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Multiplex Lateral-Flow Test Strips Fabricated by Two-Dimensional Shaping

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ABSTRACT We have fabricated paper- and nitrocellulose-based lateral-flow devices that are shaped in two dimensions by a computer-controlled knife. The resulting star, candelabra, and other structures are spotted with multiple bioassay reagents to produce multiplex lateral-flow assays. We have also fabricated laminar composites in which porous nitrocellulose media are sandwiched between vinyl and polyester plastic films. This minimizes evaporation, protects assay surfaces from contamination and dehydration, and eliminates the need for the conventional hard plastic cassette holders that are typically used to package commercial lateral-flow diagnostic strips. The reported fabrication method is novel, low-cost, and well-suited to (i) fabrication and adoption in resource-poor areas, (ii) prototype development, (iii) high-volume manufacturing, and (iii) improving rates of operator error.

KEYWORDS: multiplex • lateral flow • test strip • nitrocellulose • low-cost diagnostics • capillary flow

INTRODUCTION

The first paper-based bioassay was introduced in 1957 for the identification of glucose in urine (1). A strip of paper was impregnated with glucose oxidase, peroxidase, and 3,3′-dimethylbenzidine, dried, and then dipped in urine. Abnormal glucose levels were indicated on the strip by the development of a blue color. By the mid-1960s, this assay had been developed into a commercial product and proved popular in helping to diagnose and manage diabetes. A triple-test dipstick was introduced in 1960 comprising three spatially-distinct chemically-coated areas ("patches") that developed distinct colors in response to urinary glucose, albumin, and pH (2). 10-test dipsticks are now commercially available that test for six additional analytes, viz., leukocytes, nitrite, ketones, urobilinogen, bilirubin, and blood, plus the specific gravity of the sample (3, 4). These 10-plex dipsticks are packaged with a color-coded chart that allows the user to read out the detected quantity of a given analyte at a defined location along the length of the strip. Such multiplex dipstick tests are widely accepted by the medical community as convenient, inexpensive, and a rapid means of performing routine urinalysis.

In 1982, immunorecognition was first introduced to dipsticks by spotting and immobilizing antibodies on nitrocellulose, thereby increasing both the specificity and range of detectable analytes (5). Urine-based home-use immunoassay test kits are now commercially available for various bioanalytes, including human chorionic gonadotropin (pregnancy hormone) and 9-tetrahydrocannabinol (pharmacological agent of marijuana). Blood-based home-use dipsticks include those for cholesterol, diabetes, hepatitis C, and human immunodeficiency virus type 1 (HIV-1). Multiplex immunoassay dipsticks are in common use in clinical laboratories, such as, for instance, in screening autoimmune disease (6).

Capillary-driven lateral flow was introduced to conventional dipstick technology in 1989 (7, 8). Lateral flow eliminated the need for the incubation and wash steps of what was then the standard protocol of dipstick-based sandwich assays. It also increased the total number of captured and detected analyte molecules in a given time and thereby generally improved the lower limits of detection. These improvements were achieved by fabricating a test strip of one or more layers of porous material, typically nitrocellulose. When wetted with an analyte-containing liquid at one end of the strip, the porous material provides a motive force for the movement of liquid from wet to dry areas of the strip, with the main motive force being capillary action within the pores (9−11). This lateral flow brings an analyte-containing flow stream into contact with patches of antibodies immobilized on the nitrocellulose where immunospecific analytes bind and accumulate (10, 12). Readout of test results is usually performed optically, either by machine, such as a reflectometer, or by the unaided human eye. To generate a visual signal from noncolored analyte molecules, the analyte may be tagged with colored particles, such as colloidal gold. Hence, when tagged analyte accumulates to sufficient density and is viewed against a background of suitable contrast, such as nitrocellulose, lines or dots are detectable at one or more locations. Tags based on magnetism, phosphorescence, and fluorescence are also common but tend to be unsuited for detection by the unaided eye. The traditional rectangular nitrocellulose strip is often encased in a plastic cassette to enhance the reproducibility of fluidic control, to minimize operator error, and to mechanically clamp various components of the strip. Nitrocellulose is well-suited for lateral-flow immunoassays because of the efficient electrostatic adsorption of proteins on unblocked patches and

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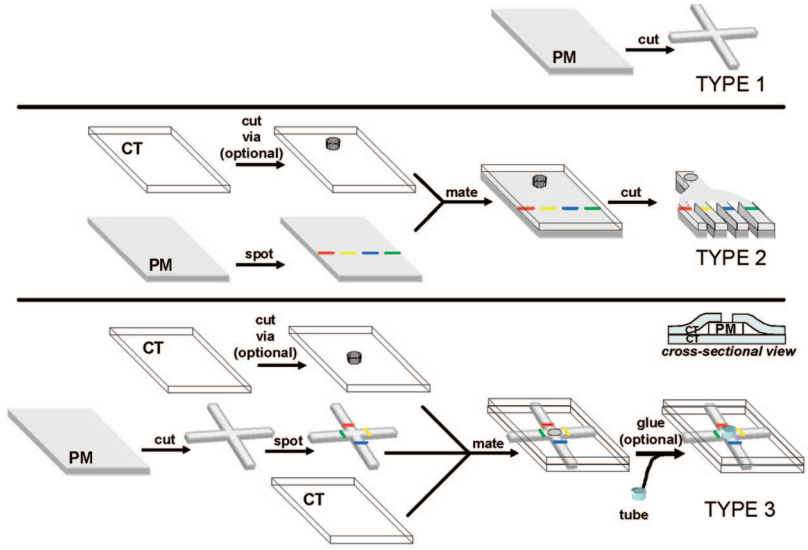

FIGURE 1. Fabrication schema. Depicted here are three distinct methods of fabrication, resulting in devices of different construction and utility: PM, porous medium (such as chromatography paper, nitrocellulose, etc.); CT, cover tape. *Type 1 fabrication scheme*: a sheet of PM is shaped directly by a computer-controlled knife plotter. *Type 2 fabrication scheme*: a sheet of CT (with or without optional via) is mated with a sheet of PM upon which the reagent, depicted here by colored lines, has been deposited; the resulting laminar composite is then shaped into a final two-dimensional form by the knife plotter. An optional cylinder may be affixed by glue or other means over the optional via, thereby providing a microscale reservoir. *Type 3 fabrication scheme*: a sheet of PM is shaped by the knife plotter into a two-dimensional form, spotted with the reagent, and then sandwiched between two sheets of CT. Fluidic access to the PM is provided by one of three methods, as described in the text. An optional cylinder may be affixed by glue or other means over these vias, thereby providing a macroscale reservoir. A photograph of the device with such an optional cylinder is given in the Supporting Information.

because of highly uniform and reproducible liquid flow (11). It is not surprising that there is a large market for diagnostics based upon nitrocellulose (10, 13).

Multiplex versions of lateral-flow assays include the Quidel duplex influenza type A and type B antigen test (14) and a triplex test for HIV-1 and the hepatitis B and C virus (15). Both devices utilize rectangular strips, upon which capture lines of probe antibodies have been spotted. The Quidel duplex test has three capture lines, one for each of the two analytes, plus a control line. The triplex test has three lines, one for each of the three analytes, and no control line.

In 2007, an alternative fabrication method was introduced by Martinez et al., who lithographically-patterned chromatography paper with hydrophobic SU-8 photoresist using conventional semiconductor process technology (16). SU-8-free hydrophilic "channels" were defined by hydrophobic SU-8-containing regions. When liquid is applied at one end of a hydrophilic channel, it flows from wet to dry regions by capillary action. Utilizing a three-branch tree pattern, three spatially-distinct flow paths were established for a single aliquot of sample placed at the base of the tree pattern. By spotting a different colorimetric reagent on each of the two branches, these workers demonstrated duplex detection of glucose and albumin; the unspotted third branch provided a control function.

We demonstrate here an alternate method of fabricating multiplexed lateral-flow assays based on two-dimensional shaping of porous media (Figure 1). This alternate method has three advantages: (i) suitability for fabrication and adoption in resource-poor areas, (ii) suitability for prototyping development work or high-volume manufacturing, and (iii) improved rates of operator error.

MATERIALS AND METHODS

Chemicals and Materials. Chromatography paper was Whatman Chr 1 (no. 3001-861). Two mil clear polyester-backed sheets of Hi-Flow Plus 135 nitrocellulose membranes (no. HF13502XSS) were from Millipore Corp., Billerica, MA. Transparent vinyl cover tape with hydrophilic adhesive (no. GL-166-clear) was a gift from G&L Precision Die Cutting, Inc., San Jose, CA. Bovine serum albumin, glucose oxidase peroxidase, and tetrabromophenol blue were from Sigma-Aldrich.

Fabrication Methods. Thin sheets of various types of porous media were shaped in two dimensions using a computer-controlled X−Y knife plotter (Graphtec FC7000-75, Western Graphtec Inc., Irvine, CA). This instrument incorporates a knife in place of the traditional ink pen. The knife rotates freely on a turret, enabling precise cutting of various features, including small-radius corners or holes (17). By appropriate adjustment of the blade angle and downward force, most media (e.g., polyester-backed nitrocellulose, conventional photocopy paper, etc.) are cut with a single pass of the knife. To cut chromatography paper without tearing, we find it necessary to employ three sequential overlapping cuts, the first two of which penetrate only part way through the chromatography paper or laminar composite. These so-called "kiss cuts" are also employed in cutting fluidic inlet holes ("vias") in laminar composites. Following cutting operations, the removal of unwanted material ("weeding") was performed manually. The one-time-only instrument setup requires ~60 s. The actual cutting of each device takes 5−15 s, depending on the nature of the media to be cut and the complexity of the shape. Weeding, if needed, takes an additional 10−100 s. The knife plotter can be programmed to cut multiple devices from single sheets up to ~1 m in width and of unlimited length.

Three different device types were fabricated from sheets of porous media, as depicted schematically in Figure 1. In the production of type 2 and type 3 devices, sheets were manually mated. The three fabrication methods each result in a device type that is distinct in both construction and utility. Prior to use, fabricated devices were stored at room temperature.

Test Protocol. An artificial urine stock solution was prepared according to Brooks and Keevil (18). Test samples were produced by adding known quantities of glucose and/or albumin to the urine stock. Type 2 and 3 devices were cut from laminar composites comprised of chromatography paper and cover tape and then manually prespotted with conventional colorimetric reagents per Martinez et al. (16): glucose was detected via the enzymatic oxidation of a chromogen, albumin was detected by the principle of the protein error of indicators (4), and the pH was assayed by methyl orange. The methyl orange patch was prepared by spotting ~0.1 mL of 7.6 μM methyl orange in a pH 3 citric buffer on the arm of a device and allowing the solution to air dry. Although chromatography paper was used as the porous medium in these assays, other media are equally suitable if not actually superior. Indeed, chromatography paper is an unconventional medium for bioassays. It was chosen here merely to demonstrate device fabrication from inexpensive materials and to replicate results obtained from devices fabricated by an alternate method (16).

A sample was added to type 2 devices by dipping an edge of the device in a pool of the sample; a sample was added to type 3 devices by spotting inlet vias with ~0.25 mL of the sample. The flow of the sample into the devices is spontaneous and immediate. Devices are completely filled within 1−4 min, depending on the size of the device. Full development of color is complete within an additional 3−4 min.

RESULTS

Three different device types have been fabricated in a variety of two-dimensional shapes (Figure 2).

Device Type 1. This type is fabricated by cutting polyester-backed nitrocellulose as received from the manufacturer. The entire top surface of the shaped device consists of exposed nitrocellulose (19).

Device Type 2. The type 2 device consists of nitrocellulose or chromatography paper, capped with polyester cover tape, which is then two-dimensionally shaped. The resulting shaped composite presents exposed nitrocellulose along the entire peripheral cut edge and/or at inlet vias. When contacted, the exposed nitrocellulose immediately and spontaneously imbibes fluids. Conversely, fluid that migrates by capillary action to a peripheral edge is subject to evaporation.

Device Type 3. The type 3 device is a type 2 device that has been further covered with cover tape such that all or part of the peripheral edge of imbibing material is covered. Three methods are utilized to provide fluidic access to the type 3 device: (i) inlet vias are cut in the cover tape, (ii) a cross section of the laminar composite is exposed by knife blade or scissors, thereby opening it to the ambient a peripheral edge of the nitrocellulose layer, or (iii) the dimensions of the capping cover tape are appropriately adjusted such that, upon mating with nitrocellulose, the cover tape falls just short of fully capping the entire exposed surface of the imbibing layer. Vias in the top surface cover tape are fabricated by one of two methods: (i) using a knife plotter, a sheet of cover tape is precut with a hole and mated with nitrocellulose to form a laminar composite, and the resulting piece is shaped in two dimensions by the knife plotter or (ii) a circular kiss cut is made in the cover tape of a shaped laminar composite, followed by the manual release of the newly cut circular portion to reveal an opening through the remaining intact cover tape.

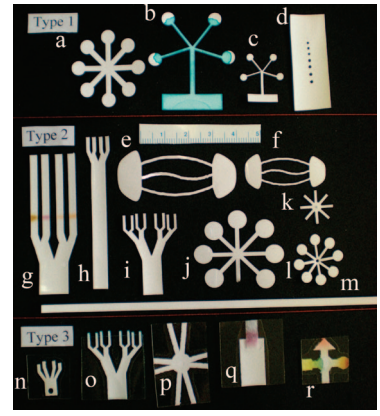

FIGURE 2. Device types fabricated by two-dimensional shaping. Details of fabrication are given in Figure 1. Top row: Type 1 devices formed by shaping uncapped sheets of nitrocellulose. The base of the device 2b has been dipped briefly in a solution of a blue dye; uniform rates of wicking of dye to each of the four arms is observed. Device 2c shows the ability to fabricate a device whose smallest dimension, the width of an arm, is 1 mm. The rectangle 2d tests the limits of hole-cutting: the knife blade was programmed to cut a series of holes of diminishing diameter, with each hole 0.1 mm smaller in diameter than the previous hole; the observed smallest hole was 0.7 mm diameter. Middle row: Type 2 devices formed by shaping laminar composites comprised of vinyl-backed nitrocellulose, capped by sheets of cover tape. The device 2g exhibits the deposition and encapsulation of four different reagents on each of four different arms. Devices 2k and 2l exhibit center vias. The long rectangular strip 2m acts, in effect, as a microchannel of a thin rectangular cross section. Bottom row: Type 3 devices formed by shaping laminar composites in a two-step process; see Figure 1 for fabrication details. Device 2p exhibits the deposition and encapsulation of eight different reagents on eight different arms; this device has a center via, which serves as an inlet port; a photograph of the device following the addition of water to the inlet port is given in the Supporting Information. Device 2o is a branching device that is fully enclosed along the perimeter except for the exposed edges of nitrocellulose on the bottom and top. Device 2n is an example of covering the entire peripheral edge of the nitrocellulose; a single via at the center serves as an inlet port. Device 2r is a triplex test for glucose, albumin, and pH; the device shown was tested with a solution of 500 mM glucose and 75 μM albumin in an artifical urine solution. Additional photographs of devices in the Supporting Information include (i) a Tygon cylinder glued to the center of a star device, thereby defining a macroscale reservoir, and (ii) a conventional lateral-flow test strip fully encased in cover tape except for exposed edges of nitrocellulose.

For each of the three device types, videos of the fluidic flow of aqueous dye are provided in the Supporting Information. In these videos, fluids are observed to migrate in a uniform fashion. We find this to be true regardless of the complexity of the device shape, size, or type. Uniformity of the flow can be inferred from Figure 2b, which shows a type 1 device that has been briefly dipped in a pool of dye and then removed just prior to complete filling of the nitrocellulose pattern. For all four arms of the device, the final location and profile of the fluid front is approximately identical (20). These shaped devices are capable of performing a duplex assay for glucose and albumin (Figure 3a) or a triplex assay for glucose, albumin, and pH (Figure 3b). The duplex results shown are similar to those of a similar device of Martinez et al. constructed by lithographic patterning (21).

glucose	0 mM	50 mM	75 mM	500mM
albumin	0 μM	1.5 μM	7.5 μM	75 μM

FIGURE 3. Results of a duplex test for glucose and albumin using a type 1 device. Glucose and albumin were added to stock solutions of artificial urine to produce test samples of known concentration indicated above. Color development of the square left arm is due to albumin; color development of the circular right arm is due to glucose; the circular center arm is a control arm. The devices were fabricated from Whatman Chr 1 chromatography paper.

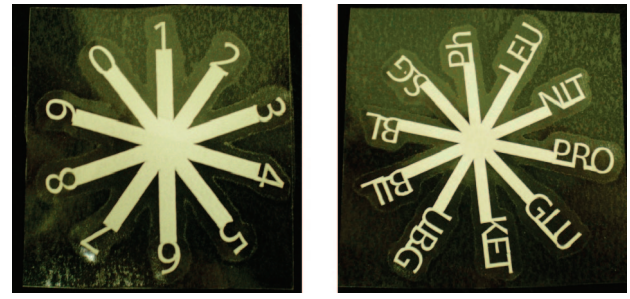

FIGURE 4. Type 3 devices directly labeled with information pertaining to the identity of a given test. Left: number information. Right: acronyms of a common 10-plex urine dipstick (LEU = leukocytes, NIT = nitrites, PRO = protein, GLU = glucose, KET = ketones, UBG = urobilinogen, BIL = bilirubin, BL = blood, SG = specific gravity of the sample). Although both star patterns are visually prominent, the actual physical shape of the two devices shown here is, in fact, *square* because of the shape of the transparent laminating cover tapes. These squares, barely discernible here, are easily and conveniently handled. Color-inverted images render the square edges visually prominent (see the Supporting Information).

DISCUSSION

Special strategies are employed to minimize operator error in the use of diagnostic test strips. For example, the 10-plex urine dipstick is fabricated as a thin rectangle upon which 10 patches, each ~1 cm^2 in area, are placed in a row ~12 cm in length. The areal size, spatial separation, and linear layout of the patches are all intentionally designed to minimize reading errors. An important additional strategy is the provision of a color chart, printed on the label of the dipstick storage container, showing (i) the location of each analyte-indicating patch on the dipstick, (ii) the name of the analyte, and (iii) the various shades of color corresponding to the detected range of the analyte quantity.

Another example of the use of error-minimizing design strategies is the standard duplex flu test. This test is performed on a rectangular strip on which two capture lines are positioned ~1 cm apart. A blue control line is situated midway between these two capture lines. If a red line appears above the blue control line, the test is positive for the type A flu antigen; if a red line appears below the blue control line, the test is positive for the type B flu antigen; if only the blue line appears, the test is negative for both A and B viral antigens. Three strategies reduce the incidence of operator error in interpreting these results. Arguably, the most important strategy is simply minimizing the number of test outcomes, in this case to one of only three possibly valid results: "red line above", "red line below", or "blue line only". The second strategy is to place directly on the test strip a printed legend of valid results that is both color-coded (red or blue) and spatially-mapped in the correct order: at the top of the legend, the letter A is printed on a red background, signifying the location and color of the positive flu A line; in the middle, the letter C is printed on a blue background, signifying the location and color of the control line; on the bottom, the letter B is printed on a red background, signifying the location and color of the positive flu B line. The third strategy is to place on the strip a printed label with arrows indicating which direction is "down", in order to reduce the chance of reading the strip upside down.

A large number of different capture antibodies can be placed on a rectangular lateral-flow strip, either as lines or as an array of spots (e.g., ref 22), but there is a practical limit to the number of lines or spots that can be interpreted by the unaided eye with ease and without error. Indeed, despite careful employment of error-minimizing strategies, operator error remains a persistent problem of CLIA-waived diagnostic tests (23–25). Additional error-minimizing strategies are warranted for multiplex assays, particularly those targeted for field and home use.

The new devices described here offer the following additional means of reducing operator error: (i) different assays are placed on different arms, thereby improving the spatial discrimination of the user; (ii) arms can be directly labeled (Figure 4); (iii) the cassetteless format of type 2 and type 3 devices eliminates operator error caused by incorrect insertion of test strips into a cassette. Moreover, eliminating the cassette is also important for reducing the cost of test kits and for rendering strips impervious to external contaminants.

Current methods of spotting and immobilizing antibodies on conventional rectangular assay strips employ fluid-dispensing nozzles. Typically, an antibody-containing solution is sprayed as a continuous line on the web of a rapidly unwinding thin film of nitrocellulose. Subsequently, a guillotine chops the membrane web into rectangles. Capture zones are thereby defined by the cut width of a rectangle and by spray dispersion. A variant of this dispense method can be applied to the fabrication method described here by including conventional $x-y$ control of the nozzle position, a typical feature of most high-performance reagent dispense systems. We have utilized $x-y$ control of our reagent dispense system to spot two-dimensionally shaped stars and find this approach to be rapid, accurate, and easily implemented.

Many commercially-available lateral-flow strips include an absorbent pad for storing reporter particles or reporter molecules. To add a similar storage element to two-dimensionally-shaped devices, reporters are spotted on exposed porous media and then capped to form type 2 or 3 devices (e.g., Figure 2g). The cap mechanically contains the reporters. Other methods exploit the storage volume of cylindrical inlet ports (e.g., parts k, n, and p of Figure 2 and Supporting

Information photograph 2) but are less attractive because of ambient exposure of the reporters.

Although it was not possible within the scope of this research to conduct tests of end-user acceptability, anecdotally we have found that type 2 and 3 devices are easily handled and operated by novices and non-technical persons alike. For example, the type 3 devices of Figure 4 are square, palm-sized, light-weight, and fully sealed against contamination, contain no sharp edges, and are easily handled. They are slightly pliable, nonflammable, impervious to delamination, and mechanically robust with respect to both tearing and folding. Because each device is only a few mils thick, large numbers of devices can be stacked, stored, and transported with ease. Flow of the liquid and the development of color are readily observed through the transparent cover tape. Because the entire flow path is visible, the physics of the device are easily comprehended. Through labeling of the arms of a star pattern, each arm is endowed with a distinctiveness that aids in the interpretation of individual results and signals the multiplex nature of the test.

The assay device we demonstrate here is fabricated by two-dimensional shaping of Whatman Chr 1 chromatography paper. The cost of paper of a large device, such as the 4 × 4 cm star device of Figure 4, is about U.S. $0.02. If the same device were to be fabricated with a nitrocellulose membrane, the cost would be about U.S. $0.27 (26). Hence, there is more than an order of magnitude difference in cost between these two media. Chr 1 is clearly a candidate for ultra-low-cost diagnostic devices. Nitrocellulose, too, may be a candidate, given the low absolute cost per device, excellent flow performance, and large existing base of knowledge of lateral-flow assays based on this media.

Knife plotters cost less than U.S. $5000. Other than bioreagents, no chemicals are used to fabricate the test strips described here, nor are semiconductor process tools used. Hence, we believe that this new method is suited for diagnostics manufacturing in *resource-poor* areas (27, 28). Also, it may be suited for use in *high-resource* areas given that (i) knife cutting enables rapid prototyping of complex-shaped devices and (ii) prototypes can be quickly advanced to high-volume manufacturing using web-based process tools, e.g., laser cutters, die cutters, bioreagent spotters, etc., for which there exists a mature supplier infrastructure.

CONCLUSIONS

Multiplexed lateral-flow strips have been fabricated by a knife plotter, a tool which is commercially available for under U.S. $5000. The fabricated strips are able to draw an analyte-containing sample across multiple capture zones, without the use of pumps, electricity, or other ancillary devices. They offer new strategies for reducing operator error associated with lateral-flow tests. Moreover, the technology for laser- and die-based cutting is commercially-available and adapted to the web-based manufacturing methods of conventional diagnostic test strips; hence, there is a facile path to high-volume manufacturing of multiplex assays by the general methods demonstrated here. These devices are of potential benefit to clinicians and patients, especially those in underserved and/or rural communities.

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Supporting Information Available: Videos of fluidic flow of dye fronts in type 1−3 devices and photographs of four devices described in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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(20) Additional tests of uniform flow were performed with eight-arm type 3 stars, which had been prespotted with granular solid inorganic dyes, a different dye for each of the eight arms. Upon application of water to the center hole of these devices, the ensuing lateral flow migrates across the lines of dye, solubilizing the dye, and generating a distinct color on each arm. In this case, the rate of advancement of the visible dye front was observed to vary from arm to arm and, in some cases, laterally across an arm (photograph in the Supporting Information). We interpret this to indicate that granules of dye deposited atop nitrocellulose are not uniformly wetted and solubilized, as might be expected for different dyes, and/or when flow occurs primarily within, not atop, the nitrocellulose.
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