REVISITING CELLULAR ASSAYS

**A SCANNING ELECTROCHEMICAL
MICROSCOPY BASED APPROACH FOR
BIOASSAYS ON MICROTISSUE ARRAYS**

ADITHYA SRIDHAR

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Adithya Sridhar

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A SCANNING ELECTROCHEMICAL MICROSCOPY APPROACH FOR BIO ASSAYS ON MICROTISSUE ARRAYS

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Chapter 0: Introduction

This chapter introduces the overall goal of this thesis work i.e. explore the use of scanning electrochemical microscopy (SECM) for biological assays. Subsequently, the main motivation behind each chapter is briefly outlined.

0.1. Goal

Modern scientific research has hugely benefited from technological advancement and like all scientific disciplines, cell biology is highly dependent on new experimental tools to push the boundaries of our understanding. A relatively new technique called scanning electrochemical microscopy (SECM) is showing promise in the field of cell biology for studying living biological systems in a non-invasive manner. Taking the previous statement about the potential of SECM at face value, this work aims to evaluate and apply the SECM as a tool for biological applications. More specifically, this thesis focuses on exploring the use of scanning electrochemical microscopy as an alternative (or complementary) method to traditional techniques (fluorescence, colorimetry etc.) for implementing biological assays on microtissue models.

0.2. Outline

Chapter 1 is intended to provide a brief introduction into the technique of SECM and the theoretical framework necessary for implementing SECM based assays along with a literature summary. The review of the literature is analysed from a biological perspective i.e., focusing on the biological relevance added by different SECM measurements reported, with respect to traditional bioassays.

Goal of **Chapter 2** is the realization of a suitable platform for the study of microtissues using SECM. Existing platform for tissue production at BIOS Lab on a chip group, based on Agarose/PDMS microwell arrays, are proven suitable for large-scale formation and culture of microtissues with control over their size and shape as determined by the microwell geometry. However, these devices are not compatible with SECM measurements due to porosity of the material and since they are processed as thick layers to improve their handling. Therefore, in this chapter we persist with the established format of microwell arrays but implement them in another material and subsequently, validate their use for microtissue production in this chapter and SECM measurements in the later chapters.

Chapter 3 explores the capability of SECM to image topography of single cells and microtissues (over time), by exploiting negative feedback mode measurements. The motivation of the topography measurements is to determine the height/shape of the microtissues in the microwells, which is expected to be pancake shaped at the beginning and evolving with cell

growth. This characterization is the basis for implementing bioassays using the SECM in the following chapters.

Chapter 4 intends to exploit oxygen measurements for conducting a first bioassay – a live/dead assay – on cancer microtissues. Prior to the assay, a the oxygen gradient around individual microwells with microtissues is to be characterized to identify optimal parameters such as scanning height and scanning rate for measurements.

In Chapter 5 a second bioassay is developed focussing on osteogenic differentiation of microtissues. This differentiation process is accompanied by the expression of alkaline phosphatase (ALP) in the cell membrane, a marker that can be detected using SECM through an indirect assay. ALP detection is, however, conducted traditionally under alkaline conditions, which is optimal for the enzymatic activity but not for cell survival. Therefore, we endeavour ourselves to design an assay to be conducted under physiological conditions by taking advantage of the collective nature of a microtissue.

Chapter 6 provides a summary of each chapter with highlights on the key results followed by recommendations and outlook for future work.

Chapter 1: Scanning electrochemical microscopy (SECM) for biological assays

This chapter serves as an introduction to the basic concepts necessary for understanding the work contained in the thesis. First, in brief, essentials of scanning electrochemical microscopy (SECM) and its operation are presented. Subsequently, the application of SECM for living biological systems is discussed using existing literature, with a focus on the biological relevance of SECM based measurements.

1.1. Introduction

An assay, as defined by Wu, “is a well-defined analytical method that contains the measurement procedure and how the measurement should be interpreted to obtain the properties of a system or an object” [1]. In this context, a biological assay or bioassay is an assay performed to evaluate the response of a living organism to a stimulus [2]. In biological laboratories, bioassays are generally performed on cell based systems, i.e. live cells, which can be either primary isolated cells or secondary immortalised cell lines.

Live cell based assays are critical for biological research, as they allow the study of whole systems and obtain complex information, which is not possible otherwise. Over the years, to enable live cell studies numerous tools and techniques have been developed such as cell culture platforms and advanced microscopy [3]. One of the commonly used tools in these studies is light microscopy, which coupled with fluorescence probes has been a mainstay in cell biology and continues to lead biological research.

However, fluorescence techniques are not without their limitations. For example, issues such as probe toxicity, photobleaching and strong illumination are present [4,5]. This provides opportunities for other techniques to serve either as an alternative or complementary method for biological assays where fluorescence techniques fall short. One such technique that is showing promise as a label-free and non-invasive way to study live cell systems is scanning electrochemical microscopy (SECM) [6-9]. This technique combines the advantages of electrochemistry with scanning probe microscopy which results in increased sensitivity of detection while providing high resolution spatiotemporal information on the electrochemical measurement.

Therefore, in this chapter, we consider SECM as a potential tool for performing biological assays. First, a brief introduction to SECM and fundamental concepts necessary for understanding the subsequent text is provided. Next, the different schemes of SECM measurements on cell systems are discussed with an up-to-date literature summary focussing on the biological relevance of SECM measurements. It is important to note that the scope of this chapter is limited to use of SECM for biological applications and its applicability for performing bio-assays. In this context, detailed information regarding SECM instrumentation along with non-biological applications is not covered and the reader is pointed to other text where relevant [10, 11, 13, 14].

1.2. Scanning electrochemical microscopy

SECM is a combination of two well-established techniques – electrochemistry and scanning probe microscopy (SPM). This combination is highly advantageous as it enables monitoring and imaging highly localised chemical reactions and gradients. The basic principles of SECM measurements are outlined below.

1.2.1. Principles of SECM

At the heart of a SECM system, is an electrochemical cell operated in a 3-electrode configuration, with the 3 electrodes being a working electrode, a reference electrode and an auxiliary (counter) electrode. A simplified way of understanding these electrode functions is as follows. First for electrochemical measurements, a potential is applied between the working and the reference electrodes resulting in a current. The working electrode is where the electrochemical reaction occurs, i.e. electron transfer by oxidation (gain of electrons) or reduction (loss of electrons), and the resulting current is detected. The reference electrode, as its name implies, provides a reference for the system and helps maintaining a stable potential that is reproducible between experiments. The opposite electrochemical reaction occurs at the auxiliary electrode which counterbalances the current at the working electrode. The reduction or oxidation reactions occur at a specific potential *vs.* the reference electrode depending on the electroactive species (referred here as reactant).

Fig 1.1. Concept of diffusion limited kinetics at the SECM tip. The electrochemical reaction and the resulting current at the UME are determined by the diffusion rates of reactant (R).

The SECM technique uses an ultramicroelectrode (UME, electrode size $\leq 25\ \mu\text{m}$) as the working electrode for studying electrochemical

reactions, which allows local measurements on a small area depending on the electrode size [11]. Understanding the current at the working UME is the key to understanding and analysing SECM measurements. The current at the UME is determined by the mass transfer kinetics of the reactant in solution [11]. In the case of a SECM system, the mass transfer is only determined (through design) by the rate at which the reactant diffuses to the UME. The point at which the current at the SECM tip is constant is referred to as the steady state. For a disk electrode of a given radius a , the steady state diffusion-limited current (i_{∞}) in bulk solution is given by the equation

$$i_{\infty} = 4nFDca$$

where n is the number of electrons transferred by the electrochemical reaction, F the Faraday constant (96,485 C mol⁻¹), c the concentration of the reactant and D the diffusion coefficient of the reactant [11]. Most SECM experiments are carried out with disk electrodes but for electrodes of other shapes similar current equations can be expressed [11]. If all other parameters are known, then from the value of this current, the concentration of the reactant in solution can be easily derived by solving from the above equation. Therefore, the described UME, in the context of biological systems, enables highly sensitive and quantitative monitoring of rapid changes resulting from cellular processes.

In addition to this 3-electrode electrochemical cell, a SECM system involves a microcontroller for scanning probe microscopy. This microcontroller is employed to move the UME in the horizontal (x , y) and vertical (z) directions. A piezo controller is also available for finer movements in the vertical direction. The mechanical resolution of the positioning controller in our SECM system (ElProScan, HEKA, Germany), for instance, is as low as 100 nm in x , y , z and 5 nm with piezo. The positioning system in combination with the UME based electrochemical cell make a scanning electrochemical microscope. The use of the SPM extends the advantages of electrochemical sensing to monitor highly localised cellular information.

1.2.2. Modes of operation

Several modes for operation of SECM are available. However, understanding the feedback mode along with substrate generation-tip collection (SG/TC) mode is sufficient for the work contained within the thesis and therefore, only these modes are introduced below. The feedback

mode is the most commonly used mode of operation and is classified into two categories – negative and positive feedback, as illustrated in Fig. 1.2.

Fig 1.2. The feedback mode is the most frequently used mode of SECM operation. Negative feedback (left) occurs when the SECM tip is close to a non-conducting surface that results in physical hindrance of diffusion resulting in a lowered current if close to the surface. Positive feedback occurs when the SECM tip is close to a conducting surface which results in redox cycling of the reactant leading to an increased current when getting closer.

1.2.2.1. Negative feedback

Consider a SECM UME in bulk solution with the tip potential set to oxidise or reduce a known electroactive reactant (e.g., ferrocene methanol or ferricyanide). The current at the tip is in the steady state, as long as the reactant diffuses to the electrode. Now, if the electrode is very close to an electrically insulating surface (e.g., polystyrene or glass), the surface presents a physical barrier which limits the amount of reactant that can diffuse to the electrode, i.e. hindered diffusion. In this configuration, a decrease in the current at the UME is observed when approaching the surface. This current decrease is referred to as **negative feedback**.

1.2.2.2. Positive feedback

Consider the same scenario as before but instead of an insulating surface, the electrode is approaching an electrically conducting surface (e.g., platinum or gold). While there is still hindered diffusion similar to the previous scenario, another phenomenon called **redox cycling** occurs. If the reactant is being oxidized at the UME, then the conducting surface does the opposite, i.e., reduces the oxidized reactant. Once reduced, the reactant can diffuse back to the UME and gets oxidized again, which results in a current increase. This cycle of oxidation at UME and reduction at the conducting surface, or vice versa, is called redox cycling. The resulting current increase is referred to as **positive feedback**.

Fig 1.3. Nano-scale constant height topographical image surface patterns on a gold compact disc using feedback mode SECM. Image was obtained using a 190 nm Pt tip with ferrocene methanol as the redox mediator at a scan rate of 500 nm/s. Reprinted with permission from Laforge et al., *Anal. Chem.*, 2009, 81, 3143-3150 [12]. Copyright (2009) American Chemical Society.

Using the SECM in feedback mode, it is possible to image a surface as shown in Fig. 1.3. Any change to the current at the UME will depend on the surface topography and properties (conducting or non-conducting). Chapter 3 contains experimental demonstration on feedback mode imaging on live cells.

1.2.2.3. Substrate generation-tip collection mode

Fig 1.4. Concept of substrate generation-tip collection mode SECM. In this mode, the electroactive species (R) is not directly present in measurement solution but is instead generated by the substrate (grey box) that is being probed and generated species is subsequently detected at the SECM microelectrode.

In the feedback mode, the SECM tip is set to oxidise or reduce an electroactive species that is already present in solution. However, in the

substrate generation-tip collection (SG/TC) mode, the oxidised or reduced species is not ubiquitous in measurement solution but is produced by the substrate being probed. Therefore, in this mode the SECM microelectrode can be positioned close to a substrate and the electroactive species detected when it is produced or released by the substrate as shown in Fig. 1.4. The SG/TC mode offers a higher sensitivity compared to feedback mode as there is no background signal from continuous oxidation or reduction of the electroactive species.

1.2.2.4. Additional concepts

A few additional concepts of SECM measurements that might be helpful for this chapter or later in the thesis are presented in table 1. A basic understanding of the aforementioned concepts is deemed sufficient to venture further into the work presented. An in-depth discussion of SECM technology is beyond the scope of this thesis but the reader is referred to other works for detailed descriptions that are of interest [10,11,13,14].

Table 1. Definition of additional terms related to SECM measurements.

TERM	DEFINITION
Approach curve	The plot of the tip current <i>vs.</i> distance to the surface when the tip is moved in the vertical (<i>z</i>) direction.
Scan rate	The speed at which the SECM tip is moved in any given direction (<i>x</i> , <i>y</i> , <i>z</i>) for a measurement. Usually given in $\mu\text{m/s}$.
Constant height mode	In constant height mode scanning, the tip is not moved in the vertical direction, i.e., scans are only performed on the horizontal (<i>x</i> , <i>y</i>) plane while the tip position on the vertical plane (<i>z</i>) remains constant.
Constant distance mode	In constant distance mode scanning, the tip is constantly adjusted in the vertical (<i>z</i>) direction to maintain a constant distance from the surface being studied.
Line scan	Simple one dimensional scan in either the <i>x</i> or <i>y</i> direction.
2D scan/imaging	2 dimensional scan in both the <i>x</i> and <i>y</i> direction resulting in a SECM image.

As discussed, the underlying principle of SECM based measurement is electrochemistry and this requires an electrochemically active molecule. In some cases, the electrochemical detection can be achieved directly as shown in section 1.3.1.2 and chapter 4 for oxygen measurements. However, direct detection is not always possible and therefore indirect strategies such as the one shown in 1.3.2.1 and chapter 5 for ALP detection need to be developed. This requirement for an

electroactive molecule should be taken into account when considering the strategies employed for biological detection described in the next section.

1.3. SECM for cell measurements

We will review the SECM measurements on cell systems by classifying them into two categories – extracellular and cellular (membrane and intracellular) measurements. This section introduces these concepts along with the corresponding literature with a focus on the detected analytes and its biological relevance.

1.3.1. Extracellular measurements using SECM

The term extracellular refers to measurements outside of the cell, as presented in Fig. 1.5. These can be either changes in the surrounding environment due to the presence of a cell or secretions from the cell - for cell communication or in response to stimuli.

Fig 1.5. Concept of SECM measurements for extracellular measurements. SECM microelectrode can be positioned close to a single cell to monitor effects of the cell on its microenvironment or molecules released for cell signalling.

1.3.1.1. Topography measurements

As seen in section 1.2.2, the feedback mode can be used for imaging topography of surfaces. The example in Fig. 1.3 demonstrates this for an inorganic surface but this concept is also directly applicable for living cells. A living cell or tissue is a 3-dimensional object that has a specific shape depending on the cell and culture surface. Normally, in cell biology, cells are grown on a flat polystyrene surface, which is referred to as monolayer culture. When cells are grown in monolayers, they tend to stretch on the surface (x , y) with protrusions (5-10 μm) in the z direction. Imaging this topography requires a redox mediator that cannot cross the cell

membrane. The cell membrane blocks hydrophilic mediators such as hexaammineruthenium(III) chloride or potassium ferricyanide which can be used for imaging topography in negative feedback mode [16]. Cellular topography measurements were first performed by Lee et al., 1990 who used potassium ferricyanide for imaging topography (see Fig. 1.6) of elodea leaf [17].

Fig 1.6. 2D scan of the top surface of an elodea leaf imaged in negative feedback mode using potassium ferricyanide. Image is projected in 3D using SECM tip current for z information. Reprinted with permission from Lee et al., *Proc. Natl. Acad. Sci. USA*, 1990, 87, 1740-1743 [17]. Copyright (1990) belongs to the authors.

Suitable mediators for biological imaging must be neutral to the cell i.e. it should not induce any adverse effects on the cell function. Additionally, if other electroactive species are present in solution then the mediator should have a distinct redox potential in order to avoid cross talk. Properties of a good mediator were assessed by Liebetrau et al., 2003 who compared several mediators for topographical imaging of neurons using 5 μm carbon fibre electrodes [18].

Apart from imaging cellular morphology, topographical imaging also presents an opportunity for studying different biological processes and environmental factors that can influence cell shape and morphology. For instance, nitric oxide (NO) is an important signalling molecule in endothelial cells, and it regulates vasorelaxation. The release of NO is accompanied by changes to cell shape. This was demonstrated by Wang et al., 2007 who imaged morphological changes to HUVECs in constant height mode using hexaammineruthenium(III) chloride reduction at a 5.6 μm carbon-fibre micro-disk electrode [19].

Environmental factors such as temperature also play a vital role in cell survival and can be assessed through cellular morphology. When cells are exposed to low temperatures for a long duration, cell death occurs. This was demonstrated by Hirano et al., 2008 using SECM, who observe swelling and rupture of HepG2 cells when incubated at 4°C for 8 h, a phenomenon consistent with apoptosis [20]. They expanded on this work further by observing the effect of antifreeze proteins (AFPs) in protecting cells from hypothermic damage. Incubation with AFPs ensures that the cellular size does not change up to 16 h suggesting stabilization of the cell membrane in the presence of AFP. Similar observations on the effects of non-physiological temperature on cellular morphology were imaged by Zhang et al., 2012 who used oxygen as the redox probe for label-free imaging of dynamic morphological changes (see Fig. 1.7.) [21].

Fig 1.7. Label-free imaging of changes to T24 cells at room temperature imaged using SECM and oxygen as the redox probe. Using this method it is possible to track changes in position of sub-cellular component (nucleus) over time, the centre of which is indicated by the crosshairs. Reprinted with permission from Zhang et al., *Chem Cent J*, 2012, 6 [21]. Copyright (2011) belongs to the authors.

Morphological changes to a cell can also be caused by chemical stimuli. Depending on exposure times and concentration, apoptosis or necrosis can be triggered, resulting in changes to cell shape [22,23]. For instance, pH-induced cell necrosis causes volume changes which can be detected by monitoring cell shape [24]. Hirano et al., 2013 employed SECM topographic measurements in combination with a micropipette for local delivery of chemicals such as drugs, while monitoring the response of the cells undergoing apoptosis [25]. Alternatively, the level of contraction is used to evaluate response of cardiomyocytes to drugs. Topography measurements are, therefore, of direct relevance for pharmaceutical testing, as illustrated in the work of Hirano et al., 2014 who quantified contractile effects of a cardiotonic drug (digoxin) using SECM [26].

1.3.1.2. Oxygen measurements

Oxygen measurements dominate literature concerning the application of SECM for live biological systems. This is not surprising since oxygen is central to metabolism in living systems [3]. For instance, oxygen is

required for generation of adenosine triphosphate (ATP) – the primary source of energy transport within mammalian and bacterial cells [3]. Therefore, monitoring oxygen and changes to its concentration provides vital information on cellular function ranging from cellular metabolism to viability and also as an indirect marker for other cellular processes. Additionally, detection of oxygen through electrochemical means is straightforward and well-established. Oxygen is electroactive and dissolved oxygen can be directly detected and quantified by reduction at a working electrode (in examples presented here, Pt is mostly used as the working electrode unless specified otherwise). This coupled with highly localised information brought by SECM has a major implication for quantitative biological assays as detailed later. For all these reasons, significant amount of SECM literature on living systems focus on oxygen measurements.

A variety of cell systems such as plants, bacteria etc. has been subject to SECM probing based on oxygen reduction. First, oxygen measurements using SECM for cellular activity were conducted on plant cells [27]. In plant systems, the metabolic energy is produced by a process called photosynthesis which requires light, while producing oxygen. An extension of this approach is towards single cells that are involved in aerobic respiration, which in contrast to plant systems actively consume oxygen for energy production. Therefore, live bacterial or mammalian cells can be imaged by observing lowered oxygen concentrations in their vicinity, when compared to bulk solution [28-30]. Similarly, more complex models such as microtissues and embryos have also been assessed for their oxygen consumption [31,32]. These examples and resulting applications such as drug testing are discussed in the following section.

Production of oxygen by plant cells upon exposure light was studied using SECM by Tsionsky et al., 1997 [33], as presented in Fig. 1.8 where changes in oxygen reduction current are recorded upon changes to illumination. Monitoring variations in this photosynthetic activity is of particular importance for studying the direct effects of environmental pollutants, such as cadmium, released into soil from industrial and daily waste [34]. Simultaneous oxygen and topographical data were also be obtained on plant systems by using dual-microdisk electrodes [35]. The high spatial and temporal resolution that the SECM brings can provide further insights. For example, response times of a cellular system not readily accessible using detection or imaging techniques can be determined using SECM.

Fig 1.8. Oxygen concentration variation in the vicinity of a plant cell monitored using SECM and the oxygen reduction. When the light is turned off, the tip current can be seen to decrease as photosynthetic process that produces oxygen is stopped. Once the light is turned back on, the tip current starts to increase, indicating resumption of photosynthesis. Reprinted with permission from Tsionsky et al., *Plant Physiol.*, 1997, 113, 895-901 [33]. Copyright (1997) American Society of Plant Biologists.

Fig 1.9. (a-c) Fluorescence images of HeLa cells before and after exposure to 20 mM KCN, (d-f) SECM images of HeLa cells before and after exposure to 20 mM KCN by measuring oxygen reduction current. The effects of KCN are seen earlier in the SECM images as it blocks respiratory activity resulting in rapid increasing oxygen concentration around the cell. Reprinted with permission from Kaya et al., *Biosensors and Bioelectronics*, 2003, 18, 1379-1383 [39]. Copyright (2003) Elsevier.

Perhaps the most interesting and straightforward application of these oxygen reduction measurements is for cell viability assays [36], since oxygen can be directly used as a marker for determining the status of a

cell. First, such live/dead assays are of interest for pharmaceutical testing as it allows non-invasive observation of the effect of drugs. For example, in bacterial systems, the effect of antibiotics was successfully tested using oxygen measurements by Kaya et al., 2001 who developed a microbial chip for assessing the bactericidal properties of streptomycin and ampicillin on *E. coli* as a function of their concentration using SECM [37]. Later, the same group extended this approach to individual HeLa cancer cells before and after exposure to different drugs [38]. As shown in Fig. 1.9, effects of cellular respiratory inhibitors (potassium cyanide and Antimycin A) can be detected earlier using SECM when compared to a standard fluorescence approach. The specified drugs inhibit oxygen consumption which is readily detected using SECM as oxygen is directly imaged whereas membrane damage on which fluorescent assays are based occurs later. Apart from the temporal gain in information, an obvious added value of this approach for live/dead assays is that it is non-invasive.

Fig 1.10. SECM based viability assay in a silicon microfluidic chip. This approach was used to test the effects of different concentrations – (a-1) control, (a-2) 1 mM, (a-3) 10 mM and (a-4) 100 mM – of NaN_3 on MCF-7 spheroids after 4 h incubation. The bottom figure (b) shows the oxygen gradient around the spheroids measured by scanning over the microtissue with SECM. Reprinted with permission from Torisawa et al., *Biomaterials*, 2005, 28, 559-566 [42]. Copyright (2005) Elsevier.

The biological relevance of the SECM based live/dead assay can be improved by extending these measurements to 3D cell culture systems, which are acknowledged to be of significance over the traditional monolayer cultures used in cell biology [40]. In that respect, Torisawa et al., 2005 developed a microfluidic cell culture system on a silicon chip which enabled SECM based drug testing on 3D culture system [41]. By integrating microfluidic channels, they were able to compare the effects of

various cancer drugs on a single chip. They later extended their work to characterize the effects of different concentrations of NaN_3 on MCF-7 spheroid systems, as illustrated in Fig. 1.10 [42]. In addition, they employed this method for evaluating the growth kinetics of cultured spheroids over time [43].

Another promising avenue of application for SECM-based oxygen measurements is the field of assisted reproductive technologies (ART), where the quality and maturity of both the gametes and the embryo must be assessed prior to *in vitro* fertilization or embryo transfer, respectively [44]. First, a novel SECM-based method has been proposed to check the quality and maturation of bovine oocytes, by monitoring the oxygen consumption of the oocytes together with the surrounding cumulus cells (cumulus oocyte complex) [45]. Typically, oocyte maturation is graded based on morphology [46], and therefore subjective criteria, while the metabolism of the cumulus cells has been acknowledged as a better and quantitative marker for oocyte maturation [47].

Fig 1.11. Changes in oxygen consumption (fmol/s) over time of vitrified-warmed blastocysts at different stages of their development. SECM measurements allow assessing quality of these embryos where traditional morphological evaluations fail. Reprinted with permission from Yamanaka et al., *Human Reproduction*, 2011, 26, 3366-3371 [54]. Copyright (2011) Oxford University Press.

Alternatively, oxygen consumption can be used as a marker for embryo quality, and this approach has proven successful for the identification of competent embryos [48]. As a result, several studies have been performed using SECM for evaluating oxygen consumption on mouse

and bovine embryo models as it allows highly localised measurements on single samples [31, 49-53]. The same SECM-based strategy has also been applied to determine the quality of cryopreserved/vitrified human embryos, after thawing, and before transfer, as presented in Figure 10 [54]. SECM approach to assessing embryo quality could help select the “best” embryos for transfer and increase the success chance in ART.

Apart from viability assays, oxygen measurements can also be used for correlating cell behaviour and physical parameters such as cell shape. For instance, Nishizawa et al., 2002 micropatterned small islands on which individual HeLa cells were grown to control the cell shape [30], and correlated the oxygen consumption of individual cells to their level of spreading. Interestingly, they were able to show that cells spreading on a surface consume significantly more oxygen than round cells.

Oxygen measurements also provide information on the cell quality by indirectly correlating oxygen consumption to other cell functions. For instance, pancreatic cell aggregates (islets) are used for treatment of diabetes and their quality is assessed prior to implantation through time consuming tests such as ELISA for insulin release. This insulin function test is normally performed on bulk solution which provides information on the whole population whereas insight into single microtissues is required. Alternatively, as shown by Goto et al., 2009 islet quality can also be assessed using respiratory activity on individual microtissues [55]. Islets with a >1.5 fold increase in respiratory activity after glucose stimulation were found to have significantly greater chance of curing diabetes in rats. Similar strategy has been employed for assessing liver function in hepatocyte spheroids [56], and correlating differentiation of embryoid bodies based on their oxygen consumption [57]. These measurements highlight the potential of SECM in applications such as regenerative medicine where the “best” microtissues need to be identified for bottom up tissue engineering.

Other examples where SECM based oxygen measurements have proven to be useful include studies of oxygen permeability in cartilage [58], beat fluctuations in cardiac cells [26], and variations in pH on the surface on rat kidney [59].

While we have highlighted the different applications of oxygen reduction measurements in cell biology, there are a few points that deserve attention. The Pt microelectrode that catalyses oxygen reduction tends to be unstable and this results in decay of oxygen reduction current over

measurement duration, as illustrated in Fig. 1.12 [60]. This is a drawback for long term stable measurements using these electrodes. While Hg coated Pt electrodes proved to be stable alternatives [16,60], the toxicity of Hg presents a huge roadblock, and this issue must be addressed before moving further with SECM based oxygen measurements.

Fig 1.12. SECM imaging of HeLa monolayer using oxygen reduction. It can be seen from the image that the oxygen reduction current decays slowly over the measurement duration. This important issue must be addressed for continuous monitoring of samples using oxygen reduction based SECM measurements. Reprinted with permission from Li et al., J. Electroanal. Chem., 2009, 628, 35-42 [61]. Copyright (2009) Elsevier.

Another main issue is that for quantitative measurements, the topography of the sample needs to be considered. The changes in oxygen current arising from live cell activity can be separated from changes due to topography, by subtraction from oxygen measurements around a dead cell, to accurately evaluate cell respiration [62]. While this approach enables decoupling respiratory activity from topography, it cannot be considered feasible as it requires sample destruction. Alternatively, through the use of microfabrication techniques [63, 64], or measurements in combination with constant distance imaging discussed in the topography section can alleviate these issues and help fully exploit the SECM based oxygen measurements [65, 66].

1.3.1.3. Cell secretion and signalling

Another vital component of the extracellular environment includes factors released by the cells for intercellular communication and in response to stimuli. For example, nitric oxide (NO) is an important signalling molecule that is released by cells to regulate different functions such as angiogenesis and cell death [67]. For instance, and as mentioned earlier, NO plays a critical role for endothelial cells. Upon stimulation with vascular endothelial growth factor (VEGF), a protein that promotes

angiogenesis, endothelial cells secrete NO. An approach for detecting this process is through topographical changes, as explained earlier [19]. Alternatively, the released nitric oxide can be detected using SECM. This, however, is not straightforward, and requires chemical modification of the Pt UME. For that purpose, Borgmann et al., 2006 applied a Ni-Porphyrin film on a Pt microelectrode, to follow NO release from HUVEC cells upon VEGF stimulation [68].

SECM based measurements could also be used for studying exocytosis in neuronal cells [69,70]. Upon stimulation, neuronal cells can release neurotransmitters, and several of these neurotransmitters, such as dopamine and acetylcholine can be detected electrochemically [71,72]. Bauermann et al., 2004 demonstrated one such application of SECM for neurotransmitter detection [73] using carbon fibre microelectrodes. Specifically, they detected adrenaline release from individual chromaffin cells upon stimulation with potassium.

SECM has also been applied for detecting extracellular reactive oxygen species [74], which play a vital role in both cellular damage and as a regulatory molecules in different processes [75]. For instance, Zhao et al., 2010 used SECM measurements of ROS generation as a probe to assess inflammatory response in single cells [76]. The same group later expanded on this work further to study the effects of cisplatin, an anti-cancer drug, in a time-lapse manner to gain insights into extracellular ROS generation upon drug exposure [77].

1.3.1.4. Other extracellular measurements

Fig 1.13. Glucose profile measured using GOx modified electrode 25 μm Pt electrode around a single fibroblast cell. This approach is complementary to oxygen for monitoring cellular activity. Reprinted with permission from Ciobanu et al., *Anal. Chem.*, 2008, 80, 2717-2727 [80]. Copyright (2008) American Chemical Society.

While oxygen is a good indicator for the overall metabolism of cells, other markers can also be detected separately. Markers such as glucose, lactate and pyruvate bring a more comprehensive picture of cell activity. However,

these compounds are not electroactive, so that their electrochemical detection implies the use of an indirect approach, with oxidase enzymes and a product of their catalyses, hydrogen peroxide is then detected electrochemically at the Pt UME. A side-product of the enzymatic degradation reaction is hydrogen peroxide, which in turn can easily be detected and quantified by electrochemistry. Two strategies have been applied for glucose detection using SECM; when the enzyme is added in solution [78], or by immobilizing it on the UME [79]. Therefore, Ciobanu et al., 2008 developed a UME biosensor for detection of glucose and lactate in the extracellular space by immobilising glucose oxidase (GOx) and lactate oxidase (LOx) on Pt electrodes [80]. As shown in Fig. 1.13, this approach can be used for mapping glucose uptake profile on single cells.

Extracellular SECM measurements have also been applied for mapping salt gradients on leaf surface [81] and for measuring transport of redox mediators across a confluent monolayer [82]. The latter technique holds potential applications for SECM in the field of organ on a chip where transport across confluent monolayers need to be assessed for different applications [83].

1.3.2. Cellular measurements using SECM

In the previous section, we have discussed the application of SECM for studying the effects of cells on their extracellular space. However, SECM is also a promising technique to probe activity and infer information regarding the cell state through measurements directly on or in the cell. The following section covers SECM based measurements on molecules present on the cell membrane and in the intracellular space.

1.3.2.1. Membrane measurements

SECM can serve as a potential tool for detecting and imaging the molecules on the cell membrane [84]. The cell membrane is an essential part of the cell that protects its interior from the extracellular environment. Next to this, the cell membrane also acts as a gateway for exchange of molecules and ions that are necessary for proper cell function. One important molecule that is found on the cell membrane is alkaline phosphatase (ALP), an enzyme hydrolysing phosphate group from various substrates [5,85,86]. ALP detection is significant as this enzyme is central in different processes such as embryonic development [87] and serves as a diagnostic marker in diseases such as cancer [88, 89]. ALP can be detected electrochemically, while it is not electroactive itself, provided the product of its enzymatic action is electroactive. This approach is useful, for instance,

to detect differentiation status of embryonic stem cells, since the ALP expression level in stem cells is an indicator of their potential to differentiate into different cell lineages [90].

One possible artificial substrate used for the electrochemical detection of ALP is p-aminophenyl phosphate (PAPP), which is converted by ALP into p-aminophenol (PAP) which can in turn be electrochemically converted to p-quinone imine (PQI) [91]. This principle was first demonstrated by Matsumae et al., 2013 to detect the differentiation status of live embryonic stem cells using SECM at the single cell level [92].

Fig 1.14. SECM based imaging of co-culture (MCF-7 and embryonic stem cells) spheroids. Based on ALP activity, it is possible to distinguish embryonic cells from cancer cells as seen on the SECM image (right). (Left) shows optical and fluorescence image of the spheroid co-culture. Reprinted with permission from Arai et al., Anal. Chem., 2013, 85, 9647-9654 [93]. Copyright (2013) American Chemical Society.

The same group extended later this approach to study the differentiation status of embryoid bodies [93]. Using the SECM, they could successfully and non-invasively identify embryoid bodies that could differentiate into cardiomyocytes based on their low ALP activity, which has potential applications in regenerative medicine. Additionally, as presented in Fig. 1.14, the same approach enabled them to map the location of embryonic stem cells in a co-culture spheroid system using SECM. These examples illustrate that SECM enables detecting ALP activity on live single cells and tissues non-invasively while traditionally ALP detection requires fixation (sample destruction). Further improvements to this approach, such as detection at physiological pH (examples here are conducted at alkaline pH as required for optimal ALP activity), would be a significant step forward for SECM based biological assays.

Apart from acting as a marker for differentiation, ALP together with the PAPP/PAP couple can be used for monitoring other cellular processes, such as signal transduction [94, 95] or for assessing gene expression in response to stimuli on transfected cells [96]. Similarly, β -galactosidase (β GAL) is another enzyme capable of hydrolysing PAPP to PAP. Using β GAL

as a reporter, Shiku et al., 2008 characterized the enzymatic activity of genetically modified yeast cells. It should be noted that using these enzymes, as mentioned above, cannot be applied to primary cells as they require cell transfection to act as a reporter system [97].

Fig 1.15. Scheme of SECM based immunoassays for detection of EGFR. In the first approach (a), an antibody with ALP or β GAL is attached to EGFR and detected using PAPP/PAP in generation-collection mode. In the second approach (b) antibodies with Diaphorase-1 which redox cycles ferrocene methanol is used for feedback mode detection. Reprinted with permission from Takahashi et al., *Anal. Chem.*, 2009, 81, 2785-2790 [98]. Copyright (2009) American Chemical Society.

Imaging of cell membrane proteins using SECM can also be achieved through immunoassays. Immunoassays involve attaching antibodies specifically to target molecules. If a molecule, such as ALP, that is detectable electrochemically can be attached to this antibody then it will enable SECM measurements on the cell surface. This was reported by Takahashi et al., 2009 using two different approaches for detecting EGFR expression [98]. Fig. 1.15 illustrates the scheme of SECM based immunoassays. In the first approach, a primary antibody is attached to EGFR while a secondary antibody with ALP or β GAL is coupled to the secondary antibody. Subsequently, using the PAPP/PAP couple and by adding PAPP in solution, the presence of EGFR can be detected. In the second approach, Diaphorase-1 (Dp) labelled antibodies are attached to EGFR. Dp interacts with ferrocene methanol and results in redox cycling close a cell which can be used for detecting the presence of EGFR on the cell membrane. More recently, Roberts et al., 2013 used an immunoassay for detecting CD-44, a signalling protein, on the cell membrane [99]. Here they coupled horseradish peroxidase (HRP) with benzoquinone as a mediator for detection at Pt microelectrodes. HRP, in the presence of hydrogen peroxide, converts hydroquinone in solution to benzoquinone which is then detected at the SECM electrode by reduction. Thus, by using

targeted antibodies for specific membrane species, such a SECM based approach can be applied for detecting and imaging virtually any protein on the cell membrane. Unlike fluorescence measurements, this detection technique is selective for cell surface detection and therefore can be useful to elucidate membrane processes.

1.3.2.2. Intracellular measurements.

Going across the cell membrane and into the cell, important regulatory molecules can be found that are essential for maintaining a healthy status and protect the cell from oxidative stress. For example, nicotinamide adenine dinucleotide (NAD) and glutathione (GSH) are two electrochemically active molecules that play vital roles in cellular metabolism and for combatting oxidative stress [100,101]. Levels of glutathione inside a cell vary depending on cell status. For example, a cell under oxidative stress has a reduced level of glutathione. Therefore, detecting and quantifying these molecules would provide a direct window into several cellular functions. However, despite being electrochemically active, these molecules cannot be easily detected directly as they cannot cross the cell membrane. Therefore, other strategies need to be developed for intracellular measurements using SECM, as illustrated in the following Fig 1.16.

Fig 1.16. Different – A) Indirect redox mediated, B) Direct and C) cell poration – for accessing intracellular information.

A first approach towards intracellular electrochemistry relies on indirect redox measurements (See Fig. 1.16A), and requires the use of hydrophobic mediators that are able to cross the cell membrane. 1, 2-naphthoquinone and menadione are two good candidates for such

measurements to probe the intracellular redox activity using SECM [102,103]. Interestingly, through imaging of cells using this approach, the location of internal organelles such as the nucleus can also be identified as these organelles are impermeable to the mediators and result in decreased activity locally.

Additionally, depending on their health status, cells have different metabolic activity and redox state. For example, cancer cells have increased metabolism, and a by-product of this enhanced metabolic activity is the generation of reactive oxygen species (ROS) [104]. As a result, the cell redox state is different, and shifted to lower glutathione concentrations from combatting ROS. Thus, it is possible to differentiate between normal and metastatic cells based on their redox activity and rates of regeneration of intracellular redox species [102]. However, a number of parameters such as cell shape, electrode-cell distance, nature of the mediator need to be considered before this can be used for differential cell analysis [105]. This approach using hydrophobic mediators is also applicable for studying the cellular detoxification process and infer information on related processes such as efflux mechanisms modulated by membrane proteins [106, 107].

Fig 1.17. Scheme of double-mediator system for intracellular analysis. Alternative to direct detection of hydrophobic mediators, this approach uses ferricyanide and menadione to detect redox activity in the positive potential window. This does not require deoxygenating the measurement solution in order to avoid interference from dissolved oxygen. Reprinted with permission from Matsumae et al., *Anal. Chim. Acta.*, 2014, 842, 20-26 [110]. Copyright (2014) Elsevier.

One of the critical considerations for these indirect measurements is the choice of the mediator. It should be noted that due to the direct interactions with glutathione, mediators like menadione can have adverse

effects on the cells due to oxidative stress [108, 109]. As a result, low concentrations must be used for SECM measurements. While detection of low concentrations is possible, the detection is performed in the negative potential window which results in interferences from dissolved oxygen. This can be overcome by deoxygenating the measurement buffer which is not feasible when working with living systems for which oxygen is vital for survival. More recently, Matsumae et al., 2014 proposed a novel mechanism using a double-mediator system to overcome this issue, which is presented in Fig. 1.17 [110]. This system can serve as an alternative way for estimating enzymatic activities in live cells.

Alternatively, more direct approaches can also be employed for intracellular measurements, as sketched in Fig. 1.16B and 1.16C. Using nanoelectrodes, it is possible to pierce through the cell membrane to access intracellular information as shown by Sun et al., 2008. Using a 42 nm Pt tip they penetrated a MCF-10A cell and performed intracellular voltammetry [111].

Another direct approach to accessing intracellular information is by poration or permeabilization of the cell membrane, after which membrane impermeable species can exit the cells and be detected in the extracellular space. For example, scanning close to a cell surface while the electrode is set to oxidise ferrocene methanol will result in negative feedback. However, if the cell membrane has been permeabilized with Triton X-100 (a detergent that is commonly used for cell permeabilization) then current changes due to the presence of the cell is lower [112]. This demonstrates that cell poration can open up the intracellular space which can then be used to study intracellular processes and quantify enzymatic activity. Gao et al., 2007 employed digitonin to chemically permeabilize the cell membrane, and studied enzymatic activity of peroxidase using SECM after addition of hydroquinone in the extracellular medium [113]. Once pores are created in the cell membrane, hydroquinone in the measurement buffer can diffuse in the cell to be converted into benzoquinone, which is finally detected at the SECM tip.

A drawback of chemical poration is that it leads to permanent damage of the cell membrane. Alternatively, electroporation, which relies on the use of pulses of electric field [114], is a better suited option for cell poration as the process of permeabilization can be reversible [115], with the cell healing rapidly. While electroporation has not been performed with SECM systems, it has achieved using microelectrodes [116]. This could be

a potential application using SECM systems as it enables localised cell poration to study intracellular processes.

1.4. Conclusions

In this chapter, an introduction into SECM was provided followed by a literature summary of the several applications of SECM on biological systems. From the literature, it can be seen that the SECM has a huge potential for implementing non-invasive biological assays and for studying biological processes that may not possible using traditional approaches such as fluorescence measurements.

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Chapter 2: Stamped Petri dishes for 3D cell culture

In this chapter, a polystyrene (PS) based tissue culture platform is proposed and realized. Microwell arrays are hot embossed on commercially available PS Petri dishes using a custom setup. The fidelity in replicating microwell arrays from a polydimethylsiloxane (PDMS) stamp (200 μm depth, 400 μm diameter) is assessed and seen to be reproducible (184 ± 2 μm depth, 395 ± 3 μm diameter) on the Petri dish surface. Subsequently, homogenous (<10% size difference) microtissue culture in the Petri dish is achieved using various cell lines (HeLa, HepG2, MCF-7, C2C12 and 4T1). Finally, the microtissues are characterized for their size and cell organisation, along with protein expression patterns which show differences (differential expression of over 40 proteins with minimum 2 fold change) in comparison to monolayer culture.

2.1. Introduction

The potential of the SECM for biological assays is discussed in detail in the previous chapter. A large focus of the existing SECM measurements on biological samples has been on monolayer cell culture. However, it would be critical to shift the focus towards 3D cell culture as they are biologically more relevant. The advantages of 3D cell culture over traditional 2D culture have been the subject of numerous reviews in the past decade [1-4]. Combining these systems require a 3D culture platform that is compatible with SECM. Ideally, measurements using the SECM will be coupled with optical microscopy for imaging the microtissues as well as for monitoring the position of the SECM microelectrode. Simultaneous microscopy is possible with standard dishware for monolayer culture such as well plates or Petri dishes for instance, which provide an open system where SECM measurements can be performed from the top along with inverted optical microscopy. 3D cell culture systems that enable homogenous and large scale tissue culture, however, are not as straightforward to combine with the SECM technique as discussed later. So while numerous platforms have been developed for 3D cell culture [5-7], not many are readily usable with SECM.

One 3D culture approach through which this combination could be realized is a platform based on microwell arrays [8, 9]. Earlier work within the BIOS group resulted in development of one such open system using microwell arrays for microtissue culture fabricated from polydimethylsiloxane (PDMS) or agarose [10]. Initially, the focus was to make use of these PDMS or agarose devices in combination with SECM for studying the microtissues. However, it quickly became apparent that these devices are not suitable for SECM measurements. While they are extremely functional for the purpose they were developed, i.e., production and culture of microtissues, they suffer from specific limitations that hinder their use with SECM systems.

PDMS and agarose are both soft materials and therefore, to improve handling, are generally processed as thick layers. However, as a consequence, microtissue imaging is only accessible from the top side since the bottom is normally too thick for optical microscopy. Therefore, aforementioned microscopy techniques cannot be combined and the SECM measurements have to be done in isolation without any optical feedback.

This by itself is not a major issue as the SECM microelectrode can be positioned and controlled precisely based on current feedback as

explained in the previous chapter [11-13]. For example, the dissolved oxygen can be used in conjunction with negative feedback (hindered diffusion) to identify the proximity to the surface. However, PDMS and agarose are also porous materials, in which oxygen can permeate. Therefore, a strong negative feedback for oxygen is difficult to obtain even close to the surface. This results in damage of the fragile SECM microelectrode if the surface is hit.

Additionally PDMS and agarose are both “academic” materials which are difficult to integrate with automated systems. Also PDMS suffers from other issues that prevent it from being accepted widely within the biological community [14]. In this context, non-porous and rigid materials such as thermoplastics are better candidates for realizing microwell arrays for tissue culture (as well as combining with SECM as an analysis technique). Taking into account all these factors, a practical question arises, viz.: why not realize a microwell array platform based on standard polystyrene (PS) dishware such as Petri dishes?

PS is a widely used and thoroughly characterized material for biological applications, which provides a rigid, non-porous and transparent platform suitable for simultaneous SECM and optical microscopy. Processing of thermoplastics is also well understood and several techniques readily available for that purpose. For example, commercially PS based devices can be mass manufactured using injection molding and thermoforming [15, 16]. Academically PS based devices are also often fabricated using hot embossing and soft lithography [17-19].

Therefore, in this chapter, we propose a simple and user-friendly PS based microwell array platform for microtissue culture and analysis. Specifically, PS microscope slides and Petri dishes are stamped with microwell arrays using hot embossing. Subsequently, large scale and homogenous microtissue culture is demonstrated using several cell lines. Finally, microtissues are compared to monolayer models in terms of protein expression patterns (differential proteomic analysis).

2.2. Experimental section

2.2.1. Materials

Polydimethylsiloxane (PDMS, Sylgard 184) is purchased from Dow Corning, The Netherlands. Perfluorodecyltrichlorosilane (FDTS) is

purchased from ABCR GmbH, Germany. Kapton film is purchased from Cole Parmer, The Netherlands. Polystyrene (PS) microscope slides are purchased from Electron Microscopy Sciences, USA. CELLSTAR® polystyrene culture dishes (35 mm diameter) are from Grenier Bio-One GmbH, Germany. Isopropyl alcohol (IPA), ethanol, paraformaldehyde (PFA), phosphate buffered saline (PBS), bovine serum albumin (BSA), triton X-100 and RIPA buffer are all obtained from Sigma-Aldrich, The Netherlands. Human cervical cancer (HeLa), mouse myoblasts (C2C12), human liver carcinoma (HepG2), human breast adenocarcinoma (MCF-7), and mouse breast cancer (4T1) cells are purchased from ATCC, Germany. Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI) medium, foetal bovine serum, penicillin/streptomycin, L-glutamine, trypsin-EDTA, Hoechst 33342, calcein AM (acetoxymethyl), propidium iodide (PI), and fluorescein phalloidin are purchased from Gibco, Invitrogen, The Netherlands. Pluronic F-127 is from BASF, USA.

2.2.2 Fabrication

2.2.2.1 Fabrication of PDMS stamps

SU-8/Si master molds for the preparation of PDMS stamps are fabricated in the clean rooms of the MESA+ Institute for Nanotechnology using standard photolithography techniques. Different molds consisting of 200 μm high SU-8 patterns on a Si wafer are used as master depending on the application. In order to improve the durability of SU-8 master and easy release of PDMS stamps, a hydrophobic coating of FOTS is applied via molecular vapour deposition [20]. PDMS stamps are produced using standard soft lithography techniques, as described extensively in the literature [21]. In brief, a two component kit consisting of pre-polymer and curing agent is mixed in different (10:1 and 5:1) weight ratios as specified. The mixture is then degassed to remove air bubbles. Following degassing, the mixture is poured onto the master mold and degassed again to remove air bubbles. It is then cured at 60°C overnight to achieve polymerisation. After polymerisation, the PDMS stamps are released from the master mold and cut into individual chips.

2.2.2.3. Hot embossing of PS microscope slides

For hot embossing of PS microscope slides, PDMS master molds with 10:1 and 5:1 ratios are used. A crude method using 1 kg weight is employed for hot embossing. First, the PS slides are placed in a glass Petri dish with a PDMS stamp on top. A 1 kg weight is placed on top of the PDMS stamp and the whole assembly is placed in the oven set to 105°C. After 2 h of

heating, the system is cooled down to room temperature, and the PDMS stamp released.

2.2.2.4. Hot embossing of PS Petri dishes

Hot embossing of PS Petri dishes is achieved using a custom setup (Fig. 2.2 and Fig. 2.3), which is described in detail in the results section of this chapter. The optimal process for hot embossing is as follows. Prior to embossing, PDMS stamps are cleaned for 10 min in ethanol or IPA in an ultrasonic bath and dried for 15 min at 60°C. The setup is then preheated to the desired temperature (180°C) using a soldering station (Tooltronics, The Netherlands). The applied temperature is monitored using an internal thermocouple that is part of the soldering iron. After preheating, the dish is placed together with the PDMS stamp in the setup while pressure is applied with the help of a screw. After 10 min of heating, the pressure is maintained but the setup is cooled down for 15 min before the PDMS stamp is released from the Petri dish. Two different designs are used for microtissue culture as shown in Fig. 2.1.

Fig 2.1. Two different designs used for embossing microwell arrays in PS Petri dishes. Design 1(left) has 126 microwells and design 2 (right) has 108 microwells with the same microwell dimensions (400 μm diameter, 200 μm depth and 800 μm spacing between wells).

Temperature calibrations of the hot embossing setup are performed by monitoring the temperature on the brass disc using an external thermocouple and comparing it to the displayed temperature on the soldering station based on the internal thermocouple of the soldering iron. The applied pressure is measured using a load cell (model 1022 load cell, Farnell, The Netherlands).

2.2.3. Interferometry

Surface and depth profiles of the patterned Petri dishes are assessed using a vertical light interferometer (TMS-1200, Polytec GmbH, Germany). A coherent monochromatic light source is used. The light is split into two beams using a beam splitter and a phase difference is introduced between the two waves. As a result of the phase difference an interference pattern is produced. Physical changes along the vertical axis of the Petri dishes further alters the phase difference which is then used to analyse the surface of the patterned Petri dishes and optimise the parameters used for hot embossing.

2.2.4. Cell culture

C2C12, HeLa, HepG2, and MCF-7 cell lines are cultured in DMEM with 10% v/v FBS, 2% v/v penicillin/streptomycin and 1% v/v L-glutamine. 4T1 cell line is cultured in RPMI with 10% v/v FBS, 2% v/v penicillin/streptomycin and 1% v/v L-glutamine. Cells are cultured in standard conditions at 37°C, 5% CO₂ in a humidified incubator. All cell lines are typically passaged every 3 days and 0.25% trypsin-EDTA in PBS is used for detachment. The cell suspensions in trypsin are centrifuged at 1500 RPM for 5 min, supernatant removed and res-suspended in their respective growth medium.

2.2.5. Spheroid formation and characterization

After hot embossing, the patterned Petri dishes are sterilized in IPA or ethanol. Subsequently, a cell-repellent coating based on Pluronic® F-127 (BASF, USA) is applied. 1% w/v (1 g in 100 mL) Pluronic® solution is prepared in DI water, and 3 mL of the solution is added to the Petri dish followed by a centrifugation step (3000 RPM, maximum acceleration and deceleration). After centrifugation, the Petri dishes containing Pluronic® solution are incubated overnight at 37°C.

Prior to cell seeding, the Pluronic® solution is aspirated and the dishes washed once with PBS. A cell suspension with 500,000 cells is seeded in the Petri dish followed by addition of 2 mL culture media. The Petri dish is then centrifuged at 2500 RPM (max acceleration, 4 deceleration) for 5 min. After centrifugation, the culture media is aspirated to remove the excess cells followed by a washing step (minimum twice) with PBS. 2 mL of fresh media is subsequently added and the Petri dish placed in the incubator for tissue formation. 24 h after cell seeding, the culture media is replaced and subsequently every 48 h.

To characterize the homogeneity of tissue culture, 3 Petri dishes per cell type are produced and images of the microtissue arrays are captured using an optical microscope (IX51 microscope, Olympus, The Netherlands). The tissue diameters are subsequently measured using Image processing software (ImageJ, NIH, USA).

2.2.6. Fluorescence staining

Cell organisation in the microtissue and monolayer is imaged using phalloidin-FITC (fluorescein isothiocyanate) and Hoechst 33342. For staining, the tissues are first pipetted out of the microwells and washed twice with PBS. They are then fixed using 4% PFA (in PBS) at 4°C for 30 min. Tissues are then permeabilized with 0.1% triton X-100 (in PBS) for 10 min at 37°C. Cells are then incubated in staining solution (1% BSA in PBS) containing 10 µg/mL fluorescein phalloidin at 37°C for 2 h. Following phalloidin staining, the cells are incubated in staining solution containing 1 µg/mL Hoechst 33342. The tissue samples are washed twice with PBS between each step. Confocal imaging of the microtissues is performed using a confocal laser scanning microscope (Nikon A1R-A1 confocal system, 40x oil immersion objective) in the BioNanoLab of the MESA+ Institute for Nanotechnology.

2.2.7. Protein analysis

Proteomic analysis is performed on 4T1 breast cancer cell line on both monolayer and microtissues produced in the Petri dish platform. One day after cell seeding, the cells and tissues are lysed with 500 µL RIPA buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Igepal CA-630, 0.25% sodium deoxycholate and protease inhibitors) at 4°C. The lysate is then processed and digested in eFASP digestion buffer (50 mM ammonium bicarbonate, 0.2% sodium dichloroacetate pH 8) to recover peptides. After phase transfer of recovered peptides, the samples are analyzed with a nanoflow HPLC instrument (U3000 RSLC Thermo Fisher Scientific) coupled on-line to a Q Exactive plus (Thermo Scientific) with a nanoelectrospray ion source. Mass spectrometry (MS) data are acquired on Q Exactive plus using a data-dependent top 20 method dynamically choosing the most abundant precursor ions from the survey scan (400-1600 m/z). Complete details regarding the sample preparation and MS analyses can be found in Appendix I. The acquired raw files are analyzed with PEAKS 7 studio software (Bioinformatics Solutions Inc., Canada) using the MASCOT 2.5 search engine against the Swissprot_mouse

database (<http://www.matrixscience.com/>, Swissprot 2014_07, 16.678 sequences).

2.3. Results

2.3.1. Fabrication

2.3.1.1. Hot embossing of PS slides

Fig 2.2. Microwells (left) and micro channels (right) hot embossed with PDMS molds on PS microscope slide using a 1 kg weight for pressure at 105°C in an oven.

Microstructures can be fabricated in PS by increasing the temperature above the glass transition of the material (95°C; reversible transition from hard to amorphous state above this temperature) and then applying pressure with a negative mold. Traditionally hard molds such as epoxy or metals are used as negatives for hot embossing. However, more recently reports in literature show that hot embossing is possible with PDMS molds. Since facilities are in place within the BIOS group for fabrication of PDMS, hot embossing of microstructures on PS with PDMS mold is chosen. Using a crude setup with a 1 kg weight it is possible to emboss clear structures on PS (see Fig. 2.2) at 105°C using PDMS. PDMS molds with 10:1 weight ratio are sufficient for embossing large structures (200 μm depth, 400 μm diameter) whereas for finer structures such as microchannels, a more rigid (5:1) PDMS mold is preferred.

2.3.1.2. Hot embossing setup for stamping Petri dishes

While microwells arrays can be made on PS slides, fabrication of structures in a Petri dish provides a direct platform for tissue culture. However, in order for a Petri dish to retain its shape, the bottom surface

must be selectively heated above the PS glass transition while keep the walls at room temperature, which is not possible in an oven, as previously used for embossing microscope slides. Therefore, a custom setup is developed to be able to emboss microwell arrays on Petri dishes to ensure that the overall shape of the dish is not lost.

The hot embossing setup (Fig. 2.3) consists of a brass disc (2.2 mm $\varnothing$; same as PDMS mold) housed in a Teflon (thermally insulating) cup. The brass disc is used to apply heat selectively to the Petri dish surface while the Teflon cup ensures that the surface of the Petri dish not in contact with the brass disc is insulated and remains below glass transition temperature. The brass disc is heated with the use of a solder iron which is connected to a soldering station. The PDMS mold used for embossing is placed in a dedicated metallic holder which can be screwed in to apply pressure (Fig. 2.3 right).

Fig 2.3. (Left) Hot embossing setup with all parts labelled and (right) setup assembly for embossing. This custom setup is used for stamping microwell arrays on commercially available PS Petri dishes. It consists of a Teflon cup for housing the Petri dish. In the centre of the Teflon cup is a brass disc that is used for selectively heating the Petri dish using a soldering station. The pressures for hot embossing can be controlled by the two screws (black and orange).

One of the critical parameters for hot embossing is the applied pressure, which in our setup, is not controlled with high precision. However to ensure reproducible pressures, only the black screw is adjusted while applying pressure and the position of the orange screw is kept constant for all experiments. Measurements with a load cell show that using this restriction the applied pressure is roughly reproducible. The pressure can be maintained in the order of 20 to 30 N (c.a. 0.5 MPa). These values are comparable to what is reported in literature for embossing with PDMS [22].

Another critical parameter for hot embossing is the applied temperature. Unlike the applied pressure, this parameter is precisely controlled using the internal thermocouple of the solder iron. However, the brass disc is in an open environment which results in heat dissipation. As a result, there is an offset between the surface temperature on the brass disc and temperature measured by the internal thermocouple. This offset is assessed using an external thermocouple placed on the brass disc to be 18°C. As shown in Fig. 2.4, the offset is constant with varying temperatures across a broad range. As long as this offset is accounted for, the exact applied temperature for the hot embossing can be controlled with precision.

Fig 2.4. Temperature calibration curve for the hot embossing setup. The applied temperature is the temperature of the internal thermocouple while the measured temperature is the temperature on the brass disc as measured using an external thermocouple. A constant offset of 18°C is measured across a broad temperature range.

Fig 2.5. Surface of Petri dish after hot embossing directly on brass disc (left), with a nickel film (middle) and with a Kapton film (right). The roughness found on the brass surface is transferred to the PS surface which makes imaging infeasible. Addition of a thick Kapton layer between the Petri dish and brass alleviates this issue.

Another factor that needs to be accounted for in the setup is the surface roughness of the brass disc. Above the glass transition

temperature, PS is amorphous and can be reshaped using the PDMS stamp. However, this reshaping is not limited to only the side with the PDMS stamp. Any roughness (cracks, scratches etc.) on the brass disc is also imprinted on the underside of the Petri dish in contact with the brass disc. As shown in Fig. 2.5, the presence of this roughness makes imaging through the PS surface infeasible. In order to correct for this, different films (nickel, polymer, thin and thick Kapton) are tried to ensure a smooth reformation of the PS after cooling. Thick Kapton film gives the best result and thus clear imaging through the PS. Therefore a thick Kapton film is always placed between the brass disc and Petri dish during hot embossing.

With the control of pressure, temperature and surface roughness accounted for, it is possible to emboss microwell arrays on commercially available PS Petri dishes using the custom setup. The next step is to assess the fidelity of the embossing process in replicating the dimensions of microwells from the PDMS to PS.

2.3.1.3. Optimisation of the fabrication protocol

As mentioned in the previous sections, the pressure cannot be controlled precisely and therefore is not altered in the following. Instead the focus is on the applied temperature along with times of heating and cooling. Applied temperature, from hereon, refers to the temperature expected on the surface of the brass disc after correction for the 18°C offset. As an example, an applied temperature of 100°C implies that the temperature on the brass disc is 100°C while the temperature set on the solder station is 118°C.

Fig 2.6. Interferometry image of a microwell fabricated at 160°C with 5 min heating and 10 min cooling. The shape of the microwell is oval rather than the circular shape on the PDMS molds.

Microwell arrays from design 1 (400 μm diameter, 200 μm depth and 800 μm spacing between wells; see Fig. 2.1) are fabricated under

different settings and characterized using white light interferometry. The variable parameters are temperature (160, 170, 180, 190°C), heating time (5, 10 min) and cooling time (10, 15 min). Three Petri dishes are made for each combination and 10 microwells from each Petri dish are imaged using WLI.

As demonstrated with microscope slides, 10°C higher than the glass transition temperature is sufficient for hot embossing fine structures. However the time required is relatively high (2 h) and in order to expedite the fabrication process, higher temperatures are chosen. However, at temperatures of 160°C and below as well as lowered embossing times fail to replicate the circular shape (see Fig. 2.6) of the microwell. Also the difference in depth of the microwell between centre and the edge of the array is greater than 20 μm .

Fig 2.7. Depth characterization of microwells for different fabrication parameters using interferometry. 3 Petri dishes and 10 microwells from each dish are characterized for each combination of fabrication parameter.

At temperatures of 170°C and above, the microwell shape is circular as targeted. At these temperatures, a heating time of 10 min results in more homogeneous structures than a heating time of 5 min. While microwells fabricated at all three temperatures (170, 180 and 190°C) can be considered homogenous, a temperature of 180°C provides slightly higher replication fidelity than 170°C. Replication fidelity is comparable between 180°C and 190°C but the higher temperature results in heating a larger surface area and this starts to have an effect on the Petri dish shape. Based on these interferometry results, the optimal settings for fabrication are determined to be 180°C with 10 min heating. The cooling time doesn't have a significant influence but 15 min cooling is chosen to allow cooling to lower temperatures before release. The results discussed

in the rest of this chapter are with the Petri dishes hot embossed at 180°C with 10 min heating and 15 min cooling. Petri dishes fabricated at this setting are homogeneous with microwell depth of $184 \pm 2 \mu\text{m}$ and diameter of $395 \pm 3 \mu\text{m}$. Fig. 2.7 shows the results of depth characterization for different setting using WLI.

Fig 2.8. 3D reconstruction of a single microwell based on interferometry measurements. Petri dish is fabricated at 180°C with 10 min heating and 15 min cooling. A smooth reformation of the Petri dish surface can be seen after hot embossing.

Fig 2.9. Scanning electron micrographs of the surface of a hot embossed PS Petri dish.

It can be seen from the 3D reconstruction of the interferometry measurements that the surface is reformed smoothly after hot embossing.

This is also confirmed with high resolution images of the Petri dish surface (Fig. 2.9) using scanning electron microscopy.

2.3.2. Microtissue production and characterization

In traditional monolayer cultures, cell-substrate interactions are the dominant effect, i.e., the cell interacts and adheres to the material surface. However, to form tissues, cell-cell interactions need to be promoted. If cells are seeded into the Petri dish immediately after embossing, they will naturally adhere to the Petri dish surface. Therefore, a protein repellent coating needs to be applied on the surface of the dish. In this instance, Pluronic® F-127, a non-ionic surfactant polyol, is used, which forms a hydrophilic layer on the hydrophobic PS surface [23]. The hydrophilic F-127 tails are protein repellent, which forces cell-cell interactions by preventing cell-substrate interactions. Through the use of this coating, microtissues are successfully produced from various cell lines (HeLa, MCF-7, HepG2, C2C12 and 4T1) in the stamped Petri dishes.

Microtissues are formed through spontaneous self-assembly. As shown in Fig. 2.10, C2C12 cells in a microwell start forming microtissues by compacting into a small cluster. After ca. 24 h, the compaction stops and microtissues with a “stable” diameter are obtained. This is observed for all the cell lines seeded. Microtissues from fast growing cell lines like HeLa start expanding after 24 h and tend to pop out of the microwells as they grow into bigger microtissues.

Fig 2.10. Spontaneous self-assembly of C2C12 microtissues due to forced cell-cell interactions from the presence of protein repellent coating on the Petri dish surface. The picture shows a time lapse of microtissues over a period of 90 min after cell seeding.

After 24 h, the tissue diameter is determined for 4 different cell lines (HeLa, HepG2, C2C12, and MCF-7). All 4 cell lines form homogenously sized microtissues with the final diameter dependent on the cell line, as shown in Fig. 2.11. For example, C2C12 microtissues ($206 \pm 34 \mu\text{m}$) are more compact than HepG2 ($311 \pm 35 \mu\text{m}$) microtissues when both cell lines are seeded initially with the same cell number.

Fig 2.11. Box-plot of the microtissue diameter 24h after seeding. All four cell lines form homogeneously ($<10\%$ deviation) sized microtissues. The microtissue diameters for each cell line are as follows - HeLa $369 \pm 33 \mu\text{m}$; MCF-7 $331 \pm 35 \mu\text{m}$; Hep G2; $311 \pm 35 \mu\text{m}$; and C2C12 $206 \pm 34 \mu\text{m}$.

HeLa microtissues from 3 Petri dishes are disassembled by trypsinisation for 10 min and a cell count shows that there are approximately 4000 cells per microtissue ($369 \pm 33 \mu\text{m}$ Ø), 24 h after cell seeding.

Fig 2.12. Bright field microscopy images of microtissues produced in stamped Petri dishes from HepG2 (left) and C2C12 (right) 24 h after cell seeding.

The cell organisation of HeLa microtissue is also imaged using confocal microscopy and compared to monolayer cell culture (see Fig. 2.13). The actin filaments in the cells are stained with phalloidin-FITC to observe the cell organisation. In the monolayer, the protein is spread

across the cell and holds the cell to the substrate through focal adhesion points. Actin is reorganised in a microtissue and is found solely in the cell-cell junctions, as expected, due to increased cell-cell interactions.

Fig 2.13. Side by side comparison of organisation of matrix protein actin (green) in a monolayer (left) and microtissue (right). The nucleus of the cell is blue as the cellular DNA is stained with Hoechst.

2.3.3. Proteomic analysis

Protein concentrations are determined from the lysates using Bradford assay before MS analysis. These data shown below in table 1 indicate that the quantity of protein expression in Petri dish platform and monolayers are comparable for similar cell numbers (11 petri dish *vs.* 1 confluent T75 monolayer). Even with 10 times lower cell concentrations, there is a high protein expression from the tissues cultured in the Petri dish platform.

Table 2.2. Protein concentrations determined by Bradford assay after cell lysis for monolayers and tissues produced in the Petri dish platform.

Sample	Protein concentration (mg / mL)	Volume (µL)
1 petri dish	5.10	100
11 petri dish	9.80	500
Confluent T75	9.65	500

After mass spectrometry, a differential protein analysis is performed to compare changes (minimum 2 fold) in protein expression between monolayers and microtissues. The full list of proteins can be found in Fig. 2.14. It is interesting to note that there is an increase in

expression of several metabolic proteins in the microtissue sample. The upregulated proteins- triosephosphate isomerase (TPIS) [24], glucose-6-phosphate isomerase (G6PI) [25], fructose-bisphosphate adolase A (ALDOA), glyceraldehyde-3-phosphate dehydrogenase (G3P), alpha-enolase (ENOA) [26], pyruvate kinase PKM (KPYM) [27]- are involved in glycolysis or gluconeogenesis, indicating increased metabolic activity in tissue samples.

Fig 2.14. Differential protein analysis comparing changes in protein expression between cells cultured in monolayers and tissue aggregates. Group 1 is the tissues and group 2 the monolayer sample. The proteins listed here show minimum 2-fold change in expression. One day after cell seeding, several metabolic, cell-matrix and redox regulatory proteins show increased expression in 3D cell culture when compared to traditional monolayer models. See appendix II for the names of each protein on the image.

Another set of proteins that show increased expression are proteins involved in cell organisation. Increased expression of ezrin (EZRI), moesin (MOES), galectin-1 (LEG1) [28], vinculin (VINC) [29] indicate cell-cell adhesion, focal adhesion and membrane to membrane docking which are essential for tissue formation. Several proteins- 14-3-3 protein gamma (1433G), fascin (FSCN1) [30, 31], prolifin-1 (PROF1) [32], src substrate cortactin (SRC8) [33] show cytoskeletal reorganisation which is expected as shown in the confocal image in Fig. 2.13. It is also interesting to note that

proteins involved in redox regulation such as peroxiderodoxin-2 (PRDX2) [34], peroxiredoxin-6 (PRDX6) [35] are upregulated as redox stress is expected in cancerous tissue [36].

2.4. Discussion

Processing of thermoplastics can be achieved using a variety of techniques mentioned briefly in the introduction. In this chapter, hot embossing has been preferred as it is cheap and straightforward for academic fabrication. It is also possible to develop a custom setup for hot embossing while this is not possible for injection molding. However, Petri dishes with microwell arrays could be easily mass manufactured using injection molding if adequate facilities are in place.

While the Petri dish platform is primarily developed for simultaneous SECM and optical microscopy, it can be easily adopted for regular biological applications. One of the advantages of using standard dishware based platform is that it is compatible with existing laboratory infrastructure. No additional equipment or facilities is needed to work with the Petri dish platform if it can be mass manufactured and delivered to the end user. An extension of Petri dish based tissue culture would be to incorporate microwell arrays in standard well plates in order to scale up for analysing large number of samples. This could be particularly interesting for applications such as drug screening.

Fig 2.15. Microwell array (20 μm $\varnothing$) hot embossed on Petri dish platform for single cell trapping. HeLa cells (stained with calcein AM) are trapped in microwells by centrifugation.

While relatively large microwell are embossed here, in order to make microtissues, this approach could be also be extended for embossing

smaller microwells which could be used for single cell trapping as shown in Fig. 2.15. This aspect is not explored in detail as the focus is to work with microtissues. However, this could be an interesting approach for trapping single cells to obtain individual and population metrics [37].

The differential protein analysis performed in this chapter is a very simple test to demonstrate the differences between monolayer and tissue aggregates. The samples analysed here are 1 day after cell seeding and tissue samples of fixed size which already shows distinct differences. As a next step, it will be interesting to study different parameters such as long term tissue culture, variations in tissue sizes to check how the Petri dish platform compares to *in vivo* samples.

2.5. Conclusion

We have demonstrated a straightforward Petri dish platform for large scale production of homogenous (<10% variation in size) microtissues from several different cell lines. The differential protein expression between our microtissues and monolayers show distinct differences for the cell line (4T1) analysed. In conclusion, the platform presented enables large scale production of homogeneous microtissues on a standard biological dishware. The platform is well suited for scanning probe microscopy on microtissues as reported in the next chapters but also has potential to be a standalone biological tool.

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Chapter 03: SECM for mapping tissue topography

In this chapter, scanning electrochemical microscopy (SECM) is used for imaging the topography of single cells, followed by microtissues formed and cultured in the Petri dish platform. First, approach curves are performed towards conducting (gold-coated) and insulating (polystyrene) illustrating the difference between the two feedback modes. Subsequently, negative feedback scanning is used for imaging topography of a single A549 cell using the same redox probe. Finally, microtissues produced in the Petri dish platform are imaged using SECM, one and two days after cell seeding, to evaluate their topography, and changes in topography, e.g., as a result of cell growth. Using the SECM measurements, we are able to determine that the microwells have a tilt which is not observable using optical microscopy. This information provides insight into microtissue properties, as well as elucidate the extent of the applicability of the platform for SECM based biological assays.

3.1. Introduction

One of the principle modes of operation for scanning electrochemical microscopy (SECM) is the feedback mode, as explained in chapter 1. In this mode, SECM measurements are performed by monitoring the current at the microelectrode, which depends on the diffusion rate of the measured species. By monitoring changes to this current because of hindered diffusion or redox cycling, as explained in Chapter 1, it is possible to estimate distances from a surface and obtain topographical information of the surface through 2D scanning.

SECM is particularly interesting for mapping topography of micro- and nanostructures, for studying surface processes, or to image patterns, without any direct contact with the surface. For example, Lee et. al, 2002 characterized the topography of 30 μm sized interdigitated electrode array by SECM scans based on negative feedback and shear force [1]. Using nanoelectrodes, it is possible to obtain high resolution nanoscale topographical information on surfaces, as demonstrated for lithium ion batteries, plastic and gold discs [2, 3]. Patterns of organic molecules, often used in biosensing applications, have also been imaged using feedback mode SECM [4].

Same principles of negative feedback i.e., hindered diffusion, using cell membrane impermeable mediators, can be easily extended for imaging topography of living systems. Studying the morphology of cells brings plethora information on various biological processes, and on the cell status and viability. Morphology is, for instance, linked to cell communication, as shown by Wang et. al, 2007 [5]. Next to this, cell apoptosis can be detected through changes in the cell shape and height [6], and cell motion could also be tracked using SECM [7]. First measurements were reported by Yasukawa et. al, 1998 who recorded the topography of a single living protoplast using ferricyanide [8]. Liebetrau et. al, 2003 explored different redox mediators for biocompatibility for imaging the topography of neuronal cells [9]. Later, high resolution images of model neurons were obtained by Kurulugama et. al, 2005 using constant distance imaging based on impedance measurements [10]. More recently, Wang et. al, 2007 monitored morphological differences in endothelial cells before and after release of nitric oxide, a key signalling molecule in the process of angiogenesis [5].

Alternatively, positive feedback, i.e., redox cycling, using membrane permeable mediators such as 1,2-naphthoquinone and menadione enables to

image subcellular organisation as not all regions of the cell equally participate in redox cycling. For instance, Liu et. al, 2000 used 1,2-naphthoquinone for imaging the redox activity of MCF-10A breast epithelial cells, and were able thereby to determine the location of the cell nucleus [11] due to lowered redox cycling in its vicinity. These and other examples in literature show the capability of using SECM measurements for evaluating cellular topography in a non-contact manner and imaging the 3D cell structure [12-15].

Applying this approach of imaging cell topography to our microtissue arrays would for instance provide insights into shape and organisation of the microtissues, and changes therein as a function of culture days. Subsequently, information on the cell growth, cell rearrangement and on the structure of microtissues could be derived from these SECM measurements, information which is not easily accessible using conventional optical microscopy. Furthermore, this topographical information can be used to decouple the effects of topography in relation to other SECM measurements based on other soluble probes, such as dissolved oxygen. Therefore, in this chapter, we aim to exploit negative feedback measurements to assess the topography of microtissues cultured in the Petri dish platform (see chapter 2) and characterize their evolution over time. First, the concept of negative and positive feedback is illustrated using approach curves towards insulating (polystyrene) and conducting (gold-coated) surfaces. Subsequently, negative feedback measurements using ferrocene methanol are performed for imaging a single A549 cell. Finally, the topography of HeLa microtissues is imaged on two different days using 2D scans and approach curves.

3.2. Materials and methods

3.2.1. Materials

Polydimethylsiloxane (PDMS, Sylgard® 184) is purchased from Dow Corning, The Netherlands. Perfluorodecyltrichlorosilane (FDTS) is purchased from ABCR GmbH, Germany. CELLSTAR® polystyrene culture dishes (35 mm diameter) are from Grenier Bio-One GmbH, Germany. Isopropyl alcohol (IPA), ethanol, phosphate buffered saline (PBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), monosodium phosphate (NaH_2PO_4), D-(+)-Glucose, 1,2-naphthoquinone and ferrocene methanol ($\text{C}_{11}\text{H}_{12}\text{FeO}$; FcMeOH) are all

obtained from Sigma-Aldrich, The Netherlands. HeLa human adenocarcinoma and A549 human lung carcinoma cell lines are from ATCC, Germany. Dulbecco's Modified Eagle Medium (DMEM), foetal bovine serum, penicillin/streptomycin, L-glutamine, and trypsin-EDTA are purchased from Gibco, Invitrogen, The Netherlands. Pluronic® F-127 is purchased from BASF, USA.

3.2.3. Fabrication

Fabrication of stamped Petri dishes is described in detail in Chapter 2. In brief, PDMS stamps are made from SU-8/Si master mold by soft lithography. Microwell arrays are hot embossed on commercially available 35 mm Petri dishes at 180°C with 10 min heating and 15 min cooling times. Hot embossing is performed using a custom setup (see chapter 2) connected to a soldering station (Tooltronics, The Netherlands).

For approach curve on a conductive surface, gold is sputtered (200W DC power, 110 sccm argon flow, and 0.005 mbar pressure) on the Petri dish using a homemade sputter setup. First, an adhesion titanium layer of 25 nm thickness is sputtered for 2 min, followed by deposition of 100 nm gold layer by sputtering for 2 min.

3.2.3. Cell culture

HeLa and A549 cells are cultured in DMEM with 10% v/v FBS, 2% v/v Penicillin/Streptomycin and 1% v/v L-glutamine at standard culture conditions (37°C and 5% CO₂). Cells are passaged using 0.25% Trypsin-EDTA (2 min incubation) and reseeded after centrifugation at 1500 RPM for 5 min.

3.2.4. Spheroid formation and culture

Stamped Petri dishes are sterilised in IPA or Ethanol followed by a cell repellent coating using 1% w/v (1 g in 100 mL) Pluronic® F-127. After overnight incubation, the coating solution is removed and the dishes washed with PBS. Circa 500,000 HeLa cells are seeded in the Petri dish followed by centrifugation and removal of excess cells.

3.2.5. Electrochemical measurements

Electrochemical measurements are performed in a three electrode configuration using an electrochemical probe scanner or SECM (ElProScan, HEKA, Germany). All measurements are performed with 10 µm Pt working, Ag wire reference and Pt wire counter electrodes in buffer solution containing 10 mM HEPES, 1 mM FcMeOH, 150 mM NaCl, 4.2 mM

KCl, 2.7 mM MgCl₂, 1 mM NaH₂PO₄ and 11.2 mM D-(+)-Glucose at room temperature. Ferrocene methanol is oxidized at +0.35 V *vs.* Ag wire. SECM approach scans are performed at 1 μm/s while 2D scans are performed at different scan rates as specified in the respective sections. For cell redox approach curves, the measurement buffer is supplemented with 100 μM 1,2-naphthoquinone which is reduced at -200 mV *vs.* Ag wire.

3.3. Results

3.3.1. Approach curves

Ferrocene methanol is a redox active compound that undergoes reversible oxidation at very low potentials. Fig. 3.1 shows a cyclic voltammogram of ferrocene methanol with an oxidation potential of +100 mV *vs.* Ag/AgCl. For the following SECM measurements, a slightly higher potential of +0.35 V *vs.* Ag wire where steady state is reached, is set for continuous oxidation of ferrocene methanol in solution and changes to this oxidation current is monitored.

Fig 3.1. Cyclic voltammogram of 1 mM ferrocene methanol using 10 μm Pt working electrode in SECM measurement buffer. Ferrocene methanol can undergo reversible oxidation and is therefore commonly used for electrochemical applications.

The steady oxidation current, as previously mentioned, is dependent on diffusion rate of ferrocene methanol to the microelectrode

tip. When the microelectrode tip is moved closer to a surface, depending on the surface properties the steady state current will change, as explained in chapter 1. When approaching an insulating surface such as PS, the diffusion of FcMeOH to the microelectrode tip is hindered, and, as result, the oxidation current of FcMeOH decreases sharply as shown by the blue line in Fig. 3.2.

PS is non-conducting and therefore does not have an influence on the oxidised ferrocene methanol. However, a conducting surface with a gold coating will result in redox cycling i.e., reduction of oxidised FcMeOH at the gold surface and simultaneous oxidation of the reduced FcMeOH at the microelectrode. Therefore, despite hindered diffusion, there will be an increase in current due to the redox cycling of FcMeOH, when the microelectrode approaches closer to the surface. This redox cycling behaviour is represented by the green line in Fig. 3.2, where the SECM tip approaches a Petri dish surface coated with a thin gold layer.

Fig 3.2. Approach curves demonstrating hindered diffusion and redox cycling effects using ferrocene methanol. As the microelectrode approaches an insulating surface such as PS there is a decrease in the steady state current due to hindered diffusion, whereas approaching a conductive surface such as gold results in a current increase due to the redox cycling. Currents are normalised to values in bulk solution.

3.3.2. Single cell imaging

FcMeOH is a redox mediator that cannot pass through a cell membrane. When a microelectrode set to oxidise FcMeOH approaches a cell surface, hindered diffusion will occur, accompanied by a current drop as seen on a PS surface. This concept is exploited for imaging the topography of the cell, as shown in Fig. 3.3. First, an approach curve is carried out towards the insulating Petri dish surface while the microelectrode is set to oxidise FcMeOH in solution. Once the surface is reached, the microelectrode is pulled back 10 μm from the surface to avoid contact with the cell while scanning. Following this, a 2D scan (scan rate 1 $\mu\text{m}/\text{s}$) is performed over an A549 cell grown on a standard 35 mm Petri dish. The structure of the cell results in changes to oxidation current, and from those changes, the cell topography can be derived.

Fig 3.3. Topography of a single A549 cell obtained by negative feedback measurements using SECM – (left) 2D SECM and (right) 3D projection using the measured current. This measurement gives an insight into the 3D structure of the cell.

3.3.3. Imaging microtissues

Similar to the scan on a single cell, 2D scans (scan rate 20 $\mu\text{m}/\text{s}$) are performed on HeLa microtissues produced in the Petri dish platform to characterize their 3D shape, one and two days after cell seeding. Optical examination (see chapter 2 and 4 for examples) of a microtissue indicates a spherical shape given the geometry of the microwell. However, the full microtissue could not be imaged, as shown in Fig. 3.4, using SECM scans, which reveals that cells are at different heights ($> 20 \mu\text{m}$) within the microwell, which indicates deeper cells in regions without any current

change in the SECM 2D picture. Additionally, in the regions where the microtissue is successfully imaged, the topography of the tissue appears to be uneven, as illustrated by the changes in current intensity.

Fig 3.4. Topography mapping of HeLa microtissues produced in the Petri dish platform, one day (left) and two (right) days after cell seeding. Although the microtissue seems to fill the microwell (from 50 to 450 μm on the images) completely when looking at it optically but cannot be imaged completely using constant height scanning due to differences in microtissue height.

Table 3.3. Relative distances of different points on the same microtissue on day 1 and day 2 after cell seeding. T1 to T6 represents different microtissues. The distances are given in μm and relative to the lowest point (represented by 0 μm) on each microtissue. 0 distance is defined to be the point at 10% reduction in measured current occurs due to hindered diffusion.

Day 1					Day 2			
Position	T1	T2	T3		Position	T4	T5	T6
Z_1	40	29	30		Z_1	26	33	3
Z_2	0	9	17		Z_2	87	45	58
Z_3	26	0	0		Z_3	25	4	18
Z_4	37	36	34		Z_4	0	0	0

Fig 3.5. Scheme of different points on the microtissue (blue circle) and PS where approach curves are performed to determine heights of the surfaces with respect to each other. Z_{ps} are points on the Petri dish surface while Z_1 to Z_4 & Z_c (centre of the microtissue) refers to different points on the microtissue, where approach curves are performed.

In order to estimate the height differences within a microwell, approach curves are performed at different locations on both the microtissue and the PS surfaces, as represented in Fig. 3.5. Using this approach, the heights of the different points with respect to the lowest point on the tissue are determined and presented in table 1. The points Z_1 , Z_2 , Z_3 , and Z_4 are relative values corrected to the lowest point among them. As can be seen from table 1, the orientation of the highest and lowest point on the microtissue varies in each microwell. Additionally, the difference in height between the lowest point and highest point in the microtissue is different between day 1 (36 μm) and day 2 (63 μm) and is seen to be increasing. It is also interesting to note that cells start growing outside the microwell at the point where the microtissue is the highest as observed optically. This difference in heights within a single microtissue could result from tilting of the microwell or from non-uniform filling of the microwell. Also the height of the microtissue centre (Z_c) from the polystyrene surface is increasing between day 1 (22 μm) and day 2 (27 μm) indicating cell growth.

3.4. Discussion

This chapter illustrates the capability of the SECM to characterize the topography of living biological systems. Information such as tissue shape and smoothness which could not be determined optically is easily observed using SECM scans. It also raises interesting points of discussion regarding the tissue culture platform and the extent of applicability of this platform for SECM measurements.

The microtissues produced in the Petri dish platform, while homogenous in terms of diameter (see chapter 2) do not have a spherical shape or smooth surface. In fact, as shown in the last section, the tissues appear to be forming unevenly. The reason for this could be the effect of hot embossing using 10:1 PDMS molds. PDMS molds are quite soft compared to the traditional rigid molds (epoxy) used for hot embossing. As a result, the pillars on the PDMS stamp may bend when pressure is applied resulting in a slight tilt of the embossed microwell as suggested earlier. This could explain the differences in height on different points of the microtissue. This bending can be observed optically when using smaller microwells (100 μm diameter and depth) whereas it is not seen with the size (400 μm diameter and 200 μm depth).

The 2D SECM scans are performed here in constant height mode based on negative feedback. This mode is not suitable for imaging structures with height differences greater than 20 μm as the effect of hindered diffusion is not seen above this height. Therefore, performing measurements using constant height mode on the Petri dish platform has some limitations.

Primarily, constant height mode is not suitable for imaging a complete microtissue. However, for certain applications this may not be necessary and simple line scans will be sufficient. Therefore, in the rest of the thesis work, due to limitation of the Petri dish platform, we restrict ourselves for all SECM assays to recordings using line scans as complete imaging would require constant distance scanning. Constant distance scanning, while demonstrated in literature, is difficult to implement using our commercial SECM setup. The technique available for constant distance scanning i.e. shear force measurements was not sensitive enough for soft substrate such as cells.

Fig 3.6. Approach curve on a single A549 cell with the microelectrode set to reduce 1,2-naphthoquinone (NQ). NQ is a hydrophilic redox species that is capable of crossing the cell membrane where it is will be redox cycled by intercellular redox molecules such as glutathione similar to redox cycling of FcMeOH on gold surfaces. Initially a negative feedback is seen as the electrode approaches the cell. However, very close to the cell ($<10 \mu\text{m}$), the NQ is able to cross the cell membrane and is redox cycled resulting in positive feedback.

Additionally, the inability to perform constant distance imaging also limits certain measurements such as positive feedback based redox measurements on the microtissues. This is shown in Fig. 3.6, using 1, 2-

naphthoquinone (NQ) which is a hydrophilic mediator that can pass the cell membrane. Inside a living cell, the reduced NQ is oxidised and subsequently reduced at the microelectrode resulting in increased current due to redox cycling. However, for these measurements, the microelectrode needs to be very close to the cell surface for redox cycling to occur. Therefore these measurements cannot be performed in constant height mode due to the severe height differences across the microtissue surface.

3.5. Conclusion

In this chapter, we use the SECM system for imaging cell and tissue topography using ferrocene methanol. First, approach curves are made towards conducting and insulating surface to demonstrate positive and negative feedback. Subsequently, negative feedback is used for imaging a single cell, followed by imaging microtissues cultured in the Petri dish platform over 2 days. Using feedback mode SECM, we are able to observe a tilt in the microwell which was not evident using optical measurements.

3.6. References

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Chapter 04: Live/dead assay using SECM

In this chapter, the Petri dish platform is combined with SECM for performing a standard live/dead assay based on oxygen measurements. First, Pt and Pt/Hg microelectrodes are characterized for stable reduction of dissolved oxygen. Due to their improved stability, Hg coated Pt microelectrodes are used to characterize oxygen consumption and oxygen gradients around a microtissue with approach curves and 2D line scans. Microtissues in the microwells show an hemispherical oxygen depleted zone extending more 200 μm from the tissue surface. Finally, 2D line scans are used for monitoring changes in oxygen gradients before and after exposure of the microtissues to a model drug (ethanol). As a control, a live/dead assay based on fluorescent staining is performed to confirm the SECM data. This chapter demonstrates that the SECM based live/dead assay can be used for non-invasive drug testing on microtissues produced in the Petri dish platform.

4.1. Introduction

Viability assays are an integral part of biological studies in order to assess the response of cell and tissues during the course of an experimental procedure. Cell health can be qualitatively observed by simply looking at the cellular morphology [1]. More commonly, however, fluorescence based approaches based on membrane impermeable dyes are used for determining cell viability. For instance, a derivative of fluorophore calcein is often used for live cell staining [2, 3]. This non-fluorescent acetomethoxy derivative can pass the cell membrane, and inside the cell is hydrolysed by esterases that are active only in live cells. Once hydrolysed into fluorescent calcein, it remains trapped inside the cell (as calcein is membrane impermeable) and a live cell can be identified by green fluorescence while dead cells do not exhibit green fluorescence [4].

Calcein is often used for live/dead assays in combination with propidium iodide (PI), which binds to nucleic acids of dead cells [5]. These assays are highly popular as they are easy to use but offer poor temporal resolution due to photobleaching. Fluorescent assays, in general, are also invasive (long incubation with PI results in all cells being PI positive) and can have adverse effect on cell health due to labelling and strong illumination required for imaging [6, 7]. Alternatively, cell health could be assessed through the cells effect on their surrounding environment.

In static cell culture, for example, the nutrient transport is diffusion limited which results in a concentration gradient around a cell or tissue depending on rate of diffusion and rate of nutrient consumption. Monitoring changes to this steady state by cellular processes can thus be directly indicative of cellular activity. In this respect, oxygen can serve as an important biomarker for viability assays [8]. Oxygen is intrinsically linked with aerobic respiration, a process that leads to generation of adenosine triphosphate (ATP), which is central for cellular metabolism. Thus, any changes to cellular metabolism will have a direct effect on oxygen concentrations around a cell. For instance, cancerous cells show an increased metabolism compared to healthy cells [9].

The key question then is whether oxygen and changes in oxygen concentrations can be reliably monitored. An electrochemical approach to this question offers a straightforward solution for monitoring dissolved oxygen concentrations in aqueous media. Oxygen is electroactive in aqueous media and is easily detected through reduction process [10]. Electrochemical detection in combination with scanning probe microscopy

would then enable assessing of localised oxygen gradients that arise due to diffusion limited oxygen transport coupled with cellular consumption of oxygen as shown in Fig. 4.1.

Fig 4.1. Concept of monitoring oxygen gradients for viability assays on microtissues. Live microtissues consume oxygen via aerobic respiration resulting in oxygen gradient around a microtissue. This gradient is directly linked to the metabolic process and provides insight into cell state. When exposed to a drug that arrests cellular activity, the gradient is lost as the cells stop consuming oxygen and the oxygen concentration around the tissue reverts to bulk oxygen concentration. The gradient is represented by change in the shades of green.

This idea was first demonstrated by Tsionsky et al. in 1997 when they used a SECM approach based on oxygen reduction for imaging the respiratory activity of living cells [11]. Since then, this work has been expanded to various biological systems from tissues to embryos as an approach for monitoring cell health and viability [12-16]. A large focus of SECM measurements on biological system have been the use of oxygen reduction measurements for applications such as drug testing [17-19]. Therefore, this approach is considered a critical first step in demonstrating the suitability of the tissue culture platform described in the previous chapter for SECM based assays.

In this chapter, microtissues are produced in the Petri dish platform and subsequently applied for SECM measurements to monitor oxygen gradients. First, Pt and Pt/Hg (for improved stability) microelectrodes are considered for electrochemical detection of oxygen. SECM approach curves are then performed on polystyrene (PS) substrate and live microtissues to establish the presence of and characterize the oxygen gradients around a microtissue. Finally, a live/dead assay is performed using ethanol as a model drug to demonstrate the capability of the platform in combination with SECM for viability screening.

4.2. Experimental section

4.2.1. Materials

Polydimethylsiloxane (PDMS, Sylgard® 184) is purchased from Dow Corning, The Netherlands. Perfluorodecyltrichlorosilane (FDTS) is purchased from ABCR GmbH, Germany. CELLSTAR® polystyrene culture dishes (35 mm diameter) are from Grenier Bio-One GmbH, Germany. Isopropyl alcohol (IPA), ethanol, phosphate buffered saline (PBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), monosodium phosphate (NaH_2PO_4), D-(+)-Glucose, mercury (II) nitrate ($\text{Hg}_2(\text{NO}_3)_2$), potassium nitrate (KNO_3) and nitric acid (HNO_3) are all obtained from Sigma-Aldrich, The Netherlands. HeLa human adenocarcinoma and Hep G2 human hepatocellular carcinoma cell lines are from ATCC, Germany. Dulbecco's Modified Eagle Medium (DMEM), foetal bovine serum, penicillin/streptomycin, L-glutamine, trypsin-EDTA, calcein AM and propidium iodide are purchased from Gibco, Invitrogen, The Netherlands. Pluronic® F-127 is purchased from BASF, USA.

4.2.3. Fabrication

Fabrication of stamped Petri dishes is described in detail in Chapter 2. In brief, PDMS stamps are made from SU-8/Si master mold by soft lithography. Microwell arrays are hot embossed on commercially available 35 mm Petri dishes at 180°C with 10 min heating and 15 min cooling times. Hot embossing is performed using a custom setup (see chapter 2) connected to a soldering station (Tooltronics, The Netherlands).

4.2.3. Cell culture

HeLa and HepG2 cells are cultured in DMEM with 10% v/v FBS, 2% v/v Penicillin/Streptomycin and 1% v/v L-glutamine at standard culture conditions (37°C and 5% CO_2). Cells are passaged using 0.25% Trypsin-EDTA (2 min incubation) and reseeded after centrifugation at 1500 RPM for 5 min.

4.2.4. Spheroid formation

Stamped Petri dishes are sterilised in IPA or Ethanol, a protein repellent coating using 1% w/v (1g in 100mL) Pluronic® F-127 is applied. After overnight incubation, the coating solution is removed and the dishes washed with PBS. Ca. 500,000 cells in culture media are seeded in the Petri dish followed by centrifugation and removal of excess cells. 2 mL of

media is then added to the Petri dish and incubated in standard culture conditions (37°C and 5% CO₂).

4.2.5. Electrochemical measurements

Electrochemical measurements are performed in a three electrode configuration using an electrochemical probe scanner or SECM (ElProScan, HEKA, Germany). Hg is electrodeposited on 10 µm Pt microelectrodes using a potential step of -0.1 V *vs.* Ag wire for 300 s in a solution containing 10 mM Hg₂(NO₃)₂ and 0.1 M KNO₃ acidified to pH 1.2 using HNO₃. For measurements on microtissues, either Pt or Pt/Hg working electrodes is used with Ag wire reference and Pt wire counter electrodes in buffer solution containing 10 mM HEPES, 150 mM NaCl, 4.2 mM KCl, 2.7 mM MgCl₂, 1 mM NaH₂PO₄ and 11.2 mM D-(+)-Glucose at 35°C. Oxygen is detected by reduction reaction at -0.6 V *vs.* Ag wire. SECM line scans are performed at different heights from microtissue surface at 10 µm/s while approach curves are obtained at 1 µm/s. The temperature for SECM measurements is controlled using TC-20 temperature control system (npi electronic, Germany). The SECM amperograms are normalised to bulk oxygen current measured at the start of each recording.

4.2.6. Fluorescence based live/dead assay

Fluorescence live/dead assay is performed using calcein AM and PI couple described earlier. First, live microtissues are stained with 1 µg/mL calcein AM in culture media. After 1 h incubation at 37°C, the medium is removed and sample washed twice with PBS. Cell death is induced using 50% v/v ethanol solution in culture medium for 45 min. After ethanol treatment, the Petri dishes are washed twice with PBS and culture media containing 1 µg/mL PI is added to the Petri dish. After 1 h incubation at 37°C and washing steps, the samples are imaged using fluorescence microscopy (X51 Olympus inverted microscope equipped with an X-cite 120 PC Hg lamp using a 2x objective).

4.3. Results

4.3.1. Hg coating for improved Oxygen detection

While Pt microelectrodes have been used for mapping O₂ gradients and O₂ based viability assays, Pt electrodes are not stable for continuous monitoring of oxygen concentration. The platinum surface on which the oxygen reduction reaction is catalysed is prone to changes (oxide coverage

on Pt surface) which lead to instability [20]. Fig. 4.2 shows this instability, where the oxygen reduction current is constantly decreasing as the electrode scans over a microwell. The effect of any slope from the Petri dish is eliminated as the same phenomenon occurs in the reverse scan. Similar observations were made by Li et al., 2009 who proposed the use of Hg coated microelectrodes for stable oxygen reduction to alleviate the drift in signal [21]. The faradic currents are more stable on Pt/Hg microelectrodes than on bare Pt tip. Therefore Hg coating is electroplated on a 10 μm Pt electrode for stable detection of oxygen gradients.

Fig 4.2. SECM scans for oxygen gradient over a single microtissue demonstrating the instability of using bare Pt tip for measurements. The oxygen reduction current decays constantly and is reduced by 20% in 240 seconds (10 $\mu\text{m/s}$ scan rate over total distance of 2400 μm) of scan time. Microwell is between 400 and 800 μm .

Hg is electrodeposited on Pt surface by chrono-amperometry when the potential is held at -0.1 V vs. Ag wire in an acidified mercury (I) nitrate solution for 300 s [22]. As shown in Fig. 4.3, Hg ions are seen accumulating around the bare Pt surface and a stable Hg layer is formed after 300 s. It is also interesting to note that the Hg ions accumulate around the Pt surface and at certain points are released back into solution. The peaks on the graph (Fig. 4.4) where the reduction current drops coincide with dispersion of Hg ions away from Pt electrode when observed optically.

Fig 4.3. Electrodeposition of mercury on bare Pt microelectrodes. Mercury ions are electrodeposited chrono-amperometrically from 10 mM mercury (II) nitrate solution by holding the potential at -0.1 V vs. Ag wire. The images show a time lapse of accumulation of mercury ions on the Pt surface with a final stable Hg layer over the bare Pt tip after 300 s.

Fig 4.4. Chrono-amperograms of Hg deposition process on bare Pt microelectrode through the reduction of mercury (II) nitrate. As mercury is deposited on the platinum surface the reduction current decreases. The deposition is also seen on the previous figure by the accumulation of elemental mercury on the Pt electrode. Regions where the reduction current increases coincide to dispersion of some of the mercury around the Pt electrode back into solution.

After deposition of Hg layer, comparisons are made between electrochemical performance of bare Pt and Pt/Hg microelectrodes by cyclic voltammetry. Hg working electrodes are well known for their wide range in the negative potential window. This is clearly seen in Fig. 4.5 which shows cyclic voltammetry (CV) in PBS solution for Pt and Pt/Hg microelectrode. The hydrogen reduction potential for bare Pt microelectrode is lower than -1 V compared to -1.4 V for Pt/Hg microelectrode. Also as expected, Hg working electrodes cannot be used in the positive potential window as they are unstable above 0 V at physiological pH due to oxidation of mercury. However, with the Hg coating, stability of oxygen reduction current improves when compared to bare platinum microelectrodes.

Fig 4.5. Cyclic voltammograms of PBS solution using bare Pt and Pt/Hg microelectrodes. Hg coated microelectrodes have a wider potential window in the negative potentials when compared to bare Pt electrodes.

4.3.2. Characterization of Oxygen gradient around microtissues

Before employing oxygen measurements for viability assay, the influence of a microtissue on oxygen reduction current and the oxygen gradient around the microtissue is characterized. First, as shown in Fig. 4.6 approach curves to the Petri dish surface and microtissues are performed with the Pt/Hg microelectrode set to $-0.6\text{ V vs. Ag wire}$ for oxygen reduction. The approach curve towards an insulating surface will change as a result of hindered diffusion and happens very close from the surface. In the case of PS, it is non-conducting and the reduction current starts decreasing around $20\text{ }\mu\text{m}$ from the surface. This value ($20\text{ }\mu\text{m}$) is as expected for microelectrodes with a RG value (ratio of glass insulator radius to microelectrode radius) of 3. In the case of a microtissue, however, the gradient in oxygen concentration starts far away from the microtissue. For HeLa microtissues with an average diameter of $369\pm 33\text{ }\mu\text{m}$ the gradient starts around $250\text{ }\mu\text{m}$ and the oxygen concentration continuous to decrease gradually as the electrode approaches a microtissue surface.

Fig 4.6. Approach curves on PS surface and 3 different microtissues. The graphs demonstrate the differences between hindered diffusion when approaching a non-conducting surface and approaching an oxygen consumer. Currents are normalized to currents at bulk concentration for each measurement. Distances are indicative of the surface of the target (PS or microtissue) i.e. 0 μm from tissue surface is different from 0 μm from PS surface as the microtissues protrude ($>20 \mu\text{m}$) out of a microwell.

Fig 4.7. Horizontal line scans performed at different heights from the microtissue surface. These line scans demonstrate the presence of an oxygen depleted region around a microwell due to microtissue metabolic activity which consumes dissolved oxygen in measurement buffer. Microwell is better 400 and 800 μm .

Second, horizontal line scans shown in Fig. 4.7 are performed at various heights above the microtissue surface and across a length of 1200

μm covering $400\ \mu\text{m}$ on either side of a microwell (diameter $400\ \mu\text{m}$). As the distance of the electrode from the microtissue surface increases, the oxygen concentration also increases as explained in Fig. 4.1. These scans indicate that there is a gradient of oxygen depleted zone around a microwell as a result of microtissue respiration. From the horizontal line scans, a distance of $15\ \mu\text{m}$ from the microtissue surface is chosen as optimal distance for the live/dead assay. At this distance, the microelectrode is $>35\ \mu\text{m}$ (as a 2 day HeLa microtissue protrudes $>20\ \mu\text{m}$ outside a microwell) from the PS surface and any influence of hindered diffusion is eliminated. These measurements when repeated with HepG2 cell line also show a similar hemispherical gradient around the microtissue.

4.3.3. Live/dead assay using SECM

After characterization of the oxygen gradient, a live/dead assay is conducted by monitoring oxygen concentrations around the microtissues. One day after seeding cells in the microwells, line scans ($15\ \mu\text{m}$ from microtissue surface) are performed across $4000\ \mu\text{m}$ covering 3 microtissues. After scans on live microtissues, the tissues are exposed to 50% v/v ethanol for 45 min which leads to cell death. After ethanol exposure, line scans are performed again over 3 microtissues. As shown in Fig. 4.7 & 4.8 B, at a height of $15\ \mu\text{m}$ with respect to the tissue surface, the oxygen reduction current decreases by c.a. 35% over the live tissues. After ethanol treatment, a flat line is observed as the oxygen reduction current is not affected due to absence of oxygen gradient around a dead tissue as there is no respiration.

The results from the SECM experiments are confirmed as shown in Fig. 4.9 C and 4.9 D using a standard fluorescence method. Prior to ethanol treatment, microtissues are stained with calcein and have a green fluorescence indicating that the cells are alive and enzymatically active. After ethanol treatment, microtissues are exposed to PI. Dead cells lose their membrane integrity and PI is able to enter the cells of the microtissues and subsequently bind to nucleic acid inside the cells. As a result, dead tissues become PI-positive (red) and calcein-negative.

Fig 4.8. A) Concept of SECM line scans for measuring oxygen gradients around a microtissue array. B) Live/dead assay using SECM. Live tissues show c.a. 35% (at 15 μm from the surface) drop in oxygen reduction current whereas as dead tissue show a flat line. C) & D) Standard fluorescent live/dead assay used as control to confirm the results of SECM experiments.

4.4. Discussion

One of the important points of discussion and a safety consideration is the use of mercury for stable detection of oxygen. Mercury and mercury compounds are known to be highly toxic [23]. As a result, it presents a roadblock for use of these electrodes in routine procedures. In this instance, Hg coating is used for proof of concept but alternatives strategies need to be explored for evaluating tissue status which does not present safety concerns for both personnel and sample.

Additionally using mercury electrodes for oxygen detection involves a 2 electron transfer that results in the production of hydrogen peroxide [19, 24]. Hydrogen peroxide is a strong oxidizer and can cause adverse effects in cell culture [25]. However, in this instance, no adverse effects as a result of the measurements is seen and previous work in literature do not report adverse effect due to the generation of hydrogen peroxide from oxygen catalysis at Pt/Hg microelectrodes [19, 21]. This is assumed to be due to the low concentrations of hydrogen peroxide produced and short term exposure. However, long term influences of these measurements might be an interesting point for study in the future.

While the faradic currents show improved stability with Hg coating, the instability in oxygen reduction current is not completely alleviated. It can be seen in the SECM recordings shown in Fig. 4.7 and Fig. 4.8 B, that there is still decay in oxygen reduction over the scan duration. This decay is significantly less than on bare Pt electrodes shown in Fig. 4.2. This decay could arise due to incomplete coverage of Hg on the Pt surface during coating or wear on the Pt/Hg surface over time.

From the oxygen gradient graph in Fig. 4.7, it can be seen that the microtissue influence on the oxygen gradient extends $<200\text{ }\mu\text{m}$ on the sides of the microwell. Since the microwells are spaced $800\text{ }\mu\text{m}$ apart, no influence of microtissues on each other can be seen. However, the SECM technique might be an interesting method to study the gradients and effects of microtissues on each other when they are spaced close enough to influence each other. This could be used for optimising distances while scaling up the microwell arrays for large scale microtissue production.

Finally, the live/dead assay performed here is done using ethanol as a model for drug. Ethanol was chosen as it is commonly used as a fixative and higher concentrations have an immediate effect on cell health. Ethanol also preserves the tissue size and shape. Therefore changes in oxygen gradient due to tissue size do not have to be accounted for. As a future step, the relevance can be increased by the use anticancer drugs with differing mechanisms to study their effects on tissue metabolic activity and on the viability of the microtissues. However, there is an issue of tissue disassembly that needs to be tackled first before more relevant assays can be implemented.

4.5. Conclusion

In this chapter, we have demonstrated the use of Pt/Hg microelectrodes for studying oxygen gradients around a microtissue. A hemispherical oxygen depleted zone is found around a microtissue. With oxygen reduction measurements we then perform a live/dead assay using SECM which are confirmed using standard fluorescent microscopy. More significantly, this chapter illustrates the compatibility of the Petri dish platform developed in chapter 2 for simultaneous scanning probe and optical microscopy.

4.6. Safety consideration

CAUTION: Mercury ions are highly toxic. Solution must be handled with protective equipment and extreme care to avoid contaminations. All solution and waste from rinsing must be collected and directed to designated heavy metal disposal.

4.7. References

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Chapter 5: Non-invasive osteogenic differentiation assay using SECM

In this chapter, production of homogenously ($206 \pm 34 \mu\text{m}$) sized C2C12 microtissues (in the Petri dish platform) is demonstrated. The C2C12 microtissues are subsequently differentiated into osteogenic lineage using bone morphogenic protein 2 (BMP-2) as a differentiation factor. The change in lineage is subsequently assessed for alkaline phosphatase activity using both scanning electrochemical microscopy (SECM) and standard colorimetric assays. Prior to the SECM assay, the PAPP/PAP system used for ALP detection is characterized thoroughly. The non-invasive SECM based differentiation assay is successfully performed on live microtissues at physiological pH. Measurements at this pH are a significant improvement over traditional approaches for ALP detection which are performed at alkaline (pH 9.5) conditions for optimal ALP activity.

5.1. Introduction

Alkaline phosphatase (ALP; EC 3.1.3.1) is an important enzyme responsible for catalysing the hydrolysis of phosphate groups from various molecules [1, 2]. First described in 1907, ALP has been found to be ubiquitous in nature and present in numerous organisms from bacteria to fungi, fish and mammals. In mammals, ALP can be found in high concentrations in several tissues that include liver, bone, kidney, lung etc. [3]. It also plays a vital role in embryonic development as well as increased expression in certain tumours [4-6]. Consequently, ALP is an essential factor for study, and serves as a marker for different biological assays.

One of the key processes where ALP is widely used as a marker is osteogenic differentiation, i.e., the differentiation of precursor cells (myoblasts, stem cells) into osteoblasts (bone forming cells) [7]. For example, C2C12 myoblasts can be triggered to differentiate into osteoblastic lineage, which is characterised by ALP expression [8, 9]. Thus ALP is considered as an early marker for osteogenesis and several strategies are readily available for its detection. The most commonly used method is an indirect approach where phosphate group is hydrolysed by the enzyme from the substrate para-nitrophenylphosphate (pNPP) to form product para-nitrophenol (pNP) [1]. The substrate pNPP is colourless while the product pNP is yellow, and this change in colour indicates the presence of ALP.

Fig 5.1. Standard colorimetric assay used for detection of ALP. The substrate pNPP (left) is colourless and is catalysed to yellow coloured pNP (right) by the enzyme.

Alternative assays include indirect fluorescence approaches, which provide improved sensitivity and allow quantification (these assays can be combined with spectrometers or fluorimeters to obtain a quantitative analysis) [10, 11]. Aforementioned colorimetric and fluorometric assays are

conducted with secreted ALP (SEAP) which is analysed from aspirated culture medium [12]. SEAP assays are convenient, straightforward and preserve the sample. However, they are limited to bulk solution and cannot be used to determine local ALP intracellular concentrations on individual cells or tissues.

Direct assessment of ALP content in individual samples can be achieved using immunostaining. In this approach, antibodies that bind to specific or non-specific isoforms of ALP can be used for tagging membrane bound ALP. The tagged ALP can then be visualised by using a chromophore or fluorophore and quantified similar to indirect techniques mentioned previously. However, immunostaining requires extensive and time-consuming sample preparation. More significantly, the biological sample (cell or tissue) has to be fixed before the assay can be carried out. Western blotting and PCR analysis are also possible analytical strategies, which however requires cell lysis. As a consequence, these direct approaches preclude any further use or continuous monitoring of the sample.

Aside from the standard biological techniques, several electrochemical alternatives have been explored over the years. Substrates based on phosphate esters such as 2-carboxy-1-naphthylphosphate, phenyl phosphate, p-cyanophenyl phosphate, ferrocenylethyl phosphate, ascorbic acid 2-phosphate etc. have been demonstrated as suitable for electrochemical detection of ALP [13, 14]. Perhaps the most straightforward electrochemical substrate is also the one commonly used in colorimetric detection – pNPP. pNP resulting from the hydrolysis of pNPP is electrochemically active and can be detected amperometrically at 1.1 V *vs.* Ag/AgCl [14]. This oxidation potential of pNP is high which makes measurements susceptible to noise. Instead of pNP, p-aminophenol (PAP) which has a lower oxidation potential (100 mV *vs.* Ag/AgCl) is widely used for electrochemical detection although the substrate p-aminophenyl phosphate (PAPP) is unstable.

These examples demonstrate the diverse strategies available for ALP detection. Out of these methods, electrochemical approach is an attractive option as it has high sensitivity and detection of zeptomolar concentrations have been demonstrated. Combining one of these electrochemical approaches with scanning probe microscopy would yield very high local information on the ALP activity of individual samples. In this context, scanning electrochemical microscopy (SECM) can be used to

detect activity and gradients of local membrane bound ALP on cell or tissue sample.

1. Enzymatic reaction

2. Oxidation reaction

Fig 5.2. Electrochemical detection of ALP using PAPP/PAP system. The electrochemical approach is similar to pNPP/pNP systems used in colorimetric assays. PAP is preferred over pNP for electrochemical detection as it has lower oxidation potential, and is thus less susceptible to noise.

Sen et. al, 2012 demonstrated the use of SECM in an electrochemical assay for detection of ALP [15]. Through the use of a microarray device they were able to capture SEAP on surface immobilised antibodies and detected the activity of the enzyme using PAPP/PAP couple. This work was further expanded on by Matsumae et. al, 2013 who employed the same PAPP/PAP system for evaluating the differentiation status of single embryonic stem cells using SECM [16]. They were able to demonstrate differences in PAP current for undifferentiated (high ALP) and differentiated (low ALP) embryonic stem cells. This work was later applied by Arai et. al, 2013 to embryoid bodies and co-culture systems to map heterogeneous ALP activity [17].

However, these measurements have been conducted in alkaline conditions (pH 9.5) which is expected as (particularly mammalian) ALP, true to its name, has optimal activity at high pH. High pH measurements are not conducive for cell culture and can have a deleterious effect on sensitive cells lines such as primary cells or stem cells. Ideally,

measurements conducted on live samples and at physiological pH would present a significant improvement. We hypothesized that this should be possible on our platform (see chapter 2) due to the collective nature of microtissues and high local sensitivity of SECM measurements.

In this chapter, we first produce C2C12 microtissues on the Petri dish platform described previously. Subsequently, C2C12 microtissues are exposed to bone morphogenic protein 2 (BMP-2) to induce osteogenic differentiation. Finally, differentiation status of both BMP-2 treated and untreated microtissues is assessed using the SECM through the indirect enzymatic assay based on PAPP/PAP couple.

5.2. Experimental section

5.2.1. Materials

Polydimethylsiloxane (PDMS, Sylgard® 184) is purchased from Dow Corning, The Netherlands. Perfluorodecyltrichlorosilane (FDTS) is purchased from ABCR GmbH, Germany. CELLSTAR® polystyrene culture dishes (35 mm diameter) are from Grenier Bio-One GmbH, Germany. Isopropyl alcohol (IPA), paraformaldehyde (PFA), bone morphogenic protein 2 (BMP-2 human, recombinant, expressed in *E. coli*), alkaline phosphatase (bovine), phosphate buffered saline (PBS), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), monosodium phosphate (NaH_2PO_4), D-(+)-Glucose are all obtained from Sigma-Aldrich, The Netherlands. C2C12 mouse myoblast cell line is from ATCC, Germany. Dublecco's Modified Eagle Medium (DMEM), foetal bovine serum, penicillin/streptomycin, L-glutamine, trypsin-EDTA is purchased from Gibco, Invitrogen, The Netherlands. Pluronic® F-127 is purchased from BASF, USA. ALP detection kit is purchased from Millipore, USA.

5.2.2. Fabrication

Stamped Petri dishes are fabricated as described in detail in Chapter 2. In brief, PDMS stamps are made from SU-8/Si master mold by soft lithography. Microwell arrays are hot embossed on commercially available 35 mm Petri dishes at 180°C with 10 min heating and 15 min cooling times. Hot embossing is performed using a custom setup (see chapter 2) connected to a soldering station (Tooltronics, The Netherlands).

5.2.3. Cell culture

C2C12 cells are cultured in DMEM with 10% v/v FBS, 2% v/v Penicillin/Streptomycin and 1% v/v L-glutamine at standard culture conditions (37°C and 5% CO₂). Cells are passaged using 0.25% Trypsin-EDTA (2 min incubation) and reseeded after centrifugation at 1500 RPM for 5 min. C2C12 cells are not allowed to form confluent monolayers.

5.2.4. Spheroid formation and culture

Stamped Petri dishes are sterilised in IPA or Ethanol followed by application of a cell repellent coating using 1% w/v (1 g in 100 mL) Pluronic® F-127. After overnight incubation, the coating solution is removed and the dishes washed with PBS. 500,000 cells are seeded in the Petri dish followed by centrifugation and removal of excess cells. 2 mL of culture medium is added to Petri dish and incubated at standard culture conditions. To induce cell differentiation, the culture medium is supplemented with 300 ng/mL BMP-2.

5.2.5. ALP detection kit

ALP detection kit consists of two components. Component A – fast red violet solution (0.8 g/L stock) and component B - Naphthol AS-BI phosphate solution (4 mg/mL) in AMPD (2-Amino-2-methyl-1,3-propanediol) buffer (2 mol/L) at pH 9.5. Prior to adding to the cell or tissue samples, the staining solution is prepared in the following ratio – 2:1:1 (component A: component B: water). For the staining procedure, the culture media is aspirated and the samples rinsed once in PBS. Subsequently the cell or tissue sample is fixed with 4% (in PBS) PFA at room temperature. Samples are fixed for 90 seconds as fixing longer than 120 s results in the inactivation of ALP. The fixative is then removed, and samples are rinsed in PBS followed by addition of staining solution. The samples are then incubated in dark at room temperature for 15 min. The staining solution is subsequently aspirated and samples are rinsed with PBS. Microtissues are imaged using IX51 Olympus optical microscope and images are processed using ImageJ (NIH, USA) image processing software.

5.2.6. Electrochemical measurements

Electrochemical measurements are performed in a three electrode configuration using an electrochemical probe scanner or SECM (ElProScan, HEKA, Germany). For all measurements, a 10 µm Pt electrode is used as working electrode along with a Ag wire reference and Pt wire counter electrodes. Both characterisation and microtissue measurements

are conducted in buffer solution containing 10 mM HEPES, 150 mM NaCl, 4.2 mM KCl, 2.7 mM MgCl_2 , 1 mM NaH_2PO_4 and 11.2 mM D-(+)-Glucose at 35°C. This buffer solution is henceforth referred to as measurement buffer. All SECM scans on microtissues are performed in constant height mode (15 μm from surface) at a scan rate of 10 $\mu\text{m}/\text{s}$. The temperature for SECM measurements are controlled using TC-20 temperature control system (npi electronic, Germany).

5.3. Results

5.3.1. Microtissue production and differentiation

Experiments performed with C2C12 cell lines in literature are normally with monolayer models. C2C12 monolayers when allowed to become confluent tend to form myotubes (multinucleated cell due to cell fusion). Therefore the first step was to test the ability of C2C12 cell line to form stable tissues in the microwells. After seeding cells in the Petri dish platform we observe that the C2C12 cells form homogenous microtissues (see Fig. 5.4) in 24 h with an average diameter of $206 \pm 34 \mu\text{m}$.

Fig 5.3. Array of C2C12 microtissues with an average diameter of $204 \mu\text{m}$ produced on stamped polystyrene Petri dishes.

Once stable microtissues were formed, it is necessary to ascertain the ability of the microtissues to differentiate into osteoblasts. Katagiri et. al first demonstrated the ability of C2C12 monolayers to differentiate (upon exposure to BMP-2) into osteoblasts and express ALP. However, in a microtissue the cells might become multinucleated (myotubes) due to close proximity and may lose their ability to differentiate into osteoblasts.

Fig 5.4. BMP-2 treated C2C12 microtissues can be qualitatively assessed for osteogenic differentiation using a colorimetric ALP detection kit. Differentiated tissues (48 h after seeding) turn red-violet due to ALP activity. Scale bar – 100 μ m.

To induce differentiation, culture media is supplemented with 300 ng/mL BMP-2 during cell seeding. Microtissues are tested (see Fig. 5.5) for ALP expression using the ALP detection kit at 24h time point for 6 days. No ALP expression is observed 24 h after cell seeding in both BMP-2 treated and control microtissues. After 48 h, BMP-2 treated microtissues show positive ALP expression as they become red-violet from ALP activity whereas no changes are seen in control microtissues. This is comparable to literature results for C2C12 monolayers which start expressing ALP 48 h after exposure to BMP-2 with ALP activity progressively increasing, and reaching peak values on day 6.

5.3.2. Electrochemical detection of ALP

5.3.2.1 Characterization of PAP detection limit

PAPP was chosen as the substrate for ALP detection due to the straightforward detection scheme described previously (see Fig. 5.3). The end product of PAPP hydrolysis by ALP is PAP. Since the idea behind the measurements is to detect ALP at lowered pH (8.5 and 7.5), it is necessary to characterize electrochemical behaviour of PAP at these pH (8.5 and 7.5) and determine its detection limit. For this purpose, known amounts of PAP is added to the measurement buffer, following which cyclic voltammograms and chrono-amperograms are recorded to determine the lower limit of detection using the SECM system. Different concentrations of PAP – 0 μ M, 10 μ M, 50 μ M, 100 μ M, 250 μ M, 500 μ M and 1000 μ M are tested at pH 7.5 and 8.5. No significant difference is seen in electrochemical behaviour of PAP between each pH as shown on Fig. 5.6 and Fig. 5.7.

Using cyclic voltammetry (see fig 6.) concentrations as low as 50 μM of PAP are easily distinguished from control (0 μM) and a linear relationship can be established to higher concentrations. Lower concentration (10 μM) is not discernible although the presence of PAP seems to have some effect on oxygen reduction (below 0 V).

Fig 5.5. Cyclic voltammograms (-100 mV to 500 mV at 100 mV/s) of varying PAP concentrations (0-1000 μM) at pH 7.5 (left) and pH 8.5 (right). Concentrations as low as 50 μM is easily distinguished from control.

Fig 5.6. PAP detection limit characterized by chrono-amperometry at pH 7.5 (left) and pH 8.5 (right). Concentrations as low as 10 μM can be detected and a linear relationship is seen between 10 μM to 1000 μM of PAP in measurement buffer.

Using chrono-amperometry (see Fig. 5.7) it is possible to detect even lower concentrations of PAP as chrono-amperometry has higher sensitivity than cyclic voltammetry. Concentrations as low as $10\ \mu\text{M}$ are easily distinguished from control and a linear relationship are established from $10\ \mu\text{M}$ to $1000\ \mu\text{M}$. These results suggest that the concentrations of PAP will not be a limiting factor for determination of ALP activity. Even with lowered ALP activity around physiological pH, detection of ALP should be possible as concentrations higher than $10\ \mu\text{M}$ are expected due to local production of PAP in the vicinity of a microtissue.

5.3.2.2. Detection of exogenous ALP activity using PAPP/PAP system

Fig 5.7. Changes in CV to measurement buffer containing $1\ \text{mM}$ PAPP after addition of external ALP. CV's are shown for pH 7.5 (top) and pH 8.5 (bottom).

Before SECM measurements are performed on C2C12 microtissues, the effect of pH on ALP activity is tested using exogenous ALP. Cyclic voltammetry is used for quick characterisation of the effect of ALP. Initially $1\ \text{mM}$ PAPP is in measurement buffer and $0\ \text{mM}$ PAP is expected to be present. After addition of ALP, the PAPP is expected to be slowly converted

to PAP with final concentrations of 0 mM PAPP and 1 mM PAP. Effect of ALP on this system is characterized at pH 7.5 and 8.5.

Changes in CVs at both pH 7.5 and 8.5 can be immediately seen after the addition of ALP. However, as expected the conversion of PAPP to PAP by ALP is faster at pH 8.5 when compared to pH 7.5. At pH 8.5 peak values for PAP oxidation are reached within 5 min as opposed to 15 min for pH 7.5. For the isoform of ALP used, optimal activity is expected at pH 9 and certain isoforms of ALP have been shown to have nearly 2-fold decrease for every unit of pH drop. These experiments demonstrate that ALP should be active, albeit lowered activity, at physiological pH and convert PAPP to PAP, which can then be used as an indirect marker for ALP.

5.3.2.3 Non-invasive SECM based detection of C2C12 differentiation

Fig 5.8. Concept of osteogenic differentiation assay of individual microtissues using SECM in substrate generation-tip collection (SG/TC) mode. Microtissue differentiating towards an osteogenic lineage express enzyme ALP, which catalyses hydrolysis of phosphate group from substrates. In this instance, substrate PAPP is hydrolysed to PAP, which is then electrochemically detected near the microtissue surface using a SECM microelectrode.

SECM measurements are conducted 6 days after cell seeding in the Petri dish platform and in measurement buffer containing 1 mM PAPP. Typically, measurements are started 10 min after addition of buffer once the temperature reaches 35°C. First the microelectrode is positioned at a constant height (15 μm) from the Petri dish surface using negative

feedback approach and dissolved oxygen reduction current. For this the tip potential is set to -0.6 V vs. Ag wire and then the tip is approached towards the PS surface till there is current drop due to hindered diffusion. After the surface has been reached the tip is pulled back $15\text{ }\mu\text{m}$ and line scans are performed at this height.

For the differentiation assay, the potential is set to $+0.35\text{ V}$ vs. Ag wire to detect PAP oxidation. These measurements are again performed at both pH 7.5 and 8.5. As the SECM tip scans over a 6 day old control microtissue (no exposure to BMP-2) no changes in current due to the presence of the microtissue is seen. However, when the same line scans are performed on microtissues that have been treated with BMP-2, changes in current levels are seen near the microtissues. This increase is correlated to the presence of ALP which converts PAPP to PAP. Thus the increase in current is directly related to the ALP activity and the presence of ALP indicates differentiation. These measurements are also confirmed using the standard colorimetric assay as standard, where BMP-2 treated microtissues turn red-violet while no colour changes are seen in control microtissues.

Fig 5.9. SECM based differentiation assay using PAPP/PAP system to detect ALP activity. There is a current increase as the SECM tip scans over a differentiated microtissue due to local formation of PAP from PAPP due to membrane bound ALP present in a differentiated microtissue. No ALP is expected in undifferentiated tissues and therefore no changes in current are seen. The SECM assays are confirmed using a standard colorimetric assay shown as insets (scale bar $200\text{ }\mu\text{m}$).

As discussed in previous sections, ALP activity is significantly higher as the pH becomes more alkaline. This can be seen in the current levels for measurements at pH 7.5 and 8.5. At pH 8.5 the increase in current due to oxidation of PAP is >200 pA whereas at pH 7.5 this increase is ~50 pA. Additionally the cells remain viable at pH 8.5 (after 2 h) as the C2C12 cells lines are immortalised and robust.

5.4. Discussion

C2C12 cell line plays a vital role in understanding osteogenesis as it is often used for characterisation of new differentiation factors. Normally the differentiation is studied on monolayers models. However, as demonstrated in this chapter, C2C12 microtissues are also capable of differentiating into osteoblasts. As discussed in chapters 1 and 2, cell culture in 3D is significantly different from 2D culture conditions and a lot closer to *in vivo* conditions. There have not been enough studies in literature with 3D culture of C2C12 and it may be interesting to study the impact of 3D culture on these cells, its effect on differentiation and whether it amplifies or diminishes the effect of differentiation factors when compared to traditional monolayer culture.

In this instance, BMP-2 does induce osteogenic differentiation; the factor is added at the time of cell seeding. This perhaps plays down initial concerns that formation of myotubes due to close proximity of the cells in the tissue might hinder differentiation. Whether this differentiation can be initiated by addition of BMP-2 after microtissue formation might be a future line of questioning.

Additionally the ALP colour change assay performed to test differentiation is qualitative. Over the course of 6 days, it was not possible to distinguish changes in colour intensity between day 2 and day 6 samples. This is assumed to be due to multiple layers of cells in the microtissues. Further quantification of the actual ALP concentrations is needed to test whether the ALP activity increases progressively over a 6 day period similar to monolayer culture.

From the exogenous ALP measurements, it is interesting to note that at both pH (8.5 and 7.5) there is decay in PAP current levels after reaching peak values. Although this decay is higher at pH 8.5 with steady state current dropping to almost half the peak steady state current by 25 min. This could be due to either the pH or ALP accelerating the

degradation of PAP to p-Quinone imine (PQI). However, no similar decay is seen in PAP only system discussed before and ALP is not expected to hydrolyse PAP further. The exact reasoning and mechanism behind this decay needs to be explored further.

Through the use of the SECM, it has been possible to perform the differentiation assay non-invasively on live cells and at physiological pH. This will be useful, for example, in regenerative medicine where these microtissues can be used as building blocks for bottom-up tissue engineering. While this in itself is an improvement over traditional assays, it still remains qualitative. The next step that is vital to explore whether this can be done quantitatively. This would be a significant development for studying the differentiation process continuously. However, it might not be ideal to use PAPP as the electrochemical substrate as it is prone to decay into PAP and is inherently unstable. While pNPP is a stable substrate it is not practical to use due to the high oxidation potential. It is therefore critical to identify an alternate substrate that is both stable and has an electrochemically active product with low oxidation potential before the SECM based assay can be fully exploited.

5.5. Conclusion

We have demonstrated formation of C2C12 microtissues followed by their osteogenic differentiation using BMP-2. Subsequently, the differentiation assay has been performed non-invasively on live cells and at physiological pH using scanning electrochemical microscopy. The SECM based assay was confirmed using a standard colorimetric assay as control. This non-invasive approach presents a significant improvement over traditional ALP assays as they are performed at pH 9.5 and on fixed cells.

5.6. References

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Chapter 6: Conclusion and perspectives

In this chapter, a brief summary of each chapter is presented. Subsequently, a critical and personal perspective on the applicability of scanning electrochemical microscopy for biological assays is provided with a few concluding remarks.

6.1. Summary

As stated in the beginning of this thesis, the objective of this work was to explore an alternative or complementary method for studying live biological systems (cells, tissues etc.). For this purpose, scanning electrochemical microscopy (SECM) was chosen for implementing biological assays on microtissue. The following section condenses the results of each chapter into a brief summary.

Chapter 1 introduced the fundamental principle of SECM system, which basically comprises of an electrochemical cell and scanning probe instrumentation. A brief theory into microelectrode operation, feedback mode measurements and SECM imaging was also provided. Finally, the literature of SECM measurements on biological systems was presented along two lines: extracellular and intracellular measurements. While a large amount of articles dealing with SECM and living systems focus on oxygen measurements, SECM has also been applied for a great variety assays such as intracellular redox measurements, differentiation assays and monitoring cell signalling to name a few.

Chapter 2 proposed a novel platform suitable for SECM measurements and microtissue formation and culture. The platform was realized by hot embossing of commercially available polystyrene (PS) Petri dishes to incorporate the required microwell array structure. PS does not suffer from limitations of PDMS and agarose, i.e., it is not porous and the bottom substrate is thin enough for simultaneous optical and scanning probe microscopy measurements. Using a custom setup to preserve the format of the Petri dish while reshaping its bottom, the fabrication process was first optimised and ideal parameters for embossing determined to be 180°C with 10 min heating and 15 min cooling. The fidelity of replication between the PDMS master and stamped Petri dish was verified with a wide array of techniques – white light interferometry, optical microscopy and scanning electron microscopy. Finally, a coating procedure was developed to create cell-repellent surface, and microtissues were produced in microwell (400 μm diameter, 200 μm depth) arrays (>100 wells) using different cell lines (HeLa, HepG2, MCF-7, C2C12, and 4T1). The microtissues produced in the platform were also found to be homogenous in size (<10% variation) 24 h after seeding. HeLa microtissues were finally imaged to characterize cell reorganisation in comparison with monolayer models, as well as 4T1 microtissues analysed and their protein expression patterns compared to monolayer models. Interestingly, metabolic and extracellular matrix proteins were found to be over-expressed (>40 proteins

with a minimum 2 fold increase) in microtissues compared to monolayer culture.

In **Chapter 3**, negative feedback mode SECM was successfully used for imaging single cells. However, similar measurements on microtissues produced in the Petri dish platform gave unexpected results. Specifically, these measurements revealed that the tissue shape presented discrepancies such as non-uniform filling of the microwells, something that could not be detected using other microscopy techniques utilized in chapter 2. This observation was attributed to the presence of a tilt in the microwells, a phenomenon that has however been seen using microscopy for microwells of smaller sizes (100 μm diameter and 100 μm depth). Due to this non-uniform filling, it was concluded that true microtissue imaging was not possible in the Petri dish platform in its current iteration and line scans were chosen as a preferred method in the subsequent chapters for microtissue analysis using SECM. Additionally, using approach curves it was determined that the microtissues protrude $>20\text{ }\mu\text{m}$ outside of the microwell which allows for constant height scanning without negative feedback from the Petri dish surface.

In **Chapter 4** a live/dead bioassay was successfully performed using SECM. For that purpose, Hg coating of the Pt microelectrode first had to be developed for stable detection of oxygen by chrono-amperometry. Subsequently, the oxygen gradient formed around HeLa microtissues in microwells was fully characterized: a hemispherical oxygen depleted zone was typically found, which extends more than 200 μm from the tissue edges. From this characterization, a height of 15 μm above the microtissue was chosen for the live/dead assay. Finally, constant height line scans were performed on HeLa microtissues before and after treatment with a model drug (ethanol) for measuring their oxygen consumption and thereby assessing tissue viability. The SECM measurement was finally confirmed using standard fluorescence assay and cell impermeable fluorescent stains. This chapter establishes the potential of SECM combined with the tissue culture platform developed in chapter 2 as a useful combination for biological assays. The SECM based live/dead assays allows for non-invasive measurements on the tissue status and will be applicable for pharmaceutical testing on relevant tissue models.

In **Chapter 5** a second bioassay is implemented, specifically a differentiation assay based on indirect detection of alkaline phosphatase (ALP) expressed during osteogenic differentiation. The assay is based on

the function of ALP to catalyse molecules with phosphate group and the product of the enzymatic reaction detected. For SECM detection, p-aminophenyl phosphate/p-aminophenol (PAPP/PAP) couple was chosen and characterized in solution, before the assay was tested on microtissues. Specifically, cyclic voltammetry and chrono-amperometry measurements were performed to determine the detection limit of PAP, which was found to be of 50 μM and 10 μM for the two techniques, respectively. C2C12 microtissues were subsequently differentiated from myogenic to osteogenic lineage by treatment with bone morphogenic protein 2 (BMP-2), which was accompanied by expression of alkaline phosphatase on the cell membrane. SECM measurements were performed six days after cell seeding on BMP-2 treated and control microtissues. Using the indirect SECM assay we were able to detect differentiation on live individual microtissues, as also found using a commercially available colorimetry assay. Following this, we performed the SECM characterization on live microtissues at different pH values (8.5 and 7.5, which corresponds to physiological conditions). Through the use of microtissues, measurements at physiological conditions could be conducted, while normally ALP detection is performed at high alkaline (9.5) pH which is not optimal for cell viability. The differentiation assay using SECM can find applications particularly in the field of regenerative medicine where the microtissues samples need to be preserved so they can later be used for bottom up tissue engineering.

6.2. Perspectives

While the previous section highlights successful achievements of this PhD work along with the SECM as a system for biological measurements, a number of points must still be considered and much to be desired before moving forward. Therefore, in this section different aspects are discussed based on my personal experience of SECM measurements. For instance, one point of contention is that the SECM assays demonstrated here have been so far only qualitative whereas quantitative approaches are necessary to be truly relevant in biology. The qualitative approach is partially out of choice but also partially out of limitations of technology. However, it should be noted that groups more experienced in SECM measurements, such as Matsue group, do report quantitative biological measurements using SECM.

In terms of the technology, one needs extensive training for implementing assays using SECM. This, of course, is the case for many techniques (e.g., confocal microscopy) used regularly in biology. However,

even with the necessary training, the technology is not yet straightforward. Most groups work with a custom setup that they have built during the course of the last decade(s), which may provide better usability. In our case, we work with a commercial system (ElProScan, HEKA, Germany), which should be made user-friendly in terms of both technology and software. This cannot be extrapolated to other commercial systems directly but the choice of our current system was based on evaluation of different options at the point (2010) of purchase. However, improvements on user experience can be expected, with feedback and commercial competition, in the coming years given that SECM commercial systems are relatively new.

Regardless of the commercial shortcomings of the technology, there are more significant points to consider in terms of implementing regular biological assays with SECM. One of the key elements required for electrochemical detection is the presence of an electroactive species, which is not always the case. Therefore, not all assays can be readily implemented, which is a stumbling block when catching up to well-established assays such as fluorescence based ones. Additionally, when an electroactive molecule is available, it is far from straightforward to detect. As discussed in chapter 4 for oxygen measurements, electrode modifications are often necessary for stable electrochemical detection. This is also seen in other work presented in literature; for example, complex modifications like attaching enzymes are needed for glucose detection or porphyrin for nitric oxide. Additionally, the resolution of SECM scans depends on the electrode size and geometry. As a result, there is no “one size fits all” solution in terms of electrodes or detection schemes. These issues when accounted for with challenges of electrode fabrication and costs makes SECM based biological assays a challenging task.

In terms of the assays and measurements presented in this thesis work, we have not been able to explore the full capabilities that the SECM offers. For instance, as discussed in chapter 3, we had to limit ourselves to line scans due to presumed tilt of the microwells from the fabrication process. As a result, all the scans were performed in constant height mode whereas some measurements such as redox activity would require constant distance imaging and analysis. For constant distance mode scanning, shear force technology was available in our system, but it is difficult to operate with cells, which due to their soft nature give poor signal quality. Constant distance analysis should be easier to implement with new technology such as the combined SECM/AFM measurement system. Further, techniques like AC impedance and combined

SICM/SECM systems should open up wider possibilities for SECM based biological assays.

Ultimately, to be a truly relevant tool for biologists, SECM should be used for addressing and solving specific biological questions, while most of the SECM based literature focus on testing and demonstrating the potential of SECM for biological applications. Perhaps the main reason over the lack of biological problems addressed is that a significant amount of time, when implementing assays, is spent addressing engineering problems such as electrode modifications or enabling platforms. In its current state, the SECM lacks a sense of monotony and ease of implementation when compared to standardised biological assays like ELISA, which restricts its use for solving research questions.

In conclusion, the SECM remains an attractive engineering tool but far from a biological reality. To overcome this, a concerted effort is required by the SECM community to standardize the well-demonstrated assays such as oxygen measurements and establish ready to use protocols. This is critical for the full potential of the SECM to be realised either as an alternative or complementary tool for biological studies.

Appendix

Appendix I: Protocol for protein analysis

Sample preparation

For proteomic analysis the cell lines or micro tissues were washed three times with 5 mL of PBS then were lysed with 500 μ L of RIPA buffer (50 mM Tris HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP40, 0.25% sodium deoxycholate and protease inhibitors) at 4°C. Following lysis, lysates were centrifuged at 14,000 rpm at 4 °C and protein concentrations were calculated by Bradford assay (Sigma Aldrich) using BSA as a standard.

	Concentration (mg / mL)	Volume (μ L)
1 petri dish tissue	5.10	100
11 petri dish tissue	9.80	500
Mono layers	9.65	500

Digestion eFASP With Passivated Ultra filtration Unit.

UF filters from Microcon UF units (YM-3 3 kDa cutoff limit; Millipore, Billerica, MA) were incubated overnight 5% v/v TWEEN-20 (T20, Sigma-Aldrich P7949). Following incubation, the filter units were rinsed thoroughly by three immersions in MS-grade water. eFASP digestion [1], [2] was realized on 50 μ g mono layers cells (4T1) or tissues estimated from Bradford assay. This samples were mixed in 50 μ L of solubilization and reducing buffer A (4% SDS, 0.2 % DCA, 50 mM DTT, 200 mM ABC) overnight at 4°C. Samples were centrifuged at 13,000 g at 20°C for 15 min. Supernatant were mixed with 200 μ L of SDS buffer exchange (8 M urea, 0.2 % DCA, 100 mM ABC, pH 8) and were transferred into Microcon UF units (YM-3 3 kDa cutoff limit; Millipore, Billerica, MA). After 30 min of 13 000 g centrifugation, the filtrate was discarded, an additional 200 μ L of exchange buffer A was deposited in each unit, and centrifugation resumed for 30 more minutes. This buffer addition/centrifugation step was repeated twice more. The reduced proteins were alkylated within the filter unit by adding alkylation buffer A (8 M urea, 50 mM iodoacetamide, and 100 mM

ABC, pH 8) and incubating at 37°C for 1 h with shaking in the dark. After 30 min of 13,000 g centrifugation, the filtrate was discarded, an additional 200 µL of exchange buffer A was deposited in each unit, and centrifugation resumed for 30 more minutes. This buffer addition/centrifugation step was repeated one more. After buffer exchange was performed with three exchanges with eFASP digestion buffer (50 mM ABC, 0.2% DCA pH 8). eFASP digestion buffer (100 µL) was added to each UF device, followed by 1 µg of trypsin (1:50 w/w). Digestion proceeded for 16 h at 37°C. Peptides were recovered by transferring the UF filter to a new collection tube and spinning at 13 000 g for 20 min. To complete peptide recovery, filters twice were rinsed with 50µL of 50 mM ABC that was collected by centrifugation.

Phase Transfer

200 µL of ethyl acetate was added to the peptide containing filtrate and was transferred to a 2 mL tube. After 2.5 µL TFA was added and was quickly vortex. White thread like precipitate may be visible for large quantities of peptides. Peptides precipitate were mixed with 800 µL of ethyl acetate and were centrifuged at 13 000 g for 10 minutes. The organic supernatant was discarded and this step was repeated twice more. Carefully remove and discard as much of the upper organic layer as possible without disturbing the boundary layer. Uncovered sample tube were placed in a thermomixer at 60°C, in a fume hood, for 5 minutes to remove residual ethyl acetate. Residual organic and volatile salts was removed by vacuum drying in a SpeedVac. This step was repeated two times with 50% methanol.

Nano HPLC Q-Exactive plus

A nanoflow HPLC instrument (U3000 RSLC Thermo Fisher Scientific) was coupled on-line to a Q Exactive plus (Thermo Scientific) with a nanoelectrospray ion source. 1 µL of peptide mixture (corresponding to 200 ng of proteins) was loaded onto the preconcentration trap (Thermo Scientific, Acclaim PepMap100 C18, 5 µm, 300 µm i.d × 5 mm) using partial loop injection, for 5 min at a flow rate of 10 µL.min⁻¹ with buffer A (5% acetonitrile and 0.1% formic acid) then separated on column (Acclaim PepMap100 C18, 3 µm, 75 mm i.d. × 500 mm) and separated with a linear gradient of 5–50% buffer B (75% acetonitrile and 0.1% formic acid) at a flow rate of 250 nL.min⁻¹ and temperature of 45 °C. The total time for an LC MS/MS run was about 240 min long and each samples were injected 3 times for statistical treatment.

Mass spectrometry

MS data was acquired on Q Exactive plus (Thermo Scientific) using a data-dependent top 20 method dynamically choosing the most abundant precursor ions from the survey scan (400–1600 m/z) for HCD fragmentation [3]. Dynamic exclusion duration was 60 s. Isolation of precursors was performed with a 1.6 m/z window and MS/MS scans were acquired with a starting mass of 80 m/z . Survey scans were acquired at a resolution of 70,000 at m/z 400 (AGC set to 10^6 ions with a maximum fill time of 180 ms). Resolution for HCD spectra was set to 35,500 at m/z 200 (AGC set to 10^5 ions with a maximum fill time of 120 ms). Normalized collision energy was 28 eV. The underfill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.3%. The instruments was run with peptide recognition mode (ie from 2 to 8 charge), exclusion of singly charged and of unassigned precursor ions enabled.

Data Analysis

The acquired raw files were analyzed with PEAKS 7 studio software (Bioinformatics Solutions Inc., Waterloo, Canada) using the MASCOT 2.5 search engine against the Swissprot_mouse database (<http://www.matrixscience.com/>, Swissprot 2014_07, 16.678 sequences). The peptide mass tolerance was set to 10 ppm and 0.02 Da for MS/MS. Variable modifications included were as follows : oxidation of methionine, tryptophan, histidine, deamidation of asparagine, glutamine, carbamidomethylation of cysteine, phosphorylation of tyrosine, serine, threonine, pyro-Glu. For high-confidence peptide identification a FDR (False Discover Rate) of 1% and a minimum of 2 ranked peptides were used for peptide filtering. Label-free relative quantification software PEAKS 7 was used to analyze RAW data files against the same mouse protein database for identifications with MASCOT 2.5 [4], [5]. Proteins were considered significantly changed between 4T1 mono layer and petri dish tissue protein samples using a $-10 \log P$ score of 20 (equivalent to a p value of 0.01), a fold change ≥ 1.5 , and using a quality value of 1.0.

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Abbreviations

ABC Ammonium Bicarbonate

AGC Automatic Gain Control

DCA deoxycholic acid

eFASP enhance Filter-Aided Sample Preparation

FDR False Discover Rate

TFA Trifluoroacetic acid

HCD Higher energy Collisional Dissociation

HPLC High Performance Liquid Chromatography

MS/MS tandem mass spectrometry

PBS Phosphate Buffer Saline

SDS Sodium Dodecyl Sulfate

Appendix II: List of proteins

Protein	Name
<u>P11276</u> /FINC_MOUSE	Fibronectin
<u>Q9Z183</u> /PADI4_MOUSE	Protein-arginine deiminase type-4
<u>Q9QUJ7</u> /ACSL4_MOUSE	Long-chain-fatty-acid--CoA ligase 4
<u>P10107</u> /ANXA1_MOUSE	Annexin A1
<u>O08553</u> /DPYL2_MOUSE	Dihydropyrimidinase-related protein 2
<u>P06745</u> /G6PI_MOUSE	Glucose-6-phosphate isomerase
<u>P48036</u> /ANXA5_MOUSE	Annexin A5
<u>Q922Q1</u> /MARC2_MOUSE	Mitochondrial amidoxime reducing component 2
<u>P05064</u> /ALDOA_MOUSE	Fructose-bisphosphate aldolase A
<u>P06797</u> /CATL1_MOUSE	Cathepsin L1
<u>P05201</u> /AATC_MOUSE	Aspartate aminotransferase, cytoplasmic
<u>Q9Z1Q5</u> /CLIC1_MOUSE	Chloride intracellular channel protein 1
<u>P12382</u> /PFKAL_MOUSE	ATP-dependent 6-phosphofructokinase, liver type
<u>P16110</u> /LEG3_MOUSE	Galectin-3
<u>P14152</u> /MDHC_MOUSE	Malate dehydrogenase, cytoplasmic
<u>P10923</u> /OSTP_MOUSE	Osteopontin
<u>P31786</u> /ACBP_MOUSE	Acyl-CoA-binding protein
<u>Q9Z0J0</u> /NPC2_MOUSE	Epididymal secretory protein E1
<u>P35505</u> /FAAA_MOUSE	Fumarylacetoacetase
<u>Q62433</u> /NDRG1_MOUSE	Protein NDRG1
<u>P47739</u> /AL3A1_MOUSE	Aldehyde dehydrogenase, dimeric NADP-preferring
<u>P18760</u> /COF1_MOUSE	Cofilin-1
<u>Q9CQ65</u> /MTAP_MOUSE	S-methyl-5'-thioadenosine phosphorylase
<u>Q99KC8</u> /VMA5A_MOUSE	von Willebrand factor A domain-containing protein 5A
<u>Q9D0F9</u> /PGM1_MOUSE	Phosphoglucomutase-1
<u>O35639</u> /ANXA3_MOUSE	Annexin A3
<u>Q00519</u> /XDH_MOUSE	Xanthine dehydrogenase/oxidase
<u>P17751</u> /TPIS_MOUSE	Triosephosphate isomerase
<u>P09411</u> /PGK1_MOUSE	Phosphoglycerate kinase 1
<u>Q9DBJ1</u> /PGAM1_MOUSE	Phosphoglycerate mutase 1
<u>Q61147</u> /CERU_MOUSE	Ceruloplasmin
<u>Q9DCD0</u> /6PGD_MOUSE	6-phosphogluconate dehydrogenase, decarboxylating
<u>P35700</u> /PRDX1_MOUSE	Peroxiredoxin-1
<u>Q7TPR4</u> /ACTN1_MOUSE	Alpha-actinin-1

OUTPUT

Journal publications

Adithya Sridhar, Albert van den Berg, Séverine Le Gac, *Non-invasive monitoring of osteogenic differentiation on microtissue arrays under physiological conditions using scanning electrochemical microscopy*, **Electroanalysis**, 2014, 26, 1881–1885.

Adithya Sridhar, Hans L. de Boer, Albert van den Berg, Séverine Le Gac, *Microstamped Petri dishes for scanning electrochemical microscopy analysis of arrays of microtissues*, **PLOS ONE**, 2014, 9, e93618.

Scanning electrochemical microscopy (SECM) for biological assays, Manuscript under preparation.

Conference proceedings

Adithya Sridhar, Albert van den Berg, Séverine Le Gac, *In situ non-invasive electrochemical monitoring of microtissue differentiation in microwell arrays*, **Microtas conference 2013**, Freiburg (Germany), 27 Oct.-31 Oct. 2013.

Adithya Sridhar, Hans de Boer, Albert van den Berg, Séverine Le Gac, *Stamped Petri dishes for 3D cell culture and drug testing*, **Microtech. Med. Biol. 2013**, Marina del Rey (CA, USA), 10-12 Apr. 2013.

Invited talks (presented by Dr. Séverine Le Gac)

Microfabricated and microfluidic tools for tissue production and study, **Advances in Tissue Regeneration 2013 conference**, Lattrop (The Netherlands), 14-15 Nov. 2013.

Revisiting standard cellular assays using non-invasive scanning probe technique, **AFM Cost workshop**, University of Twente, Enschede (The Netherlands), 22 March 2013.

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Adithya Sridhar, Hans de Boer, Albert van den Berg, Séverine Le Gac, *In situ non-invasive cellular assays using scanning electrochemical microscopy (SECM)*, **ICBME 2013**, Singapore, 4-7 Dec. 2013.

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Samenvatting

Zoals in het begin van dit proefschrift beschreven was het doel van dit project om alternatieve of aanvullende methodes te vinden voor het bestuderen van levende biologische systemen (cellen, weefsel, etc.). Scanning electrochemical microscopy (SECM) werd gekozen om bio-analyses toe te passen op micro-weefsels. Hieronder worden de resultaten van elk hoofdstuk in het kort samengevat.

Hoofdstuk 1 introduceert het fundamentele principe van een SECM-systeem bestaande uit een elektrochemische cel en een scanning probe opstelling. Aanvullend wordt een korte theoretische inleiding gegeven over het bedienen van micro-elektrodes, metingen in de feedback mode en SECM-afbeeldingen. Tenslotte wordt literatuur over SECM-metingen aan biologische systemen gepresenteerd, opgedeeld in extracellulaire en intracellulaire metingen. Terwijl een groot aantal artikelen die toegespitst zijn op SECM en levende systemen vooral zuurstofmetingen laten zien werd SECM onder andere gebruikt voor een groot aantal verschillende analyses zoals intracellulaire redoxmetingen, differentiatie-analyses en het detecteren van celsignalering.

Hoofdstuk 2 beschrijft een nieuw platform dat geschikt is voor SECM-metingen en het vormen van micro-weefsels. In dit platform werden microputjes gerealiseerd door hot embossing in commerciële polystyreen (PS) petrischalen. PS kent niet de beperkingen van bijvoorbeeld PDMS en agarose doordat het niet poreus is en de onderkant van het substraat dun genoeg is om optische en scanning probe microscopie-metingen te kunnen doen. Hierbij werd een speciale set-up gebruikt die het formaat van de petrischalen kan behouden en waarbij de microstructuren alleen in de bodem gemaakt werden. De optimalisatie van het fabricageproces liet zien dat de beste resultaten voor embossing werden bereikt bij 180°C met een 10 min verwarmingsfase en een 15 min afkoelingsfase. De nauwkeurigheid van de afdruk van de PDMS master op de petrishaal werd geverifieerd door een aantal technieken zoals wit-licht-interferometrie, optische microscopie, en rasterelektronenmicroscopie. Uiteindelijk werd nog een coating-procedure ontwikkeld om een cel-afstotend oppervlak te creëren en micro-weefsels werden gevormd in een rooster van microputjes (400 µm doorsnede, 200 µm diepte, >100 putjes) van verschillende cellijnen (HeLa, HepG2, MCF-7, C2C12, en 4T1). De micro-weefsels die in het platform

gekweekt werden, hebben 24 uur na het zaaien een homogene grootte (<10% variatie). HeLa micro-weefsels werden optisch bekeken om de celreorganisatie te karakteriseren en om deze te vergelijken met monolaagmodellen. Bovendien werden 4T1 micro-weefsels geanalyseerd en werd hun eiwit-expressie bekeken ten opzichte van modellen met een monolaag. Bijzonder interessant was dat in micro-weefsels een overexpressie zichtbaar was van metabolische en extracellulaire matrixeiwitten (>40 eiwitten die minstens 2 keer zo hoog zijn) vergeleken met monolaag culturen.

In **Hoofdstuk 3** werd de negatieve feedback mode van de SECM gebruikt om afzonderlijke cellen te visualiseren. Vergelijkbare metingen op micro-weefsels gaven onverwachte resultaten: de vorm van de weefsels week af zoals de niet-uniforme vulling van de microputjes. Dit kon echter niet gedetecteerd worden met de microscopie-technieken die in hoofdstuk 2 werden toegepast. Deze observatie werd toegeschreven aan een lichte schuinstand van de microputjes, een fenomeen dat al bij kleinere microputjes gezien werd (100 μm diameter en 100 μm diepte). Door deze niet-uniforme vulling werd geconcludeerd dat een echte visualisatie van de micro-weefsels in deze petrischalen en met deze configuratie niet mogelijk was. Daarom werden lijnscans als analysemethode voor microweefsels met SECM verkozen en werd deze methode in de volgende hoofdstukken toegepast. Aanvullend werden benaderingscurves gebruikt om te bepalen dat de microweefsels >20 μm uit de microputjes uitstaken waardoor constante-hoogtescanning vanaf het petrischaaloppervlak zonder negatieve feedback gedaan kon worden.

In **hoofdstuk 4** werd een live/dead bio-analyse met de SECM succesvol uitgevoerd. Eerst werd een methode ontwikkeld om een Quick-coating op de platina micro-elektrode aan te brengen met het doel om zuurstof op een stabiele manier te kunnen meten met chronoamperometrie. In de volgende stap werd de zuurstofgradiënt in de omgeving van HeLa micro-weefsels in de microwelletjes gekarakteriseerd. Hieruit werd een hemisferische zuurstof-uitputtingszone zichtbaar welke zich uitstrekte tot meer dan 200 μm vanaf de zijanten van de weefsel. Door deze karakterisering is een hoogte van 15 μm boven het microweefsel gekozen voor de live/dead analyse. Uiteindelijk werden scans met een constante hoogte boven HeLa micro-weefsels uitgevoerd, voor en na de behandeling met een model-medicijn (ethanol), om hun zuurstofconsumptie te meten en hierdoor de levensvatbaarheid van de weefsels te bepalen. De resultaten van de SECM metingen werden

bevestigd met een standaard fluorescentie analyseen cel-impermeabele fluorescente kleuringen. Dit hoofdstuk laat het potentieel van SECM zien, gecombineerd met het weefselcultuur-platform beschreven in hoofdstuk 2 als een adequate combinatie voor bio-analyses. De SECM live/dead analyse maakt niet-invasieve metingen aan weefsels mogelijk en zou toegepast kunnen worden voor farmaceutische testen aan relevante weefselmodellen.

Hoofdstuk 5 beschrijft de implementatie van een tweede bio-analyse: een differentiatie-analyse gebaseerd op de indirecte detectie van alkaline fosfatase (ALP) dat een rol speelt bij osteogene differentiatie. De analyse is gebaseerd op de functie van ALP om moleculen met een fosfaatgroep te katalyseren en om het product van de enzymatische reactie te detecteren. Voor de detectie met SECM werd het p-aminophenyl fosfaat/p-aminophenol (PAPP/PAP)-paar gekozen en in oplossing gekarakteriseerd voordat de analyse werd getest op de micro-weefsels. Cyclovoltammetrie en chronoamperometrie werden toegepast om de detectielimiet van PAP te bepalen, welke voor de twee technieken respectievelijk 50 μM en 10 μM bleek te zijn. In de volgende stap werden C2C12 micro-weefsels behandeld met de botvormende proteïne 2 (BMP-2) wat in de expressie van alkalische fosfatase op het celmembraan resulteerde. SECM metingen werden zes dagen na het zaaien van de cellen doorgevoerd op BMP-2 behandelde micro-weefsels en een controle. Deze indirecte SECM analyse maakte het mogelijk om op levende individuele micro-weefsels differentiatie te detecteren zoals al met commerciële colorimetrische analyses werd aangetoond. Vervolgens voerden wij de SECM-karakterisering op levende micro-weefsels bij verschillende pH waarden (8.5 en 7.5, welke overeenkomen met fysiologische condities) uit. Door het gebruiken van micro-weefsels konden derhalve metingen bij fysiologische condities uitgevoerd worden terwijl normaliter ALP gedetecteerd wordt bij een basische pH (9.5) welke niet optimaal is voor de levensvatbaarheid van cellen. De SECM-gebaseerde differentiatie-analyse zou toegepast kunnen worden op het gebied van de regeneratieve geneeskunde waar monsters van micro-weefsel bewaard moeten worden voor latere bottom-up weefselkweek.

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At the eleventh hour, coincidentally 11 hours before the book is due to the printers, I start writing the most important section and the first one people flip over to. Admittedly, this should have been done earlier but the lack of time gives me an excuse to resort to a trite but true statement. My time as a PhD and it's culmination in the form of this book would not have been possible without the help and support of numerous people.

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Apart from this, one of the memorable things during this time has been the pub quizzes. I have been part of many great teams – Thunderbunnies, BRINF and sexual harassment pandas – and had a lot of fun! Hope we can keep quizzing! Also in the last year and half, I have been playing cricket at Hengelo in the summer. This has been enjoyable and I want to thank everyone at the club.

I also need to thank my many friends outside of Enschede. Varun, Aditya and George, we have remained good friends from our time in Oxford. I always have fun hanging out with you guys and on our road trips. Hope we can do another one soon! Also to my friends in India, who have been entertaining and spamming me daily on different WhatsApp groups – you know who you are. Finally thanks to my mom, uncle and aunt for all their support and trouble they have had to deal with during my education. I hope all your support and motivation come to fruition with me obtaining the doctoral degree.

Cheers,

Adithya

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