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Research review paper

Opportunities for visual techniques to determine characteristics and limitations of electro-active biofilms

João Pereira [a,b], Sam de Nooy [a,b], Tom Sleutels [a,c], Annemiek ter Heijne [b,*]

[a] Wetsus, European Centre of Excellence for Sustainable Water Technology, Oostergoweg 9, 8911, MA, Leeuwarden, the Netherlands
[b] Environmental Technology, Wageningen University, Bornse Weilanden 9, 6708, WG, Wageningen, the Netherlands
[c] Faculty of Science and Engineering, University of Groningen, Nijenborgh 4, 9747, AG, Groningen, the Netherlands

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A B S T R A C T

Optimization of bio-electrochemical systems (BESs) relies on a better understanding of electro-active biofilms (EABfs). These microbial communities are studied with a range of techniques, including electrochemical, visual and chemical techniques. Even though each of these techniques provides very valuable and wide-ranging information about EABfs, such as performance, morphology and biofilm composition, they are often destructive. Therefore, the information obtained from EABfs development and characterization studies are limited to a single characterization of EABfs and often limited to one time point that determines the end of the experiment. Despite being scarcer and not as commonly reported as destructive techniques, non-destructive visual techniques can be used to supplement EABfs characterization by adding in-situ information of EABfs functioning and its development throughout time. This opens the door to EABfs monitoring studies that can complement the information obtained with destructive techniques. In this review, we provide an overview of visual techniques and discuss the opportunities for combination with the established electrochemical techniques to study EABfs. By providing an overview of suitable visual techniques and discussing practical examples of combination of visual with electrochemical methods, this review aims at serving as a source of inspiration for future studies in the field of BESs.

1. Introduction

The increasing world population, global warming due to the increased greenhouse effect and depletion of fossil fuel reserves are making sustainable energy and research recovery technologies, such as recovery of energy and nutrients from wastewater, more pressing matters (Borole et al., 2011; Lahiri et al., 2022). Bio-electrochemical systems (BESs) have gained substantial interest in the past two decades as they provide a new way to recover resources (e.g. nutrients) and energy from wastewater (Das, 2017; Kiran and Patil, 2019). BESs are systems that make use of microorganisms that are able to use electrodes as external electron acceptors (exoelectrogens) or electron donors (electrotrophs) for chemical conversions (Babauta et al., 2012; Santoro et al., 2017). These systems include the Microbial Fuel Cell (MFC) and Microbial Electrolysis Cell (MEC) for energy recovery in the form of electricity or hydrogen, and Microbial Electrosynthesis Cell (MES) for production of fuels or chemicals from CO_2 (Logan et al., 2006). They all base their working principle on electro-active microbial communities, with the difference that MFCs and MECs rely on exoelectrogens (at the anode), while MESs rely on eletrotrophs (at the cathode) (Logan et al., 2019; Thapa et al., 2022).

Electro-active biofilms (EABfs) are a conglomerate/community of electro-active bacteria that develop on the surface on an electrode (Erable et al., 2010). These bacteria catalyze the conversion between electrical energy and chemical energy. Because of their crucial role in BESs, providing the most suitable operating conditions for bio-catalysis has been the focus of many studies (Choi and Chae, 2013; Jadhav and Ghangrekar, 2009; Lee, 2018). We frequently see research resulting in the improvement of BESs performance using more suitable materials and optimized electrode designs to improve the interaction between EABfs and electrode surface (Caizán-Juanarena et al., 2019; Chong et al., 2019; Hindatu et al., 2017; Schröder et al., 2015). However, being electro-active bacteria the key player that determine the exchange between electrical and chemical energy, it is pivotal not only to study the behavior of electro-active bacteria and EABfs as a response to operational conditions e.g. electrode designs and electrode current/potential, but also the relation between their characteristics to improved performance.

* Corresponding author.
E-mail address: annemiek.terheijne@wur.nl (A. ter Heijne).

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Several types of techniques, including electrochemical, visual, and chemical analyses, have been used to study EABfs. These techniques provide a wide range of information about EABfs: they give insights in, e.g., microbial activity, biofilm composition, structure and thickness, mass transfer limitations and conductivity (Bartosch et al., 2003; Lusk et al., 2016; Pepè Sciarria et al., 2019). Electrochemical techniques are used to determine the general performance indicators of EABfs, being the relationship between electric current and potential. Examples of commonly used electrochemical techniques are Cyclic Voltammetry (CV), potentiostatic control (also called chronoamperometry), and Electrochemical Impedance Spectroscopy (EIS), which can be performed at different stages of EABfs growth, and provide information about microbial activity, the presence of redox active compounds, and charge storage (de et al., 2021; Droop, 1966; He and Mansfeld, 2009; Strycharz et al., 2011; ter et al., 2015). Chemical analyses are also frequently used in BESs to assess the concentration of substrate and/or products in the bioreactor. Linking these concentrations to the electrons exchanged at the electrode(s) gives information on the (coulombic) efficiency of anodes and cathodes. Besides, these chemical techniques can be used on EABfs themselves, to evaluate the composition of the biofilm by means of elemental analysis (with an elemental analyzer) or quantification of protein and polysaccharides present in the extracellular matrix of the biofilm (using Pierce BCA protein Assay Kit for proteins, and the phenol-sulphuric acid method for polysaccharides) (Pereira et al., 2021). Well-known techniques to visualize biofilms have also been adopted and adapted to study electro-active biofilms on an electrode (Azeredo et al., 2017). Among others, the use of Confocal Laser Scanning Microscopy (CLSM) and Optical Coherence Tomography (OCT) has been reported in EABfs works as tool to monitor biofilm thickness, investigate biofilm composition and to localize microbial species and activities in the biofilm structure.

Even though there are many techniques to study EABfs, many of the available techniques are destructive. This means that the biofilm needs to be sacrificed to perform a given analysis and that the ongoing study needs to be interrupted and cannot be resumed after the analysis. As a result, using these destructive techniques means that EABfs cannot be monitored during the experiments and that these biofilms are monitored during their operation only using a typically "safe" and repetitively reported set of techniques. Added value can be brought to the field of BESs when in-situ techniques are used to visualize EABfs, since these can perform online monitoring of biofilm characteristics, and follow biofilm developments over time. Table 1 gives an overview of techniques that can be used to visualize biofilms on electrode surfaces, based on the criteria that are relevant for biofilm monitoring and characterization: quantification, 3D distribution, destructivity, and the possibility to detect/target specific compounds of interest. This table aims at providing a non-exhaustive summary on the general characteristics of the techniques that will be discussed in detail in this review. These six techniques, namely Confocal Laser Scanning Microscopy (CLSM), Optical Coherence Tomography (OCT), Raman Microscopy, Scanning Electron Microscopy (SEM), Scanning Transmission X-ray microscopy (SXRM) and Magnetic Resonance Imaging (MRI), have been selected due to their potential to add valuable information on electrochemical data and the positive trade-off between image quality and ease of use. More criteria, that are not included in Table 1, can be discussed when evaluating the suitability of a visual technique: its working methodology including the time invested for samples preparation and for visualization, and the equipment and operating costs. The information learned on EABfs from the use of other more costly and very sensible techniques is acknowledged and, therefore, some are mentioned and briefly discussed in section 3.

Table 1
Techniques that can be used to visualize electro-active biofilms. In green (✓), the aspects that can be covered with each technique; in red (x), the ones that are not.

Techniques	Quantify	3D distribution	Non-destructive	Specific compounds
Confocal Laser Scanning Microscopy (CLSM)	✓	✓	✓* / ×	✓
Optical Coherence Tomography (OCT)	✓	✓	✓	×
Raman Spectroscopy	✓	✓	✓	✓
Scanning Electron Microscopy (SEM)	×	×	×	×
Scanning Transmission X-ray microscopy (SXRM)	✓	✓	✓	✓
Magnetic Resonance Imaging (MRI)	✓	✓	✓	×

*CLSM is non-destructive when using auto-fluorescent samples and no specific compounds are stained.

Each visual technique provides specific information on the biofilm, at diverse resolutions and on different aspects of the biofilm. Their advantages and disadvantages for biofilm characterization differ: most techniques can be used for biofilm quantification, since they give insights in the 3D distribution of biofilms, while others are limited to 2D imaging and can require more destructive sampling procedures. From the techniques included in Table 1, CLSM is the most universal as it allows for visualization, quantification, 3D imaging and characterization of biofilm composition with a single apparatus. For a quick biofilm visualization, SEM is an appropriate technique as it allows for a qualitative description of the biofilm development on an electrode providing insights on the structure of the biofilm including shapes and distribution on an electrode surface. MRI and OCT allow for 3D imaging, to determine the biofilm distribution and its volume, and they have the advantage of not destroying the sample. Due to their non-destructive features, Raman and STXM are suitable options when aiming at studying the biofilm composition. Even though their positive points, each technique has its intrinsic shortcomings. Therefore, choosing a suitable technique is challenging and a careful weighing is needed to assess what a certain technique can offer to meet the goal of the study.

EABfs can be very challenging to study as these biological matrixes, in which electro-active bacteria are embedded, have unique composition and mechanical properties (depending on the operating conditions such as feed concentration and electrode potential). Moreover, the composition of this matrix is continuously changing with time as EABfs grow on an electrode, which makes characterizing EABfs and predicting their performance even more challenging. Therefore, using visual techniques to detect specific compounds present in the extracellular matrix and to visualize the distribution of the biofilms as a function of time opens opportunities to better understand performance results obtained e.g. from an electrochemical measurement in electro-active biofilms. For example, monitoring the current profile during continuous polarization on an anode is a typical measure of the activity of EABfs. This activity can, for example, be associated with the amount of biofilm on the anode. Therefore, relating the activity of the biofilm with its thickness on the anode is an example of the added value derived from the combination of electrochemical and visual techniques. Besides, this combination helps understanding how biofilm growth is affected by the operating conditions. High activities are also linked to a high concentration of c-type cytochromes on the membrane of electro-active bacteria (Reguera, 2018; Zhang et al., 2018). Thus, staining these compounds and visualizing the biofilm with a suitable technique supplement and support the information derived from the electrochemical measurements. Moreover, the activity of EABfs can also be affected by other factors such as biofilm density, the microbial community the biofilm is composed of, and the positioning of species in the biofilm structure. For that purpose, visual techniques can be used to image the morphology and cellular density in the biofilm structure, identify species that are part of the biofilm and mapping their disposition in the biofilm. Especially the combination of electrochemical and visual techniques allows to acquire more knowledge about EABfs.

In this review, we describe visual techniques that have been used for EABfs studies combined with electrochemical techniques and discuss what information has been obtained. For the techniques introduced in Table 1, we provide a brief description of basic principles, how they have been applied to study electro-active biofilms (including limitations and practical implications) and what information/knowledge has been gained from their use. We also discuss other less often used visual techniques and summarize their applications to investigate EABfs. Finally, by providing an overview of suitable visual techniques and discussing practical examples of combination of visual with electrochemical methods, this review aims at serving as a source of inspiration for future studies in the field of BESs.

2. Techniques for visualization of EABfs and outcomes of their use

Visual techniques in BESs are plentiful and cover very wide-ranging aspects of biofilms (Hu et al., 2005; Li et al., 2016; Zhang et al., 2019). In this chapter, we will discuss six different techniques and their application for EABfs: CLSM, OCT, Raman, SEM, STXM and MRI. Some more versatile techniques are a better choice for analyzing diverse biofilm characteristics such as thickness and composition, and others stand out due to their high resolution. In many cases, these visual techniques provide additional information to electrochemical techniques, instead of offering an alternative way to measure similar characteristics of EABfs. In fact, the combination of these visual and electrochemical techniques gives more reliable and/or more comprehensive information on EABfs.

2.1. Confocal laser scanning microscopy (CLSM)

Confocal Laser Scanning Microscopy (CLSM) is suitable for real-time, non-invasive and in-situ measurement of biofilm characteristics. It is often considered as the most powerful visual technique for biofilms (Azeredo et al., 2017). CLSM uses a laser to excite fluorescent molecules (fluorophores) and it measures, subsequently, the light emitted when electrons fall back to their ground energy state (Franklin et al., 2015). It makes use of a pinhole to filter out light that is not in the optimal focal plane, also known as out-of-focus light. Due to the pinhole, the sub-micrometer resolution is high enough to visualize single cells. CLSM allows 1) imaging of live and hydrated samples, 2) sectional visualization of samples without invading the sample, 3) performing 3D analysis of molecules and cells (Neu et al., 2010; Schlafer and Meyer, 2017; Tejedor-Sanz et al., 2017). For EABfs, this translates in observing individual components such as proteins, polysaccharides and nucleic acids, pH mapping, viability and activity of cells, thickness, and 3D structures. Even though the ability to visualize biofilm samples at different depths, the penetration depth of the laser is one of the limitations linked to the use of CLSM. Samples thicker than 200 μm easily absorb all the laser light, leading to loss of visibility. The penetration depth is also affected by the presence of impurities in the samples such as sand, clay or precipitates (Palmer et al., 2006). A non-destructive visualization of biofilm structures with CLSM depends on the auto-fluorescence of the biofilm. Since biofilm samples have typically weak auto-fluorescence signals, their visualization with CLSM is dependent on the use of fluorescent probes and dyes. These probes, which are genetic sequences that bind to specific genome fragments (or to mRNA to target the expression of specific proteins), can be used to quantify the biofilm amount on an electrode. Biofilm samples can also be stained with dyes (generally chemical reaction-based interaction) to investigate its composition: for example, dyes to assess the species present in the biofilm and dyes to investigate the ratio of live/dead cells and proteins and polysaccharides content in the Extracellular Polymeric Substances (EPS). The use of these probes and dyes is not reversible. Once these bind to and/or react with their target compounds, namely DNA and/or mRNA and proteins and/or polysaccharides in the EPS, these can often not be unbound from the biofilm structure without affecting the activity and development of the biofilm on an electrode. On the one hand, these probes and dyes can be added to the biofilm for visualization at any moment of an ongoing experiment. Moreover, with this approach, the electrode does not necessarily need to meet the requirements for in-situ visualization as the study will not be resumed after biofilm staining. On the other hand, besides their costs and toxicity, the use of several fluorescent probes on a biofilm sample needs to ponder overlapping of the emission spectra of the fluorophores (which may reduce the number of possible probes and dyes combinations to study one biofilm sample). In a non-destructive approach, the visualization can be performed in-situ with genetically modified bacteria: for example, with electro-active bacteria that incorporate a fluorescent probe such as Green Fluorescence Protein (GFP). However, in-situ visualization of electro-active bacteria with an incorporated fluorescent probe requires a suitable transparent electrode with a flat surface to allow high resolution imaging.

Even though the need to dye biofilm samples, the use of this technique in EABfs studies is widely reported (Dong et al., 2021; Kim et al., 2004; Nevin et al., 2008; Yang et al., 2019). By including CLSM in their works, Franks et al., 2009 and Richter et al., 2009 were able to image the growth of *Geobacter sulfurreducers* biofilms and to determine the biofilm thickness on the electrode. Monitoring the growth of biofilm is an important tool to calculate biomass yields and to relate the amount of biofilm with produced current (so-called microbial specific activity). In addition to monitoring biofilm growth, CLSM has also been used to investigate the viability of the bacteria present on the electrode by means of Live/Dead kits (Marsili et al., 2008; Reguera et al., 2006; Takenaka et al., 2001). Sun et al., 2016 combined Live/Dead staining with electrochemical measurements on an anodic biofilm and showed that the decreasing produced current at the anode was caused by a fast accumulation of dead cells in the electro-active biofilm (Fig. 1). Since the thickness of the biofilm can also be measured with CLSM, the relation between biofilm thickness, maximum activity of the electro-active biofilm and the presence of dead cells was also reported in this study: maximum activity was reached when the biofilm thickness was approximately 20 μm, and it decreased (due to the accumulation of dead cells) as the biofilm grew up until a final thickness of 45 μm.

CLSM also allows to identify species present in the biofilm and their positioning on the electrode by using Fluorescent In-Situ Hybridization (FISH) (Azeredo et al., 2017; Das, 2017; Franklin et al., 2015; Neu et al., 2010). The information obtained using FISH can be used to give insights in how the accumulation of dead biomass and minority and/or unfavorable positioning of electro-active species on the electrode affects performance. The composition of the biofilm matrix also plays a role in the performance of EABfs (Cao et al., 2011; Vu et al., 2009). Schmidt et al., 2017 and Esteve-Núñez et al., 2008 have used CLSM to image *G. sulfurreducens* biofilm and study the importance of c-type cytochromes in electron transfer mechanisms and their auto-fluorescent properties. As recognized in literature, CLSM is thus a versatile and powerful technique that allows linking electrochemical data with the presence of redox compounds in EAB, composition of biofilm matrix (for example, proteins and polysaccharides), cells viability, and the mapping of microbial species on the electrode.

2.2. Optical coherence tomography (OCT)

Optical Coherence Tomography (OCT) is an imaging technique based on the scattering of light. This technique uses near-infrared light, and the light reflected from the samples is analyzed with an interferometer (Aumann et al., 2019). Based on its working principle, the delay in the reflected light has already been used to study flow and diffusion phenomena in colloidal suspensions (Weiss et al., 2015). OCT has a micrometer resolution and it allows imaging of large sample areas (several millimeters) without the use of fluorescent probes (Li et al., 2016; Neu and Lawrence, 2015). Even though in-situ visualization,

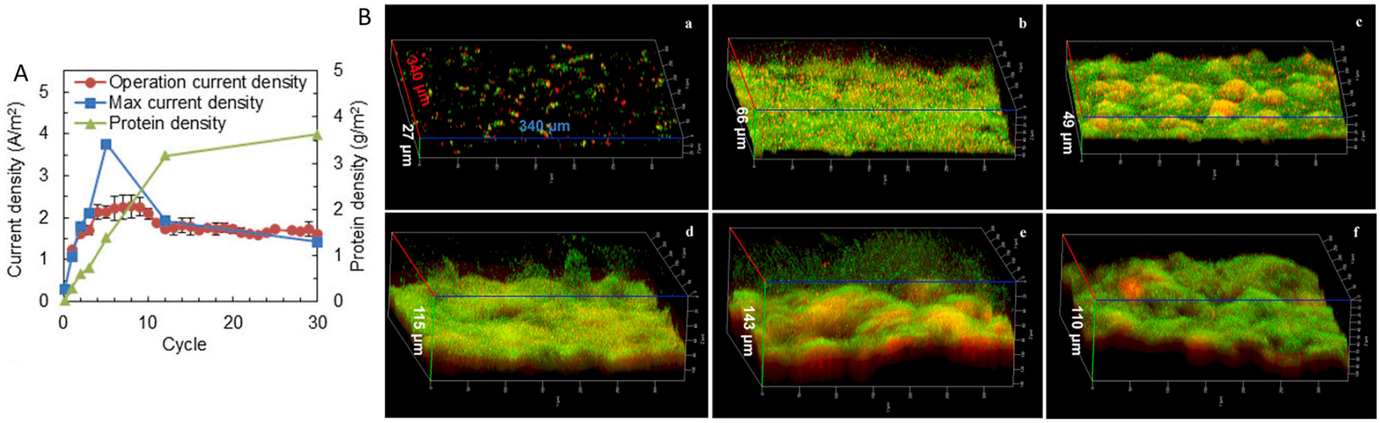

Fig. 1. A) Current density as a function of time (in number of cycles) and B) Anode biofilms of *G. sulfurreducens* PCA visualized with CLSM after Live/Dead staining with BacLight™ Bacterial Viability Kit. Green are live and red are dead cells at sequential growth phases: a) beginning of initial phase (beginning of cycle 1), b) end of initial phase (end of cycle 1), c) fast cell accumulation (cycle 2), d) maximum activity (cycle 5), and e and f) mature phase (cycle 12 and 30, respectively) (adapted from Sun et al., 2016). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

quantification and 3D imaging are possible, the penetration depth of the OCT's signal is limited to around 2 mm thick samples and samples composition cannot be assessed.

The use of OCT as a tool to quantify the biofilm volume over time on an transparent anode has been reported by Molenaar et al., 2018. This work validated the use of this visual technique as a non-invasive and in-situ analysis to study EABfs. In this work, 54 scans to the transparent electrode with biofilm were taken with OCT, and then processed with a Matlab script that isolated and counted the biofilm pixels. The thickness of the biofilm on the electrode was calculated by averaging the 54 pixel counts and dividing this average by the pixel size. The biofilm volume was calculated by multiplying the thickness with the electrode surface area. By facilitating biofilm monitoring on an electrode, it allowed for linking biofilm growth/formation to local conditions and overall system performances. Positioning and morphologic changes on the biofilm structure as a response to operating conditions can also be determined. In a study that aimed at understanding the effect of intermittent anode potential on the morphology of EABfs, Pereira et al., 2021 used OCT to describe the response of the electro-active biofilms to this anode potential regime. In this study, irregular and patchy biofilm structures were observed on the anodes controlled with an intermittent anode potential, and regular and flat biofilm structures were observed on the anodes controlled with a continuous anode potential (Fig. 2). By combining potentiostatic operation with OCT measurements and chemical analysis of the biofilms at the end of the experiments, a higher production of EPS by the intermittent EABfs was observed and quantified. Besides, measuring the acetate concentration in the analyte and the amount of the biofilm on the electrode allowed to calculate biomass yields, which were higher for the intermittent EABfs.

Xi et al., 2006 showed that it is also possible to obtain 3D images of the volume of the biofilm with OCT. More recently, Pereira et al., 2022 have identified mass transfer limitations in bio-anodes by monitoring the thickness of the biofilm at three different anode potentials and acetate concentration. In this study, acetate diffusion rates in bio-anodes that can be used for modelling EABfs have also been reported.

2.3. Raman microscopy

Raman can be used to determine the chemical composition and molecular structure of a biofilm (Zhang et al., 2019). Raman microscopy uses monochromatic light and measures the scattering patterns of the light. Since the frequency of the scattered light differs per compound, the chemical composition of the biofilm can thus be assessed. It is a non-destructive method capable of real-time detection (Franklin et al., 2015). Raman is a highly sensitive technique to detect neutral chemical bonds such as C—C, C═C and C—H, and it has a very high resolution (in the order of microns) without the need for staining. For some measurements though, the equipment needs to be optimized before use due

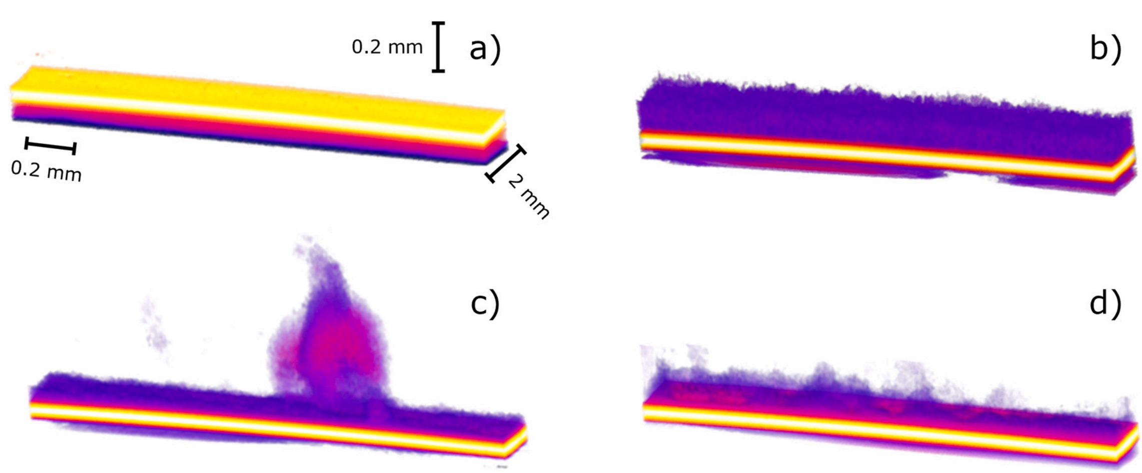

Fig. 2. OCT visualization of biofilm morphology on a transparent glass electrode coated with Fluorine Tin Oxide: a) bare electrode, b) continuous anode potential, and c and d) intermittent anode potential regimes in which patchy forms can be observed (adapted from Pereira et al., 2021).

to the weakness of the Raman effect (i.e., hardly detected changes in the vibration mode of chemical bounds) and fluorescence of a given sample may readily distort the spectrum (Schechter et al., 2014). Besides, even though biofilms share similar compounds e.g. DNA, proteins, polysaccharides and lipids, the vibration of the chemical bounds of a given compound varies among species (Maquelin et al., 2002). Therefore, a more accurate and valid use of Raman to determine biofilm composition usually requires the recognition of a vibration pattern and the creation of library for the specie of interest.

Raman has been used to monitoring EABfs development at different growth stages based on the Raman resonance effect of c-type cytochromes (Virdis et al., 2012) (Fig. 3). In this study, they showed that the redox state of cytochromes can be determined without interfering with the biofilm structure and used to measure the activity of the electro-active biofilm.

In a follow up study, they related the oxidation state of the cytochromes with biofilm thickness (Virdis et al., 2014). They observed that cytochromes remained homogenously oxidized at early and middle stage of biofilm development (10 and 57 days, respectively) when the biofilm had a thickness of 70 μm. In the later stages (80 days) when the biofilm reached a thickness of 100 μm, the cytochromes were in a reduced state. This ability to monitor the redox state of cytochromes adds essential information to better understand electric characteristic of biofilm, and here it suggests electron transfer limitations as thick biofilms could not exchange the electrons with an oxidized redox compound or electrode. More works have reported the use of Raman to show the presence of a redox gradient caused by cytochromes in the biofilm monitor, and to characterize *G. sulfurreducens* biofilms during electricity generation for both wild and mutant strains (Krige et al., 2019; Lebedev et al., 2014).

Besides cytochromes, Keleştemur and Avci, 2018 used Raman to determine the concentration of protein and polysaccharides in EPS and to describe changes in the composition of polysaccharides into glycoproteins in EPS.

2.4. Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) is mostly used for qualitative analysis of biofilms (Vyas et al., 2016; Yang et al., 2019). SEM is based on spraying the sample with electrons, which will bounce back to a detector that will then produce an image of the surface of the sample. SEM allows for visualization at nanometers scale. However, drawbacks of the use of this technique are the sample preparation that requires a pre-treatment/fixation, which may alter the structure of the sample, and the detection of the reflected electrons from non-smooth surfaces, which makes imaging rough surfaces of biofilms very challenging. However, this has been tackled by combining SEM with advanced segmentation methods to get better image quality. Vyas et al., 2016 applied machine learning to be able to calculate the area of a biofilm by distinguishing biofilm structure from the surface on which the biofilm had been developed.

SEM has been used in BESs not only to visualize electro-active bacteria but also electrode surfaces (Choi and Chae, 2013; Marsili et al., 2008; Read et al., 2010), and the adhesion/interaction of the biofilm on different electrode surfaces (Bond and Lovley, 2005; Kim et al., 2014; Torres et al., 2010). In a study on anodic EABfs, Katuri et al., 2020 concluded that the electrode surface characteristics had a noticeable effect on the biomass adhesion, activity and morphology. They reported that the produced current on an anode was linked to the presence and growth of electro-active bacteria on the anode surfaces and that the

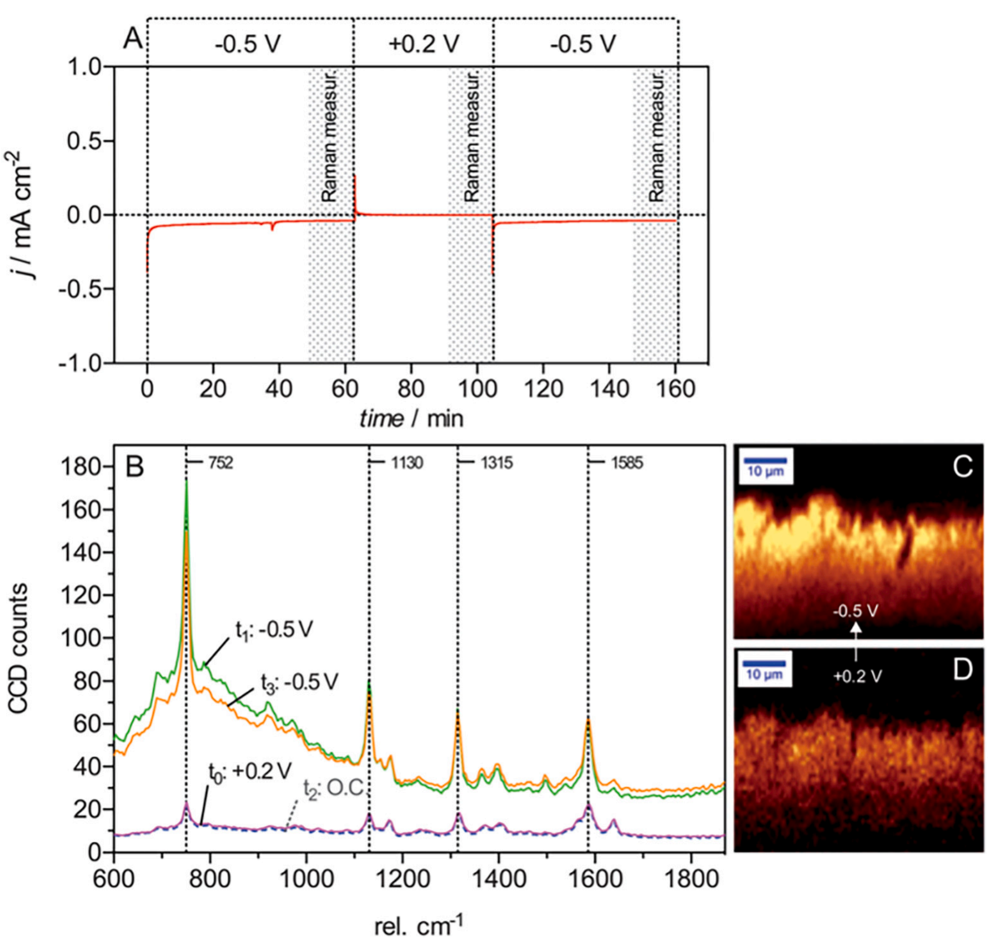

Fig. 3. A) The reduced and oxidized states of the c-type cytochromes were tested by controlling the anode at −0.5 and + 0.2 V vs. Ag/AgCl, respectively; B) Reduced (green and orange traces, t_1 and t_3) and oxidized (purple and blue traces, t_0 and t_2) states in biofilm under non-turnover conditions; C and D) Cross-sectional images of the biofilm at −0.5 V and + 0.2 V vs. Ag/AgCl, respectively (adapted from Virdis et al., 2012). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

produced current was promoted by hydrophilic surfaces, especially at early stages of biofilm development (Fig. 4). From all the studies that use SEM to study EABfs, this study is here described given the combination of SEM with CLSM to determine the biofilm thickness. Particularly, the homogenous distribution of the biofilms on the electrode was visualized with SEM, and when later combined with CLSM, a thickness of approximately 22 μm was determined.

SEM can also be coupled with Energy-Dispersive X-ray spectroscopy (SEM-EDX) to investigate the composition of precipitates and the elemental composition of biofilm samples. Even though this approach is not commonly found in literature, the composition of the biofilm could be used to derive an experimental biomass elemental formula towards a more accurate mass balance in the bioreactors.

2.5. Scanning transmission X-ray microscopy (STXM)

Scanning Transmission X-ray Microscopy (STXM) makes use of soft X-ray absorption to provide information on chemical bonding, charge state and magnetic state of the elements present on the analyzed samples (Neu et al., 2010; Santini et al., 2015). Therefore, it allows to quantitatively determine the composition of biofilms in terms of proteins, polysaccharides, lipids and nucleic acids and how they are distributed. STXM is non-invasive, has a nanometer resolution and can be applied to hydrated samples owing to the fact that X-rays penetrate water. However, due to its low penetration depth, sectional visualization of the biofilm is very challenging (up to a maximum of around 300 nm thickness) (Zhang et al., 2019).

Due to this low penetration depth, the use of STXM in the field of BESs is in a premature phase. However, the potential of this technique has been acknowledged in other studies with biofilms and there are some reports of the use of STXM in combination with other visual techniques. In these, Carrel et al., 2018, Carrel et al., 2017used STXM to visualize the morphology and provided biofilm volume profiles and indicated that the biofilms were exposed to shear stress, which led to non-homogeneous growth. They observed more growth in low shear stress regions, and evaluated the effect of mass transfer of nutrients and electron acceptors on the growth of the biofilm. Used here as a practical example on how to take advantage of combining different techniques, Lawrence et al., 2003 combined STXM with CLSM and Tomography Electron Microscopy (TEM) to obtain 3D structural and compositional information on biofilms. TEM was used to get structural information at high resolution, CLSM with fluorescent probes provided compositional information, and STXM was used to add information on the composition of macromolecules without probes (Fig. 5). The mass transfer limitation and biofilm composition outcomes mentioned above are also of interest to understand the performance of EABfs. Therefore, benefits on the use of STXM and replications of this combination approach are to be expected in the field of BESs.

2.6. Magnetic resonance imaging (MRI)

Many nuclei of atoms carry a quantum mechanical spin and thus a magnetic moment (Bartacek et al., 2016). If a strong polarizing magnetic field is used on those nuclei they become magnetized. By irradiating the nuclei with a specific frequency, the Larmor frequency, a measurable magnetic resonance signal is created. This signal can be used to determine the structure of large molecules. Because the energy involved in this process is very low, the technique is suitable for analysis of living and hydrated objects. MRI provides information on the dynamics of water and transport properties in biofilms such as mass transport and oxygen diffusion. Therefore, this technique can be used for modelling biofilm processes and diffusion (Das, 2017). However, MRI has mainly been used in biofilm research to form a 2D or 3D image of the biofilm to show structural biofilm features (Franklin et al., 2015; Neu et al., 2010).

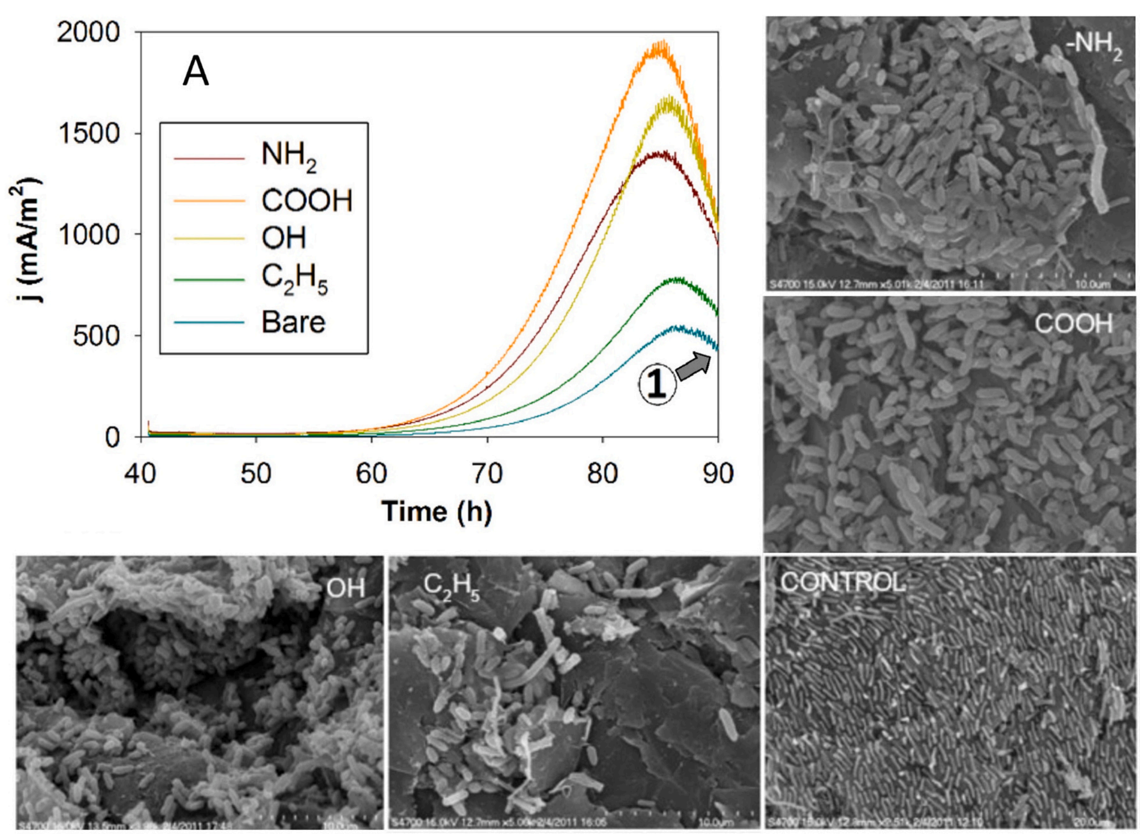

Fig. 4. Current density as a function of time (A) and SEM comparison of the adhesion of early stage biofilms (90 h, identified with an arrow in A) to electrode surfaces with different functional groups (adapted from Katuri et al., 2020).

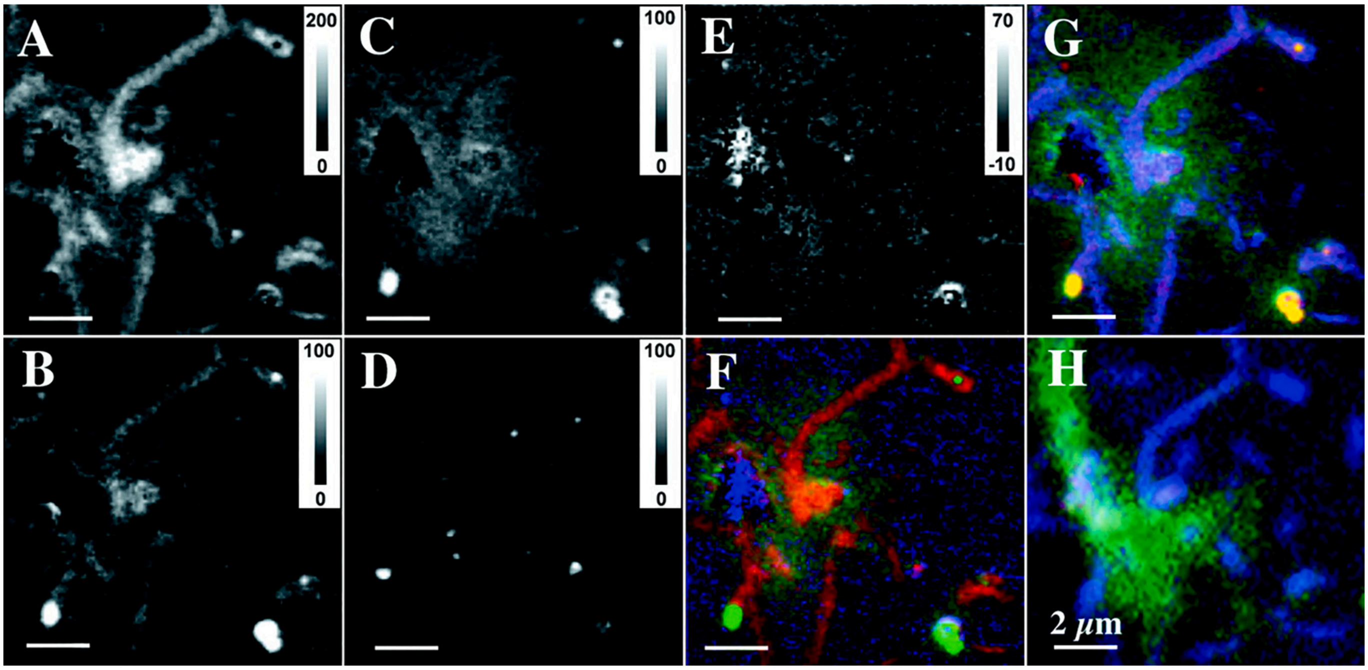

Fig. 5. STXM imaging of A) proteins, B) lipids, C) polysaccharides, D) carbonate and E) nucleic acid in the biofilm; F) is a colour mapped image showing proteins (red), polysaccharides (green), and nucleic acids (blue), whereas G) shows lipids (red), polysaccharides (green), and proteins (blue) – both F and G derived from a STXM image sequence of the biofilm. H) shows a CLSM image of the same region using probes for EPS (green) and nucleic acids (blue) (adapted from Lawrence et al., 2003). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

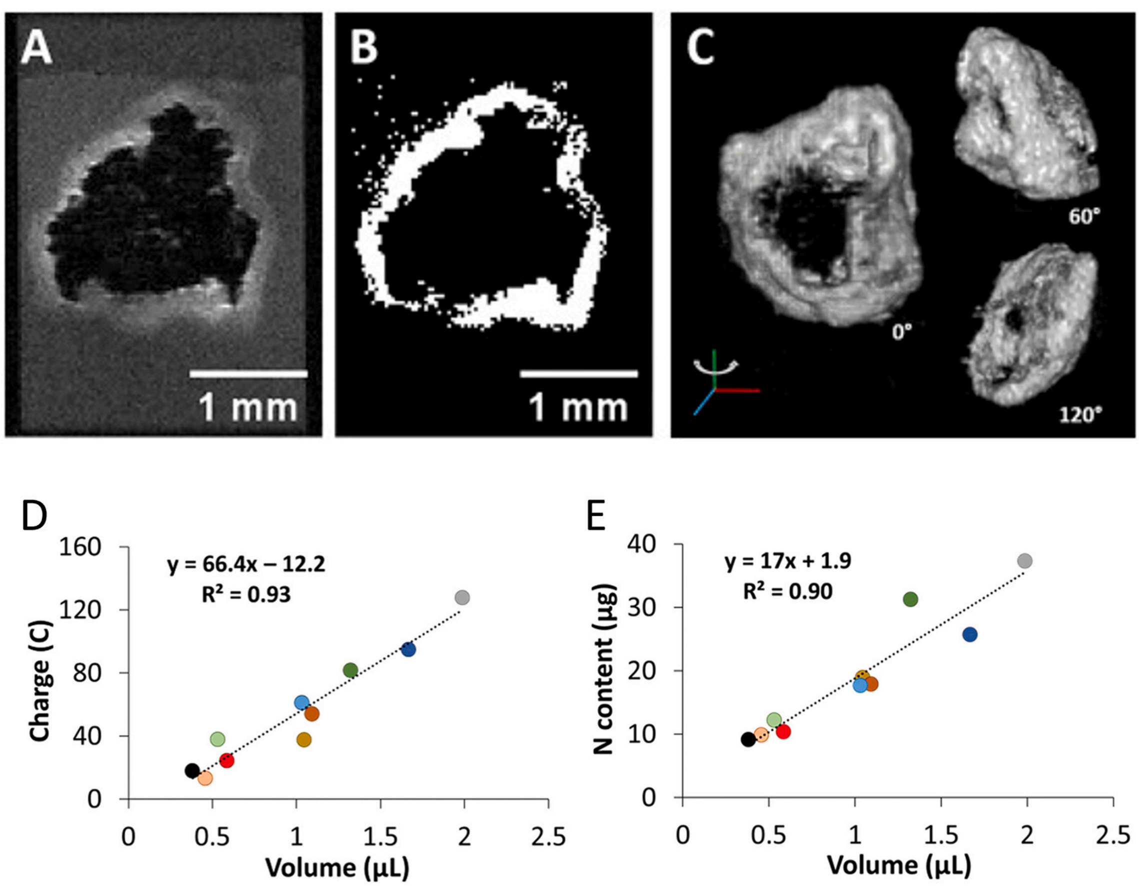

Fig. 6. A) Cross-sectional MRI image of a granular bio-anode, B) thresholding to select voxels that contain biofilm and C) 3D reconstruction of the biofilm, D) linear relation between produced charge and biofilm volume, and E) linear relation between total nitrogen and biofilm volume (adapted from Caizán-Juanarena et al., 2019).

For biofilm imaging, magnetic field used can vary from 0.7 to 14.1 Tesla (Bartacek et al., 2016). Caizán-Juanarena et al., 2019 used a magnetic field of 14.1 T to get (28 μm)^3 resolution 3D images of EABfs (Fig. 6) at the early stage (days 3 and 4), middle stage (days 6 and 7) and late stage (day 11 to 22). In the same study, 2D images to distinguish biofilm water from the bulk water were taken, and biofilm volume was also determined. The correlation between the total produced electric charge with the biofilm volume obtained with MRI and total nitrogen content reported in this study places MRI in an advantageous position among the techniques that allow in-situ monitoring of biofilm growth, as MRI can also be used to quantify proteins (and other nitrogen containing molecules), and it gives information on the distribution of the biofilm and on the interaction between the biofilm and the electrode surface.

At the expense of the 3D image resolution, lower magnetic field of 0.7 Tesla can also be used to perform in-situ observation of the development of the biofilm (Bartacek et al., 2016). With these works, MRI was used to successfully determine the biofilm distribution and its volume with the biggest advantage of not destroying the sample. In fact, MRI at low magnetic fields has been used in in-situ measurements on EABfs grown on activated carbon granules (Renslow et al., 2014; Renslow et al., 2010). However, the resolution was not high enough to determine the roughness of activated carbon granules nor to visualize bacterial growth in the inner macro-pores of the granules.

2.7. Opportunities for visual techniques to study EABfs

All visual techniques are tools to increase our understanding of the combination of biofilm and electrode in BESs. The list of techniques used to visualize biofilms can be further expanded with techniques that have had little application in BESs. Table 2 describes some other visual techniques that extend the opportunities to study EABfs and the six visual techniques described above. These other visual techniques include Light Microscopy (LM), Transmission Electron Microscopy (TEM), Two Photon-Laser Scanning Microscopy (TP-LSM), Structured Illumination Microscopy (SIM), Atomic Force Microscopy (AFM), Blink Microscopy (BM), Nano-Secondary Ion Mass Spectrometry (SIMS), Scanning Electrochemical Microscopy (SECM), and Cryo Electron Microscopy (Cryo-EM). A short description of their applications in biofilms studies and pros and cons are given (Table 2). LM is a very basic visual technique to allow cells visualization, whereas other techniques such as TEM and AFM allow for structural investigation of the biofilm. More robust techniques, namely TP-LSM, SIM, NanoSIMS, SECM and Cryo-EM, provide more detailed information on the biofilm structure and composition, but these are also more laborious and require image processing. Overall, this table, together with the six techniques described above, shows that the opportunities for EABfs visualization are widely available and can match several study purposes.

When a single visual technique is not enough to meet the aims of a given study, combining more than one visual technique can help overcoming shortcomings and eventual incompatibilities with the experimental set-up. Besides the described example of the combination of three visual techniques provided in Section 2.5, two more scenarios are given here. These intend to show possible approaches and the benefits of combining different visual techniques and electrochemical techniques. These should therefore not be seen as strict and defined set of techniques but rather a source of inspiration for readers to select techniques and find their own opportunities. Moreover, we encourage readers to explore other techniques to detect the electrochemical characteristics of EABfs (Gimkiewicz and Harnisch, 2013; Harnisch and Rabaey, 2012; You et al., 2015), alternative approaches to monitor EABfs growth (Millo, 2015; Millo, 2012), advanced microscopy techniques (Golden et al., 2018; Grohmann and Vaishampayan, 2017), and integrate upcoming techniques in EABfs studies.

As a first example, the qualitative visualization of EABfs with SEM could be supplemented with AFM to allow determining conductivity and mapping specific proteins. By combining these two visual techniques,

Table 2
Overview of possible techniques for biofilm visualization.

Technique	Application in (EA) biofilms	Advantages	Disadvantages	References
CLSM	Morphology and composition of the biofilm, and identification of species	Offers a wide range of visualization opportunities in one equipment, sub-micrometer resolution	Dyes and probes are destructive and expensive, long staining procedures can be required	(Reguera et al., 2006; Sun et al., 2016)
OCT	Monitoring biofilm growth and morphology on electrode surfaces	In-situ and fast measurement of large biofilm areas (millimeters)	No information on the composition of the cells and low resolution (micrometer)	(Molenaar et al., 2018; Pereira et al., 2021)
Raman	Target specific compounds in the biofilm structure	Non-destructive and micrometer resolution	Expensive, laborious and it requires the creation of a library to study a specie of interest	(Krige et al., 2019; Virdis et al., 2012)
SEM	Visualization of cell structures on electrode surfaces	Nanometer resolution, allows elemental analysis with EDX	Not suitable for wet samples, destructive sample preparation	(Bond and Lovley, 2005; Katuri et al., 2020)
STXM	Identification and quantification of compounds present in the biofilm	Determination of a wide range of compounds of interest and high resolution (nanometer)	Low penetration depth and expensive	(Carrel et al., 2017; Lawrence et al., 2003)
MRI	Morphology and quantification of biofilm structures	Allows for a non-destructive visualization, high resolution (micrometers)	Nanometer resolution images require destructive approach, expensive	(Caizán-Juanarena et al., 2019; Renslow et al., 2014)
LM	Bacterial growth and morphology on electrode surfaces; spot areas of interest in complex biofilms	Easily covers large biofilm surface areas, cheap and fast	Low magnification and resolution, resulting in limited information	(Rabaey et al., 2004)
TEM	Spatial arrangement biofilm, cellular structure; spot areas of interest in complex biofilms	Very high resolution (better than SEM)	Needs destructive sample preparation, only dehydrated samples, and time consuming	(Kim et al., 2004; Lawrence et al., 2003; Zakaria et al., 2018)
TP-LSM	Spatial distribution of active biomass and ions in biofilms (similar to CLSM)	Deep sample penetration and less fluorophores bleaching	Needs probes and a laborious procedure	(Hu et al., 2005; Neu et al., 2010)
SIM	Imaging of structural details	High resolution (up to 120 nm), allows use of conventional fluorophores	Needs probes, limited penetration depth (lower than CLSM),	(Neu and Lawrence, 2015)

(continued on next page)

Table 2 (*continued*)

Technique	Application in (EA) biofilms	Advantages	Disadvantages	References
			susceptible to errors in digital image analysis	
AFM	Determination of biofilm structure, cell quantification, visualization of individual molecules	Non-destructive	Limited surface area scanned, sensitive to external physical and electrical noise	(Azeredo et al., 2017; Schechter et al., 2014; Sivasankar et al., 2018)
BM	Imaging cellular substructures	High resolution (nanometer scale)	Not all fluorophores can be used, imaging in z-direction is limited	(Agrawal et al., 2013; Neu and Lawrence, 2015)
NanoSIMS	Imaging cells, biofilm morphology and composition, and active biomass	High resolution (nanometer scale)	Destructive approach, expensive, and laborious procedure and data interpretation	(Chadwick et al., 2019; He et al., 2017)
SECM	Detection of redox compounds and quantification of mediators involved in bacterial interactions	Microns scale resolution and in-situ measurements	Requires a suitable probe and a very precise positioning between probe and sample	(Caniglia and Kranz, 2020; Darch and Koley, 2018)
Cryo-EM	Structural and compositional characteristics of biofilms	Macromolecular structure determination, high resolution (angstrom scale)	Destructive approach due to cryogenic temperatures	(Filman et al., 2019)

the performance of EABfs could be related to their amount, distribution, and shape on an electrode (with SEM) and linked to the conductivity and activity of cytochromes (with AFM) in specific areas of the biofilm. Therefore, on a surface with several working electrodes, the activity of EABfs could be mapped and used as visual strong evidence to explaining performance. As a second example, we discuss the opportunities of combining CLSM with TEM and/or cryogenic electron microscopy and flow cytometry. Operating a bio-electrochemical reactor in continuous mode on the visualization stage of an CLSM equipment and scanning EABfs on an electrode allows to monitor growth and to visualize the structure of the biofilm. Even though its versality, allowing to assess live/dead cells and FISH analysis, CLSM would not be the most suitable visual technique to quantify and study planktonic cells in such a set-up. Here, sampling the electrolyte and using other microscopy analysis would give opportunities for a more complete characterization of planktonic cells. To this end, TEM and/or cryogenic electron microscopy could be used to provide information on cellular structure and flow cytometry used to quantify the number of planktonic cells. By combining several visual techniques, this would allow a more complete understanding of the anode/cathode as a whole system and a higher accuracy in mass balances.

Even though this review focusses on the study of EABfs, it is relevant to highlight that the use of visual techniques to study single cells is also possible and of crucial importance. When looking at individual cells, specific characteristics can be identified and separated from the properties of the whole biofilm regarding structure, composition and/or microbial community. Therefore, the biocatalysis of single cells can be studied and insights given on the maximum performance of a single electro-active microorganism (Jiang et al., 2013). Besides the use of visual techniques, single cells present in EABfs can also be identified with microbiological techniques that target DNA and, when targeting RNA, insights on genomics and proteomics can be gained (Mollaei et al., 2021; Orellana et al., 2014).

Finally, the combination of any of the visual techniques with performance indicators from electrochemical measurements will lead to additional insights to better understand EABfs behavior compared to one technique on itself. The list of positive outcomes of this combination is long and important when one aims to control, characterize and scale-up BESs: 1) quantify biofilm amounts on an electrode to calculate microbial specific activities of electro-active biofilms and biomass yields, 2) measure biofilm composition to study electron storage mechanisms and make a more accurate charge balance in EAB (ter Heijne et al., 2020), 3) determine biofilm density at different sheer stresses and calculate diffusion rates and identify mass transfer limitations thereof, 4) identification of active areas in the biofilm structure and determining which species are playing the key role in those, 5) chemically characterize the composition of the biofilm as a response to different feeding and electrode potentials regimes, and 6) study the adhesion of biofilms to different electrode surfaces.

Even though electrochemical and visual techniques can stand alone, merging the advantages of these techniques creates a very solid and powerful tool for understanding and gaining more information on electro-active biofilms. At first instance, this combination of techniques is a reliable source of knowledge, but in the long-run, this combination is the path that needs to be followed to provide the best operating conditions to electro-active bacteria and EABfs and to steer their catalysis towards the improvement of BESs performance.

3. Conclusions

The list of techniques available for biofilm visualization on an electrode is extensive. This wide range of techniques allows researchers to choose the most suitable technique to match the purpose of the study. Up until now, we have assisted a repetitive use of a limited set of techniques in the field of BESs. Despite the valuable information reported by its use, with this review we encourage researchers to refresh their approach in their coming works by showing the results and insights derived from the combination of electrochemical and visual techniques. Steps forward in the field of BESs depend on combination approaches discussed in this review and other possible combinations of the described techniques.

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CRediT authorship contribution statement

João Pereira: Conceptualization, Investigation, Validation, Writing – original draft, Writing – review & editing. **Sam de Nooy:** Investigation, Validation, Writing – review & editing. **Tom Sleutels:** Conceptualization, Writing – review & editing. **Annemiek ter Heijne:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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