# On-Chip Dielectrophoretic Separation and Concentration of Viable, Non-Viable and Viable but Not Culturable (VBNC) *Escherichia coli*

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## ABSTRACT

Although bacterial culture remains the gold standard for detection of viable bacteria in environmental specimens, the typical time requirement of twenty-four hours can delay and even jeopardize appropriate public health intervention. In addition, culture is incapable of detecting viable but not culturable (VBNC) species. Conversely, nucleic acid and antibody-based methods greatly decrease time to detection but rarely characterize viability of the bacteria detected. Through selection by membrane permeability, the method described in this work employs positive dielectrophoresis (pDEP) for separation and purification of viable and VBNC species from water and allows concentration of bacteria for downstream applications.

***Keywords*:** viable but not culturable (VBNC), *Escherichia* coli C3000, bacterial viability, ATP production, membrane integrity, metabolic capacity

## 1 INTRODUCTION

During disinfection or in the presence of a stress environment, bacteria may enter a viable but non-culturable (VBNC) state in which growth and replication ceases but viability and pathogenicity are maintained [1-4]. This unique quality increases the need for an assay which can reliably and specifically detect viable organisms without cultivation. The VBNC state has been induced under a number of conditions including low temperatures, starvation, and in the presence of certain chemicals. Bacteria in this state are characterized by an inability to form colonies under standard plating procedures while maintaining an intact membrane capable of preventing the uptake of certain dyes such as propidium iodide. Confirmation of viability can be achieved through resuscitation in specialized broth media [5-8].Separation and concentration of viable bacteria by dielectrophoresis (DEP) has previously been demonstrated [9-13], however, to date, no research has been done to characterize the behavior of viable but not culturable (VBNC) *Escherichia* coli species in the presence of high frequency electric fields. A key parameter governing the effect of such fields is the Clausius-Mossotti factor ($F_{CM}$). In positive dielectrophoresis, $Re[F_{CM}]$ is greater than 0 and the dielectrophoretic constant of the particle is greater than that of the medium; here, the particle moves "up the field gradient" toward regions of greatest electric field. In negative dielectrophoresis, $Re[F_{CM}]$ is less than 0 and the dielectrophoretic constant of the particle is less than that of the medium so the particle moves toward regions of lowest field. Upon cell death and subsequent membrane permeability, cell conductivity increases by ~$10^4$ as inner cell composition more closely resembles that of the surrounding medium, resulting in a greater Re $[F_{CM}]$.

The dielectrophoretic force (F) experienced by a given colloidal particle can be approximated by equation (1) for spherical particles, where $\varepsilon_0$=vacuum dielectrophoretic constant, r=particle radius, $E_{rms}$=root mean square value of electric field, $Re[F_{CM}]$ is the real part of the Clausius-Mossotti factor (equation 2), $\varepsilon^*_p$=relative permittivity of particle, and $\varepsilon^*_m$=relative permittivity of medium.

$$F=2\pi\varepsilon_0\varepsilon_m r^3 Re\,[F_{CM}]\,\nabla|E_{rms}|^2 \quad (1)$$

$$Re[F_{CM}]=(\varepsilon^*_p-\varepsilon^*_m)/(\varepsilon^*_p+2\varepsilon^*_m) \quad (2)$$

Because dielectrophoretic separation of live and dead *Escherichia coli* is dependent on various cell morphologies and components including its non-spherical shape, a model was experimentally generated to predict the behavior of bacteria when subjected to various media and frequencies. These factors were then tested to compare the behavior of VBNC species to that of live and dead cells.

## 2 MATERIALS AND METHODS

### 2.1 Bacterial Preparation

*Escherichia coli C3000* (ATCC# 15597) was grown overnight at 37°C in nutrient broth then centrifuged at 3000 rpm for five minutes and rinsed three times in distilled water ($dH_2O$). The resulting pellet was then resuspended and incubated for six days in one of the following:

(a) Nutrient broth at 37°C
(b) 70% Isopropanol (IPA)
(c) 0.5, 5, 50 or 500 µM copper sulfate ($CuSO_4$) in 0.9% NaCl at 25°C (to induce VBNC)

After incubation, samples were centrifuged at 3000 rpm for five minutes, then rinsed three times in $dH_2O$ for culture and membrane integrity testing in Brain Heart Infusion (BHI) broth, and finally incubated at 37°C for 24 hours to resuscitate. Samples were assayed for culturability, cell membrane integrity, and ability to resuscitate prior to initial resuspension and after six days incubation in nutrient broth, IPA, or $CuSO_4$. To determine limits of detection, samples were then serially diluted from $10^{-1}$ to $10^{-8}$ in either $dH_2O$ or BHI broth. All testing was completed in triplicate.

## 2.2 Induction and confirmation of VBNC

To quantify levels of untreated bacteria, 100 µL of bacteria diluted to $10^{-4}$ in dH2O was plated on MacConkey agar with sorbitol (SMAC). At days 2 and 6, 100 µL of each sample was also plated undiluted to assess loss of culturability. Serial dilutions of resuscitated bacteria were also plated to verify expected concentrations and derive calibration curves for viability assay.

Membrane integrity was observed with the LIVE/DEAD *Bac*Light bacterial viability assay (Promega Cat No. L7012). A 2X solution consisting of 1 µL 3.34 mM Syto 9 and 1 µL 20 mM propidium iodide in 1 mL dH2O was mixed with equal parts of each sample. Viable cells were then excited using a QBC ParaLens Advance LED objective consisting of a blue LED (385-480 nm) for excitation and a longpass filter (>510 nm) to view both viable (green) and nonviable (red) cells. Ten images were captured for each sample and cells were counted manually.

## 2.3 Dielectrophoretic Separation and Detection

Dielectrophoresis was performed on a modified flow array (Fig. 1)) customized by Applied Biophysics (Part No 8F10E, Troy, NY). Normally configured with 10 active 250 µm diameter electrodes per region (Fig. 2), photoresist was omitted during fabrication to produce eight individually addressable electrodes per array at the base of the 100 µL flow channel (50 mm x 5 mm x 0.4 mm). Alternating current (10 $V_{p-p}$) was applied to the electrode via a laptop-controlled frequency generator (Velleman, Inc PCGU1000). Flows from 3 mL luer-lock syringe were controlled using a syringe pump (New Era NE1000). For the purposes of this work, a small region centered on the electrode edge was visualized by LED microscopy with a 20X objective. Images were captured by a ScopeTek 2.0M pixel CCD camera and MiniSee software and analyzed with ImageJ software for background subtraction[14].

To determine the parameters for optimal separation of live and dead *Escherichia coli*, dielectrophoresis was initially performed at a range of frequencies in $10^{-5}$ to $10^{-2}$ concentrations of PBS diluted in deionized water (conductivities = 2.7, 3.2, 15.46, and 154.0, µS/cm, respectively) and flowed for 50 µL/min for two minutes for each frequency tested. An image was then captured at the conclusion of the flow period and manually counted in two regions, at the electrode edge and nearing the electrode center. Counts at the former (pDEP) and latter (nDEP) were represented as a percentage of all cells collected for both live and dead populations (pDEP % and nDEP%). An estimate of the dominating DEP force was then derived by equation (3) for live and dead populations at each tested frequency. Bacteria treated with 500 µM CuSO4 in 0.9% NaCl were then separately but identically subjected to dielectrophoretic conditions to assess their behavior as compared to live and dead bacteria.

$$DEP\% = pDEP\% - nDEP\% \quad (3)$$

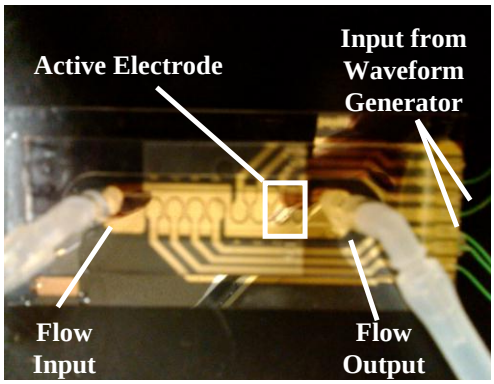


Figure 1. Modified Applied Biophysics flow array employed for bacterial dielectrophoresis.

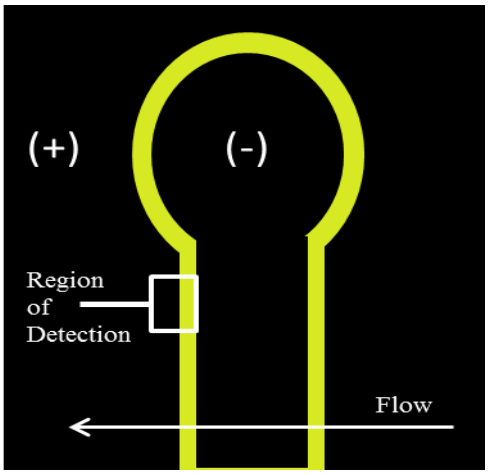


Figure 2. Illustration of single electrode within the flow array.

# 3 RESULTS AND DISCUSSION

## 3.1 Viability Testing

Plating results revealed initial bacterial concentration equal to 5 x $10^7$ cfu/mL. Limit of detection as determined by lowest dilution producing colonies was $10^{-6}$, equivalent to 50 cfu/mL. Limit of detection for the LIVE/DEAD assay was $10^5$ cfu/mL. A linear relationship comparing viable cell counts and culture results was statistically significant ($R^2$=0.9943)

for concentrations ranging from $10^5$-$10^7$. At concentrations greater than $10^7$, cells were too numerous to accurately count. At concentrations below $10^5$, no cells were detectable. Within the linear range, each cell in the observation region could be extrapolated to represent 2.1 x $10^6$ cells/mL ($R^2$=0.9805). This relationship was used to estimate concentrations of viable cells for $CuSO_4$ treated samples.

## 3.2 Induction and detection of VBNC

For $CuSO_4$ treated samples, 0.5 and 5 µM $CuSO_4$ were found to have no significant effect on culturability or viability as compared to normal growth. At 50 µM $CuSO_4$, the number of culturable cells decreased by a factor of approximately $10^3$, however no significant decrease in viable cells was noted. At 500 µM $CuSO_4$, culture resulted in no colonies, however, the presence of cells with compromised membranes was scarce (Figures 3a and b).

Comparatively, the membranes of cells incubated in IPA were compromised throughout the population. Treatment with IPA resulted in full removal of both culturable and viable cells within two days incubation (Figure 3c), and this loss remained following the resuscitation procedure. The sample incubated in BHI at 37°C showed minimal variation in viability and culturable ($10^8$-$10^9$ cfu/mL) over six days or after the resuscitation procedure. However, 500 µM $CuSO_4$ reduced culturability by approximately $10^3$ cfu/mL after two days incubation and completely after six days. Despite this, the number of viable cells in 500 µM $CuSO_4$ decreased but only to about $10^6$ cfu/mL after six days. The resuscitation procedure was successful, with both culturable and viable cell counts resuming to >$10^8$, in good agreement with the positive control. Despite lacking in culturability, viable cell counts for these treated samples stained with LIVE/DEAD assay were equivalent to $10^6$ cells/mL. After resuscitation, cell levels as determined by culture and membrane integrity were all approximately $10^8$ cells/mL.

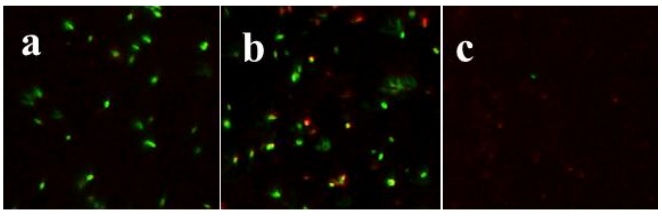


Figure 3. Visualization of intact membranes as indicated by LIVE/DEAD viability assay. A) Live, untreated cells showed were fully viable with maintained membranes (green). B) After treatment with $CuSO_4$, a scarce number of cells demonstrated compromised membranes (red) however the majority are intact. C) Treatment with IPA resulted in compromised membranes for all but one cell.

## 3.3 Dielectrophoretic Separation and Detection

When resuspended in $10^{-5}$ PBS (3.2 µS/cm), both live and dead cells behaved similarly, demonstrating pDEP at frequencies greater than 1 kHz. Cell separation was achieved in both $10^{-3}$ and $10^{-4}$ PBS (Figure 4). Here, pDEP was noted for live cells at frequencies above 23 and 4 kHz, respectively, while nDEP remained dominant for dead cells up to 41 and 6 kHz, respectively. No dielectrophoresis was observed in $10^{-2}$ PBS (Figure 4). Because the largest range of frequencies under which separation could be observed was detected with $10^{-3}$ PBS, this media was used exclusively for subsequent VBNC studies.

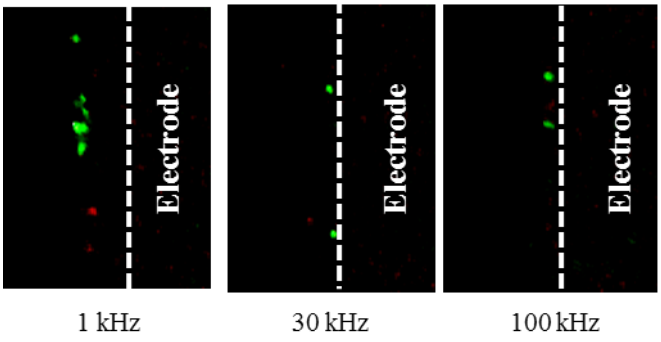


Figure 4. Dielectrophoresis of live and dead bacteria in $10^{-3}$ PBS. At 1 kHz, both live and dead bacteria were repelled away from the electrode edge (white dotted line), indicative of dominating nDEP forces. At 30 kHz, dead bacteria remained under nDEP forces while live bacteria were attracted to the electrode edge (pDEP). At 100 kHz, both live and dead bacteria were subjected to pDEP forces.

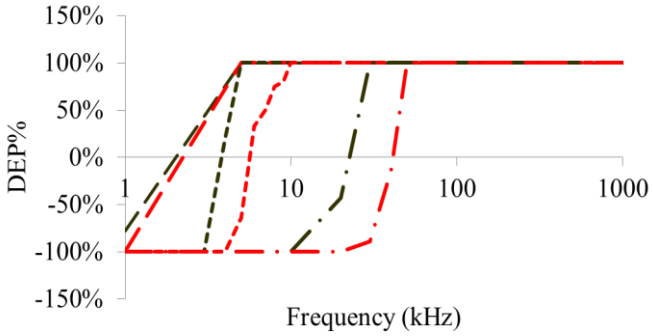


Figure 5. Dielectrophoretic behavior of live (green) and dead (red) cells for $10^{-5}$ (— —), $10^{-4}$ (- -), and $10^{-3}$ (— · —) PBS. DEP% less than zero is indicative of nDEP whereas DEP% greater than zero suggests pDEP.

$CuSO_4$-induced VBNC suspended in $10^{-3}$ PBS behaved almost identically to live cells, with nDEP observed for lower frequencies and pDEP occurring at frequencies above 20 kHz (Figures 6 and 7). This initial data suggests that dielectrophoresis may offer a simple and predictive mechanism for isolation of viable cells regardless of culturability.

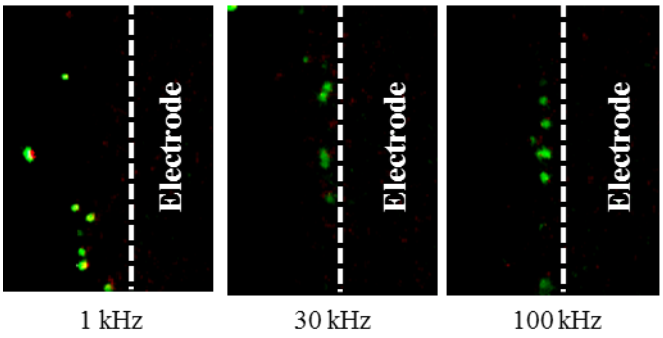


Figure 6. Dielectrophoresis of VBNC. Similar to live bacteria, VBNC bacteria displayed nDEP at 1 kHz and pDEP for frequencies greater than 20 kHz.

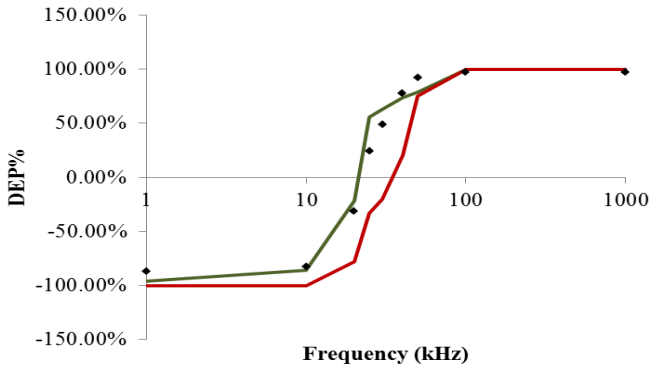


Figure 7. DEP% for both live (green) and dead (red) bacteria was negative in the range of 1-10 kHz. The crossover frequencies for live and dead bacteria were 23 kHz and 41 kHz, respectively. Above the crossover, all bacteria were positively attracted to the electrode edge. Dyanmics and crossover frequenices of VBNC species ( • ) were consistant with those of live bacteria.

## 4 CONCLUSIONS

The VBNC state, determined by comparison of culture and membrane integrity, was successfully induced by incubation in 500 µM $CuSO_4$. Bacteria in this state were determined undetectable by culture but were successfully resuscitated to initial concentration. Upon determining the optimal conditions for cell separation, an assessment of the dielectrophoretic behavior of VBNC cells revealed behavior most similar to that of live cells. Based on this data, we suggest that dielectrophoresis may be a reliable and more suitable mechanism for viable cell isolation, especially where concerns of VBNC presence are of issue. Although further trials will be necessary to explore the breadth to which these results are applicable, this initial report shows promise for this technological application.

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