A Dielectrophoresis-activated Multi-Well Plate for Label-Free High-Throughput Drug Assessment

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ABSTRACT

Dielectrophoresis (DEP) offers many advantages over conventional cell assays such as flow cytometry and patch clamp techniques for assessing cell electrophysiology as a marker for cancer studies and drug interaction assessment. However, despite the advantages offered by DEP analysis, uptake has been low, remaining largely in the academic arena, due to the process of analysis being time-consuming, laborious and ultimately allowing only serial analysis on small numbers of cells. In this paper we describe a new method of performing DEP analysis based on laminate manufacturing methods. These use a 3D "well" structure similar in size and pitch to conventional microtiter well plates, but offer electrodes along the inner surface to allow easy measurement of cell properties through the whole population. The result can then be determined rapidly using a conventional well-plate reader. The nature of the device means that many electrodes, each containing a separate sample, can be tested in parallel, whilst the mode of observation means that analysis can be combined with simultaneous measurement of conventional fluorimetric well-based assays. Here we benchmark the device against standard DEP assays, then show how such a device can be used (a) to rapidly determine the effects both of ion channel blockers on cancer cells and antibiotics on bacteria, and (b) to determine the properties of multiple subpopulations of cells within a well simultaneously.

1. Introduction

One of the key technologies in high throughput drug assaying is the use of multi-well plates that can perform a large number of experiments in parallel. For cell-based high-throughput screening, a model organism is placed in all wells, while a different drug candidate is added to each single well. By means of a marker, such as pH indicators or fluorescence markers, it is established if a candidate drug interacts with the model organism in the desired way. Since a large number of experiments can be performed and compared simultaneously, well plates enable the screening of large compound libraries for suitable drug candidates.

One challenge in high-throughput screening is finding suitable assay markers that do not interact with the model organism and are easy to evaluate in a small sample volume. One method that eliminates the need for chemical markers is *dielectrophoresis* (DEP). DEP is the induced motion of particles in non-uniform electric fields. This technique can be applied to the manipulation, separation and analysis of cellular and viral particles (1-3). Particles experiencing such forces can be made to exhibit a variety of motions including attraction to, and repulsion from, regions of high electric field (termed positive and negative DEP respectively) by changing the frequency of the applied electric field. Since bio-particles such as bacteria, cells or viruses have characteristic dielectric properties, dielectrophoresis can be used to distinguish between different types of bacteria, to detect changes in cell cytoplasmic properties, and to detect whether cells are viable or non-viable. The main factors influencing the dielectric properties of a bioparticle are the surface charge, the membrane capacitance and the conductivity of the cytoplasm. If drugs such as antibiotics change any of these factors, the dielectric properties of the cell change and can be detected. This change can be used to distinguish between cells that are resistant or sensitive to a drug, for example (4).

By exploiting the fact that different particles may experience forces acting in different directions when all other factors are the same, researchers have been able to use DEP to analyse and separate mixtures of cells on electrode arrays. For example, work has demonstrated that DEP can be used to examine the effect of drugs on cells, such as the effect of nystatin on erythrocytes (5) or the response of

neutrophils to activation by chemotactic factors (6). Since populations of particles may experience forces acting in different directions within specific frequency windows, separation has been demonstrated for mixtures of viable and non-viable yeast cells (7), and CD34+ cells from bone marrow, which can be separated from human blood (8). Other demonstrations of cell separation have been made in separating breast cancer cells from blood, erythrocytes with and without malarial parasite infection, B- and T- lymphocytes, and different types of viruses in solution (9-12). Studies using bacteria (4), algae (13), cancer cells (14, 15) have demonstrated that DEP can be used to detect the manner in which a drug interacts with a bio-particle. This makes DEP a very valuable tool for screening applications. Previous DEP studies have used electric fields generated by planar (effectively 2D) electrodes etched from gold across the surface of a microscope slide. However, these conventional dielectrophoretic chips are not well suited for large numbers of parallel experiments due to the complex nature of electrode fabrication, the very low effective volumes in which DEP is effective (of the order of nanolitres), and the difficulty in making simple observations of many cells (which are typically microscope-based). An automated measurement system has recently been developed that use "crossover" measurements to estimate the dielectric properties of cells by image processing (16) but the system can only perform serial experiments (i.e. one drug candidate at a time) and its output is limited to the electrical properties of the membrane.

In this paper, we describe the implementation of a new form of DEP device that provides simple measurements of large numbers of cells, whilst also allowing a high degree of parallelisation in a format directly compatible with current multi-well standards for both plate readers and cell automation. The new technology (DEP-Well) uses a conductor/composite laminate to construct electrodes with similar dimensions, but which can be structured around the walls of a hollow cylinder. DEP-Well technology is based on a laminate of alternating layers of insulating and conducting material. Conducting layers are connected to opposing phases of a signal generator. The layers are patterned to connect to different vertical via-hole connections of the device depending on the connection to phase or ground and the signal is input to the via-hole, connection all electrodes of the same polarity. Channels are drilled

through the laminate, and the walls of the resulting wells have electrodes of alternate potential separated by insulating layers all along the wall, so that each hole is equivalent to a "rolled-up" two-dimensional electrode array. This arrangement encloses a much larger volume than planar electrodes on a similar scale, and a large number of holes can be drilled through a single laminate to form multi-well plates. Originally developed for flow-through applications (17), we have developed an advanced version with a base enabling the measurement of DEP force of the population in the well (18). The advantage of DEP-Well technology is the dramatic increase in electrode area around a given volume; in a well 3mm deep and 0.7mm diameter, can process a volume of more than 1μL with a total electrode area of more than 6.5mm^2; some 20 times larger than for conventional DEP electrodes with a similar footprint. Furthermore, as the DEP-Well devices are effective in a much larger volume than conventional DEP arrays, and can be constructed in parallel, the throughput of the device is much higher. Finally, the radial symmetry of the electric field within the device simplifies the tools needed to perform analysis of DEP behaviour considerably, so that measurements can be made using a standard well-plate reader. Together with the possibility of low cost fabrication for DEP-Well devices, the technology allows large numbers of experiments to be performed in parallel on well-plate systems.

2. Method

The method used to measure the DEP effect is an absorption measurement technique similar to those found in many conventional well-plate readers (and shown schematically in Figure 1A). When the electrodes are energized, particles are either pulled from the centre of the well by positive DEP, or repelled into the center of the well by negative DEP. In the former case, the evacuation of particles from the well reduces the absorption along the center of the well, whist the particles collected at the wall disappear from the field of vision. In the case of negative DEP the reverse is true, as particles are pushed from the edges and collect in the centre. Although the wells are typically about 1mm in diameter, the thickness of the electrode layers (17μm), and spacing between them (75μm), are comparable to electrode dimensions made by conventional photolithographic methods. A light source is

used to illuminate the well, which is then observed from the opposite side of the well (systems have been shown to be effective with both upright and inverted microscopes). When the electric field is applied, particles are either pulled from the visible region of the well, causing the number of particles at the well center (and hence the light absorbance) to be reduced, or particles are pushed from the well wall causing light absorbance of the well to be increased. By quantifying these changes in light intensity due to the redistribution of particles, it is possible to rapidly determine both the magnitude and direction of the particles in the well. In cases of small cells such as bacteria, the field gradient is insufficient to move particles from the center of the well, such that the center region does not change absorbance. Whilst measuring the absorbance from the whole well can give good results, it is possible to reduce noise by specific evaluation the change in particle concentration in the outer 30% of the radius of the well. This region contains the particles nearest the well (and the electrodes); as the volume scales as the square of the radius, this region contains 64% of the total volume of the well.

We have developed well plates on two footprints, as shown in Figure 1B. The first, termed the "spectra-chip", consists of 80 wells grouped into 20 clusters of four wells each. The clusters have common-connected ground planes interleaved with independent power rails, allowing different frequencies to be applied to each cluster (arranged as 19 separate frequencies plus a control cluster). The second format, the "spectra-plate", consists of 1536 wells arranged in clusters of 16, 32 and 64 in different regions of the plate to provide different potential functions (such as spectrum determination on 16-well clusters or simple binary testing on larger clusters), and which fits a standard 1536-well format. Both formats share common construction details, including identical well construction methods and well operability.

Plates were constructed of twelve layers of 17μm-thick copper interleaved with 75μm-thick polyimide, through which wells were drilled with 2.25mm spacing between adjacent well centres. Well diameters between 0.8-1.2mm have been investigated. The well base consisted of a 1mm polyimide sheet, which was bonded to the structure by using high-temperature-curing resin. All exposed copper

surfaces were gold-plated to ensure biocompatibility. The thermal characteristics of the materials were chosen to ensure that the plates and chips are suitable for autoclaving.

In order to perform the analysis, the activity in each well was monitored through a Nikon Eclipse E400 microscope (Nikon, Tokyo, Japan) fitted with an AVT Dolphin camera (Stadtrota, Germany). The total light intensity across each well was determined by recording the light transmitted through the well on a microscope equipped with an AVT Dolphin video camera (Stadtroda, Germany). The change in intensity was determined using a MATLAB (The Mathworks Inc, Natick, USA) script that compared the average greyscale value of the well before the field was applied to the value after the field was applied. To allow evaluation of the change over time an image was recorded every 5 sec. Parameters measured included the maximum and minimum change in light intensity across the radius of the well, as well as the rate of change of light intensity along the radius, and the net change across the whole well. Of these parameters, the net change provided the most accurate match to predictions derived from existing dielectrophoretic data. Wells were typically energised with a $10V_{pk-pk}$ signal over the range 10kHz-20MHz at 5 points per decade, with each point typically being analysed for two minutes. Fluid motion due to heating effects and electro-osmotic fluid flow were observed to increase as the applied frequency approached 1kHz; becoming disruptive to data acquisition below 4kHz. To ensure reliable data collection, 10kHz was chosen as the lower limit for experiments.

To ensure DEP-Well plates are compatible with standard well plate readers, the 1536 spectra-plate prototype was monitored using a Tecan (Zurich, Switzerland) Safire II well plate reader. Reading spectra-plates using a commercial well-plate reader is advantageous since it eliminates the need for specialist hardware for data collection; in order to integrate spectra-plates into a commercial plate reader system, the only requirement is an extra electronics module added to the reader in order to generate the signals required for DEP analysis.

As the spectra-plate is thinner than commercial well plates, plates were mounted on the empty frame of a 96-well plate with the wells removed so that only the outer rim remained. The margins of the plate were protected with high temperature Kapton tape. The frame was then glued to the tape using epoxy

glue. The plates were tested in absorbance mode. The plates were initially mapped as a commercial 1536 well plate with increased thickness. A plate with 1.2mm-diameter wells was analysed using two light beam widths (1000μm and 1500μm), allowing the effect of alignment of the plate to be examined. In order to calibrate the plate, the base underneath some wells was darkened with black permanent marker, which was successfully recovered by the reader. As the wider light beam (1500μm) had a slightly larger beam diameter then the well, alignment is less critical as the beam always overlaps the well. Since the plate always absorbs a part of the wider beam, the absorbance values should be slightly higher with the wider beam. However, when the two methods were compared, the results indicated an overall increase of absorption of only 2% caused by the larger beam being absorbed by the board. When the absorption measurements were repeated a number of times, the standard deviation was only 1.25%, which is well within acceptable limits.

3. Results

3.1 Standard Yeast Assay

For a number of years, the yeast cell has been used as a de facto standard cell type for DEP characterisation (e.g. 19-23). In order to benchmark the DEP-Well system, the well plates were used to characterise a suspension of yeast cells (*S. cerevisae*) resuspended in a solution consisting of 280mM mannitol solution adjusted to a final conductivity of $3mSm^{-1}$ by adding small amounts of 100mM phosphate buffered saline (0.01 M phosphate buffer, 2.7mM KCl, 137mM NaCl, pH 7.4), and a cell concentration of 10^8 ml^{-1}. The experiment was then repeated using yeast cells that were first heat-treated at 80°C for 5 minutes prior to analysis. The solutions were inserted into the electrode wells and energized with a $10V_{pk-pk}$ signal over the range of frequencies from 5kHz-20MHz at 5 points per decade.

Figure 2a shows the change in light intensity (points) scaled to fit a DEP spectrum generated for yeast using data published by Huang et al. (19) (line), together with the same curve for non-viable yeast. As can be seen, the well-plate method provides an excellent match to published data, with slight differences in numbers potentially being accounted for by differences in yeast strain or culture conditions being used

in our experiments as compared to those in the published data. A summary of these parameters is shown in Table 1. Note that there is a single outlier point in the spectrum of dead yeast at 250kHz; this occasional phenomenon is caused by the appearance or movement of bubbles in the well. Such events can easily be detected by image processing due to the wide variation in light intensity around individual rings, enabling rogue points to be identified and repeated.

As with the other sets of experimental data presented in this paper, the data presented show the results from a single experimental run; whilst all experiments were repeated a number of times, the accuracy of the measurement system means that single-run data is useable without need for averaging.

3.2. Effect of chemical agents on Jurkat cells and bacteria

One of the principal requirements of high-throughput electrophysiology is the investigation of the effects of drugs such as ion channel blockers. In order to test the DEP-Well system for this, we treated Jurkat leukaemic lymphoblasts with three ion channel blockers to observe the changes in cell electrical properties. Three ion channel blockers were selected; quinine, verapamil and NPPB, which block channels relating to the transport of potassium, calcium and chloride respectively.

Experiments were performed to investigate the effects of quinine, verapamil and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) on human leukaemic T cell lymphoblast (Jurkat E6.1) cells. Jurkat cells were grown in RPMI-1640 medium that was supplemented with 10% heat-inactivated foetal bovine serum, 2 μM ml^{-1} glutamine, and 100 units mL^{-1} penicillin-streptomycin. Cell lines were cultured in a humidified incubator with 5% CO_2 and 95% air at 37°C. All cell culture reagents were obtained from Invitrogen, Paisley, UK. For the ion channel blocker treatment, the cell concentration was adjusted to 10^6 cells mL^{-1}. One aliquot was left untreated; the others were incubated for 2 h with either 0.1 mM Quinine, 20 μM Verapamil or 10 μM NPPB. Afterwards the cells suspensions were centrifuged at 500 g for 3 min, washed and resuspended in a conductivity medium consisting of 8.5% (w/v) sucrose plus 0.3% (w/v) glucose. A conductivity of 3 mS m^{-1} was achieved by adding small amounts of phosphate buffered saline (composition as before). The cells were analysed using the DEP-Well system and the

resulting frequency-response spectra were analysed using a two-shell model; a plot of the high-frequency best-fit data is shown in Figure 3A. Solvent controls were performed (not shown) and showed no difference to the control data.

Cell modelling indicated that NPPB and Verapamil decrease the cytoplasmic conductivity from 0.7 Sm^{-1} to 0.4 Sm^{-1} and 0.45 Sm^{-1} respectively while Quinine increases the cytoplasmic conductivity to 1.1 Sm^{-1}. Ion transport can be either passive due to diffusion through ion channels along the concentration gradient or active through ion pumps against the concentration gradient under the consumption of energy. Verapamil blocks calcium channels and prevents calcium from moving through those channels from the outside of the cell into the cytosol of the cell. At the same time, active Ca^{2+} pumps transport Ca^{2+} out of the cell, decreasing the concentration of calcium, leading to the drop in cytoplasmic conductivity that was measured by DEP. In a healthy cell, the intracellular concentration of K^+ is 140 mM, whilst the extracellular concentration is 5 mM, leading to K^+ diffusing out of the cell through the ion channels. The Na^+-K^+ pump transports potassium into the cell in exchange for pumping sodium out of the cell. Once the K^+ ion channels are blocked by quinine, K^+ ions cannot diffuse out of the cell any more and the ion concentration increases. This increase in cytoplasmic conductivity can be seen in the dielectrophoretic spectrum. In order to control the cytosolic pH, a Na^+-driven Cl^--HCO_3^- exchanger couples the influx of Na^+ and HCO_3^- to an efflux of Cl^- and H^+. Once the Cl^- channels are blocked using NPPB, the concentration of Cl^- keeps decreasing due to the ion exchanger pumping Cl^- out of the cell.

In order to examine the effect of cell cise on the ability of the system to detect cellular changes due to drug action, *E. Coli* was analysed before and after treatment with the antibiotic Polymyxin B. A colony was taken from an agar plate using a wire loop and cultured for 18h in nutrient broth no. 3 (Sigma Aldrich, UK). The culture was split to keep approximately half of the culture as a control sample, while the other half was treated for 4h with 40μg/mL Polymyxin B (Sigma Aldrich, UK). 3mL of the culture was centrifuged at 6000g for 3 min and the pellet was re-suspended in 1 mL of 280 mM mannitol (Sigma Aldrich, UK) adjusted to an electric conductivity of 5mSm^{-1} by adding a small amount of phosphate buffered saline. This was repeated twice to wash off the culture medium. Since the change in

light intensity in the well was normalised against the light intensity prior to energising the electrodes, any change in cell number due to the action of Polymyxin B would have no change on the efficacy of the system.

The strength of the DEP force depends on the size of the particle, and so smaller particles such as bacteria are only affected closer to the wall of the well. Therefore only a ring covering the outer 40% of the well radius was analysed. The resultant spectra are shown in figure 3B. The primary change in electrophysiological properties between the control and the treated sample indicated that the Polymyxin B has disrupted the membrane of the *E. coli,* causing a reduction in cytoplasmic conductivity from $0.35Sm^{-1}$ to $0.05Sm^{-1}$ indicating the antibiotic has interfered with the membrane function. This technique has proven demonstrably faster than conventional methods of the determination of antibiotic activity.

3.3. Recognition of multiple populations

One significant advantage of the DEP-Well technique is the ability to discriminate between different populations on the basis of their dielectric properties. Work by Broche et al. (20) indicated that multiple subpopulations of cells could be identified within a DEP spectrum by identifying multiple dispersions, an approach later used to identify cells in different stages of apoptosis (24). Whilst this approach was developed for positive DEP analysis only, our studies have shown that the same approach can be used to detect multiple subpopulations within the wells. In order to demonstrate this, viable and non-viable yeast cells similar to those described in section 4.1 were mixed so that the solution contained known, fixed ratios of cell concentrations. Concentrations were determined after heat treatment to ensure the cell ratios were correct at times of mixing. The mixture was then analysed using the DEP-Well system, and curves fitted to the resulting spectra in order to verify the efficacy of multiple population extraction. Specifically, the data were fitted to the models of live and dead yeast determined in section 4.1, with the key best-fit parameter being the ratio of these two spectra required in order to fit the data. Graphs showing the net DEP response of 9:1, 1:1 and 1:9 mixtures of live:dead cells be seen

in figure 4. The system was found to be capable of identifying the two populations accurately; once identified, it is possible to then and extract their characteristics using the same methods outlined in section 3.1. When the ratios of the two superimposed spectra were determined, it was found that the system could predict the premixed ratios with 1% accuracy, except where the ratio was large (9:1) where it typically overestimated the smaller population by up to 5%. This demonstrates that the DEP-Well system is capable not only of identifying these separate populations, but of extracting their properties as well. This is of great significance for assays where an agent transforms the cells in some way, and the degree to which this has occurred needs to be determined; examples of this include the measurement of cell toxicity (the IC50 assay) and measurement of the progress of cell death by apoptosis.

4. Conclusion

The well-plate method presented here offers many advantages over conventional, serial DEP analysis. If cells are well-characterised (such as the yeast cells in our example) so that the differences between stressed and unstressed cells are known then the determination of the effects of drugs on a cell sample can be made using the application of a single frequency, across an entire well plate. Such an approach is particularly effective in conditions where the unaffected population experiences positive dielectrophoresis whilst the affected population experiences negative dielectrophoresis, or vice versa. Alternatively, different areas of multi-well plates can be energised with different frequencies in order to determine the complete spectra of many cell lines simultaneously.

In conclusion, we have developed a method of bringing DEP analysis directly to high-throughout screening and rapid assay applications, using a low-cost and mass-producible technology allowing the simultaneous analysis of many populations of cells. This finally allows DEP to be used as a method for the extraction of vital biophysical data for drug discovery and analysis in applications throughout the biotechnological sector.

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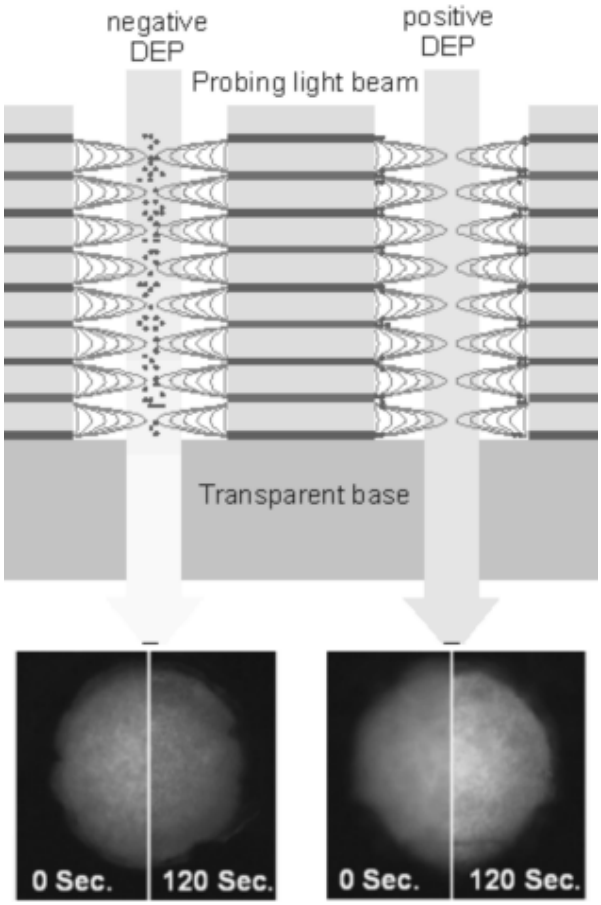
Figures:



Figures 1: The DEP-Well system allows DEP analysis to be applied to multi-well assays. (A) The electrodes are energised at a range of frequencies, eliciting a response of particles contained within the well of either positive or negative DEP. The intensity of light passing through the well indicates the magnitude and sign of the force. (B) The DEP-Well formats devised are (i) the smaller spectra-chip(size 37x23.5mm) which can be energised by up to 19 parallel frequencies on four wells each, and (ii) the larger 1536-well plate (size 127x86mm) on a standard well plate template. (C) Close inspection of the inside of a 1.2mm-diameter well shows the gold-plated conducting electrode “stripes” that surround the inside of each well.

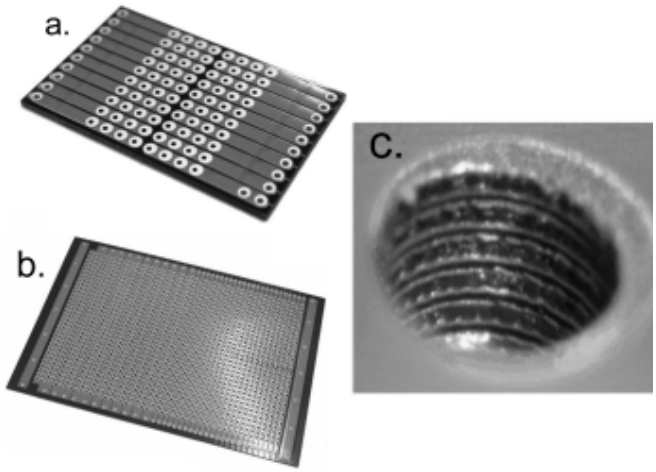


Figure 2. DEP-Well spectra of (a) viable and (b) non-viable, heat-treated yeast cells suspended in a 280mM mannitol solution adjusted to 3 mSm^{-1} by adding phosphate buffered saline. The dots represent data recorded by the DEP-Well system; the line indicates predicted behaviour according to data published by Huang et al [1992].

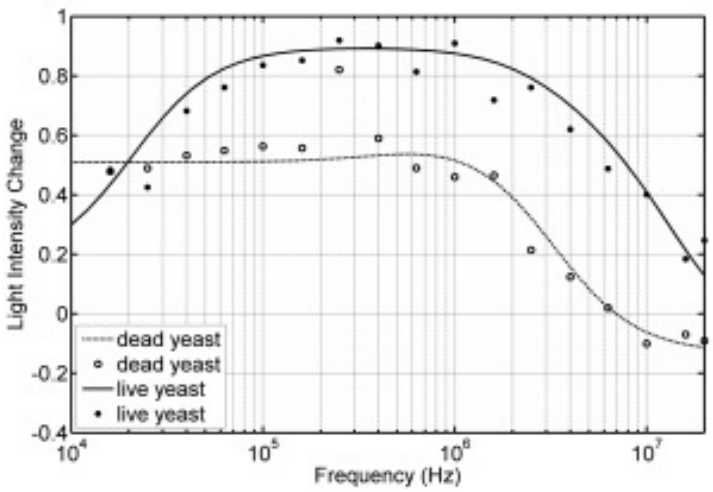


Figure 3. Using the DEP-Well system to observe the effects of chemical agents on cells. (A) Jurkat cells were treated for 2 h with several ion channel blockers. The changes in the dielectrophoretic response at high frequencies indicate how the cytoplasmic conductivity of the cells changes in comparison to a control sample.(B) DEP-Well spectra of *E. Coli* before and after 4h treatment with 40µg/mL Polymyxin B and suspended in a 280nM mannitol solution adjusted to 5 mS/m by adding phosphate buffered saline. The downwards shift in the response at high frequencies indicates a loss of cytoplasm conductivity whilst the similar shift at low frequencies indicates that the membrane conductivity has increased.

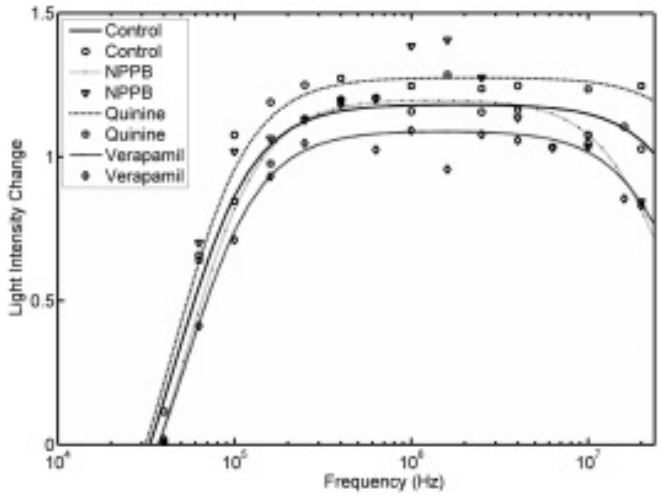
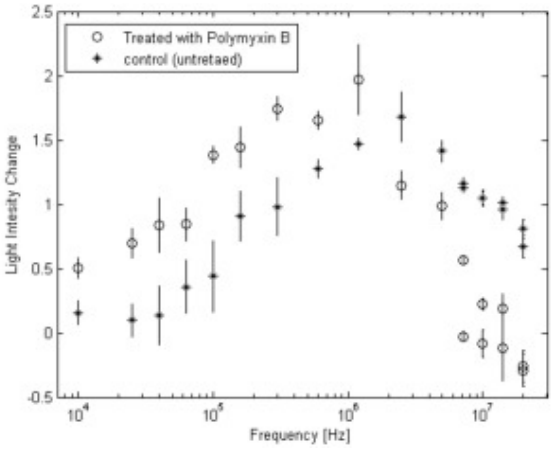


Figure 4. Defined mixtures of viable and non-viable yeast were made up and frequency-response spectra were recorded using DEP. Examples of the recorded spectra are shown for ratios of (a) 10:90 (b) 50:50 and (c) 90:10 dead-live yeast respectively. The best fit lines were determined by fitting the sum of the two best-fit lines in figure 2. The magnitudes of the scaling factors for the two populations were then used to determine the viability of the cell population.

Figure 5



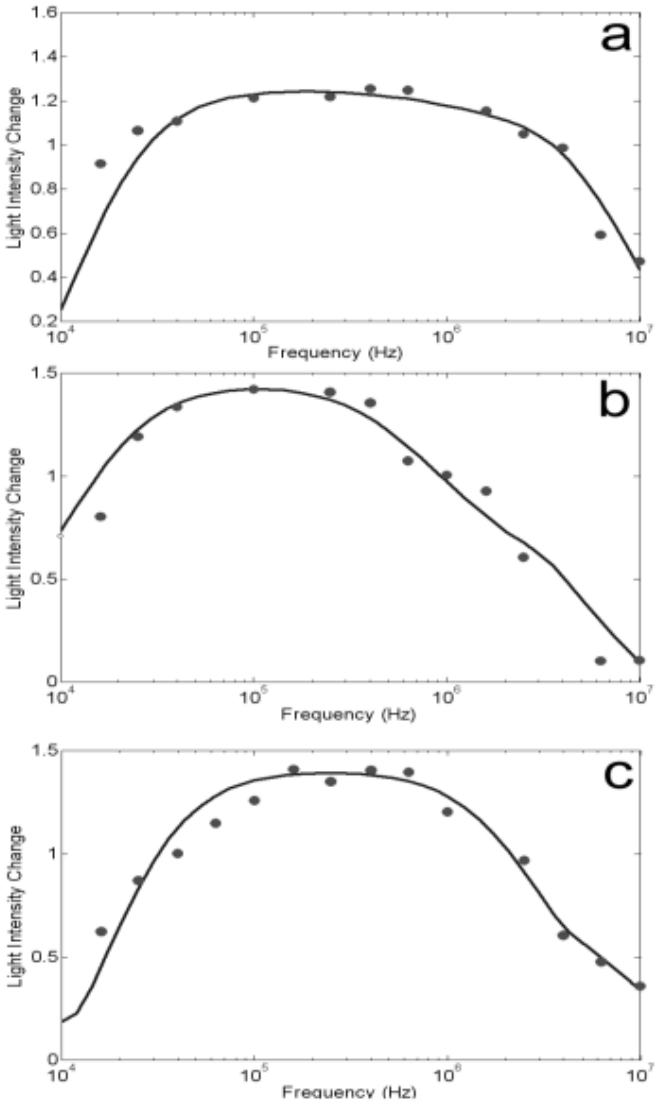

a
b
c
Light Intensity Change
Frequency (Hz)
Frequency (Hz)
Frequency (Hz)

Figure 6

TABLE CAPTION

1: Dielectric properties of viable and non-viable yeast determined using well electrodes, compared to literature values [19].

	Non-viable		Viable	
Parameter	Well data	Literature	Well data	Literature
Cytoplasmic Conductivity (Sm^{-1})	7.00E-03	7.00E-03	0.16	0.2
Cytoplasmic Relative Permittivity	50	50	50	50
Conductivity of Membrane (Sm^{-1})	1.60E-04	1.60E-04	2.50E-07	2.50E-07
Relative Permittivity of Membrane	6	6	6	6
Conductivity of Cell Wall (Sm^{-1})	1.50E-03	1.50E-03	1.40E-02	1.40E-02
Relative Permittivity of Cell Wall	60	60	60	60
Radius of Cell (m)	3.50E-06	3.50E-06	4.00E-06	4.00E-06
Membrane Thickness (m)	8.00E-09	8.00E-09	8.00E-09	8.00E-09
Cell Wall Thickness (m)	2.50E-07	2.50E-07	2.20E-07	2.20E-07

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