

Accurate High-Speed Liquid Handling of Very Small Biological Samples

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ABSTRACT

Molecular biology techniques require the accurate pipetting of buffers and solutions with volumes in the microliter range. Traditionally, hand-held pipetting devices are used to fulfill these requirements, but many laboratories have also introduced robotic workstations for the handling of liquids. Piston-operated pumps are commonly used in manually as well as automatically operated pipettors. These devices cannot meet the demands for extremely accurate pipetting of very small volumes at the high speed that would be necessary for certain applications (e.g., in sequencing projects with high throughput). In this paper we describe a technique for the accurate microdispensation of biochemically relevant solutions and suspensions with the aid of a piezoelectric transducer. It is suitable for liquids of a viscosity between 0.5 and 500 milliPascals. The obtainable drop sizes range from 5 picoliters to a few nanoliters with up to 10000 drops per second. Liquids can be dispensed in single or accumulated drops to handle a wide volume range. The system proved to be excellently suitable for the handling of biological samples. It did not show any detectable negative impact on the biological function of dissolved or suspended molecules or particles.

INTRODUCTION

Conventional dispensing systems are commonly based on piston-operated pumps. The piston is either manually or electrically moved inside a cylinder to displace the liquid that is contained within a fixed or removable pipet tip. When the liquid is ejected, a drop forms at the tip of the pipet with adhering forces holding the drop in place until it is eventually released by gravitational force. These forces are composed of surface tensions between the liquid, the material from which the pipet tip is made and the surrounding air. These tension forces have to be minimized to decrease the liquid volume. One means of accomplishing this is to miniaturize the pipet tip. The radius of a drop decreases faster (rate = radius $\times 10^{-3}$) than the corresponding tension forces (rate = radius $\times 10^{-1}$), so a limit is soon reached. When using a 10- μm -wide pipet

tip, a liquid with a surface tension of 50 mN/m—like an aqueous solution—forms a drop with a volume of approximately 30 nl (0.03 μl) and a diameter of about 400 μm . Since nozzles of that size are mechanically sensitive and tend to clog, another approach is simply to touch the surface of the receiving vessel directly with the pipet tip after the drop has formed, but before it falls off due to its own weight. Again, problems arise: the pipet tip has to be positioned very carefully and the piston movement inside the pipet cylinder has to be controlled with extreme accuracy. Furthermore, the surface tension and viscosity of the liquid in combination with the wetting properties of different surface materials lead to unacceptable volume deviations.

To handle extremely small volumes of liquids accurately, the problems presented by tension forces and wetting properties must be circumvented. A way to overcome the difficulties is to apply inkjet printer technology (2), and with the aid of a piezoelectrical transducer, a small liquid volume is ejected through a tiny nozzle. As shown in Figure 1, the element is mounted on a capillary that is completely filled with the liquid. While applying voltage, the element squeezes the capillary, which quickly increases the pressure in the liquid. Since the liquid is almost incompressible, a pressure wave is produced that travels toward the nozzle at sonic speed. The nozzle transforms the pressure wave into a movement of the liquid, so that a fine droplet is ejected at a very high acceleration (typically 100 000 $\times g$). Due to the loss of the ejected volume, the elevated pressure quickly decreases causing the pressure wave to last for only a couple of microseconds. The speed of the process implies that hardly any wetting of the nozzle occurs. The droplet is ejected into the air (Figure 2) at a speed of about 4 m/s until it hits the target surface. At the same time the displaced volume in the nozzle is immediately replaced by liquid from a reservoir due to capillary forces.

Depending on the bore diameter of the nozzle (20–100 μm), droplet volume ranges from a few nanoliters (10^{-3} μl) to as little as 5 pl (5×10^{-6} μl) with a very high reproducibility.

The piezoelectrical transducer is supplied with a control

unit that permits the release of single droplets from the nozzle as well as "machine gun"-like droplet ejection at frequencies up to 10000 Hz. This makes it possible to pipet larger volumes as multiple single droplets. Liquids and suspensions with viscosities between 0.5 mPa (methanol) and 100 mPa (mineral oil) as well as pure mercury have been successfully pipetted with high accuracy.

Nucleic acids, proteins and whole bacterial cells with susceptible appendages were pipetted to test the applicability of the operating device and its impact on biologically active samples.

MATERIALS AND METHODS

Drop Size Reproducibility

A solution of $\alpha^{32}\text{P}$ -ATP (Amersham Buchler, Braunschweig, FRG) with a high specific activity was prepared and poured into the reservoir of the Microdrop dispenser (Microdrop GmbH, Norderstedt, FRG). A 1.85 nl per drop nozzle was used, and single droplets were ejected directly into

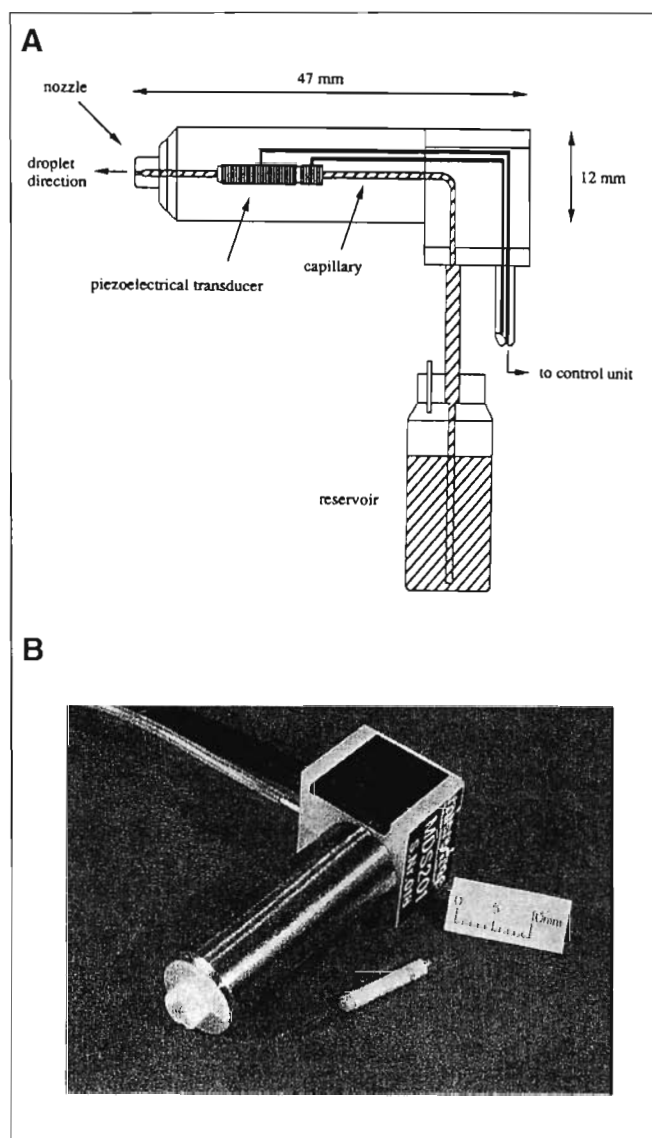


Figure 1. (A) Schematic drawing and (B) real view of the Microdrop dispenser.

counter vessels filled with scintillation cocktail. Two sets of 24 samples were pipetted and subsequently counted in a Packard Tri-Carb counter (Canberra-Packard, Frankfurt, FRG).

Pipetting RNA

A solution of tRNA^{Phe} from yeast (Boehringer Mannheim, Mannheim, FRG) was diluted in TE buffer to a final concentration of 0.2 $\mu\text{g}/\mu\text{l}$. After removal of a reference volume, 50 μl of the tRNA solution were pipetted into a sterile microcentrifuge tube at a drop frequency and volume of 2 kHz and 500 pl, respectively, using the Microdrop dispenser. The pipetted tRNA was then examined with the untreated sample on a 14% denaturing polyacrylamide gel after a 2-h run at 20 V/cm and staining with ethidium bromide.

Pipetting DNA

Similarly, double-stranded, supercoiled, circular plasmid DNA was subjected to the pipetting procedure. The test solution contained 0.5 $\mu\text{g}/\mu\text{l}$ of plasmid pDR720 (4100 bp; Pharmacia Biotech, Freiburg, FRG) in TE buffer. After the procedure, the plasmid and the control sample were loaded on a 0.8% agarose gel and run at 10 V/cm in TAE buffer (0.04 M

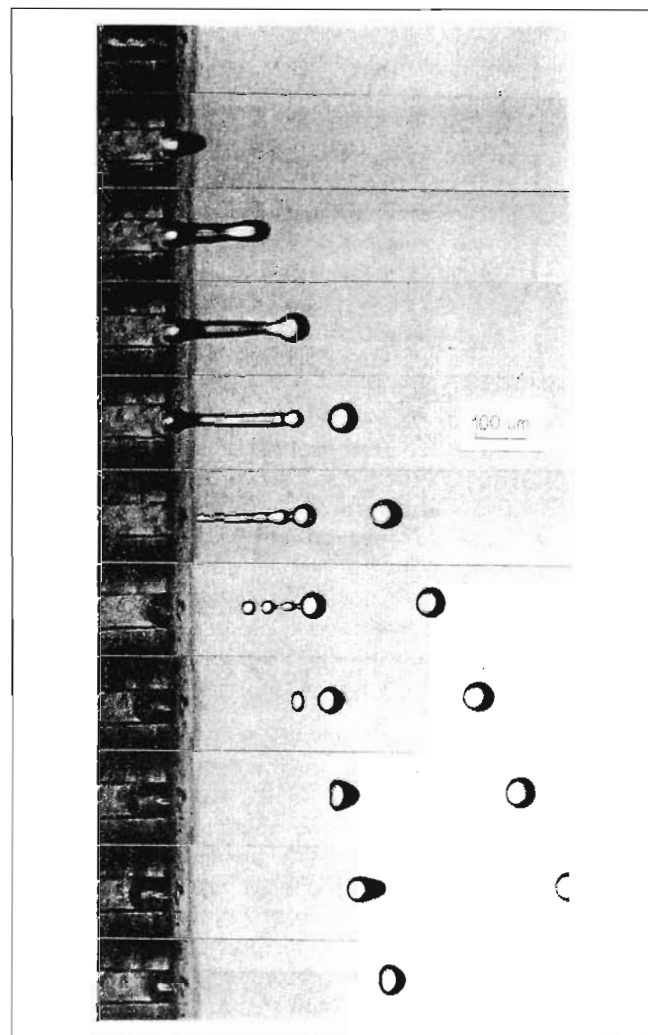


Figure 2. Dosing of water (viscosity 1 mPa) at a rate of 5000/s. The set of frames was taken with help of a stroboscope: the droplets that are ejected from the nozzle at the left seem to stand still. The tail that is formed during ejection immediately disappears and a perfectly round droplet flies away.

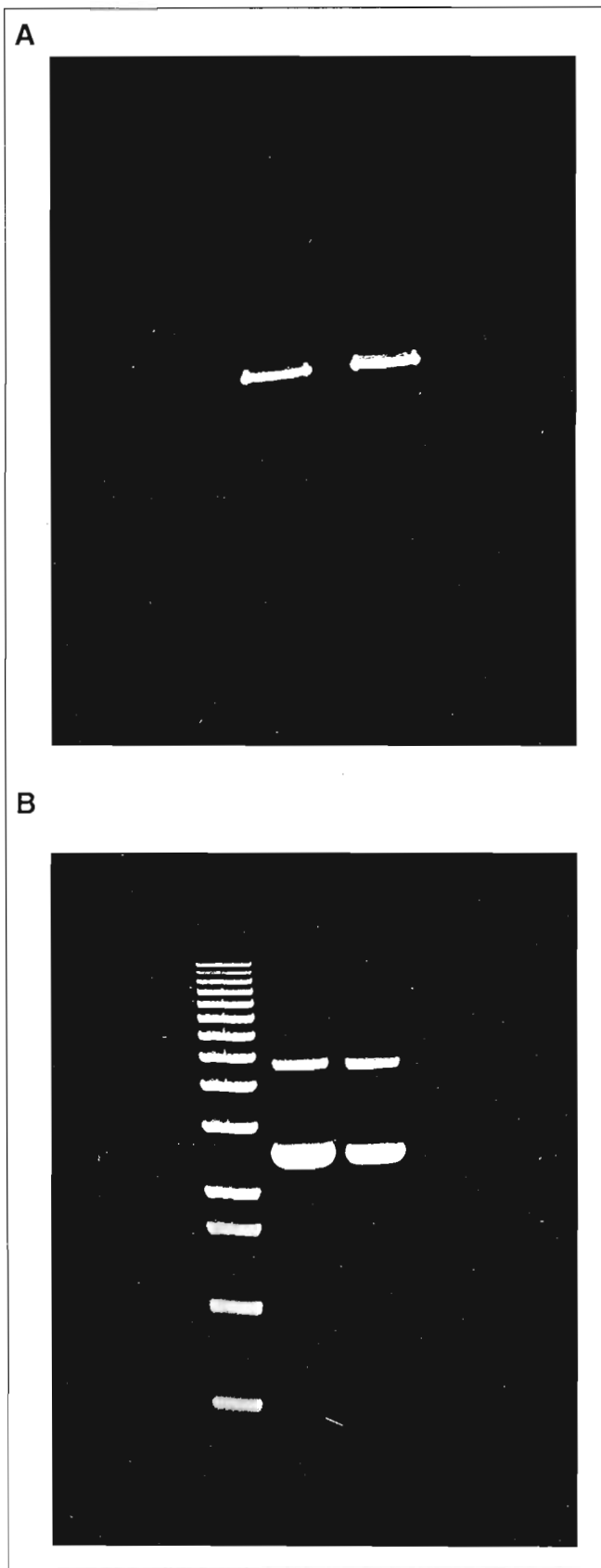


Figure 3. Transfer RNA and plasmid DNA before and after Microdrop pipetting. Untreated and pipetted yeast tRNA^{Phe} (Figure 3A, left lane and right lane, respectively) was analyzed on a 16% denaturing polyacrylamide gel. Plasmid pDR720 was run on a 0.8% agarose gel (Figure 3B). Lanes from left to right: marker, untreated plasmid, pipetted plasmid. The ratio of closed circular and supercoiled plasmid remains unchanged after pipetting.

Tris-acetate, 0.001 M EDTA) for 1 h. The gel was examined after staining with ethidium bromide.

Pipetting Enzymes

To test the impact of the pipetting procedure on biologically active enzymes, 25 units of *Taq* DNA Polymerase (Stratagene, Heidelberg, FRG) were diluted to a final concentration of 2.5 U/ μ l in 1 \times standard polymerase chain reaction (PCR) buffer (10 mM Tris-HCl [pH 8.3], 50 mM KCl, 1.5 mM MgCl₂, 0.01% [wt/vol] gelatin). The solution was divided into two portions, one of which was kept on ice as a control. The other volume was used to fill the reservoir of the Microdrop dispenser. After pipetting the enzyme solution into a fresh Eppendorf tube (Eppendorf-Netheler-Hinz, Hamburg, FRG) at a drop frequency and volume of 2 kHz and 500 μ l, respectively, 2.5 units of this preparation were added to a standard PCR reaction mixture containing 1 \times PCR buffer, 200 μ M each deoxyribonucleoside triphosphate (dNTP), 0.1 μ g of template DNA (plasmid pQB1; Reference 6), 0.3 μ M primer 1 (5'CGCGTAAAGCGTTGAAACTT3') and primer 2 (5'GATCGGGTTTTGACCCTGAG3'), respectively. Another aliquot of the same solution was supplemented with the control enzyme. Control and test solutions were processed in a Trio Thermoblock cycler (Biometra, Göttingen, FRG) under the following temperature cycling protocol: 0.5 min at 94°C, 1 min at 55°C and 1 min at 72°C for 29 cycles; 7 min at 72°C for 1 cycle.

Samples (5 μ l) were loaded on a 0.8% agarose gel, run for 45 min at 10 V/cm, stained with ethidium bromide and photographed.

Pipetting Bacteria

Cell viability. *Escherichia coli* K-12 HB101 cells (7) were grown in LB medium in a shaker flask to a density nearly equal to 10⁶ cells/ml. The cell suspension was then divided into a control and a test volume. The latter (2 ml) was pipetted using the Microdrop dispenser at a droplet volume of about 2 nl. Control and test suspensions were further diluted 1:10⁴ with fresh medium, plated on Luria-Bertani (LB) agar plates and grown overnight. The number of colonies from pipetted and untreated bacteria was compared.

Sowing of bacterial cells. In another experiment the dispenser was used to sow bacterial cells at distinct positions on an agar plate. A suspension of *E. coli* HB101 cells (ca. 5 \times 10⁵ cells/ml) was filled into the reservoir of the Microdrop dispenser and single droplets with a volume of 2 nl were "shot" onto the surface of a pre-warmed LB agar plate. The distance between the nozzle tip and the agar surface was adjusted to approximately 5 mm. After the release of a droplet, the plate was moved 2 mm in one direction with the help of a compound slide rest, and the next droplet was ejected by manually triggering the control unit. The plate was grown in the incubator overnight and photographed the following day.

Impact on sensitive surface structures. *E. coli* K-12 HB101 cells harboring the plasmid pPKL4 (3) were aerobically grown in LB medium with 100 μ g/ml ampicillin until an OD₆₀₀ of 0.7 was reached. The cells were harvested in a benchtop centrifuge, and the pellet was washed twice with cold (4°C) phosphate-buffered saline (PBS). After resuspending and keeping the cells in the same volume of PBS for 1 h, a small volume was removed and kept on ice as a control. The remaining suspension was pipetted into a cold microcentrifuge

tube using the Microdrop dispenser at a drop frequency and volume of 2 kHz and 500 pl, respectively. Five microliters from the control and the test sample were put on a carbon film-coated nickel grid placed on a paper towel. The cells were negative-stained with a drop of a 1% uranyl acetate solution for 2 min at room temperature, dried and photographed in a transmission electron microscope.

RESULTS

The impact of the pipetting process on different biologically active solutions and suspensions with the Microdrop dispenser was tested. Yeast tRNA^{Phe} that had been subjected to the process did not show any difference compared to the untreated control as proven by denaturing polyacrylamide gel electrophoresis (Figure 3A); no traces of breakdown products were visible.

Also plasmid DNA is not affected by the forces occurring during the droplet ejection as judged from the gel (Figure 3B). The ratio of supercoiled, covalently closed circular and nicked circular DNA appeared to be unchanged compared to the control DNA.

Sequencing and PCRs have been automated and carried out in robotic workstations (1,8–10). The reactions are commonly started by the addition of the most sensitive compound to the mixture—the DNA polymerase. Therefore *Taq* DNA Polymerase was pipetted with the Microdrop dispenser, and the effect of the procedure on the enzyme was subsequently determined in a standard PCR. The comparison with a control reaction did not reveal any differences between treated and untreated *Taq* DNA Polymerase. The amount of PCR product obtained with the pipetted enzyme is identical with the yield of the control polymerase (Figure 4).

The comparison of the total numbers of bacterial colonies prior to and after pipetting with the Microdrop dispenser showed no effect on cell viability. The colony numbers were identical within the borders of standard deviation. The distribution of bacterial cells on agar surfaces is easily done with the dispenser. Single cells can be sowed to form colonies

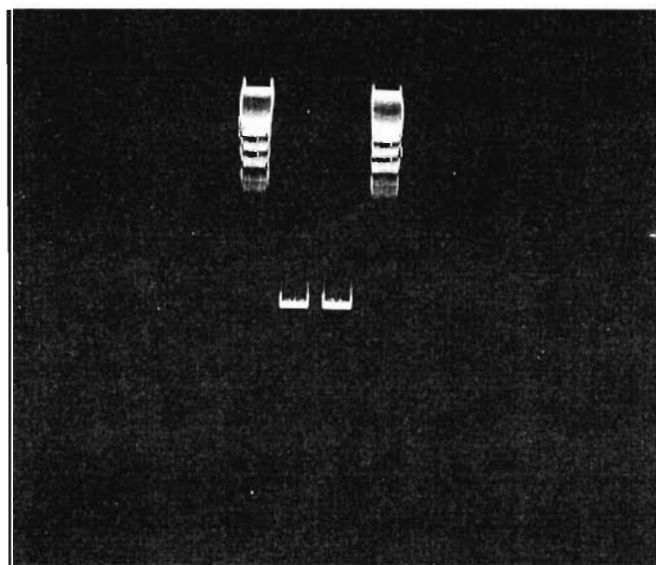


Figure 4. PCR-amplified DNA on a 0.8% agarose gel. Lanes 1 and 4 carry a DNA marker. The 480-bp DNA in lane 2 was produced with the untreated *Taq* DNA Polymerase (control). Lane 3 shows DNA produced by the pipetted enzyme.

(Figure 5). Clogging of the 100- μ m dispenser nozzle was not observed during 30 minutes of constantly pipetting bacteria.

Type I fimbriae are adhesive threadlike appendages on the surface of certain *E. coli* strains that are highly sensitive to mechanical forces. These protein structures can easily be made visible in the electron microscope by negative staining. They are usually shortened and disrupted by mechanical forces, e.g., short sonication, due to built-in “shearing points” (5). To be sure that the fimbriae under investigation were type I fimbriae and not more rigid structures like P pili (4), a normally “bald” strain, *E. coli* HB101, was used to express the plasmid pPKL4 containing the complete *Fim* gene cluster (3). Cells that had undergone the pipetting procedure in the Microdrop dispenser were compared using an electron microscope with those that had been kept on ice. The bacteria were starved in PBS during the experiment to prevent the synthesis

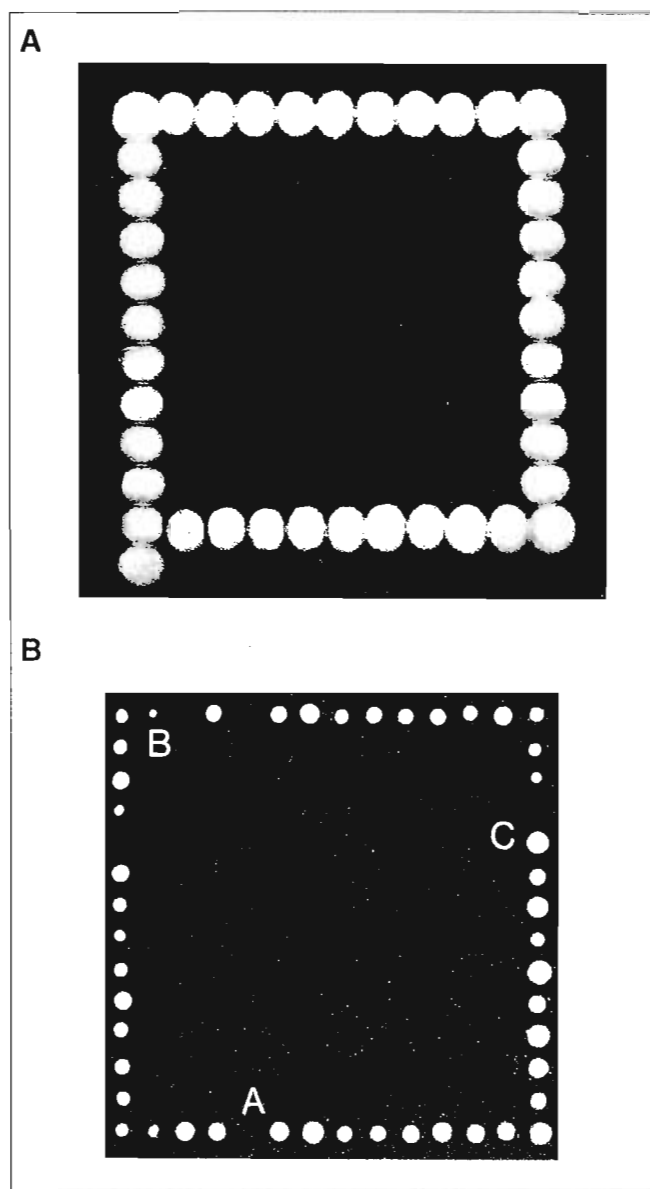


Figure 5. Sowing of bacterial suspensions with different cell densities. High cell concentrations ($>10^6$ cells/ml) give uniform colonies (Frame A) that can be placed very accurately on the agar surface. Frame B shows colonies obtained from a diluted suspension (5×10^3 cells/ml). The droplet that hit position A did not contain a viable cell, the one at position B carried one cell and the colony at C grew up from probably more than a single cell.

and formation of new fimbriae after having been pipetted.

No difference of the fimbriation was visible between control cells and those that had been pipetted. The average number of fimbriae per bacterial cell and also the length of the appendages was identical to both control and test cells (Figure 6).

The drop volume could be shown to vary less than 4% by counting radioactively labeled samples. This corresponds to a maximum size deviation of 75 pl with 1.85 nl droplets and 0.2 pl with the smallest possible drops (5 pl).

DISCUSSION

The Microdrop dispenser, a new pipetting device for accurately handling very small volumes at high speed, was initially developed for industrial applications (1). Biological samples are not affected by the high energy forces occurring during the operation of the device, as shown by pipetting RNA, DNA, enzyme and whole bacterial cells. The dispenser can handle viscous solutions, for example, enzyme storage buffer containing glycerol as well as real suspensions at high accuracy without clogging.

The accuracy of the pipetting method is more than sufficient for molecular biology applications. The smallest drops that can be ejected from the nozzle are only fivefold larger than a bacterial cell and much smaller than eukaryotic cells. When handling these small volumes, a maximum deviation of 4% means 2×10^{-13} l or just 1/5 of a bacterial cell.

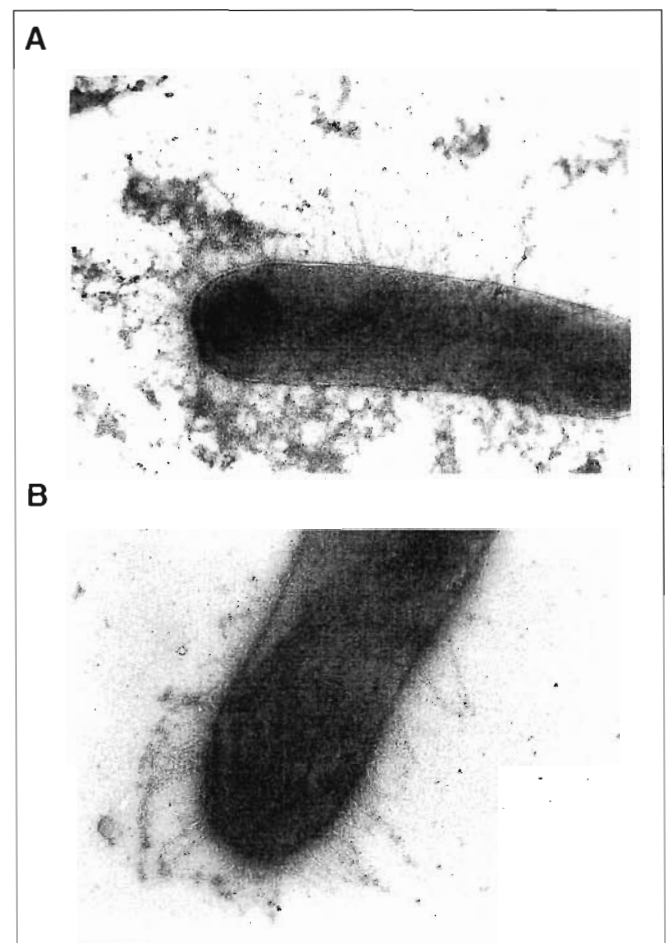


Figure 6. *E. coli* cells carrying fimbrial surface appendages before (A) and after (B) the pipetting procedure.

The speed at which droplets can be ejected allows handling of typical enzyme reaction volumes (e.g., at an ejection frequency of 10 kHz, it would take 0.1 s to pipet a volume of 10 μ l or 1 s for 100 μ l for a droplet volume of 10 nl). Since the dispenser unit is lightweight and small, it would be easy to mount it on a robotic arm. A construction of that kind in combination with a programmable control unit (i.e., a personal computer) could be used to carry out sequencing reactions or diagnostic analyses automatically at high speed and accuracy compared with available robotic workstations.

A common problem with automated workstations is the release of the liquid from the (disposable) pipet tips. The tips usually have to touch the inner surface of the reaction tubes carefully in order to transfer the complete sample volume. To avoid cross contamination, the tips have to be replaced for every pipetting step. These problems simply do not exist when using the Microdrop dispenser. Another imaginable application would be the distribution of single cells on agar plates. By accurately moving either the plate or the dispenser in small steps, cells could be placed on the surface, forming separated colonies at maximum density.

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