**Biofilms in Orthopaedic Infections: A Review on Laboratory Methods**

ALEX C. DIBARTOLA, 1 MATTHEW C. SWEARINGEN, 2 JEFFREY GRANGER, 3 PAUL STOODLEY, 2,3,4 and DEVENDRA H. DUSANE 2\*

1College of Medicine, 2Department of Microbial Infection and Immunity,

3Department of Orthopaedics; The Ohio State University, Columbus, Ohio, USA; and

4National Centre for Advanced Tribology, Faculty of Engineering and the Environment, University of Southampton, Southampton, UK

\*Corresponding author

Devendra H. Dusane,

760 Biomedical Research Tower (BRT),

460 West, 12th Avenue, Columbus, OH 43210,

U.S.A.

E-mail: Devendra.Dusane@osumc.edu

**ABSTRACT**

Bacterial infection after hardware implantation in orthopaedic surgery is a devastating issue as it often necessitates increased hospital costs and stays, multiple revision surgeries, and prolonged use of antibiotics. Due to the nature of hardware implantation into the body, these infections are commonly in the form of attached biofilms. The current literature on a range of methodologies to study clinically explanted infected orthopaedic hardware, with potential biofilm, in the laboratory setting is limited. General methods include traditional and advanced culturing techniques, microscopy imaging techniques, and techniques that manipulate genetic material. The future of diagnostic techniques for infected implants, innovative hardware design, and treatment solutions for patients all depend on the successful evaluation and characterization of clinical samples in the laboratory setting. This review will provide an overview of current methods to study biofilms associated with orthopaedic infections, as well as provide insight into future directions in the field.

Key words:orthopaedic; periprosthetic joint infection; biofilm; antibiotics; laboratory methods; agar encasement; microscopy

**INTRODUCTION**

Bone and joint infection affect millions of people worldwide. Bone tissue infections, namely osteomyelitis, septic arthritis, and prosthetic joint infections (PJI), represent some of the worst complications of orthopaedic surgery. The main pathways of infection for osteomyelitis, septic arthritis, and PJI are from bacteremia, or when the infection is transmitted from direct inoculation of tissue, such as following injury, surgery or implantation of a foreign body, such as joint replacement (1-3). Joint replacement has become one of the most common surgical procedures in orthopaedics and contributes significantly to improving the quality of life of elderly (4-6). It is predicted that the percentage of the elderly population affected by bone diseases will double by 2020 (7). Specifically, as the number of total joint arthroplasty and internal fixation procedures continues to rise, the threat of infection following surgery has significant clinical implications.

Infection after orthopaedic surgery can be devastating for the patient and the physician alike. Infectious complications after total joint replacement may require an exhaustive course of hardware explanation, prolonged doses of single or multiple intravenous antibiotics, and intensive revision surgery (8-10). Prolonged antibiotic usage can lead to establishment of antibiotic resistance bacterial communities. In addition, infectious complications are commonplace after orthopaedic trauma procedures (11). However, aside from the human impact, infection after surgery can also increase length of hospital stay, hospital costs, and readmission rates (12).

Early and correct diagnosis of infection is extremely necessary; therefore, diagnostic methods have received increasing attention in recent years. In addition, it is now well established that bacteria common in PJIs are typically organized in the form of a biofilm. Biofilms are microbial communities adhering to a surface by means of a self-generated exopolysaccharide matrix (13-15). Biofilms are most commonly formed by bacteria including staphylococci and Gram negative pathogens but can also be formed by fungal pathogens and be single as well as mixed species. Four characteristic steps are necessary for biofilm formation to occur: attachment, micro-colony formation, maturation, and dispersal (16, 17). The definitive characterization of an infection as a biofilm is based on the unequivocal demonstration (usually only possible by direct microscopy) of matrix-enclosed microbial communities within or upon the affected tissues or surface (18). In addition, biofilm growth mechanisms alter bacterial phenotypes and foster tolerance to many classes of antibiotics, as well as host immune defenses (19-22). Thus, continued and increasing study of biofilms has been imperative to the development of treatments for these hard to manage infections. Along the same lines, there has also been a growing body of scientific publications related to orthopaedic infection and biofilms, as clinicians and researchers alike seek understanding, novel treatments, and preventative measures (Fig. 1).

Given the commonality of hardware implantation in orthopaedic practice and the propensity of bacteria to grow in biofilm format on foreign implanted material, infection after orthopaedic surgery often takes a biofilm format (Fig. 2), (23-25). Equally important, biofilms have been shown to complicate and intensify numerous other types of clinical infections outside of Orthopaedics. For example, urethral stents, suture material, endotracheal tubes, and cardiovascular devices have all been shown to harbor biofilm (13, 26-33). It is clear that biofilms are common place in many clinical infections.

The normal microbiota of the skin is the most common source of bacteria detected in PJIs and other orthopaedic infections. Molecular methods based on 16S rRNA gene sequence-based methods have revealed that infections are commonly polymicrobial (34, 35). Despite the increased utilization of molecular techniques, culture methods are indispensable for determination of antibiotic susceptibility, and they are an important means of confirming results obtained by culture-independent methods. However, over the last decade it has become apparent that routine clinical culture often returns false negatives (36, 37). In addition, as routine inflammatory markers often fail to discriminate trauma; osteolytic reaction to wear particles, foreign body inflammation and infection, the Musculoskeletal Infection Society (MSIS) has developed a set of criteria for the diagnosis of PJI from a set of clinical signs despite a culture negative test (38). Importantly, a number of studies have addressed different methodological issues that may have an impact on the yield of positive cultures (39).

Methods exist to study laboratory generated biofilm samples, and new ones are in constant development (40). Unfortunately, the detection of biofilm infections *in vivo* and the analysis of clinical samples of biofilms *in vitro* are seriously hampered by complex culturing methods and a paucity of methodology to analyze clinical samples. In this review we focus on some of the methods commonly employed in the research laboratory to understand the biofilm bacteria associated with periprosthetic joint infections.

**DIRECT CULTURING IN BROTH OR ON AGAR**

Direct culture employs the use of standard microbiological culturing techniques with sterile swabs, loops, or toothpicks. Explanted materials are usually first dip rinsed (although the number of rinse steps and degree of agitation is not standardized) using sterile phosphate buffered saline (PBS) to remove loosely adhered host tissue, surgical debris, blood, and planktonic or contaminating bacteria (41). This step is imperative in removing planktonic bacteria and leaving behind only the strongly adhered biofilm. The clinical samples may then be swabbed and then spread onto selective growth media, or inoculated into liquid broth media such as Brain Heart Infusion (BHI) or Tryptic Soy Agar/Broth. This method is limited by the current understanding of where on clinical samples biofilm grows. For larger samples in particular, it may be difficult to culture/swab the entire sample.

**AGAR ENCASEMENT CULTURING METHOD (AECM)**

The agar encasement culturing method (AECM) is a simple but novel method used to encase retrieved orthopaedic hardware components in a thin film of warm molten agar (Fig. 3). Once the agar solidifies, bacterial colonies grow to confirm the location of biofilms on the surface of explanted components (42). This is important in mapping the location and association of biofilm on the clinical implants or components with surface types. Briefly, the explanted materials are first rinsed with PBS to remove non-adherent bacteria and other host debris. Subsequently a vigorous shaking may be done with a rinse that is subsequently used for DNA extraction or spread plating on selective growth media for bacterial isolation and identification. After the rinse step, warm molten agar media such as TSA or BHI is dripped onto the hardware components to encase them in a film of agar. A plastic serological pipette (50 – 100 ml) pipette works well, as smaller sizes tend to have narrow diameters that promote rapid agar solidification. Generally, BHI agar works well, however this method may be applied to a wide array of selective growth media to isolate specific bacteria or a group of bacterial communities. For instance, Mannitol salt agar supplemented with phenol red may be used to selectively identify *S. aureus*. Two to four applications of thin films of agar media are often required to get complete coverage. Samples may then be incubated at suitable growth temperatures and CO2 concentrations. A humidified chamber at 37°C, 5% CO2 for up to 12 days is suitable to allow for bacterial growth of most slow growing bacterial communities. Close attention to incubation humidity is essential because of the thin agar layer is prone to rapid dehydration. Once bacterial growth appears, digital photography is used at different time intervals to determine colony outgrowth. In addition, large (500 – 2000 ml) sterile clear plastic or glass containers are generally useful for visual analysis. In addition to being able to map the location of biofilm, another advantage of this method is due to the fact that as some bacterial outgrowth is inside agar (not all on the surface), it might be possible to culture both aerobes and anaerobes. For example, these aerobic conditions have yielded culture of *Bacteriodes fragilis* from an infected suture (41). The presence of this strict anaerobe was corroborated by 16S RNA gene phylogenetic sequencing and anaerobic clinical culture.

There are some limitations associated with this culturing method. First, sub-culture of colonies encased in agar may prove difficult. However, it is possible to use a sterile toothpick or a disposable sterile plastic or metal loop to carefully penetrate the agar and select an individual colony. In addition, further processing of orthopaedic samples for microscopy to determine the biofilm structure after AECM is not possible. Furthermore, wear and metal analysis would require removal of the agar.

**IMMERSION CULTURE**

In a similar method to AECM, immersion culture involves the use of molten agar based growth media. In this method, the retrieved implant is completely immersed in agar in an appropriately sized glass vessel such as a beaker or a measuring cylinder (i.e. for a stemmed femoral hip component). After identical rinse steps to those used in AECM, large volumes of growth media are used to completely immerse the clinical components. Once the agar media is set and after an appropriate incubation period, colony outgrowth is assessed (Fig. 4). The immersion method works best with smaller orthopaedic components, as larger components require greater volumes of media and may obscure visual analysis due to opacity. In the immersion method, addition of molten agar from the bottom of the culturing vessel helps avoid dispensing the agar directly onto the suspected biofilm. As in the AECM, colony outgrowth in the immersion method is implicative of biofilm presence, assuming vigorous and multiple rinse steps. One limitation to this method is that visualization of biofilm growth is sometimes difficult due to opacity and volume of the growth medium. In addition, bacteria removed during the pouring of the agar may form colonies in the residual growth media, obscuring outgrowth from the implant surface.

**CULTURING AFTER SONICATION/RINSING**

Biofilms are difficult to remove from surfaces due to their ability to produce sticky exopolymeric substances (EPS). Therefore, even numerous saline rinses often leave residual biofilm bacteria adherent to the implant materials behind. Sonication is one of the laboratory research methods that can effectively remove biofilm whilst retaining bacterial viability. In this method, orthopaedic implant materials/samples are placed in sterile collection vessels. Explanted materials are first rinsed with sterile PBS to remove loosely adhered host tissue, surgical debris, blood, and planktonic bacteria. Samples are then submerged in PBS, sonicated, and then the PBS “sonicate” collected for plating. Time duration for sonication of the orthopaedic explants varies in different labs. For example Evangelopoulos et al. (2013) suggest 1-minute is effective for the removal of adherent biofilms from implant materials (43). In another study however, Trampuz et al. (2007) and Holinka et al. (2011) use 5-minute sonication with intermittent vortexing steps to remove biofilms from prosthetic components (44, 45). The sonicate may then be subjected to direct culture on selective growth media. In addition, the PBS sonicate may be analyzed for presence of bacteria using molecular or microscopic techniques.

Similar to the AECM and agar immersion method, the main drawback of this technique is that the rinse steps are not standardized with respect to the removal of planktonic and attached biofilm bacteria. In addition, the technique is time-consuming and often requires large quantities of growth media.

**FLUORESCENT MICROSCOPY**

Fluorescent in-situ hybridization (FISH) allows direct quantification and visualization of biofilm on clinical samples, and may be used with fluorescent microscopy or confocal laser scanning microscopy (CLSM) (Fig. 5A-B). While numerous methods have been developed to take advantage of FISH protocols [catalyze reporter deposition FISH (CARD-FISH), rRNA FISH, multiplex-FISH, multicolor-FISH, peptide nucleic acid FISH (PNA-FISH), and 3D-FISH], the process remains the same at its core (24, 46-51). Fluorescently tagged probes for specific and unique nucleic acid sequences are introduced to biofilm samples, and after hybridization, probes may be visualized under fluorescent microscopy, thus identifying specific bacterial species or genera. With an always increasing availability of probe development, FISH’s utility in sample analysis to detect specific bacterial species will only increase (52).

Once such target for fluorescently labelled probes include the exopolysaccharidesthat serve as part of the structural components in the complex extracellular matrix (ECM) created by bacteria in the biofilm growth conditions (53, 54). This may be exploited when imaging biofilms using fluorescent laser scanning microscopy. Fluorescent probes, such as wheat germ agglutinin-Alexa Fluor® 488 (WGA488, green), have be used to stain biofilms in clinical specimens (Fig. 5C). WGA is a lectin specific for poly-N-acetyl glucosamine (pNAG), a component of extracellular polymeric slime (EPS) produced by *ica+* staphylococci (55). In addition to staining the ECM components, propidium iodide (PI, red), SYTO 59 (red) or SYTO 9 (green) nucleic acid stains may be used to identify bacterial cells in biofilms. Since lectins can also stain host matrix components, a combination of lectin staining with nucleic acid staining for cells is preferred. This allows for the co-localization of stains that often provides more data for the identification of biofilm. The overarching goal of this method is to stain a component of the biofilm or bacteria using a fluorescent probe.

Limitations of this method include the inability to identify non-fluorescently labeled components of bacteria and/or biofilms, and the difficulty of differentiating biofilm ECM from bacteria. However, while some stains such as Calcofluor White (CFW), Concanavalin A (Con A), and tetramethyl-rhodamine conjugate stain the ECM and some components of bacteria, stains such as SYTO 9 will only stain the ECM, allowing for ECM and bacteria differentiation. One final limitation is the limited ability of fluorescent microscopy to generate 3D spatial data related to biofilms.

**CONFOCAL LASER SCANNING MICROSCOPY (CLSM)**

Confocal laser scanning microscopy (CLSM), when combined with FISH probes, has added advantage to better evaluate the three-dimensional structure of biofilms (56). Briefly, after clinical biofilm samples are preserved (4% paraformaldehyde in PBS overnight at 4°C, and subsequently in 50% ethanol in PBS at -20°C the following day), sections are cut, and imaged using CLSM (41). Many imaging software are available for image procurement, processing, and analysis. The most commonly used ones are ImageJ ([*https://imagej.nih.gov/ij*)](https://imagej.nih.gov/ij)), IMARIS ([*www.bitplane.com/imaris*](http://www.bitplane.com/imaris)), and COMSTAT ([*www.comstat.dk*)](http://www.comstat.dk)). In addition, one other advantage of CLSM is its ability to be run in reflective mode, allowing it to better characterize and identify the surface of implant materials.

Limitations of FISH and CLSM for the imaging of clinical samples relate to the fact that these techniques are often most beneficial for small sample sizes. Analyzing larger samples may prove difficult and expensive. In the analysis of orthopaedic hardware samples specifically, large areas with possibly rich biofilm deposition may simply remain “un-imaged”. While smaller samples (less than 1cm2) may be imaged in their entirety, decisions on which areas of larger samples to image must be made using either clinical suspicion or laboratory protocols. Finally, as only fluorescently labeled structures may be imaged using FISH and CLSM, many important extracellular components of biofilms remain undefined using this method of study (57).

**SCANNING ELECTRON MICROSCOPY (SEM)**

Scanning electron microscopy (SEM) is useful due to its extremely high resolution and its ability to readily resolve single bacterial cells. In addition, it can be readily zoomed without the need to change objectives as in light and fluorescent microscopy. SEM involves use of focused electron beams to scan specimens and deliver signals related to surface characteristics. SEM has been used in numerous applications to image *in-vitro* biofilms on orthopaedic hardware (58, 59). In addition, SEM has been successfully used to evaluate biofilms on clinically infected hardware samples in the oral-maxillary-facial surgery fracture literature (60). When combined with energy dispersive x-ray analysis (EDS), SEM can also be used for surface elemental analysis which may be useful for analyzing the surface chemistry of metallic implants and/or their wear particles. In addition, FISH-CLSM has been used with SEM to create impressively detailed images of endodontic biofilms (61).

Some limitations related to SEM are similar to the other imaging techniques discussed. For example, knowing which areas of larger samples to image remains burdensome. In addition, it has been shown that bacteria and biofilm surface characteristics may lose shape during dehydration and imaging procedures, providing difficult to understand information on biofilm and EPS structures (62). In such cases, non-invasive techniques such as fluorescent staining associated with CLSM may be a better option. In addition, most SEM imaging chambers have size limitations for samples.

**POLYMERASE CHAIN REACTION (PCR) BASED MOLECULAR TECHNIQUES**

Due to failure to culture methods for the detection of many chronic biofilm infections (culture negative bacteria) and the lack of sensitivity and accuracy of classic culture techniques, many researchers have explored the use of PCR techniques for the detection and identification of biofilm bacteria associated with orthopaedic hardware infections.

**DNA EXTRACTION, PCR AND 16S rRNA GENE SEQUENCING**

Extraction of DNA from clinical biofilms has become a useful technique to identify precisely which bacterial species are present in sometimes complex clinical samples. Once again, rinse steps prior to bacterial DNA extraction are imperative to make sure only biofilm bacteria are identified. However, even the detection of DNA from planktonic cells may provide useful. Large clinical samples are rinsed with large volumes of PBS to remove host tissue debris and loosely adhered or planktonic bacteria. Sonication aids in biofilm removal from larger samples, and DNA may be extracted from the “sonicate”. Alternatively, smaller tissue samples may be directly used for DNA extraction methods. Many DNA extraction procedures exist and often include a centrifugation component (63, 64). It is helpful to elute DNA in UltraPure™ DNase/RNase-free distilled water to prevent contamination of other genetic material and enzymes.

After DNA isolation, DNA may be amplified using PCR or real-time PCR (RT-PCR), the difference being that RT-PCR allows for the quantification of relative amounts of DNA in multiple samples. In addition, DNA samples may be subjected to PCR using primers specific for the 16S rDNA variable regions, therefore allowing amplification of species specific regions. Purified amplicons may then be used to prepare DNA libraries that can be sequenced to differentiate between species (41).

One major limitation of using DNA extraction, amplification, and sequencing techniques to study biofilms is the high sensitivity of PCR to amplify even small amounts of DNA (65). In addition, bacterial DNA can contaminate even sterile surgical grade irrigation fluids and sampling containers (41, 66). Sequencing results sometimes indicate the presence of hundreds of bacterial species in varying amounts, necessitating the need to introduce somewhat arbitrary cut off points to distinguish false positives. It then becomes difficult to differentiate between bacterial contamination (from many sources including the patient skin, the operating room environment, and the research lab) and actual infective clinical bacteria. Furthermore, free DNA from dead bacteria may also contaminate samples. Using reverse transcriptase to amplify mRNA from targeted species overcomes this issue by specifically identifying only active bacteria (67). Also, as mRNA is more labile than genomic DNA, it may also somewhat overcome the issue of contaminating DNA.

**COMBINATION OF METHODS**

To most confidently evaluate orthopaedic samples for the presence of biofilm, the use of a combination of the methods discussed will yield the most constructive results (Fig. 6). For instance, upon the last rinse step prior to using AECM, the rinsate PBS may be centrifuged, pelleted, and a DNA extraction may be performed on the resulting sample. This may allow for molecular technique results to be corroborated with results obtained from culturing methods (41). Similarly, PCR based techniques on rinsates may be combined with fluorescent or CLSM imaging using specie specific FISH probes to validate data.

**CONCLUSIONS AND FUTURE DIRECTIONS**

Infection in orthopaedics specifically, and surgery in general, has major consequences for the patient and healthcare system. While evaluation of biofilms in the *in vitro* laboratory setting is imperative to the understanding of biofilm growth mechanisms, antibiotic resistance patterns, and materials susceptibility, research has progressed to necessitate methodologies to evaluate clinical samples of suspected biofilm infections for diagnostic purposes.

Biofilms are particularly important to orthopaedic infection due to the commonality of hardware implantation. In the infected patient with suspected hardware biofilm, surgeons must often decide to explant hardware and re-implant at a later date (2-stage revision surgery), or “wash-out” the infected wound and remove biofilm, leaving hardware in place (1-stage revision surgery) (10, 68-70). Due to the inability to visualize biofilms location on implanted hardware, focusing biofilm removal in any one area is difficult. However, through mice models of infected orthopaedic hardware, there has been some success with using *in vivo* bioluminescent and fluorescent imaging combined with computed tomography to locate biofilm and inflammatory response (71). And while bioluminescent bacteria and fluorescent imaging may not be used translationally in humans, laboratory study using a similar model may aid in correlating biofilm location with an inflammatory response.

In addition, while the vast majority of biofilm in orthopaedic infection focuses on the role biofilm plays in relation to implanted hardware, some recent evidence suggests biofilms presence on soft tissue graft material used in orthopaedic surgery. For instance, soft tissue surgery is common in the sports medicine orthopaedic arena. Cartilage repair, ligamentous reconstructions, and tendon repair are all susceptible to infection and biofilm. For example, DNA extractions, PCR, and FISH analysis have demonstrated *Propionibacterium acnes* on ACL grafts material (72, 73). This evidence suggests that implantation of foreign hardware is not required for a biofilm growth format. Further research should focus in part on rapid diagnostic techniques for biofilms, possibly through detection of ECM components on such samples removed for non-traumatic failure, as indolent biofilm infection may play a role.

In addition to methodology for the study and diagnosis of infections involving biofilms, techniques continue to be developed to treat and/or prevent infection in orthopaedics. Recently, it has been shown that bathing anterior cruciate ligament grafts in vancomycin can statistically significantly reduce clinical infection (74). In addition, a large amount of evidence has supported the use of new clinical tests, such as alpha-defensin, to diagnose joint and hardware infection in total joint replacement (75-78). However, while measurement of inflammatory markers may be extremely helpful in diagnosis, these markers are unable to yield information about growth mechanisms. Discovery of inflammatory markers that are upregulated secondary to biofilms specifically would be a breakthrough in biofilm diagnostics.

Without continued research and development of existing and novel laboratory techniques to study clinical biofilm samples in orthopaedics, improvement in the diagnostic and therapeutic arena will not improve. Strong and comprehensive laboratory methodology to develop better diagnostics generally and biofilm diagnostics specifically is certainly at the forefront of the battle against biofilms in orthopaedic infections.

**FIGURE LEGENDS**

Fig. 1. Accumulated publications retrieved using key words ‘biofilm’ and ‘orthopaedic’ (from 1987), derived from PubMed (<http://www.ncbi.nlm.nih.gov/pubmed/)> on 1st January 2017.

Fig. 2. Reconstructed knee showing patellar, femoral and tibial components depicting hypothesized stages in the establishment of a biofilm of *S. aureus*. Single cells of *S. aureus* enters the surgical site (a), the cells rapidly aggregate through physical interaction (b), the aggregate switch to the biofilm phenotype, producing a protective EPS (c) followed by attachment of the aggregates to the knee components and formation of mature biofilms (d).

Fig. 3. Mapping biofilms *ex-vivo* and grown *in-vitro*. (A) MRSA colony outgrowth (arrows) from the rim of a retrieved femoral head from a PJI patient indicated for revision. The femoral head was overlaid with Brain Heart Infusion (BHI) agar following the agar encasement method (AECM). (B) A heat map of *in-vitro* growth of bioluminescent *S. aureus* SAP231 shows most biofilm growth in a similar location. Red indicates actively metabolizing biofilm. The rim is less polished than the more polished articulating surface suggesting that surface finish is an important factor.

Fig. 4. (A) Retrieved femoral component from a chronically infected total knee arthroplasty. Colony outgrowth from the surfaces is heterogeneous and is concentrated in four patches (indicated by dashed lines). Note there are few colonies on the lower part of the component where it would articulate with the polyethylene component of the tibial tray. Individual colonies can be counted from different images and fields of view to give CFU/cm2. (B) Colony forming units (CFU/cm2) measured from images of components in the agar encasement. P1 and P2 are different patients. P1 data for each component are from 4 fields of view and P2 is from 8 fields of view. The large SD illustrates the heterogeneity in biofilm distribution on the component.

Fig. 5. (A) *S. aureus* biofilms (green) stained with sau-Cy3 FISH probe attached to the polyethylene component (shown in blue imaged by reflected light) from a chronically infected ankle arthroplasty and (B) High magnification image of periprosthetic tissue associated with the ankle. The nucleic acid stain SYTO 59 (red) is used for all bacteria and sau-Cy3 is used for *S. aureus* (green). *S. aureus* appear yellowish green since they take up both the stains. One cocci only took up SYTO 59 (shown by arrow) so it is not *S. aureus* and suggests that the biofilm was polymicrobial. The hazy red staining between the cells is possibly low density eDNA in the EPS. (C) Confocal laser scanning image demonstrating biofilm bacteria on a piece of suture material recovered from a clinically infected patient. Red cocci are surrounded by green wheat germ agglutinin-Alexa Fluor® 488 stain (staining poly-N-acetyl glucosamine) at 63x magnification suggesting the biofilm was staphylococcal. *S. aureus* was cultured from aspirate and identified by 16S RNA gene sequencing (41).

Fig. 6. Schematic of research laboratory methods being used or under development for studying biofilms associated with orthopaedic periprosthetic infections.

**CONFLICT OF INTEREST**

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