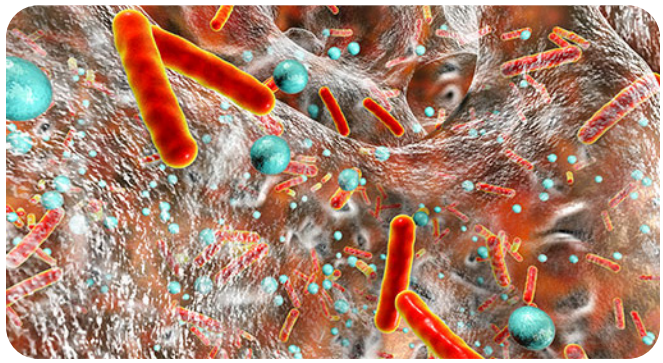
# Tackling biofilm formation

## Abstract/Introduction

Microbial biofilm formation has important implications for human health and disease. Biofilms on indwelling devices such as implants, blood and urinary catheters, heart valves and endotracheal tubes represent a persistent source of pathogenic microbes that can invade the human body and cause serious illness. Biofilms are also important virulence factors involved in antimicrobial resistance and extended survival of microbes. Although the mechanisms of biofilm formation, growth, and antimicrobial resistance have been investigated by the research community, further studies are needed to help identify therapeutics that are effective in preventing formation or impairing biofilm integrity. This article provides an overview of novel approaches to improve our understanding of biofilms and enhance the diagnosis and treatment of bacterial implant infections.

## Microbial biofilm formation

Biofilms are communities of single or multiple microbial species that form on a range of biotic and abiotic surfaces. Although mixed-species biofilms predominate in most environments, single-species biofilms often exist in a variety of infections and on the surface of medical implants.[1] The initial process of biofilm formation involves irreversible attachment to a surface by microorganisms. Following attachment, bacteria undergo further adaptation to life in a biofilm; the attached cells will begin cell division to form microcolonies and produce the extracellular polymeric substances (EPSs) that define a biofilm. This facilitates attachment and matrix formation, resulting in an alteration in the phenotype of the organisms with respect to growth rate and gene transcription.[2]



The EPS is a vital component of bacterial biofilms. It consists primarily of polysaccharides, is highly hydrated, and accounts for as much as 90% of the biofilm mass.[3] This self-produced extracellular matrix assists the bacteria by providing structural support, antimicrobial resistance, sequestering of nutrients, and preventing dehydration.[3] Microbes living in a biofilm also acquire other benefits which are not available in planktonic life, such as physicochemical advantages, multi-species synergisms, and rapid gene transfer.[4] Biofilms also confer a measurable decrease in antimicrobial susceptibility.[4]

## Biofilms on medical devices

Biofilms have great significance for public health due to the increased resistance of biofilm-associated organisms to antimicrobial agents and the potential for these organisms to cause infections in patients. There are numerous factors considered to be responsible for biofilm resistance, including restricted penetration of antimicrobials into a biofilm, decreased growth rate, and expression of possible resistance genes.[5]

Medical device infections are often linked to colonization of a device by microbes. It is often difficult to detect microbial colonization and in some cases it can go undetected for years, whereas in others it can have life-threatening urgency.[4] Indwelling medical devices (e.g., contact lenses, central venous catheters and needleless connectors, endotracheal tubes, intrauterine devices, mechanical heart valves, pacemakers, peritoneal dialysis catheters, prosthetic joints, tympanostomy tubes, urinary catheters, and voice prostheses) can act as a "bridge" between the nonsterile outside environment and the sterile inside environment of a patient.[4] Indwelling devices such as urinary catheters are frequently associated with microorganisms that originate from the skin of the patient or healthcare providers.[4] Indeed, many bloodstream and urinary tract infections are associated with indwelling medical devices and, therefore, are often biofilm associated.[6] Once in use, colonization of a medical device surface can be difficult to treat if the bacteria have become resistant to antibiotics, and in many cases successful treatment of persistent infection may require surgical removal of the device and/or surgical debridement.

Understanding biofilms and identifying therapeutics that can effectively prevent biofilm formation or impair biofilm integrity is therefore key to reducing the risk of patient infection. Analytical methods are needed to understand how biofilms form, their biochemical composition, and where they are localized. Here, we present several novel approaches for understanding biofilm formation and integrity.

## High-throughput screening

Identifying compounds that impair biofilm formation may prove useful for clearing infections or preventing relapse. However, few approaches have successfully identified such agents. To address this, researchers developed a high-throughput screen for potentiators of clotrimazole,[7] a common therapy for Candida infections, including vaginitis and thrush. *Candida albicans* is an opportunistic pathogen which forms drug-resistant biofilms that contribute to the recalcitrance of disease.

The screen was performed against *C. albicans* biofilms grown in microtitre plates to target the most resilient forms of the pathogen. Biofilm growth, in individual wells of 384-well plates, was measured using the metabolic indicator alamarBlue® and a Revvity EnVision® microplate reader. More than 120,000 small molecule compounds were screened and those that enhanced the activity of clotrimazole or acted on the biofilms alone were identified as hits. Hits from the screen were subsequently validated for their ability to inhibit biofilms alone, and in the presence of clotrimazole. The team believes this targeted, small molecule approach may have important therapeutic and industrial applications to help further understand biofilms and make currently available antifungals more effective. For example, the clotrimazole potentiators identified in this screen have the potential to improve dosing regimens, decrease the acting concentration, and combat resistance. Moreover, since the hits identified in the screen have activity against biofilms, they may be useful in treating other biofilm-related fungal infections.

## High-content screening

An alternative to traditional plate-based biofilm assays is to use a high-content screening approach to help visualize and quantify biofilm integrity and identify anti-biofilm coatings or treatments. One such application of this approach was used to analyze endotracheal tube (ET)-associated biofilms, which represent a persistent source of pathogenic bacteria that can invade the lower airways, colonizing the lungs and causing ventilator-associated pneumonia (VAP).[8] Current methods for inhibiting and removing these biofilms are not widely effective in controlling the microorganism layers on the ET surface.

The researchers used a high-content screening assay to evaluate the anti-biofilm capacity of five bacteriophages for both removing and preventing *Pseudomonas aeruginosa* biofilms on the device surface. A Revvity Operetta® CLS™ High-Content Analysis System was used to collect images and the biofilm-covered areas were analyzed using Harmony® High-Content Imaging and Analysis Software. Subsequently, two phages were selected as cocktail components and applied as a preventive strategy to inhibit bacteria colonization in a dynamic biofilm model which simulated endotracheal intubation. The researchers suggest this approach demonstrates that, with the development of new coating strategies, phage therapy has the potential to control ET-associated biofilms.

Another issue in the field has been the lack of a rapid, highly sensitive, systematic approach to screen biofilm formation and investigate the core EPS composition. To address this, Ó Cróinín *et al.*[3] used high-content screening microscopy to

quantitatively measure the effect of a variety of inhibitors of EPS integrity of adherent Campylobacter jejuni, the leading cause of bacterial gastroenteritis, biofilm in aerobic conditions (Figure 1). Adherent biofilms were induced in Revvity PhenoPlate 96-well microplates by aerobic incubation. Automated confocal microscopy was carried out using a Revvity Opera® Phenix high-content screening system. The use of two fluorescent dyes allowed for the quantification of the effect of biofilm size and density for a metabolically active living bacterial population as well as the extracellular DNA (eDNA) EPS component of the biofilm. Image analysis was performed using the Revvity Columbus Image Data Storage and Analysis System.

This model offers the opportunity to be easily adapted to phenotypically screen and characterize adherent biofilm of large numbers of fresh isolates of a variety of bacterial pathogens and to study biofilm formation by pathogens on a much larger scale.

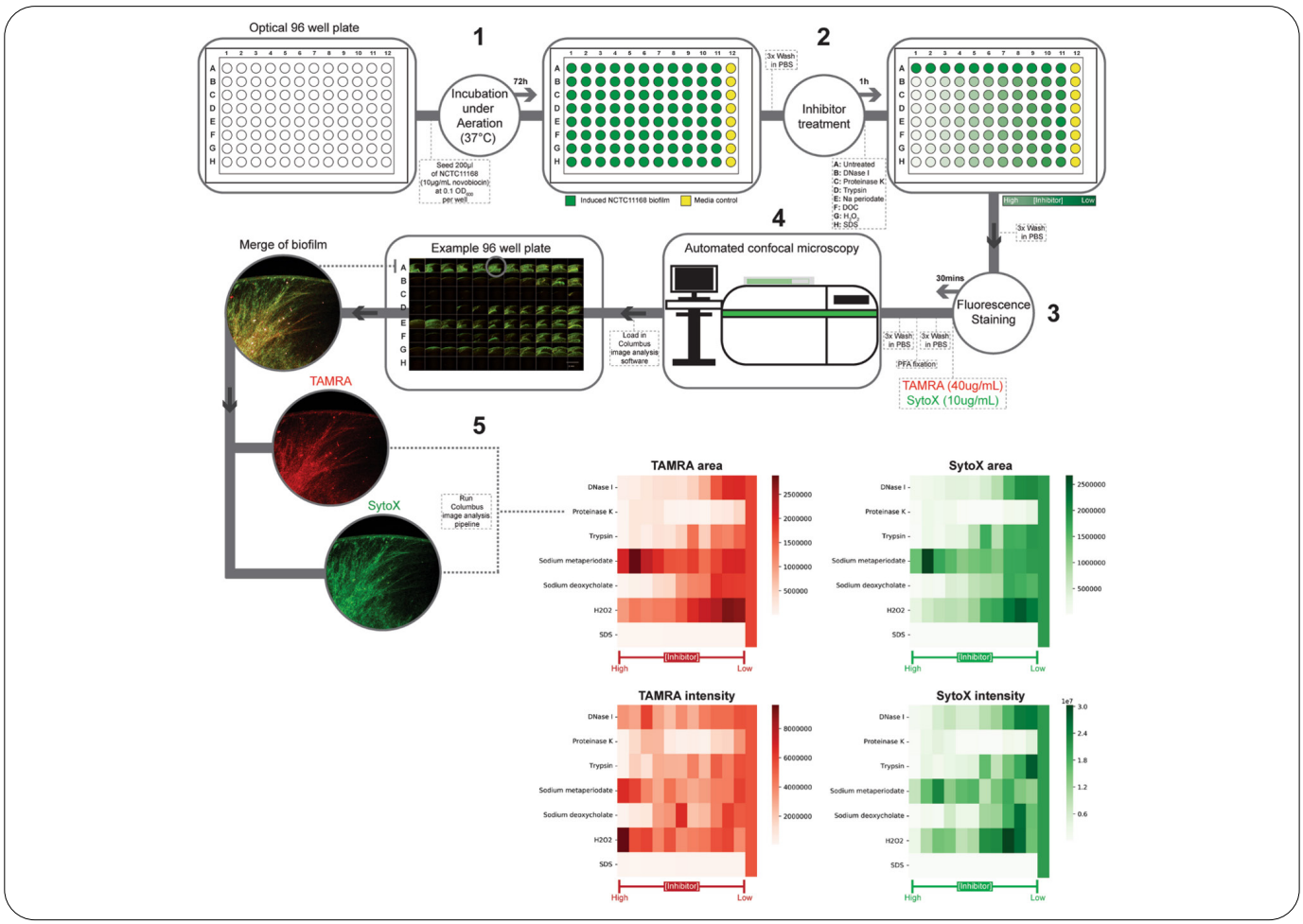



Figure 1. Workflow of high-content screening approach to investigate the effect of a range of inhibitors on the structure, composition and integrity of Campylobacter biofilm under aeration. (1) Bacterial overnight cultures of NCTC11168 (Induced with 10 µg/mL novobiocin) were equalized to an OD600 of 0.1 in MH broth (supplemented with 10 µg/mL novobiocin) and 200 µL were seeded into all of the wells of an optical 96-well plate. The cultures were incubated for 72h at 37°C aerobically (21% $O^2$) to induce adherent biofilm formation. (2) Each row of the 96-well plate was selected for addition of an inhibitor to be tested for its effect on biofilm integrity. After 72h incubation, the adherent biofilms in all 96 wells were washed once with PBS in aseptic conditions followed by addition of 100 µL MH broth (+10 µg/mL novobiocin) into all wells except for the first well of each row. For each inhibitor, 200 µL of the highest concentration was dissolved in MH broth (+10 ug/mL novobiocin) and was added into the first well of each row. The inhibitors used in this study and their highest concentrations were DNAseI (25U/mL, Sigma), sodium (meta)periodate (4 mg/mL, Sigma), proteinase K (20 mg/mL, Promega), trypsin (0.05%, Gibco), H2O2 (30% pure, Sigma) and sodium deoxycholate (10%, Sigma). Using a multiwell pipettor, 100 µL of the medium in the first well of each row was diluted in a 1:2 serial dilution along the row to a final volume of 200 µL. The adherent biofilms were then incubated at 37°C for 1h in the presence of inhibitors. (3) The metabolically active bacteria were stained for 30 mins with PBS containing 40 µg/mL 5-TAMRA-SE followed by counterstaining of dead bacteria and extracellular DNA structures with PBS containing 10 µg/mL SytoX green dye for a further 30 minutes. (4) Automated confocal microscopy was carried out using an Opera Phenix (Revvity) high-content screening microscope using a 5x/0.16 NA air objective. Images were acquired for channels using laser channels 561 nm (TAMRA) and 488 nm (SytoX). (5) A high-throughput image analysis approach for quantification of TAMRA and SytoX intensity and biofilm area was developed using the Columbus image data and analysis system. Heatmaps of mean biofilm area intensities across three biological replicates were generated to provide a rapid readout of biofilm inhibition. Image credit: Whelan, M.V.X., Simpson, J.C. & Ó Cróinín, T.[3]

## *In vivo* imaging

Accurate diagnosis and treatment of biofilm infections remains a challenge due to difficulties in locating and identifying bacterial pathogens situated deep within tissues. Fluorescent imaging is a non-invasive technique that can been used for *in vivo* localization and identification of organisms and biofilms.

In a recent study, researchers compared the ability of two fluorescent probes to localize *Staphylvococcus aureus* biofilm infections on spinal implants.[9] The team used Revvity's IVISbrite™ *S. aureus* Xen36 strain and performed *in vivo* bioluminescence and fluorescence imaging using a Revvity IVIS® SpectrumCT (Figure 2).


Figure 2. Three-dimensional bioluminescence and fluorescence imaging of spinal implant infection. At 72 hours following PI, the mice were subjected to 3D-CT with bioluminescence and fluorescence imaging. Two representative infected mice are shown. The 1D9-680 probe signal colocalizes with the bioluminescent bacterial signal on the infected implant. The Vanco-880W probe signal colocalizes with the infected implant but is also detected in the pelvic and abdominal cavities. Image credit: Park, H.Y., Zoller, S.D., Hegde, V. et al.[9]

The most successful probe – 1D9-680 (composed of the anti-IsaA IgG1 antibody conjugated to the Revvity near infrared fluorophore NIR680) was then used to guide surgical debridement of the infected tissue and implant retrieval. This study shows the ability of targeted fluorescent imaging (TFLI) technology to enhance the diagnosis and treatment of bacterial implant infections.

## Conclusion

The formation of biofilms on medical devices can pose a serious threat to patient health. Efforts are needed to further our understanding of biofilms and identify therapeutics that are effective in preventing biofilm formation or impair biofilm integrity. High-throughput screening assays have been shown to successfully identify biofilm inhibitors, while high-content screens can help researchers visualize and quantify biofilm integrity and identify anti-biofilm coatings or treatments. Furthermore, *in vivo* imaging can be used to enhance the diagnosis and treatment of bacterial implant infections. Applying these approaches to future studies could aid our understanding of biofilms and help develop or enhance treatments in the future.

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