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In Vitro Model Systems for Exploring Oral Biofilms: From Single-Species Populations to Complex Multi-Species Communities

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Abstract

Numerous *in vitro* biofilm model systems are available to study oral biofilms. Over the past several decades, increased understanding of oral biology and advances in technology have facilitated more accurate simulation of intraoral conditions and have allowed for the increased generalizability of *in vitro* oral

biofilm studies. The integration of contemporary systems with confocal microscopy and 16S rRNA community profiling have enhanced the capabilities of *in vitro* biofilm model systems to quantify biofilm architecture and analyze microbial community composition. In this review, we describe several model systems relevant to modern *in vitro* oral biofilm studies: the constant depth film fermenter, Sorbarod perfusion system, drip-flow reactor, modified Robbins device, flowcells, and microfluidic systems. We highlight how combining these systems with confocal microscopy and community composition analysis tools aids exploration of oral biofilm development under different conditions and in response to antimicrobial/anti-biofilm agents. The review closes with a discussion of future directions for the field of *in vitro* oral biofilm imaging and analysis.

Introduction: Importance of *in vitro* model systems to the study of oral biofilms

Microorganisms form dynamic multi-species biofilm communities on numerous surfaces in the human oral cavity (Marsh 2009). Over time, oral biofilms change in composition and architecture as component microbes interact with each other, the environment, and the host (Lamont et al. 2018). Oral biofilm communities can be extremely resilient; redeveloping rapidly after physical perturbations (e.g. brushing or flossing) and chemical treatments (e.g. application of mouthwash) (Marsh 2010). Furthermore, certain ecological and environmental conditions can alter the microbial composition and behavior of oral biofilm communities resulting in dental caries and periodontal disease (Aas et al. 2008; Peterson et al. 2013; Marsh 2018). Dental caries and periodontal disease are among the most prevalent of human diseases (Petersen et al. 2005) ranking 1 and 11 in a 2016 ranking of global health burden of 328 diseases (Vos and Collaborators 2017). In 2016, an estimated 2.44 billion people had active dental caries while about 750 million suffered from periodontal disease worldwide (Vos and Collaborators 2017).

While clinical studies are the gold standard for evaluating approaches to control oral biofilms, implementing such studies can be costly and logistically demanding (Martin-Kerry et al. 2015). By contrast, *in vitro* biofilm systems offer a relatively less challenging platform for exploratory, fundamental, and applied studies to close knowledge gaps in human oral biofilms prior to clinical studies. For example, *in vitro* biofilm model systems have been used to demonstrate how biofilm formation, succession, and/or architecture respond to environmental challenges (Kolenbrander et al. 2006; Hojo et al. 2009), and to evaluate candidate antimicrobials (Corbin et al. 2011). Many of the available *in vitro* biofilm systems can be adapted to simulate multiple *in vivo* conditions representative of the human oral cavity (Coenye and Nelis 2010; Yu et al. 2017). The closer the *in vivo* mimicry, the more generalizable the results gathered from *in vitro* model systems are likely to be.

An additional advantage of *in vitro* model systems is the ability to alter one parameter at a time, thus providing a powerful strategy for studying how biofilms develop (Fernandez et al. 2017). These experiments can provide clues into how component species interact with each other within the oral cavity and enable the characterization of potential keystone pathogenic species in biofilm development (Hajishengallis et al. 2012). For example, when considering investigations into understanding how oral species interact with one

another, using a two stage chemostat system and a defined ten species biofilm community, Bradshaw and colleagues showed the absence of the promiscuous coaggregating organism *Fusobacterium nucleatum*, resulted in significant changes in biofilm community representation (Bradshaw et al. 1998). Other examples of how *in vitro* model systems have been used in fundamental and applied oral biofilm research are detailed in Table 1.

In this review we describe the relevance of *in vitro* biofilm models to oral health and disease research and provide a distillation of previously established models used to develop defined single-species, defined multi-species, and complex multi-species (i.e. microcosm) oral biofilms. We also focus on select biofilm models that can be integrated with confocal microscopy and 16S rRNA community profiling. This integration enables the study of biofilm growth under conditions representative of the oral cavity. A particular focus of discussion will be on biofilm models that are open (constant delivery of fresh media), multiple-throughput (allowing for concurrent side by side testing) and that use small volumes to conduct experiments. Furthermore, we discuss the impact and potential clinical relevance of *in vitro* oral biofilm model systems, their limitations, and future directions for *in vitro* oral biofilm model research.

Past and Present: Oral *In Vitro* Biofilm Models

From early oral biofilm models developed in the mid 1900's (Dietz 1943; Pigman et al. 1952), that followed from relatively primitive models in the late 19th century (Tang et al. 2003), and throughout the ensuing decades, newer conceptual designs improved upon their predecessors. From a historical perceptive, *in vitro* oral biofilm studies using model systems can be characterized by transitions in foci from fundamental to applied studies within three main arenas: (**1**) understanding the development of single-species biofilms, (**2**) exploring environmental and cell-cell interactions in defined multi-species biofilms, and finally, (**3**) studies of complex multi-species biofilms. In each arena, fundamental studies of biofilm development provide the framework for applied studies, such as the effects of antimicrobial or anti-biofilm interventions, resulting in insights into the potential application to improving oral healthcare. It should be noted that there are fewer fundamental and applied *in vitro* periodontal disease models compared to cariogenic models, partly because of the increased complexity of simulating subgingival plaque (Walker and Sedlacek 2007; Velsko and Shaddox 2018). Research in multi-species (microcosm) biofilms has recently gained traction due to technological advancements and methodologies that enable investigators to measure biofilm outcomes such as community membership with 16S rRNA profiling and measuring biofilm architecture captured by a confocal microscope (Rudney et al. 2012; Fernandez et al. 2017).

Among the earliest examples of *in vitro* oral biofilm model systems was an "artificial mouth" developed by Pigman and colleagues to study early carious lesions using extracted teeth (Pigman et al. 1952). This model was particularly notable because it was arranged vertically, and sterile media was drip-fed over an extracted human tooth inoculated with pooled human saliva and housed in an acrylic box. The media reservoir was positioned above the extracted tooth and media delivered with a hypodermic needle. This experimental setup focused on identifying conditions that favor cariogenesis; Pigman's model is arguably

an ancestor to contemporary drip-fed systems (discussed later in this review). From the 1950's to the 1960's, many *in vitro* oral studies improved Pigman's artificial mouth system, by including an incubator cabinet and sterilization with ethylene oxide (Pigman et al. 1955; Pigman et al. 1962; Pigman and Newbrun 1962). From a fundamental perspective, these studies linked common dietary sugars, e.g. glucose and sucrose, to cariogenicity. From an applied standpoint, anti-cariogenic effects of compounds and dentifrice slurries could be evaluated by treating tooth enamel with anti-caries agents concomitantly with conditions that would favor cariogenesis.

Artificial mouth model variants have been used extensively over the years since the mid 1980's, most frequently by Sissons' group (Sissons et al. 1985; Sissons et al. 1991; Sissons et al. 2000). Their artificial mouth system, called the "Multiple-plaque Artificial Mouth" (MAM), was developed from designs by Russell and Coulter (Russell and Coulter 1975) and Dibdin and co-workers (Dibdin et al. 1976). The MAM is experimentally flexible and reproducible, and is compatible with computer-controlled systems (Sissons et al. 1991; Wong et al. 1994; Sissons et al. 2000). Contributions and advancements by Sisson's group and other research groups to the development of artificial mouth systems and oral biofilm research (and in particular, dental caries research) are described in further detail in an informative review by Tang and colleagues (Tang et al. 2003).

From the 1960's onwards, investigators identified and characterized many key microbial species associated with oral diseases (Listgarten 1965; Keyes 1968; Gibbons and Fitzgerald 1969; Tanner et al. 1979). Consequently, biofilm model studies from the 1970s to present often focused on single-species surface-attachment/biofilm development or dual-species interaction studies using key microbial species (Russell and Coulter 1977; Noorda et al. 1986; Bos et al. 1996; Wright et al. 1997). For example, biofilm model systems have improved understanding of coaggregation. Notably, using an *in vitro* flowcell biofilm model that used 25% pooled human saliva as the sole nutrient source, Palmer and colleagues evaluated biofilm development by three species known to coaggregate with one another: *Streptococcus gordonii*, *Streptococcus oralis*, and *Actinomyces oris*. Independently, *A. oris* and *S. oralis* were shown to poorly form biofilms within the model system; however, dual-species cultures of *A. oris* and *S. oralis* formed more abundant biofilms (Palmer et al. 2001). The role of coaggregation in biofilm development has since been further explored, using *in vitro* biofilm models (Foster and Kolenbrander 2004; Nagaoka et al. 2008; Periasamy and Kolenbrander 2009).

In part due to limitations with the ability to identify microorganisms in complex microcosm communities, as well as the interest in the behavior of specific oral pathogens/species, many studies in the 1990s and 2000s were restricted to the development of oral biofilms containing one or a few species. While single or small consortium biofilm model systems can play an important role in uncovering the behavior of individual or small groups of species (as mentioned above), studies of such communities provide limited understanding of how natural oral multi-species microbial communities function in their native environment (Rudney et al. 2012). Natural oral biofilms exist as a dynamic ecosystem with estimates of the total number of indigenous

153 species ranging in the hundreds (Avila et al. 2009). In complex multi-species communities, the behavior of
154 a single species can be modified by other species in a community to behave in a way distinct from its
155 behavior when alone. Emphasizing this point, Sissons remarked in his review of oral biofilm model systems:
156 “an attempt to explain plaque behavior based on the properties of monocultures can be regarded
157 somewhat as heroic” (Sissons 1997). However, through broad technological advancements in the last
158 decade, most notably advances in microscopy and 16S community profiling, investigators have acquired
159 tools and methods to better characterize multi-species or microcosm biofilms (Tan et al. 2017). In recent
160 years, many fundamental validation and protocol studies emerged to gauge reproducibility and provide
161 preliminary microbiological results from *in vitro* oral microcosm biofilm (Edlund et al. 2013; Samarian et al.
162 2014; Klug et al. 2016). Specifically, studies using *in vitro* oral microcosm biofilm models have enabled the
163 measurement of different biofilm outcomes, such as biofilm architecture, microbial community profiles, and
164 taxonomic spatial distribution (Luo et al. 2019; Roder et al. 2020).
165
166 To provide historical context, this review describes *in vitro* model systems that have been developed and
167 adapted over the last fifty years. Particular attention is given to selected drip-fed and flow-fed model
168 systems which have been used in oral biofilm studies by various research groups (**Fig. 1**). Static
169 microplate-based systems, which generally expose developing biofilms to minimal fluid flow, are not
170 discussed as these types of biofilm systems were recently reviewed by Azeredo and colleagues (Azeredo
171 et al. 2017). Drip-fed systems deliver nutrient semi-continuously, whereas flow-fed systems deliver a
172 constant flow of nutrients. The drip-fed systems discussed are the constant depth film fermenter (CDFF),
173 the Sorbarod perfusion system, and the drip-flow biofilm reactor. The flow-fed systems that are discussed
174 are the modified Robbins device (MRD), flowcells, and microfluidic systems, of which we describe the
175 Bioflux™ in detail. Many of these systems possess attributes that make them appealing candidates as
176 model systems for modern oral biofilm studies. All the model systems discussed in this review are
177 compatible to varying degrees with confocal microscopy and have or can conceivably be manipulated to
178 harvest biofilm cells for microbial community profiling using culture-dependent techniques and/or modern
179 culture-independent (next-generation sequencing) methods (**Fig. 1**). Finally, all systems can be set up for
180 multiple-throughput studies, and some require only relatively small volumes for experiments. A summary of
181 the discussed model systems is presented in **Table 2**.
182
183 **Advancements in *In Vitro* Model Systems for Oral Biofilm Research**
184 Over the years, *in vitro* biofilm models, including drip-fed and flow-fed model systems, have been modified
185 to better reflect the characteristics of the oral environment. One particularly important modification replaced
186 traditional bacteriologic culture medium with either artificial saliva such as, “McBain medium”, variations of
187 “SHI medium” (McBain et al. 2005; Tian et al. 2010; Lamont et al. 2021), other artificial saliva types such as
188 those highlighted by Pratten and colleagues (Pratten et al. 1998), or human saliva (Yaari and Bibby 1976;
189 Palmer et al. 2001). Biofilms grown in artificial saliva or pooled human saliva will likely better represent *in*
190 *vivo* plaque as the bacterial composition is influenced by selective pressure of the physical-chemical
191 properties and nutrients of human saliva, rather than artificial media Indeed, over 10 years of research

published by the Kolenbrander group using *in vitro* oral biofilm models has highlighted the utility of using pooled 25% human saliva as a growth medium to study complex interactions between oral bacteria in biofilms (Kolenbrander 2011).

In addition to the relevance of growth medium composition, growth of biofilms under different shear is important for simulating salivary or gingival crevicular flow (Blanc et al. 2014; Fernandez et al. 2017). The composition of exhaled breath can also be mimicked by delivering a gas mixture consisting of 95% atmospheric air and 5% carbon dioxide (Dibdin et al. 1976). Lastly, the choice of a substratum that represents human enamel or dentin should be considered. Hydroxyapatite and glass are two surfaces commonly used to represent oral hard surfaces. While glass may seem to be less relevant than hydroxyapatite for oral biofilm studies, a study comparing the differences of *S. sanguinis* biofilm growth on both surfaces, on which an acquired pellicle (i.e. conditioning film) had also formed, found no difference in resultant biofilm development (Elliott et al. 2005). The authors concluded that the generation of a conditioning film reduced the influence of differences in substratum surface properties. Indeed, many papers have described the use of saliva (artificial or pooled human saliva) to "condition" glass surfaces to generate an acquired pellicle to enhance bacterial adhesion for subsequent biofilm studies (Foster and Kolenbrander 2004; Tsutsumi et al. 2016). With the development of *in vitro* biofilms that are increasingly representative of biofilms in the oral cavity, investigators will gain a better platform to observe the role oral biofilm plays in disease.

Once an *in vitro* model system has been validated and optimized for a dental biofilm study, the cost to maintain the system and serially perform multiple runs decreases significantly. Compared to *in vivo* based research (Martin-Kerry et al. 2015) proof of concept and testing for efficacy of new anti-biofilm agents through *in vitro* model systems will likely be time and cost effective. Another advantage of using *in vitro* oral biofilm models is that oral biofilm communities can be relatively easily developed. *In vitro* systems can be extremely versatile: nutrient availability, flow, the introduction of defined species, and time can be strategically controlled to help answer specific research questions regarding biofilm architecture, cellular organization, and mechanisms associated with biofilm growth (Roder et al. 2020).

Drip-fed Biofilm Models

Constant Depth Film Fermenter

The constant depth film fermenter (CDFF) was first described by Peters and Wimpenny in 1988 as a means to develop freshwater biofilms at a defined thickness (Peters and Wimpenny 1988). The reason for maintaining biofilms at a constant depth is to achieve a steady state biofilm within a reactor where measurable properties do not change significantly over time (Kinniment et al. 1996). Mechanically, the CDFF is a chamber housing a rotating turntable on the bottom (for a graphical representation, see McBain, 2009). The rotating turntable holds customizable sampling pans where each pan contains plugs made of a material on which biofilms develop. To distribute media to each plug, media is drip-fed from above via inlets as the disc rotates. Spent media is collected in a waste outlet located below the disc. The CDFF keeps

biofilms at a constant depth using a scraper blade that removes excess biofilm biomass and spent media as the disc rotates. The initial model described by Peters and Wimpenny held 25 plugs to support biofilm development (Peters and Wimpenny 1988) while later models had the capacity of up to 75 plugs (Deng et al. 2005).

While initially used to study freshwater biofilms (Peters and Wimpenny 1988), the CDFF has been applied successfully to the development of *in vitro* oral biofilms (McBain 2009; Hope et al. 2012). The CDFF has been used extensively for single-species (Zanin et al. 2005; Metcalf et al. 2006), defined consortia (Fan et al. 2012), and oral microcosm studies (Hope et al. 2002; McBain et al. 2003; Abdulkareem et al. 2015). CDFFs are particularly well-equipped to conduct studies of antimicrobial challenges on mature oral biofilms, and for monitoring the growth of biofilms. Biofilm can be grown on the plugs in the same chamber and assigned to treatment or control groups during or post-growth. Specifically, plugs can be removed from the device and then treated (Hope et al. 2002) or treatment(s) can occur while the plugs are within the device (Deng et al. 2005). For example, Deng and colleagues grew *S. mutans* on dentin plugs in a split CDFF chamber that was simultaneously treated with sodium fluoride or sodium fluoride/chlorhexidine formulations after the biofilm had matured (Deng et al. 2005). Sodium fluoride/chlorhexidine formulations conferred the greatest kill, lactic acid reduction, and remineralization of dentin compared to sodium fluoride alone. In another study, Feldman and coworkers monitored dual-species *C. albicans* and *S. mutans* biofilm development on pre-treated hydroxyapatite discs (Feldman et al. 2017). The discs were coated with a membrane designed to slowly release thiazolidinedione-8, a quorum sensing quencher. Biofilm development was hindered on discs containing the quorum sensing quencher. When considering these and other papers using the CDFF, it has been, and still is, a valued *in vitro* model system to study oral biofilms.

Sorbarod Perfusion System

In the mid 1990's, Hodgson and colleagues developed a perfused *in vitro* model system that was called the Sorbarod perfusion system (also referred to as a Sorbarod biofilm fermenter system) (Hodgson et al. 1995). There are multiple structural variations of this system that have been published, but all use Sorbarod filters as the material on which biofilms develop. Sorbarod filters are cylinders that contain a roll of cellulose fibers and the cylinders are approximately 10mm in diameter and 20 mm in length (Budhani and Struthers 1997; McBain 2009). Sorbarods can be loaded into supports such as tubing (Hodgson et al. 1995), syringes (Rickard et al. 2008), or an engineered device that can support multiple Sorbarods (McBain et al. 2005), and exposed to flowing media. Harvested Sorbarods can be used to perform viable counts and biofilms on the Sorbarod fibers can be imaged. Another benefit of this model system is the high surface area to volume ratio, which maximizes the amount of biofilm that can form. During an experiment, gas or fluid can be collected to track cell numbers, volatile sulfur compounds, and cell-signaling molecules (Hodgson et al. 1995; Spencer et al. 2007; Rickard et al. 2008).

A Sorbarod perfusion system can be used for anaerobic and microcosm biofilm studies which require extended run times to achieve dynamic steady states (McBain 2009). In a study by McBain and colleagues

(McBain et al. 2005), multiple Sorbarod devices were inoculated with saliva from human volunteers and supplied with artificial saliva nutrient. Dynamic stability was achieved after two to three days, with high bacterial diversity and presence of anaerobic species. McBain and coworkers concluded that the Sorbarod system was effective at maintaining a stable and reproducible oral biofilm community over multiple days (McBain et al. 2005). In an oral malodor study by Spencer and colleagues, a microcosm derived from dorsal tongue scraping was used as inoculum to grow representative communities that produce volatile sulfur compounds (Spencer et al. 2007). Biofilm development was studied over 96 hours and quasi steady states were achieved by 48 hours. The community composition of developed biofilms resembled that of the original dorsal tongue scrapings. Overall, Spencer and colleagues demonstrated the viability of the Sorbarod system for maintaining a stable tongue microcosm community.

Drip-Flow Biofilm Reactor

The drip-flow biofilm reactor was first described by Xu and colleagues in the late 1990's as a means to develop *P. aeruginosa* biofilms (Xu et al. 1998). Unlike the CDFF and Sorbarod systems, the drip-flow biofilm reactor is unique in that it is positioned at an angle and media is dripped from above at the apex of the reactor. During use, the media flows downward coating a glass microscope slide or a detachable coupon. The coupon can be made from various materials, allowing investigators the flexibility of choosing a substratum on which a biofilm can develop (Gomes et al. 2018). The gravity-assisted flow of media creates a low shear environment that can be adjusted by elevating or depressing the angle of the system. At the bottom of the reactor is an outlet where effluent media traverses into a waste receptacle. An excellent review with informative diagrams and detailed descriptions of the use of drip flow biofilm reactors is presented by Goeres and colleagues (Goeres et al. 2009). When considering analysis of biofilms developed in the system, care must be applied in sampling biofilms over a large surface area whether it be imaging or harvesting biomass for further testing. As demonstrated by Xu and colleagues, oxygen availability can influence heterogeneity of *P. aeruginosa* biofilms and if media flow across the slide is not uniform, then the development of a heterogeneous biofilm is possible (Xu et al. 1998).

Several studies have used the drip flow reactor to model single-species and multi-species oral biofilms. For example, two single-species studies used the drip flow reactor to test the efficacy of antimicrobial agents on *S. mutans* biofilm development (Brambilla 2017; Williams et al. 2017) Williams and colleagues used silver loaded into polymethyl methacrylate (PMMA) sheets, which were cut into rectangular coupons; Brambilla and colleagues used chlorhexidine loaded into dentin bonding systems. Williams and colleagues demonstrated that silver PMMA coupons were able to resist *S. mutans* biofilm formation in short-term washouts, but not long-term washouts. As described by Brambilla and colleagues, chlorhexidine loaded dentin adhesion bonding agents demonstrated variable results, leading authors to suspect the variable chemical composition of the dentin binding systems masked the effects of chlorhexidine. Drip flow reactors have also been used for dentifrice studies on mature oral multi-species microcosm biofilms (Ledder et al. 2010; Ledder and McBain 2012). In those studies, oral microcosm biofilms were grown over 24 or 48 hours,

followed by treatment regimens delivering dentifrice slurries every six hours for six days. The dentifrice treatments reduced culture counts and affected oral biofilm community alpha diversity.

Flow-fed Biofilm Models

Modified Robbins Device

Based on an earlier design called the Robbins device, the modified Robbins device (MRD) (McCoy et al. 1981), facilitates the study of biofilms under flow. The MRD uses individual coupons affixed to plugs that then can be inserted into ports that run along the length of a device. The coupons can be made of different materials such as those used in dental prostheses or hydroxyapatite (Blanc et al. 2014). A peristaltic pump provides unidirectional media flow across all ports after coupons are inoculated. Biofilm development occurs on the surfaces of the coupons as the system runs. Plugs containing coupons can be removed aseptically over time and replaced with plugs containing fresh coupons. The number of sampling ports of the MRD varies by design. For example, commercially available low pressure and small volume MRDs are available that range from 12-25 ports. Thus, longitudinal studies of biofilms can be performed, although, as with the CDFF, Sorbarod system, and the drip-flow biofilm reactor, it is not possible to perform repeated *in situ* biofilm visualizations of the same biofilm sample over time and only endpoint imaging can be performed (**Fig. 1**). Coupons with the supporting plug and associated biofilm must be removed to be visualized microscopically.

The MRD has been used extensively to study oral biofilms, with many studies demonstrating its reproducibility at developing oral biofilms (Honraet and Nelis 2006; Coenye et al. 2008; Noiri et al. 2008; Sliepen et al. 2010; Blanc et al. 2014; Yassin et al. 2016). The system and its detachable coupons proved to be particularly useful in evaluating the efficacy of antimicrobials and materials primed with antimicrobial. For example, in the study by Yassin and colleagues (Yassin et al. 2016), MRD coupons were prepared from a mixture of polymethyl methacrylate and sodium fluoride to create a copolymer that can be used for dentures while also releasing fluoride ions passively while worn. The investigators observed that three-species (*C. albicans, L. casei*, *S. mutans*) biofilm growth was inhibited by 10-fold on coupons containing the fluoride compared to biofilm growth on coupons that did not. Conversely, biofilm can be treated after biofilm development to evaluate effectiveness of an antimicrobial (Coenye et al. 2008). In 2008, Coenye et al. grew mono-species biofilms of *C. albicans, S. mutans*, *S. aureus*, and *P. aeruginosa* in a stainless steel MRD. After growth, the biofilms were treated with NitrAdine™, sonicated to remove biofilm from the coupons, and plated to determine efficacy of treatment in preventing regrowth. Similarly, Blanc *et al*. developed multi-species biofilms on hydroxyapatite coupons to test antimicrobial efficacy of chlorhexidine, cetylpyridinium chloride, and sodium fluoride mouthwash rinses (Blanc et al. 2014).

Flowcells

Of the six model systems described in this review, flowcells are among the smallest in physical size (**Table 2**). Due to the compactness of the system, flowcells use small volumes of inocula and media for biofilm experiments. Oral biofilms can be studied at the end of an experiment using a confocal microscope

(endpoint studies, for example by Foster et al., 2004) or at different times, for example during treatment with antimicrobials (Corbin et al. 2011) (**Fig. 1**). An example of a flowcell system built in-house for oral biofilm studies was described by Palmer & Caldwell in the mid-1990s (Palmer and Caldwell 1995). The main advantage of using flowcells to study oral biofilms is the capability of studying changes to biofilm community composition and architecture over time (Fig. 1). For imaging, this can be accomplished because the substratum of the flowcell is often glass. Using confocal or even epifluorescence microscopy (for less-detailed studies), the accumulation of biofilm biomass can be monitored at different times following inoculation.

The flowcell has played a prominent role in oral biofilm research. For example, in 2004, Foster and colleagues used flowcells to test the efficacy of antimicrobials on oral biofilms. The authors grew single species *S. gordonii* biofilms in saliva-conditioned flowcells and treated them with commercially available mouthwashes (Foster et al. 2004). The study indicated that different active ingredients within mouthwashes differed in antimicrobial efficacy. Later, Foster and colleagues used the same type of saliva-conditioned flowcells for consortia biofilms containing four oral species and showed that biofilm formation can depend on whether the microorganisms form coaggregates with each other in the planktonic phase (Foster and Kolenbrander 2004). The flowcell has also been used in studies to test pellicle formation on glass compared to hydroxyapatite. Elliott and colleagues showed that the two surfaces were similar and had no effect on biofilm attachment (Elliott et al. 2005). Another study used flow cells to image in real-time biofilm development of the oral pathogen *Candida albicans* (McCall and Edgerton 2017). McCall and Edgerton compared wild type and hyperfilamentous *Δhog1 C. albicans* strains in their ability to attach to the flow cells and develop biomass during the 18-hour growth. The gene *hog1* is activated by oxidative stress, osmotic stress and heavy metal stress resulting in hyphal filamentation (Su et al. 2013). McCall and Edgerton demonstrated that the wild type *C. albicans* had twice the attachment rate of the *Δhog1* mutant, but formed biofilms of lesser biomass, suggesting that cellular detachment is integral for biomass accumulation.

Microfluidic Model Systems

Microfluidics involves the engineered delivery of fluids on the sub-milliliter levels through microchannels (Sackmann et al. 2014). A significant advantage of *in vitro* microfluidics systems over other *in vitro* model biofilm systems is the much smaller amounts of inoculum that are needed (Samarian et al. 2014). This is especially advantageous if sample volume is limited or reagents are expensive. Additionally, the systems are compact and require low energy costs to run. Microfluidic biofilm model systems have become increasingly popular in oral biofilm studies as they can be used to perform culturing, bioinformatics, and microscopy (Gashti et al. 2016; Mira 2018).

One commercially available microfluidic system is the Bioflux™ system, manufactured by Fluxion Biosciences (San Francisco, CA). The Bioflux™ is a continuous flow microfluidic system used by investigators to model oral biofilms (Tao et al. 2011; Ding et al. 2014; Volgenant et al. 2016). The system consists of three main parts: consumable microfluidic plates, a controller, and a software control interface

(Samarian et al., 2014). The software control interface regulates the flow rate, the total runtime, and determines which pumps are active. A pressure top that is fixed to the top of the consumable plates creates an airtight environment within the Bioflux™ plate, allowing pressure to be applied only from the controller. This forces fluid from inlet well to output well at a fixed rate. A viewing port exists between the inlet and outlet wells, where biofilms develop under the prescribed flow rate. The Bioflux™ plate, similar to flowcells, can be imaged with inverted microscopy techniques during biofilm growth or after maturation (**Fig. 1**).

Of all the systems described in this review, the Bioflux™ requires the least amount of media and inocula. Oral biofilms have been developed overnight at 0.2 dynes/cm^2, requiring 380 uL of media per sample and as little as 50 µL of inoculum. Volumes required were calculated from the Bioflux™ software interface. The low volumes required are especially advantageous for studies using donations of bodily fluid for media and/or inoculum. Another advantage of the Bioflux™ system is its throughput. With evenly-distributed flow supplied by a computerized pneumatic pump and a heating plate that covers the entirety of the plate, multiple biofilms can be produced in parallel under the same environmental parameters. Additionally, the atmospheric composition of the airtight environment within the Bioflux™ can be controlled by fitting a Bioflux™ controller with a pressurized gas cylinder containing a defined gaseous mixture. Different plate formats contain 3, 8, or 24 channels which enable replicates of oral biofilms to be developed in parallel. Given the dimensions of the Bioflux™ plates, which are compatible with microplate holders, both endpoint and live imaging of oral biofilm development is possible (**Fig. 1**).

First described in 2010, Benoit and coworkers used the throughput advantage of the Bioflux™ system to screen the effectiveness of several antimicrobials on *P. aeruginosa* PAO1 biofilms (Benoit et al. 2010). Over the last decade, the Bioflux™ system has been adapted for oral biofilm architecture and community studies (Ding et al. 2010; Dong et al. 2012; Samarian et al. 2014; Fernandez et al. 2017). In 2013, Nance and coworkers developed overnight microcosm biofilms seeded from salivary inoculum and tested the antimicrobial effectiveness of cetylpyridinium chloride (CPC) (Nance et al. 2013). Using LIVE/DEAD™ staining, a dose-response viability gradient was observed between .001% and .5% w/v CPC. Also, in the study, Nance and coworkers established that the Bioflux™ system was capable of developing an oral biofilm that was compositionally similar to early supragingival plaque. A standardized protocol for developing oral multi-species biofilms using the Bioflux™ system was described by Samarian *et al*. in 2014. The Bioflux™ system also has been used to study the effects of different antimicrobial compounds on oral biofilms. For example, Luo *et al.* evaluated the effect of stannous fluoride on oral multi-species biofilm architecture (Luo et al. 2019). Lastly, the Bioflux™ system has been used in single-species studies. Ding and coworkers. grew single-species *S. mutans* biofilms with flowing media and tested the antimicrobial peptide bactenecin (Ding et al. 2014). The authors observed a significant decrease in viability. In another study using the Bioflux™, Dong *et al.* showed that development of *S. mutans* biofilms in subminimum inhibitory concentrations of chlorhexidine or sodium fluoride altered the biofilm architecture and

development in subminimum inhibitory concentrations of tea polyphenols reduced biofilm biomass (Dong et al. 2012).

Integration of *In Vitro* Oral Model Systems with Microscopy and Bioinformatics

Since the first biofilm model systems were described in the 1950s, innovations in methodologies have enhanced the generalizability of oral biofilms grown *in vitro*. Today, investigators can cultivate an *in vitro* oral biofilm that is compositionally similar to the microbial community of plaque (Rudney et al. 2012; Nance et al. 2013). The ability to generate representative communities is critical if the desired outcome is to generalize results to human subjects. Two disciplines where technological advancements have significantly augmented the value of laboratory model systems are microscopy and bioinformatics, particularly in the domain of 16S rRNA bacterial community profiling. Microscopy is essential for the study of biofilm architecture, whereas bioinformatics techniques are becoming increasingly popular for characterizing the taxonomic diversity and function of biofilm microbial communities as a whole.

Confocal Microscopy

Several different microscope technologies are available to study oral biofilms each with advantages and disadvantages. While not the focus of this review, a useful review of microscopy and image analysis has been published by McNamara and colleagues (McNamara et al. 2017). Here, we will focus on the use of the confocal microscope, which was first used to describe biofilms in 1991 (Lawrence et al. 1991). Using a confocal microscope, investigators can capture oral biofilm architecture and simultaneously gain insight into cell viability or species location (Zaura-Arite et al. 2001; Cuadra-Saenz et al. 2012; Ruangcharoen et al. 2017). Instead of destructively removing oral biofilm for downstream quantification, confocal microscopy enables *in situ* quantification by taking optical sections of a biofilm and subsequently generating 3D renderings using the optical sections. This can be performed for single-species biofilms, a defined multi-species consortium, or complex microcosm biofilms. For example, instead of culturing and harvesting biofilm to determine colony forming units (CFU), a confocal microscope can take a digital snapshot of a biofilm stained with viability stains (e.g. a mixture of SYTO-9 stain and propidium iodide stain, which are part of the commercially available LIVE/DEAD™ staining system). In this scenario, the amount of viable (membrane intact) and inactive/dead (membrane compromised) cells or biofilm biomass can be quantified while the biofilm remains attached to the substratum. This approach has advantages because determining CFUs may underestimate true viability due to the destructive nature of the biofilm harvesting process and/or inadequate cell removal from the surface. However, it should be noted that the use of viability stains is not without potential problems which include possible issues with differential staining (Netuschil et al. 2014).

A key advantage of confocal microscopy over other forms of microscopy, is the ability to discern complex biofilm architecture, the properties of the contained cells, and spatial arrangement of biofilm species. In non-targeted (i.e. non species-specific) fluorescence studies, confocal microscopy has been used to identify distribution of viable and non-viable cells in multi-species oral microcosm biofilms developed within

a CDFF (Hope et al. 2002). Using LIVE/DEAD staining, Hope and colleagues demonstrated that the basal layer of an untreated oral multi-species biofilm contained more non-viable cells compared to the surface. In targeted (i.e. species-specific) fluorescence studies, the spatial position of a specific species within a multi-species biofilm can be determined (Palmer et al. 2001; Thurnheer et al. 2019). For example, Robert Palmer and coworkers used fluorescently-labeled antibodies to discern the spatial arrangement of oral *Streptococcus gordonii, Streptococcus oralis,* and *Actinomyces oris* in single-species and dual-species biofilms developed in pooled human saliva (Palmer et al. 2001). These biofilms were grown in flowcells where the only potential perturbation to the biofilms was from labeling with antibodies after growth. Another notable study using an *in vitro* model system and confocal microscopy was performed by Thurnheer and colleagues who grew biofilms containing six species on hydroxyapatite disks in 24-well polystyrene cell culture plates and used fluorescent in situ hybridization (FISH) to discern their spatial arrangement (Thurnheer et al. 2019). This work showed that FISH, in combination with the optical sectioning capabilities of a confocal microscope, enabled the analysis of spatial arrangement of numerous species and had the potential to investigate alterations in biofilm species arrangement in response to environmental challenges. Understanding these biofilm structures and cellular arrangements could be important to biofilm control. Thus, considerable effort has been dedicated to identifying a disease-associated motif seen in biofilm architecture and its possible role in pathogenesis. With this in mind, a recent paper by Kim and colleagues identified corona-like biofilm architectures formed by when *S. mutans* developed biofilms with other oral species and these architectures could enhance the pathogenic potential of *S. mutans* in biofilm communities (Kim et al. 2020).

With modification, certain *in vitro* model systems can be adapted to monitor changes in biofilm architecture over time (Fig. 1). To image a developing biofilm over time, the model system must be capable of growing an oral biofilm on a surface that can be simultaneously imaged with microscopy techniques as the system is running. Indeed, a recent study by Paula and coworkers explored the dynamics of *S. mutans* biofilm formation from microcolonies to biofilm superstructures (Paula et al. 2020). Using a modified flow cell that can house hydroxyapatite discs containing attached *S. mutans*, biofilm development was monitored with a confocal microscope taking images every 20 minutes.

To maximize information derived from imaged *in vitro* biofilms, the application of appropriate downstream computational analytics is required to describe the spatial position of fluorescently labeled biofilm species. Many analytical software packages are publicly available and offer a multitude of outcome measures. Alternatively, customized in-house analysis can be performed. A computing environment such as MATLAB (Natick, MA, USA) is necessary for the latter alternative and its successful implementation is described in more detail by Beyenal and colleagues (Beyenal et al. 2004). Furthermore, the commonly used biofilm image analysis program COMSTAT, which was originally coded in MATLAB (Heydorn et al. 2000), provides users a graphical user interface to analyze confocal data. A more recent analytical tool built using the MATLAB environment is the Biofilm Architecture Inference Tool (BAIT), developed by Luo and colleagues (Luo et al. 2019). BAIT can import confocal image stack data and perform various image thresholding

algorithms prior to image analysis. One method, named the biovolume elasticity method (BEM), identifies thresholds that more accurately define biofilm edges (Luo et al. 2018). Post-processed image stacks can then be quantified for various architectural descriptors including: biovolume, surface area, fluffiness, total number of objects, connectivity, and convex hull porosity. Viability can also be evaluated if the confocal stack possesses two channels. For combining optical sections collected by confocal microscopy and the subsequent image rendering of biofilms, commercially available software, such as Imaris (Zurich, Switzerland) and Volocity (Puslinch, Ontario), can be used to give further insight into architectural features of oral biofilms. Open-source software imaging programs software such as ICY (de Chaumont et al. 2012) and BioimageXD (Kankaanpaa et al. 2012) are also available to render biofilms from confocal image stacks.

16S rRNA Community Profiling

Since its inception in the 1970's, 16S rRNA gene sequencing technology has become extremely useful in studying bacterial phylogeny and taxonomy (Woese and Fox 1977; Weisburg et al. 1991; Konstantinidis and Tiedje 2007). Given that all bacteria possess and require the 16S rRNA gene, it is an excellent target for identifying and analyzing community membership (Clarridge 2004; Aas et al. 2005; Petti et al. 2005). Furthermore, 16S rRNA sequences from bacterial species are readily available on public and curated repositories such as GenBank, Greengenes, RDP, and SILVA for comparative sequence analyses (Balvociute and Huson 2017; Benson et al. 2018). Depending on the length of the 16S rRNA gene sequence that is analyzed and the variable regions covered, for which there are nine "hypervariable regions" (labelled V1 – V9) in the 16S rRNA gene, identities can be assigned to a taxonomic rank often to the genus or species level (Chakravorty et al. 2007; Janda and Abbott 2007). With more hypervariable regions sequenced within a read a higher resolution taxonomic assignment can be achieved. Prior to the advent of next-generation sequencing (NGS), investigators relied upon culture-dependent techniques, such as culturing on agar to isolate bacteria for identification, or older culture-independent (molecular) technologies (e.g. Sanger sequencing of cloned 16S rRNA gene sequences or denaturing gradient gel electrophoresis) that produced relatively low read counts of 16S rRNA sequences and/or limited species resolution for *in vitro* oral microcosm biofilm studies (**Fig. 1**). With NGS, massively parallel and deep sequencing capabilities have emerged, enabling the oral microbiome to be quickly characterized (Behjati and Tarpey 2013).

The development of NGS and endeavors to study increasingly more complex *in vitro* oral biofilm microcosms has also coincided with a shift in the focus on the pathogenicity of oral biofilms from individual species associated with disease to understanding the disease-causing ability of microbial communities (Li et al. 2016; Vogtmann et al. 2018). Substantial evidence indicates that multiple species, and their interactions with the host and one another, are responsible for propagating pathways for soft and hard tissue destruction seen in periodontal disease and caries (Negrini et al. 2019; Wade and Prosdocimi 2020). For instance, Whitmore and Lamont reviewed the role mitis group streptococci play in the recruitment of successional pathogenic species such as *Porphyromonas gingivalis* and *Actinobacillus*

actinomycetemcomitans (Whitmore and Lamont 2011). Another review by Banas and Drake, emphasized the perspective shift away from *S. mutans* being the lone causative agent to caries, but rather a relative contributor within a complex oral microbiome (Banas and Drake 2018). Thus, the present challenge is to identify microbial community profiles, not individual species, most associated with disease.

Pertinent to this review, the incorporation of NGS approaches with biofilm model systems is relatively new and there are a variety of factors and challenges that must be considered when considering NGS studies of *in vitro* biofilm model systems. Critically, there have been numerous NGS platforms used for the 16S rRNA profiling of biofilm communities. Choice of sequencing platform depends on the investigator's research questions and involve trade-offs between read length, read depth, sequencing depth, and accuracy. Sequencing platforms relevant to oral biofilm studies are listed below in **Table 3**, although this is not an exhaustive list since NGS technologies that offer insufficient or unnecessary read length (e.g. 20Kb read lengths offered by PacBio) for 16S rRNA gene sequencing are excluded. The choice of sequencing platform heavily influences which hypervariable regions can be included in one contiguous read. The longer the read length, the more hypervariable regions can be included. Some platforms offer paired-end reads (**Table 3**), which can be joined to create a longer fragment, but a trade-off between read-length and sequence overlap for accuracy must be considered. Hypervariable region selection can also influence interpretation of results and taxonomic resolution (Barb et al. 2016; Teng et al. 2018; Bukin et al. 2019). This consideration is accentuated for oral streptococci where species are difficult to differentiate due to the limited amount of variation in the hypervariable regions of the 16S gene (Mukherjee et al. 2018).

When considering the collection of biofilm material to analyze the community composition of an oral biofilm grown *in vitro*, investigators must first harvest and prepare biofilm cells from their model system to be analyzed with NGS technologies. This process will vary by model system and may involve using physical treatments to harvest biofilm cells. For example, in the Bioflux™ system, this involves removing biofilm material from substratum with high shear (Samarian et al. 2014). With the modified Robbins device (MRD), sonication could be used to remove biofilm cells from coupons (Coenye et al. 2008). Unlike cell culturing techniques, the destructive nature of removing biofilm is less of a concern for 16S rRNA community profiling. Ultimately, the objective is to retrieve a cross-sectional snapshot of the oral biofilm community composition at the time of harvesting.

Several oral biofilm studies have utilized NGS technologies to characterize the microbial community within biofilms that were developed using *in vitro* model systems. Velsco and Shaddox described a static system where they collected plaque samples from healthy and periodontitis-affected individuals (Velsko and Shaddox 2018). Plaque samples were used to inoculate hydroxyapatite discs and grown statically over eight days. The resultant communities were sequenced with Illumina MiSeq and characterized with the software QIIME (Caporaso et al. 2010; Velsko and Shaddox 2018). They concluded that periodontitis-derived plaque resulted in communities that differed from communities derived from healthy individuals' plaque samples, as determined by weighted UniFrac measures. In another study, Klug et al. used 454

pyrosequencing to determine community diversity and survivorship after enamel-dentin slabs worn by volunteers were removed and placed in biofilm reactors (Klug et al. 2016). They discovered general survivorship of the biofilm community and diversity was maintained from after removal to 48 hours after growth in the biofilm reactor. Fernandez and colleagues studied the effect of shear force on oral communities derived from saliva, tongue, and plaque-based inoculum (Fernandez et al. 2017). After harvesting biofilm communities grown in a microfluidics *in vitro* model system, the samples were sequenced with Ion Torrent sequencing platform. The group discovered that after overnight growth, bacterial communities shifted to a community with less alpha diversity compared to its starting inoculum. Taken together, these studies highlight the application of different NGS technologies and demonstrate its relevance in various *in vitro* oral biofilm model system studies.

Concluding Remarks and Future Directions

The miniaturization of *in vitro* platforms operating on the microscale, combined with integration with imaging and 'omic' technologies, as well as a greater understanding of the biology of oral biofilms have reinvigorated the appeal of laboratory biofilm model systems. A PubMed search using the search terms "*in vivo* model system oral biofilm" and "*in vitro* model system oral biofilm" indicates that laboratory-based models are more commonly used in the realm of oral biofilm research than animal-based models. This observation has held steady in the last 25 years. This popularization of laboratory-based systems is likely owed to technologies that can be tethered to model systems, such as confocal microscopes (Valm et al. 2012) and 16S community profiling approaches (Azevedo et al. 2009). Combined with decreasing costs, *in vitro* biofilm model systems have become an appealing option for multi-species oral biofilm studies.

The future directions of *in vitro* model systems could involve a shift from developing representative dental plaque within the system to transplanting already-developed *in vivo* plaque into the system. For example, Fernandez and coworkers described a cariogenic model using *ex situ* methods that involve human participants wearing non-invasive oral prostheses housing enamel specimens (Fernandez et al. 2016). I*n vitro* model systems could also incorporate a biological substratum for biofilm development, such as that developed using tissue culture techniques.

There are multiple surfaces in the intraoral cavity including hard and soft palate, tongue, subgingival, buccal, and teeth. Glass and hydroxyapatite are representative of the hard surfaces of teeth, but are a poor model for attachment and development of subgingival plaque (Cieplik et al. 2019). There is a disparity in volume of research involving epithelial substratum in oral diseases, thus periodontal biofilm models are lacking (Walker and Sedlacek 2007). This is due to the relative difficulty of cell culture techniques over use of glass or hydroxyapatite. Epithelial cells are the preferred substratum for periodontal models as they more adequately represent the substratum of subgingival plaque (Guggenheim et al. 2009). As demonstrated by Guggenheim and colleagues, an epithelial substratum can actively model the interaction between host immune cells and oral microbial biofilm cells. This is important to consider in periodontal models where *in*

vivo microbial cells at the periodontal tissue interface trigger host immune response and then mount evasion or defense mechanisms.

Outcomes	Fundamental Study (Reference)	Applied Study (Reference)	Model System(s) Used
Cariogenesis*	D-glucose and sucrose induce caries (Pigman et al. 1962).	Fluoride slurry inhibits enamel softening (Pigman and Newbrun 1962).	Artificial Mouth
Single-Species Biofilm	*S. mutans* biofilms fed sucrose induces caries (Deng and ten Cate 2004).	Chlorhexidine in dentin bonding systems may inhibit *S. mutans* biofilm formation (Brambilla 2017).	Constant Depth Film Fermenter, Drip-Flow Reactor
Defined-Species Biofilm	*S. oralis* and *A. oris* biofilm growth was enhanced when co-cultured compared to when alone (Palmer et al. 2001).	*C. albicans*, *L. casei*, *S. mutans* mixed-species biofilm growth inhibited 10-fold on MRD coupons containing fluoride compared to coupons containing no fluoride.	Flowcells, Modified Robbins Device
Microcosm Biofilm	Community composition of *in vitro* biofilms can reflect that of microcosm donor (McBain et al. 2005).	Nisin retarded multi-species biofilm development without cytotoxicity to human cells (Shin et al. 2015).	Sorbarod Perfusion, Bioflux™

In conclusion, the development and validation of new *in vitro* biofilm model systems for applied oral biofilm research is a continual effort, especially with changing paradigms, perspectives, and capabilities in microbiological research techniques. The biggest challenge thus far in translating *in vitro* model system findings into clinical practice has been the difficulty to form *in vivo*-like biofilms in a laboratory setting. Enhancing older "classic" model systems or creating newer model systems and combining such models with new or improved technologies is allowing investigators to move closer to mimicking natural oral biofilm states and providing tools to measure oral biofilm outcomes more accurately.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Author's Contribution Statement

All authors contributed to the preparation of this manuscript.

Table 1. Examples of Fundamental & Applied Research of *In Vitro* Oral Biofilms. Studies that improve the understanding of the biology of oral biofilms are considered fundamental. Applied studies, on the other hand, are studies that focus on interventions to control oral biofilms.

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673 * Not an oral biofilm outcome but listed to provide historical context and highlight the shift of focus to oral biofilm outcomes.

674 **Table 2. Open System Biofilm Models Relevant to Oral Biofilm Research.** Examples of biofilm model
675 systems that have been used for the study of oral biofilms. General properties of each system are
676 described, along with each system's nutrient delivery classification, number of biofilms that can be grown
677 per model, and volumetric scale.

Biofilm Model	Classification	General Properties	Number of Biofilms Grown Per Model ×	Volumetric Range
Constant depth film fermenter (Peters and Wimpenny 1988)	Drip-fed	-Scraper blade to smear media and keep biofilm at constant depth -Keeps biofilm in a steady state -Rotating disc embedded with plugs -Plug composites can be modified to simulate a different substratum -Can run for weeks	**25** (Peters and Wimpenny 1988), **75** (Deng et al. 2005)	Liters
Sorbarod perfusion system (Hodgson et al. 1995)	Drip-fed	-Cellulose filter substratum -Media perfuses through filter material -Can run for day(s)	**1** (Hodgson et al. 1995), **5** (McBain et al. 2005)	Liters to Milliliters
Drip-flow biofilm reactor (Xu et al. 1998)	Drip-fed	-Reactor angled to allow drip to flow continuously across substratum -Gravity-assisted flow simulates low shear -Typically run for day(s)	**4-6***	Liters to Milliliters
Modified Robbins device (McCoy et al. 1981)	Flow-fed	-Individual coupons or discs as substratum -Coupons customizable by investigator -Can run for day(s) or weeks	**12, 25*†**	Liters
Flowcells (Palmer 1999)	Flow-fed	-Rubber or silicone spacer bound by glass coverslips -Amenable to microscopy	**1-4*‡**	Liters to Milliliters

		-Can use other substrata but may be less compatible with microscopy -Typically run for a day		
Bioflux™ (Benoit et al. 2010)	Flow-fed	- Microplates with microfluidic channels between wells -Software-controlled pneumatic pump -Glass-bottomed substratum - Used with inverted microscope -Typically run for a day	**3,8,24**§	Milliliters to Microliters

678 ˣ Refers to the number of biofilms that can be grown for sampling per device and these can be in the same channel/vessel
679 (constant depth film fermenter, modified Robbins device, or sorbarod perfusion system) or spread across multiple channels/vessels
680 in one device (drip flow biofilm reactor, flowcells, and Bioflux™ system).
681 * Commercially available through Biosurfaces Technologies Corporation.
682 † Commercially available through Tyler Research Corporation.
683 ‡ Commercially available through Stovall Life Science, Inc.
684 § Commercially available through Fluxion Biosciences.

685 **Table 3. Sequencing Platforms for 16S rRNA Community Profiling.** Compatible next-generation
686 sequencers that have been used to characterize an oral microcosm biofilm grown *in vitro* are listed. The
687 sequencing chemistry, expected read length, sequencing depth, and consensus accuracy of each platform
688 is also described.

Sequencing Platform (Reference)	**Sequencing Chemistry**	**Read Length**	**Sequencing Depth**	**Consensus Accuracy**
454 GS FLX+* (Nance et al. 2013; Kistler et al. 2015; Koopman et al. 2015)	Pyrosequencing	Up to 1000bp	700 Mb	99.997
Illumina MiSeq (Koopman et al. 2016) (Agnello et al. 2017)	Sequencing by synthesis	2x150, 2x250, 2x300	4.5-5.1 Gb, 7.5-8.5 Gb, 13.2-15 Gb	80% bases > 99.9 75% bases > 99.9 70% bases > 99.9
Illumina HiSeq (Edlund et al. 2013)	Sequencing by synthesis	2x125	450-500 Gb	80% bases > 99.9
IonTorrent PGM (Fernandez et al. 2017)	Ion semiconductor	Up to 400bp	Up to 2 Gb	>99.0

689 * Technology is no longer supported by manufacturer.

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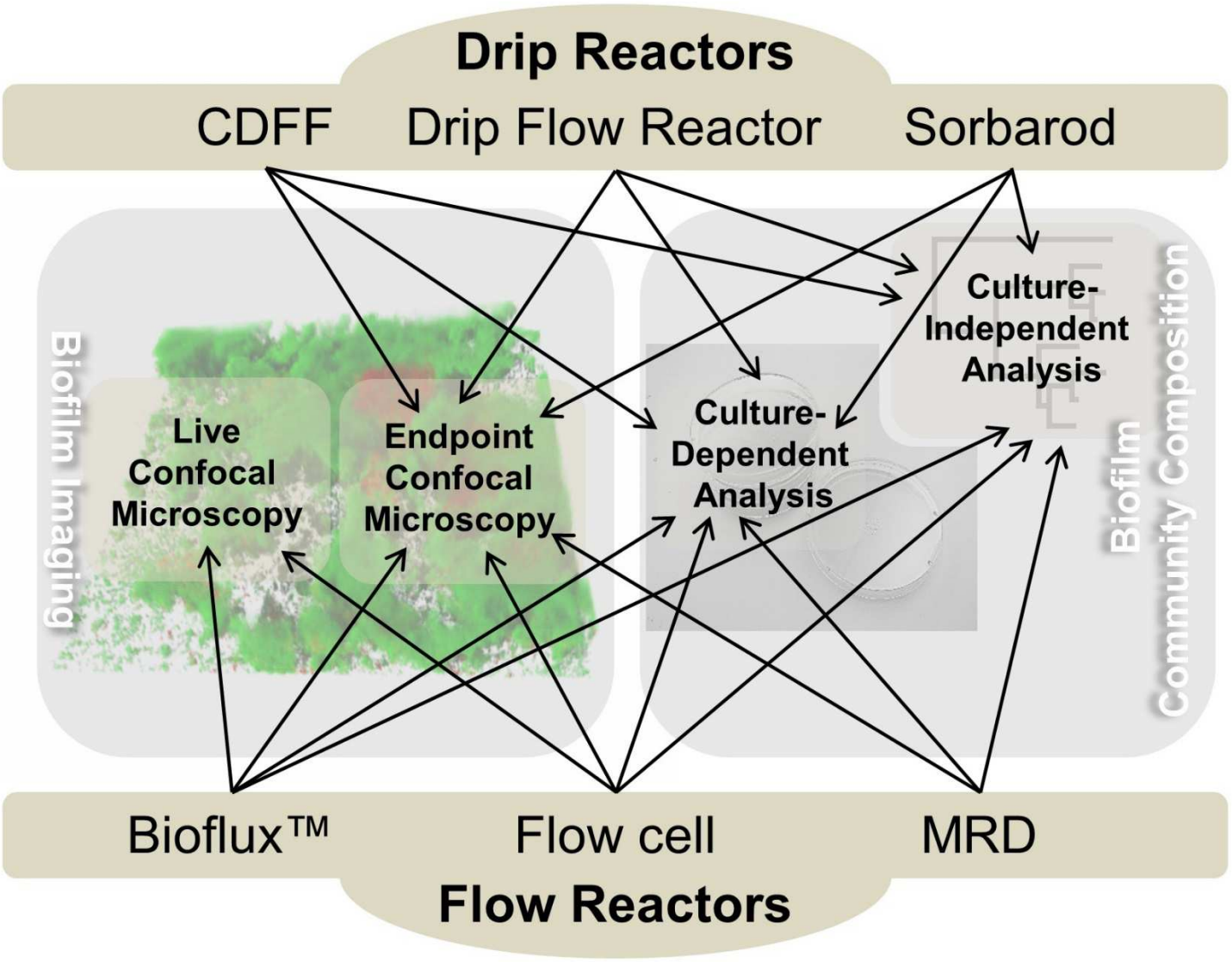
Fig 1. Diagram showing different types of drip reactors and flow reactors described in this review. These have been, or could conceptually be, integrated with a confocal microscope to image biofilms in 3D at the end of an experiment ("Endpoint Confocal Microscopy") and/or image repeatedly in 3D over time during an experiment for spatiotemporal analyses ("Live Confocal Microscopy"). Both confocal microscopy approaches have the potential for the spatial analysis of single or multiple species. Some of these systems have or could also conceivably be combined with culture-dependent approaches and/or culture-independent techniques to study the community composition of oral biofilms.



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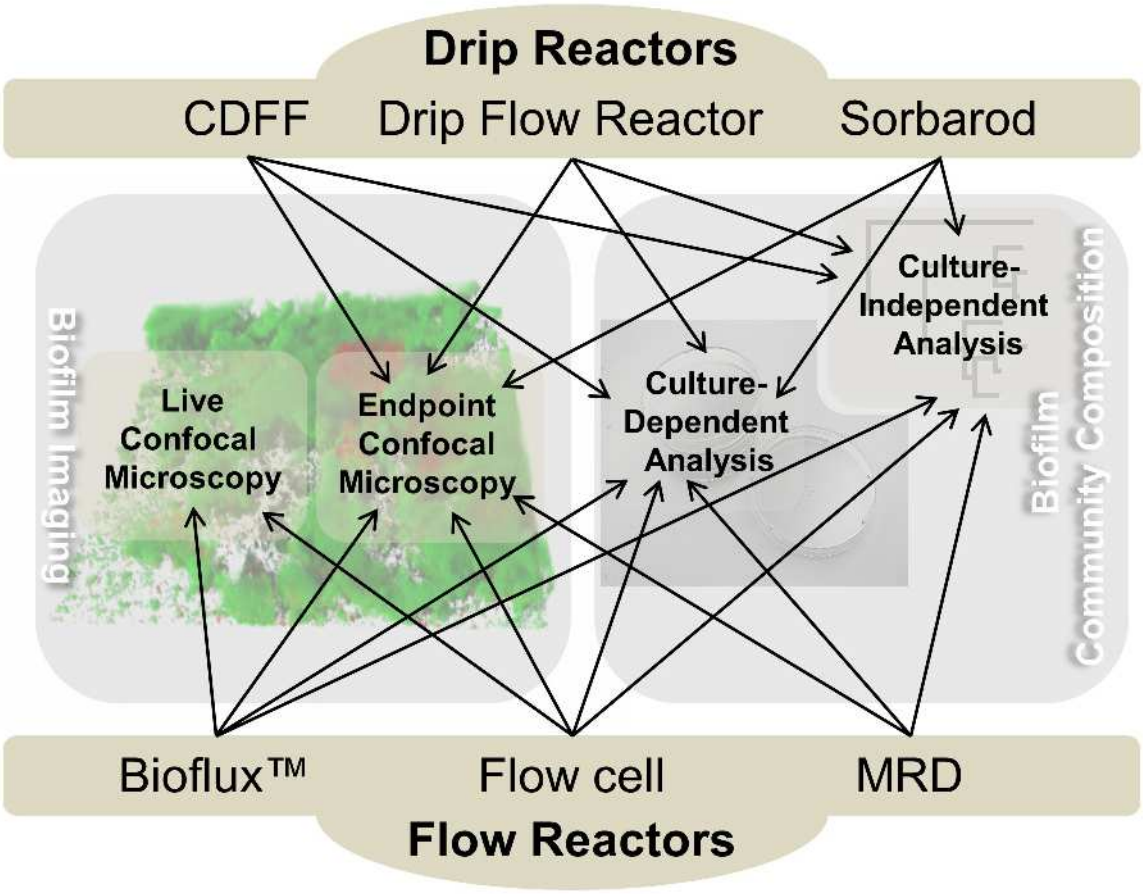

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