Whitesides, G.M. Diagnostics for the developing world: microfluidic paper-based analytical devices. *Anal. Chem.* **82** (1), 2-10, (2010).
5. Cate, D. M., Adkins, J. A., Mettakoonpitak, J., & Henry, C. S. Recent developments in paper-based microfluidic devices. *Anal. Chem.* **87** (1), 19-41 (2015).

6. Li, X., Ballerini, D. R., & Shen, W. A perspective on paper-based microfluidics: Current status and future trends. *Biomicrofluidics.* **6**, 011301 (2012).
7. Lisowski, P., & Zarzycki, P. K. Microfluidic paper-based analytical devices (μPADs) and micro total analysis systems (μTAS): Development, applications and future trends. *Chromatographia.* **76**, 1201-1214 (2013).
8. Pollock, N.R., *et al.* A paper-based multiplexed transaminase test for low-cost, point-of-care liver function testing. *Sci. Transl. Med.* **4** (152), 152ra129 (2012).
9. Mentele, M.M., Cunningham, J., Koehler, K., Volckens, J., & Henry, C.S. Microfluidic paper-based analytical device for particulate metals. *Anal. Chem.* **84** (10), 4474-4480 (2012).
10. Weaver, A.A., et al. Paper analytical devices for fast field screening of beta lactam antibiotics and antituberculosis pharmaceuticals. *Anal. Chem.* **85** (13), 6453-6460 (2013).
11. 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. *Anal. Chem.* **80** (10), 3699-3707 (2008).
12. Martinez, A.W., Phillips, S.T., & Whitesides, G.M. Three-dimensional microfluidic devices fabricated in layered paper and tape. *Proc. Natl. Acad. Sci. USA.* **105** (50), 19606-19611 (2008).
13. Vella, S.J., *et al.* Measuring markers of liver function using a micro-patterned paper device designed for blood from a fingerprick. *Anal Chem.* **84** (6), 2883-2891 (2012).
14. Nie, Z., Deiss, F., Liu, X., Akbulut, O., & Whitesides, G.M. Integration of paper-based microfluidic devices with commercial electrochemical readers. *Lab Chip.* **10** (22), 3163-3169 (2010).
15. Martinez, A.W., *et al.* Programmable diagnostic devices made from paper and tape. *Lab Chip.* **10** (19), 2499-2504 (2010).
16. Connelly, J.T., Rolland, J.P., & Whitesides, G.M. "Paper machine" for molecular diagnostics. *Anal. Chem.* **87** (15), 7595-7601 (2015).
17. Schonhorn, J.E., Fernandes, S.C., Rajaratnam, A., Deraney, R.N., Rolland, J.P., & Mace, C.R. A device architecture for three-dimensional, patterned paper immunoassays. *Lab Chip.* **14** (24), 4653-4658 (2014).
18. Fernandes, S.C., Logounov, G.S., Munro, J.B., & Mace, C.R. Comparison of three indirect immunoassay formats on a common paper-based microfluidic device architecture. *Anal. Methods.* **8** (26), 5204-5211 (2016).
19. Deraney, R.N., Mace, C.R., Rolland, J.P., & Schonhorn, J.E. Multiplexed, patterned-paper immunoassay for detection of malaria and dengue fever. *Anal. Chem.* **88** (12), 6161-6165 (2016).
20. Abramoff, M., Magalhaes, P.J., & Ram, S.J. Image processing with ImageJ. *Biophotonics Int.* **11** (7), 36-42 (2004).
21. Derda, R., *et al.* Multizone paper platform for 3D cell cultures. *PLoS ONE.* **6** (5), e18940 (2011).
22. Mace, C.R., & Deraney, R.N. Manufacturing prototypes for paper-based diagnostic devices. *Microfluid. Nanofluidics.* **16** (5), 801-809 (2014).
23. Liu, H., & Crooks, R.M. Three-dimensional paper microfluidic devices assembled using the principles of origami. *J. Am. Chem. Soc.* **133** (44), 17564-17566 (2011).
24. Kalish, B., Tsutsui, H. Using Adhesive patterning to construct 3D paper microfluidic devices. *J. Vis. Exp.* (110), e53805 (2016).
25. Scida, K., Cunningham, J.C., Renault, C., Richards, I., & Crooks, R.M. Simple, sensitive, and quantitative electrochemical detection method for paper analytical devices. *Anal. Chem.* **86** (13), 6501-6507 (2014).
26. Lewis G.G., DiTucci, M.J., Baker, M.S., & Phillips, S.T. High throughput method for prototyping three-dimensional, paper-based microfluidic devices. *Lab Chip.* **12** (15), 2630-2633 (2012).
27. Kalish, B., & Tsutsui, H. Patterned adhesive enables construction of nonplanar three-dimensional paper microfluidic circuits. *Lab Chip.* **14** (22), 4354-4361 (2014).
28. 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 Chip.* 2015, **15** (23), 4461-4466 (2015).

