

# Short Technical Reports

## Protein Microdeposition Using a Conventional Ink-Jet Printer

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### ABSTRACT

Many recent bioanalytical systems based on immunologic and hybridization reactions in a mono- or bidimensional microarray format require technology that can produce arrays of spots containing biospecific molecules. Some microarray deposition instruments are commercially available, and other devices have been described in recent papers. We describe a system obtained by adapting a commercial ink-jet printer and used to produce mono- and bidimensional arrays of spots containing horseradish peroxidase on cellulose paper. In a few minutes, it was possible to obtain bidimensional arrays containing several thousands of spots with a diameter as low as 0.2 mm, with each of which requiring only a few nanoliters of the enzyme deposition solution. The quantity of enzyme in each spot was evaluated with a chemiluminescent reaction and a charge-coupled device-based, low-light imaging luminograph. The chemiluminescence measurements revealed that the reproducibility of the enzyme deposition was satisfactory for analytical purposes, with the variation coefficients being lower than 10% in almost all cases.

### INTRODUCTION

Bioanalytical microsystems such as biosensors and immuno- and hybridization assays in microarray format (1–5,7,11–13,16) often require the use of a technology that can dispense quantities in the order of nanoliters of a solution containing biospecific probes (enzymes, antibodies, receptors or DNAs) onto a given solid support. The technology must allow the rapid and highly reproducible deposition of many sample spots in small areas as to obtain a spot density of 10–100 spots/cm<sup>2</sup> or more. A few rather expensive instruments for this purpose are commercially available. Some recent papers have described the design and construction of devices for the production of mi-

Table 1. Chemiluminescent Signal Obtained for Spots of Different Sizes (mean value  $\pm$  SD, n=8)

Spot Diameter (mm)	Spot Area (mm <sup>2</sup> )	HRP (pg)	CL Signal ( $\times 10^3$ photons/s/pixel)	Total CL Signal ( $\times 10^5$ photons/s/spot)
1.6	2.01	43.6	1.2 $\pm$ 0.1	4.6 $\pm$ 0.3
1.4	1.54	33.3	1.3 $\pm$ 0.1	3.7 $\pm$ 0.3
1.2	1.13	24.6	1.4 $\pm$ 0.1	3.0 $\pm$ 0.3
1.0	0.79	17.0	1.5 $\pm$ 0.2	2.1 $\pm$ 0.3
0.8	0.49	10.9	1.3 $\pm$ 0.1	1.2 $\pm$ 0.1
0.6	0.28	6.1	1.4 $\pm$ 0.2	0.8 $\pm$ 0.1
0.4	0.13	2.7	1.9 $\pm$ 0.6	0.4 $\pm$ 0.1
0.2	0.03	0.7	3.4 $\pm$ 1.6	0.2 $\pm$ 0.1

croarrays of biospecific molecules on various types of solid supports (6,18).

This report evaluates the performance of a simple and inexpensive device for the microdeposition of biospecific molecules on cellulose or plastic supports, which is obtained by adapting a commercial ink-jet printer for protein microdeposition. Horseradish peroxidase (HRP) was used as a model protein since it can be easily imaged and quantified using chemiluminescent (CL) detection. Both the amount and spatial distribution of the HRP deposited on the solid support were evaluated using a charge-coupled device (CCD)-based, low-light imaging luminograph (8–10,17). The most appropriate experimental conditions were defined in terms of the physical-chemical parameters of the printing solution, such as surface tension and chemical composition. The final goal was to obtain a simple system for the microdeposition of enzymes or other biospecific molecules, characterized by high reproducibility and rapidity.

### MATERIALS AND METHODS

#### Ink-Jet Printer

A DeskJet 600 ink-jet printer, based on thermal ink-jet technology, and HP 51629A black ink cartridges (Hewlett-Packard, Boise, ID, USA) were used. The black ink was removed; the cartridge was extensively washed with distilled water, filled with the deposi-

tion solution using a microsyringe and was then properly sealed following the procedure reported in a commercial ink refill kit for HP 51629A cartridges (Rotring, Hamburg, Germany).

#### Deposition Solution

HRP (EC 1.11.1.7, type VI-A, 1100 U/mg) was purchased from Sigma (St. Louis, MO, USA). A 1 mg/mL stock solution obtained by dissolving HRP in 0.1 M Tris-HCl buffer, pH 8.6, was used to prepare the deposition solution that contained 1  $\mu$ g/mL of HRP. The surface tension of this solution was adjusted to a value similar to that of the conventional black ink by adding 1.5 mM SDS in 10 mM NaCl as a surfactant. Surface tensions were measured using a Sensadyne<sup>®</sup> 6000 surface tensionmeter (Chem-Dyne Research, Mesa, AZ, USA) based on the maximum bubble pressure method.

#### Sample Deposition

The printing pattern was defined by a standard word-processing program. The solid support was then printed using the cartridge filled with the deposition solution and by selecting the highest-quality printer settings. Frames of full dots of uniform or different diameters (ranging from 0.2–1.6 mm) were used as sample spots. Different solid supports were tested, including conventional cellulose paper of various weights (ranging from 30–80 g/m<sup>2</sup>), cellulose filter paper, nylon sheet, pho-

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tographic gelatin paper, tissue paper and ink-jet transparency film. Compatibility was evaluated for the adaptability of the support to the mechanics of the printer and also for the deposition quality that is related to the surface properties of the solid support. After sample deposition, the paper was air dried and then stored at 4°C for short periods (1–2 weeks) or at -20°C for longer periods of up to six months.

### Chemiluminescence Measurements

The enzymatic activity of the HRP deposited on the solid supports was evaluated using a luminol/H<sub>2</sub>O<sub>2</sub>/*p*-iodophenol chemiluminescent substrate (ECL™, Amersham Pharmacia Biotech, Buckinghamshire, England, UK) (14,15). For the cellulose paper and other permeable supports, the printed side was covered with adhesive transparent paper and then placed on ab-

sorbent paper wetted with the CL substrate. For the non-permeable supports, a microsyringe was used to place the substrate directly onto the printed side of the support. The CL signal from the HRP was measured using a Night Owl LB 981 luminograph (EG&G Berthold, Bad Wildbad, Germany), which is a high-performance, low-light imaging system equipped with a cooled, back-illuminated CCD camera. In all cases, the CL measurement was performed with the printed side of the support turned towards the CCD camera.

This system permitted a quantitative evaluation of the amount of enzyme deposited and its spatial distribution on the solid support. Light emission was measured by one-minute acquisitions taken at different times after the addition of the CL substrate, for up to 30 min. The results were expressed as photons/s/pixel, and the CL signal was integrated over the spot areas to obtain the

total photons emitted from the sample spots. The background signal was measured over areas where no HRP was deposited and its value was subtracted from the measured CL intensities.

### RESULTS AND DISCUSSION

Conventional black ink cartridges can be successfully used for HRP microdeposition. Empty cartridges can be washed to completely remove the black ink and then filled with the deposition solution without compromising the performance of the printer. The minimum volume of deposition solution required for printing was about 2 mL. Any unused solution could be recovered, except for the small volume (200–300 µL) contained in the cartridge print head. The control of the inner pressure is important for determining the proper operation of the cartridge because it

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prevents ink from leaking. The refilling kit includes a priming pump that is used for pressure equalization (by forcing a known volume of air into the cartridge) before sealing off the cartridge. After sealing, the cartridge is ready for printing. In principle, the use of a multicolor cartridge instead of the black one could allow the deposition of arrays containing multiple (up to three) reagents. However, at least for this printer model, the design of the original HP multi color cartridges prevents their being refilled.

The filling solution must mimic the ink used by the cartridge manufacturer. The main problems affecting printing operation and quality concern the wetting of the deposition solution in relation to the solid support used. These properties were accounted for by considering the surface tension value of the solution as a representative physicochemical parameter. The surface tension of the ink was 55 dyne/cm, when measured at  $25 \pm 1^\circ\text{C}$ , and the surface tension value of water or conventional buffers ranged from 65–72 dyne/cm. We therefore added SDS as a surfactant to obtain a surface tension value similar to that of the ink. We found that a 1.5 mM SDS solution containing 10 mM NaCl has the required surface tension value of 55 dyne/cm. The effect of the surfactant on the enzyme activity was determined in solution by comparing the CL intensities obtained for samples of HRP 1  $\mu\text{g}/\text{mL}$  in the deposition solution and in the Tris-HCl buffer. The results showed that in the presence of SDS, the CL intensity was lowered by about 15%, which can be from a decrease in the enzyme activity or, more likely, from a slight interference of SDS with the CL reactions.

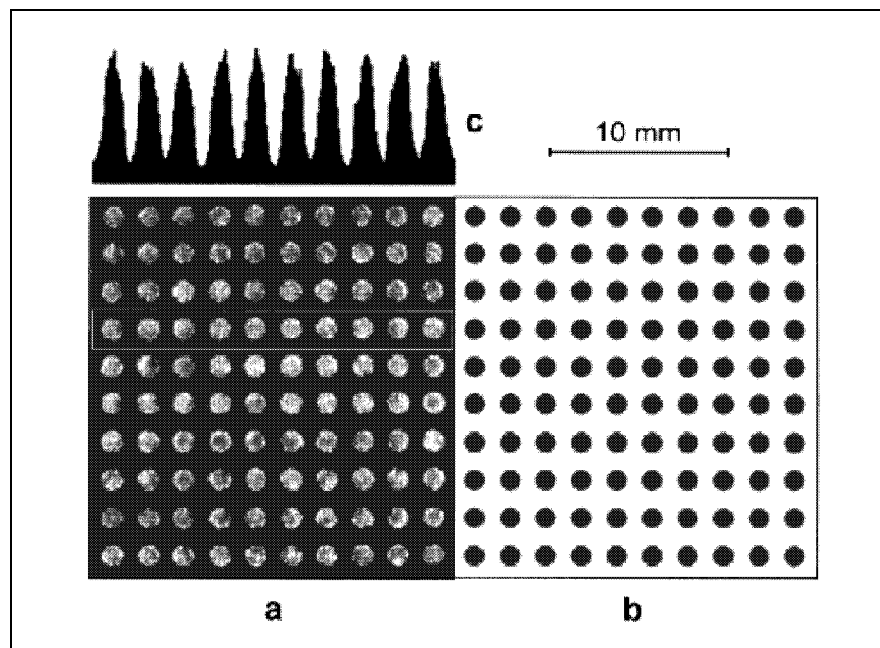
As for the solid support, the best results for the intensity and spatial localization of the CL signal were obtained using conventional cellulose paper. A direct addition of the CL substrate on the printed side of the paper always led to a poorly localized CL signal because it partially washed away the enzyme. To overcome this, the printed side of the cellulose paper was covered with transparent adhesive paper, and the CL substrate was added by placing the printed sheet over absorbent paper that was wetted with the substrate. In this

way, the CL substrate could diffuse through the solid support, which results in a much less pronounced washing away of the enzyme and a more localized CL signal. The lightest cellulose paper (weight 30  $\text{g}/\text{m}^2$ ) gave the fastest diffusion of the CL substrate, with the maximum CL signal intensity being reached in 3–6 min, while the other cellulose papers required 10–15 min.

All the data reported here were obtained using the lightest cellulose paper as solid support. Other types of supports turned out to give less satisfactory results. In particular with the non-permeable ones (ink-jet transparency and photographic gelatin paper), the CL substrate had to be added on the printed side and resulted in a poorly localized CL signal because the HRP washed away. A suitable chemical immobilization procedure of the enzyme would thus be required to use these non-permeable supports for microdeposition with the developed system.

We tested the performance of the system by printing frames of full dots with a diameter of 1 mm. A sheet containing several thousands of sample spots can be obtained in a few minutes.

The amount of enzyme deposited in each spot was evaluated by weighing the printer cartridge before and after printing a large known number of spots. We calculated that each 1 mm diameter spot required an average of 17 nL of deposition solution, which corresponded to approximately 17 pg of HRP. The quality of the deposition was evaluated by the spatial distribution of the signal derived from the HRP-catalyzed chemiluminescent reaction that is shown in Figure 1, together with a black ink printout of the deposition pattern. The analysis of the CL image and the CL intensity two-dimensional plot (also reported in Figure 1) showed a homogeneous distribution of the enzyme in each spot (the variation coefficient of the CL emission from the spots was lower than 10%,  $n=100$ ). This result demonstrated that the developed system can be properly optimized and used for the deposition of biospecific proteins that must be homogeneously distributed to achieve good performance in immunoassays and other biospecific methods based on microarrays. The stability of the enzyme on the cellulose paper was determined by measuring the



**Figure 1.** Image of the CL signal from a bidimensional array of HRP spots. The CL image (a) was obtained from a bidimensional array of 100 HRP spots (diameter 1.0 mm) deposited in a  $20 \times 20$  mm area of light cellulose paper (weight 30  $\text{g}/\text{m}^2$ ). The intensity of the CL signal was  $(3.0 \pm 0.3) \times 10^5$  photons/s/spot (mean value  $\pm$  SD,  $n=100$ ). A black ink printout of the deposition pattern (b) is also shown. The 2-D plot (c) of the CL emission along the highlighted row of the array demonstrates the homogeneous distribution of HRP in the different spots.

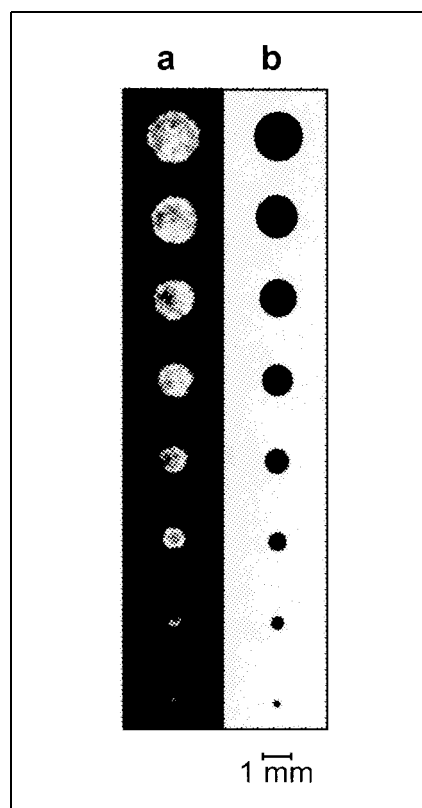
CL signal after different storage periods at 4°C. The CL signal was quite stable for 2–3 weeks of storage, and then decreased. On the other hand, storage for up to 6 months at -20°C did not affect the enzyme's activity.

Figure 2 shows the image of the CL emission obtained from an array of full dots of different diameters (ranging from 0.2–1.6 mm), together with a black ink printout of the same array. Table 1 reports the mean values of the CL signal measured for this array (referred to the image pixel or spot area) as a function of spot diameter, spot area and amount of HRP deposited in each spot. The amount of enzyme per spot was calculated on the basis of the value of 17 pg obtained for the 1 mm diameter spot, assuming that the amount of HRP is directly proportional to the spot area. The mean CL intensity values were obtained by analyzing eight spots of each size; all

the CV were lower than 10%, except for the two smallest size spots.

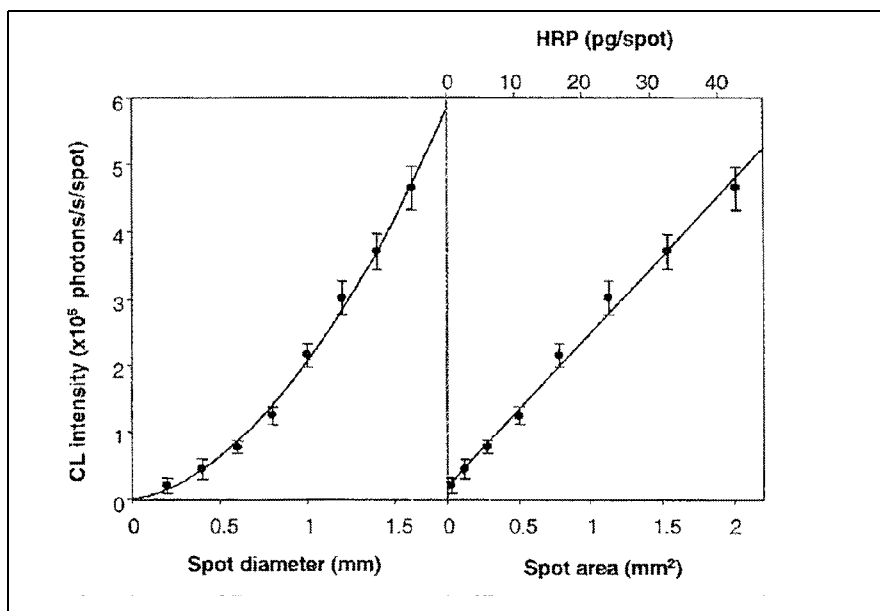
Figure 3 shows the relationship between the CL signal, expressed as total emitted light per spot area, and the spot diameter, the spot area and the amount of HRP. As expected, the total CL signal showed a linear relationship with the spot area, but not with the spot diameter. This linear relationship was quite good within the studied range (spot diameter from 0.2–1.6 mm), indicating that the amount of HRP deposited in each spot really was proportional to the spot area. Conversely, the measured CL signal per image pixel was nearly constant for all spot sizes, as expected. The only values of the CL signal for image pixel that were significantly different from the others were those obtained for the two smallest size spots (diameter 0.2 and 0.4 mm). However, these values are less significant because of their high variation coefficients. The scarce reproducibility in the CL intensity values of these spots may be attributed to at least two different factors. First, the system used to add the CL substrate by layering the printed paper with an absorbent paper wetted with the CL substrate might not have been ideal: that is, differences in the diffusion of CL substrate or in the structure of the paper could have compromised the reproducibility of the system, particularly with the smallest spots. Moreover, the measure of the CL intensity requires exact definition of the image area corresponding to each spot, which can be difficult for the smallest ones because such areas are of only a few pixels. Even in the case of the larger spots, some of the variability observed in the CL intensities could have been from differences in the CL substrate diffusion rather than from a variation in the amount of HRP deposited in each spot.

Another aspect regarding the ink-jet printing technology should be addressed. Each spot is printed by jetting many tiny droplets individually, which is different from commercial microdeposition devices. The DeskJet 600 ink-jet printer is based on thermal ink-jet technology, i.e., a thin layer of ink is vaporized within the printing head to form a bubble that expels a small drop of ink through an orifice or nozzle. We did not experience clogging problems



**Figure 2. Image of the CL signal from an array of various-size HRP spots.** The CL image (a) shows the spatial distribution of the emission due to the HRP-catalyzed CL reaction in an array of HRP spots with diameters ranging from 1.6–0.2 mm. The CL intensities measured from these spots are reported in Table 1. The panel (b) shows the deposition pattern used to obtain the spots.

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**Figure 3. Correlation between CL signal and spot diameter, spot area and amount of HRP.** The left graph shows the relationship between the measured CL intensities and the spot diameter. The right graph shows the linear relationship between the CL intensities and the spot area (lower scale) and the amount of HRP deposited in each spot (upper scale). The curves represent quadratic (left,  $R^2 = 0.995$ ) and linear (right,  $R^2 = 0.992$ ) fittings of the data. The data and the associated standard deviations are those reported in Table 1.

caused by heat-aggregated proteins (HRP is a relatively thermostable enzyme, and we could use the same cartridge for several days). However, we cannot exclude the idea that some thermal degradation could occur for less thermostable molecules. Preliminary data obtained in our laboratory (not shown) suggested that antibodies (IgG, IgM) could also be deposited with this device, with no detectable loss of their recognition capacity.

In conclusion, these data demonstrate that a commercially available ink-jet printer can be adapted to protein microdeposition for analytical purposes, which offers a rapid, reproducible and easy-to-perform protein deposition on cellulose paper. Linear or bidimensional arrays of circular spots or more complex printing patterns can be easily obtained. This printer could also be used with non-permeable solid supports that will require a suitable chemical immobilization procedure of the protein, and for the deposition of other biospecific molecules, such as nucleic acids. Therefore, this device represents a low-cost alternative to the commercially available microdeposition devices, even if it presents some limitations concern-

ing the nature of the biospecific molecule (which should be relatively thermostable) and the spot size (the minimum spot size is 0.2–0.4 mm). We think that the availability of this microdeposition technology will enlarge and encourage the development of new microarray-based bioanalytical assays.

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