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Cytology microarrays

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Abstract. The use of high throughput genetic and expression platforms are generating many candidate diagnostic markers and therapeutic targets for a wide variety of clinical conditions. Tissue microarrays can be used for the evaluation of the utility of many of these markers. However, tissue microarrays can suffer from the limitations associated with sampling and sectioning tissues. We introduce a novel microarray technique based on cell suspensions. Multiple slides can be made, all of which are equally representative of the initial sample. A robotic device was designed that can deposit 60 distinct spots of cytological material on a glass slide. Each spot of cells deposited in this manner may correspond to a unique source. Controlling the number of cells per spot, their distribution within the spot and the size of the spot can be achieved by modifying the viscosity of the cell solution or regulating the amount of fluid deposited. A fully automated analysis of quantitatively stained microarray samples has been performed to quantify the number of cells per spot, the size of spots and the DNA amount per cell in each spot. The reproducibility of these parameters was found to be high.

Keywords: Cytometry, microarray, high throughput screening, molecular markers

1. Introduction

Over the past decade and a half a number of high throughput array techniques have revolutionized the study of biological samples from gene expression [4] to glycan binding [2]. DNA microarrays, cell microarrays, and tissue microarrays have allowed for cheaper and faster analysis crucial to the genomic era [15]. The tissue microarray, invented by Kononen et al. in 1998 [7], is one of the most useful of these high throughput techniques. It has applications in both research and clinical settings, for in situ gene expression and immunohistochemical and FISH analysis [11].

Constructed by placing an array of paraffin embedded tissue cores into a recipient block, tissue microarrays (TMA) can hold up to several hundred tissue samples per slide [15]. The TMA does have drawbacks, as it is labour intensive and requires considerable skill to produce error-free recipient blocks. Commercial TMA building instruments are expensive [10] and the technique is not applicable to cell suspension samples, whether for research (cell lines), or pathological diagnosis (sputum, blood, cervical, etc.)

We have developed a novel microarray technique, the cytology microarray (CMA), where cell suspensions are deposited on a glass slide in an array pattern. The process illustrated below is quick, reproducible, and applicable to a wide range of cell suspension materials.

2. Materials and methods

2.1. Material

The twenty nine lung and cervix tumour cell lines used in these experiments were purchased from ATCC[1] (Manassas, VA, USA), and three normal cell lines were purchased from Cambrex Bio Science (Walkersville, MD, USA). The cells were grown in conventional conditions as recommended by ATCC and Cambrex Bio Science until harvesting. MEM and RPMI cell growth media supplemented with 10% foetal bovine serum were used. Following several passages, the monolayer of cells were mechanically harvested and suspended in cell medium. After centrifugation, the medium was removed and the cell pellet was fixed with 70% Ethanol.

The clinical specimens used in preparation of CMA were Bronchial Alveolar Lavage (BAL) specimens

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[1] American Type Culture Collection.

	1	2	3	4	5	6	7	8	9	10	11	12
A	HTB-55	HTB-57	HTB-58	HTB-59	HTB-119	HTB-168	HTB-171	HTB-174	HTB-175	HTB-177	HTB-178	HTB-182
B	HTB-55	HTB-57	HTB-58	HTB-59	HTB-119	HTB-168	HTB-171	HTB-174	HTB-175	HTB-177	HTB-178	HTB-182
C	CRL-1848	CRL-2170	CRL-5800	CRL-5804	CRL-5807	CRL-5809	CRL-5810	CRL-5826	*SAEC*	CRL-5928	CRL-5920	*NHBE*
D	CRL-1848	CRL-2170	CRL-5800	CRL-5804	CRL-5807	CRL-5809	CRL-5810	CRL-5826	*SAEC*	CRL-5928	CRL-5920	*NHBE*
E	HTB-33	HTB-34	HTB-35	CRL-1550	CRL-1594	CRL-7850	CRL-7920	HTB-33	HTB-34	HTB-34	HTB-35	*CHEC-EC*

Fig. 1. The arrangement of 32 cell lines deposited onto a glass slide: The types in Italics have been purchased from Cambrex, whereas the rest have been purchased from ATCC.

and liquid based cervical cytology specimens. BAL specimens were collected during bronchoscopy. After centrifugation the cells were fixed with SedFix fixative from Surgipath (www.surgipath.com/region/ca). Cervical specimens were collected with a cervical brush into the PreservCyt solution from CYTYC Corp. (Marlborough, MA 01752, USA) and after centrifugation the cell pellet was fixed with 70% Ethanol. The cell pellets were washed several times with 70% Ethanol before resuspension and deposition onto the slide in an array pattern.

2.2. *Cytology microarray preparation*

Microarray slides were prepared using thirty two different human cell lines in an arrangement shown in Fig. 1. A fully automated robotic spotter (Fig. 2a) developed by Cancer Imaging Department (BC CRC, Vancouver, Canada) was used to prepare cytology microarray slides by depositing a drop of cell suspension onto a microscope glass slide. To improve cell adhesion, the positive charge of the slides (Histobond slides, Raymond A. Lamb Ltd) was enhanced by briefly rubbing them against plastic material. The head of the robotic machine contains 60 micropipettes arranged in an array of 5 × 12. The centre to centre distance between the tips of the micropipettes is 4.5 mm. 15 μl of the cell suspension were placed in 96 well trays that matched the dimensions of the micropipettes array. The micropipettes were dipped into the sample wells 4 times for 1–2 seconds; this not only loaded the micropipettes with the cell suspensions by capillary

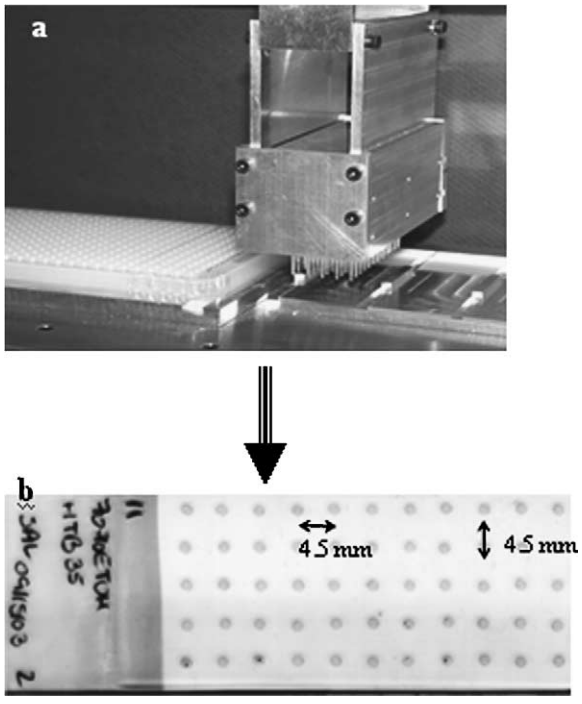

Fig. 2. (a) The automated cytology microarray depositing device. (b) The scan of a stamped and stained glass slide after preparation.

action, but ensured that the cell suspensions remained evenly mixed between deposition runs.

The robotic head positioned itself above a glass slide and then deposits small amount of cell suspension by briefly touching the glass slide (Fig. 2b). The robotic head could then proceed to the next glass slide and deposit a similar small amount of the cell suspension or

return to the multiwell plate to refill.

To illustrate the representativeness and equivalence of cytology microarrays, we prepared regular smears and cytospins from existing cell line suspensions. The smears were made by dropping a few drops of cell suspension between two glass slides and then pulling the two slides apart. The cytospin slides were prepared by placing 2–3 drops of cell suspension into a funnel and spun using a Cytospin 3 Cytocentifuge (Shandon Scientific Limited, Cheshire, England).

Slides from the different preparation methods were stained with a modified Feulgen–Thionin quantitative nuclear stain frequently recommended for use in automated image cytometry. These slides were post fixed with mixture of methanol, formaldehyde and acetic acid (85:10:5 by volume) for forty five minutes, treated with acid hydrolysis (5 N HCl) at 26°C for one hour and stained using a Feulgen–Thionin stain for one hour [6,14].

Image acquisition was performed using the Oncometric Cyto-Savant automated quantitative image cytometer (Department of Cancer Imaging, British Columbia, Cancer Agency, Vancouver, Canada) [11, 13]. Slides were exhaustively scanned and the images of each nuclei from all of the 60 spots were stored in the computer memory. Approximately 110 features were calculated for each nuclear image by the instrument. These features describe the amount and distribution of stained DNA in the nuclei as well as their location on the slide. Values of the following feature measurements were calculated for each spot:

- The total number of cells
- The total mean DNA amount per nuclei
- Mean size per nuclei per spot.

3. Results

From a qualitative observation of the spotted slides we found that the best results were obtained if the micropipettes were refilled after every deposition. A high consistency in size and shape of the spots was achieved (Fig. 2b) as long as external conditions (temperature or humidity) were not dramatically altered. Particular care was necessary with certain clinical samples, as some tended to clump (cervical) blocking the micropipette, or the presence of other material (mucus) in the samples impeded binding to the slide. Each stamped slide was found to be comparable and every spot was found to be a very good representation of the original sample. It was found that the extent of contact

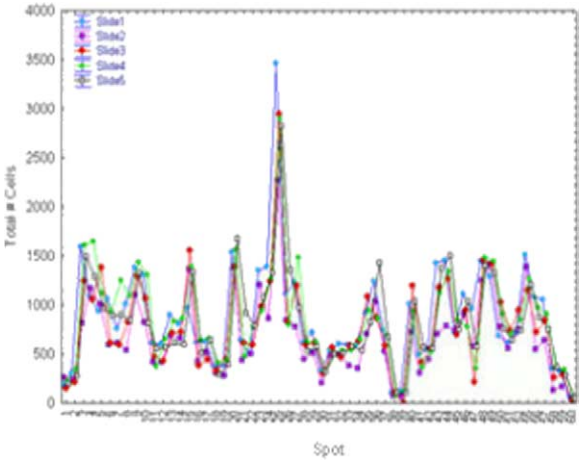

Fig. 3. Five different slides showing the total number of cells per dot per slide.

time between the micropipette tip and the glass slide, as well as the concentration of specimen inside the pipettes determined the volume of sample deposited. Therefore keeping the touch time uniform was important in producing an acceptable degree of reproducibility.

The quantitative analysis of the Feulgen–Thionin stained slides demonstrates that the total cell number per spot was consistent over each of the five selected slides. Since the concentration of sample was kept the same throughout the experiment, we can conclude that the volume of sample deposited per spot was also consistent throughout each depositing event accounting for uniform cell count results (Fig. 3).

Using the integrated optical density of a nucleus [4,5] the DNA amount of each cell was measured, and from that the variation and mean DNA value per cell was calculated per spot; shown in Fig. 5. The nuclear area of cells (the number of connected pixels forming an object which has the spectral and shape properties of nuclear material) was determined in each spot, and from that the mean value and variation of nuclear size over each spot is demonstrated in Fig. 4.

Observation of cell count (Fig. 3), nuclear size (Fig. 4) and mean DNA amount (Fig. 5), show the extent of overlap between the results obtained from the 5 different slides and the significant repeatability of morphological densitiometric features using CMA. The variability present in groups 39, 53 and 54 for slides 2 and 5 is a result of too few cells present in the deposited volume; the large error bars, therefore, are caused by a small sample size. Also, the variations between 60 spots are mainly due to different sample material.

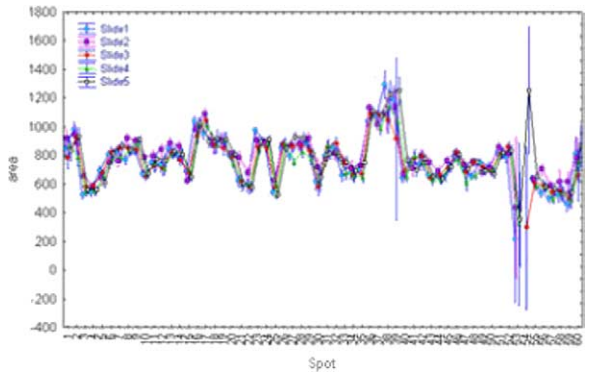

Fig. 4. Five different slides showing the variation of mean nuclear size per dot per slide.

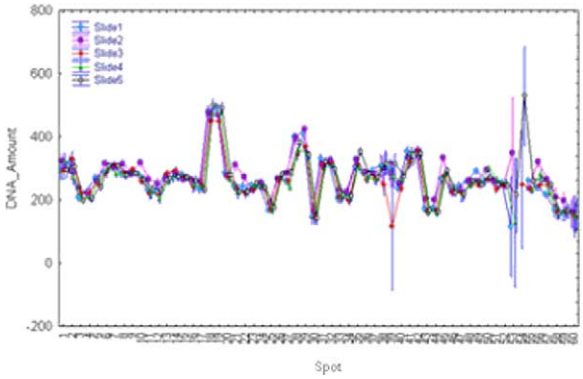

Fig. 5. Five different slides showing the variation of mean DNA amount per dot per slide.

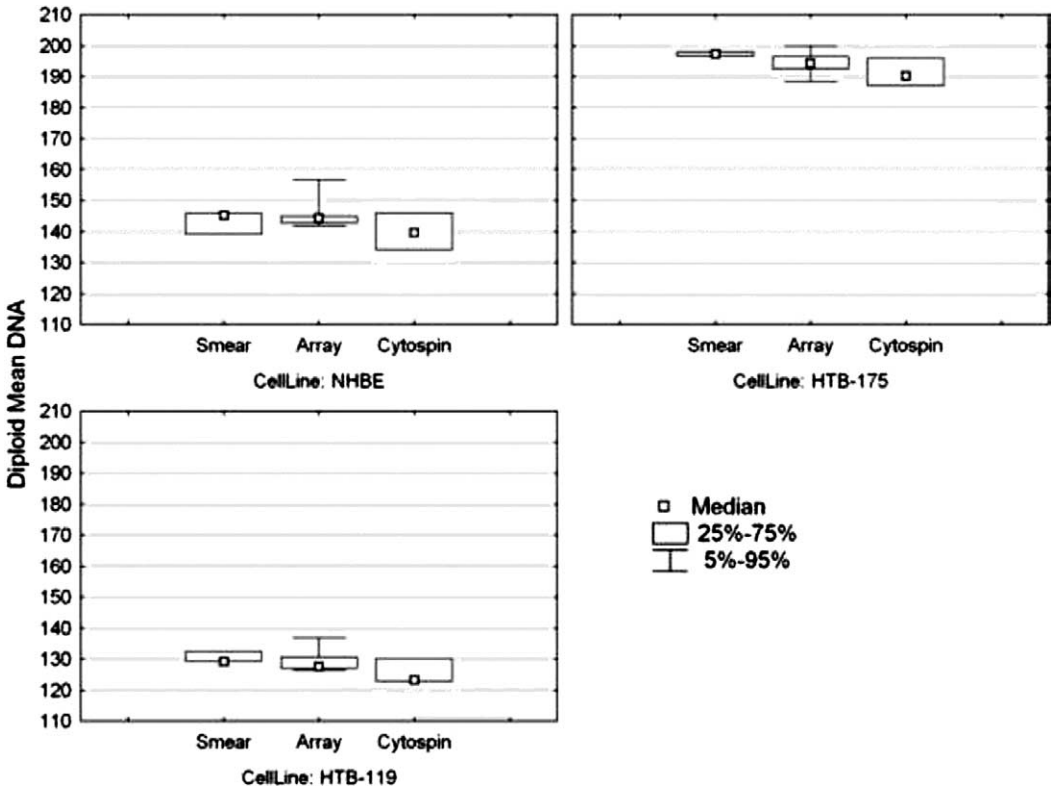

Fig. 6. Three different cell lines showing diploid mean DNA amount for three different preparation methods (smear, array and cytospin). Note the variation between samples is much larger than the variation within or between sample depostions.

No systematic variance between CMA, cytospins and smears was observed in a quantitative analysis of these samples. The Mean DNA amount of diploid cells in all three techniques shows no statistical differences and we can conclude that all three techniques are equally representative of the original sample in this regard (Fig. 6). This is further substantiated from our observations of the coefficient of variation (CV) of the tetraploid group which shows the variability between all three techniques is less than that between the sample types (Fig. 7). The percentage of S-phase cells in the cytospins, smears and CMA are equivalent (Fig. 8). With respect to these measures the sample deposition methods are equivalent, except that the number of cells in smears and cytospins is much larger than the number of cells in the CMA.

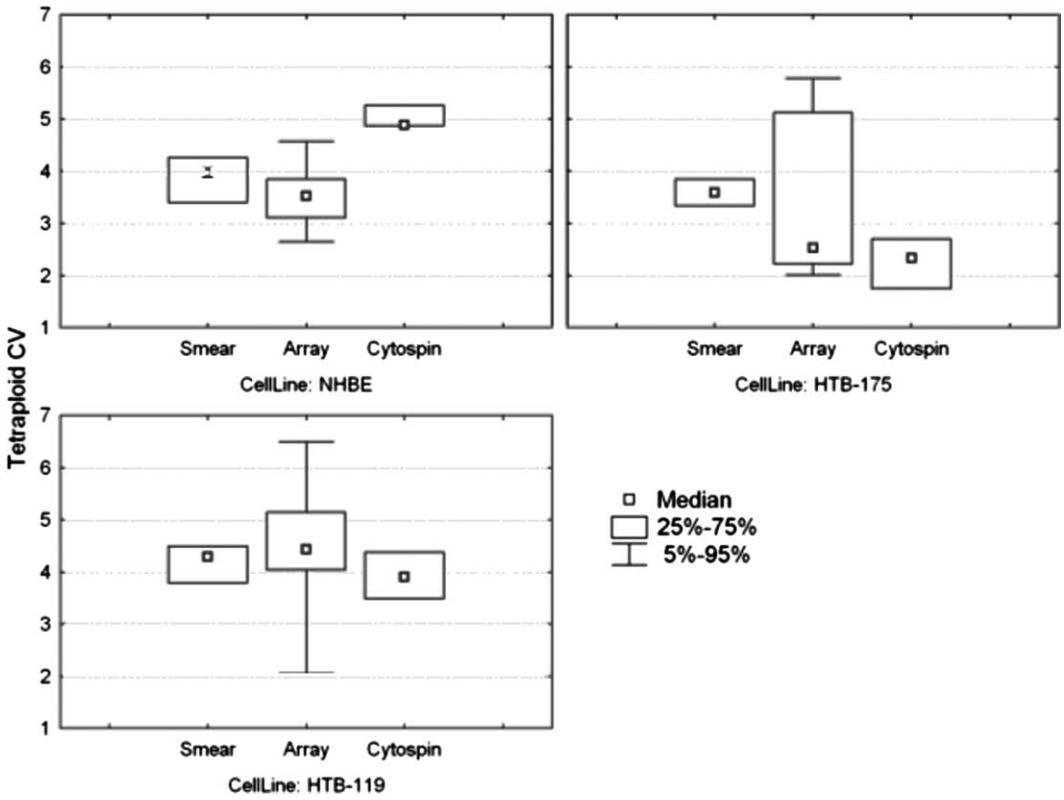

Fig. 7. Three different cell lines showing tetraploid Coefficient Variation (CV) for three different preparation methods (smear, array and cytospin).

To ensure that there was no cross-contamination between the samples an array of alternating empty and full wells were run and then stained with the Feulgen–Thionin stain as described in methods. The resulting slide was then scanned using the automated image cytometer, and the results are shown in Fig. 9. The coloured pixels in this figure correspond to individual cells as captured by the automated scanning system. Using the computer scan of our microscope slides, we were able to show that cells were confined almost exclusively within a definite spot boundary, due to the strong cohesion forces between the cells and the slide upon printing. A photographic example of a typical spot is presented in Fig. 10. It was quantitatively measured that an uncontained cell can get no further than about 0.45 mm from the outer boundary of a spot, when the diameter of spots is roughly about one millimetre. No cells were found in the blank spots that were initially spotted using a solution of 70% ethanol confirming that there was no cross contamination between the spots.

An example of a CMA slide with clinical samples stained with the Papanicolau staining method is presented in (Fig. 11).

MIB-1 immunostaining on cytological specimens is not commonly used, although it would be very valuable for the assessment of tumour proliferation before treatment [3,12]. To evaluate the use of CMA with immunohistochemical techniques we stained all cell line CMAs for antigens MIB-1 using a DakoCytomation Ki-67 Monoclonal Mouse, Clone MIB-1 according to laboratory standards. As expected, across all cell lines we found dot like nuclear, and some diffuse cytosolic, staining. A strong signal was detected in all proliferating cell lines. Positive stained cells, as well as, total number of cells per spot were counted. The results of MIB immunostaining were expressed as a percentage of stained cells in relation to the total number of cells within each spot. Similarities in the percentage of positive cells within the same cell line can be noted (Fig. 12).

4. Discussion

Microarray techniques: TMA, CMA and DNA microarrays have rapidly become an indispensable tool for researchers and clinicians. The cytology microarray outlined above is an important addition to these ar-

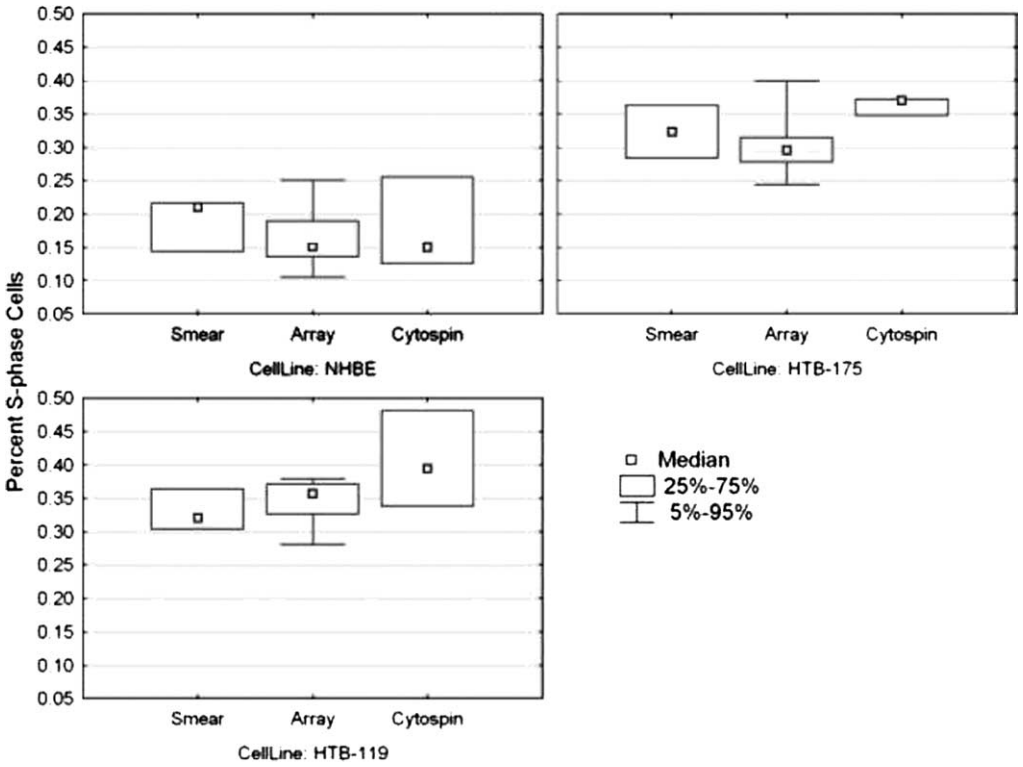

Fig. 8. Three different cell lines showing percentage of S-phase cells for three different preparation methods (smear, array and cytospin).

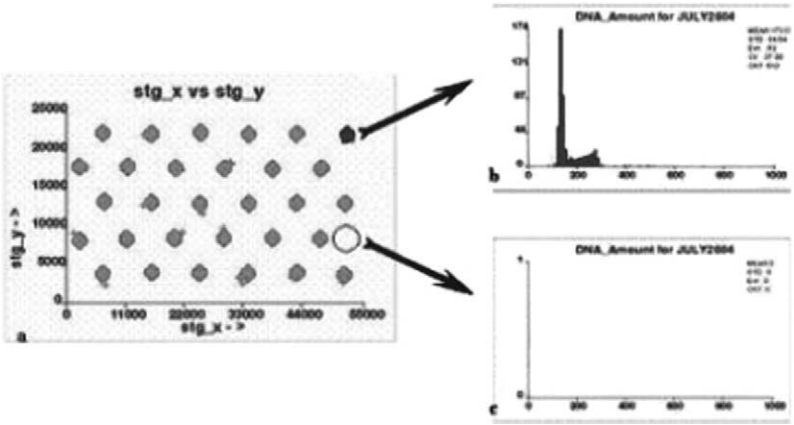

Fig. 9. An example of a slide prepared by our automated depositing device, stained by quantitative Feulgen staining technique, scanned by the Oncometrics Imaging Company Cyto-Savant automated data analysis system, and analyzed by in house developed software (a). The "+" sign is used by the software to indicate the presence of a single scanned cell. It can be seen that the cells tends to stay in close proximity of their deposited spot. The histogram of the DNA amount of one of the spots is shown in (b). The DNA amount of the blank spot is shown in (c), zero DNA amount is recorded as excepted.

ray techniques. CMA allows the rapid analysis of high sample volumes that characterize microarray techniques, to be applied to cell suspension samples, be they clinical material or cell lines. While there has been some attempt to create TMA-like arrays for cell lines [1,2,16], the CMA offers many advantages: it is simple, fast, and easily reproducible (Figs 3–5). Despite differences in cell count, the variability of the quantitative analysis of all three deposition methods is negligible in which case we can conclude that all three de-

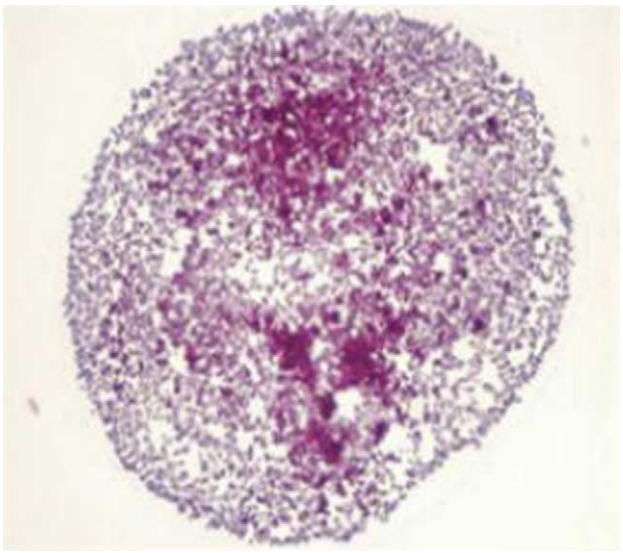

Fig. 10. CRL-2170 Human cell line after deposition onto the slide and stained with H&E Stain.

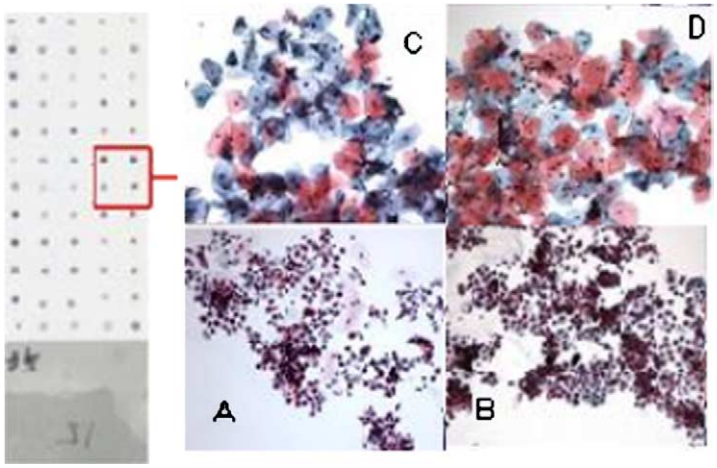

Fig. 11. Percentage of MIB-1 positive cells in relation to the total number of cells within each spotted cell line.

position methods are truly comparable to the original sample. Furthermore, the cells are minimally disturbed (not sectioned or heated), allowing for more types of analyses to be performed, such as DNA ploidy and structural characterization [13].

One of the issues with TMAs is the large amount of data that must be interpreted with each slide (typically each spot is assessed visually) [15]. The analysis of CMA presented here is fully automated. This not only speeds the process along, which allows for greater and more efficient throughput. It also opens up the possibility for data mining processes to be applied to any studies done. Our fully automated technology allows for rapid testing of new biological and molecular markers which can be used in conjunction with current cervical cancer screening methods [3,8].

As the array dimensions and spot number are controlled merely by altering the pattern of micropipettes, the CMA is very flexible and can be designed to meet diverse experimental applications. We have created arrays of 12 spots to over 500 spots (data not shown); the former can be used for internal quality control for clinical samples slides, an important element in clinical investigations [16].

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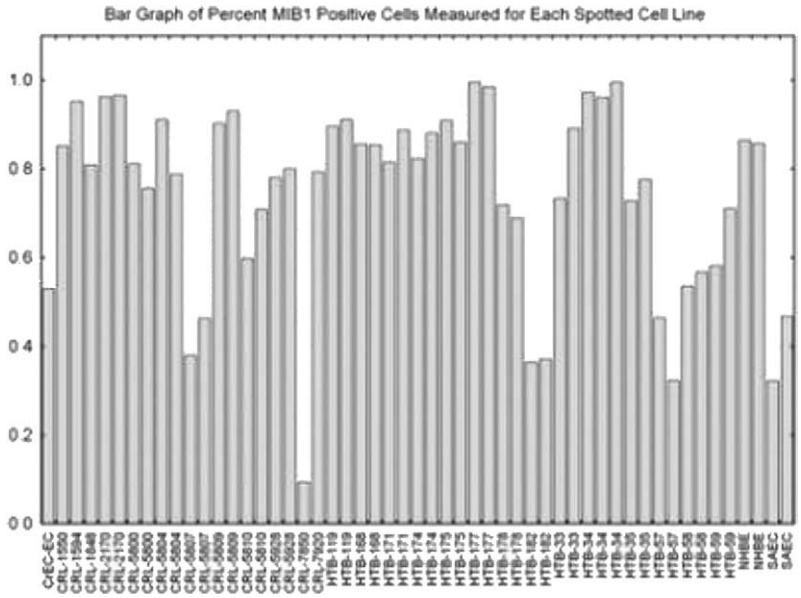

Fig. 12. Five by twelve cytology microarray spotted slide, a magnified (20×) image of 2 bronchial lavage sample spots (A, B) and 2 liquid based cervical cytology spots (C, D) stained with Papanicolau stain.

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