Published in final edited form as:

Sens Actuators B Chem. 2018 December 10; 276: 472–480. doi:10.1016/j.snb.2018.08.137.

Single-cell electro-phenotyping for rapid assessment of *Clostridium difficile* heterogeneity under vancomycin treatment at sub-MIC (minimum inhibitory concentration) levels

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Abstract

Current methods for measurement of antibiotic susceptibility of pathogenic bacteria are highly reliant on microbial culture, which is time consuming (requires > 16 hours), especially at near minimum inhibitory concentration (MIC) levels of the antibiotic. We present the use of single-cell electrophysiology-based microbiological analysis for rapid phenotypic identification of antibiotic susceptibility at near-MIC levels, without the need for microbial culture. *Clostridium difficile* (*C. difficile*) is the single most common cause of antibiotic-induced enteric infection and disease recurrence is common after antibiotic treatments to suppress the pathogen. Herein, we show that de-activation of *C. difficile* after MIC-level vancomycin treatment, as validated by microbiological growth assays, can be ascertained rapidly by measuring alterations to the microbial cytoplasmic conductivity that is gauged by the level of positive dielectrophoresis (pDEP) and the frequency spectra for co-field electro-rotation (ROT). Furthermore, this single-cell electrophysiology technique can rapidly identify and quantify the live *C. difficile* subpopulation after vancomycin treatment at sub-MIC levels, whereas methods based on measurement of the secreted metabolite toxin or the microbiological growth rate can identify this persistent *C. difficile* subpopulation only after 24 hours of microbial culture, without any ability to quantify the subpopulation. The application of multiplexed versions of this technique is envisioned for antibiotic susceptibility screening.

Graphical Abstract

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Keywords

single-cell analysis; bacteria; antibiotic; minimum inhibitory concentration; dielectrophoresis; electrorotation

1. Introduction

The global rise of antimicrobial resistant bacterial strains and antibiotic persistence has spurred an urgent need for technologies capable of rapidly screening susceptibility of particular strains to key antibiotics, preferably without requiring time consuming microbial culture steps. The unavailability of such technologies, especially within point-of-care or resource-poor settings, has been directly implicated in the widespread prescription of broad-spectrum antibiotics [1], since current methods based on microbial culture to assess antibiotic susceptibility require 48–72 hours to go from sample to answer [2]. While conventional microbiological assays, including broth dilution, e-test strips, and disk diffusion [3] are highly reliable indicators of antibiotic susceptibility, their use of turbidity to measure microbial growth, limits their sensitivity to 10^7 CFU/mL levels of bacterial sample and growth times of 16–20 hours are required for detectable signals [4]. Molecular methods based on amplification of genomic DNA that are essential for the purpose of screening during infectious outbreaks [5] are not practical for broad-spectrum screening, since the genetic markers for emerging antibiotic-resistant strains are not often known, while the high frequency of genetic mutation under selective environmental pressure causes genetic markers to be less reliable [6], with mutations that may not be well-correlated to antibiotic susceptibility [7], and with combinatorial small effects of resistance genes that are difficult to measure [8]. While antibiotic susceptibility assessment should be possible based on monitoring alterations in microbial phenotypic properties, the sensitivity and specificity of these techniques in identifying and isolating persistent microbial subpopulations after antibiotic treatment, at levels close to their minimum inhibitory concentration (MIC) has not been demonstrated.

Various microfluidic platforms have been implemented to track antibiotic susceptibility of microbials by using culture-dependent and culture-independent methods. In this context, electrophysiology-based methods are especially powerful since electrical polarization is highly sensitive to size and shape variations of the microbial cell, while different subcellular regions, such as the cell wall, membrane and cytoplasm respond within different frequency ranges to enable distinction of the cell region affected by the antibiotic in a culture-independent manner [9]. Additionally, the altered polarization frequency response can be used towards dielectrophoretic (DEP) isolation of live microbial subpopulations after critical

levels of particular antibiotics, for downstream analysis. In prior work, antibiotic-sensitive versus antibiotic-resistant strains of *Staphylococcus epidermidis* [10], as well as those of *E.coli* [11] were distinguished based on conductivity differences of their cell wall region after antibiotic treatment, as deduced by their dielectrophoretic collection spectra. In another study [12], the gentamicin resistant *Staphylococcus epidermidis* strain was reported to have substantially lower dielectrophoretic mobility under DC fields versus the respective susceptible strain. In another study of great microbiological relevance [13], dielectrophoretic focusing enabled highly sensitive optical monitoring of *E.coli* for polymyxin susceptibility, which enabled multiplexing and automated parallel testing of multiple drugs. In other studies on *Mycobacterium smegmatis* [14], [15], the low frequency DEP spectra where cell wall characteristics dominate, were used to show distinct differences in conductivity for active versus dormant and non-viable strains. Since microbial cells can undergo either elongation or swelling after treatment with various antibiotics [16], [17], the respective shape changes interfere with image-based detection of cell division [18]. However, these shape changes can be sensitively probed based on electrical polarization [19]. In a recent study, the susceptibility of *E. coli* to the antibiotic Cephalexin [20] and of several gram-negative bacteria to β -lactam antibiotics [21] were tracked by using positive DEP (pDEP) to fix bacterial cells at well-defined positions along the focal plane for enabling facile and accurate estimation of cell length and elongation rate. While there has been some prior work on antibiotic susceptibility analysis by dielectrophoresis [22–24], this work has not been performed at near-MIC antibiotic levels, for determination of alterations to the electrophysiological phenotype within a few hours of treatment, as performed in the current work using single-microbial phenotyping.

The persistence of a small fraction of microbials to antibiotics has been attributed to heterogeneity in microbial responses at critical antibiotic levels [25]. Hence, subpopulations can emerge even though the starting sample is an isogenic population [26]. *Clostridium difficile* (*C. difficile*) infection (CDI) is a toxin mediated intestinal disease [27], which is the single most common cause of antibiotic-induced enteric infection associated with increased morbidity and in-hospital fatalities [28]. Metronidazole and vancomycin are the most commonly used antibiotics for CDI treatments, but patients often experience disease recurrence after standard antibiotic treatments [29] and a great majority of patients with suppressed *C. difficile* levels during antibiotic treatments were identified as asymptomatic carriers of *C. difficile* within weeks of completion of the treatment [30]. Additionally, some *C. difficile* strains have demonstrated resistance to metronidazole [31]. Traditional metrics to quantify antibiotic susceptibility based on the half maximal inhibitory concentration (IC50) or minimum inhibitory concentration (MIC) are end-point measurements, which are not suited towards detecting heterogeneity in responses within a fluctuating environment. Singlecell techniques, on the other hand, for electrophysiology-based phenotypic analysis within a droplet [32] or by using impedance [33], [34], [35], or electro-rotational analysis [36], [37], are capable of quantifying subpopulations, but they have not been applied towards studying microbial heterogeneity after antibiotic treatments. In prior work, we have demonstrated that *C. difficile* cell wall capacitance measurements can identify subtle alterations in S-layer glycoproteins, which can occur due to strain-based morphological differences [38] or due to cell wall reconstitution under probiotic interactions [39], and

which are correlated to the colonization ability of *C. difficile* [40]. In the current study, we demonstrate that alterations in cytoplasmic conductivity gauged based on the pDEP level and quantified using single-cell electro-rotational (ROT) analysis can serve as a rapid, sensitive and specific means to quantify the persistent live *C. difficile* subpopulation after sub-MIC level vancomycin treatment, whereas measurement of the microbiological growth rate or secreted metabolite toxin requires 24 hours of culture to identify this persistent *C. difficile* subpopulation, with no ability for its quantification.

2. Experimental

2.1. Microbial culture and antibiotic treatment

Highly toxigenic *C. difficile* (HTCD) VPI 10463, low toxigenic (LTCD) ATCC 630, and nontoxigenic (NTCD) VPI 11186 strains (ATCC, Manassas, VA) were cultured in brain heart infusion (BHI) broth (BD BBL Brain Heart Infusion) at 37°C for 20 hours under anaerobic conditions. Antibiotic treatment at 1 mg/L and 2 mg/L vancomycin levels (note: mg/L equals µg/mL) was performed on the HTCD strain with $\sim 10^6$ cells/mL in the culture media, for a period of up to 4 hours to ensure complete disinfection. Strain growth following antibiotic treatment was measured based on absorbance at 600 nm (in triplicate) using a spectrophotometer (Eppendorf AG, Germany) at 0, 4, 8, and 24 hours post-treatment. Toxin production was determined using a *C. difficile* TOX A/B II ELISA (Techlab, Blacksburg, VA) as per user instructions and absorbance (at 450 nm–620 nm) on the Epoch microplate reader (BioTek, Winooski, VT). Two-way ANOVA with Tukey's multiple comparison test were performed with Prism (Graphpad, San Diego, CA).

2.1. Dielectrophoresis and electro-rotation measurements

Following vancomycin treatment for 4 hours, the BHI broth was replaced with 8.8% sucrose water and re-adjusted with BHI broth to optimize the medium conductivity at $105 \pm 5\%$ mS/m for electro-rotation (ROT) and dielectrophoresis (DEP) analysis. For ROT experiments, a PDMS (Poly-di-methyl-siloxane) microfluidic chamber with inlet and outlet tubing connected to flow control systems was bonded on a glass chip that was pre-patterned with planar hyperbolic-shaped gold electrodes arranged within a quadrupole pattern. Electrical connections were enabled using a printed circuit board with pins for independent energization of electrodes (0.1–40 MHz). A four-channel arbitrary waveform generator (TGA12104–Thurlby Thandar Instruments) was programmed to apply 90 degree phase shifted signals to adjacent electrodes. Highly parallelized electrorotational measurements[41] of ~ 20 *C. difficile* cells per chip were observed under Zeiss Z1 microscope and recorded by Hamamatsu CMOS camera at 100 fps (frames per second). The average rotation rate at each frequency was obtained by intensity thresholding [42] to identify cell edges for analyzing 80–100 cells per treatment group or strain. All the rotation rates were normalized with respect to the maximum rotation rate of untreated *C. difficile*, for comparison purposes, while the model fits were performed using rpm of the rotation rates. For dielectrophoresis (DEP) measurements, PDMS micromolding was used to fabricate microchannels with sharp lateral constrictions (1000 µm to 15 µm) and bonded to a glass coverslip for easy microscopic viewing of DEP behavior. Using Pt electrodes at the inlet and outlet, AC fields were applied over a wide-frequency range (50 kHz–1 MHz) by utilizing a

power amplifier [43] for particle trapping towards or away from high field points at the constriction tips. The trajectory of the unlabeled *C. difficile* after each antibiotic treatment was observed under this field, as high frame per second movies to quantify the DEP velocity [44]. The data acquisition was automated to enable the capture of movies at each frequency within about ten seconds, with the entire frequency spectrum completed within about 5 minutes. The same cells can be measured multiple times under the DEP field since we use the electrode-less DEP technique under high frequency AC fields, with applied fields less than 300 V_{pp}/cm, thereby obviating electro-permeabilization effects, which we confirmed through viability analysis on microbials under the DEP field. The DEP and ROT analysis were conducted on the microbials following the log phase stage of their culture period, to ensure insensitivity of the analysis to the temperature of the culture media and time for the culture.

3. Theory and Calculation

3.1. Dielectric model fits to compute electrophysiology

Based on the rod shape of *C. difficile*, we employed the ellipsoidal single shell model [45], [46], [47] to extract dielectric properties for the cell envelope (wall plus membrane) and cytoplasm of *C. difficile*. A rotating electric field induces dipoles on the target bio-particles that are suspended in electrolytic medium. If angular velocity of the rotating field is substantially greater than the time for dipole formation on the particle, then the resulting lag between the dipole moment and the electric field causes a torque on the particle, thereby resulting in asynchronous rotation within the field. We employed effective moment method to derive expressions for the net torque on an ellipsoid in AC electric field. In all these calculations, frequency of applied field is high enough to ensure that particles respond to the time averages. For planar electrodes (x-y), the time averaged torque in the z-direction ($\langle T_z \rangle$) and effective dipole (p_{eff}) are given by:

$$\langle T_z \rangle = \frac{1}{2} \operatorname{Re} \left[(p_{eff})_y E_{0,x}^* - (p_{eff})_x E_{0,y}^* \right] \quad 1$$

$$(p_{eff})_\alpha = 4\pi abc \epsilon_m K_\alpha E_{0,\alpha} \quad \alpha = x, y \quad 2$$

In order to generate a torque, there is need for a phase difference between the applied electric field ($\vec{E}$) and dipole ($\vec{p}$). For this purpose, the electric field is represented in its complex phasor form, with real and imaginary parts. Hence, $\vec{E}$ represents electric field, E^* represents complex conjugate of the electric field, and E_0 represents intensity of the electric field. The term " $K_{\alpha,0}$ " is the spheroidal complex Clausius Mossotti factor for a single layer particle (similar to fCM for spherical particles) along α direction. a, b, and c are the axis lengths in different Cartesian directions (x, y, z) and ϵ_m is the permittivity of medium. For a rotating electric field $E_x = E_0$ and $E_y = \pm jE_0$, the torque in z direction will be:

$$\langle T_z \rangle = -\frac{2\pi abc}{3} \epsilon_m E_0^2 \text{Im}[K_x + K_y] \quad 3$$

In the single-shell ellipsoidal model, ϵ_s represents permittivity of the shell, and ϵ_p represents permittivity of the particle core that is enveloped by the shell. For an ellipsoidal particle with a single shell, this can be written as:

$$K_\alpha = K_{\alpha,1} = \frac{(\epsilon_s^* - \epsilon_m^*) + 3\gamma K_{\alpha,0}(\epsilon_s^* + L_\alpha(\epsilon_m^* - \epsilon_s^*))}{3(\epsilon_m^* + L_\alpha(\epsilon_s^* - \epsilon_m^*)) + 9K_{\alpha,0}\gamma L_\alpha(1 - L_\alpha)K_{\alpha,0}(\epsilon_s^* - \epsilon_m^*)} (\alpha = x, y) \quad 4$$

Here, “ γ ” is the ratio of volume of the core to the total spheroid, “ $K_{\alpha,0}$ ” is the spheroidal complex Clausius Mossotti factor and L_α is the “depolarizing factor” along the α axis:

$$K_{\alpha,0} = \frac{(\epsilon_p^* - \epsilon_s^*)}{3[\epsilon_s^* + (L_\alpha(\epsilon_s^* - \epsilon_m^*))]} \alpha = x, y \quad 5$$

The depolarization factor along major axis (L_x) and minor axis (L_y), for a particular particle eccentricity “ e ” are given by:

$$L_x = \frac{bc}{2a^2e^3} \left[\ln\left(\frac{1+e}{1-e}\right) - 2e \right] \quad 6$$

$$L_y = \frac{1 - L_x}{2} \quad 7$$

$$e = \sqrt{1 - \frac{b^2}{a^2}} \quad 8$$

For Gram-positive *C. difficile*, the dielectric properties of the shell or cell envelope are dominated by its cell wall, due to its single-layer membrane. The core particle for *C. difficile* is formed by its cytoplasmic region. Hence, we can use ϵ_w^* (to denote cell wall) in place of ϵ_s^* ; ϵ_{cyto}^* in place of ϵ_p^* ; and media properties: ϵ_{media}^* for ϵ_m^* . The respective complex permittivity values (ϵ^*) are related to their dielectric permittivity (ϵ) and conductivity (σ) based on angular frequency (ω) and imaginary unit (j): $\epsilon^* = \epsilon + j\sigma/\omega$. In this manner, the permittivity and conductivity of the cell wall and cytoplasmic regions were computed by solving Eq. 3 and fitting the parameters in Eq. 4 using the geometrical information about cell axis and cell wall thickness, which were measured from TEM images and using established

values for cell envelope (σ_w and ϵ_w) and cytoplasm (σ_{cyto} and ϵ_{cyto}). The relative permittivity and conductivity of medium were set to 78.5 and 105 mS/m, respectively.

4. Results and Discussion

4.1. *C. difficile* functionality after vancomycin treatment at near-MIC levels

To confirm MIC levels, we plot measurements of the *C. difficile* growth rate (Figure 1a) after vancomycin treatment (1 mg/L to 50 mg/L levels) and quantification of the secreted toxin (Figure 1b) after vancomycin treatment (2 mg/L and 1 mg/L levels), with untreated *C. difficile* serving as the positive control. We choose 2 mg/L and 1 mg/L vancomycin treatment levels, since these are close to the previously reported MIC level [48]. Based on comparison to the control (untreated *C. difficile*), it is clear that while the 2 mg/L treated sample is completely deactivated, the 1 mg/L treated sample exhibits an active subpopulation, which becomes apparent only after 24 hours of culture. Significant differences in growth between untreated, 1 mg/L, and 2 mg/L vancomycin treated *C. difficile* growth 24 hours post-inoculation were confirmed by two-way ANOVA with Tukey's multiple comparison posttest ($p < 0.0001$ in each case). Hence, we infer for the highly toxigenic *C. difficile* (HTCD) VPI 10463 strain, 2 mg/L (or 2 μ g/mL) vancomycin is at the MIC level and 1 mg/L (1 μ g/mL) vancomycin is at the sub-MIC level. Antibiotic concentrations in excess of 2 mg/L did not demonstrate any increase in growth suppression. Subsequently, we seek to identify this persistent active subpopulation at sub-MIC levels at the earlier time points and quantify its relative levels by following alterations in the microbial cytoplasmic conductivity.

4.2 Identifying the heterogeneity by dielectrophoresis

The deactivation of *C. difficile* after vancomycin treatment can possibly be identified based on the associated reduction in its cytoplasmic conductivity, as measured by the substantial drop in polarizability of the microbial cell under an electric field. To validate this hypothesis, we carried out DEP analysis after varying levels of vancomycin treatment, as shown within Figure 2. Using the device configuration of Figure 2a, the microfluidic flow cell is used to place *C. difficile* cells just outside the constriction region after which the field is turned ON for recording the DEP velocities of single microbial particles towards or away from the constriction tip in the 50 kHz to 1 MHz region, as per negative DEP (nDEP) in Figure 2b or positive DEP (pDEP) in Figure 2c. The respective images of DEP transport after ~30s at 400 kHz shows uniform levels of strong pDEP for untreated *C. difficile* (Figure 2e), consistent with strong field-induced polarization due to high cytoplasmic conductivity. On the other hand, the 2 mg/L vancomycin treated *C. difficile* sample exhibits uniform levels of nDEP (Figure 2f). We attribute the insubstantial field-induced polarization to the significantly reduced cytoplasmic conductivity of the deactivated microbial. However, at intermediate levels of 1 mg/L vancomycin treated *C. difficile* (Figure 2g), the majority population shows nDEP, while a minority subpopulation shows pDEP. Based on this, we infer that the DEP data agrees with the growth and toxin production data, to indicate persistence of a live *C. difficile* subpopulation after treatment at 1 mg/L levels. The measured frequency spectra of the DEP velocities of Figure 2d show distinct differences for each treatment group, confirming the substantial drop in field-induced microbial polarizability that is consistent

with reduction in cytoplasmic conductivity and reduction of cell aspect ratio (major to minor axis).

4.3 Quantifying the heterogeneity by electro-rotation

Electro-rotational (ROT) spectra enable sensitive determination of the DEP crossover frequencies, since shifts in the peak for counter-field rotation correspond to inflection from nDEP to pDEP behavior, while shifts in the peak for co-field rotation correspond to inflection away from strong pDEP behavior. In Figure 3, we seek to establish the correlation of ROT spectral shifts to known phenotypic differences between respective strains: highly toxigenic *C. difficile* (HTCD) VPI 10463 strain with higher cell wall roughness and higher shape eccentricity versus the low toxigenic *C. difficile* (LTCD) ATCC 630 strain and non-toxigenic *C. difficile* (NTCD) VPI 11186 strain [38]. The rotational rates at different time intervals (Figure 3a-3c) for computing the ROT spectra (Figure 3d) was acquired using the device set-up of Figure 3e for the three *C. difficile* strains, using homogenous samples. It is apparent from the ROT spectra of Figure 3d that the peak for counter-field rotation, which corresponds to inflection from nDEP to pDEP behavior, is shifted towards higher frequencies from HTCD to LTCD to NTCD. Consistent with prior DEP work [38], we attribute this observation to the reduction in *C. difficile* cell wall capacitance due to lower surface roughness for HTCD (Figure 3d) versus LTCD (Figure 3e) and even lower roughness for NTCD (Figure 3f) strains. It is also clear that the HTCD strain shows a higher frequency value for the counter-field peak and consistently higher rotational rates at all measured frequencies versus other strains, which is directly linked to its higher polarizability that results from the higher eccentricity of its prolate ellipsoidal shape [9]. The dielectric parameters determined by fitting the ROT spectra of Figure 3 to a single-shell model (Table 1a), show a gradual reduction in ϵ_w from HTCD to the LTCD and NTCD strains that is consistent with the increasing counter-field rotation peak and a reduction in σ_{cyto} that scales with rpm level for co-field rotation. The above correlations allow us to establish the basis for utilizing ROT spectral shifts and ROT rates towards assessing vancomycin-induced alterations to the HTCD strain, especially since its ROT spectra in Figure 3 exhibits the highest sensitivity to its phenotype versus other strains. Since ROT spectra measure the rotational rate of individual microbial cells over the entire frequency range, they are capable of discerning heterogeneity in microbial phenotype and quantifying the proportion of each subpopulation exhibiting the heterogeneity. Comparing spectra for untreated VPI 10463 *C. difficile* (HTCD) to that obtained after treatment with vancomycin at 2 mg/L (so-called “viable” or “live” versus “non-viable” or “dead” based on growth curve in Figure 1a), Figure 4a shows that the counter-field ROT peak is shifted to higher frequencies (consistent with upshifting of crossover to pDEP in Figure 2d), while the co-field peak is shifted to lower frequencies (~3.5 MHz shift). The latter observation is consistent with the reduced field-induced polarizability of microbial cytoplasm that we inferred in Figure 2d, based on reduced pDEP levels after microbial deactivation. Fitting of the ROT spectra using a single-shell model (continuous red and black curves in Figure 4a), confirms a significant reduction in cytoplasmic conductivity (σ_{cyto}), from 0.46 S/m for untreated *C. difficile* (“live”) to 0.275 S/m for 2 mg/L treated *C. difficile* (“dead”), as per the fitted parameters summarized in Table 1b. Consequently, the lowered cytoplasmic conductivity causes inflection away from pDEP at earlier frequencies and downshifting of the co-field ROT peak, which can serve as a

sensitive indicator of vancomycin-induced *C. difficile* disinfection. Examination of the ROT spectra for the 1 mg/L vancomycin-treated *C. difficile* cells in Figure 4b shows far higher standard deviations in rotation rates (blue diamond and green square data points considered together) versus that obtained for 2 mg/L treated *C. difficile* cells, especially for the co-field ROT peak region (marked as region of interest). For instance, at 10 MHz, the 1 mg/L vancomycin treated sample shows a combined rpm range of 480–680 rpm versus the far lower range of 688–720 rpm for the untreated sample and relatively lower range of 465–530 rpm after 2 mg/L vancomycin treatment (all ranges within one standard deviation of the mean). This heterogeneity is consistent with DEP responses for the 1 mg/L treated *C. difficile* sample, wherein one set of cells showed pDEP and another set showed nDEP at 0.4 MHz (Figure 2g). Choosing 10 MHz as the particular frequency of interest for identifying and quantifying the subpopulations after 1 mg/L vancomycin treatment, since it is in the 3.5 MHz region between the co-field peak of the 2 mg/L (“non-viable”) and untreated (“viable”), we plot the raw ROT data as the number of cells at each significant increment of rpm range (Figure 5a). Histograms were plotted using this data in Figure 5b to determine: (i) the proportion of each subpopulation; and (ii) the mean and standard deviation of rpm values (including at least 85% of measured cells) for each subpopulation. The mean rpm values for each of these subpopulations from Figure 5b are compared to mean rpm values obtained from statistically relevant histograms for untreated and 2 mg/L vancomycin treated *C. difficile* in Figure 5c–5e. Based on 100 analyzed cells in Figure 5b, it is clear that we could fit a lower rpm histogram that is denoted as subpopulation 1 (Sub 1), which is the majority subpopulation (~65%) that resembles the rpm ranges observed for “non-viable” or “dead” cells (Figure 5d); as well as a higher rpm histogram that is denoted as subpopulation 2 (Sub 2), which is the minority subpopulation (~35%) that resembles the rpm ranges observed for “viable” or “live” cells (Figure 5e). A number of instances in prior work [37, 41] have shown a steady reduction in rotational rate upon cell de-activation under heat or antibiotic treatment, since lowering of cytoplasmic conductivity can cause a gradual drop in polarizability and rotation rate. Histograms similar to Fig. 5b can be plotted at other frequencies in the “region of interest” (7.5–30 MHz) to separate the subpopulations based on similar shifts in their co-field rotation rates, with the blue diamond symbols of Subpopulation 1 corresponding to the fitted ROT curve for 2 mg/L treated or “non-viable” *C. difficile* and the green square symbols of Subpopulation 2 corresponding to the fitted ROT curve for untreated or “live” *C. difficile* (Fig. 4b). Through fitting the ROT spectra of each subpopulation in Fig. 4b to the single shell ellipsoidal model (fitted parameters in Table 1b), we confirmed that the cytoplasmic conductivity of the majority subpopulation after 1 mg/L vancomycin treatment (65% of cells corresponding to Sub 1 after 4 hours of 1 mg/L vancomycin treatment) was fit to a value of 0.3 S/m that is close to the respective value of 0.275 S/m for deactivated *C. difficile*, whereas for the minority subpopulation (35% of cells corresponding to Sub 2 after 4 hours of 1 mg/L vancomycin treatment) the fit cytoplasmic conductivity of 0.42 S/m is close to the respective value of 0.45 S/m obtained for functional *C. difficile* (untreated sample). The mechanism for vancomycin action on microbials is attributed to inhibition of cell wall synthesis and the associated leakage of cytosol upon cell wall interruption. Our observations indicate that vancomycin treatment at levels above MIC (2 mg/L) clearly upshifts the DEP crossover frequency (Fig. 2d) and the counter-field rotational peak (Fig. 4a), which indicates reduction of dielectric permittivity of

its cell wall (ϵ_w), as per Table 1b. However, upon sub-MIC level vancomycin treatment (1 mg/L), the shifts in DEP crossover frequency (Fig. 2g) and the counter-field rotational peak (Fig. 4b) are no longer a reliable indicator of a reduction in wall permittivity (ϵ_w). As per the counter-field region of Fig. 4b, while the red curve (untreated *C. difficile*) is significantly different from black curve (2 mg/L treated *C. difficile*), there is no significant difference in the measured rotation rates between subpopulation 1 (blue diamond symbols) and subpopulation 2 (green square symbols), which highlights that reduction in cell wall permittivity (ϵ_w) is not reliable. On the other hand, the shift in the co-field peaks are clearly apparent in Fig. 4b, with the subpopulation 1 (blue diamond symbols) resembling the trend for 2 mg/L treated *C. difficile* (black curve) and the subpopulation 2 (green square symbols) resembling the trend for untreated *C. difficile* (red curve). Since the electrorotation response in the peak co-field region is highly sensitive to cytoplasmic conductivity (σ_{cyto}), we infer that these alterations are a more reliable indicator of modifications to *C. difficile* versus the wall permittivity (ϵ_w). This set of DEP observations identifying heterogeneity (Fig. 2g) and ROT results quantifying the proportion of each subpopulation (Fig. 5a & 5b), along with the growth curve (Fig. 1a) and toxin production (Fig. 1b) for 1 mg/L treated *C. difficile* after 24 hours, allows us to infer that ROT spectra can quantify the minority “live” subpopulation at sub-MIC levels after just a few hours of treatment, whereas the growth and toxin-based methods require 24 hours. It is noteworthy that since the antibiotic treatment of *C. difficile* was conducted in the culture media for 4 hours to ensure complete deactivation, the measured proportion of ~35% live cells after 1 mg/L vancomycin treatment includes the persistent live *C. difficile* subpopulation, as well as additional microbial levels that may occur due to *C. difficile* growth in the microbial culture media for four hours.

5. Conclusions

For the purpose of enabling rapid assessment of antibiotic susceptibility on pathogenic microbes, we present an electrophysiology-based single-cell technique for gauging phenotypic alterations of toxigenic *C. difficile* (VPI 10463) after treatment with near MIC (minimum inhibitory concentration)-levels of vancomycin. Specifically, we demonstrate that disinfection of *C. difficile* after vancomycin treatment at MIC levels (2 mg/L), as validated by microbiological growth assays, can be ascertained rapidly based on reduction in microbial cytoplasmic conductivity, which reduces the level of positive dielectrophoresis (pDEP) and down-shifts the co-field electro-rotation (ROT) peak to lower frequencies. Furthermore, this single-cell electrophysiology technique can identify and quantify the persistent live *C. difficile* subpopulation after 4 hours of vancomycin treatment at sub-MIC levels (1 mg/L), whereas methods based on measurement of the secreted metabolite toxin or the microbiological growth rate can identify this persistent *C. difficile* subpopulation only after 24 hours of microbial culture and are unable to quantify the subpopulation. The electro-phenotyping technique described here differs significantly from prior work by its use of single-microbial analysis to rapidly quantify the emerging subpopulations after MIC-level antibiotic treatments, with no need for microbial culture to amplify their numbers. Microbiological assays or electro-phenotyping methods that do not utilize single microbial analysis are not capable of: (i) quantifying subpopulations as we observed at near-MIC levels, which likely get averaged out in methods that measure whole cell populations; and

(ii) rapidly ascertaining antibiotic susceptibility at near-MIC levels due to their need for ~24 hours of microbial culture that is necessary to amplify the microbial numbers to determine the growth curve. Hence, this electro-phenotyping technique is best suited for situations wherein there is a need at near-MIC levels, to rapidly ascertain antibiotic susceptibility and quantify any emerging subpopulations. Based on the reported sensitivity within short analysis times, we envision that multiplexed versions of this technique can be applied for antibiotic susceptibility screening.

Acknowledgments

Funding: This work was supported by funds from the NIH Grant 1R21AI130902-01 and from University of Virginia's Global Infectious Diseases Institute.

Biography

Ali Rohani recently completed his Ph.D. dissertation research in Electrical Engineering at the University of Virginia focusing on signal analysis aspects of electrokinetic manipulation within microfluidic systems. His contributions include the development of an electrical tweezer for enabling highly-parallelized electrorotational measurements and the design of nanoslit devices for creating ion conductivity gradients to enhance the spatial extent of negative dielectrophoresis. He is currently a Research Associate in the Biomedical Data Sciences area.

John Moore is a final year Ph.D. student in Electrical Engineering at the University of Virginia, specializing on AC electrokinetics for microbial analysis within microfluidic systems. His contributions include development of co-culture and analysis platforms for studying microbiota interactions on *Clostridium difficile*.

Yi-Hsuan Su completed her Ph.D. dissertation research in Electrical Engineering at the University of Virginia, with a focus on microfluidics for microbiological analysis. Her contributions include microfluidic platforms for measurement of dielectrophoretic forces on single cells and microbes. She is currently employed as a scientist with ZeptoLife, Inc.

Victoria Stagnaro conducted the reported research as part of her undergraduate thesis and recently completed her undergraduate degree in Biomedical Engineering at University of Virginia. She is currently employed as a scientist with Luna Innovations, Inc.

Circle Warren is an Associate Professor of Infectious Diseases within the School of Medicine at University of Virginia. Her lab specializes in developing clinical diagnostics and therapeutics for antibiotic resistant infections.

Nathan Swami: Nathan Swami is a Professor of Electrical & Computer Engineering at the University of Virginia. His group specializes in label-free microfluidics for sorting, bioanalysis and cytometry of single cells and biological nanostructures, with a focus on impedance and deformability-based methods. He seeks to impact detection systems within point-of-care and resource-poor settings for enabling precision medicine, personalized healthcare and environmental monitoring systems.

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Highlights

- Microbial-culture independent antibiotic susceptibility assessment using electrophysiology
- Susceptibility to MIC vancomycin levels identified by cytoplasmic conductivity alteration
- Electro-phenotyping based antibiotic susceptibility benchmarked by broth dilution assay
- Rapid identification (< 4 hours) of live subpopulation after sub-MIC level vancomycin
- Single-cell phenotyping to quantify live subpopulation after sub-MIC level vancomycin

Figure 1.
(a) Growth of vancomycin-treated *C. difficile* with culture time; (b) toxin production of vancomycin-treated *C. difficile* measured as function of culture time.

Figure 2.

(a) Device configuration to measure DEP velocity under: (b) nDEP and (c) pDEP. (d) DEP velocity versus frequency (50 kHz – 1 MHz) for single *C. difficile* cells at indicated vancomycin treatment levels. Example images after 30s of DEP at 400 kHz for: (e) Untreated; (f) 2 mg/L treated and (g) 1 mg/L treated *C. difficile*.

Figure 3.

Electro-rotational (ROT) spectra of: highly toxigenic *C. difficile* (HTCD) VPI 10463 strain, low toxigenic *C. difficile* (LTCD) ATCC 630 strain and non-toxigenic *C. difficile* (NTCD) VPI 11186 strain to quantify rotational rates at different time points (Figure 3a-3c) for computing the ROT spectra (Figure 3d), using the device set-up (Figure 3e). The TEM micrographs show decreasing cell wall roughness levels for HTCD (Figure 3f), LTCD (Figure 3g) and NTCD (Figure 3h) strains (Scale bar = 0.2 μm). All the rotation rates were normalized with respect to the maximum rotation rate obtained for untreated *C. difficile*. The curves are based on single-shell model fits (Eq. (1) – Eq. (8)) using parameters from Table 1a.

Figure 4:

(a) ROT shows up-shifting of counter-field peak (corresponding to crossover frequency towards pDEP) and downshifting of co-field peak (corresponding to crossover away from pDEP) for 2 mg/L vancomycin treated versus untreated *C. difficile*, (b) 1 mg/L vancomycin treated *C. difficile* shows two distinct populations (see Fig. 5 and associated text for analysis), with subpopulation 1 (blue diamond symbols) resembling the fit observed for 2 mg/L vancomycin treated *C. difficile* (black curve) and subpopulation 2 (green square symbol) resembling the fit observed for untreated *C. difficile* (red curve). The curves are

based on single-shell model fits (Eq. (1) – Eq. (8)) using parameters from Table 1b. Rotation rates in Figure 4a-4c were normalized with respect to the maximum rotation rate obtained for untreated *C. difficile*.

Figure 5:

(a) Rotation rates at 10 MHz for *C. difficile* (total of 100 cells) after 1 mg/L vancomycin treatment suggest two subpopulations based on the histograms plotted in (b) for their quantification: subpopulation 1 (Sub 1) of 65 cells with lower rpm range (480–580 rpm for 85% cells with estimated mean of 523 rpm) and subpopulation 2 (Sub 2) of 35 cells with a higher rpm range (610–680 rpm for 85% cells with estimated mean of 645 rpm). (c) Comparison of the respective rpm ranges for 2 mg/L vancomycin treated *C. difficile* wherein complete loss of viability was assessed (Fig. 1) vs. untreated *C. difficile* wherein no loss of

viability was assessed (Fig. 1), indicates significantly lower rpm for non-viable (465–530 rpm for 85% cells estimated mean of 498 rpm) versus viable *C. difficile* (688–720 rpm for 85% cells with estimated mean of 704 rpm), suggesting the resemblance of Sub 1 after 1 mg/L vancomycin treatment to non-viable or dead *C. difficile* and Sub 2 after 1 mg/L vancomycin treatment to viable or live *C. difficile*. This is further illustrated in (d) that shows similarity of rpm of the majority Sub 1 to the 2 mg/L vancomycin treated *C. difficile* and of the minority Sub 2 to the untreated *C. difficile* (e). The double sided arrows in (b) & (c) represent variation in the rotation rates within each sample (mean $\pm$ 1 standard deviation).

Table 1:

Fitted dielectric parameters (goodness of fit indicated by Root Mean Square Error or RMSE) based on the ROT spectra in **Figure 3 (Table 1a)** and **Figure 4 (Table 1b)** using Eq. (1) – Eq. (8) to determine conductivity and permittivity of *C. difficile* cell envelope (σ_w and ϵ_w) and cytoplasm (σ_{cyto} and ϵ_{cyto}). The geometric parameters used in the fit include: Major Axis = 3.6 μm , Minor Axis = 0.57 μm , Cell Envelope Thickness (Wall + Membrane) = 35 nm.

(a) Comparison of different *C. difficile* strains

(b) Comparison of *C. difficile* (VPI 10463) after different levels of vancomycin treatment

Electrophysiology Parameters	VPI 10463 (HTCD)	ATCC 630 (LTCD)	VPI 11186 (NTCD)
RMSE (error)	0.03957	0.02148	0.05180
$\epsilon_w (\times \epsilon_0)$	7	3.5	2
σ_w (S/m)	2.50×10^{-5}	2.00×10^{-6}	3.3×10^{-5}
$\epsilon_{cyto} (\times \epsilon_0)$	55	50	45
σ_{cyto} (S/m)	0.46	0.16	0.32

Electrophysiology Parameters	Untreated <i>C. difficile</i> (VPI 10463)	2 mg/L Vancomycin treated <i>C. difficile</i> (after 4 hours)	1 mg/L Vancomycin treated <i>C. difficile</i> (subpopulation 1)	1 mg/L Vancomycin treated <i>C. difficile</i> (subpopulation 2)
RMSE (error)	0.03957	0.08612	0.06390	0.08157
$\epsilon_w (\times \epsilon_0)$	7	4	5	3.5
σ_w (S/m)	2.50×10^{-5}	3.00×10^{-5}	3.15×10^{-5}	2.15×10^{-5}
$\epsilon_{cyto} (\times \epsilon_0)$	55	55	55	55
σ_{cyto} (S/m)	0.46	0.275	0.3	0.42