Studying Bacterial Biofilms Using Cellular Impedance

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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 microenvironments, 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, and/or proteins, the EPS matrix encapsulates the 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.

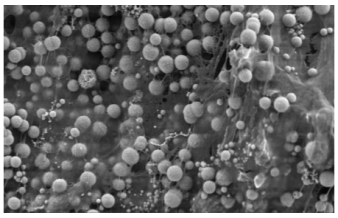

Figure 1. Example of a bacterial biofilm. Electron micrograph of *Staphylococcus aureus* cells (spheres) enmeshed in their secreted polymeric matrix.

Besides playing critical roles in human dental plaque and cavities, chronic infections, rejection 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, further increasing their economic impact. Though developing drugs to treat biofilms – or prevent their formation in the first place – is of critical importance, the colorimetric assays traditionally used for studying biofilms are inefficient/low throughput, are incompatible with orthogonal assays (i.e. samples are destroyed by the analysis process), and only provide end point data. Herein we describe how impedance monitoring by xCELLigence® Real-Time Cell Analysis (RTCA) instruments overcomes each of these limitations, enabling a quantitative and continuous evaluation of biofilms via an assay that is both label-free and totally automated.

Cellular Impedance Explained

The functional unit of the xCELLigence® RTCA impedance assay is a set of gold microelectrodes fused to the bottom surface of a microtiter plate well **(Figure 2)**. When submersed in an electrically conductive solution (such as buffer or growth medium), the application of a weak electric potential across these electrodes causes electric current to flow between them. Because this phenomenon is dependent upon the electrodes interacting with bulk solution, the presence of adherent cells at the electrode-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. Importantly, numerous studies have demonstrated that cell health and behavior are not affected by the gold microelectrode surfaces or the electric potential (which is only 22 mV and is only applied intermittently, at a user-defined frequency).

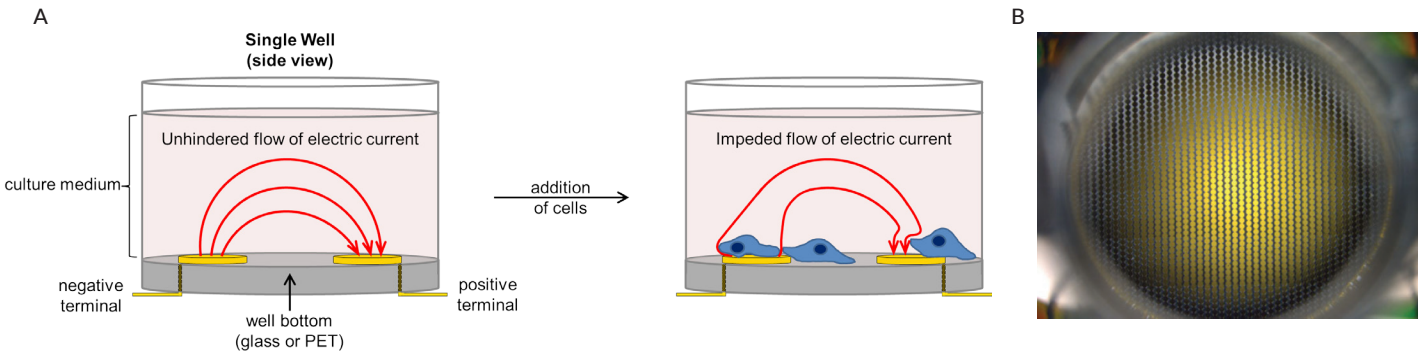

Figure 2. Overview of cellular impedance apparatus. (A) A side view of a single well from an ACEA electronic microtiter plate (E-Plate®) is shown before and after cells have been added. Neither the electrodes nor the cells are drawn to scale (they have been enlarged for clarity). In the absence of cells electric current flows freely through culture medium, completing the circuit between the electrodes. As cells adhere to and proliferate on the electrodes 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 electrodes are actually an interdigitated array that covers >75% of the well bottom. Though cells can be visualized directly on the gold electrode surfaces, special E-Plates® with an electrode-free region in the middle of the well (not shown here) are also available to facilitate microscopic imaging.

Using Impedance to Study Bacteria

To date, 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 ACEA's electronic microtiter plates (E-Plates®) does in fact result in an impedance signal that increases over time, with the kinetics being consistent with what is observed in orthogonal assays. Believing that impedance monitoring is extremely well suited for both basic and applied biofilm research applications, we recently 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:

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

The results of these studies have now been published[5], and are serving as the foundation for our ongoing bacterial biofilm research. By presenting a few specific case studies, below we summarize our optimized methodologies for using xCELLigence® RTCA to probe different facets of biofilm biology and anti-biofilm 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 please see Ferrer *et al.*[5] Bacteria were taken from -80°C storage and streaked onto trypticase soy agar plates. After 24-48 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 TSBG (TSB supplemented with 0.25% (w/v) glucose). These diluted stocks were used to seed an E-Plate® 96 (cat.# 05232368001), as described in below sections.

Instrument Prep and Measuring Background Impedance

Experiments were conducted using an xCELLigence® RTCA MP (multi-plate) instrument (cat.# 00380601040) 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, etc.) 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 (i.e. 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 described above were added to wells that already contained the 100 µL of TSBG used for background measurement (giving a final volume of 200 µL/well). Biological and/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 the course of 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, in theory, 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. In order 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 to prevent their formation in the first place. Towards this end, we evaluated the capacity of different clinically-relevant antibiotics to inhibit biofilm formation inside E-Plates. 100 µL of TSBG supplemented with different antibiotics (at concentrations ranging from 62.5 ng/mL to 32 µg/mL) was added to each well of an E-Plate® 96, and the background impedance was measured. Diluted *S. aureus* 240 cultures (described above) were then 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 Cell Index is defined as: % Cell Index = $[(\text{Cell Index})_{\text{with drug}}/(\text{Cell Index})_{\text{without drug}}]$x100. The lowest antibiotic concentration that inhibited biofilm formation (yielding a Cell Index value less than 0.05) was considered to be the biofilm minimum inhibitory concentration (Bio-MIC).

Identifying Drugs that Disrupt Established Biofilm

Beyond inhibiting the initiation/formation of biofilms, agents that can disrupt already established biofilms are needed. To see if xCELLigence® RTCA can be used in screens 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 and then antibiotics were added. Specifically, 100 µL of TSBG culture medium was used for background measurement, and 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. At this point 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 a total of 25 hours.

Results

Signal Amplitude and Reproducibility

Consistent with what has been reported in previous publications, the impedance signal observed here for *S. epidermidis* and *S. aureus* are consistently ~10 fold lower than what is typically observed for eukaryotic cells. This could be a consequence of (*i*) bacterial cells being smaller and therefore providing a thinner insulating layer that is less capable of impeding current flow, (*ii*) bacterial cells packing less uniformly and less densely than eukaryotic cells (thereby leaving more avenues for electric current to flow between electrodes), or (*iii*) some combination thereof. 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 non-biofilm producing (CECT 231) strains of *S. epidermidis* were grown side-by-side. As seen in **Figure 3B**, 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 next grown alongside an isogenic mutant which lacked the sarA gene that regulates the production of both proteinaceous and polysaccharidic components of biofilms. Importantly, 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 indeed 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 the above experiments collectively demonstrate that EPS influences the impedance signal, they don't provide an explanation for why this is the case. One possibility is that the EPS components themselves 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/tightly. 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 6 hours and remains constant out to 12 hours, both 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. Wheather

EPS components impede current flow directly, or whether EPS is causing the bacterial cells to interact with the E-Plate® electrodes more tightly, is actively being investigated.

Collectively, the above results are consistent with a model wherein both the cells and the EPS of a biofilm contribute to the impedance signal that is monitored by xCELLigence®. This is a valuable finding as it sheds light on the types of questions that can be probed using Real-Time Cell Analysis.

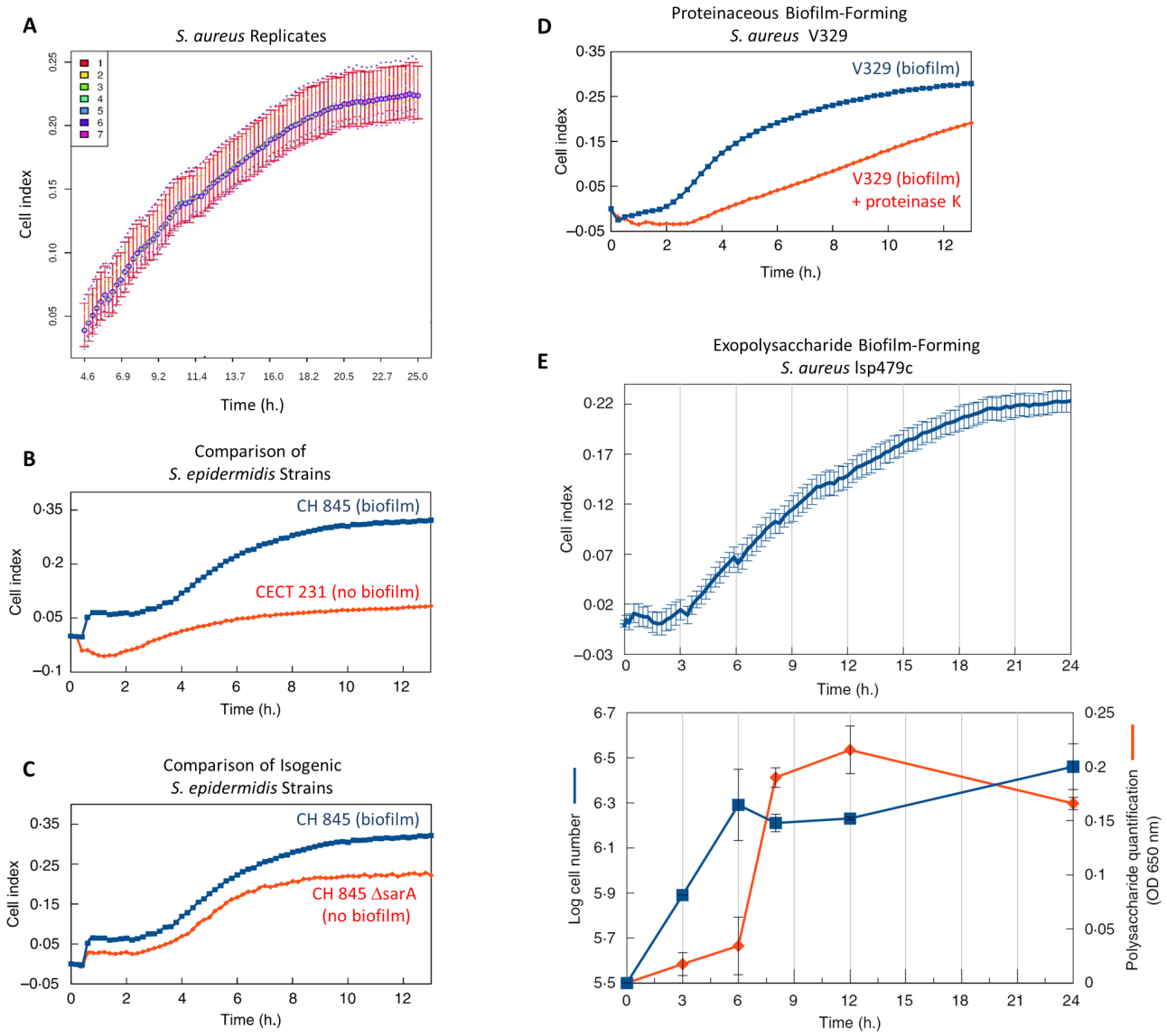

Figure 3. Assessing biofilm signal reproducibility, and the impact of EPS on the impedance signal. (A) The intra-experiment variability of biofilm-derived impedance signals was evaluated by monitoring seven technical replicates of *S. aureus*. The mean value and confidence intervals are plotted here; 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* lsp479c.

Screening for Biofilm Blocking Agents

By including antibiotic in the growth media at the time that *S. aureus 240* was seeded into E-Plate® wells, the capacity of RTCA to identify biofilm blocking activity was evaluated. As seen in **Figure 4A**, though each of the 10 antibiotics that were tested displayed prophylactic activity, 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 in the first place.

Of very high clinical 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 very infection they are trying to treat. Importantly, this bifurcated behavior is readily detectable, and quantifiable, using RTCA. While at concentrations of 4-32 µg/mL vancomycin is found to suppress *S. epidermidis* 43040 biofilm growth, at concentrations of 62.5 ng/mL-1 µg/mL biofilm growth is stimulated **(Figure 4B)**.

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. As shown in **Figure 4C**, at higher concentrations cloxacillin displayed a robust biofilm disruption activity. At lower concentrations biofilm disruption still occurred, though with slower kinetics; given a longer assay window it is likely that complete disruption would still be observed at these lower drug concentrations.

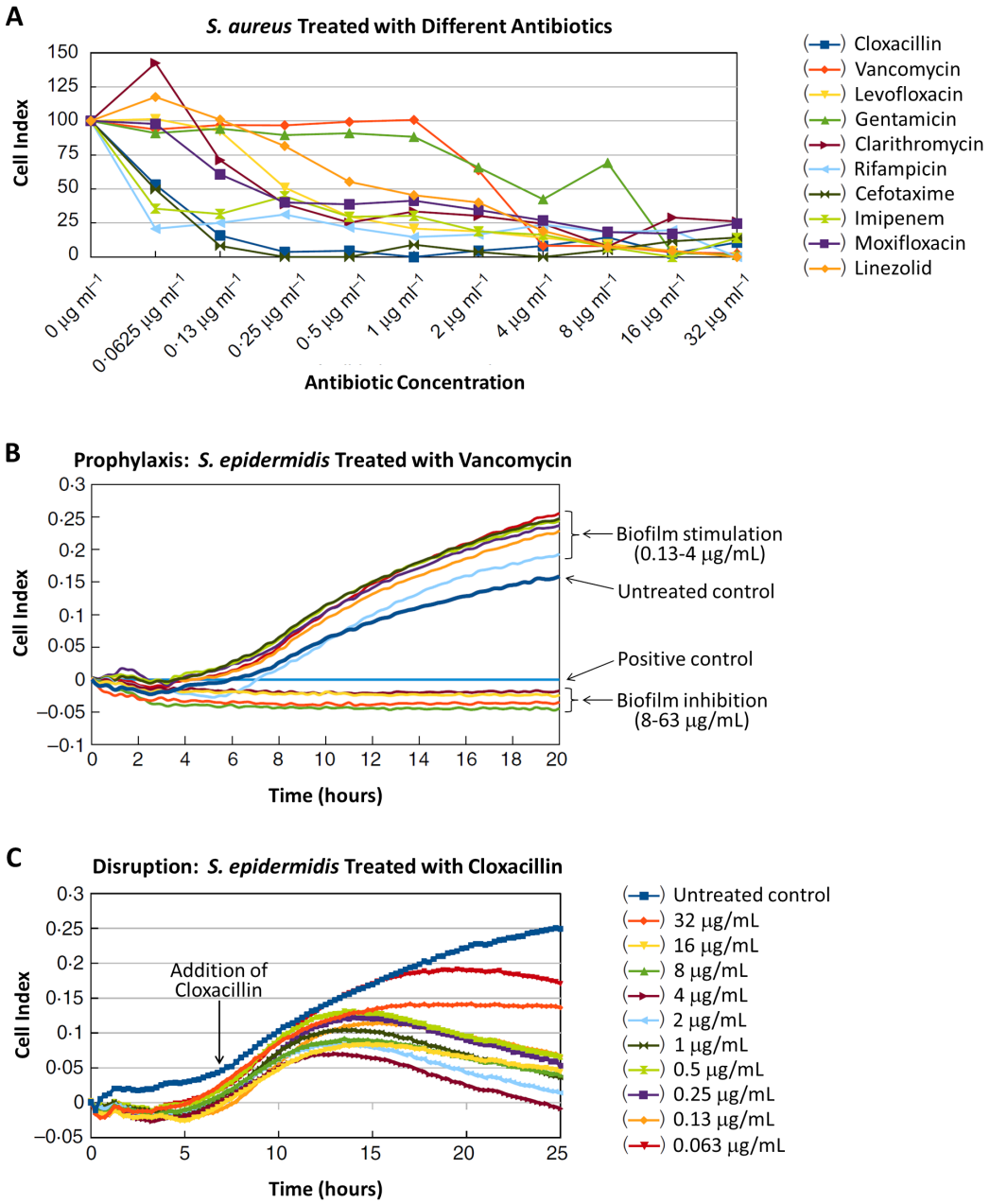

Figure 4. Using RTCA to screen for drugs that either prevent biofilm formation or disrupt established biofilms. (A) Ten different antibiotics (each represented by a different colored line) were evaluated for their ability to prevent *S. aureus* 240 from forming biofilm. Antibiotics were present at different concentrations from the moment that bacteria were seeded into wells. 20 hours after seeding, the cell index was measured and compared to the untreated control. The % Cell Index plotted here is simply $[(\text{Cell Index})_{\text{with drug}}/(\text{Cell Index})_{\text{without drug}}]\text{x}100$. **(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 higher concentrations the antibiotic cloxacillin is able to disrupt an established biofilm. See text for details.

Conclusion

Herein we have demonstrated the utility of xCELLigence® RTCA for a few basic and applied applications in biofilm research. The protocol described here 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 makes it easy to make quantitative comparisons between different strains and treatments, with both the bacterial cells and their EPS being evaluable. Achieving such a detailed and nuanced picture of biofilm dynamics using traditional endpoint assays would be prohibitively costly in terms of man hours, and would not provide the same level of reproducibility. The benefits summarized in this application note are highlighted by four xCELLigence® biofilm papers[5-8] that have been published (by independent groups) in the past few months alone.

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