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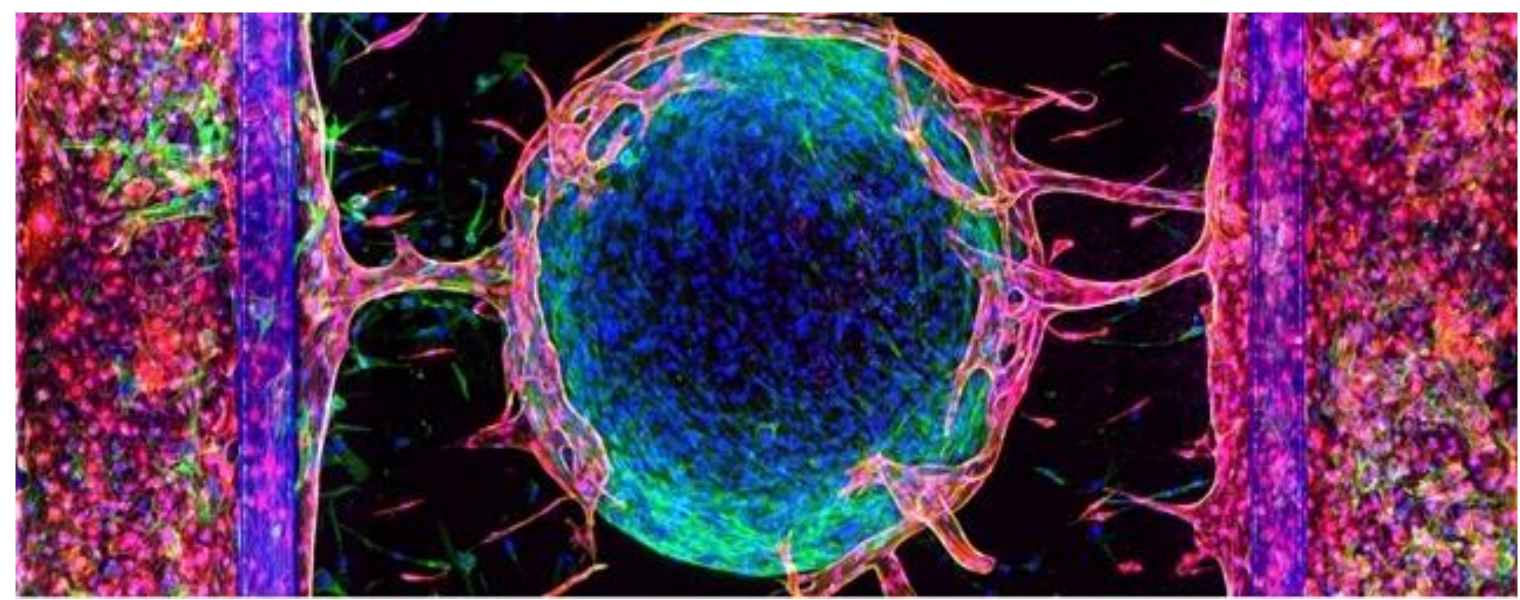

VALIDATION OF IMPEDANCE SPECTROSCOPY AS A TOOL FOR MEASURING CELLS IN A CULTURE

Lana Cleuren

Promotors:

Prof. Sally McArthur — CSIRO
Ph.D. Candidate Sorel De León — CSIRO
Ir. Evelyne Wirix — University College PXL

[Bachelor Thesis] year 2019-2020

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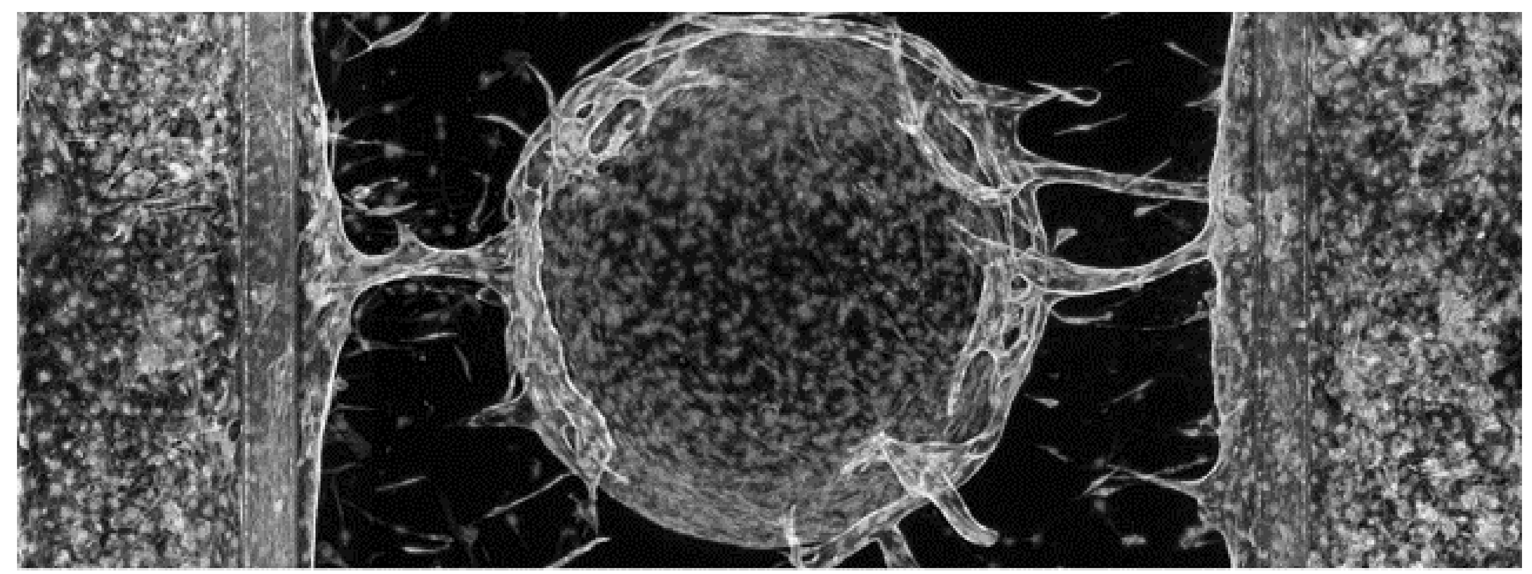

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Acknowledgment

As part of the Agro- and Biotechnology program at the university college PXL Green & Tech, located in Belgium, a bachelor thesis had to be completed. This bachelor thesis included finishing a project that was centred on a Biotechnological research question, with a special focus on research and techniques focussed on cells and genes. This internship was allowed to happen abroad, and to broaden my experiences I opted to complete my bachelor thesis at CSIRO (Commonwealth Scientific and Industrial Research Organisation), located in Clayton, Melbourne in Australia.

The focus of this bachelor thesis is: “The validation of impedance spectroscopy as a tool for measuring cells in a 3D culture”. During this internship it was planned to research impedance spectroscopy on different kinds of cell cultures, such as 2D, 2.5D and 3D. First, impedance measurements were to be made of 2D and 2.5D cultures daily over a time period of ten days and images were made using brightfield microscopy to monitor the cells’ growth. After which the samples were to be fixed and stained so that images could be made with confocal microscopy. Secondly several 2D, 2.5D and 3D cell cultures were to be seeded together and fixed at different points in time to examine cell proliferation, after which they would be stained and examined using confocal microscopy. A third experiment was planned, in which the influence of the thickness of the collagen layer in 3D cell cultures during impedance measurements would be examined. This technique is used in the hope of validating the impedance spectroscopy measurement techniques.

Due to unexpected circumstances caused by the global pandemic COVID-19 and the national shut-down of the labs, the project was interrupted and not all experiments were able to be performed. The first experiment, regarding impedance spectroscopy performed on 2D and 2.5D cell cultures was completed and brightfield microscopy images were made of the cultures daily. The samples were also fixed and stained, but due to the lab shut-down, confocal microscopy could not be completed. This report was constructed from data collected prior to the shutdown.

The successful completion of this project was made possible by a lot of people. First and foremost, I would like to thank my coordinators and promotors, both international and national, Prof. Sally McArthur, Ir. Evelyne Wirix and Ph.D. candidate Sorel De León. Because of their excellent guidance and feedback, I was able to bring this project to a successful conclusion. I would also like to thank the international coordinator of PXL, Kris Moors, for her help in arranging my international internship and her regular check-ins for my wellbeing abroad. I would also like to extend my gratitude to my other co-workers at CSIRO and the other Ph.D. students for offering their help and make me feel welcome during the duration of my internship. Lastly, I would like to thank my parents and my sister for their support during the period of my internship.

I am very grateful to have been given the opportunity to complete my internship at the renowned company CSIRO and for my bachelor thesis to be of use to future research regarding the usage of impedance spectroscopy to monitor 3D cell cultures.

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(05/06/2020)

Validation of impedance spectroscopy as a tool for measuring cell in a culture

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Abstract

Cell culture models are being used increasingly in the biotechnological field to study various biological and metabolic processes that happen in cells, tissues, organs and organisms as a whole.

Here, cell populations are researched *in vitro* and isolated from surrounding tissues and their influences. Recently, these cell cultures have evolved from classic one-layer 2D models to more complex 3D models, in which several cell layers or even cell types are allowed to interact with each other. This to better replicate the physiology of tissues and organisms. In order to work with these complex cultures there is need for an accurate and trustworthy measurement technique that gives information about the cell number, viability, confluence and distribution of the cells within the culture. This measurement needs to happen in real-time and be non-destructive to the sample so that the 3D cell culture can be monitored throughout its growth cycle. A promising upcoming technique is impedance spectroscopy, in which the culture is modelled as an electrical circuit. This technique is commonly used in 2D cell cultures but has yet to be successfully implemented on 3D cell culture models. This due to the added complexity of their setup and the proper integration of the measurement electrodes within the culture. In this study the reproducibility and variability of impedance measurements performed on 2D and 2.5D was tested with the electrodes placed directly beneath the culture. The cultures were also imaged to investigate the location of the cells and their structure relative to the electrodes. While this study was able to successfully compare 2D and 2.5D cell cultures in terms of variability, reproducibility and validation using brightfield microscopy images, some of the more complex experiments had to be cancelled due to COVID-19. In regard to 2D cell cultures it was found that, while impedance spectroscopy is able to give a good representation of the observed cells in regards to dispersion and confluence of the cells, it is too sensitive to the natural variability in cells and cell cultures. This makes reproducibility and validation of the results difficult due to the natural cell cycle and the cell location relative to the electrode surface. These challenges that already present themselves in 2D cell cultures make the difficulty in using this measurement technique with 3D cultures all the more apparent. The main challenge this paper brings forward is how the natural occurring variability in cell cultures needs to be considered when using the highly sensitive impedance spectroscopy. Future research should focus on quantifying this variability. This knowledge will likely prove useful in interpreting impedance data, which will help when transferring these experiments from 2D to a more complex 3D culture model. If these challenges are overcome, the usage of impedance-based measurement systems will provide real-time, non-invasive and quantitative tracking of 3D cell culture systems.

Keywords: Impedance spectroscopy, Brightfield microscopy, 2D cell cultures, 2.5D cell cultures, 3D cell cultures.

Introduction

When studying biological and metabolic processes that occur within cells, tissues, organs and organisms scientist often start by looking at the smallest unit, the cell. To study these behaviours cell culture models are used, in which different cell types can be isolated from the structure of surrounding tissue and examined in a separate, isolated population.

Cell culture models

The term "cell culture" refers to an *in vitro* technique, where eukaryotic single cells are grown as independent units. The type of cells used for this are most often animal cells (Arshadchaudry, 2004).

Before starting a cell culture, cells have to be isolated from a solid block of living tissue or a cell suspension. This isolation can be realised using enzymes (collagenase, trypsin, elastase, hyaluronidase, and protease) that digest the proteins that hold the cells together in the tissue. After this, the loosely bound cells can be separated mechanically. Subsequently, the cells are grown and maintained outside of their natural environment, under controlled conditions (Figure 1) (Hu et al., 2016).

To survive in this artificial environment the cells require a medium or substrate containing all the essential nutrients, hormones, growth factors and gases. The physio-chemical environment also needs to be monitored and adjusted for the cells (pH, temperature, osmotic pressure). Lastly, depending on the cell type a suitable vessel needs to be selected for adherent or suspension cultures (Alberts, Johnson, Lewis, & Al., 2002; Arshadchaudry, 2004).

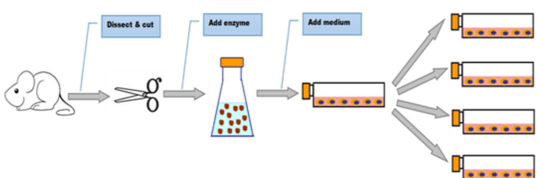

Figure 1 Creation of a primary cell culture (Cloud-Clone Corp., 2016).

Applications of cell cultures

Growing, maintaining, and studying cell cultures has been a fundamental aspect in various research fields of biology and has many applications in biotechnology.

By studying cell cultures our understanding of biological systems (i.e. cells, tissue, and organs) has vastly improved. Cell cultures make it possible to study the cell's metabolism and physiology much more accurately compared to *in vivo* systems. Improving our understanding of these processes in the cell has helped with the development and manufacturing of: viral vaccines, proteins, hormones, enzymes and other medicinal products (Alberts et al., 2002; Arshadchaudry, 2004).

The development of cell and tissue cultures is an important alternative to animal testing or in most cases adds a vital step in the preclinical studies of new medicine. Before the use of cell cultures was standard, it was customary to test a new drug on animal subjects straight after the exploratory phase, even when not all properties and side effects of the new drugs were known. Nowadays, the new drugs are first tested on cell cultures of various relevant cell types and the effects on the metabolism, behaviour and vitality of the cells is first monitored. If suitable, the testing can then be moved from *in vitro* testing in cell cultures to *in vivo* testing on animal test subjects (Artaud, Kara, & Launay, 2019; Zurlo, Rudacille, & Goldberg, 2002).

Another vital advantage that cell cultures offer is the consistency and reproducibility of experiments and results (Arshadchaudry, 2004).

Cell cultures in 2D vs 3D

In traditional cell cultures the cells are grown on flat plastic dishes where the cells will form a monolayer. We will refer to this form of cell culture as a two-dimensional (2D) cell culture. This technique is commonly used in

molecular biology, stem cell research and tissue engineering.

When researching basic life science, drug discovery, cancer biology and regenerative medicine, however, 2D cell cultures proved to be lacking. While it is possible to study varying combinations of cell types and structures in this 2D *in vitro* setup, it is an inadequate representation of *in vivo* conditions. Monolayers of cells do not possess the same behaviour and responses as cells in tissues and organs where the cells are able to have interactions with each other, with the surrounding matrix or cell scaffold in all three dimensions (Figure 2, collagen-coated glass 2D) (Barrila, Radtke, Crabbé, & Al., 2010; Brajša, Trzun, Zlatar, & Jelić, 2016; Duell, Cripps, Schembri, & Ulett, 2011).

This is where 3D cell cultures made their entrée. By simulating a three-dimensional environment cells grow and interact with their surroundings in all three dimensions. In this way, researchers are hoping to better reproduce the spatial organisation and diffusional properties of cells in the body (Figure 2, Collagen gel 3D). By mimicking the *in vivo* environment better, these 3D cell cultures have been shown to produce a biochemical and biomechanical micro-environment (Baker & Chen, 2012; Duval et al., 2017). These microenvironments will hopefully lead to a better representation of how cells function in certain tissues. By studying and understanding these models researchers can try to understand better what happens when the functioning of these cell types are disrupted by disease and drugs (Baker & Chen, 2012; De León, Pupovac, & McArthur, 2020; Duval et al., 2017).

In order to grow 3D cell cultures, a scaffold or matrix is generally used to host and support the cells. Nevertheless, 3D cells can also be grown in a scaffold-free manner using suspension methods. Some of the most common ways 3D cultures are generated use following methods: hydrogels, solid scaffolds, magnetic levitation, low-adhesion plates, nanoparticle facilitated magnetic levitation, and hanging drop plates (Bhattacharya et al., 2012; Hsiao & Tung, 2012; Mapanao et al., 2018).

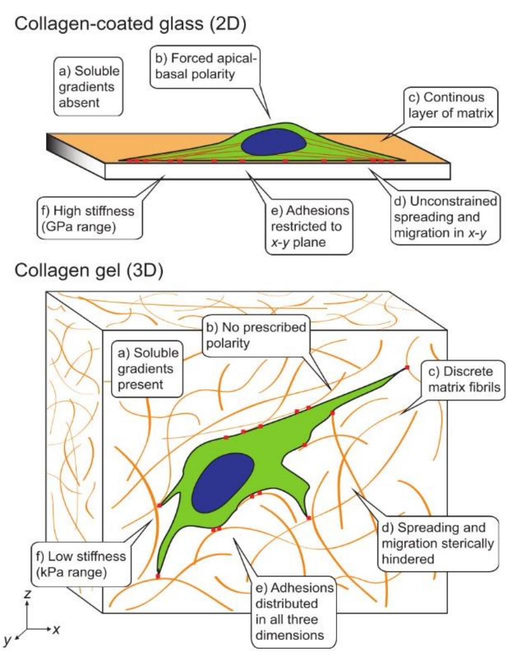

Figure 2 How 3D culture microenvironments alter cellular cues in comparison to 2D cultures (Baker & Chen, 2012).

Alternatively, cells can also be grown in 2.5D, which uses a matrix from 3D cultures, but instead embedding the cells inside the matrix, the cells are grown on top of it. This allows the cells some limited freedom since they are able to partly migrate into the matrix. These 2.5D models help with the understanding of cells that not necessarily grow embedded in a matrix and cell migration. For example, keratinocytes cells form a monolayer when seeded on top of a collagen matrix. This principle is used to build skin models (Bonnet, 2018; Li & Kilian, 2015; Pebworth, Cismas, & Asuri, 2014; Randall, Jüngel, Rimann, & Wuertz-Kozak, 2018; Teimouri, Yeung, & Agu, 2018).

Monitoring of cell cultures

When working with cell cultures it is important to monitor the cell number, viability, and distribution of the cells during their growth cycle and when exposed to external stress stimuli. Especially with 3D cell

cultures since they take a relatively long time to grow into a mature, fully differentiated 3D tissue. To guarantee that the cells are growing correctly, it is vital to accurately monitor the cells in the culture (Baker & Chen, 2012; De León et al., 2020; Duval et al., 2017; Edmondson, Broglie, Adcock, & Yang, 2014).

It is preferable to have monitoring techniques that do not disrupt or destroy the cell culture and do not rely on the usage of staining or other labelling processes so that cell growth can be followed in real-time without having to kill the cells.

The problem with 3D cell cultures is that they differ quite a bit from 2D cell cultures. These differences range from physiological, structural, to metabolite behaviour. Unfortunately, this also means that many of the traditional monitoring techniques developed for the monitoring of 2D cell cultures are not suitable to monitor 3D cell cultures (Brajša et al., 2016; De León et al., 2020; Verjans, Doijen, Luyten, Landuyt, & Schoofs, 2018).

Due to the forming of multiple cell layers on top of each other, bright field microscopy, often used for monitoring monolayer 2D cell cultures, is not a suitable technique for real-time monitoring of 3D cell cultures. This is because the technique does not penetrate the sample deep enough to provide clear images. The two techniques that are most often used for visualisation and validation of 3D models are end-point histology, where the cells are only examined at the end of their growth, and fluorescence microscopy, which only allows real-time monitoring for up to 72 hours, which is not enough for long term cultures. To overcome this, researchers have found that genetically modifying the cells to express green fluorescence protein (GFP) has allowed them to monitor the cultures overtime. Nevertheless, it cannot be ensured that other basic cell behaviour remains unchanged by the genetical modification. (De León et al., 2020; Dmitriev, 2017; Duval et al., 2017; Soboleski, Oaks, & Halford, 2005; ThermoFisher Scientific, 2019).

There is need for a new technique and methodology for the monitoring of 3D cell cultures in real-time.

Possible monitoring techniques for 3D cell cultures

It is possible to measure several parameters of cell health, such as oxygen level, glucose, CO_2, and pH of cell culture media, indirectly without disturbing the sample. In large scale cultures and bioreactors this can be done downstream or in the media with a range of traditional sensors. It is also possible to do this with the usage of transducers which convert one form of energy into another form (Surface acoustic wave (SAW) transducer, micro-electro-mechanical systems (MEMS) sensors, interdigitated ultrasound transducers, electrophysiological transducers based on a field-effect EIS, (bio-)chemical transducers). In this way, signals arising from changes in the cells and media can be converted into measurable parameters (Dantism, Takenaga, Wagner, Wagner, & Schöning, 2015; Modena, Chawla, Misun, & Hierlemann, 2018; Piro, Mattana, & Reisberg, 2018; Poghossian, Ingebrandt, Offenhäusser, & Schöning, 2009).

These techniques are, however, most suited for 2D cell culture models as the media can be easier to extract and measure, plus all the cells are exposed to the same media. In 3D cell cultures, the media does not necessarily reflect the behaviour of the cells as well as it does in 2D. This is because in 3D cultures, cells that are on the outer layer may differ vastly from the ones in the middle due to the different exposure they may have to the cell media around them. When 3D cultures are grown larger than 500 µm they undergo central necrosis, as the nutrients and oxygen supply reaching the centrum is limited, the pH is low, and waste accumulates. The outer layer of the culture stays more viable. In these

cases measuring the media may not represent all the cells in the culture, but only the cells in the outer viable layer since they are actively interacting with the media (Dantism et al., 2015; Modena et al., 2018; Verjans et al., 2018).

Trans-epithelial electrical resistance (TEER) measurements addresses this problem by measuring the permeability of cellular tight junctions. By doing so, epithelial, and endothelial cell health, as well as membrane integrity can be studied. As the name implies, however, research using TEER is mostly done on monolayer and differentiated 2D cultures of epithelial cell types. While a culture model of a monolayer of epithelial cells shares some characteristics with 3D cell culture models, the technique has not been used much on 3D cell culture models (Chen, Einspanier, & Schoen, 2015; Schmitz et al., 2018; Srinivasan et al., 2015).

Impedance spectroscopy

Electrical impedance spectroscopy (EIS) shares the same core principles as TEER. A frequency sweep is done, during which changes in current/voltage are measured. The electrodes are positioned beneath the cell culture, making the current pass through the cells, giving information about the electrical resistance and capacitance of the cells (Figure 3). In this way, cell barrier function, integrity, and cell proliferation can be monitored in real-time. By regularly performing impedance measurements during the cells' growth cycle the spreading, proliferation, migration and death in the culture can be observed (Benson, Cramer, & Galla, 2013; Groeber et al., 2015; Srinivasan et al., 2015).

However, so far this method has only proven to be a trustworthy technique for monitoring 2D cultures as cells are seeded directly on top of the electrode or are in another way in direct contact with the electrode. Because the electrode frequency sweep is very sensitive the presence of cell culture media or collagen matrices can have an influence on the impedance spectroscopy readings (Benson et al., 2013; Groeber et al., 2015).

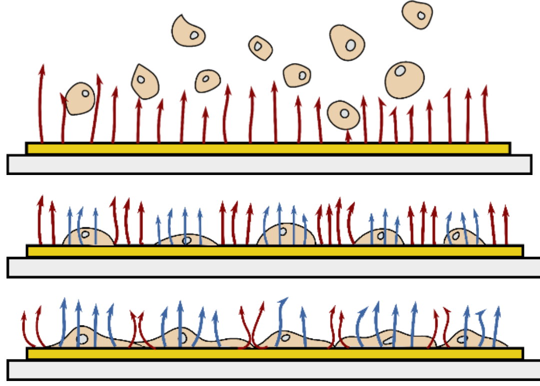

Figure 3 Graphic representation of the changes in impedance measurements depending on the state on the cells seeded on top of the electrode. Red and blue arrows represent the current going from the electrode through the cell (Image created by Sorel De Leòn, 2020).

The sheer complexity of the electrical circuit in the 3D cell cultures makes the usage of impedance spectroscopy as a measurement tool difficult. Mainly the placement of the electrodes within the cell culture model and the impact of this integration into the culture are difficult hurdles to overcome. Feasible results have been gathered when the electrodes are placed on the sides of the cell cultures or above and below the culture (Figure 4). It is still unclear if the impedance measurements prove to be accurate if the electrodes are just placed below the culture (Benson et al., 2013; De León et al., 2020; Groeber et al., 2015).

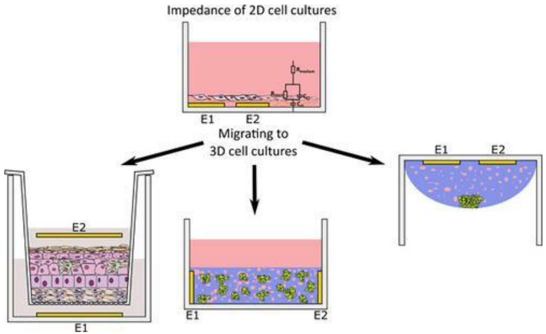

Figure 4 Possible integration methods of impedance spectroscopy electrodes into 3D cell culture models (De León et al., 2020).

While this technique is very promising and has already been applied in numerous cell culture studies, mainly 2D models, it is still not yet suited for accurate, replicable, and standardised measurements for 3D cells and tissue cultures.

Validation of impedance spectroscopy with other techniques

As impedance spectroscopy is a relative new technique with a lot of aspects that are still unknown, there is a need for validation. This means that the results gathered with impedance spectroscopy have to be cross-validated and referenced with results gathered using other more traditional techniques.

Impedance measurements could give an indication about the following parameters in a cell culture: the number of cells present, the spatial organisation of the cells, the number of live and dead cells, cell proliferation rate or response of cells to an external stimuli (i.e. wound healing). To validate these results there is need for a known technique that gives trustworthy information about these characteristics. As microscopic imaging techniques, such as bright field microscopy or confocal microscopy, are the gold standard these are good techniques to cross validate with. The variety of microscopic techniques and applications makes them the perfect tool to address and validate all the parameters that impedance spectroscopy measures (De León et al., 2020; Graf & Boppart, 2010).

By studying the cell cultures under the microscope at different time frames it is possible to monitor the cells' growth, life cycle, and dispersion in the culture. These conclusions can then be compared with the data gathered with impedance spectroscopy performed on the same cultures. By comparing these different kinds of data, the impedance measurements can be validated and a better understanding of impedance spectroscopy in 2D, 2.5D and 3D cell cultures can be achieved.

Interpretation of impedance spectroscopy

Measurements made with impedance spectroscopy of cell cultures are usually analysed by plotting the changes observed in the impedance magnitude and phase in respect to the frequency.

The phase shows the delay/shift the input signal is experiencing compared to the output signal and it gives information about the fundamental behaviour of the system (i.e. resistive, capacitive, or inductive). Cells have a combination of a resistive and capacitive behaviour, blocking the current coming from the electrode (Figure 5, B). The more confluent the cells are on top of the electrode, the more the graph will differ from the wells serving as control (Rahman, Lo, & Bhansali, 2009).

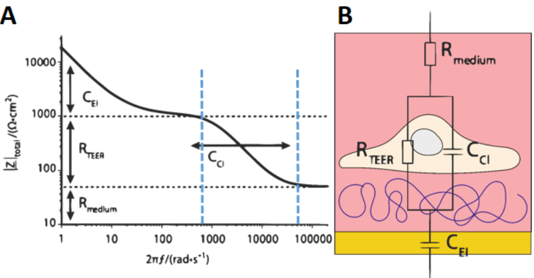

Figure 5 (A) Schematic impedance spectrum of a cell monolayer at different frequencies. (B) Equivalent electrical circuit diagram for a cell monolayer (Benson, 2013).

Understanding the fundamental behaviour of the system in the magnitude may be a bit more complex. However, it is well established that for cell cultures in 2D frequencies from 1kHz to 100kHz give information about the behaviour of the cells in the system (Figure 5, A). As the cells grow on the surface of the electrode the magnitude in this frequency window usually shows an increase in wells containing cells, while this effect is not visible in wells containing only media. This suggests the cells have electrical properties that can be observed in this frequency range. Low frequencies do not penetrate the material above the electrode enough to give out valuable information. This is because the

electrons that are send out do not possess enough energy to travel far, thus information gathered at this low range of the spectrum are related to the adsorption of proteins at the immediate surface of the electrode. Frequencies between 10^3 Hz and 10^6 Hz can penetrate and measure the elements directly above the electrode, meaning cells and media (Figure 5, A). Here the difference between control wells and wells with cells will be observed. Frequencies higher than this will represent measurements of the solution above the cells. This explains the shape of the magnitude graph where all measurements start and end more or less the same. The magnitude gives more information about the size and confluence of the cells present on top of the electrode (Benson et al., 2013).

Another useful analysis of impedance data is a culture-time analysis in which a culture is measured for multiple times during a couple of days, as it can give information about how the cells are proliferating over-time and the cells' viability. If the cells are not shown to be proliferating, this technique can also be used as an indication of cell death. The data is normalised using the data of wells that serve as controls. The x-axis contains the time, while the y-axis represents the normalised magnitude. In this report only over-time data for a frequency of 31.2 kHz ($3.12e^{+04}$ Hz) is shown. This frequency was chosen because it is known to correlate with information about the cell behaviour as described above (Figure 5, A).

The most important questions that can be answered with the data analysis are:

- Can a difference be measured between the wells that contains cells and the wells that act as a control?
- How well do the cell samples match each other?

This will answer questions regarding the reproducibility of the experiment and the growth variability that can be observed in cells.

Material and methods

For the completion of this project following materials and methods were used.

General equipment

During this project transformed cell lines derived from human tissue were used. Due to this the experiment was conducted in a biosafety cabinet type II (Labconco Logic, Class II Type A2).

All equipment and the biosafety cabinets were regularly disinfected with ethanol (80%) to avoid contamination.

After usage, the biosafety cabinets were switched to a UV-light mode for 30 minutes to sterilise the cabinet before the next usage.

Sample identification

During this project, all experiments were conducted with the cell line BJ-5Ta (ATCC CRL-4001). This cell line is derived from neonatal human foreskin. The cell type is fibroblasts immortalised with hTERT and is adherent. These type of cells need to be contained at a biosafety classification 1 (ATCC, 2018).

Cell culture maintenance

The cell cultures were maintained in a culture medium containing a 4:1 mixture of Dulbecco's medium (DMEM) (4 mM L-glutamine, 4.5 g/L glucose and 1.5 g/L sodium bicarbonate) and Medium 199. The medium was also supplemented with Hydromycin B (0.01 mg/ml) and 10% Foetal Bovine Serum (FBS) (Sigma-Aldrich Co. LLC., Germany). The instructions for this growth medium are from the company ATCC who delivered the original cell line (ATCC, 2018).

The cells are kept in a CO_2-incubator (Binder) at 37°C and a CO_2 level of 5%.

Cells are to be passaged regularly (1:2 to 1:3 twice weekly) into 75 cm^2 flasks or when the cell concentration reached between 8 X 10^3 and 1 X 10^4 cells/cm^2. During this process, after the old media is removed, the cells are

washed with phosphate buffered saline (DPBS) (200 mg/L KCl; 200 mg/L KH_2PO_4; 8000 mg/L NaCl; 2160 mg/L $Na_2HPO_4^{-}7H_2O$; pH 7.0 – 7.3) (Life Technologies GmbH, Darmstadt, Germany). The cells are then loosened from the culture flask with trypsin-EDTA (0.25%, Life Technologies GmbH, Darmstadt, Germany) which breaks the bonds between the cells and the flask and the cells themselves.

The cells are then incubated with the trypsin-EDTA for 6 minutes at 37°C to help break all bonds. After this the trypsin-EDTA is neutralised by adding more culture medium. This mixture is later removed by spinning the cells down for 5 minutes at 250 xG in a centrifuge (Thermo Scientific, Heraeus Megafuge 8 Centrifuge) after which the supernatant is removed.

After resuspension of the cells in 2-5 ml of media, the cells are passaged. Depending on the confluence previously, the ratio at which the cells are passaged may vary.

The medium has to be renewed every 2 to 3 days (ATCC, 2018).

For the complete protocols, please refer to Annex 1: Preparing of culture media; Annex 2: Changing of culture media; and Annex 3: Cell culture passaging.

Impedance spectroscopy of 2D and 2.5D cell cultures and fixing of samples after 10 days

For the impedance measurement experiments the following well-plate was used: Electric cell–substrate impedance sensing (ECIS) 8 well chamber with single circular electrode (0.049 sqr.mm) (SDR Scientific) (Figure 6).

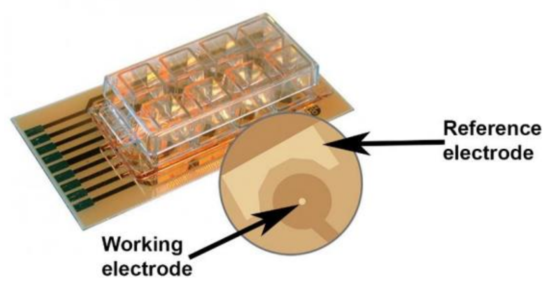

Figure 6 ECIS 8 well chamber with in-plane electrodes (SDR Scientific).

The wells were constructed on a polyethylene terephthalate (PET) sheet, on which the electrodes were placed. Eight wells with a surface area of 0.8 cm^2 were glued on top of it. The unit contained a gold reference electrode (RE) and a gold working electrode (WE) with a diameter of 250 µm.

During this experiment, the wells were first treated with 200 µl of 10 mM L-cysteine (Sigma-Aldrich) in reverse osmosis (RO) water for 10 minutes, then rinsed with RO water twice. The wells were then filled with duplicates of different conditions (media, 2D cells, collagen and 2.5D cells).

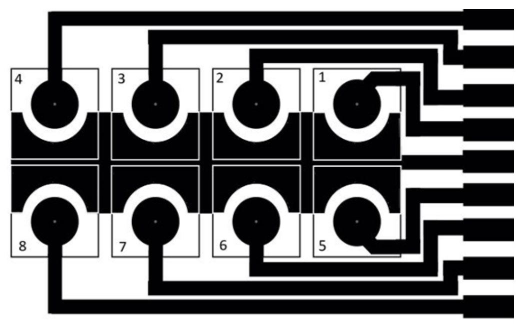

Figure 7 ECIS 8 well chamber with single circular electrode (Biophysics, 2020).

Well 1 and 2 were filled with a collagen layer and topped up with media to serve as a blank for the 2.5D wells. Well 3 and 4 contained the same collagen layer and 30000 cells were seeded on top of the collagen to create a 2.5D cell culture that was then topped up with media. Well 5 and 6 contained regular media and served as a blank for the 2D wells. Well 8 and 7 contained 3000 cells in a 2D culture (Figure 7).

During the following ten days the impedance spectrum was recorded every 24 hours. Before the measurements were performed, the wells were left at room temperature for 30 minutes. This was done to stabilise the temperature of the media and the wells. During this time images were made of the wells using brightfield microscopy.

The well system was then connected through cables with crocodile clips to a MFIA Impedance Analyser (Zurich Instruments AG Switzerland). The impedance analyser was controlled using a custom software initially developed by Dr. Steve Beguin and later customised for this work by Sorel De Leòn in Matlab R2016b (MathWorks company, USA). The data was analysed using Matlab R2016b and a python script written in a Jupyter Notebook. The impedance spectrum was collected from 10 Hz to 5 MHz. To get the impedance of the system, a small sinusoidal voltage (10 mV) was applied for a few milliseconds.

On the tenth day the wells and collagen cultures were fixed using formaldehyde (3.7%) and stored in DPBS until the staining could occur.

For the complete protocols, please refer to Annex 4: Seeding of cells in electrode wells; Annex 5: Impedance measurements; and Annex 6: Fixing of 2D, 2.5D and 3D cell cultures.

Impedance spectroscopy and fixing of 2D, 2.5D and 3D samples at 24h

During this experiment, the wells were also filled in four different ways and tested in duplicates to minimise the risk of faulty results.

Well 1 and 5 were filled with a 3D cell culture. Cell suspension and cell media was mixed with the collagen mixture and seeding into the wells. Once the collagen with the cells inside had set, they were topped up with media. Well 2 and 6 were used to make 2.5D cell cultures in the same way as the previous experiment describes. Well 3 and 7 contained 2D cell cultures. Well 4 and 8 contained collagen layers to serve as a blank (Figure 7).

The impedance was measured at 0 hours, 4 hours, and 24 hours after seeding. The wells were then fixed using formaldehyde (3.7%) and stored in DPBS until the staining could occur.

For the complete protocols, please refer to Annex 7: Seeding of 2D, 2.5D and 3D cell cultures in well plate for fixing after 24h and imaging with confocal microscopy; Annex 5: impedance measurements; and Annex 6: Fixing of 2D, 2.5D and 3D cell cultures.

Note that these samples were fixed for later research using confocal microscopy in a next experiment. These samples were not analysed during this project. Therefore the data gathered from impedance spectroscopy was also not used and will not be discussed further in this paper.

Staining of samples

Samples were stained in two ways during this project.

After impedance measurements were gathered over 10 days the collagen samples were removed and fixed for later usage. The empty electrode wells were stained with sterile trypan blue and incubated for 2 minutes after which images were made using the brightfield microscope. All wells that contained cells were stained. This was done to see whether some cells had migrated through the collagen and attached to the bottom of the well.

All fixed collagen samples that were stored in the 48 wells plate and the electrode wells from the 24 hours impedance spectroscopy were stained with a mixture containing phalloidin (1:40) and Hoechst (1 µg/ml) which were diluted in DPBS.

The stained samples were then stored in the refrigerator to be used for confocal microscopy in a later project. In this project

the samples were not analysed due to a shutdown of the labs as a result of the COVID-19 pandemic.

Results & Discussion

Impedance spectroscopy of 2D cultures over 10 days

During this experiment two electrode wells were seeded with cells and topped with cell culture media. Over the course of ten days the impedance spectroscopy was executed at the same time daily. Measurements on day four and five were skipped due to the weekend. All measurements were plotted along with the controls. The controls are wells containing only cell culture media. Images of the wells were made during the 30 minutes wait time after the wells are removed from the CO_2-incubator using bright field microscopy.

Following is a presentation and discussion of the measurements and pictures taken during this over-time analysis. In the graphs, the measurements in blue represent the impedance of the wells that served as blanks, containing only media. The first well (media 1) is represented by a solid line, the second well (media 2) by a dotted line. Measurements of the wells containing cells in a 2D culture are represented by purple lines. Once again, the first well (2D 1) is indicated by a solid line, and the second well (2D 2) by a dotted line.

The first measurement was conducted four hours after the cells were seeded into the wells (Figure 8). On the microscope pictures the cells still appear quite round in shape, indicating that they have not yet attached to the well's surface (Figure 8, 4h Well 1 and 2). The fact that the cells have not yet attached to the electrode explain why the impedance measurements show little to no difference between the wells with (purple lines) and without cells (blue lines) (Figure 8, 4h Magnitude and Phase graphs). For impedance to be measured cells need to be attached on top of the electrode or the current sent out from the impedance analyser just passes through the media directly above it (Figure 3).

After 24 hours (Figure 8) the cells have begun to attach to the well's surface, which can be observed by their shape, as the cells have flattened and spread (Figure 8, 24h Well 1 and 2). The graphs made using the impedance data show that the second well containing 2D culture (dotted purple line) is different from the control wells (blue lines), while 2D well 1 (solid purple line) is still very similar to the control wells (Figure 8, 24h Magnitude and Phase graphs). This difference can be observed in both the magnitude and the phase graphs. This makes sense when the microscopic images are compared to one another. In sample 2D 2 (Figure 8, 24h Well 2), it can clearly be seen that there are more cells present on top of the electrode. The impedance magnitude around 10 kHz increases because of this, this is an expected result for 2D cultures (Figure 8, 24h Magnitude graph). While sample 2D 1 (solid purple) still closely resembles the control wells a minor divergence from the control lines can be observed in the magnitude graphs, showing that even a small amount of cells on the electrode still seems to have an influence on the measurement (Figure 8, 24h Magnitude graph).

After 48 hours (Figure 8) passed the difference between the two wells containing cells seems to have lessened in the magnitude graph (Figure 8, 48h Magnitude graph), as well as in the phase graph (Figure 8, 48h Phase graph). The changes in magnitude of the impedance measurements in regard to the control wells can be explained by the fact that now the frequency window is actually picking up on the presence of cells in 2D 1. The phase graph also shows a more comparable graph line between well 1 and well 2 (Figure 8, 48h Phase graph). In the brightfield microscope images it can be observed that cells in well 1 have now

covered the electrode more and show a comparable result in the impedance measurements (Figure 8, 48h Well 1). This can be explained by the cells gradually covering the surface of the wells and trying to achieve an equal confluence throughout the well, leading to a more consistent covering of the electrode.

The next measurement took place after 72 hours (Figure 8) and the difference between control wells and cell samples wells in both graphs can still be observed (Figure 8, 72h Magnitude and Phase graphs). The graphs of the samples' wells do not match up quite as well as they did at the 24-hour mark, but the difference is so small that it is not significant at this point in time. Because the difference is so small it is hard to designate a single cause for it. Most likely it can be explained by the individuality of cells in the population and their differences. It is possible that the orientation of the cells have an influence on the impedance measurements (location of nucleus, cell spurs) in regard to the electrode, as different cell components have a different level of resistance to the frequency passing through the cell (Figure 8, 72h Well 1 and 2).

However, this can only be verified by staining the cells at this time interval and examining the samples using a more accurate microscopic imaging technique, such as confocal microscopy. While this was designed

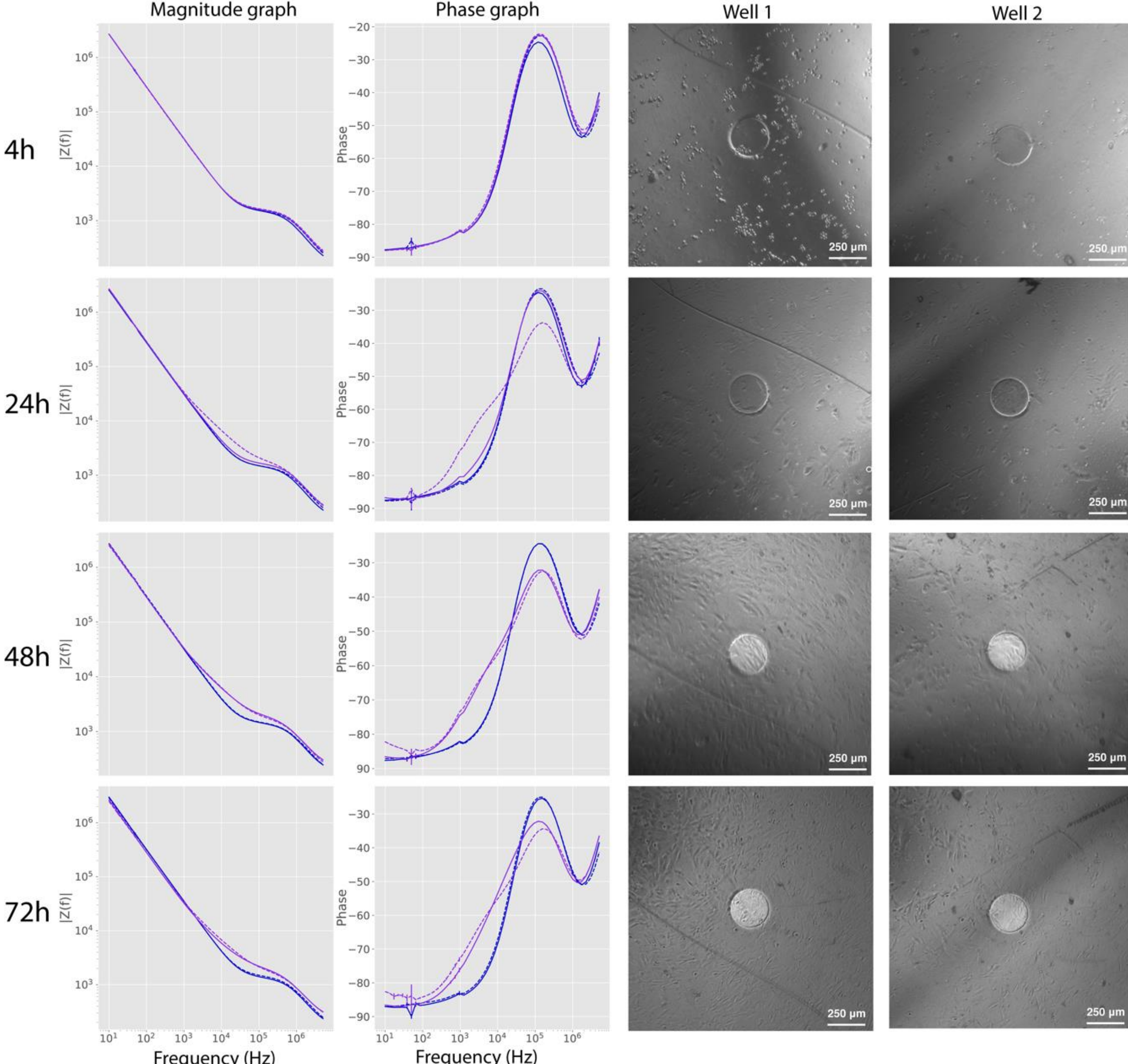

Figure 8 Plotted data of 2D wells and media blank wells 4, 24, 48 and 72 hours after seeding with corresponding brightfield microscopic images.

to be undertaken within this study and the samples were prepared, the data was not able to be collected due to COVID-19 related lab restrictions.

Measurements from day 4 and 5 after seeding were not collected due to it being the weekend. This causes the measurement 6 days after seeding to have significant and seemingly sudden differences from the 72 hours measurement.

Six days (Figure 9) after seeding the microscopic images show that the cells in well 1 (Figure 9, 6d Well 1) seem to be more confluent than the cells in well 2 (Figure 9, 6d Well 2). However, the cells in well 2 appear to be larger in size (Figure 9, 6d Well 2). In the phase graph of well 1, a phase change from -90 to 0 can be observed which indicates a change from a capacitive to a more resistive behaviour, represented by a higher peak (Figure 9, 6d Phase graph). This more resistive behaviour could be caused by the higher confluence, causing tighter junctions between the cells compared to well 2. While in well 2 the bigger cells and more spaced out orientation could be responsible for the more capacitive behaviour.

As said before experiments with more accurate imaging using confocal microscopy could help verify these assumptions, but these experiments, while planed, could not be completed due to the COVID-19 shutdown.

At the 7-day mark (Figure 9) the phase peak of well 1 lowered slightly, while the phase peak of well 2 has risen from around -42 to -35 and is now above the graph of well 1 (Figure 9, 7d Phase graph). In the magnitude graph well 1 is now above well 2 while the reverse was observed the previous day (Figure 9, 7d Magnitude graph). On the microscopic pictures it is clearly visible that the cells in both wells are getting overly confluent and may be losing some vitality (Figure 9, 7d Well 1 and 2). It is possible that cells in well 2 may be deteriorating at a faster rate than the cells in the other well due to their higher confluence at the 72 hour mark and this could be correlated with their decrease in magnitude of well 2 (Figure 19, 7d Magnitude graph).

After 8 days (Figure 9) both the magnitude as well as phase of well 1 does not seem to have changed much from the previous day. The phase graph of well 2 seems to regress a little bit and take on a similar shape of graph to well 1 (Figure 9, 8d Phase graph). The magnitude graph shows little to no change for both wells (Figure 9, 8d Magnitude graph). Both wells are now becoming overly confluent and are starting to lose cell vitality (Figure 9, 8d Well 1 and 2).

Nine days (Figure 9) after seeding the magnitude graphs of the two wells seem to be almost overlapping (Figure 9, 9d Magnitude graph). While the phase graphs are not overlapping completely, they are more similar to each other than they have been in a while (Figure 9, 9d Phase graph). This similarity observed in the graphs is mirrored in the microscopic images where the cells seem to be very similar in shape and have a similar confluence (Figure 9, 9d Well 1 and 2).

After 10 days (Figure 9) the graphs show a very similar shape in both the magnitude as well as the phase graphs (Figure 9, 10d Magnitude and Phase graph). After this much time has passed since seeding the cells are overconfluent in both wells and both are starting to deteriorate at a similar pace. This causes the small differences between the two wells to lessen. The microscopic images resemble each other a lot and the previous observed differences in confluency and cell size has decreased (Figure 9, 10d Well 1 and 2).

After these impedance measurements were taken the wells were fixed, stained and stored for later analysis with confocal microscopy, which was not possible to be performed in this experiment due to COVID-19.

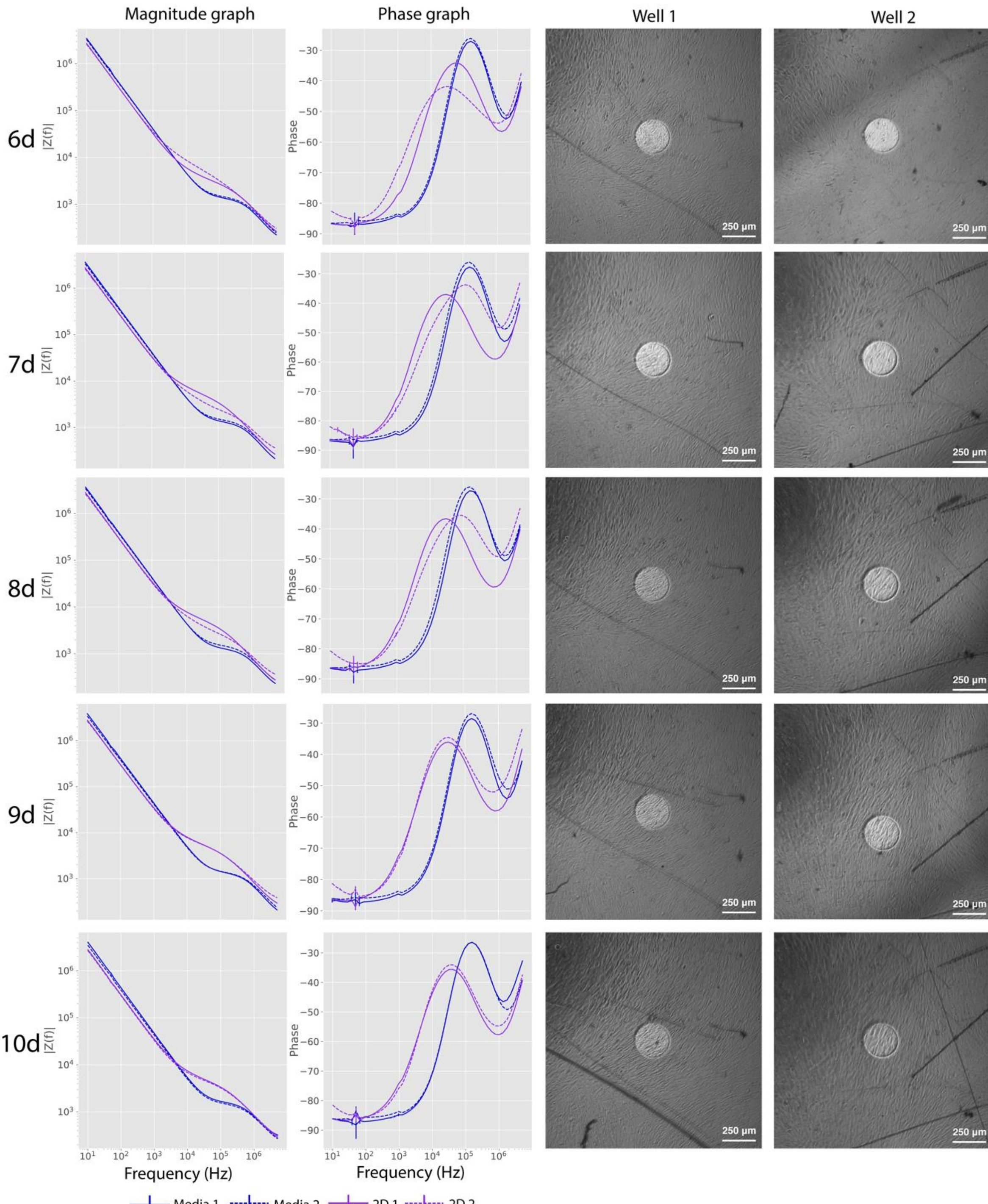

Figure 9 Plotted data of 2D wells and media blank wells 6, 7, 8, 9 and 10 days after seeding with corresponding brightfield microscopic images.

Since the two wells, which in theory contain the same cell culture, result in different impedance measures we can conclude that reproducibility is hard. Which in turn would make it hard to make conclusions on how cells react to a certain condition or drug treatment.

The observed variability is likely the result of differences in cell behaviour, because, while similar, no two cells are exactly the same in growth patterns and behaviour. This small difference between cells and cell populations on top of the electrode caused big differences when measuring impedance of a cell culture.

While we can, from a logical point of view, say that this is a phenomenon that is universally observed in cell research, previous studies using impedance spectroscopy have not discussed the problem in depth.

While the experimental setup of the research of Catríona M. Dowling in 2014 is quite similar to the one used in this paper there are some key differences that might explain why the individual cellular differences are not discussed (Dowling, Ors, & Kiely, 2014). This research also uses a fibroblastic type of cell and the cells are monitored in individual wells that contain a circular electrode. They used triplicates of each well setup they were testing. A key difference however is that, in this research, samples were only kept for 24 hours to monitor the adherence and proliferation of recently seeded cells. This short time frame might explain why the variability of individual cells are not discussed, since we only observed major differences after the first 24 hours. Minor differences between the wells in this short time frame were easily negated by implementing error bars in the charts relaying the results (Dowling et al., 2014).

In another experiment executed by Trong Binh Tran in 2016, also using fibroblastic cells, experiments are again only done in a short time frame of 24 hours (Tran, Baek, & Min, 2016). Again, triplicates were used here to negate the possibility of faulty or false measurements. A noteworthy aspect of this experiment is that all measurements are adjusted to normalise the systematic differences that may possibly occur between the wells. It is possible that the normalising also made the variability of cells less prominent in the data analysis (Tran et al., 2016).

So far the variability that can be observed between cells has not been touched upon when studying the usage of impedance spectroscopy for monitoring cell cultures. Perhaps this is because other experiments did not observe the cultures long enough for this phenomenon to become noticeable or because a difference in experimental setup makes it less prominent. It could also be possible that it was simply not mentioned by the researchers.

While it is hard to make these assumptions and guesses on why it has not been previously discussed, especially since the smallest difference in experimental setup can have a big impact on impedance measurements, we can look at our own experiment and try to look for ways to reduce the impact of it.

One possible solution that might lessen these differences is the size of the electrode. When using a bigger electrode more area of the population is measured and causes a more representative read-out of the population's characteristics. While this will not remove all variability, it will hopefully lessen the variability a little, making it easier to assess the population's characteristics using impedance spectroscopy and lead to representative conclusions made from impedance readings.

Impedance spectroscopy of 2.5D over the duration of 10 days

Concurrently with the previous experiment, wells containing 2.5D cell cultures were also measured and examined using impedance spectroscopy and brightfield microscopy.

The impedance measurements from the wells containing a blank, collagen and cell culture media, and the wells containing the collagen with cells on top do not show a lot of difference in results over-time. The electrode probably has difficulty reading signals from the collagen matrix.

To illustrate this a graph was made at the 48-hour mark (Figure 10) using the impedance measurements of all wells that served as blanks, meaning wells containing only media and wells containing media and collagen. On these graphs it is clearly visible in both the

magnitude as the phase graphs that there is no difference in the spectrum between the two types of wells any different from each other (Figure 10, Magnitude and Phase graphs). The collagen layer is probably not attached on the electrode but instead 'floats' in the media. A thin layer of media between the electrode and the collagen disrupts the measurement and causes the analyser to read the well as containing only liquid.

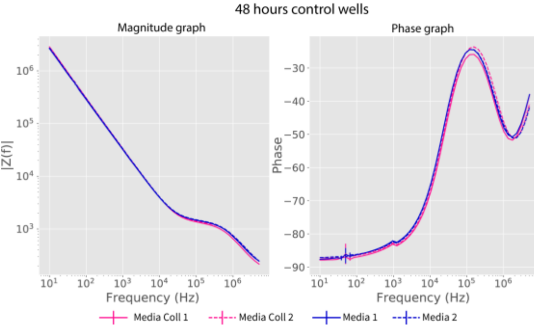

Figure 10 Plotted data of all wells serving as controls, measured 48 hours.

Because of this phenomenon the cells that were seeded on top of the collagen layer were not measured during this experiment. Their plotted data shows no difference between the blanks, no matter the point in time the measurement was taken (Figure 11).

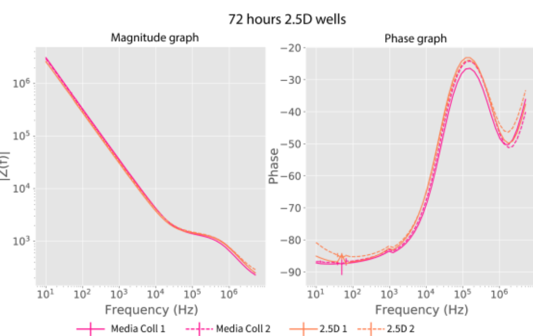

Figure 11 Example of plotted data of 2.5D wells and collagen/media blank wells 72 hours after seeding.

In future experiments a solution must be found for this problem that makes the collagen layer stick directly on top of the electrode.

It is also noteworthy that as the cells were reaching confluence the collagen layer seemed to fold in on itself. This was probably caused by the tension caused by the cells on top (Figure 12). This caused it to be even harder to gather accurate data as the

collagen tended to float to a corner of the well and not stay on top of the electrode during measurements and making microscopic images.

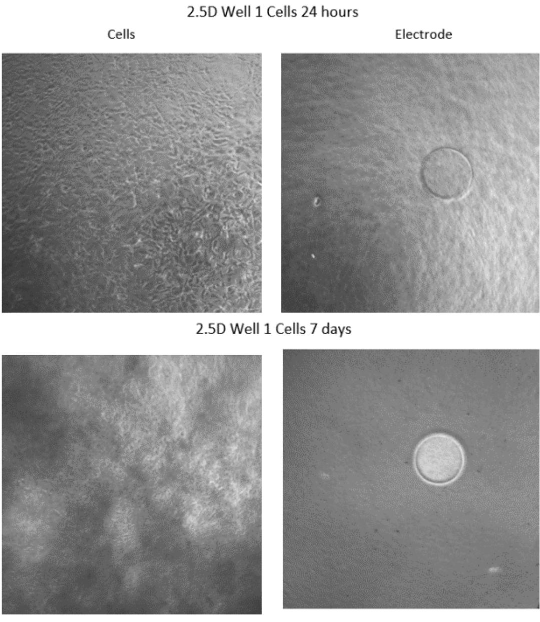

Figure 12 Comparison of well 1 24 hours after seeding and 7 days after seeding. Microscope focus on cells on top of the collagen and focus on the electrode.

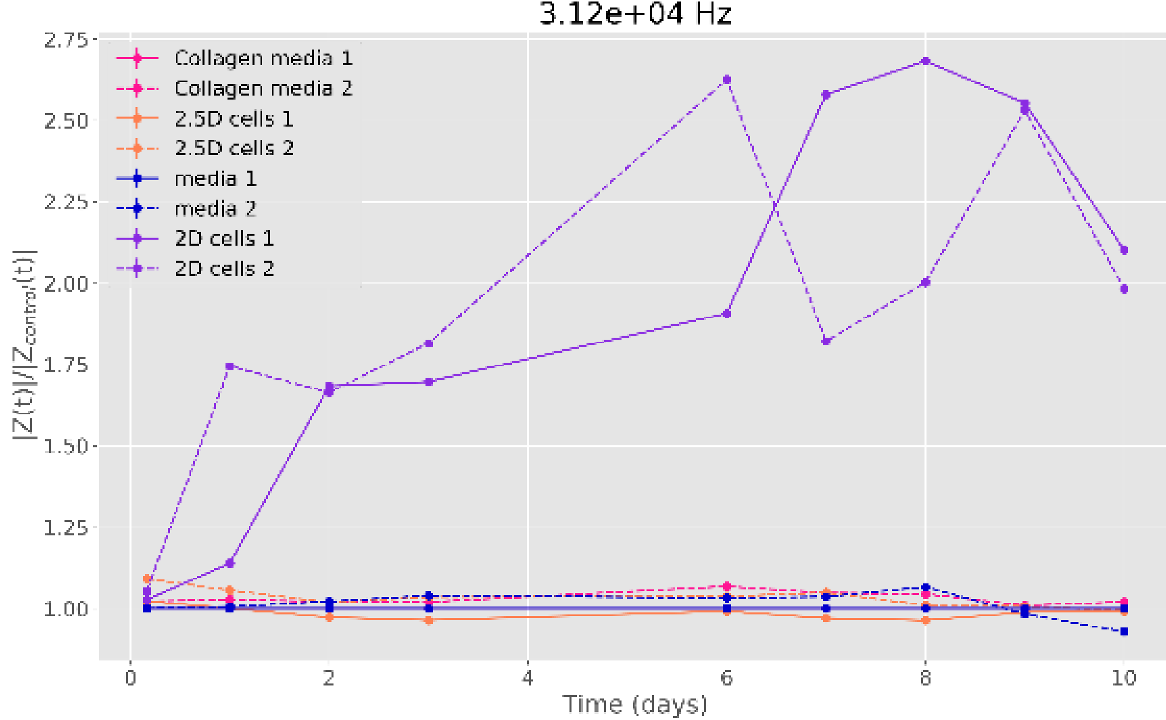

Figure 13 Plotted data of the over-time analysis of all wells over the course of ten days using the 3.12e^{+04} Hz frequency.

Changes in impedance over time

To see the evolution of the measured magnitude of the samples over the period of 10 days, an over-time analysis was made (Figure 13). The data of all samples was combined into one graph where the x-axis contains the timeframe of all the days on which measurements were taken. The y-axis contains the Magnitude of the samples, normalised using the data of the wells that served as blanks. The 2D wells were normalised using the blank wells that only contained media, and the 2.5D wells using the wells that contained media and a collagen layer. In the graph the collagen media wells are represented with a pink colour, where well 1 is a solid line and well 2 is dotted. This trend continues with the blank media wells in blue, the 2D wells in purple and the 2.5D wells in orange.

As discussed with previous results the two kinds of blanks (pink and blue) give very similar readings, indicating that the collagen layer is not properly registered by the electrode. Because of this the measurements of the 2.5D wells (orange) does not show any variations either, even though the cells on top of the collagen showed growth proven by the microscopy pictures and the contraction of the collagen. The 2D wells (purple), however, show a big variation. While both wells differ significantly they both show roughly the same pattern in the beginning and the end of the experiment. Well 2 (dotted) shows an earlier rise in Magnitude than well 1 which is consistent with the earlier conclusions at the 24-hour mark (Figure 9, 24h Magnitude). After two days the measurements overlap again, only to show very different behavior in the later days. These observations are the same as earlier discussed in the 'Impedance spectroscopy of 2D cultures over 10 days' section. At the end of the experiment both wells seem to have a similar decline in Magnitude measurement, indicating the wellbeing of the cells is worsening as they have become overconfluent in the same environment for too long.

Conclusion

Regarding impedance spectroscopy with 2D cell cultures measured by an electrode below the culture it can be said that, to properly serve as a reference, the experiments need to be repeated using a bigger electrode. The current size of the electrode caused minor differences between two wells that were expected to be the same, to have a big impact on the measured data. While all parameters of the duplicate wells were kept the same throughout the 10 days of the experiment, the readings received from the impedance spectroscopy were not very comparable. When, in future research, using a bigger electrode this problem may be resolved as a bigger surface area may negate small differences such as the diffusion of the cells on the wells surface, their orientation and shape.

However, it is a given that there will always be differences between duplicate wells, and while the bigger electrode might make the measured variability smaller, it will not make it go away. Cells always have individual characteristics, and even cells of the same kind and grown in the same circumstances will not have the same duplication rate, growth rate, time to attach or cell spread. Further, variabilities related to pipetting or clusters of cells may have an effect too. This makes it so that similar populations will not show the same confluence, spread or coverage as another population.

The variability of cells and cell populations makes it hard to have a 100% reproducibility in cell culture research. The experiment would have to be repeated many times, and the data would have to be combined in a graph that shows the level of variability that can be expected in a healthy cell cultures so that the right conclusions can be drawn when using impedance spectroscopy to make statements about the state of a cell culture.

This is already hard to achieve with the, relatively, simple 2D cell cultures, but using this measurement technique on the more complex 2.5D and 3D cell cultures poses many challenges. In this research, the experiment using 2.5D cell cultures has not led to the gathering of usable data, as the nature of the collagen layer in the wells did not allow accurate readings.

The design of these kinds of experiments, in 2D as well as 3D, needs to be carefully thought through, and the experimental setup needs to be adjusted accordingly to what one hopes to measure and research in their experiment.

Some of the differences observed in this experiment are sometimes hard to pinpoint on a specific cause. In future research, usage of more accurate microscopic techniques to image the cells can help make it easier to explain abnormalities in the gathered data with impedance measurements, such as with confocal microscopy. Sadly, it was not possible to do this during this experiment due to COVID-19 complications.

Overall, it can be said that experiments using cell cultures can always count on a certain amount of variability being present due to the very nature of cells and cell populations. Finding accurate and representative ways to measure these cultures is a difficult process that requires many repeats of experiments to try and tune out the natural variability occurring.

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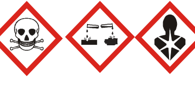
Annexes

Annex 1: Protocol: Preparing of culture media

- Ensure proper PPE is worn and disinfect gloves regularly.
- Disinfect the Biosafety cabinet Type II (Labconco Logic, Class II Type A2) with 80% ethanol (H225, P 210).

- Disinfect all needed materials and bring them into the biosafety cabinet:
  - Dispenser pipette (Thermo Scientific Matrix)
  - Micropipettes (Thermo Scientific Finnpipette F1)
  - Graduated pipette (50 mL) (Falcon Serological Pipet)
  - Micropipette tips (Interpath Services, Aerosol Barrier tips, Pre-sterilized)
  - Falcon tube (50 ml) (Falcon Polypropylene conical tube)
  - Vacuum filter pump with flask (Thermo Scientific Nalgene Filtration Products)
- Disinfect all needed chemical containers and bring them into the biosafety cabinet:
  - DMEM (Dulbecco's medium) (4 mM L-glutamine, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate)
  - Medium 199
  - Hydromycin B (H330-300-310-318-334, P260-284-264-262-280-361+364-261-304+340-310-301+310-330-361-302+350-321-305+351+338-304+341-342+311)



  - Foetal Bovine Serum (FBS) (10%)
- Take a waste beaker and add a little bit of bleach (10%, H31-34-50-41, P1/2-28-45-50-61) to it, then disinfect and bring into the biosafety cabinet.

- Take the DMEM culture media flask (500 mL) and remove 100 mL from it with a dispenser pipette using a 50 mL graduated pipette. Dispose the 100 mL in the liquid waste beaker.
  !!! Take caution not to touch anything with the pipette to avoid contamination!!!
  If you touch the flask or any other surface change the pipette.
- Add 100 mL of Medium 199 into the DMEM flask with a new 50 mL graduated pipette. Keep the previous warning in mind.
- Resuspend the mixture with a 50 mL pipette or mix by hand (gently swirl the flask).
- Remove 50 mL from the DMEM/199 mixture and dispose it in the liquid waste beaker.
- Add 50 mL of FBS (Foetal Bovine Serum).
- Resuspend the mixture with a 50 mL pipette or mix by hand (gently swirl the flask).
- Retrieve Hydromycin B from the refrigerator.
  - The stock mixture is 50 mg/mL
  - The concentration needed in the final 500 mL of culture media is 0.01 mg/mL
  - Calculation:

$$C_1 * V_1 = C_2 * V_2$$
$$50 \frac{mg}{ml} * V_1 = 0.01 \frac{mg}{ml} * 500\ ml$$
$$V_1 = 0.1\ ml = 100\ \mu l$$

- Resuspend the mixture.
- Filter the media with a 0.2 mm filter and a vacuum flask to ensure the media is sterile.
  - Set up the vacuum flask and filter.
  - Pour the media into the container on the top in one fluid motion.

    - Close the lid on the top.
- Prepare aliquots in 50 mL falcon tubes with media as needed for later usage.
- Store in the fridge until needed.
- Clean out Biosafety cabinet of materials and disinfect with 80% ethanol.
- Close the Biosafety cabinet and turn on the UV lamp for 30 minutes to sterilize the cabinet.

Annex 2: Protocol: Changing of culture media

- Ensure proper PPE is worn.
- Put the culture media in the warm water bath (VWR Grant JB Nova) at 37°C.
- Disinfect the Biosafety cabinet Type II (Labconco Logic, Class II Type A2) with 80% ethanol (H225, P 210).

- Disinfect all needed materials and bring them into the biosafety cabinet:
  - Dispenser pipette (Thermo Scientific Matrix)
  - Graduated pipette (25 ml) (Falcon Serological Pipet)
- Disinfect all needed chemical containers and bring them into the biosafety cabinet:
  - Cell culture media (DMEM, 199 medium, Hydromycin B, FBS)
- Take a waste beaker and add a little bit of bleach (10%, H31-34-50-41, P1/2-28-45-50-61) to it, then disinfect and bring into the biosafety cabinet.

- Retrieve the cell culture flasks from the CO_2-incubator (Binder).
- Pay attention to media colour to exclude contamination.
- Check state of cells under the microscope (Nikon Eclipse TS100) (confluence, shape, number alive/dead, …)
- Bring the flask into the biosafety cabinet and remove the old media. Do this by pouring it into the liquid waste beaker in one fluid motion.
  !!! Do not let the two recipients touch each other!!!
  !!! Keep the culture flask upside down when pouring away the old media!!!
- Close the lid of the cell culture flask to avoid contamination.
- Retrieve the warmed cell media from the warm water bath.
  !!! Ensure that the media has warmed up enough!!!
- Take a 25 mL graduated pipette and add 20 mL of new media to the culture flask.
  !!! Add the media on the side on the culture flask. Do not pour the media directly onto the cells as this may damage them!!!
- Close the cell culture flask and store in the CO_2-incubator.
- Clean out Biosafety cabinet of materials and disinfect with 80% ethanol.
- Close the Biosafety cabinet and turn on the UV lamp for 30 minutes to sterilize the cabinet.

- **REPEAT PROCES EVERY TWO DAYS.**

Annex 3: Protocol: Cell Culture Passaging

Sample identification

- Organism: *Homo sapiens, human*
- Cell Type: Fibroblast immortalized with hTERT
- Tissue/Origin: Foreskin
- Disease status: normal
- Identification code : BJ-5ta (ATCC® CRL-4001™)
- Biosafety level: 1

Method

a. **Harvesting of cells**

- Ensure proper PPE is worn.
- Put the culture media and the DPBS in the warm water bath (VWR Grant JB Nova) at 37°C.
- Retrieve the Trypsin (0.25%, H315-319-334-335, P261-305-351+338-342-311) from the fridge and set it out to thaw.
- Disinfect the Biosafety cabinet Type II (Labconco Logic, Class II Type A2) with 80% ethanol (H225, P 210).

- Disinfect all needed materials and bring them into the biosafety cabinet:
  - Dispenser pipette (Thermo Scientific Matrix)
  - Micropipettes (Thermo Scientific Finnpipette F1)
  - Graduated pipette (50 mL, 25 ml, 10ml, 5 ml) (Falcon Serological Pipet)
  - Micropipette tips (Interpath Services, Aerosol Barrier tips, Pre-sterilized)
  - Falcon tube (50 ml, 15 ml) (Falcon Polypropylene conical tube)
  - Culture flasks (Thermo Scientific, Nunc EasYflask 75 cm^2, Nunclon Delta Surface)
- Disinfect all needed chemical containers and bring them into the biosafety cabinet:
  - Cell culture media (DMEM, 199 medium, Hydromycin B, FBS)
  - DPBS (1x, H319-335-315, P280-302+352-304+340-305+351+338)
  - Trypsin (0.25%, H315-319-334-335, P261-305-351+338-342-311)

- Take a waste beaker and add a little bit of bleach (10%, H31-34-50-41, P1/2-28-45-50-61) to it, then disinfect and bring into the biosafety cabinet.

- Retrieve the cell culture flasks from the CO_2-incubator (Binder).
- Pay attention to media colour to exclude contamination.
- Check state of cells under the light microscope (Nikon Eclipse TS100). Check the confluence, shape, number alive/dead, … of the cells.
- Bring the flask into the biosafety cabinet and remove the old media. Do this by pouring it into the liquid waste beaker in one fluid motion.
  - !!! Do not let the two recipients touch each other!!!
  - !!! Keep the culture flask upside down when pouring away the old media!!!
- Close the lid of the cell culture flask to avoid contamination.
- Retrieve the warmed cell media from the warm water bath.
- Wash away the remaining media by adding **10 mL of DPBS** into the flask.
  - !!! Do not let the two recipients touch each other!!!
  - !!! Add the DPBS on the side on the culture flask. Do not pour the media directly onto the cells as this may damage them!!!
- Discard the DPBS by pouring it into to liquid waste beaker. Keep previous warnings in mind

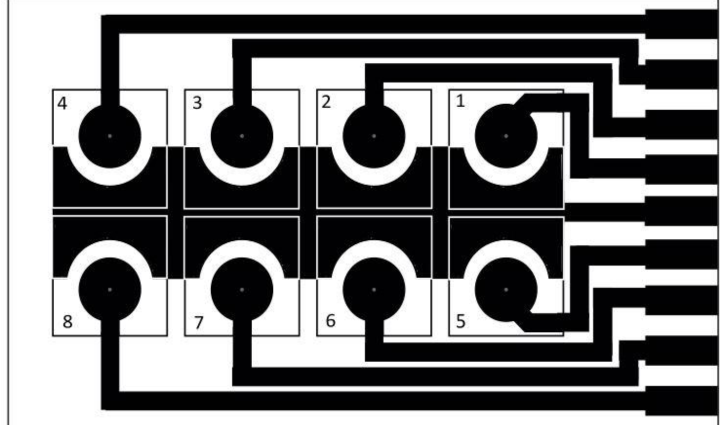
- Add **3 mL of Trypsin** in the cell culture flask. Gently shake the culture flask in a North-South, East-West way to make sure the surface is covered.
- Incubate for **6 minutes on 37°C** in the CO_2-incubator.
- Check under the microscope if all the cells have detached from the surface and are floating freely.
    - If they are still attached, incubate for a little longer
    - If the cells are still in clusters, hold the flask at an angle and firmly hit the side of the flask with a flat hand.
- Deactivate the trypsin by adding **4 mL of media** to the flask.
    - Rinse the sides of the flask when adding the media
    - Pipet the mixture up and down a few times, rinsing the sides. This ensures that most cells are gathered.
- Transfer the cell suspension to a falcon tube (15 mL)
- Centrifuge the mixture for **5 minutes at 250 XG** in the centrifuge (Thermo Scientific
- Heraeus Megafuge 8 Centrifuge).
- Remove the supernatant by pouring it in the liquid waste beaker in one fluid motion.
- Resuspend the pellet in **X mL of media**. Make sure that the pellet is completely dispersed, and no flakes remain.
    - X mL is dependent on what ratio of cells you want in your new cell culture and the confluence of your last culture.
    - For example, if you want to seed the cells 1/5 of what they were in the previous flask you resuspend the pellet in 5 mL of media and add 1 ml of cell suspension in the new cell culture flask.
- Take a new cell culture flask.
- Add **20 mL of media** into the cell culture flask.
- Homogenise the cell suspension and add **X mL (µl) cell suspension** into the culture flask.
- Close the flask and shake them gently in a North-South, East-West motion.
- Label the flask accordingly (cell type, date, name operator, passage number, cell count).
- Place culture flask in the incubator.
- Clean workspace.

Annex 4: Protocol: Seeding of cells in electrode wells

Sample identification

- Organism: *Homo sapiens, human*
- Cell Type: Fibroblast immortalized with hTERT
- Tissue/Origin: Foreskin
- Disease status: normal
- Identification code : BJ-5ta (ATCC® CRL-4001™)
- Biosafety level: 1

Wells identification



Well 1: Collagen + Media
Well 2: Collagen + Media

Well 3: 2.5D 30k cells
Well 4: 2.5D 30k cells

Well 5: Media
Well 6: Media

Well 7: 2D 30k cells
Well 8: 2D 30k cells

Method

Preparation of collagen wells

- Ensure proper PPE is worn.
- Disinfect the Biosafety cabinet Type II (Labconco Logic, Class II Type A2) with 80% ethanol (H225, P 210).

- Disinfect all needed materials and bring them into the biosafety cabinet:
    - Dispenser pipette (Thermo Scientific Matrix)
    - Micropipettes (Thermo Scientific Finnpipette F1)
    - Graduated pipette (50 mL, 25 ml, 10ml, 5 ml) (Falcon Serological Pipet)
    - Micropipette tips (Interpath Services, Aerosol Barrier tips, Pre-sterilized)
    - Falcon tube (50 ml, 15 ml) (Falcon Polypropylene conical tube)
    - Small tubes (1 ml) (Thermo Scientific, Nunc EasYflask 75 cm^2, Nunclon Delta Surface)
    - ECIS 8 well chamber with single circular electrode (0.049 sqr.mm) per well. PET substrate (MFIA 500 kHz / 5 MHz Impedance Analyzer, Zurich Instruments)
    - Syringe (50 ml) (Terumo Syringe)
    - Syringe filter (PALL Corporation, Acrodisc Syringe filter 25 mm, w/ 0.45 µM Supor Membrane)
- Disinfect all needed chemical containers and bring them into the biosafety cabinet:
    - Cell culture media (DMEM, 199 medium, Hydromycin B, FBS)
    - L-Cysteine (100%)
    - RO water (100%)
    - Foetal Bovine Serum (FBS) (10%)

- Take care in storing the chemicals correctly in the cabinet:
  - Bring **EMEM, FBS, L-Glut and Sodium Bicarb** into the hood and store them in the tube rack.
  - The **Collagen** needs to be kept at a cold temperature (ice tray).
- Put **Culture media** in the warm water bath (VWR Grant JB Nova) at 38°C.
- Take a waste beaker and add a little bit of bleach (10%, H31-34-50-41, P1/2-28-45-50-61) to it, then disinfect and bring into the biosafety cabinet.

- **This protocol is closely related to the 'Protocol Cell Passaging'. For easy integration, the Trypsin and DPBS Buffer should be warmed up in the warm water bath as well.**
- Prepare L-Cysteine solution in RO water in a 15 ml tube.
  - Weigh **0.009 g of L-Cysteine.**
  - Depending on the volume weighted recalculate how much RO water needs to be added with following volume:

$$n\ g = 121\frac{g}{mol}(MW) \times 10 \times 10^{-3}\frac{mol}{L}(mM) \times 8 \times 10^{-3}L = 9.6928\ mg = 0.009\ g$$

$$mL = 1/\left(121\frac{g}{mol}(MW) \times 10 \times 10^{-3}\frac{mol}{L}(mM) \times \frac{1}{n\ g}\right) = 9.6928\ mg$$

  - Bring the solution inside the Biosafety hood and filter it with the 50 mL syringe and filter system into a new tube.
- Add **200 µl L-Cysteine** solution to each well.
- Incubate at room temperature for **10 minutes**.
- Wash the wells with **RO water** 10x (200 µl).
  - Repeat step **3 times.**
- Take a 1 mL tube.
- Prepare the reagents for creating the collagen 2.5D wells.
  - In total a volume of 500 µl is wanted in each well
  - 4 wells will contain 200 µl of collagen → 800 µl collagen solution needed

Total volume (ml)			**1000 µl**
Chemical	**Stock**	**Final concentration**	**µl**
10 x EMEM	10	0.84 x	84
FBS	100	9.28 %	92.8
L-Glut (200 mM)	200	1.48 mM	7.4
Culture media			445.14

- Keep the 1 mL tube with the solution on ice.
- Put the media back in the warm water bath to reheat it to 37°C.
- Add collagen and the sodium bicarb into the mixture:
  - Colour change should occur!!!

Total volume (ml)			1000 µl
Chemical	**Stock**	**Final concentration**	**µl**
~~10 x EMEM~~	~~10~~	~~0.84 x~~	~~84~~
~~FBS~~	~~100~~	~~9.28 %~~	~~92.8~~
~~L-Glut (200 mM)~~	~~200~~	~~1.48 mM~~	~~7.4~~
~~Culture media~~			~~445.14~~
Collagen (1.2 mg/mL)	9	3 mg/mL	333.34
Sodium Bicarb	7.5 %	0.28 %	37,34

- Quickly add **200 µl of the collagen mixture** into 4 wells.
  - Well 1,2,3 and 4.
- Let the collagen set for by placing the wells for **30 minutes in 37°C** in the CO_2-incubator.

Passaging and counting of cells in culture

- **!!! SEE PROTOCOL CELL PASSAGING!!!**
- After the cell pellet has been resuspended do following steps:

- Take **30-50 µl of cell suspension** and set aside in a small tube.
- Mix **10 µl of cell suspension and 10 µl of Tryptan blue** together.
- Add **10 µl of the mixture** into each chamber of the automated cell counter slide (Invitrogen Countess cell counting chamber slides).
- Let the automated cell counter (Invitrogen Countess II) count the cells on both sides of the slide and calculate the average number of cells:

$$\frac{X_1 + X_2}{2} = X_m$$

- Needed: 30000 cells → X cells/ml

$$X_m\ cells/mL \qquad n\ cells$$
$$\frac{n\ cells}{X_m\ cells/mL} = \frac{n}{X_m}\ cells$$
$$\rightarrow \frac{30000}{X_m} mL$$

Calculate how much mL of cell suspension needs to be added to the wells to have 30000 cells. → **Y volume**

Seeding of 2D and 2.5D wells

- Ensure that the collagen has set in the wells.
- Add **Y mL of cell suspension** in 2 of the collagen wells.
  - Well 3 and well 4 → These are the 2.5D wells
  - The other two wells (1 and 2) with collagen will serve as blanks.
- Add **Y mL of cell suspension** in 2 of the empty wells.
  - Well 7 and well 8 → These are the 2D wells.
  - The other two empty wells (5 and 6) will serve as blanks.
- Top up the cells with media in the following way:
  - Well 1-2: Media collagen: 500 µl – 200 µl collagen = ………. µl media
  - Well 3-4: 2.5D 30k cells: 500 µl – 200 µl collagen – Y µl CS = ………. µl media
  - Well 5-6: Media: 500 µl of media
  - Well 7-8: 2D 30k cells: 500 µl – Y µl CS = ………. µl media

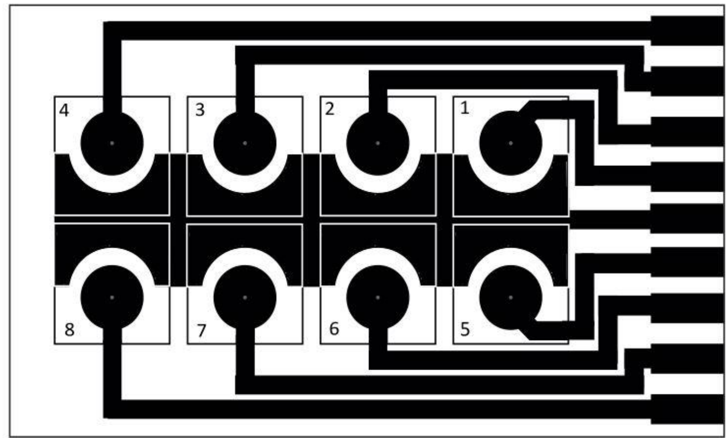
- Place the wells in a container in the 37° CO_2-incubator.
- Clean out Biosafety cabinet of materials and disinfect with 80% ethanol.
- Close the Biosafety cabinet and turn on the UV lamp for 30 minutes to sterilize the cabinet.

Annex 5: Protocol: Impedance measurements

Sample identification

- Organism: *Homo sapiens, human*
- Cell Type: Fibroblast immortalized with hTERT
- Tissue/Origin: Foreskin
- Disease status: normal
- Identification code : BJ-5ta (ATCC® CRL-4001™)
- Biosafety level: 1

Wells identification



Well 1: Collagen + Media
Well 2: Collagen + Media

Well 3: 2.5D 30k cells
Well 4: 2.5D 30k cells

Well 5: Media
Well 6: Media

Well 7: 2D 30k cells
Well 8: 2D 30k cells

Method

- Ensure proper PPE is worn.
- Put the culture media in the warm water bath (VWR Grant JB Nova) at 37°C.
- Disinfect the Biosafety cabinet Type II (Labconco Logic, Class II Type A2) with 80% ethanol (H225, P 210).
- Disinfect all needed materials and bring them into the biosafety cabinet:
    - Micropipettes (Thermo Scientific Finnpipette F1)
    - Micropipette tips (Interpath Services, Aerosol Barrier tips, Pre-sterilized)
    - ECIS 8 well chamber with single circular electrode (0.049 sqr.mm) per well. PET substrate (MFIA 500 kHz / 5 MHz Impedance Analyzer, Zurich Instruments)
- Disinfect all needed chemical containers and bring them into the biosafety cabinet:
    - Cell culture media (DMEM, 199 medium, Hydromycin B, FBS)
- Take a waste beaker and add a little bit of bleach (10%, H31-34-50-41, P1/2-28-45-50-61) to it, then disinfect and bring into the biosafety cabinet.
- Retrieve the electrode well plate (ECIS 8 well chamber with single circular electrode (0.049 sqr.mm) per well. PET substrate (MFIA 500 kHz / 5 MHz Impedance Analyzer, Zurich Instruments) from the CO_2-incubator (Binder).
- Pay attention to media colour to exclude contamination.
- Check state of cells under the light microscope (Nikon Eclipse TS100). Check the confluence, shape, number alive/dead, … of the cells.
- Bring the well plate into the biosafety cabinet.
- Set a timer for **30 minutes**.
- **Remove 200 µl of media** from each well.

    - This can be more or less. The goal is to have a semi-equivalent amount of media in each well.
    - Be careful not to touch the bottom in the 2D wells or the collagen.
    - Change your tip for every well to avoid contamination
- Close the lid of the cell culture flask to avoid contamination while you wait for the 30 minutes to go by.

- **During these 30 min:**
- Take pictures of each well using brightfield microscopy.

- After the 30 minutes have gone by take the well plate to the impedance analyser (MFIA 500 kHz / 5 MHz Impedance Analyzer, Zurich Instruments).
- Turn on the impedance analyser.
- On the computer:
    - Open the program 'BIOLAB'.
    - Select the program 'MAIN' and click 'RUN'.
    - Select 'Change folder'.
    - Select 'Zurich Impedance Analyzer'.
    - A separate screen opens up
- Hook up the well plate to the Impedance analyser.
    - The black 'reference' electrode is attached in the middle.
    - The red 'measuring' electrode is switched around to measure each well.
        - Measure in following order: Well 5 – 6 – 7 – 8 – 4 – 3 – 2 – 1.
- Click 'Measure' in the program.
    - Select 'hold' to compare the plots while measuring.
- Save the plot once it has finished measuring.
    - The main folder should contain the date on which the experiment is started and a brief description.
    - In the main folder separate folders containing the hours on which was measured should be made (24h, 48h, …)
- Measure each well 3x to rule out false measurements.
- Change the colour gradient of the plot with each different well to make later comparison easier.
- Once all wells are measured and all plots saved, close the program, and turn off the impedance analyser.
- Return the well plate to the Biosafety Hood.
- Retrieve the media from the warm water bath.
- Add **200 µl of media** to all wells.
- Store the well plate in a container back into the CO_2-incubator at 37°C.
- Clean out Biosafety cabinet of materials and disinfect with 80% ethanol.
- Close the Biosafety cabinet and turn on the UV lamp for 30 minutes to sterilize the cabinet.

Annex 6: Protocol: Fixing of 2D, 2.5D and 3D cell cultures

Sample identification

- Organism: *Homo sapiens, human*
- Cell Type: Fibroblast immortalized with hTERT
- Tissue/Origin: Foreskin
- Disease status: normal
- Identification code : BJ-5ta (ATCC® CRL-4001™)
- Biosafety level: 1

Method

- Ensure proper PPE is worn.
- Disinfect the Biosafety cabinet Type II (Labconco Logic, Class II Type A2) with 80% ethanol (H225, P 210).
- Disinfect all needed materials and bring them into the biosafety cabinet:
    - Micropipettes (Thermo Scientific Finnpipette F1)
    - Dispenser pipette (Thermo Scientific Matrix)
    - Micropipette tips (Interpath Services, Aerosol Barrier tips, Pre-sterilized)
    - 48 wells plate
    - Sterile tweezers
- Take a waste beaker and add a little bit of bleach (10%, H31-34-50-41, P1/2-28-45-50-61) to it, then disinfect and bring into the biosafety cabinet.
- Retrieve the electrode wells plate (SDR Scientific) from the CO_2-incubator (Binder) and bring it into the Biosafety cabinet).
- Remove all of the media from all wells with a micropipette and discard it in the liquid waste beaker.
- Inside the Biosafety hood, transfer the 2.5D/3D cell cultures (collagen layer) into empty wells of the 48 wells plate with tweezers.
    - Be careful not to damage the samples too much with the tweezers!!!
- Transfer the 48 wells plate and the electrode wells plate to the chemical hood before undertaking the next steps.
- Add **200 µl of 3.7% formaldehyde** to the wells containing the samples and all the wells of the electrode wells plate.
    - Formaldehyde (3.7%, H317-341-350, P280-302+352)
    - The samples need to be submerged, so add more if needed.
- Put the 48 wells plate on the automated shaker (Rotex) for 30 minutes.
- Put the electrode wells plate on the automated shaker for 15 minutes.
    - Do not let it shake too hard!
- In the meantime, clean out Biosafety cabinet of materials and disinfect with 80% ethanol. Close the Biosafety cabinet and turn on the UV lamp for 30 minutes to sterilize the cabinet.
- Remove all of the formaldehyde.
- Wash the samples **3 times** with **350 µl DPBS 1x**:
    - DPBS (1x, H319-335-315, P280-302+352-304+340-305+351+338)
    - For the 48 wells plate:
        - Add 350 µl of DPBS to each well
        - Put the wells plate on the automated shaker for 5 minutes.

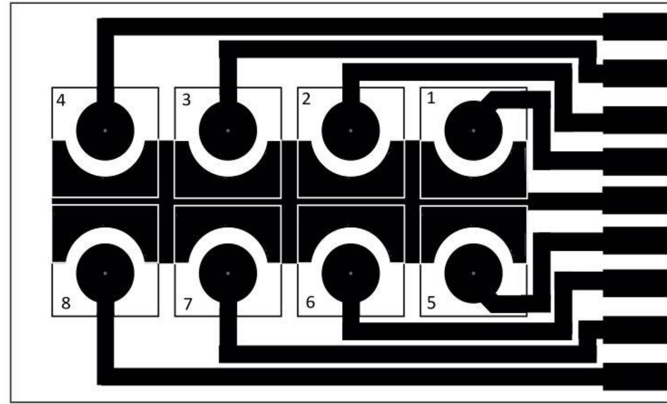
        - Remove all the DPBS.
        - Repeat 3 times
    - For the electrode wells plate:
        - Add 350 µl of DPBS to each well
        - Remove all DPBS
        - When pipetting, avoid touching the bottom of the well where cells are an do not empty the pipet directly on top of the cells, instead aim for the side of the well.
        - Repeat 3 times (5min shake not necessary)
- Add 500 µl of DPBS to each well.
- Add proper identification to the wells lid.
- Wrap parafilm around both wells plates to avoid the DPBS evaporating and the wells drying out.
- Store the wells plate in the fridge.
- Clean up the chemical hood.

Annex 7: Protocol: Seeding of 2D, 2.5D and 3D cell cultures in well plate for fixing after 24h and imaging with confocal microscopy

Sample identification

- Organism: *Homo sapiens, human*
- Cell Type: Fibroblast immortalized with hTERT
- Tissue/Origin: Foreskin
- Disease status: normal
- Identification code : BJ-5ta (ATCC® CRL-4001™)
- Biosafety level: 1

Wells identification



Well 1: 3D
Well 5: 3D

Well 2: 2.5D
Well 6: 2.5D

Well 3: 2D
Well 7: 2D

Well 4: Collagen
Well 8: Collagen

Method

- Ensure proper PPE is worn.
- Disinfect the Biosafety cabinet Type II (Labconco Logic, Class II Type A2) with 80% ethanol (H225, P 210).
- Disinfect all needed materials and bring them into the biosafety cabinet:
  - Dispenser pipette (Thermo Scientific Matrix)
  - Micropipettes (Thermo Scientific Finnpipette F1)
  - Graduated pipette (50 mL, 25 ml, 10ml, 5 ml) (Falcon Serological Pipet)
  - Micropipette tips (Interpath Services, Aerosol Barrier tips, Pre-sterilized)
  - Falcon tube (50 ml, 15 ml) (Falcon Polypropylene conical tube)
  - Small tubes (1 ml) (Thermo Scientific, Nunc EasYflask 75 cm^2, Nunclon Delta Surface)
  - ECIS 8 well chamber with single circular electrode (0.049 sqr.mm) per well. PET substrate (MFIA 500 kHz / 5 MHz Impedance Analyzer, Zurich Instruments)
  - Culture flasks (Thermo Scientific, Nunc EasYflask 75 cm^2, Nunclon Delta Surface)
  - Syringe (50 ml) (Terumo Syringe)
  - Syringe filter (PALL Corporation, Acrodisc Syringe filter 25 mm, w/ 0.45 µM Supor Membrane)
- Disinfect all needed chemical containers and bring them into the biosafety cabinet:
  - Cell culture media (DMEM, 199 medium, Hydromycin B, FBS)
  - L-Cysteine (100%)
  - RO water (100%)
  - Foetal Bovine Serum (FBS) (10%)

- L-Glutamine (200 mM)
    - Sodium Bicarbonate (7.5%, H320, P264-305+351+338-337+313)
    - EMEM (10x)
    - Collagen (1.2 mg/ml)
- Take care in storing the chemicals correctly in the cabinet:
    - Bring **EMEM, FBS, L-Glut and Sodium Bicarb** into the hood and store them in the tube rack.
    - The **Collagen** needs to be kept at a cold temperature (ice tray).
- Put **Culture media** in the warm water bath (VWR Grant JB Nova) at 38°C.
- Take a waste beaker and add a little bit of bleach (10%, H31-34-50-41, P1/2-28-45-50-61) to it, then disinfect and bring into the biosafety cabinet.

- **This protocol is closely related to the 'Protocol Cell Passaging'. For easy integration, the Trypsin and DPBS Buffer should be warmed up in the warm water bath as well.**
- Prepare L-Cysteine solution in RO water in a 15 ml tube.
    - Weigh **0.009 g of L-Cysteine.**
    - Depending on the volume weighted recalculate how much RO water needs to be added with following volume:

$$n\ g = 121\frac{g}{mol}(MW) \times 10 \times 10^{-3}\frac{mol}{L}(mM) \times 8 \times 10^{-3}L = 9.6928\ mg = 0.009\ g$$

$$mL = 1/\left(121\frac{g}{mol}(MW) \times 10 \times 10^{-3}\frac{mol}{L}(mM) \times \frac{1}{n\ g}\right) = 9.6928\ mg$$

    - Bring the solution inside the Biosafety hood and filter it with the 50 mL syringe and filter system into a new tube.
- Add **200 µl L-Cysteine** solution to each well.
- Incubate at room temperature for **10 minutes**.
- Wash the wells with **RO water** 10x (200 µl).
    - Repeat step **3 times.**

Preparation of collagen and 2.5D wells

- Take a 1 mL tube.
- Prepare the reagents for creating the collagen 2.5D wells.
    - In total a volume of 500 µl is wanted in each well
    - 4 wells will contain 200 µl of collagen → 800 µl collagen solution needed

Total volume (ml)			**800 µl**
Chemical	**Stock**	**Final concentration**	**µl**
10 x EMEM	10	0.84 x	67.2
FBS	100	9.28 %	74.24
L-Glut (200 mM)	200	1.48 mM	5.92
Culture media			356.112

- Keep the 1 mL tube with the solution on ice.
- Put the media back in the warm water bath to reheat it to 37°C.
- Add collagen and the sodium bicarb into the mixture:
    - Colour change should occur!!!

Total volume (ml)			800 µl
Chemical	**Stock**	**Final concentration**	**µl**
~~10 x EMEM~~	~~10~~	~~0.84 x~~	~~67.2~~
~~FBS~~	~~100~~	~~9.28 %~~	~~74.24~~
~~L-Glut (200 mM)~~	~~200~~	~~1.48 mM~~	~~5.92~~
~~Culture media~~			~~356.112~~
Collagen (1.2 mg/mL)	9	3 mg/mL	266.672
Sodium Bicarb	7.5 %	0.28 %	29.6

- Quickly add **200 µl of the collagen mixture** into 4 wells.
  - Well 2, 6, 4 and 8.
- Let the collagen set for by placing the wells for **30 minutes in 37°C** in the CO_2-incubator.

Passaging and counting of cells in culture

- **!!! SEE PROTOCOL CELL PASSAGING!!!**
- After the cell pellet has been resuspended do following steps:

- Take **30-50 µl of cell suspension** and set aside in a small tube.
- Mix **10 µl of cell suspension and 10 µl of Tryptan blue** together.
  - Tryptan Blue: ≥ 80 %, H351, P201- 202 – 280 – 308+313)

- Add **10 µl of the mixture** into each chamber of the automated cell counter slide (Invitrogen Countess cell counting chamber slides).
- Let the automated cell counter (Invitrogen Countess II) count the cells on both sides of the slide and calculate the average number of cells:

$$\frac{X_1 + X_2}{2} = X_m$$

- Needed for 2D and 2.5D: 30000 cells → X cells/ml

$$X_m \; cells/mL \qquad n \; cells$$
$$\frac{n \; cells}{X_m \; cells/mL} = \frac{n}{X_m} \; cells$$
$$\rightarrow \frac{30000}{X_m} mL$$

Calculate how much mL of cell suspension needs to be added to the wells to have 30000 cells. → **Y volume**

- Needed for 3D: 75000 cells → X cells/ml

$$X_m \; cells/mL \qquad n \; cells$$
$$\frac{n \; cells}{X_m \; cells/mL} = \frac{n}{X_m} \; cells$$
$$\rightarrow \frac{75000}{X_m} mL$$

Calculate how much mL of cell suspension needs to be added to the wells to have 75000 cells. → **Z volume**

Preparing and seeding of 3D wells

- Take a 1 mL tube.
- Prepare the reagents for creating the collagen 3D wells. **(Only add EMEM, FBS, L-Glut)**
    - In total a volume of 500 µl is wanted in each well
    - 2 wells will contain 200 µl of collagen → 400 µl collagen solution needed

Total volume (ml)			**800 µl**
Chemical	**Stock**	**Final concentration**	**µl**
10 x EMEM	10	0.84 x	67.2
FBS	100	9.28 %	74.24
L-Glut (200 mM)	200	1.48 mM	5.92

- Keep the 1 mL tube with the solution on ice.
- Prepare the cell suspension (see previous part)
    - Needed for 3D: 75000 cells → X cells/ml

$$X_m\ cells/mL \qquad n\ cells$$

$$\frac{n\ cells}{X_m\ cells/mL} = \frac{n}{X_m}\ cells$$

$$\rightarrow \frac{75000}{X_m} mL$$

Calculate how much mL of cell suspension needs to be added to the wells to have 75000 cells. → **Z volume**

- Add collagen and the sodium bicarb into the mixture.
    - Colour change should occur!!!
- Add the needed cells in the media.

Total volume (ml)			**1000 µl**
Chemical	**Stock**	**Final concentration**	**µl**
~~**10 x EMEM**~~	~~10~~	~~0.84 x~~	~~67.2~~
~~**FBS**~~	~~100~~	~~9.28 %~~	~~74.24~~
~~**L-Glut (200 mM)**~~	~~200~~	~~1.48 mM~~	~~5.92~~
Collagen (1.2 mg/mL)	9	3 mg/mL	266.672
Sodium Bicarb	7.5 %	0.28 %	29.6
Culture media			356.112

- Quickly add **200 µl of the mixture** into 2 wells.
    - Well 1 and 5.
- Let the collagen set for by placing the wells for **30 minutes in 37°C** in the CO_2-incubator.

Seeding of 2D and 2.5D wells

- Ensure that the collagen has set in the wells.
- Add **Y mL of cell suspension** in 2 of the collagen wells.
    - Well 2 and well 6 → These are the 2.5D wells
- Add **Y mL of cell suspension** in 2 of the empty wells.
    - Well 3 and well 7 → These are the 2D wells.
- Top up the cells with media in the following way:
    - Well 1-5: 3D 75k cells: 500 µl – 200 µl collagen = ………. µl media

- Well 2-6: 2.5D 30k cells: 500 µl – 200 µl collagen – Y µl CS = ………. µl media
- Well 3-7: 2D 30k cells: 500 µl – Y µl CS = ………. µl media
- Well 4-8: Media collagen: 500 µl – 200 µl collagen = ………. µl media

- Place the wells in a container in the 37° CO_2-incubator.
- Clean out Biosafety cabinet of materials and disinfect with 80% ethanol.
- Close the Biosafety cabinet and turn on the UV lamp for 30 minutes to sterilize the cabinet.

Annex 8: Protocol: Staining of 10-day samples for Brightfield microscopy with Trypan blue and cleaning up electrode wells for re-usage

Sample identification

- Organism: *Homo sapiens, human*
- Cell Type: Fibroblast immortalized with hTERT
- Tissue/Origin: Foreskin
- Disease status: normal
- Identification code : BJ-5ta (ATCC® CRL-4001™)
- Biosafety level: 1

Method

- Ensure proper PPE is worn.
- Disinfect the Biosafety cabinet Type II (Labconco Logic, Class II Type A2) with 80% ethanol (H225, P 210).
- Disinfect all needed materials and bring them into the biosafety cabinet:
    - Dispenser pipette (Thermo Scientific Matrix)
    - Micropipettes (Thermo Scientific Finnpipette F1)
    - Graduated pipette (50 mL, 25 ml, 10ml, 5 ml) (Falcon Serological Pipet)
    - Micropipette tips (Interpath Services, Aerosol Barrier tips, Pre-sterilized)
    - ECIS 8 well chamber with single circular electrode (0.049 sqr.mm) per well. PET substrate (MFIA 500 kHz / 5 MHz Impedance Analyzer, Zurich Instruments)
    - 48 wells plate
    - Syringe (50 ml) (Terumo Syringe)
    - Syringe filter (PALL Corporation, Acrodisc Syringe filter 25 mm, w/ 0.45 µM Supor Membrane)
    - Sterile tweezers
    - Sterile 1.5 ml tube
- Disinfect all needed chemical containers and bring them into the biosafety cabinet:
    - RO water (100%)
    - Trypan Blue (H350, P280-201-202-308+313-405-501)
    - DPBS (1x, H319-335-315, P280-302+352-304+340-305+351+338)
    - Trypsin (0.25%, H315-319-334-335, P261-305-351+338-342-311)

- Take care in storing the chemicals correctly in the cabinet:
    - Bring **EMEM, FBS, L-Glut and Sodium Bicarb** into the hood and store them in the tube rack.
    - The **Collagen** needs to be kept at a cold temperature (ice tray).
- Put **Culture media** (DMEM, 199 medium, Hydromycin B, FBS) in the warm water bath (VWR Grant JB Nova) at 38°C.
- Take a waste beaker and add a little bit of bleach (10%, H31-34-50-41, P1/2-28-45-50-61) to it, then disinfect and bring into the biosafety cabinet.
- Remove all of the media from all 10 days wells with a micropipette and discard it in the liquid waste beaker.
- Inside the Biosafety hood, transfer the 2.5D/3D cell cultures (collagen layer) into empty wells of the 48 wells plate using tweezers.
    - Be careful not to damage the samples too much with the tweezers!!!

- Transfer the 48 wells plate and the electrode wells plate to the chemical hood (Dynaflow) before undertaking the next steps.
- Add **200 µl of 3.7% formaldehyde** to the wells containing the samples in the 48 wells plate.
    - Formaldehyde: 3.7%, H317-341-350, P280-302+352
    - The samples need to be submerged, so add more if needed.

- Put the 48 wells plate on the automated shaker (Rotex) for 30 minutes.
  FOLLOW THE PROTOCOL 'FIXING of 2D, 2.5D and 3D cell cultures' FURTHER FOR THESE COLLAGEN SAMPLES. These samples will not be stained with trypan blue.
  The rest of this protocol is performed on the electrode wells.
- Sterilize trypan blue by filtering it with a syringe filter.
- Prepare trypan blue solution (1:1) using 500 µl sterile trypan blue and 500 µl sterile DPBS. Do this in a sterile 1.5 ml tube.
- Add trypan blue solution to the electrode wells and incubate for 2 minutes at room temperature.
- Take pictures of all the wells using the brightfield microscope.
- Add trypsin to the wells and leave to incubate for 30 minutes.
- Wash the trypsin out of the wells with DPBS (2 times).
- Was the DPBS out of the wells with RO water (3 times).
- Wash the wells with ethanol (3 times).
- Wash the wells with RO water (3 times).
- Remove RO water and store the wells for later tests

Annex 9: Protocol: Staining of 24 hours- and collagen samples for confocal microscopy

Sample identification

- Organism: *Homo sapiens, human*
- Cell Type: Fibroblast immortalized with hTERT
- Tissue/Origin: Foreskin
- Disease status: normal
- Identification code : BJ-5ta (ATCC® CRL-4001™)
- Biosafety level: 1

Method

- Ensure proper PPE is worn.
- Retrieve the fixed samples from the refrigerator.
- Retrieve all needed material and store near the chemical safety cabinet (Dynaflow):
    - Micropipette (Thermo Scientific, Finnpipette F1)
    - Micropipette tips (Interpath Services, Aerosol Barrier tips, Pre-sterilized)
    - Falcon tube (50 ml, 15 ml) (Falcon Polypropylene conical tube)
- Retrieve all needed chemicals and store near the chemical safety cabinet (Dynaflow):
    - DPBS (1x, H319-335-315, P280-302+352-304+340-305+351+338)
    - Triton X-100 (0.1%, H315-319, P264-280-302+352-305+351+338-332+313-337+313-362)
    - Hoechst (1 μg/ml, H302-315-319, P264-280)
    - Phalloidin (1:40, H300-310-330, P260-262-264-280-284)
- Remove DPBS from the wells of the electrode well plate and the 48 wells plate.
- Permeabilize the samples with 0.1% Triton X-100 in DPBS. Add 300-400 μl to the 48 wells plate and 200 μl to the electrode wells.
- Put the samples on the shaker (Rotex) for one hour.
- Wash all wells with DPBS and put them on the shaker for 5 minutes. Repeat this step 3 times.
- Dilute phalloidin stock solution (1:40) and Hoechst stock solution (1 μg/ml) in DPBS. !!! Wrap the tubes containing the reagents and the mixture in aluminium foil to avoid light penetration!!!
    - Depending on how many samples need to be fixed the amount of staining mixture needed will vary. Calculate the needed volume.
- Add 200 μl of the solution to the 48 wells plate and 150 μl to the electrode wells.
- Wrap the well plates in aluminium foil to avoid light penetration.
- Incubate for 2 hours on the shaker at low speed.
- Wash all wells with DPBS and put them on the shaker for 5 minutes. Repeat this step 3 times.
- Store the wells in DPBS in the refrigerator until confocal microscopy can be performed.

Additional information

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