Application Note

Cell Analysis

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Studying Bacterial Biofilms Using Cellular Impedance

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Abstract

This application note describes the use of Agilent xCELLigence real-time cell analysis (RTCA) instruments for impedance monitoring. The xCELLigence was used to overcome limitations of traditional colorimetric assays, enabling a label-free and totally automated approach for quantitative and continuous evaluation of biofilms.

Introduction

In addition to living in a free-floating planktonic state within aqueous environments, bacteria can also colonize biotic and abiotic surfaces at liquid-solid and air-solid interfaces. Within these micro-environments, secreted chemical messengers are used to coordinate gene expression profiles across the colony, thereby promoting survival.[1,2] A common adaptation of these communities, which can be comprised of hundreds of different bacterial species, is the secretion of extracellular polymeric substances (EPS), consisting of polysaccharides, nucleic acids, lipids, teichoic acids, or proteins. The EPS matrix encapsulates bacterial cells and protects them from the environment (Figure 1). The ability to form these biofilms is a key virulence factor because the EPS matrix facilitates bacterial evasion of host immune responses, and also enhances the antibiotic resistance of bacteria as much as 1,000-fold.

In addition to playing critical roles in human dental plaque and cavities, chronic infections, rejection of the polymeric matrix of artificial implants, and food poisoning, bacterial biofilms are also responsible for a large percentage of livestock diseases, and cause fouling of industrial air and water handling systems. Developing drugs to treat or prevent biofilms is critically important. But the colorimetric assays traditionally used for studying biofilms are inefficient or low-throughput, incompatible with orthogonal assays (samples are destroyed by the analysis process), and only provide endpoint data.

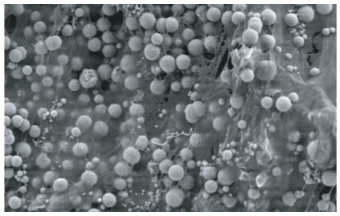

Figure 1. Example of a bacterial biofilm. Electron micrograph of *Staphylococcus aureus* cells (spheres) enmeshed in their secreted polymetric matrix.

Cellular impedance explained

The functional unit of the xCELLigence RTCA impedance assay is a set of gold biosensors fused to the bottom of a microplate well (Figure 2). When submersed in an electrically conductive solution (such as buffer or growth medium), applying a weak electric potential across these biosensors causes electric current to flow between them. Because this phenomenon is dependent on the biosensors interacting with bulk solution, the presence of adherent cells at the biosensor-solution interface impedes current flow. The magnitude of this impedance is dependent upon the number of cells, the size of the cells, and the cell-substrate attachment quality. Studies have demonstrated that cell health and behavior are not affected by the gold biosensor surfaces nor the electric potential (only 22 mV and only applied intermittently, at a user-defined frequency).

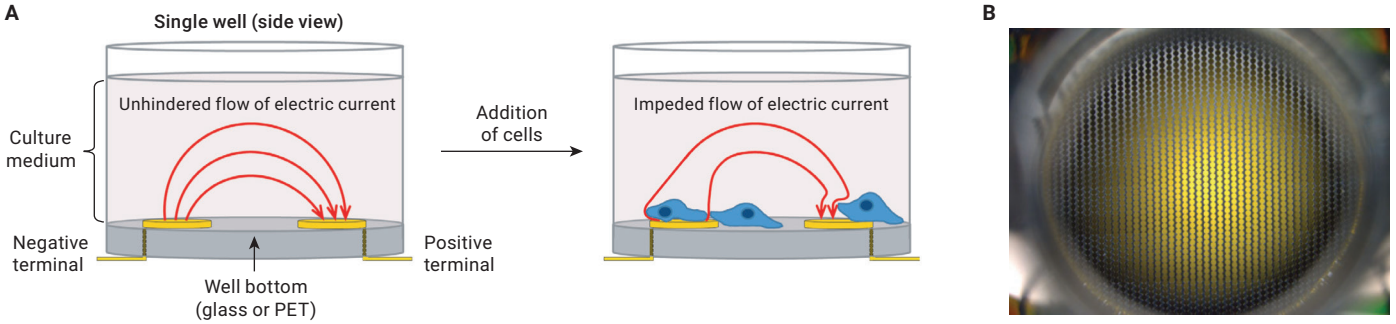

Figure 2. Overview of cellular impedance apparatus. (A) A side view of a single well from an Agilent electronic microplate (E-Plate) is shown before and after cells have been added. Neither the gold biosensors nor the cells are drawn to scale (they have been enlarged for clarity). In the absence of cells, electric current flows freely through culture medium, completing the circuit between the gold biosensors. As cells adhere to and proliferate on the biosensors, current flow is impeded, providing an extremely sensitive readout of cell number, cell size, and cell-substrate attachment quality. (B) Photograph looking down into a single well of an E-Plate. Note that, in contrast to the simplified scheme in part A, the biosensors are actually an interdigitated array that covers >75% of the well bottom. Though cells can be visualized directly on the gold biosensor surfaces, special E-Plates with a biosensor-free region in the middle of the well (not shown here) are also available to facilitate microscopic imaging.

Using impedance to study bacteria

The xCELLigence RTCA has found widespread use for studying eukaryotic cells in applications that range from GPCR agonism/antagonism and oncology drug discovery to immunotherapy potency analysis and predictive toxicology. Until recently, however, RTCA has been used only sparingly for studying prokaryotes, with just a handful of papers being published over the past few years.[3,4] These cursory studies demonstrated that growth of different strains of *Staphylococcus aureus* on Agilent E-Plates does result in an impedance signal that increases over time. The kinetics are consistent with what is observed in orthogonal assays. Believing that impedance monitoring is well suited to basic and applied biofilm research applications, we undertook more in-depth studies aimed at optimizing assay conditions and exploring the nuances of how biofilms affect the impedance signal. Two key questions we were interested in answering were:

- Do bacterial cells and their secreted EPS both contribute to the impedance signal?
- Can impedance monitoring be used to quantify the efficacy of drugs for preventing biofilm formation or causing disruption of an established biofilm?

The results of these studies have been published,[5] and are serving as the foundation for ongoing bacterial biofilm research. By presenting specific case studies, we aim to summarize our optimized methodologies for using xCELLigence RTCA to probe different facets of biofilm biology and antibiofilm drug screening.

Protocol: Using xCELLigence RTCA to study biofilms

Bacterial species and culture conditions

We have successfully analyzed *Staphylococcus aureus* and *Staphylococcus epidermidis* using xCELLigence RTCA. For a complete list of the specific strains analyzed see Ferrer; *et al.*[5] Bacteria were taken from –80 °C storage and streaked onto trypticase soy agar plates. After 24 to 48 hours of growth at 37 °C, individual colonies were used to inoculate cultures of trypticase soy broth (TSB). After overnight shaking at 37 °C, cultures were diluted to OD_{650} = 0.175 using TSB supplemented with 0.25% (w/v) glucose (TSBG). These diluted stocks were used to seed an E-Plate 96, as described in the following sections.

Instrument preparation and measuring background impedance

Experiments were conducted using an xCELLigence RTCA MP (multiple plates) instrument that was housed in a standard tissue culture incubator set to maintain 37 °C. Housing the xCELLigence instrument in a hypoxia chamber or in different atmospheric compositions (CO_2, and others) is also possible. After adding 100 µL of TSBG to each well of an E-Plate 96, the plate was placed inside the xCELLigence instrument, and the RTCA software was used to record the background impedance (that is, the inherent electrical resistance of growth media in the absence of cells) for each well.

E-Plate seeding, data acquisition, and data plotting

For a typical experiment, 100 µL of the diluted *Staphylococcal* cultures were added to wells that contained the 100 µL of TSBG used for background measurement (giving a final volume of 200 µL/well). Biological or technical replicates were set up in triplicate. After placing the E-Plate 96 back into the xCELLigence instrument, the RTCA software was programmed to record impedance measurements every 15 minutes over 24 hours. Impedance values were plotted using the unitless parameter Cell Index, which is defined as $(Z_n–Z_b)/15$, where Z_n and Z_b are the impedance values in the presence and absence of cells, respectively. Data were typically plotted as the average Cell Index ± the standard deviation.

Evaluating the impact of proteinaceous biofilm on the impedance signal

The impedance signal produced by a bacterial biofilm could be a consequence of both the cells and the EPS. The molecular constituents of EPS, and the relative ratios of constituent molecules, differ from one bacterial species to the next. To assess how a protein-rich matrix affects impedance, E-Plate wells containing *S. aureus* V329 were supplemented with proteinase K (at a final concentration of 100 µg/mL) at the time of seeding. This enzyme does not affect *Staphylococcal* cell wall integrity, but does partially degrade the extracelluar proteinaceous matrix.

Identifying drugs that prevent biofilm formation

A critical approach to fighting biofilms is preventing their formation. We evaluated the capacity of different relevant antibiotics to inhibit biofilm formation inside E-Plates. TSBG (100 µL) supplemented with different antibiotics (at concentrations ranging from 62.5 ng/mL to 32 µg/mL) were added to each well of an E-Plate 96, and the background impedance was measured.

Diluted *S. aureus* 240 cultures (described above) were added to each well, and biofilm formation was monitored by recording impedance values every 15 minutes for 24 hours. The prophylactic efficacy of each drug was analyzed by plotting, for each drug concentration, the percentage Cell Index 20 hours post cell seeding. Percentage CI is defined as: % Cell Index = $(CI_{with\ drug}/CI_{without\ drug}) \times 100$. The lowest antibiotic concentration that inhibited biofilm formation (yielding a CI value less than 0.05) was considered to be the biofilm minimum inhibitory concentration (Bio-MIC).

Identifying drugs that disrupt established biofilms

Beyond inhibiting the initiation/formation of biofilms, agents that can disrupt established biofilms are needed. To see if xCELLigence RTCA can be used to screen for drugs that possess this biofilm disruption activity, *S. epidermidis* 43040 biofilms were first grown in E-Plates until they reached their exponential growth phase; then, antibiotics were added. Specifically, 100 µL of TSBG culture medium was used for background measurement, then an additional 75 µL of TSBG containing overnight cultures was added to each well to reach a final OD_{650} = 0.0875. Impedance was recorded every 15 minutes for nine hours, allowing the cultures to reach mid log phase with a robust impedance signal. Then, the E-Plate was removed from the instrument, and 25 µL of an antibiotic solution (at concentrations ranging from 62.5 ng/mL to 32 µg/mL) was added to each well. After placing the plate back into the instrument, impedance was recorded every 15 minutes for 25 hours.

Results and discussion

Signal amplitude and reproducibility

Consistent with what has been reported in previous publications, the impedance signal observed for *S. epidermidis* and *S. aureus* were consistently ~10 fold lower than what is typically observed for eukaryotic cells. This could be a consequence of:

- Bacterial cells being smaller, and therefore providing a thinner insulating layer that is less capable of impeding current flow
- Bacterial cells packing less uniformly and less densely than eukaryotic cells (thereby leaving more avenues for electric current to flow between electrodes)
- A combination of the two

Despite this weaker signal, the reproducibility of the data is acceptable, as evidenced by the standard deviation between seven technical replicates (Figure 3A).

Bacterial cells and EPS both contribute to the impedance signal

To evaluate how EPS affects the impedance signal, biofilm-producing (CH 845) and nonbiofilm-producing (CECT 231) strains of *S. epidermidis* were grown side-by-side. Figure 3B shows that the biofilm-producing strain displayed a much more robust impedance signal. To confirm that this difference is specifically caused by the presence or absence of EPS, CH845 was grown alongside an isogenic mutant. This mutant lacked the sarA gene that regulates the production of both proteinaceous and polysaccharidic components of biofilms. When assessed by optical density measurements in liquid culture, this mutation did not cause a significant difference in growth rate compared to WT (data not shown here). The fact that the ΔsarA mutant displayed a mitigated impedance signal demonstrates that EPS does contribute, either directly or indirectly, to the impedance signal being measured by xCELLigence RTCA (Figure 3C). A similar result was obtained with the V329 and V329 ΔsarA strains of *S. aureus* (data not shown). As an alternative means of evaluating the impact of EPS on the xCELLigence RTCA impedance signal, the proteinaceous biofilm-producing *S. aureus* V329 was grown in medium supplemented with or without proteinase K. Though it did not impact the rate of bacterial growth in a planktonic assay (data not shown), the presence of proteinase K reduced V329's impedance signal (Figure 3D).

While these experiments collectively demonstrate that EPS influences the impedance signal, they do not provide an explanation for why this happens. One possibility is that the EPS components impede the flow of electric current. Alternatively, the observed impact of EPS might simply be a consequence of the extracellular matrix tethering more cells to the plate bottom, or tethering cells to the electrodes more closely. To investigate these possibilities, the growth of exopolysaccharide biofilm-producing *S. aureus* Isp479c was monitored in three side-by-side assays using impedance, cell counting, and polysaccharide quantification (Figure 3E).

Although the number of cells peaks at six hours and remains constant up to 12 hours, the impedance signal and the total polysaccharide content increase substantially over this time frame. This clearly indicates that the xCELLigence assay is monitoring more than just the number of cells present in a biofilm.

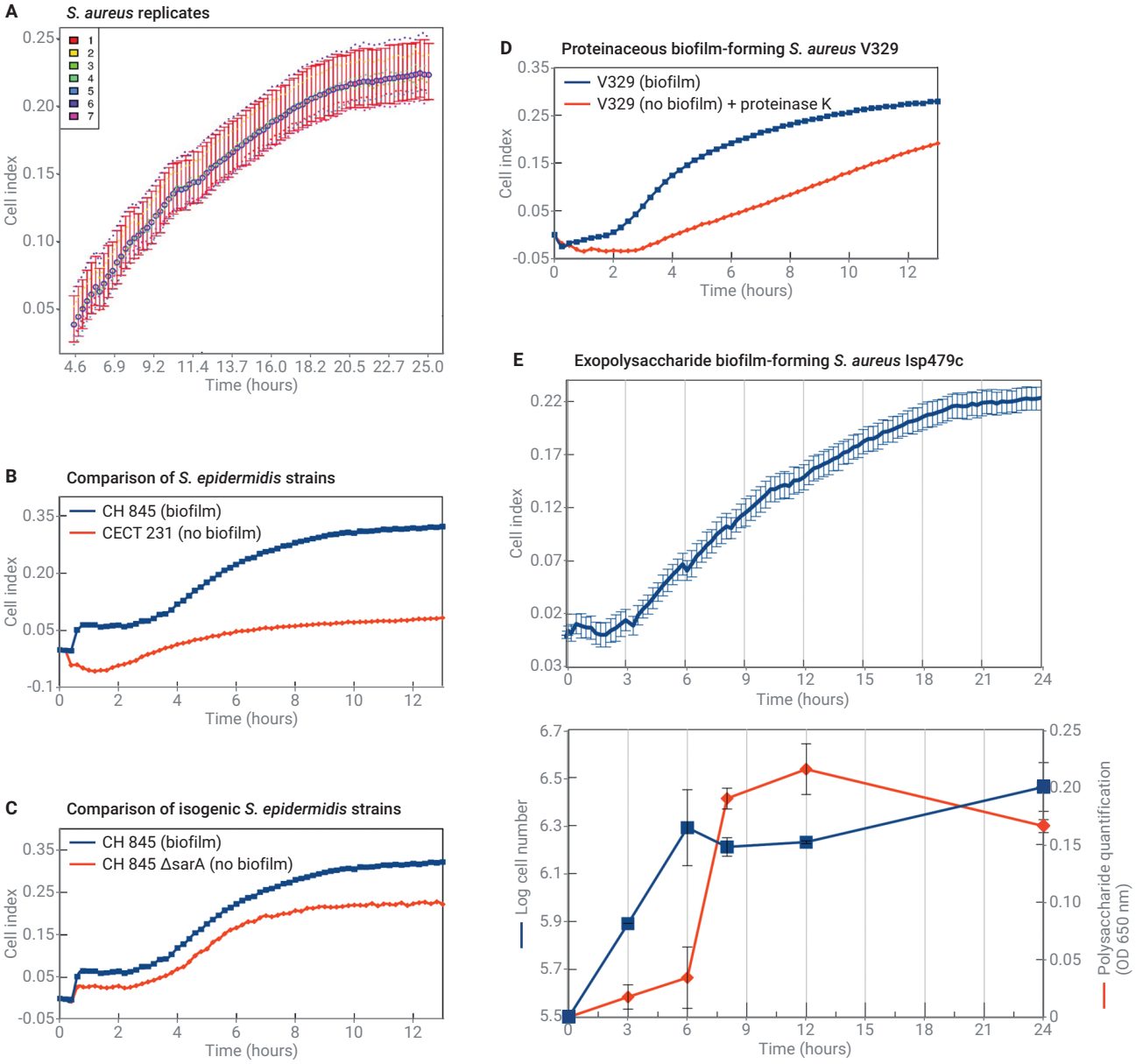

Figure 3. Assessing biofilm signal reproducibility, and the impact of EPS on the impedance signal. (A) The intra-experiment variability of biofilm-derived impedance signals was evaluated by monitoring seven technical replicates of *S. aureus*. The mean value and confidence intervals are plotted here. The average standard deviation relative to the mean was 12%. (B) Comparison of *S. epidermidis* strains that produce (CH845) or do not produce (CECT231) EPS. (C) Comparison of the biofilm-producing CH845 strain of *S. epidermidis* with an isogenic ΔsarA mutant that is deficient in EPS production. (D) Monitoring proteinaceous biofilm formation by the V329 strain of *S. aureus* in the presence or absence of proteinase K. Although it does not reduce the number of cells present (data not shown here), inclusion of proteinase K in the culture media mitigates the impedance signal associated with V329 growth. (E) Side-by-side comparison of the impedance signal, total cell number, and polysaccharide content during growth of the exopolysaccharide biofilm-producing *S. aureus* Isp479c.

Laboratories are investigating whether EPS components impede current flow directly, or if EPS is causing the bacterial cells to interact with the E-Plate biosensors more tightly.

Collectively, the results are consistent with a model where both the cells and the EPS of a biofilm contribute to the impedance signal monitored by the xCELLigence instrument. This is a valuable finding, as it sheds light on the types of questions that can be examined using real-time cell analysis.

Screening for biofilm-blocking agents

By including antibiotic in the growth media when *S. aureus* 240 was seeded into E-Plate wells, the capacity of RTCA to identify biofilm-blocking activity was evaluated. As shown in Figure 4A, each of the 10 antibiotics that were tested displayed prophylactic activity, but they did this with differing levels of efficacy. While cefotaxime completely destroyed the biofilm-associated signal at a concentration of 0.25 µg/mL, linezolid required a 128-fold higher concentration to accomplish this. As a proof of principle, this experiment demonstrates the utility of RTCA as a tool for drug screens aimed at preventing biofilms from forming.

Of high relevance is the finding that, within particular concentration ranges, some antibiotics can actually promote biofilm growth. Being able to characterize this unwanted behavior is critical for preventing physicians from unwittingly exacerbating the infection they are trying to treat. This bifurcated behavior is readily detectable, and quantifiable, using RTCA. At concentrations of 4 to 32 µg/mL, vancomycin is found to suppress *S. epidermidis* 43040 biofilm growth; however, at concentrations of 62.5 ng/mL to 2 µg/mL, biofilm growth is stimulated (Figure 4B).

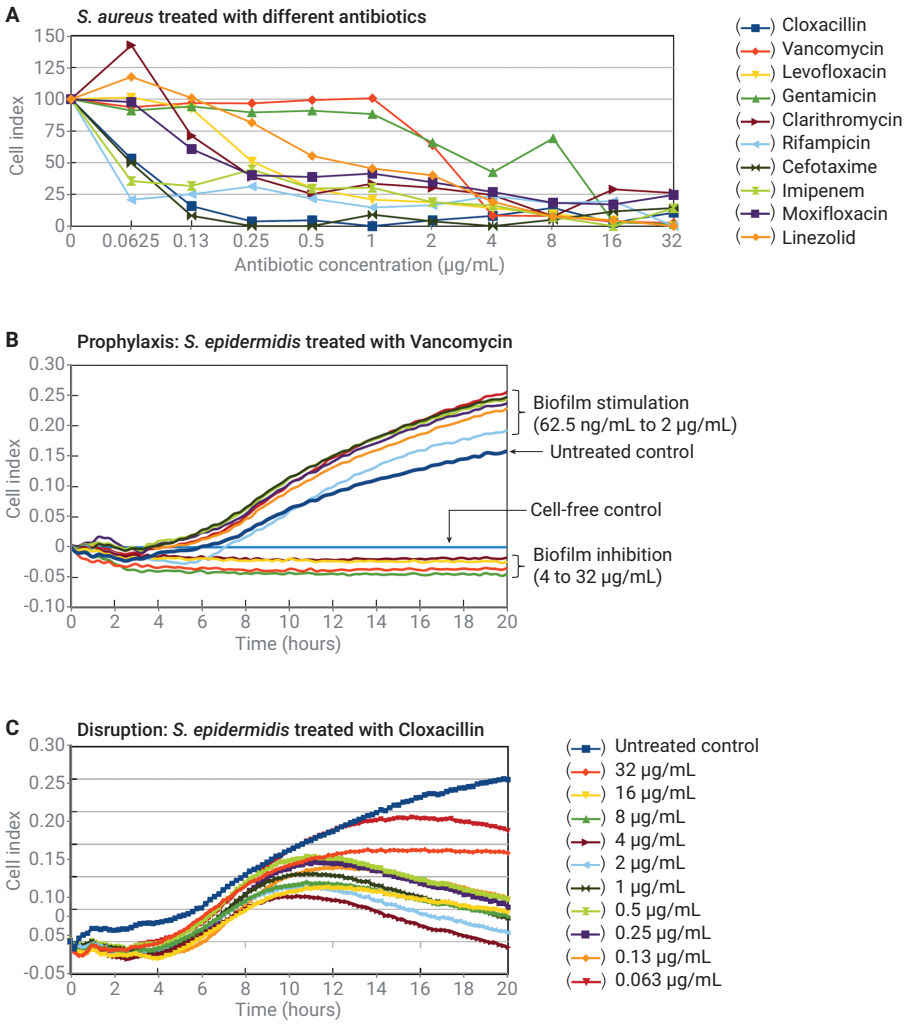

Figure 4. Using RTCA to screen for drugs that either prevent biofilm formation or disrupt established biofilms. (A) Ten different antibiotics (each represented by a different colored line) were evaluated for their ability to prevent *S. aureus* 240 from forming a biofilm. Antibiotics were present at different concentrations from the moment that bacteria were seeded into wells. Twenty hours after seeding, the CI was measured and compared to the untreated control. The %CI plotted here is simply $[(\text{Cell Index})_{\text{with drug}}/(\text{Cell Index})_{\text{without drug}}] \times 100$. (B) Testing for prophylactic activity. Depending on its concentration, vancomycin either inhibits or stimulates the growth of *S. epidermidis* 43040 biofilm. (C) Testing for disruption activity. At concentrations above 0.13 µg/mL only cloxacillin and rifampicin are able to induce partial distruption of the *S. epidermidis* biofilm.

Screening for biofilm-disrupting agents

By allowing a biofilm of *S. epidermidis* 43040 to become established, and subsequently treating it with antibiotics, biofilm-disrupting activity was probed. Figure 4C shows that at concentrations of 0.125 to 8 µg/mL, cloxicillin and rifampicin were able to induce partial disruption of the biofilm, with the CI dropping by ~60% in the most extreme case. The inability of the 10 tested antibiotics to cause complete biofilm disruption:

- Is consistent with the known antibiotic resistance of biofilms
- Demonstrates the importance of testing drug efficacy against the biofilm (rather than planktonic) state, and
- Highlights the need for more effective drugs

Conclusion

We have demonstrated the utility of xCELLigence RTCA for a few basic and applied applications in biofilm research. This protocol involves substantially less work than traditional assays: bacteria are simply seeded into an E-Plate, after which data acquisition is continuous and automatic. The real-time nature of the xCELLigence data enables quantitative comparisons between different strains and treatments, with both the bacterial cells and their EPS being evaluable.

Achieving a detailed and nuanced picture of biofilm dynamics using traditional endpoint assays would be costly in work time, and would not provide the same level of reproducibility. The benefits summarized in this application note are highlighted by six xCELLigence biofilm papers[5–10] that have been published (by independent groups).

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