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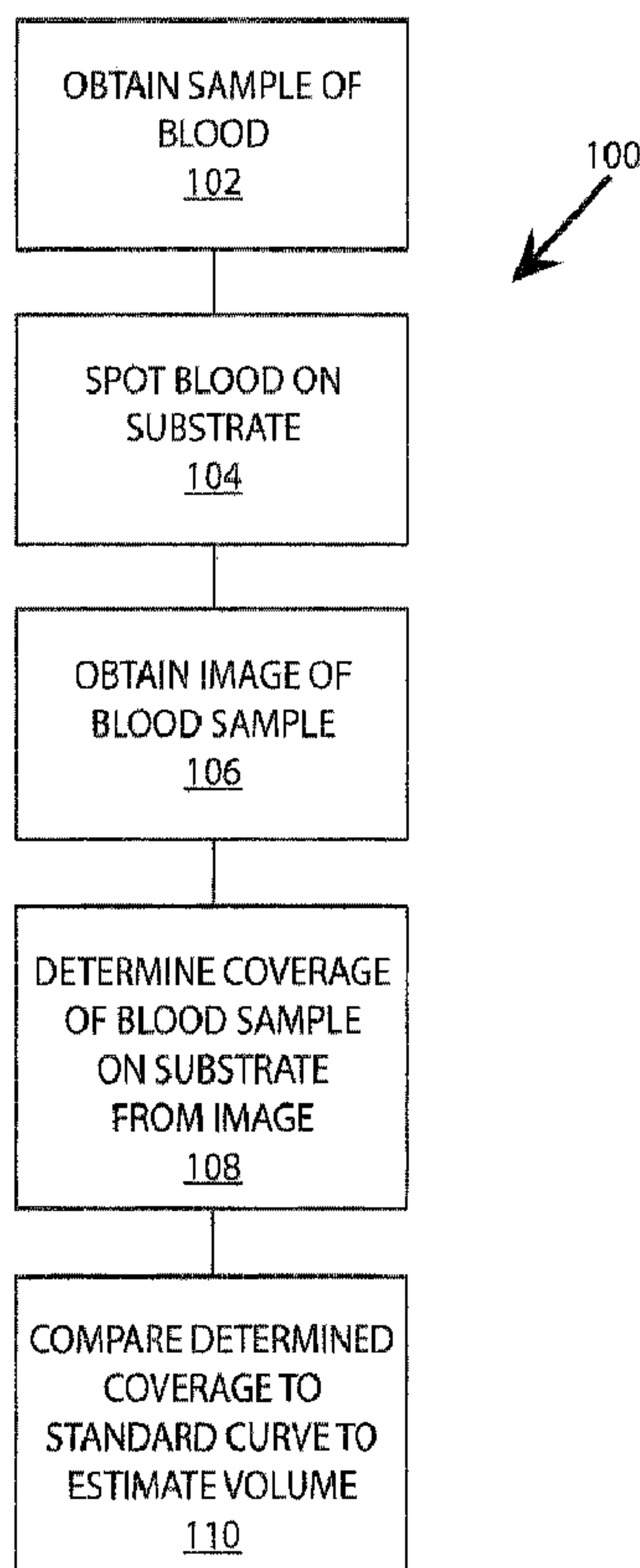


Fig. 2

**(57) Abrégé/Abstract:**

Methods and devices for analysing blood sample volumes are provided. In particular, the disclosure provides a method for estimating the volume of a blood sample on a substrate including the steps of acquiring an image of the blood sample. A coverage

**(57) Abrégé(suite)/Abstract(continued):**

or area of the blood sample may be obtained from the image and compared to a standard curve to obtain a volume estimate of the blood sample. The disclosure also proves a device for scanning one or more blood samples on a substrate. The device includes three layers that may be assembled to hold multiple samples between the layers. The device also includes labels and may be disassembled for decontamination and reloading of samples.

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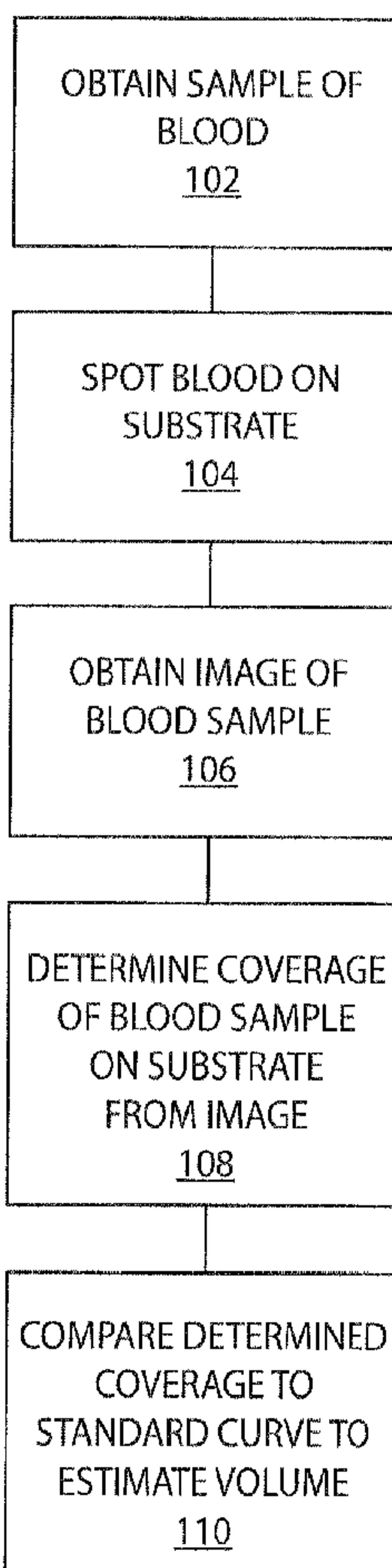
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(54) Title: METHODS AND DEVICES FOR QUANTITATING BLOOD SAMPLES



(57) **Abstract:** Methods and devices for analysing blood sample volumes are provided. In particular, the disclosure provides a method for estimating the volume of a blood sample on a substrate including the steps of acquiring an image of the blood sample. A coverage or area of the blood sample may be obtained from the image and compared to a standard curve to obtain a volume estimate of the blood sample. The disclosure also proves a device for scanning one or more blood samples on a substrate. The device includes three layers that may be assembled to hold multiple samples between the layers. The device also includes labels and may be disassembled for decontamination and reloading of samples.

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Fig. 2

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1        **METHODS AND DEVICES FOR QUANTITATING BLOOD SAMPLES**

2        The present disclosure relates generally to methods and devices for blood  
3        sampling, and more particularly to methods and devices for estimating blood sample  
4        volumes. More specifically, the disclosure relates to a method for estimating the  
5        volume of blood samples collected on a filter or other substrate. The disclosure also  
6        more specifically relates to a device that may be used for holding filters and other  
7        substrates for scanning images of the dried blood samples that will be used for  
8        quantitating the collected blood.

9        Blood samples are routinely taken in clinics, hospitals or specialized labs by  
10      trained professional for diagnostic purposes. A more cost effective and less invasive  
11      alternative to traditional venipuncture method is collecting blood by finger stick on a  
12      filter paper. The blood is then dried and sample known as dried blood spot (DBS) can  
13      be stored or processed as required. The DBS can then be used for analysis of various  
14      small molecules, metabolites, proteins etc. DBS is a powerful blood sampling  
15      procedure as it allows collection of blood at any time or place. No special training is  
16      required to collect the blood and the blood can be stored or shipped at ambient  
17      temperature for a period of time.

18        One major drawback associated with DBS is the difficulty in quantitating the  
19      analytes as the volume of blood loaded cannot be ascertained when directly loaded  
20      onto the filter paper in non-lab settings. Volumetric application of blood is not  
21      practical when collecting samples in the field. A number of factors influence the  
22      spread of blood on a substrate like filter paper. The hematocrit of blood greatly  
23      influences the spread of blood on the filter paper (higher hematocrit blood spreads  
24      less compared to low hematocrit blood). Also there is differential spread of the blood  
25      due to capillary effect (blood spreads more on thinner paper compared to thicker  
26      paper). Finally, the chromatographic effect results in uneven distribution of blood  
27      components (some blood components may move faster than others). So, one area of  
28      the DBS may have different composition than another area. Therefore, the often used  
29      practice of punching out specific sizes of blood spots may not be as accurate as  
30      processing the whole blood sample entirely collected on the filter.

31        Different methods have been proposed to overcome the difficulty in  
32      quantitating the analytes from DBS. One method proposes quantitating amount of

1 endogenous potassium levels to calculate the hematocrit of loaded blood. While this  
2 method reports accurate estimation of hematocrit, it requires additional processing  
3 and analysis of blood samples and filter paper. Another method uses diffuse reflection  
4 to estimate the hematocrit of blood in the DBS to allow for sample volume correction.  
5 While accurate, this method requires additional expensive lab equipment, sample  
6 processing and specialized software to analyze the DBS.

7 Accordingly, there exists an urgent need in the relevant field for a technique  
8 that provides accurate estimation of blood volume. Such techniques should be capable  
9 of being performed in a cost effective manner, as the whole purpose of DBS  
10 technology is to reduce the expenses associated with blood collection, storage and  
11 shipment. Such techniques should also allow for estimation of the blood volume in the  
12 entire spot, so that analytes can be quantitated accurately.

13 Furthermore, current devices known in the art used to scan blood samples,  
14 including dried blood spots on filter paper, suffer from problems such as high risk of  
15 contamination of samples, difficulty in keeping multiple samples organized, isolating  
16 samples from human exposure, and limited ability to quickly, efficiently and  
17 consistently process multiple samples or batches of samples. Accordingly, there also  
18 exists a need in the relevant field for devices that overcome these limitations of the  
19 prior art, including devices that may be used in conjunction with the novel techniques  
20 disclosed herein.

21 Embodiments of the present disclosure provide methods and devices for  
22 processing blood samples. In particular, the present disclosure provides novel  
23 methods of estimating the volume of blood samples collected on filters and other  
24 substrates in a consistent and accurate manner. This is essential for quantifying  
25 analytes in blood samples collected in different settings, including non-laboratory  
26 settings. The methods disclosed herein address the major technical problem of  
27 quantification associated with an otherwise powerful blood sampling method that  
28 allows collection, storage and transport of blood in the field in a minimally invasive  
29 and cost-effective manner.

30 Accordingly, in one embodiment, the disclosure provides a method for  
31 estimating the volume of blood samples comprising the following steps: obtaining a  
32 sample of blood (e.g., by sticking a finger with lancet to get blood sample); spotting

1 the blood sample on a substrate; obtaining an image of the blood sample; determining  
2 an approximate coverage or area of the image of the sample; and comparing the  
3 determined approximate coverage or area to a standard curve to determine an  
4 estimated volume of the blood sample.

5 In one embodiment determining the approximate coverage or area of the  
6 image of the sample comprises calculating a coverage ratio of the blood sample on the  
7 substrate, wherein calculating the coverage ratio of the blood sample on the substrate  
8 preferably comprises counting pixels in the image of the blood sample.

9 In another embodiment calculating the coverage ratio of the blood sample on  
10 the substrate comprises counting pixels in an image of a blank substrate, wherein  
11 calculating the coverage ratio of the blood sample on the substrate preferably  
12 comprises determining a ratio of the number of pixels counted in the image of the  
13 blood sample to the number of pixels counted in the image of the blank substrate.

14 In one embodiment the standard curve comprises data from two or more blood  
15 samples of known volumes plotted against data of approximate coverages or areas of  
16 the two or more blood samples determined from images of the two or more blood  
17 samples. In such embodiment the two or more blood samples of known volumes  
18 preferably comprise samples with varying hematocrits.

19 In another embodiment the image of the sample of blood is obtained with a  
20 scanner or a camera.

21 In yet another embodiment the substrate comprises a filter comprising paper.

22 The present disclosure also provides devices for holding blood samples  
23 collected on filter or other substrates. The devices provide the benefits of ease of use,  
24 simplified and more efficient decontamination, reduction of mistakes when handling  
25 samples due to human error, the ability to easily log samples and keep records of  
26 samples, and increased durability over previous devices. With respect specifically to  
27 scanning of samples, the devices provide the advantages of the ability to fit virtually  
28 any known scanner or similar imaging device, the ability to allow for uniform and  
29 consistent sample spacing and scanning distance, and ease of cross-comparison  
30 between different samples. Thus, the devices can provide the ability to perform more  
31 efficient and accurate scanning in a reduced amount of time over previously known  
32 devices.

1        Accordingly, in one embodiment, the disclosure provides a device for  
2        scanning filters (dried blood samples) comprising: a first layer comprising one or  
3        more transparent portions; a second layer comprising one or more holes, wherein said  
4        one or more holes are formed through the second layer and are sized to each receive a  
5        dried blood sample filter; and a third layer comprising one or more raised portions;  
6        wherein said one or more transparent portions of the first layer overlap with the one or  
7        more holes of the second layer and the one or more raised portions of the third layer  
8        when the first, second and third layers are aligned and stacked on top of each other  
9        with the second layer between the first and third layers.

10        In one embodiment each of the one or more raised portions of the third layer  
11        fits into each of the one or more holes in the second layer. In such embodiment the  
12        raised portions of the third layer preferably are sized to compress a dried blood spot  
13        filter against the first layer and within a hole of the second layer when the first,  
14        second and third layers are aligned and stack on top of each other with the second  
15        layer between the first and third layers.

16        In one embodiment the first, second and third layers are configured to be  
17        securely assembled to one another such that the second layer is positioned between  
18        the first and third layers. In such embodiment the first, second, and third layers  
19        preferably are secured by an attachment mechanism selected from the group  
20        consisting of one or more screws, one or more bolts, one or more nails, a chemical  
21        adhesive, a tape, one or more elastic bands, and combinations thereof.

22        In one embodiment at least one of the first, second or third layers comprises  
23        plexiglass.

24        In one embodiment the first, second and third layers are substantially  
25        rectangular in shape and substantially the same size.

26        In one embodiment the first layer is entirely transparent.

27        In one embodiment the one or more raised portions on the third layer comprise  
28        acrylic discs.

29        In one embodiment the device further comprises one or more labels for  
30        identifying the dried blood sample filters. In such embodiment the labels preferably  
31        comprise one or more codes comprising one or more of letters, words, numbers,  
32        colors, bar codes, and matrix bar codes, and/or are removable.

1        In yet another embodiment the one or more holes in the second layer are  
2        uniformly sized and/or uniformly spaced apart from one another

3        The features, functions, and advantages that have been discussed can be  
4        achieved independently in various embodiments of the present disclosure or may be  
5        combined in yet other embodiments, further details of which can be seen with  
6        reference to the following description and drawings.

7        *Other features, functions and advantages of the present disclosure will be or*  
8        *become apparent to one with skill in the art upon examination of the following*  
9        *drawings and detailed description. It is intended that all such additional systems,*  
10        *methods, features, and advantages be included within this description, be within the*  
11        *scope of the present disclosure, and be protected by the accompanying claims.*

12        Many aspects of the disclosure can be better understood with reference to the  
13        following drawings. The components in the drawings are not necessarily to scale,  
14        emphasis instead being placed upon clearly illustrating the principles of the present  
15        disclosure. Moreover, in the drawings, like reference numerals designate  
16        corresponding parts throughout the several views.

17        Fig. 1 shows blood spots of specific known volumes on filter paper.

18        Fig. 2 shows a flow diagram of an exemplary embodiment of a method for  
19        estimating blood volume disclosed herein.

20        Fig. 3 shows images of blood spots obtained using a variety of backgrounds.

21        Fig. 4 shows two images of a dried blood spot with the spot pixels selected in  
22        one image and the surrounding clear pixels selected in the other image.

23        Fig. 5 shows an exemplary graph of pixel coverage versus blood spot volume.

24        Fig. 6 shows exemplary data and corresponding standard curves of pixel  
25        coverage versus blood volume generated for blood samples from three different  
26        subjects.

27        Fig. 7 shows an exemplary bar graph of pixel coverage versus blood volume  
28        generated from the combined results of blood samples from three different subjects.

29        Fig. 8 shows an exemplary standard curve of pixel coverage versus blood  
30        volume generated from the combined results of blood samples from three different  
31        subjects.

1 Fig. 9 shows a top view of an exemplary embodiment of a first layer of a plate  
2 reader device disclosed herein.

3 Fig. 10 shows a perspective view of an exemplary embodiment of a first layer  
4 of a plate reader device disclosed herein.

5 Fig. 11 shows a top view of an exemplary embodiment of a second layer of a  
6 plate reader device disclosed herein.

7 Fig. 12 shows a perspective view of an exemplary embodiment of a second  
8 layer of a plate reader device disclosed herein.

9 Fig. 13 shows a top view of an exemplary embodiment of a third layer of a  
10 plate reader device disclosed herein.

11 Fig. 14 shows a perspective view of an exemplary embodiment of a third layer  
12 of a plate reader device disclosed herein.

13 Fig. 15 shows a top view of an exemplary embodiment of labels of a plate  
14 reader device disclosed herein.

15 Fig. 16 shows a top view of an exemplary embodiment of an assembled first  
16 layer and second layer of a plate reader device disclosed herein.

17 Fig. 17 shows a top view of an exemplary embodiment of an assembled third  
18 layer and labels of a plate reader device disclosed herein.

19 Fig. 18 shows a top view of an exemplary embodiment of an assembled plate  
20 reader device disclosed herein.

In the following description, reference is made to the accompanying drawings, which form a part hereof, and in which is shown, by way of illustration, various embodiments of the present disclosure. It is understood that other embodiments may be utilized and changes may be made without departing from the scope of the present disclosure.

# *Method for Estimating Volume of Blood Sample*

27 In a preferred embodiment, the methods provided herein may be used to  
28 estimate the volume of blood in a dried blood spot on a paper filter or other substrate.  
29 While not wishing to be bound by theory, it is believed that blood spread on filters  
30 primarily is influenced by the capillary effect (i.e., the spread is inversely proportional  
31 to the thickness of the paper), chromatographic effect (i.e., how fast or slow the blood  
32 components spread through the filter) and the hematocrit (i.e., high hematocrit blood

1 spreads less and vice versa). In a preferred embodiment, the HemaForm™ filter (Spot  
2 On Sciences, Inc.) was selected because its unique design allows spread of blood  
3 evenly and the filter paper thickness is consistent. It is believed that this results in  
4 more consistent blood sampling as a result of reduced hematocrit and  
5 chromatographic effects. The results obtained were reproducible and consistent with  
6 these filters; however, this technology may be applied to other types of filters too.

7 The present disclosure is based, in part, on the hypothesis that the spread of  
8 the blood on the filter would be proportional to the volume of blood being spotted.  
9 We can exploit this property to measure the volume of blood. First, specific  
10 incremental volumes of fresh blood were spotted on different HemaForm™ filters and  
11 visually analyzed after overnight drying. Fig. 1 depicts eight different HemaForm  
12 filters with varying known volumes of blood spotted on the filters. Specifically, the  
13 known volumes of blood spotted increase from left to right, top to bottom in the  
14 images in Fig. 1. Thus, a quick visual inspection of the spotted filters reveals that the  
15 spread of the blood on the filter is proportional to the volume of the blood spotted on  
16 it. As this confirmed the correctness of the hypothesis, the next step was to actually  
17 measure the spread of blood. Since there are no methods currently available to  
18 correlate the spread to the volume of blood in a dried blood spot, it therefore became  
19 necessary to develop a novel analytical tool that would enable the spread to be  
20 quantitated.

21 Our method allows consistent and accurate estimation of blood volume loaded  
22 onto a substrate (e.g., filter paper), which is essential for quantifying analytes in blood  
23 samples collected in non-lab settings. This method addresses a major technical  
24 problem of quantification associated with an otherwise powerful blood sampling  
25 method that allows collection, storage and transport of blood in the field in a  
26 minimally invasive and cost-effective manner.

27 Fig. 2 is a flow chart of an exemplary embodiment of the present invention,  
28 wherein a method 100 is provided for estimating the volume of a blood sample. It  
29 should be noted that any process descriptions or blocks in flow charts should be  
30 understood as representing modules, segments, portions of code, or steps that include  
31 one or more instructions for implementing specific logical functions in the process,  
32 and alternate implementations are included within the scope of the present disclosure

1 in which functions may be executed out of order from that shown or discussed,  
2 including substantially concurrently or in reverse order, depending on the  
3 functionality involved, as would be understood by those reasonably skilled in the art  
4 of the present disclosure.

5 As is shown in block 102 of Fig. 2, a sample of blood is obtained from a  
6 human or animal patient by any method known in the art. This may include traditional  
7 venipuncture, wherein blood is obtained directly from a patient's vein. The blood may  
8 also be collected via a finger prick or via a "prick" of any other part of the patient's  
9 body. Blood collected via this method is typically obtained from blood capillaries  
10 near the surface of the skin by piercing the skin with a lancet or similar device. The  
11 blood from the "finger prick" may be collected into a capillary tube and then  
12 dispensed onto a filter or directly spotted onto the filter paper.

13 As is shown in block 104 of Fig. 2, the sample of blood obtained or a portion  
14 of the sample of blood obtained is spotted or otherwise placed onto a substrate. The  
15 substrate may be any material known in the art that is capable of retaining a sample of  
16 blood. In a preferred embodiment, a paper filter may be used as the substrate. Paper  
17 filters, including Guthrie cards, HemaForm filters and others, are well known in the  
18 art for their use in dried blood spot sampling. In certain embodiments, the substrate  
19 may be a composite material and/or may be coated, for example, with silica.

20 In block 106 of Fig. 2, an image is obtained of the spotted blood sample. The  
21 image may be obtained with any imaging device known in the art, preferably after the  
22 blood has dried completely. Thus, a camera, a scanner, or other similar imaging  
23 devices may be used successfully with the disclosed methods. Selection of an  
24 appropriate imaging device may include such considerations as ease of use, the need  
25 for a fixed platform for acquiring multiple images, the ability to process multiple  
26 filters, image quality, and the ability to control and manage various imaging settings  
27 and controls.

28 In one embodiment, a camera may be used to obtain the image. Typically, the  
29 camera will be mounted on a tripod or other device to hold it steady and to obtain  
30 images that are consistent and reproducible. In an alternative embodiment, a scanner  
31 may be used to obtain the image. For example, an HP Photosmart 1300 or other  
32 similar device may be used to obtain the image. For any imaging device used, it may

1 be beneficial to adjust the resolution of the acquired image to a preferred or  
2 standardized resolution. For example, a resolution of 600 dpi may be used. Resolution  
3 may also be controlled and adjusted via software, as is discussed below. The image  
4 obtained may be provided in a digital format such that it may be viewed on a  
5 computing or other electronic device. This further allows the image to be viewed with  
6 image editing or image analysis software.

7 In block 108 of Fig. 2, an approximate coverage or area of the blood sample  
8 on the substrate is determined from the image of the blood sample. For purposes of  
9 this disclosure, the coverage of the blood sample may be measured or estimated in  
10 terms of absolute area or, alternatively, as a ratio of areas, such as percent coverage.  
11 In a preferred embodiment, the determination of the coverage may be performed by  
12 counting pixels in the image. This is preferably done with the use of image analysis  
13 or image editing software. Numerous software programs for this purpose are known  
14 in the art (e.g., GIMP2, Adobe Photoshop).

15 Prior to obtaining a pixel count, various settings of the software may need to  
16 be adjusted to obtain an optimal image for counting the pixels. For example, it may be  
17 beneficial to adjust the resolution of the images. Using a fixed resolution across all  
18 images may help to ensure accurate results. Further optimization can be carried out by  
19 choosing the right threshold for pixel selection with the GIMP2 image analysis  
20 program. A lower threshold may often lead to non-specific selection, whereas a higher  
21 threshold may interfere with color selection of pixels.

22 It may also be beneficial to optimize resolution in order to minimize the  
23 shadow interference in the images. Shadows observed along peripheries of the  
24 substrate (e.g., shadows along the “petals” of the HemaForm™ filter) may interfere  
25 with pixel selection during pixel analysis and counting. Alternatively, or in addition to  
26 adjusting settings in the scanning software for this purpose, a variety of backgrounds  
27 may be used in the images in order to minimize these effects, as is shown in Fig. 3.

28 The software may be utilized to count only those pixels that correspond to the  
29 portion of the image that actually depicts the blood spot. Thus, any pixel that covers  
30 or at least partially overlaps with a blood spot may be counted. Alternative  
31 methodologies for determining whether an individual pixel should be counted may be  
32 used, so long as the methodology is consistent with respect to other images or data to

1 which the image may be compared. In certain embodiments, the absolute number of  
2 pixels that correspond to the blood spot may be used to determine the area of the  
3 blood spot. The pixels representing the blank areas of the filter (i.e., the portions of  
4 the filter to which the blood has not spread) may also be counted. From this additional  
5 count, a total number of pixels may be acquired for use in the area or coverage  
6 calculation or, by adding to the pixels counted in the blood spot and comparing to a  
7 known number of pixels corresponding to an entire blank filter, to ensure accurate and  
8 precise pixel counting.

9 In alternative embodiments, a “percent coverage” area may be determined, for  
10 example, by additionally counting the pixels representing a blank substrate (e.g.,  
11 filter) in an image of the blank substrate. It is important that the image of the blank  
12 substrate be acquired with the same settings (e.g., resolution) as the image of the  
13 blood spot for accurate and meaningful comparison of the two images. To obtain a  
14 percent coverage calculation, a ratio of the number of pixels counted in the blood spot  
15 to the number of pixels counted in the blank filter is determined.

16 As is shown in block 110 of Fig. 2, the coverage or area of the blood spot  
17 determined in block 108 may be compared to a standard curve, table, chart or other  
18 data to calculate the volume of the blood spot. Such a standard curve, table, chart or  
19 other data may include data on blood samples with known volumes. For example, a  
20 series of blood samples of different known volumes may be spotted separately on  
21 substrates. Images of each of the spotted blood samples may be obtained, along with  
22 area or percent coverage calculations obtained from the images. These calculated  
23 areas or percent coverages may then be plotted against or otherwise compared to the  
24 known volumes to obtain a standard curve, table, chart, etc. A best-fit line may be  
25 used to assist with comparing the data. For any subsequently obtained blood samples,  
26 an estimation of the volume of the blood sample may be determined by comparing the  
27 area of percent coverage of the blood sample to the standard curve or other data  
28 generated in block 110. It may further be necessary to update the data in the standard  
29 curve as necessary to ensure its accuracy. Moreover, it may be desirable that the  
30 known samples used to generate the standard curve vary in other characteristics, for  
31 example, hematocrit, to correct for possible effects of such characteristics on the  
32 spreading area of the blood sample on the substrate.

1    Example 1: Generation of a Standard Curve

2        A standard curve may be generated by the following exemplary method. A  
3    series of exact known volumes of fresh blood were spotted onto HemaForm™ filters  
4    and dried overnight. For imaging, a camera was fixed onto a tripod. The camera  
5    settings were adjusted for optimum resolution, and all of the images were then taken  
6    keeping the settings fixed. Pixels were counted using the image analysis program  
7    GIMP2. This software is an ideal choice because it is freely available from the  
8    internet and it has proven successful in carrying out analyses (though other image  
9    analysis programs like Adobe Photoshop etc. may work equally well). For reference,  
10   an image of a blank HemaForm™ filter was acquired, and the total number of pixels  
11   corresponding to the blank filter was determined from the image. The pixels in the  
12   dried blood spot and the surrounding empty (clear) region of the filter around the  
13   blood spot were also selected and counted, as is shown in Fig. 4.

14       Next, the pixel coverage was determined by dividing the pixels in the spot by  
15   the total pixels in the blank filter. Fig. 5 shows the results of the pixel covered vs.  
16   volume of blood spotted. The slope of a best-fit line fitted to the data points was  
17   determined, as is also shown in Fig. 5. To test the accuracy of the line in Fig. 5,  
18   estimations of the blood sample volumes obtained from the slope of the line were then  
19   compared to actual known volumes. Our volume estimations were within 1-5  $\mu$ l of the  
20   actual volumes spotted, thereby demonstrating the efficacy of the method. However,  
21   with additional refinements to the analysis, even greater accuracy may be achieved.

22   Example 2: Generation of a Combined Standard Curve

23       A combined standard curve may be obtained by the following exemplary  
24   method. A scanner (e.g., HP Photosmart 3100) was selected based on the  
25   considerations mentioned above. Image resolution was also optimized with the  
26   GIMP2 image analysis program to improve the quality of pixel analysis and counting.

27       Bloods from three different individuals varying in age, gender and hematocrit  
28   was analyzed to account for the differences in the spread of blood. Fresh blood from  
29   the three subjects was spotted on filters at known different volumes. Images of these  
30   spotted blood samples were acquired and used to calculate the pixels covered. From  
31   this data, three separate standard curves were generated, as is shown in Figs. 6 and 7.  
32   A combined standard curve, shown in Fig. 8, was also generated from which the slope

1 was determined. The combined curve was used to determine the volumes of unknown  
2 samples.

3 Prediction accuracy when using the methods disclosed herein is quite high,  
4 and estimated volumes may generally fall within 2-3 $\mu$ l or less of the actual volumes.  
5 Hematocrit was found to have an effect on the obtained measurements. Thus, for very  
6 low and very high hematocrit levels, the calculated volume may generally fall within  
7 5% the actual volume spotted. Blood lower in hematocrit may tend to spread further  
8 because there is a greater amount of plasma in a given volume. Conversely, a higher  
9 hematocrit blood may typically spread less due to a lower amount of plasma in a  
10 given sample size. Thus, measurements obtained using the disclosed methods may  
11 directly correlate with the volume of plasma in the blood. Further, metabolite levels  
12 measured from whole blood provides a more accurate measure of disease state or  
13 progression and also allow for a more consistent comparison with plasma.

14 Validation of the accuracy of the standard curve may be accomplished by  
15 spotting known volumes on the filter. Blinded analyses are typically performed in  
16 these cases, such that the volumes are not known to the person carrying out the  
17 volume analysis. Table-1 shows a sample of some volumes that were analyzed by  
18 blinded analysis. As can be seen from Table 1, all estimated volumes fall within 0.2 –  
19 3.5 $\mu$ l of the actual known volumes.

Subject	Calculated volume using image analysis ( $\mu$ l)	Actual volume loaded on filter ( $\mu$ l)
1	8.64	9.00
2	11.3	10.00
3	19.26	20.00
4	18.89	20.00
5	36.5	33.00
6	43.06	40.00
7	43.1	40.00
8	48.46	50.00
9	49.80	50.00
10	52.11	50.00

20

21

Table-1

1    Example 3: Method for Estimation of Blood Volume

2        In a preferred embodiment, estimation of blood volume may be performed as  
3        follows. This technique is based on calculating the pixels in the DBS image. The  
4        volume of blood in the spot is measured by comparing its pixels-against-pixel count  
5        to a standard curve of DBS of known volumes. The step by step procedure is  
6        described below:

7        **Step 1: Pixel calculation**

8            (a) Scanning DBS

9        To count the pixels in a DBS, the first step is to scan the DBS. DBS samples  
10      are scanned along with a blank filter (on which blood has not been spotted) on the  
11      scanner. The scanning resolution is set to a fixed setting –this can be any setting but  
12      once chosen, should be kept same for all analyses. For example, a resolution of 600  
13      DPI may be used.

14            (b) Image analysis using GIMP 2 image analysis software

15        This software is available for free download from the internet. The scanned  
16      DBS is cropped using the selection tool and the pixels are separately counted for the  
17      exact blood spot area and the blank area. Pixels are also counted for a blank filter  
18      likewise.

19            (c) Actual pixel coverage

20        Percent pixels in the spot are calculated by determining the ratio of pixels in  
21      the spot to the overall pixels in the blank filter.

22        **Step 2: Preparation of the Standard Curve**

23        Collect blood from different individuals (ideally with a varying hematocrit) in  
24      heparin/EDTA tubes. The freshly collected blood is then spotted in exact known  
25      volumes ranging from 5 to 60  $\mu$ l individually (in triplicate samples) to prepare DBS  
26      of known volumes. These are left to dry overnight followed by scanning as described  
27      in (1) above. The pixel coverage is calculated and the standard curve is created for the  
28      pixel coverage against the volume. The slope is determined for the standard curve.

29        **Step 3: Calculation of Blood Volume in the DBS**

30        Pixel coverage is calculated for a DBS of unknown volume as described in  
31      step (1) above and the volume is calculated on the basis of the slope determined as  
32      discussed in step (2).

1

*Plate Reader Device*

2        The devices disclosed herein may be used for holding filters on which blood  
3        samples have been collected (e.g., DBS). In a preferred embodiment, this disclosure  
4        provides a device for scanning or imaging multiple blood samples quickly and  
5        without cross-contamination and with minimal scanning-related shadow artifacts. The  
6        device also provides the benefits of keeping samples organized, holding them securely  
7        in place for scanning, and *isolating the sample from human exposure as much as* possible.  
8        Additional advantages of the device include that it may fit virtually any  
9        scanner, is easy to use, and is durable. Because it may keep sample space and  
10       scanning distances uniform, it will allow for cross-comparison of samples scanned  
11       from one batch to the next. This may greatly reduce time spent trying to get samples  
12       scanned exactly the same way or having to adjust algorithms to calculate volumes and  
13       other characteristics of blood samples. The device is made of separate components  
14       that may be assembled for scanning and disassembled for decontamination and  
15       sample preparation. The device may also assist in logging and identification of  
16       samples as it can keep a record of which samples have been scanned via an  
17       incorporated label template.

18       In another embodiment, the present disclosure provides a device for scanning  
19       dried blood spots on a filter that may be utilized with all methods disclosed herein. In  
20       a preferred embodiment, the device enables scanning of multiple dried blood spots  
21       simultaneously. As is depicted in Figs. 9-15, the device may include up to four parts  
22       that may be assembled or otherwise positioned together.

23       Figs. 9-10 depict an exemplary embodiment of a first layer 10 of the device.  
24       The first layer 10 typically will comprise a flat or substantially flat piece of material.  
25       The first layer 10 may include a first surface 14 and a second surface 16 on opposing  
26       sides of the layer. Preferably, the first layer 10 will be of uniform thickness. In a  
27       preferred embodiment, the first layer may comprise a sheet of plexiglass. The first  
28       layer 10 may include one or more transparent portions 12. The one or more  
29       transparent portions 12 may be of the same or varying sizes, shapes, diameters, etc.  
30       The spacing of the transparent portions 12 may be uniform in one or more dimensions  
31       of the first layer 10. Alternatively, the transparent portions 12 may be randomly or  
32       irregularly spaced. In certain embodiments, the first layer may be entirely transparent.

1        A second layer 20 of the device is shown in Figs. 11-12. The second layer will  
2 typically comprise a flat or substantially flat piece of material. The second layer 20  
3 may include a first surface 24 and a second surface 26 on opposing sides of the  
4 second layer. Preferably, the second layer 20 will be of substantially uniform  
5 thickness. In a preferred embodiment, the second layer 20 may comprise a sheet of  
6 plexiglass. One or more holes 22 are formed in second layer 20. The holes may  
7 extend entirely through second layer 20 (i.e., extend from a first surface 24 to a  
8 second surface 26), or alternatively may only extend partially through second layer  
9 20. The one or more holes 22 may be of the same or varying sizes, shapes, diameters,  
10 etc. The spacing of the holes 22 may be uniform in one or more dimensions of the  
11 second layer 20. Alternatively, the holes 22 may be randomly or irregularly spaced.

12        In a preferred embodiment, the holes 22 should be sufficiently sized such that  
13 each hole may receive a dried blood sample, as is shown in Fig. 16. Typically, such a  
14 blood sample will be contained on a paper filter 28 or other similar substrate. Thus,  
15 each of the one or more holes 22 should be large enough that a filter 28 or similar  
16 substrate containing a blood sample can lay flat within the hole. In a preferred  
17 embodiment, the dimensions of the hole will closely match or be slightly larger than  
18 the dimensions of the filter or substrate such that any movement or shifting of the  
19 filter or substrate within the hole will be limited or eliminated.

20        Figs. 13-14 show a third layer 30 of the device. The third layer 30 will  
21 typically comprise a flat or substantially flat piece of material. The third layer 30 may  
22 include a first surface 34 and a second surface 36 on opposing sides of the third layer.  
23 Preferably, the third layer 30 will be of substantially uniform thickness. In a preferred  
24 embodiment, the third layer 30 may comprise a sheet of plexiglass. This layer  
25 includes one or more raised portions 32 extending upward from a first surface 34 of  
26 the third layer. The raised portions 32 may be of any size, shape or color. They may  
27 be formed integrally from the third layer 30 or may alternatively be separate  
28 components that are attached to the third layer 30 via any adhesive mechanism known  
29 in the art. The raised portions 32 may comprise any suitable material, including  
30 acrylic, plastics, metals, etc. In a preferred embodiment, the raised portions 32 may  
31 comprise acrylic discs. In a preferred embodiment, the raised portions 32 may be

1 sized such that each raised portion fits into a hole 22 in the second layer 20 when the  
2 second and third layers are aligned and stacked on top of each other.

3 Fig. 15 shows one or more labels 40 that may be used with the device. The one  
4 or more labels may each be separate, or, alternatively may be included on a template  
5 42 of multiple labels. In either case the labels should be removable from the device.  
6 Thus, the one or more labels, or a template containing the labels, may be configured  
7 to lie over or rest upon one or more layers of the device. Alternatively, the one or  
8 more labels, or a template containing the labels, may include an adhesive that permits  
9 both secure attachment of the labels to one or more layers of the device, as well as  
10 removal of the labels from the device. The labels and/or template containing the labels  
11 may comprise any suitable material, including paper, transfer plastic, cardboard,  
12 plexiglass, etc. The labels may comprise a code 44 for identifying a specimen (e.g.,  
13 blood sample, filter, substrate, etc.) held within the device. One or more types of  
14 codes 44 may be used, including letters, words, numbers, colors, bar codes, matrix bar  
15 codes, or any combination thereof.

16 In a preferred embodiment, each of the first, second and third layers will  
17 comprise a substantially similar size and shape. Preferably, the layers will be sized  
18 such that they may be used in conjunction with a standard scanner or other imaging  
19 device. Thus, in certain embodiments, the layers may be substantially the same length  
20 and width as a piece of typing paper. Such a size permits the device to sit on the bed  
21 of a scanner such that any specimens within the device may be scanned. The first,  
22 second and third layers may comprise the same or different materials. Preferably, the  
23 layers will comprise plexiglass or other plastics.

24 The layers, as well as the labels, are configured to be assembled together to  
25 form the device 1, as is shown in Figs. 16-18. The layers, when assembled, may  
26 simply be aligned and stacked such that the length and width of each layer aligns or  
27 substantially aligns with the lengths and widths of the other layers. Alternatively, the  
28 layers may be securely assembled by an attachment mechanism, including screws,  
29 bolts, nails, chemical adhesives, tape, elastic bands, or combinations thereof. In one  
30 embodiment, the raised portions 32 on the third layer 30 fit tightly into the holes 22 in  
31 the second layer such that the layers are held snug. The assembly may also be  
32 securely held on the edges with tape.

1        When the device is assembled and all parts are aligned, the various  
2    components of each layer should also align with one another. That is, the transparent  
3    portions 12 of the first layer 10 should align with the holes 22 of the second layer 20,  
4    the raised portions 32 of the third layer 30, and the labels 40. The layers will typically  
5    be stacked with the second layer between the first and third layers. Thus, each of the  
6    one or more raised portions 32 of the third layer 30 will fit into a hole 22 of the  
7    second layer, while each one or more transparent portions 12 of the first layer will  
8    cover the holes 22 on the opposite surface of the hole from the surface where the  
9    raised portions are inserted. Further, when the device is assembled and a filter or other  
10   substrate is also inserted in a hole (as is shown in Fig. 16), the raised portions will  
11   hold the filter within the hole and compress it against a transparent portion of the first  
12   layer (as is shown in Fig. 18). Thus, the raised portions 32 preferably will extend the  
13   full depth of the hole or nearly the full depth of the hole. Further, the insertion of the  
14   raised portions in the holes also provides a secure assembly of the device by limiting  
15   relative movement of the second and third layers.

16        When assembled, the labels 40, or a template 42 containing the labels, will  
17   typically be attached to or otherwise lie adjacent to the third layer, as is shown in Fig.  
18   18. However, the labels or template may also be attached, adhered, or otherwise lie  
19   adjacent to the first or second layers also.

20   **Example 4: Exemplary Characteristics of Device Components**

21        In an exemplary embodiment, the components of the device may have the  
22   following characteristics. The first layer comprises an 8x11" piece of plain plexiglass  
23   (Fig. 9). The second layer comprises an 8x11" piece of plain plexiglass with  $\frac{3}{4}$ " holes  
24   drilled into it (Fig. 11). The third layer comprises an 8x11" piece of plain plexiglass  
25   with  $\frac{3}{4}$ " colored acrylic discs attached to it that correspond to the  $\frac{3}{4}$ " holes drill in the  
26   second layer (Fig. 13). The labels are included on a removable template (comprising  
27   paper and/or transfer plastic) for labeling the samples (Fig. 15). The labels comprise a  
28   number system.

29   **Example 5: Assembly of the Device**

30        In an exemplary embodiment, the components of the device are assembled as  
31   follows. The first and second layers are attached together in such a way that the  
32   transparent portions of the first layer correspond with the holes in the second layer

1 (Fig. 16). A blank piece of filter paper is placed a hole of the second layer and the  
2 remaining holes are filled with filters containing dried blood spots. The third layer and  
3 label template are assembled (Fig. 17) and placed on top of the first and second  
4 layers, such that each label corresponds to the appropriate filter or blood sample.  
5 Also, the raised portions (acrylic discs) of the third layer compress the filters down  
6 within each hole so than an accurate scan can be made of 100% of the surface area of  
7 each specimen. The whole assembled device (Fig. 18) is then placed in the scanner  
8 and scanned.

9 It should be emphasized that the above-described embodiments of the present  
10 disclosure, particularly, any “preferred” embodiments, are merely possible examples  
11 of implementations, merely set forth for a clear understanding of the principles of the  
12 disclosure. Many variations and modifications may be made to the above-described  
13 embodiment(s) of the disclosure without departing substantially from the spirit and  
14 principles of the disclosure. All such modifications and variations are intended to be  
15 included herein within the scope of this disclosure and the present disclosure and  
16 protected by the following claims.

17

## CLAIMS

2. What is claimed is:

3        1. A method for estimating the volume of a blood sample comprising the  
4 following steps:

5 obtaining a sample of blood;

6 spotting the sample of blood on a substrate;

7 obtaining an image of the sample of blood;

8 determining an approximate coverage or area of the image of the sample; and

9 comparing the determined approximate coverage or area to a standard curve to

10 determine an estimated volume of the sample of blood.

11 2. The method of claim 1, wherein determining the approximate coverage or area  
12 of the image of the sample comprises calculating a coverage ratio of the blood sample  
13 on the substrate, wherein calculating the coverage ratio of the blood sample on the  
14 substrate preferably comprises counting pixels in the image of the blood sample.

15 3. The method of claim 1 or claim 2, wherein calculating the coverage ratio of  
16 the blood sample on the substrate comprises counting pixels in an image of a blank  
17 substrate, wherein calculating the coverage ratio of the blood sample on the substrate  
18 preferably comprises determining a ratio of the number of pixels counted in the image  
19 of the blood sample to the number of pixels counted in the image of the blank  
20 substrate.

21 4. The method of claim 1, wherein the standard curve comprises data from two  
22 or more blood samples of known volumes plotted against data of approximate  
23 coverages or areas of the two or more blood samples determined from images of the  
24 two or more blood samples and wherein the two or more blood samples of known  
25 volumes preferably comprise samples with varying hematocrits.

26 5. The method of any one of claims 1-4, characterized by one or more of the  
27 following features:

28 (a) wherein the image of the sample of blood is obtained with a scanner or a  
29 camera; and

30 (b) wherein the substrate comprises a filter comprising paper.

31 6. A device for scanning filters for dried blood samples C

1        a second layer comprising one or more holes, wherein said one or more holes are  
2        formed through the second layer and are sized to each receive a dried blood sample  
3        filter; and

4        a third layer comprising one or more raised portions;

5        wherein said one or more transparent portions of the first layer overlap with the  
6        one or more holes of the second layer and the one or more raised portions of the third  
7        layer when the first, second and third layers are aligned and stacked on top of each  
8        other with the second layer between the first and third layers.

9        7. The device of claim 6, wherein each of the one or more raised portions of the  
10      third layer fits into each of the one or more holes in the second layer.

11        8. The device of claim 7, wherein the raised portions of the third layer are sized  
12      to compress a dried blood spot filter against the first layer and within a hole of the  
13      second layer when the first, second and third layers are aligned and stack on top of  
14      each other with the second layer between the first and third layers.

15        9. The device of any one of claims 6-8, wherein the first, second and third layers  
16      are configured to be securely assembled to one another such that the second layer is  
17      positioned between the first and third layers.

18        10. The device of claim 9, wherein the first, second, and third layers are secured  
19      by an attachment mechanism selected from the group consisting of one or more  
20      screws, one or more bolts, one or more nails, a chemical adhesive, a tape, one or more  
21      elastic bands, and combinations thereof.

22        11. The device of any one of claims 6-10, characterized by one or more of the  
23      following features:

24            (a) wherein at least one of the first, second or third layers comprises  
25      plexiglass;

26            (b) wherein the first, second and third layers are substantially rectangular in  
27      shape and substantially the same size;

28            (c) wherein the first layer is entirely transparent; and

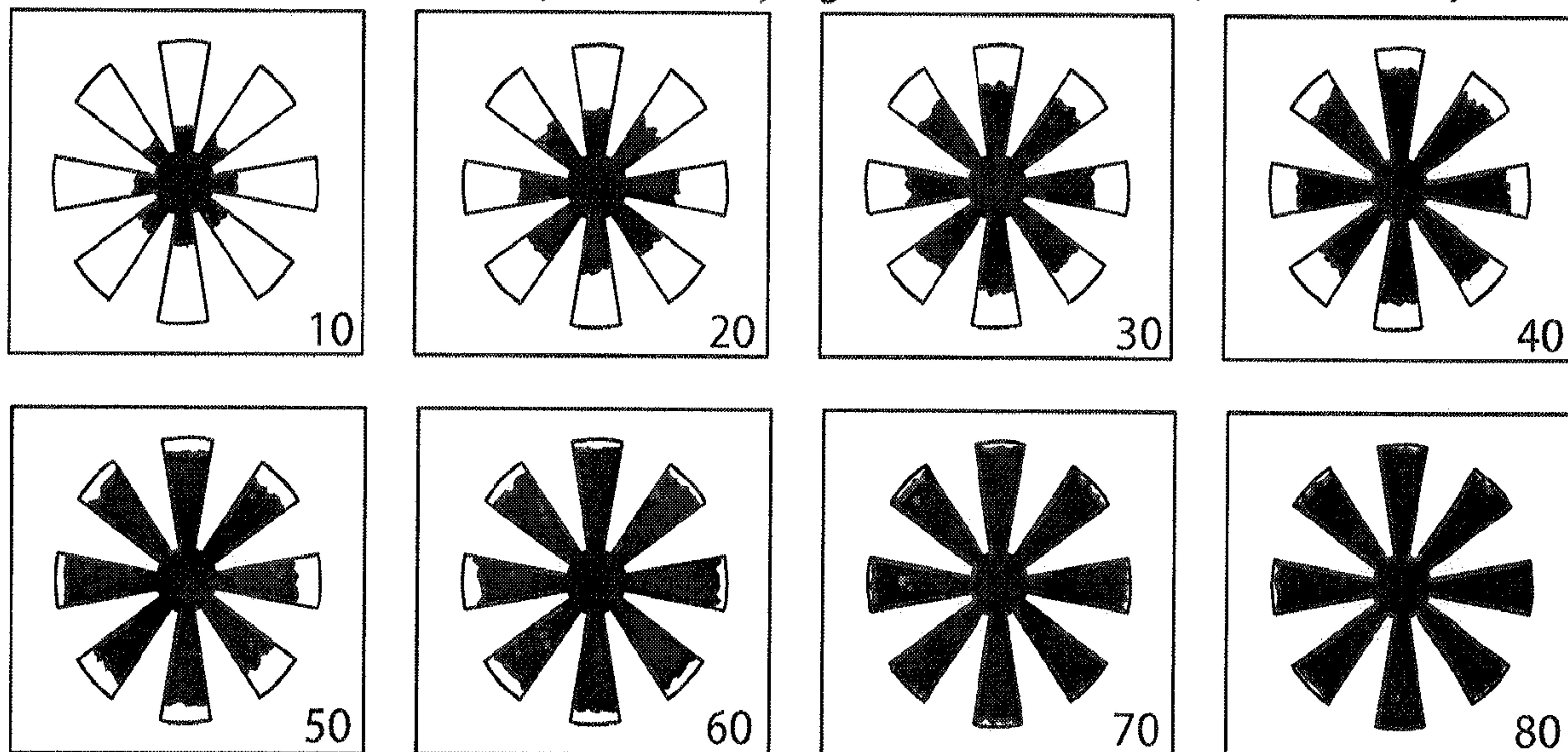
29            (d) wherein the one or more raised portions on the third layer comprise acrylic  
30      discs.

31        12. The device of any one of claims 6-11, further comprising one or more labels  
32      for identifying the dried blood sample filters.

- 1        13. The device of claim 12, wherein the labels comprise one or more codes
- 2        comprising one or more of letters, words, numbers, colors, bar codes, and matrix bar
- 3        codes.
- 4        14. The device of claim 12, wherein the one or more labels are removable.
- 5        15. The device of any one of claims 6-14, wherein the one or more holes in the
- 6        second layer are uniformly sized and/or uniformly spaced apart from one another.
- 7

1/15

**DBS (on the Hemaspot 80)- varying known volumes (-as indicated)**



**Fig. 1**

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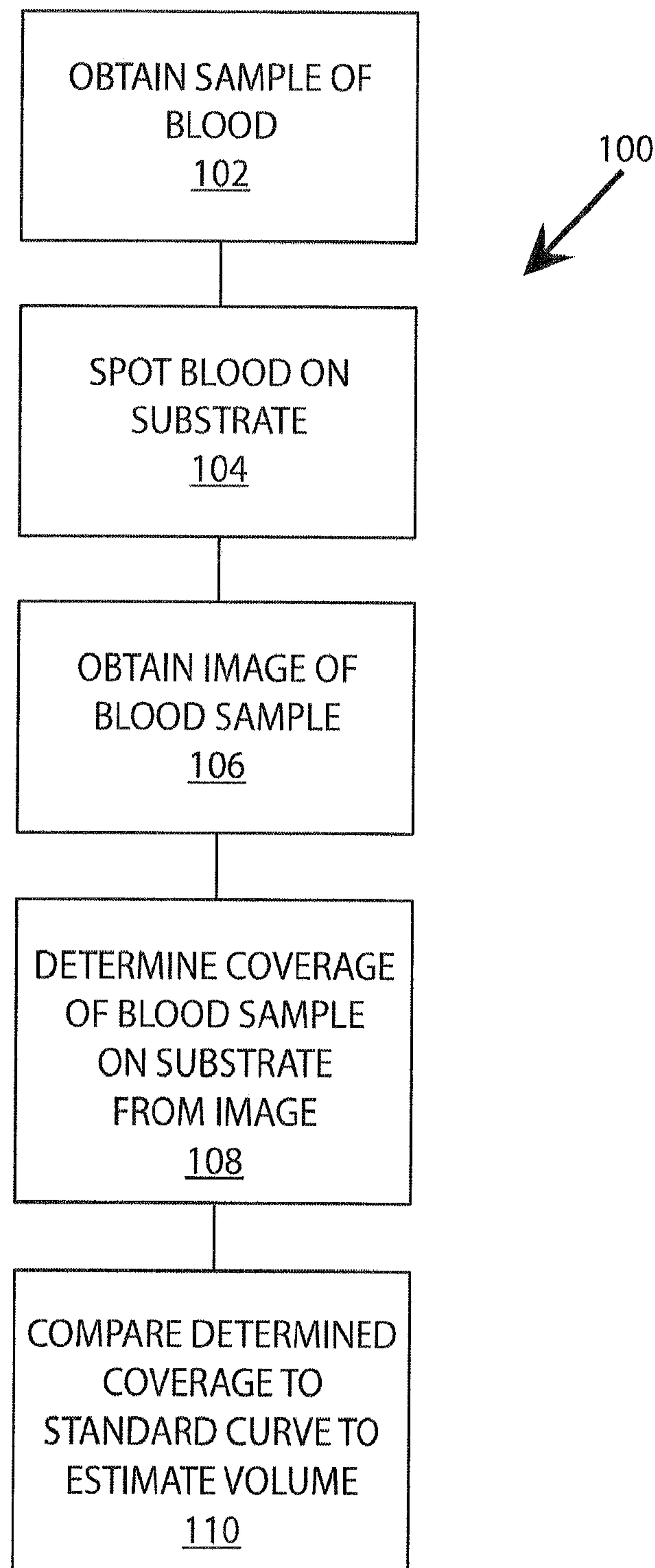


Fig. 2

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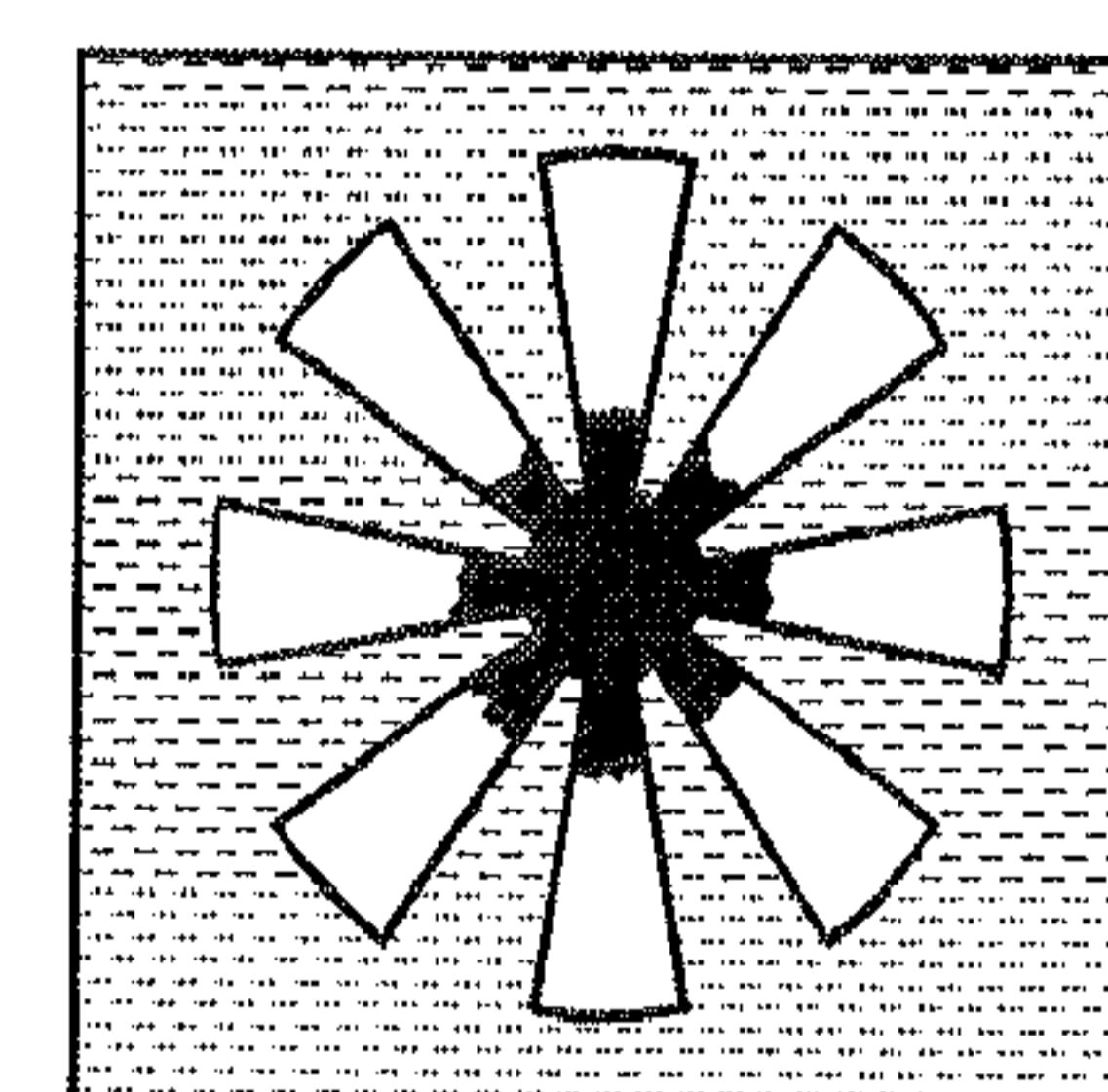
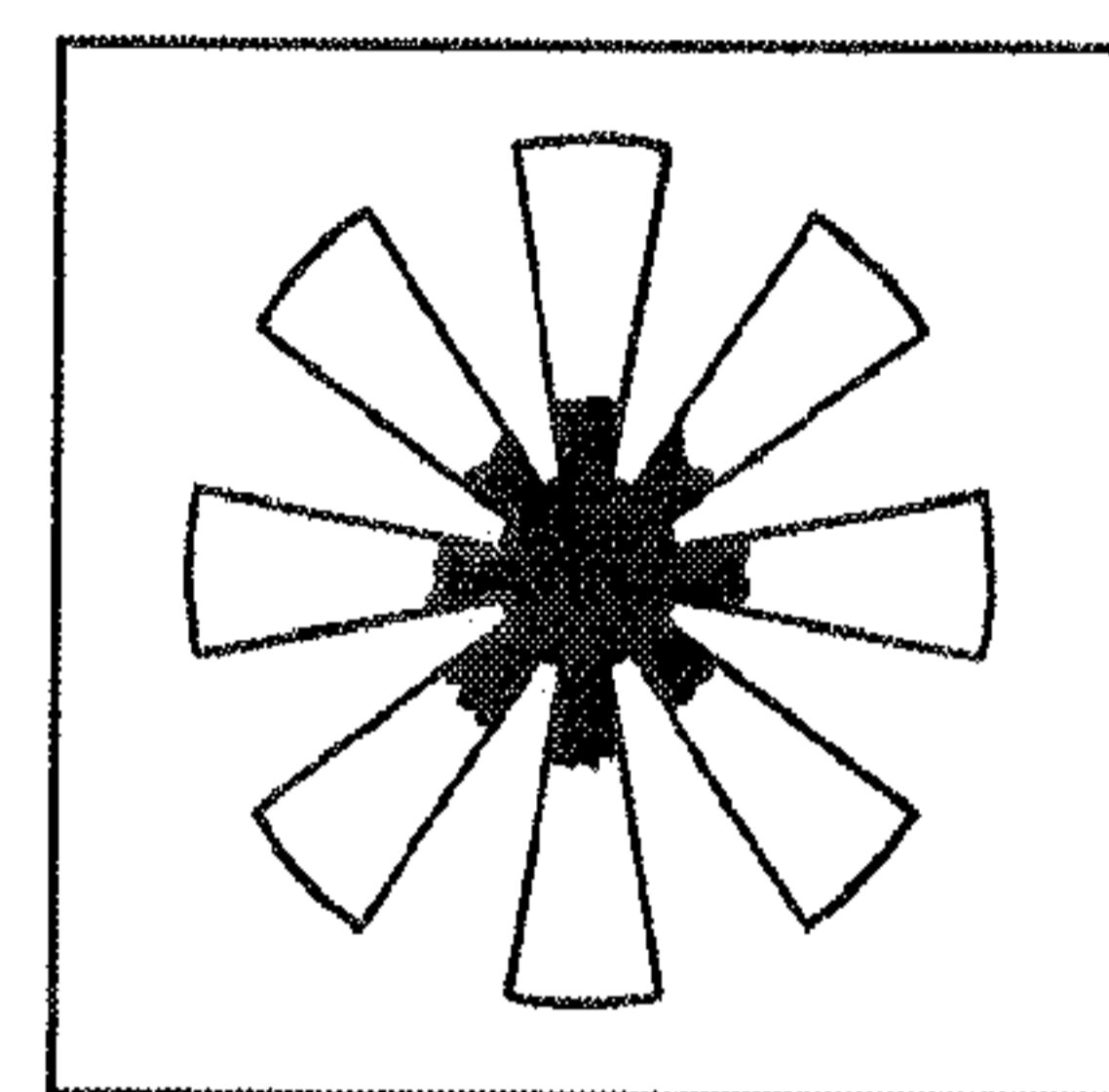
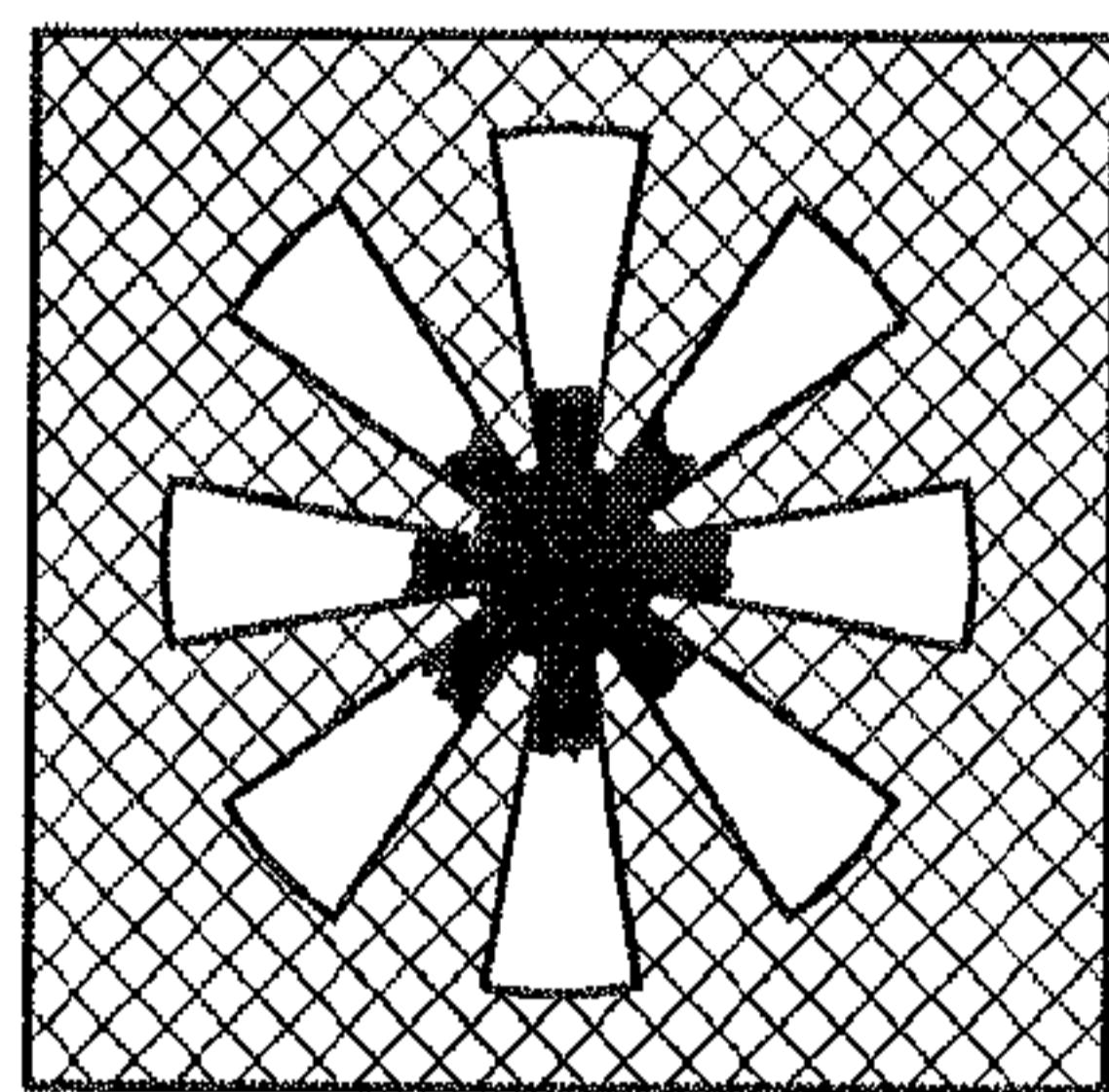
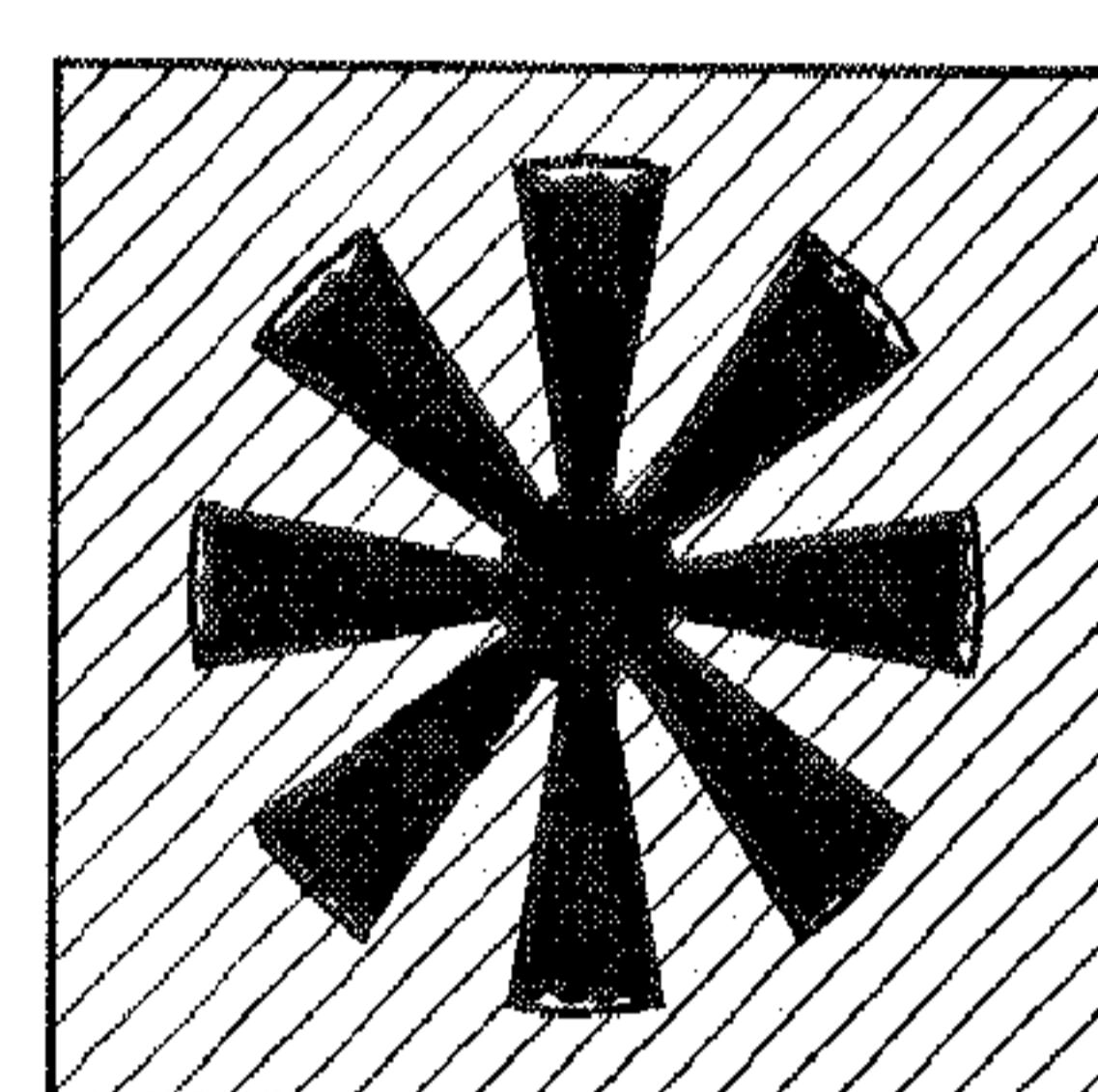
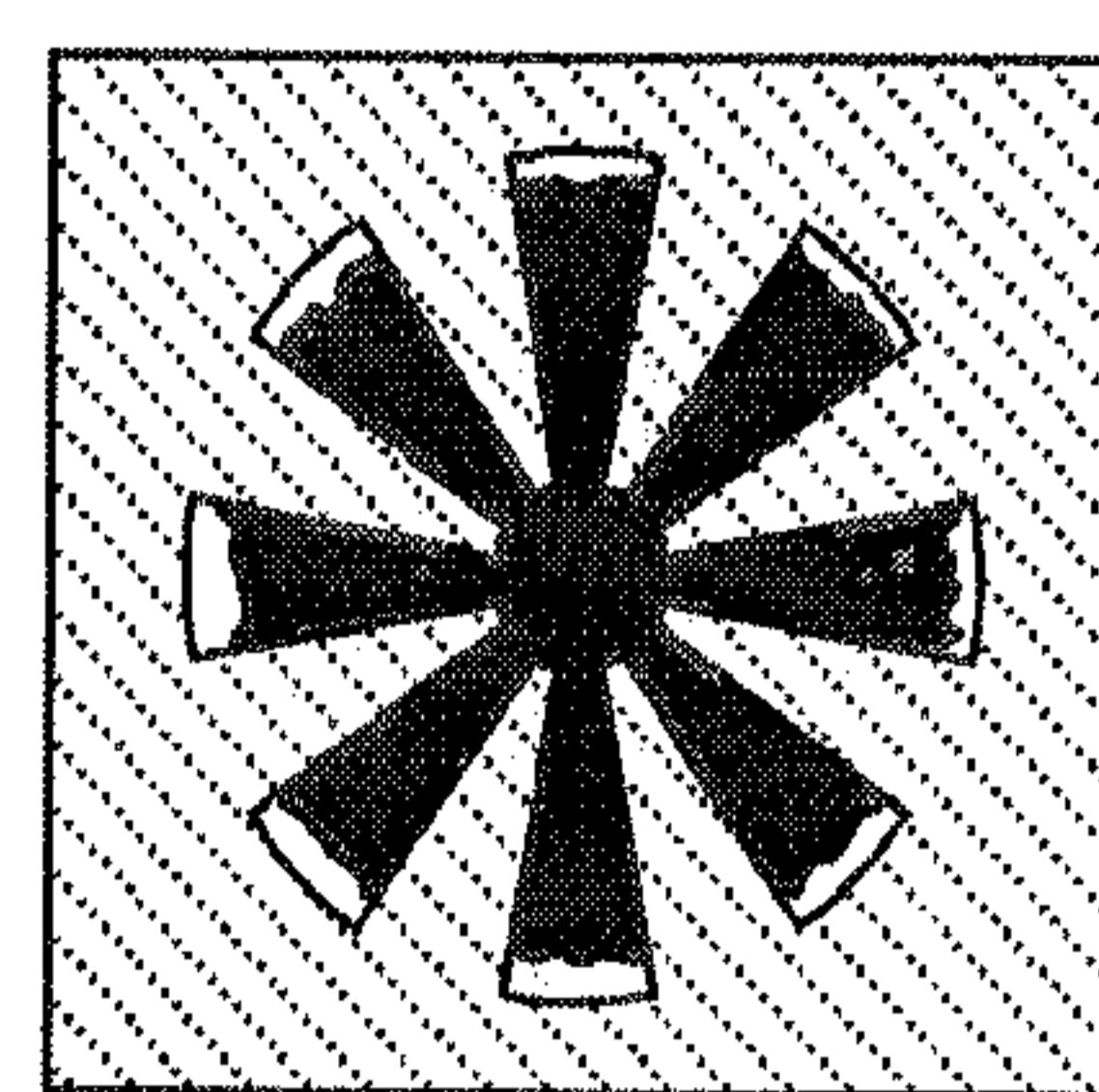
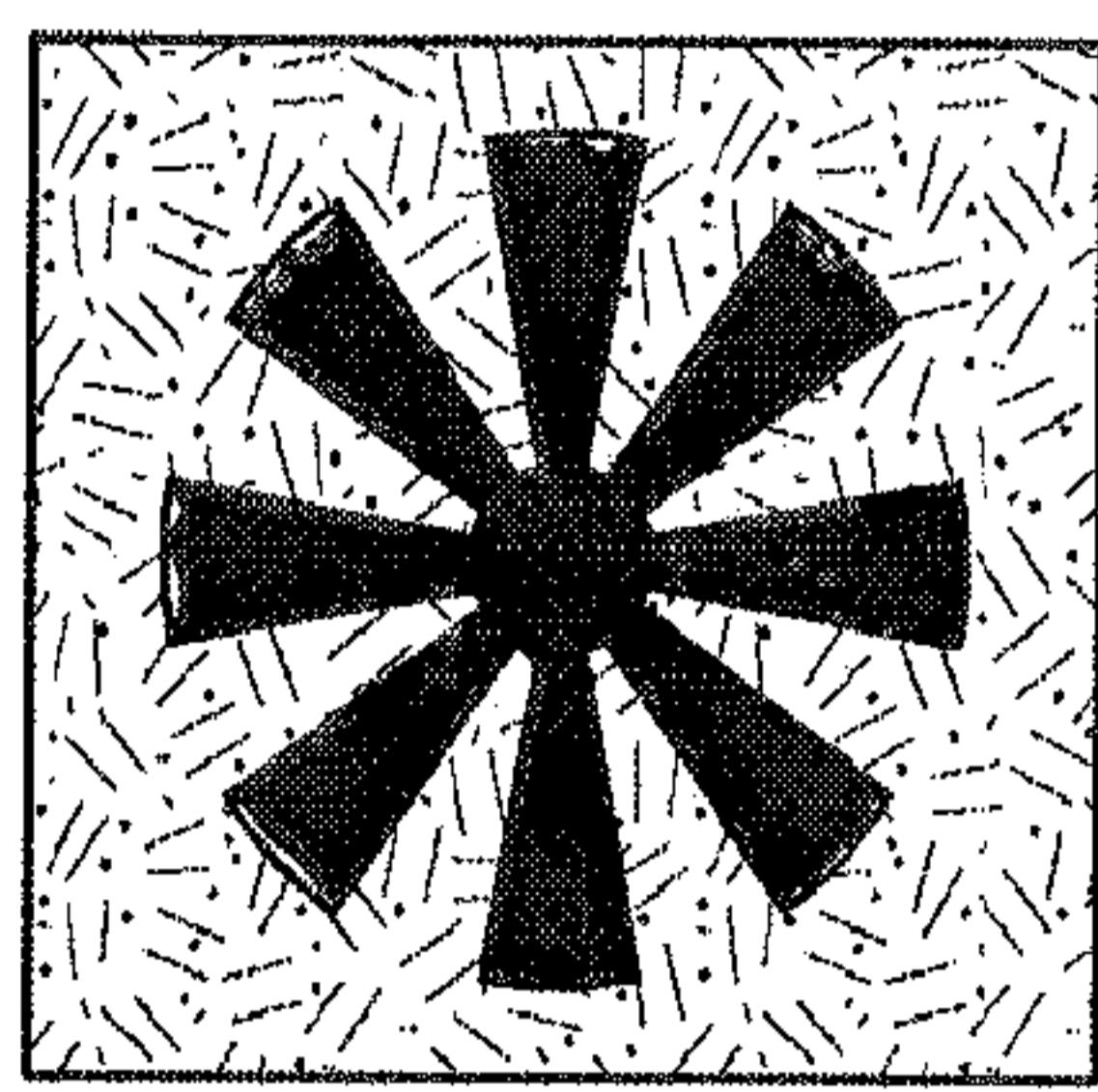
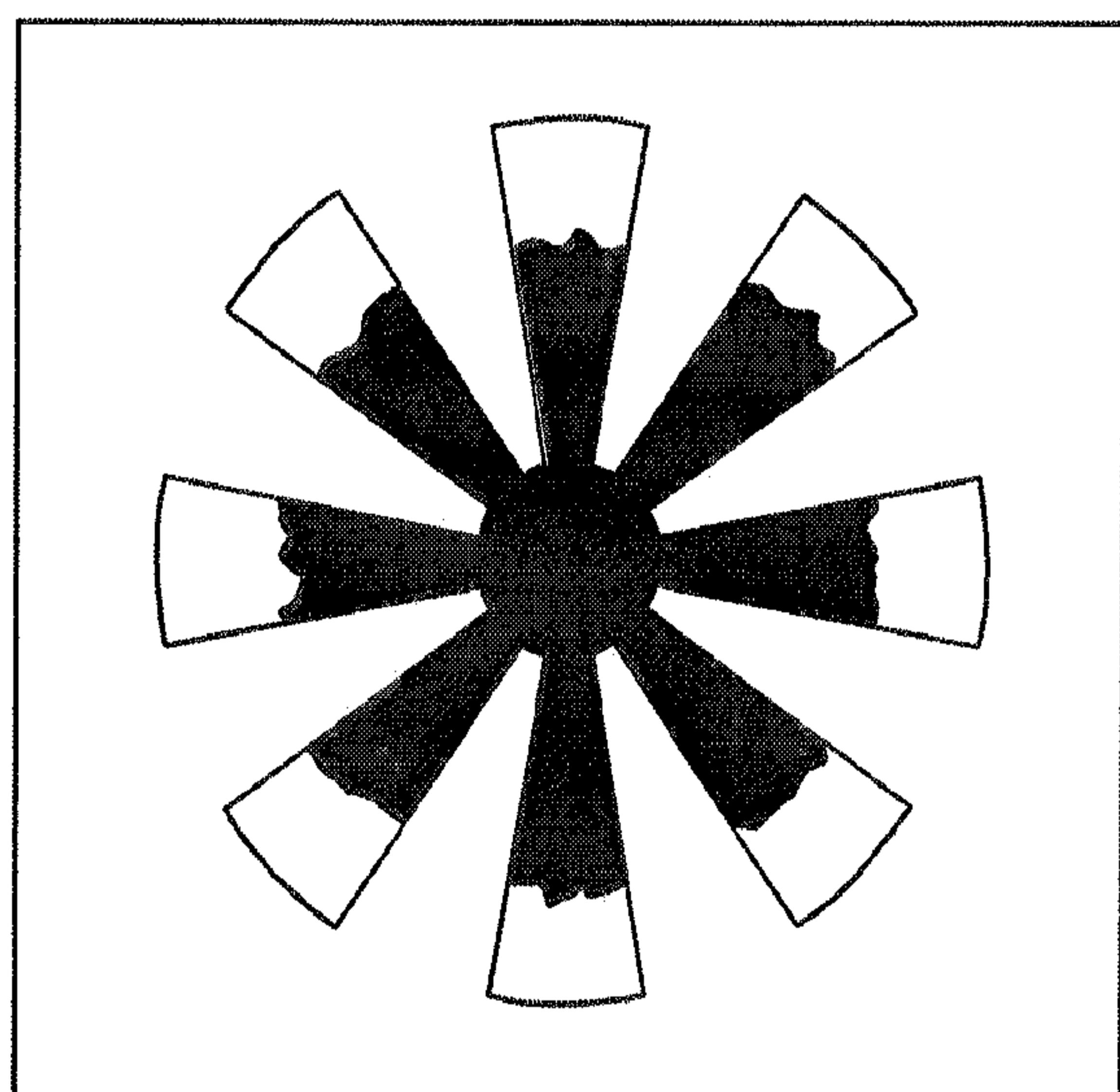


Fig. 3

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Spot pixels selected



Clear pixels selected

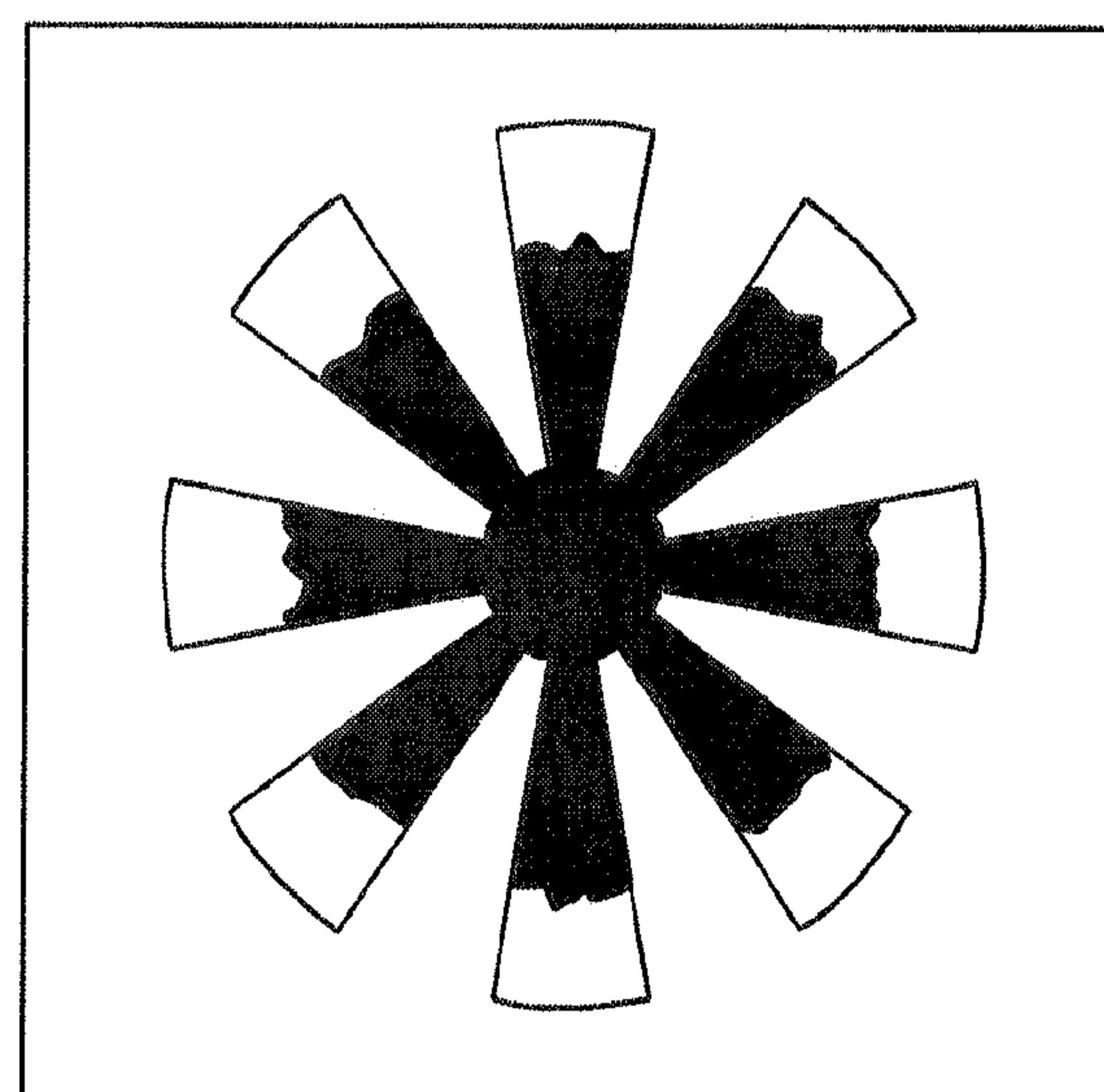


Fig. 4

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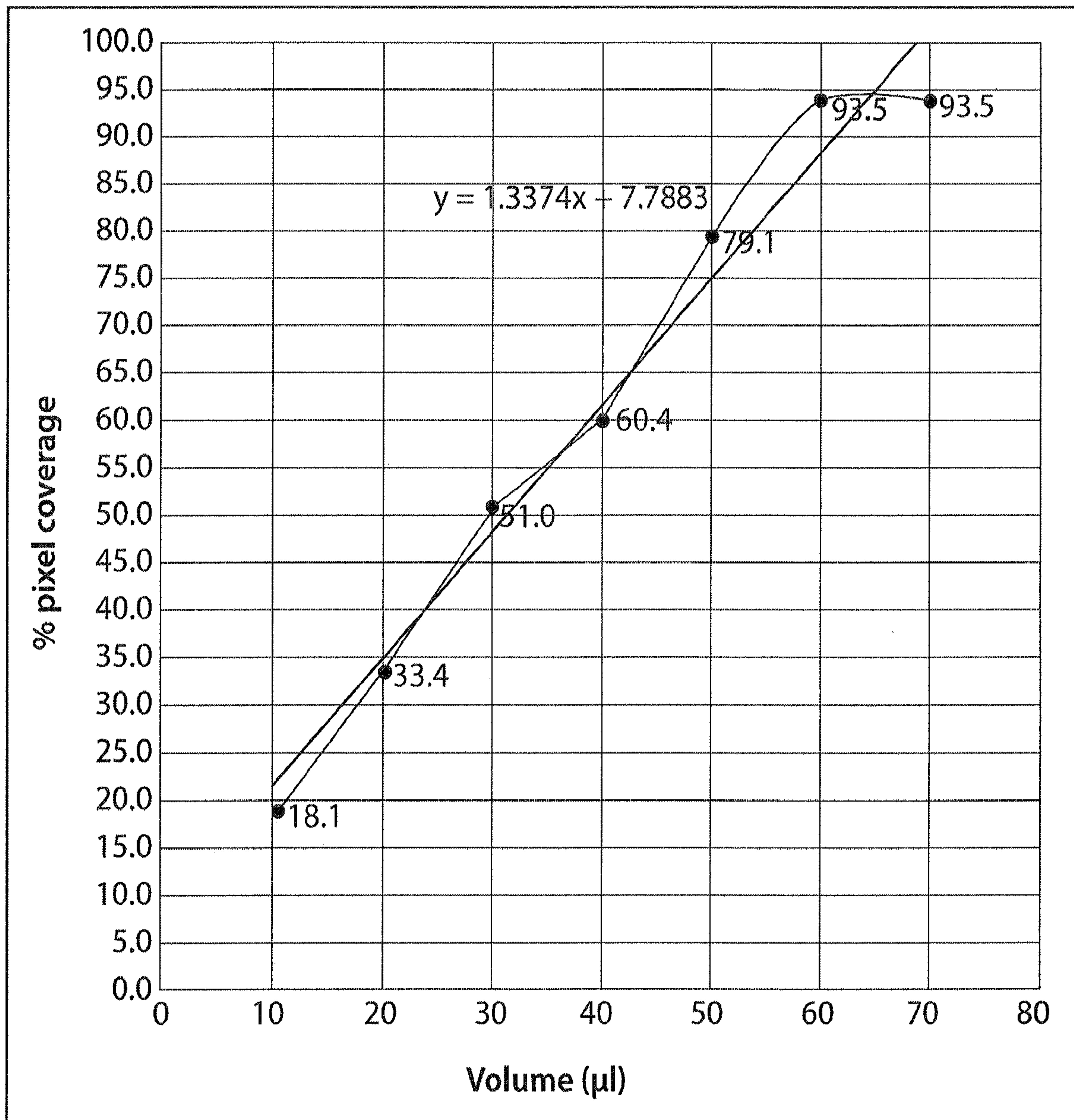


Fig. 5

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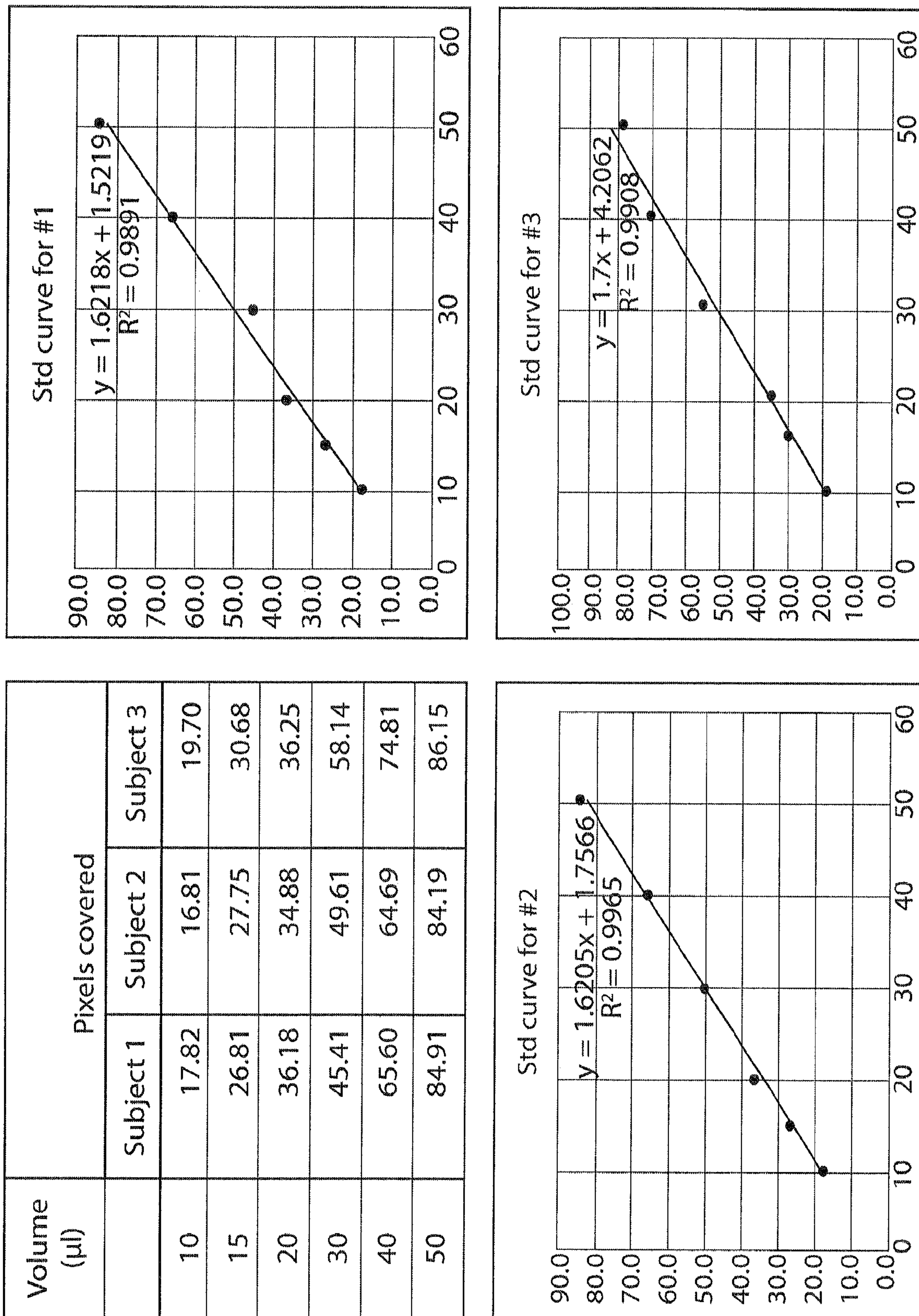


Fig. 6

7/15

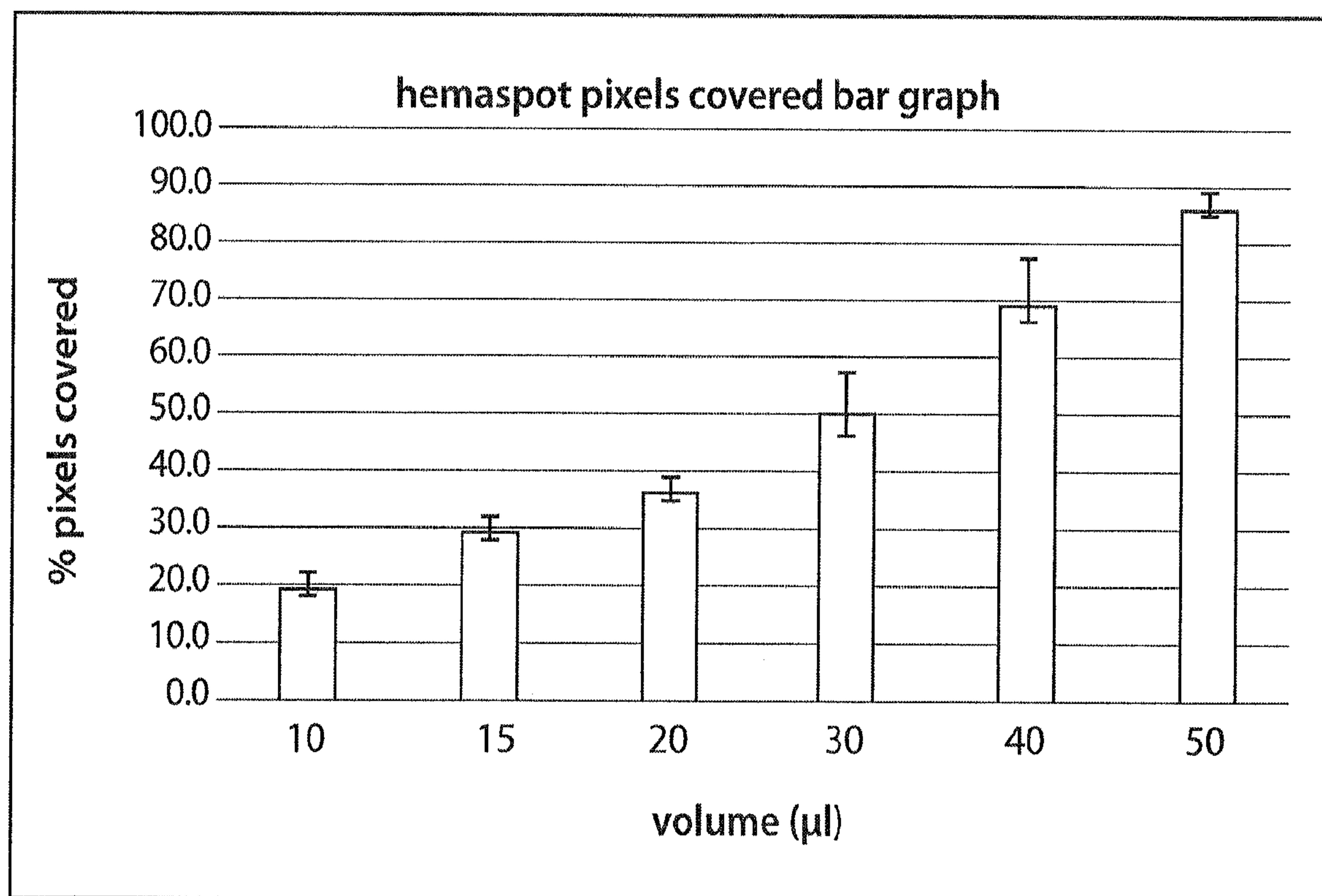


Fig. 7

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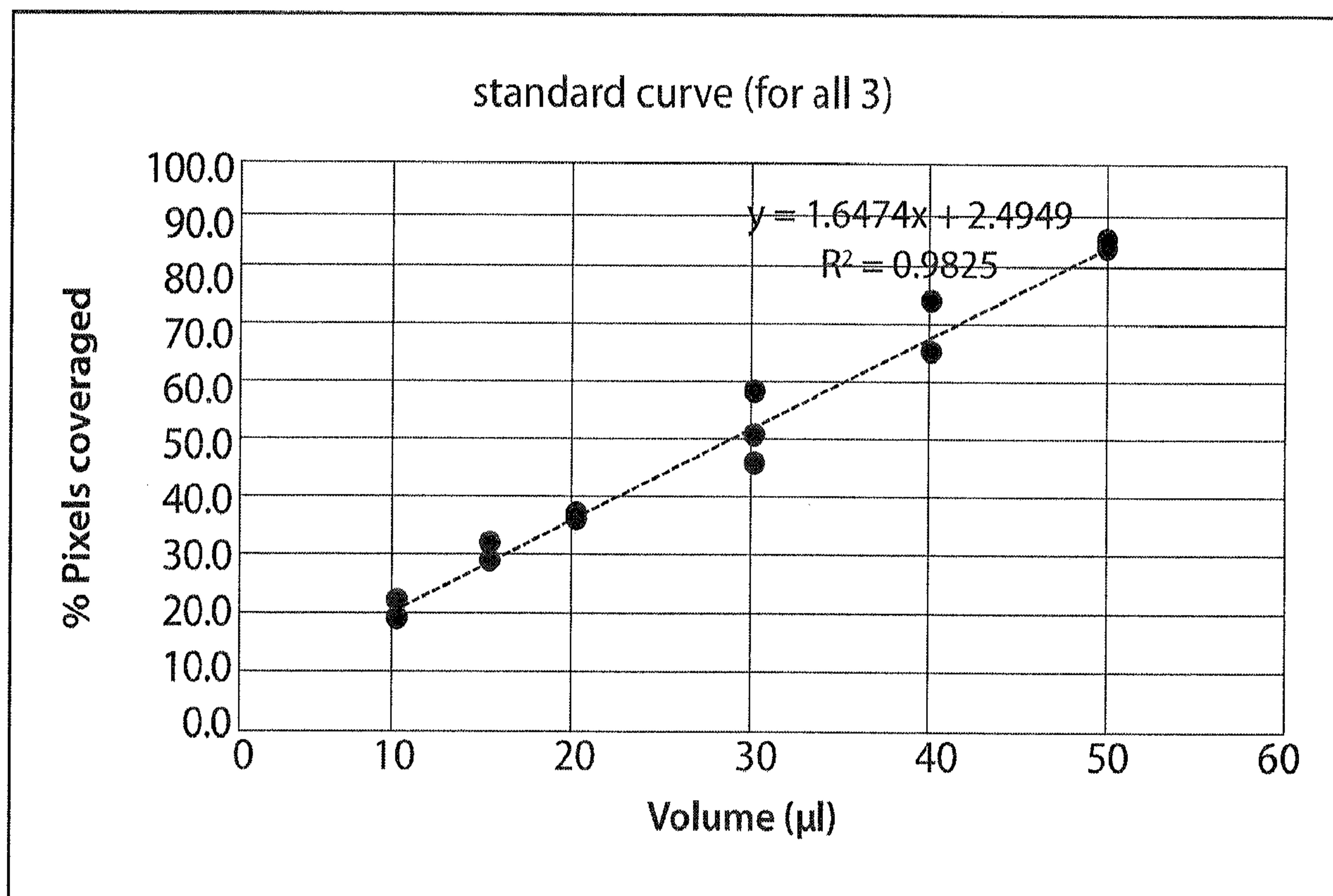


Fig. 8

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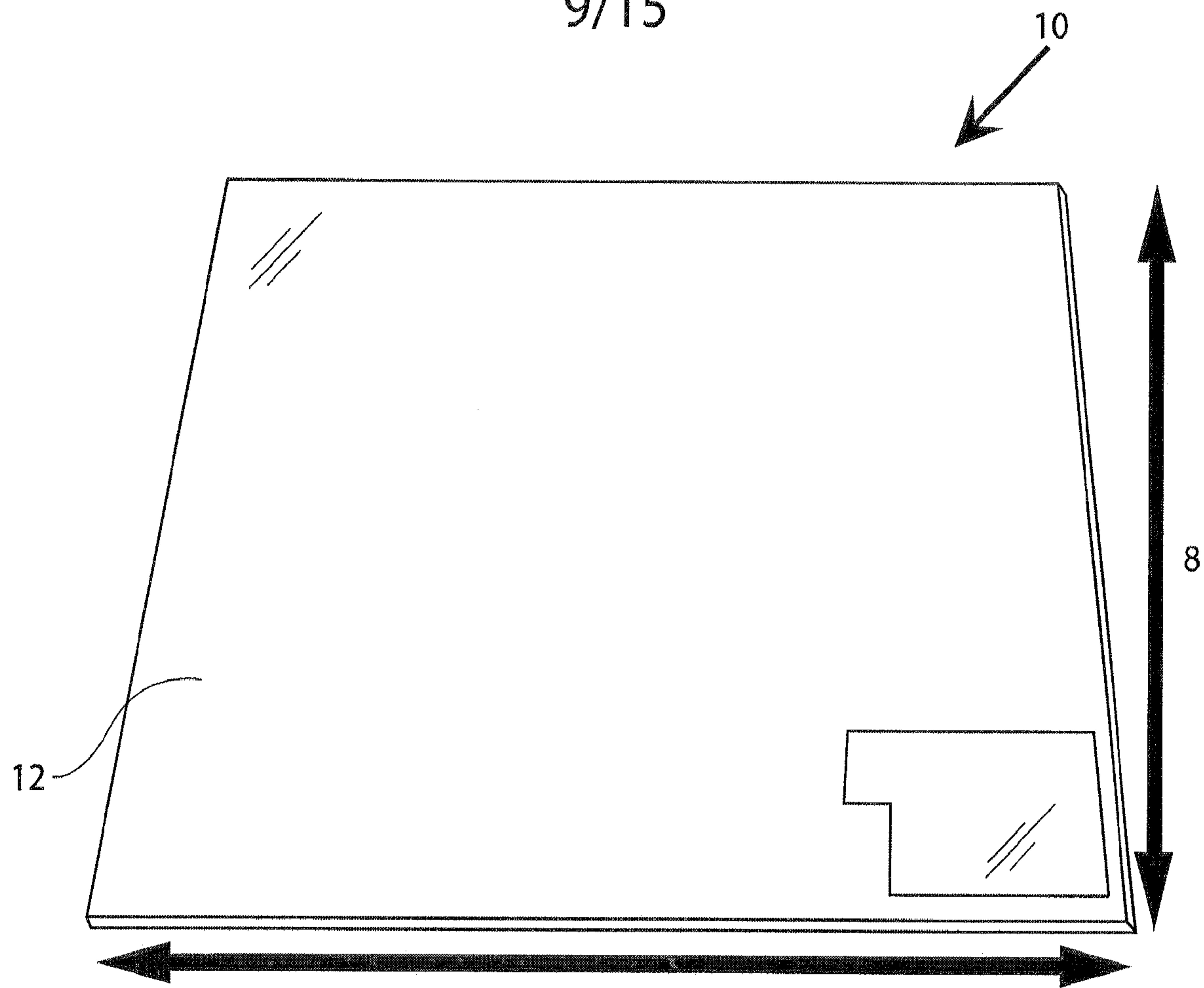


Fig. 9

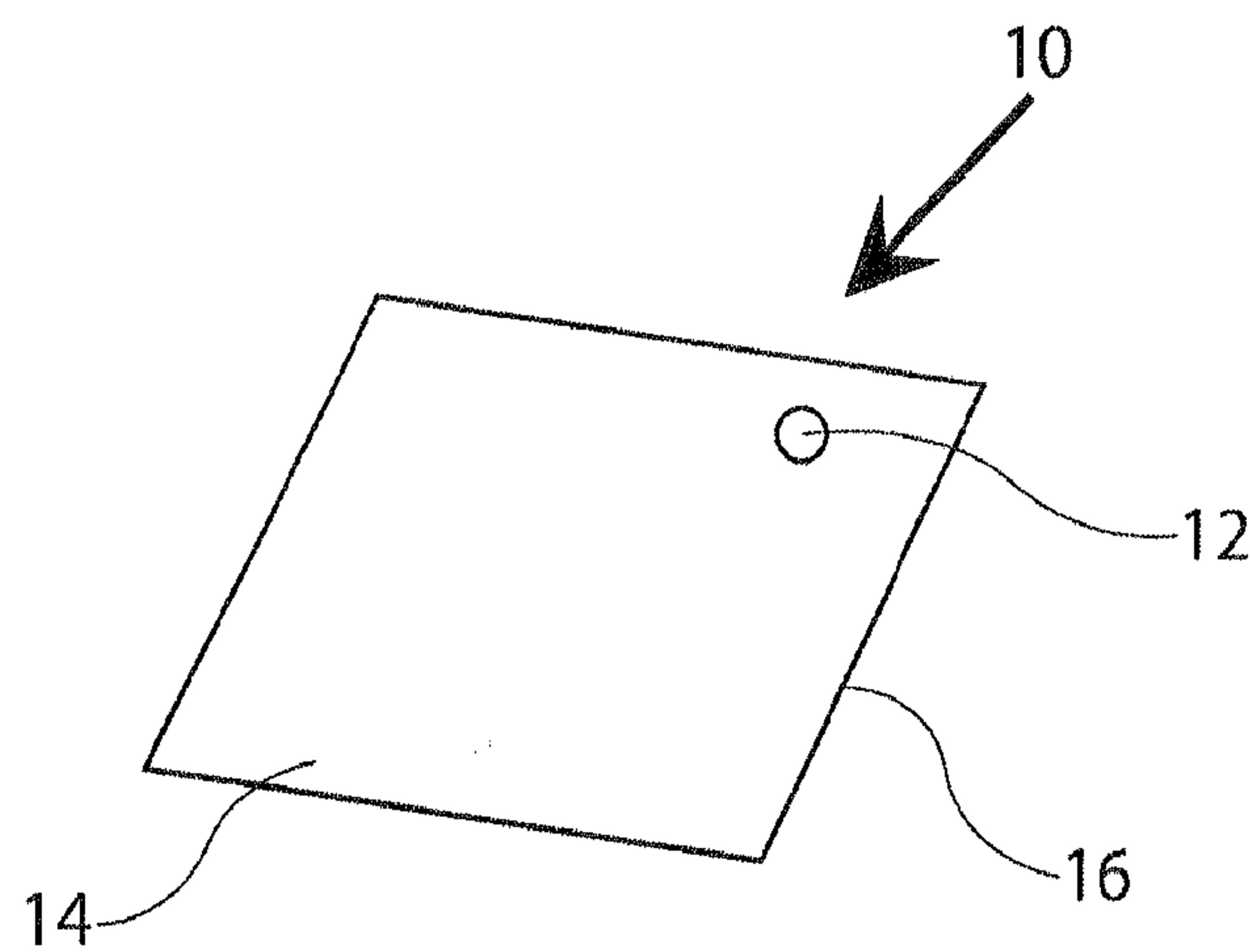


Fig. 10

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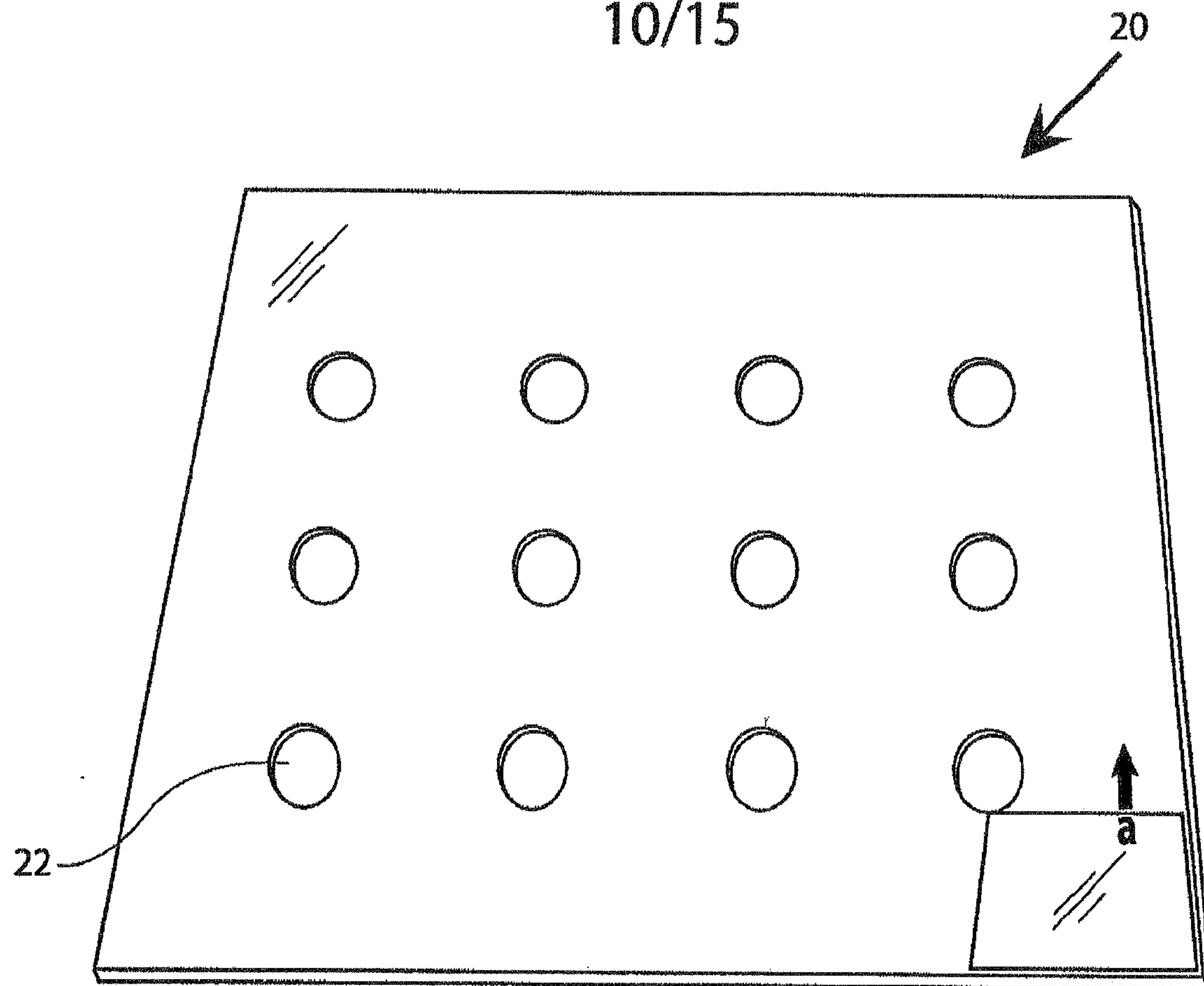


Fig. 11

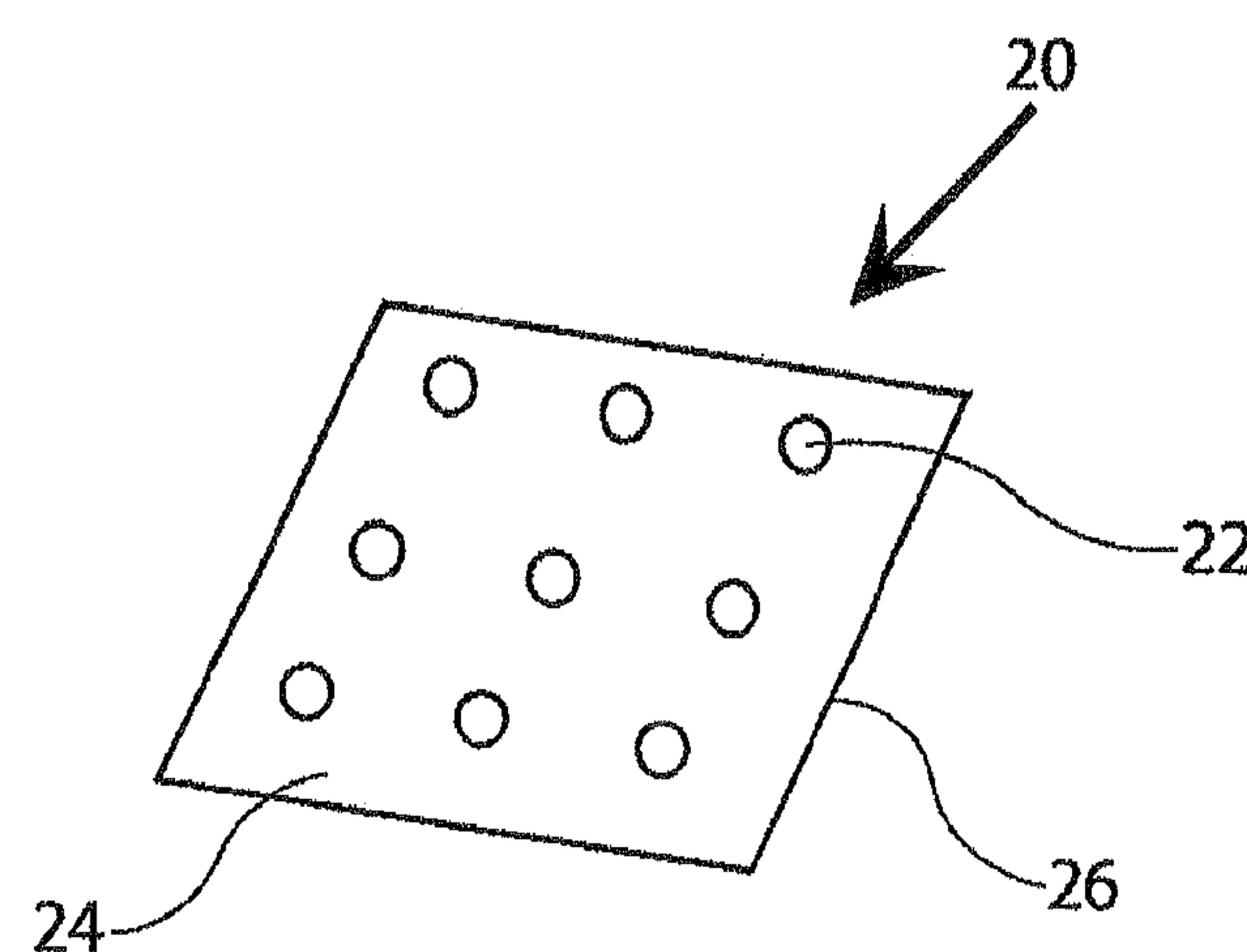


Fig. 12

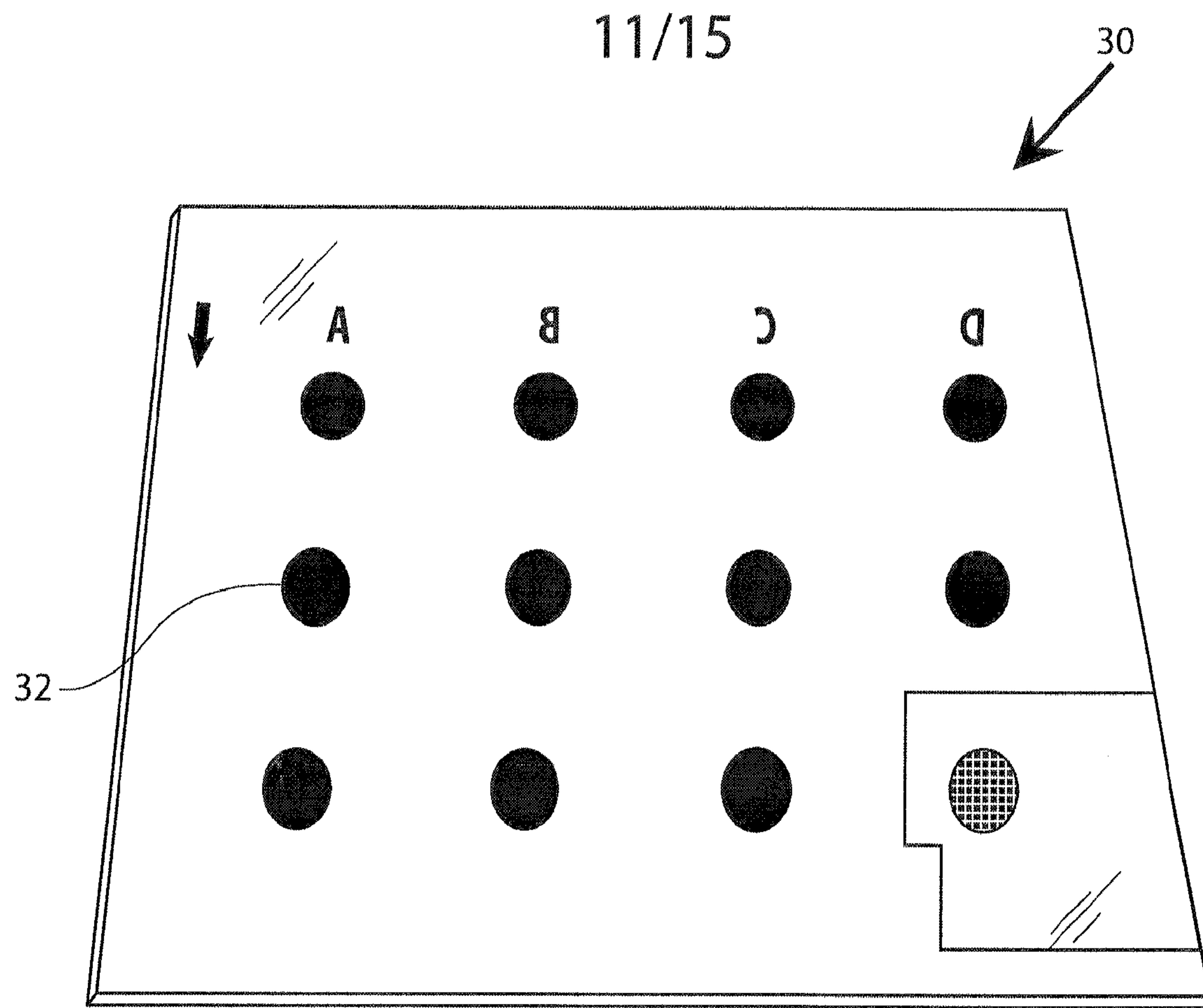


Fig. 13

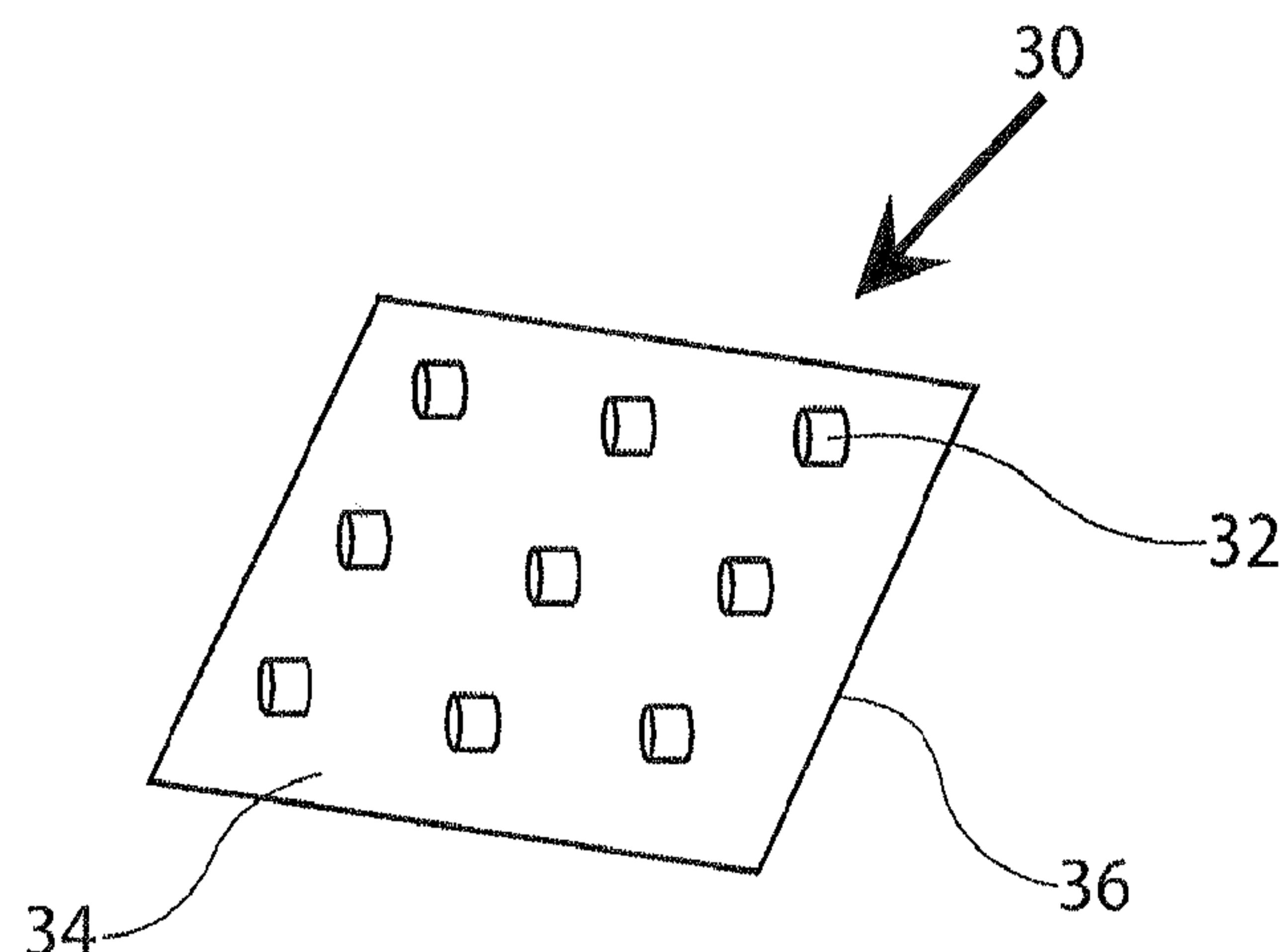


Fig. 14

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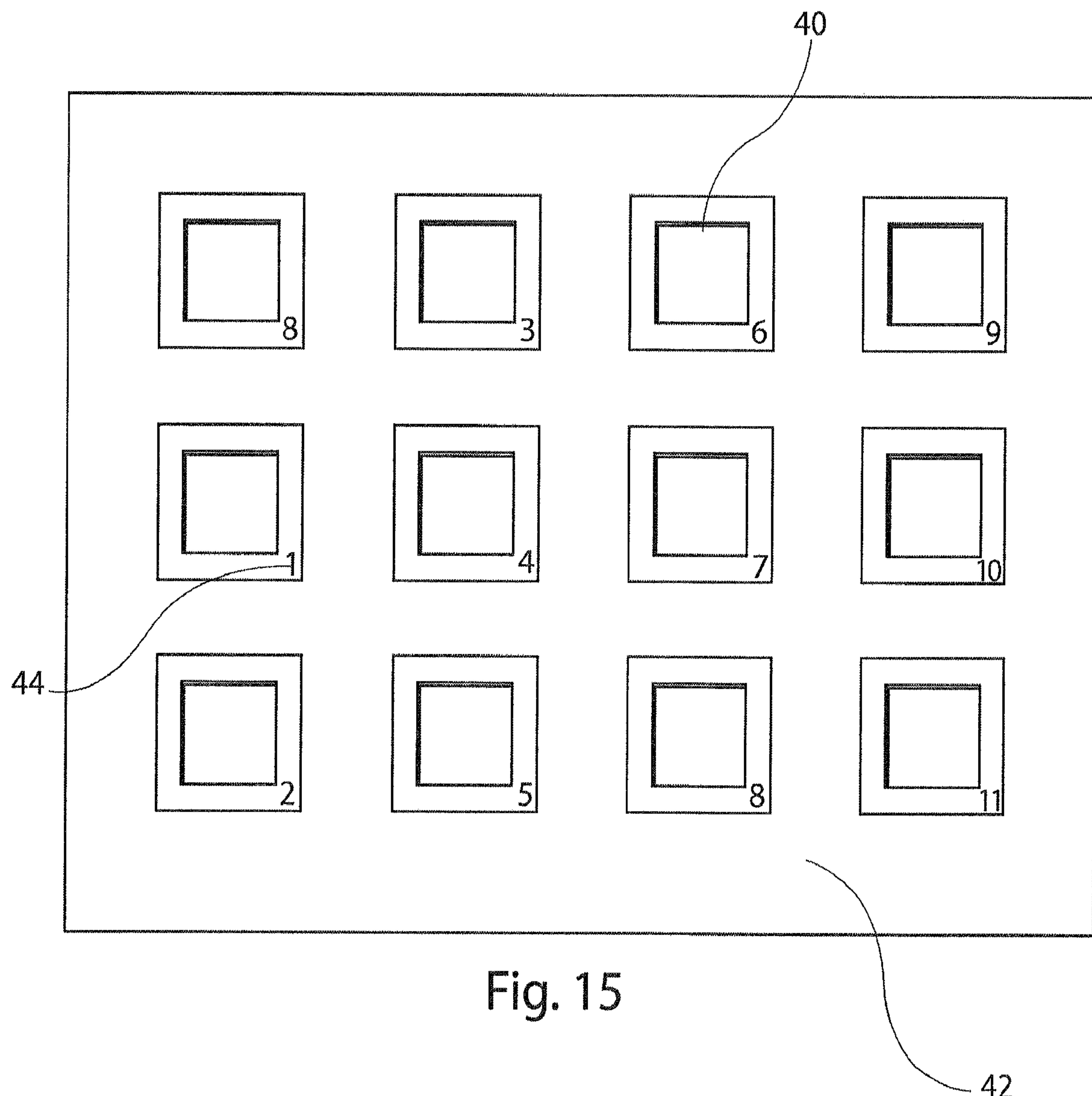


Fig. 15

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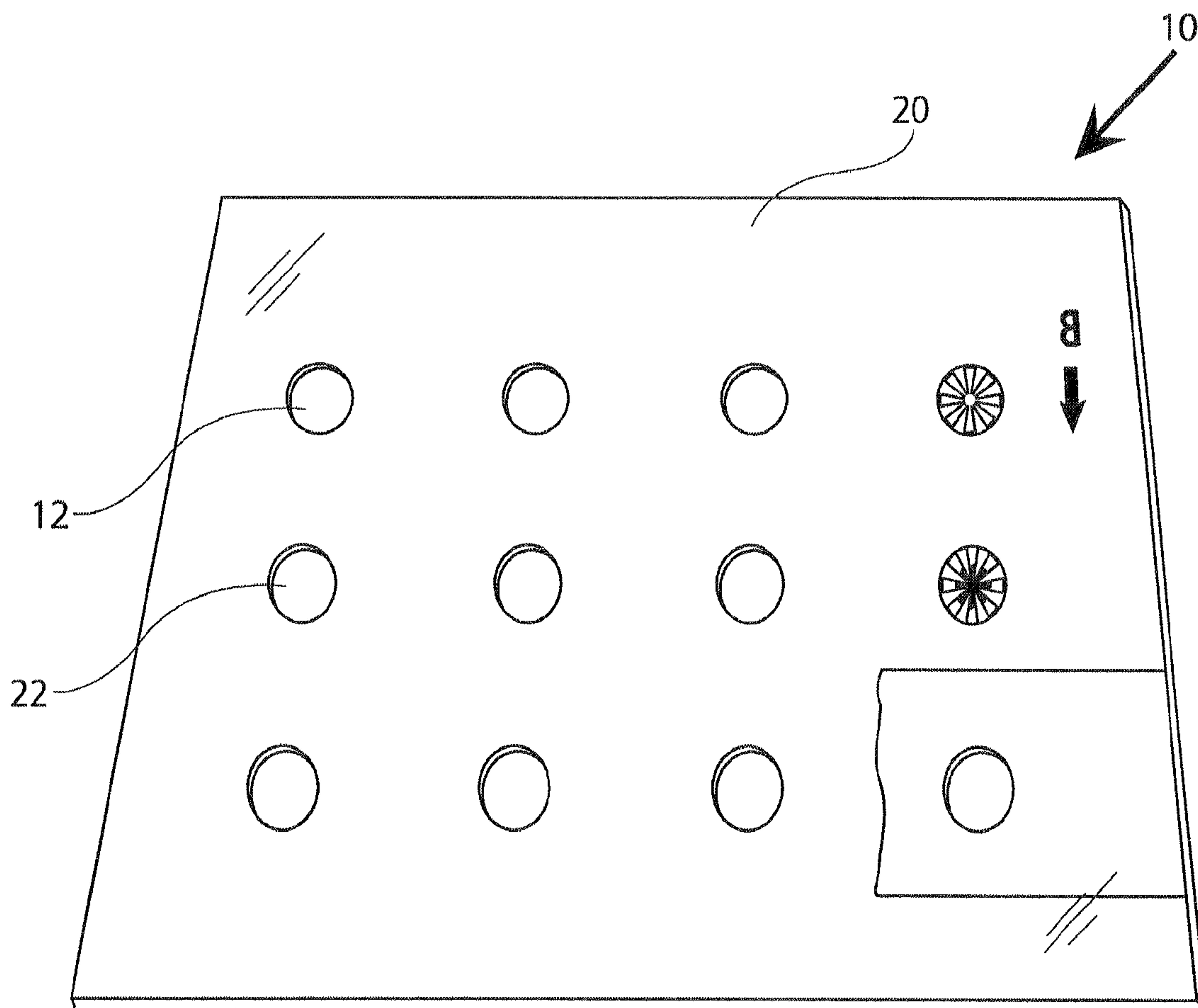


Fig. 16

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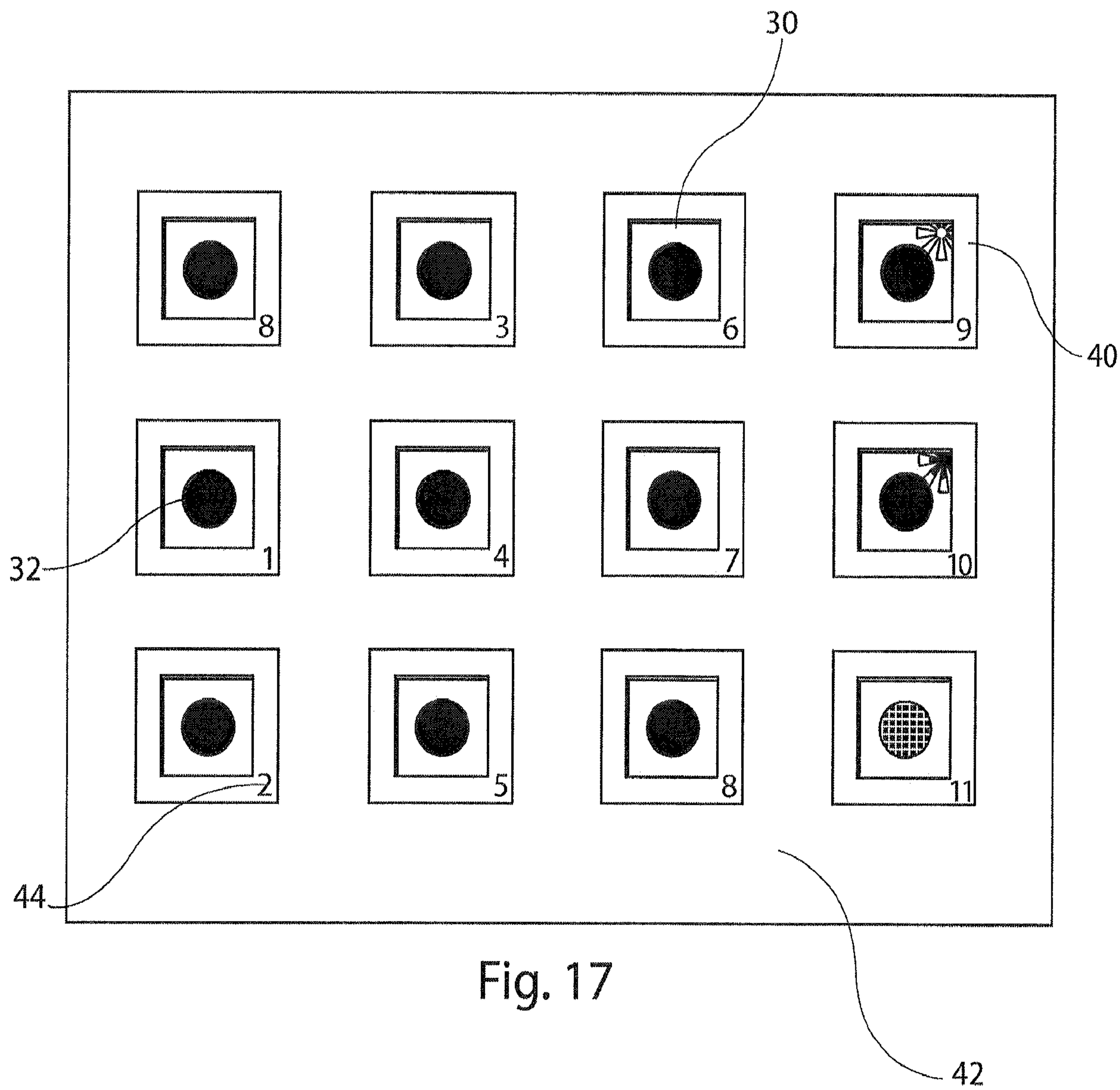


Fig. 17

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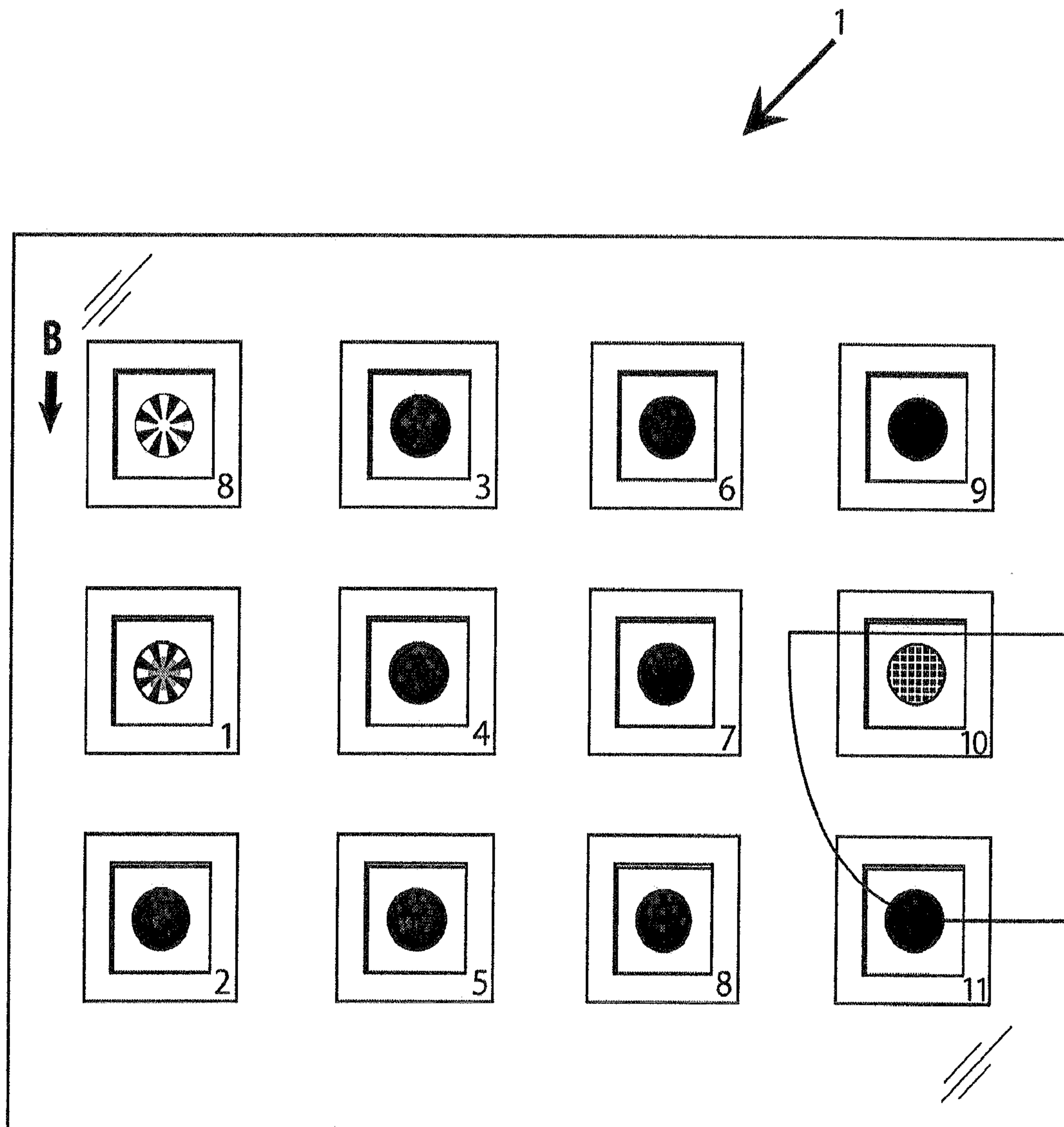


Fig. 18

OBTAINT SAMPLE OF  
BLOOD  
102

100



SPOT BLOOD ON  
SUBSTRATE  
104

OBTAINT IMAGE OF  
BLOOD SAMPLE  
106

DETERMINE COVERAGE  
OF BLOOD SAMPLE  
ON SUBSTRATE  
FROM IMAGE  
108

COMPARE DETERMINED  
COVERAGE TO  
STANDARD CURVE TO  
ESTIMATE VOLUME  
110

Fig. 2