REPlicating RAPid Microfluidics: self-replicating printer for hydrophobic pattern deposition.

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ABSTRACT

Paper based microfluidics broadens the use of point-of-care devices to applications and situations where cost is an important restriction. This study focuses on the REPRAP PRUSA i3 Printer which can print itself a part that combined with an infusion pump extends the capabilities of this printer which can now create different types of hydrophobic patterns on an abundant, renewable substrate: paper. Different flow rates of the syringe pump and printing velocities are combined to optimize the resolution of this new manufacturing process. Besides different papers are used to print patterns to either check the influence of the paper type on the printing resolution and to choose the more suitable paper to build blood typing assays. The resolution improves decreasing flow rate and increasing printing velocity to a minimum value approximately 10% higher than the needle diameter. The printer working with a G25 needle prints microfluidic patterns that can be used evaluate the blood type on different types of chromatographic papers. Two blood types (A- and O-) are evaluated with this new approach with results equivalent to traditional methods, validating its feasibility in the clinical practice. This novel printing method for paper-based microfluidics manufacturing does not require specialized equipment or skills, it is fast and inexpensive and thus, can help to introduce the advantage of health-care in areas where access to health systems is not guaranteed.

Keywords: Lab on a Chip, 3D printer, Paper-based microfluidics, Blood type assay, Point of Care Testing (POCT)

Abbreviation

REP	RAP REPlicating RAPid
RBC	Red Blood Cell
RGB	Red Green Blue
ECF	Elemental Chlorine Free

1. Introduction

The REPRAP open source self-replicating 3D printer started as a British initiative to develop a 3D printer that can print most of its own components, and is now an open source collaborative project [1]. All designs produced by the project are released under a free software license, the GNU General Public License[2] . Rep-rap is part of these new additive-manufacturing technologies which are recognized as the base of the next industrial revolution. The standard manufacturing methods are replaced by novel-manufacturing processes. Health services are also changing due to new technologies, which can provide health-care near the patient. PH, glucose or pregnancy assays on paper strips were the first commercial use of paper in the diagnostics industry. In 2007, Whiteside's Group proposed the use of microfluidic manufacturing processes (soft-lithography) to define micro-channels on paper for the first time, which now is called paper-based microfluidics. This enables the spread of microfluidic advantages: low volumes, low energy consumption and quick turnaround results to economies with limited resources [3].

These characteristics are specially relevant for blood typing tests. This test should be performed, when a blood transfusion is needed to prevent the antibodies of the patient from destroying the cells of the transfused blood. This is called a transfusion reaction, and it occurs immediately when incompatible blood is transfused causing serious health problems. Blood transfusions are normally required in non programmed medical situations. Shen's research group proposed the use of paper based microfluidic devices in these situations [4], [5]. Patterning methods initially developed by Whitesides and co-workers which used expensive hydrophobic barriers such as SU8 were changed to more accessible products such as wax [3], [6], [7].

Nilghaz et al. pointed out the effectiveness of using paper-based point-of-care devices to extend medical care in low resource settings was

previosuly [8],[9]. As remarked for World health Organization (WHO), these type of sensors should be Affordable, Sensitive, Specific, Userfriendly, Rapid and Robust, Equipment-free and Deliver to the users who need them. Taking the first letter of these terms WHO called these type of sensors: ASSURED. While "SSURED" propierties can be provided by conventional microfluidics with embedded sensors [10],[11]. Paper-based analytical devices (uPADs) can provide fast and reliable medical results near the patient without the need of an important infrastructure can be more afordable than the previous glass or silicon based microfluidic devices.

uPADS share paper as a support material with lateral flow assays or dip sticks, but they have hydrophobic patterns that allow multiple test at the same time. The definition of these patterns can be done using a particular type of printers (wax printers which can only use wax as a hydrophobic barrier and achieve resolutions of around 850 microns[12], [13] .

To achieve better resolutions, researchers has used other methodologies such as plasma etching [14],knife cutting [15], laser cutting [16], flexographic printing [17] or injecked printing [18]), but in all these cases the machinery involved is more expensive than a REPRAP printer.

Pearce et al. introduced the possibility to define wax microfluidic patterns using as a primary framework a REPRAP printer[19]. This study focuses on the REPRAP PRUSA i3 Printer which can print itself a part that combined with an infusion pump extends the capabilities of this printer which can now create different types of hydrophobic patterns on an abundant, renewable substrate: paper.

The approach of using an ink injector, which can acommodate different syringe length allows to test different hydrophobic agents not only wax and use of more cost effective products such as alkyl ketene dimer (AKD)[20]. The use of hydrophobic ink avoids the heating step needed to melt the wax before printing. This shortens the manufacturing time and adds flexibility to the system. These findings extend those since the presented experiments in our study deepen in the printing resolution achievable with REPRAP printers.

2. Materials and methods

This section describes the methodologies used to modify the REPRAP printer to print microfluidic patterns on paper to obtain a blood typing assay.

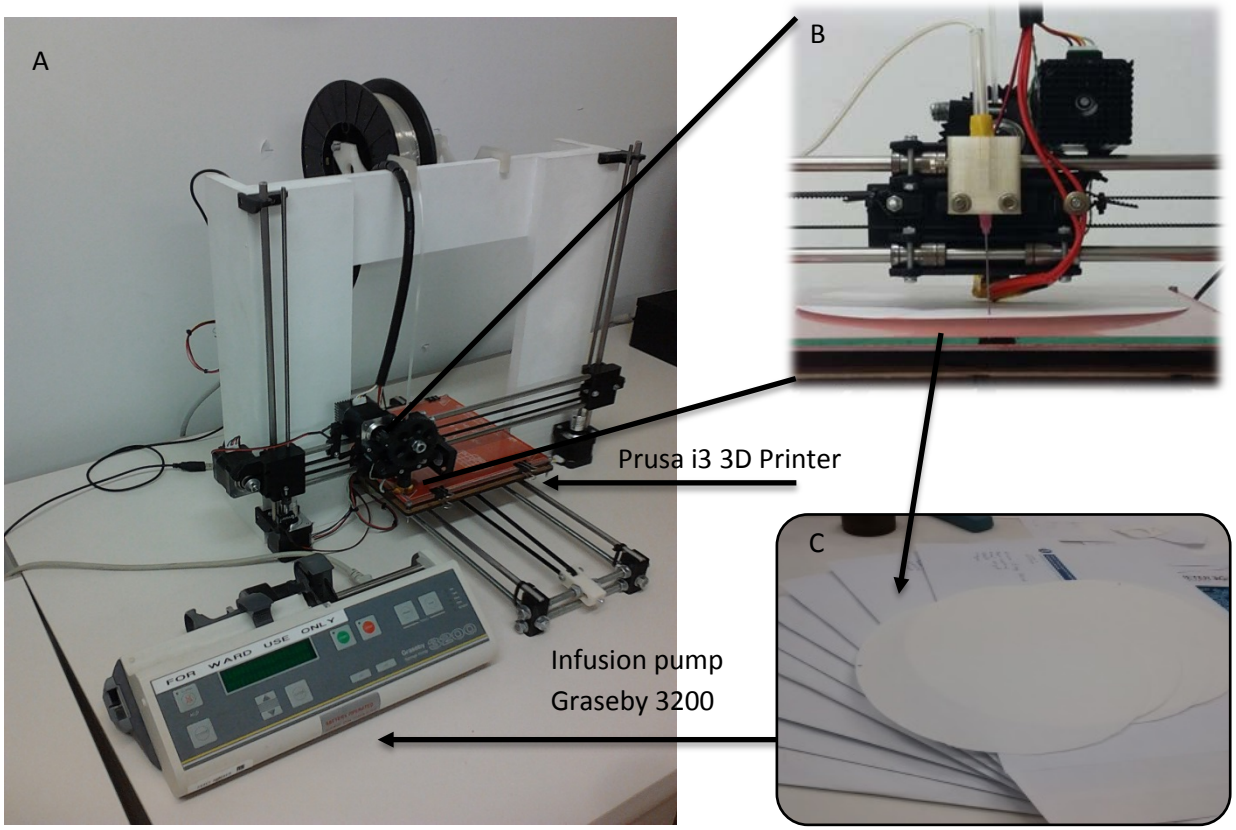


Figure 1 (a) Schematic of the one-step plotting method for patterning paper with the use of a Prusa i3 printer and a infusion pump Graseby 3200. (b) Detail of the needle adaptor at the Prusa i3. (c) Photo of a manufactured sisal paper.

2.1. Design and manufacturing microfluidic patterns

The PRUSA i3 Printer, which was originally designed to manufacture 3D parts by using additive manufacturing technology, was modified to print the hydrophobic barrier needed to define the paper-based microfluidic designs. The part shown in Figure 2 was used to replace the 3D printer extruder. This part was designed to adpat and fix in place the needle adaptor with a pipe in the 3D printer as shown in Figure 1b. Since, there are at least two different heights of standard needles, the part has two trenches that can regulate the height at which the needle is positioned over the printing substrate.

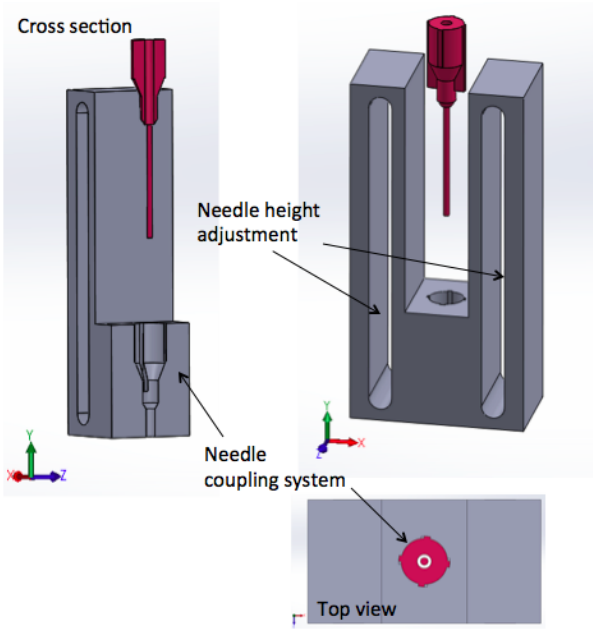


Figure 2 ·3d printed part to position the ink injector.

In contrast to 3D printed parts, printing microfluidic patterns only required placing the ink needle at different *XY* locations and expanding the

z axis to move the ink injector between points without a contour.

Patterns were produced by using the 2D geometry software Inkscape v. 0.91. Geometries were transformed into *x–y* contours and exported as G code by using the J Tech Photonics Laser Tool in Inkscape, the G code being imported into the printer's firmware Repetier.

While the 3D printer plotted the contour, the infusion pump Graseby 3200 was connected to the ink injector in order to control the flow rate of injected ink (see Figure 1a). The pipe should always be free of air bubbles in order to avoid disrupting continuity in the printed pattern.

Hydrophobic barriers to define the patterns on the paper were produced by using Fennosize G7020F (KERIMA), which is a commercially available mixture of alkylketene dimer (AKD and colophony. AKD is an alkaline sizing agent synthesized from fatty acids that is commonly used to provide hydrophobicity to papers whereas colophony is a tree resin that is normally used to make it resistant to fluid penetration but was employed here as a natural dye for easier viewing of the patterned channels.

The printer injector was fitted with 32 mm long needles with blunt end of G25 or G20 type according to ISO 6009. Table 1 shows the inner and outer diameters of the two syringes used. After printing, the microfluidic ink patterns were then heated in an oven at 100 °C for 10 minutes as a curing treatment.

Table 1. Syringe dimensions according to ISO6009.

Syringe	Outside diameter	Inside diameter
G25	0.51 mm	0.25 mm
G20	0.91 mm	0.61 mm

Lines and channels were printed onto the different paper specimens in order to assess the resulting resolution, see Figure 3a. The minimum line width was determined by using a dektop scanner to capture an image that was converted into grayscale for measurment at different points with ImageJ 1.49v software, see Figure 3b.

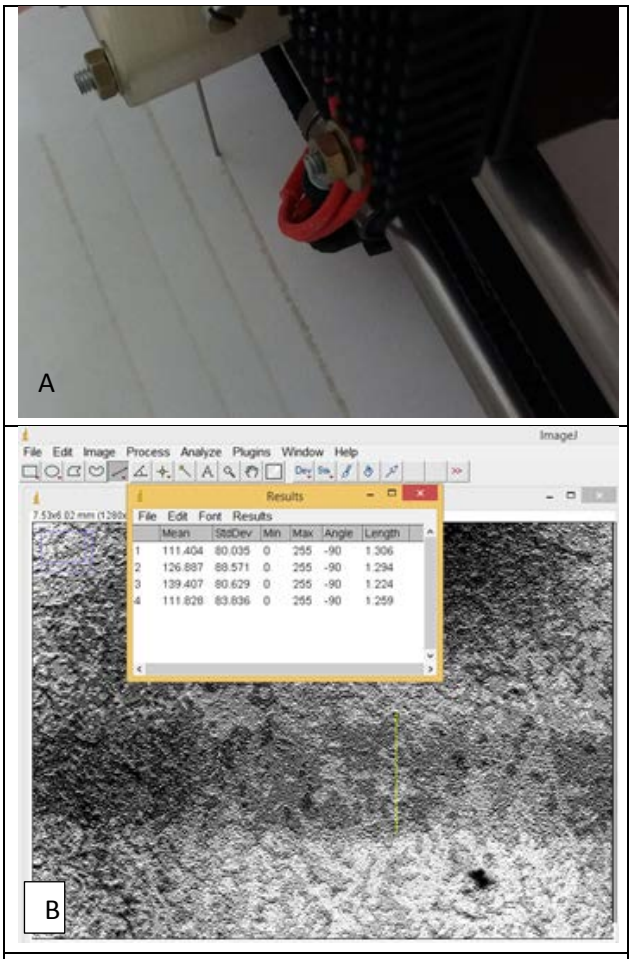


Figure 3 (a) Printed line width (b) ImageJ greyscale image

2.1. Paper Manufacturing

Table 2 shows a summary of the papers used and their main characteristics. Chromatography Whatman papers (W) and homemade sisal based paper were used for comparison (S).

Table 2. Papers

Paper	Refining (rpm)	Average Pore size (μm)	Basis Weight (g/m^2)
SB50	0	23.3	52.41± 1.76
SB100	0	19.8	99.35± 8.05
S2000_50	2000	22.05	52.35± 2.16
S2000_100	2000	8.70	107.03± 2.71
S6000_50	6000	7.2	50.04± 1.37
W5	-----	2.5	98.40± 0.59
W40	-----	8	88.18± 0.58

W 1	-----	11	86.35± 0.63
W41	-----	20	85.76± 0.40

For home made paper, the cellulosic raw material used elemental chlorine free (ECF) bleached sisal pulp obtained at the CELESA mill in Tortosa (Spain). The pulp was disintegrated for 30,000 revolutions (according to standard ISO 5263) and then refined in a PFI mill at different revolutions (0; 2,000 and 6,000) according to standard ISO 5264. The refining process was a mechanical treatment altering the structure of the cell walls to increase interfibre bonding capacity and, the mechanical properties of the resulting paper, see Figure 1c. Handsheets at different basis weight or grammages (50 and 100 g/m2) were formed according by the Rapid-Köthen method according to ISO 5269 and conditioned at 23 ºC, 50% relative humidity according to ISO 187 for 48 h before physical testing,.

2.3 Blood typing assay manufacturing using a modified RepRap printer

As can be seen in Figure 4, the printer was used to print a cross-pattern on two different types of papers (Whatman and home made Sisal based paper).

The cross-pattern was printed with a pump flow rate of 1 ml/h and a printing velocity 800 mm/mine using a G25 needle. The pattern was then cured in a oven at 105ºC for 30 min. The test were washed with 5 ml of saline solution.

The cross-pattern was used to accommodate the three antibodies required to perform the ABO blood test and a control region intended to avoid any influence of the lightning conditions or the camera setting on the results.

The pattern dimensions were optimized to ensure complete filling within a reasonable time (<30 seconds). The cross was filled with 15-30 µL fresh thumb pricked blood diluted 1:1 with 15-30 µL of Phosphate Buffered Saline (PBS) from Sigma Aldrich, Figure 4 (b). Then 1.5 µL of DiaClon Monoclonal IgM antibodies Anti-A, Anti-B and Anti-D from Bio-Rad was deposited with a pippete onto each square area of the microfluidic patterns. The volume of antibodies used, 1.5 µL, was previously confirmed to exclusively wet the square of each antibody region and hence to avoid cross-talk between antibodies. Figure 4 (c) shows the labels for each antibody area and illustrates its dimensions.

Any agglutinated lumps of RBCs formed by reaction of the noncorresponding grouping antibodies with the blood cannot be washed with saline solution, (see the video in the Supplementary Information). Rather, they are trapped in fiber web and stain the cross after the cleaning step. If no heamoglutination occurs, RBCs can be washed off the fibers to clear the paper virtually completely. Figure 4 d shows the 3D printed manufactured part used in all the test to wash the blood tests. This part allows the placement of the test in the same location and to wash with the same amount of saline solution. As can ben seen from Table 3 (and Figure 6) the experimental results obtained after the washing step were as expected.

Compared to conventional slide ABO blood typing test where results are read by visual inspection, a picture was taken and the grayscale intensity on each of the zones was compared to the grayscale intensity on the control zone using ImageJ software.

All experimental values were studied using Grubbs stadistic, to eliminate the non valid values. Thus, experimental errors were calculated as mean and standard deviation in accordance with the respective standards.

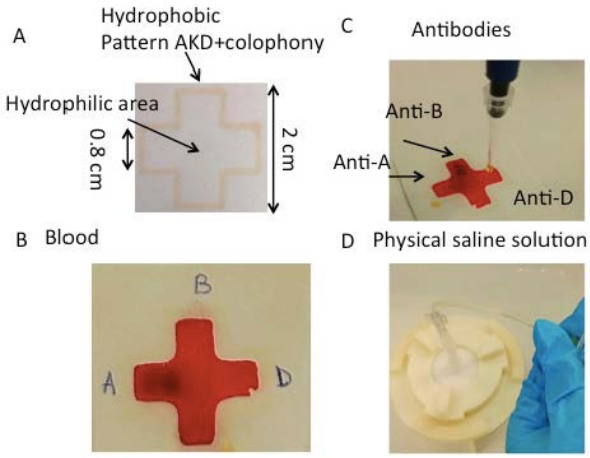
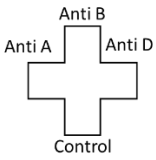
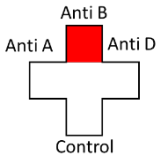
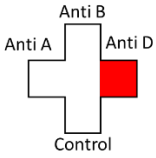
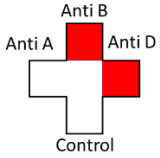
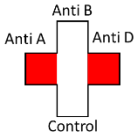
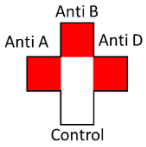
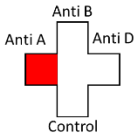
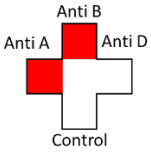
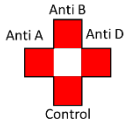


Figure 4 (a) Hydrophobic pattern for ABO blood typing (b) Blood sample deposition (c) Antibodies deposition (d) Washing step with saline solution

Table 3. Blood Test results

Test		Test	
O-		**B-**	
O+		**B+**	
A+		**AB+**	
A-		**AB-**	
NOT VALID			

3.Results and discussion

3.1. Pattern definition analysis

The influence of three variables was examined in order to maximize resolution on Whatman 5 paper. The printer head velocity, which was changed along its range (0-800 mm/min). The infusion pump flow rate up (0.1-6 ml/h) and different two different needle diameters (G20 and G25).

Figure 5 (a) and (b) show the variation of line width after deposition and curing as a function of flow rate. As can be seen, line width initially decreased with decreasing flow rate and ten levelled off at a minimum value approximately 10 % higher than the needle diameter. Thus, the minimum width was 0.7 mm with the G25 needle and 1.1 mm with G20 needle (see Figure 5 (b)).

As can be seen form Figures 5 (c) and (d), line width decreased with increasing printing speed but levelled off at a value proportional to the diameter of the injection needle. The dependence of line width on needle diameter is clearly apparent from Figure 5 (e), where the printing speed was changed at two different flow rates for the same needle diameter; as can be seen, the smallest width achieved was limited by the outer diameter of the needle in both cases.

Using an ink injector allows one to test alternative hydrophobic agents that are more cost effective than wax (e.g., alkyl ketene dimer, AKD). Also, it increases the resolution of the REPRAP printer. Based on the results, line width was minimal with complete penetration of the paper thickness (a pump injection flow rate of 1 ml/h) and the highest printing speed (800 mm/min).

3.2. Influence of paper properties on pattern definition

One other key parameter for microfluidic pattern definition is the minimum channel width. Its influence was examined by printing several rectangles on sheets of cured Whatman paper and examining their scanned images. Figure 4f shows a rectangle immediately after curing and scanning. The image was grayscaled and the distance between hydrophobic barriers measured with ImageJ. This test was performed on Whatman paper 1 and 5 in order to examine the influence of pore size. Figure 5 (f) shows the channel width obtained by using blunt end needles of two different diameters. The G25 and G20 needles were used to create a channel theoretically 1 and 1.5 mm wide, respectively. In both cases, the rectangle was printed at 1 ml/h at 800 mm/min, the resulting line width being only about 0.4 mm. As expected, the thinnest line was obtained with the thinner needle. Also, pore size was scarcely influential on channel width; thus, resolution was similar with Whatman paper 1 or 5 despite the large difference in pore size (11 μm versus 2.5 μm). Based on the results, pore size over the studied range (2.5–11 μm) had no critical effect on pattern line width.

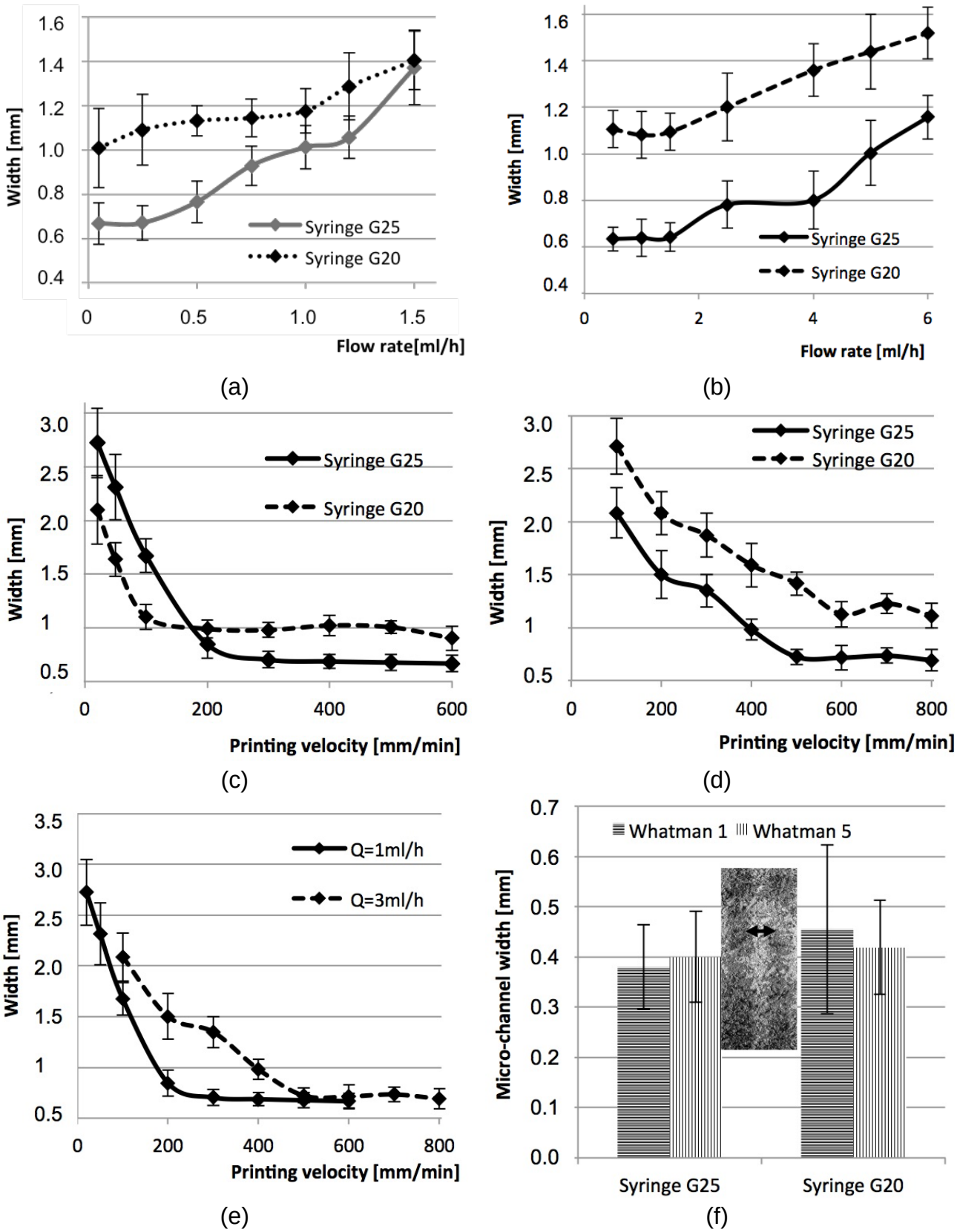


Figure 5 Printing at constant velocity. (a) V= 200 mm/min (b) 500 mm/min. (c) Printing at constant flow rate. 1ml/h (d) 3ml/h, in both cases G25 needle provides smaller line width (e) Printing with the G25 needle at different flow rates. According to the results, the optimum parameters are 800 mm/min and 1 ml/h (f) Minimum channel width vs. needle type

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3.3. Influence of paper type on blood fluency

In most blood based tests, blood not interacting with the reagents must be removed from the regions where no reaction has occurred. Washing the paper with saline should suffice to ensure that its grayscale intensity will exceed 50. Eventhough paper propierties did not define the printer resolution, they are key in this step.

Tests with pure blood samples (SA) and 1 blood:1 PBS diluted samples (SPA) were prepared. Figure 6 shows an example of test in each different paper of a photograph taken after washing with saline for 30 s, using a USB portable microscope under identical lighting conditions. Later the grayscale intensity level was measured using ImageJ and plotted in Figure 7.

Diluted samples provided acceptable white levels (gray intensity levels above 60) with only 30 s of washing in all paper specimens. With undiluted samples, however, grayscale intensity levels after the same cleaning time were low even in those specimens that were clear when stained with diluted blood (e.g., S2000_100 and S6000_50, all of which had a grayscale level close to 50). Based on these results, we chose to use diluted blood on the pattern in order to ensure that the test results could be obtained with only 30 s of washing.

The previous procedure was also applied to Whatman paper 5, 40, 41 and 1, and to chromatography paper typically used in microfluidic based applications [12]–[17]. Table 5 and 6 summarize the greyscale intensity level for diluted blood in different papers.

Table 5. Grayscale intensity level after cleaning of sisal-based papers

	Type	50 g/m²	100 g/m²
RGB	0 rev	77,10 ± 4,53	74,46 ± 3,38
(SPA)	2000 rev.	68,99 ± 4,71	65,69 ± 3,99
	6000 rev.	63,68 ± 6,31	**40,08 ± 6,57**

Based on these results, Whatman paper, the intensity level of which is typically low and similar to S6000_100, was excluded from further testing because it would have required longer washing and hence greater amounts of saline —or might even have led to confusion in reading the test results.

Tabla 6 Grayscale intensity level after cleaning procedure in Whatman papers.

	W5	W40	W1	W41
RGB (SPA)	42,61 ± 5,40	46,39 ± 5,04	50,62 ± 5,33	49,72 ± 4,75

Its easy cleaning led us to use sisal based paper to print the cross pattern of Figure 4 for ABO blood typing. A– and O– type fresh thumb pricked blood was provided by healthy volunteers with a known blood type. A– blood should only aggregate on the Anti-A reagent zone and O– should not aggregate. Figure 6 compares the gray intensity level with that of the control area in different paper specimens and Figure 7 quantifies these results using grayscale intensity level.

The control arm of the cross from the device shows the color intensity level to be reached by each arm not reacting with the antibodies. The test was done on five different sisal paper specimens. As can be seen from Figure 6, all antibodies did not react with O– blood; as a result, the gray intensity level was similar to that of the control area in all cross arms —however, the paper specimens subjected to strong refining (S6000_50 and S2000_100) exhibited greater variability and the results to the naked eye were confusing due to the color intensity of washed areas was still strong. In these papers the pore size was similar to RBC diameter (see Table 2).

Similar results were obtained with A– blood samples. Thus, A– blood aggregated with Anti-A reagent, which was placed on the left arm of the cross, and decreased grayscale intensity as a result. Figure 7 shows the absolute value of the difference in gray intensity between each region and the control area. Papers with pore sizes twice the RBC diameter and low refining showed difference values less than 10 in the negative tests but exceeded 15 in the positive tests, providing clear results for users.

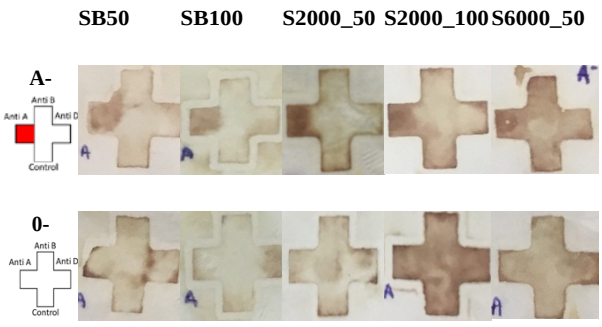
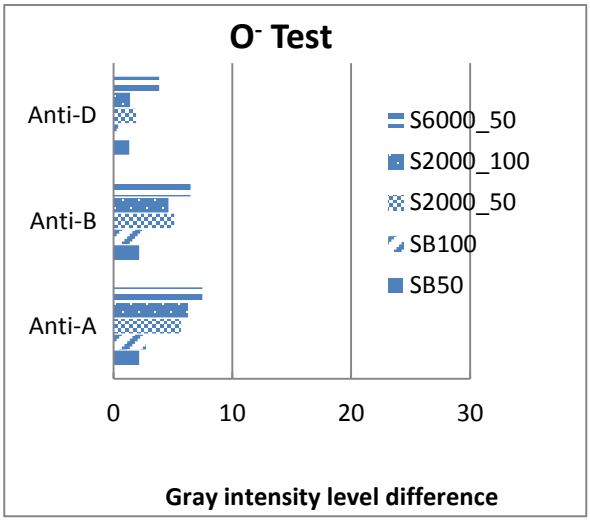
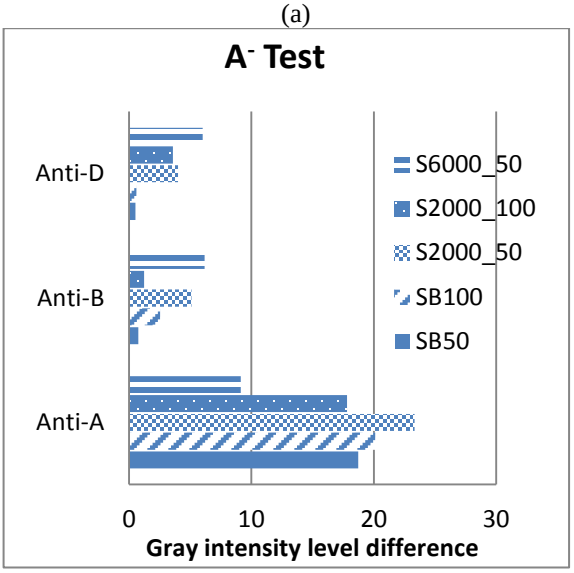


Figure 6 ABO blood typing of sisal paper based microfluidic



(a)



(b)

Figure 7 % Grayscale intensity level for (a) O- blood type test (b) A- blood type test

Unrefined 100 g/m^2 sisal paper (SB100) exhibited a marked difference in gray intensity in the positive tests but a small difference in the negative tests. Since this paper needs no refining, is inexpensive to produce in terms of energy; compared to S2000_50 which does provide reasonable results. The results testify to the potential of sisal paper in combination with the proposed printing method for developing low cost point-of-care tests.

4.Conclusions

The purpose of the actual study was to show that paper-based diagnostics can be manufactured using a self replicating 3D printer and an infusion pump for printing microfluidic patterns with AKD based inks on renewable non-wood sisal papers. The results of this research has been used to prepare ABO blood typing tests, with complete agreement to the results obtained from conventional tests.

An important implication of these findings is that the resolution of this printing methodology is directly proportional to needle diameter. The needles used allowed patterns for evaluating up to three different antibodies to be printed and primary blood typing tests be performed with results exactly matching those of the conventional tests

This truly low-cost, straightforward, efficient paper micropatterning technology thus constitutes a major addition to the existing array of paper production devices for point-of-care bioapplications, especially in remote regions and resource-limited environments (e.g., small laboratories, private clinics).

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