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Chapter

A Purpose-Built System for Culturing Cells as *In Vivo* Mimetic 3D Structures

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Abstract

Culturing cells in 3D is often considered to be significantly more difficult than culturing them in 2D. In practice, this is not the case: the situation is that equipment needed for 3D cell culture has not been optimised as much as equipment for 2D. Here we present a few key features which must be considered when designing 3D cell culture equipment. These include diffusion gradients, shear stress and time. Diffusion gradients are unavoidably introduced when cells are cultured as clusters. Perhaps the most important consequence of this is that the resulting hypoxia is a major driving force in the metabolic reprogramming. Most cells in tissues do not experience liquid shear stress and it should therefore be minimised. Time is the factor that is most often overlooked. Cells, irrespective of their origin, are damaged when cultures are initiated: they need time to recover. All of these features can be readily combined into a clinostat incubator and bioreactor. Surprisingly, growing cells in a clinostat system do not require specialised media, scaffolds, ECM substitutes or growth factors. This considerably facilitates the transition to 3D. Most importantly, cells growing this way mirror cells growing *in vivo* and are thus valuable for biomedical research.

Keywords: clinostat, functionality, hypoxia, spheroids and organoids, *in vivo* mimetic, culture time, minimisation of infections, direct observation, media change

1. Introduction

Cells are pre-programmed to carry out certain functions – this represents their potential. At the same time, they are sensitive to physical or biological changes in their surrounding environment and will modify their function accordingly – and this becomes their constantly changing actuality. Cells *in vivo* are surrounded by an extracellular matrix (ECM) and are actively communicating with other cells. This forms part of an active environment which modifies and integrates their activities.

In the early days of cell culture in the 1950's the focus was to get cells to propagate rapidly and reliably in flasks, facilitated by the destruction of their ECM. With the realisation that cells grown in 3D conditions are more mimetic of human cell

biology, the focus has changed away from getting the cells to propagate to getting the cells to function (physiologically). These two conditions mark the extremes in a spectrum of cellular activity [1].

Bearing this in mind, there are a number of factors that should be considered when changing this focus and transitioning from the classical 2D cell culture to 3D cell culture. These factors not only indicate which 3D culture systems could be expected to be advantageous over others but also indicate which should generate data that is more representative of the *in vivo* performance of such cells.

Perhaps, the most significant difference between 2D and 3D culture is the establishment of longer diffusion gradients for the majority of cells and thus the cells will experience significantly different levels of oxygen, CO_2, nutrients and waste products.

Related to the diffusion gradients is the amounts of various compounds in the environment around cells: cells in 2D are typically exposed to levels of for example O_2 and glucose which are not seen in the intact, healthy organism.

Another very significant difference will be the establishment of channels of communication between cells that are not only juxtapositioned but also further away. In 2D, immortal cells are typically passaged roughly every week (and shorter for faster growing cells). At the end of this cycle, cells are usually treated with enzymes or cocktails (containing trypsin, collagenases or other compounds) that damage proteins protruding from the plasma membrane and which dissolve or fragment the ECM. Similar cocktails may be used to produce cell suspensions from tissue biopsies, or these biopsies may be pressed through a mesh to 'liberate' cells. All these treatments release proapoptotic factors, damage cells and have a significant impact on gene expression. Cells will attempt to repair this damage and recover, but need time to do so. So, the final factor to consider is time.

While there are numerous publications illustrating that 3D cell culture can mimic functionalities of human tissues, perhaps one of the most graphic is shown in **Figure 1** where a freshly extirpated human liver biopsy and 29 day old C3A spheroids have been biosynthetically labelled with [^{35}S]-methionine and their proteins extracted and run on high resolution two-dimensional gels (IPG-SDS). Notice that, not only are the proteins expressed in very similar amounts, but also that their post-translationally modifications are very similar.

For many purposes in medical research, what is needed is a model system that accurately reflects what happens in the living organism - more often than not a human being – to shed light on many different processes, whether normal physiology or what goes wrong in disease, or infections by microorganisms, or the effects of compounds during treatment or poisoning.

3D cell culture promises to offer what is needed, but the field is still relatively new and many of the products used are small modifications of existing products that have been available for many years and as such, many have not been ideally suited to the purpose.

2. A purpose-built system for culturing cells

For these reasons, when we started to design some equipment specifically designed to support 3D cell culture. In doing so, we used four aims to guide the process. These were:

1. Use *in vivo* functionality as a yardstick

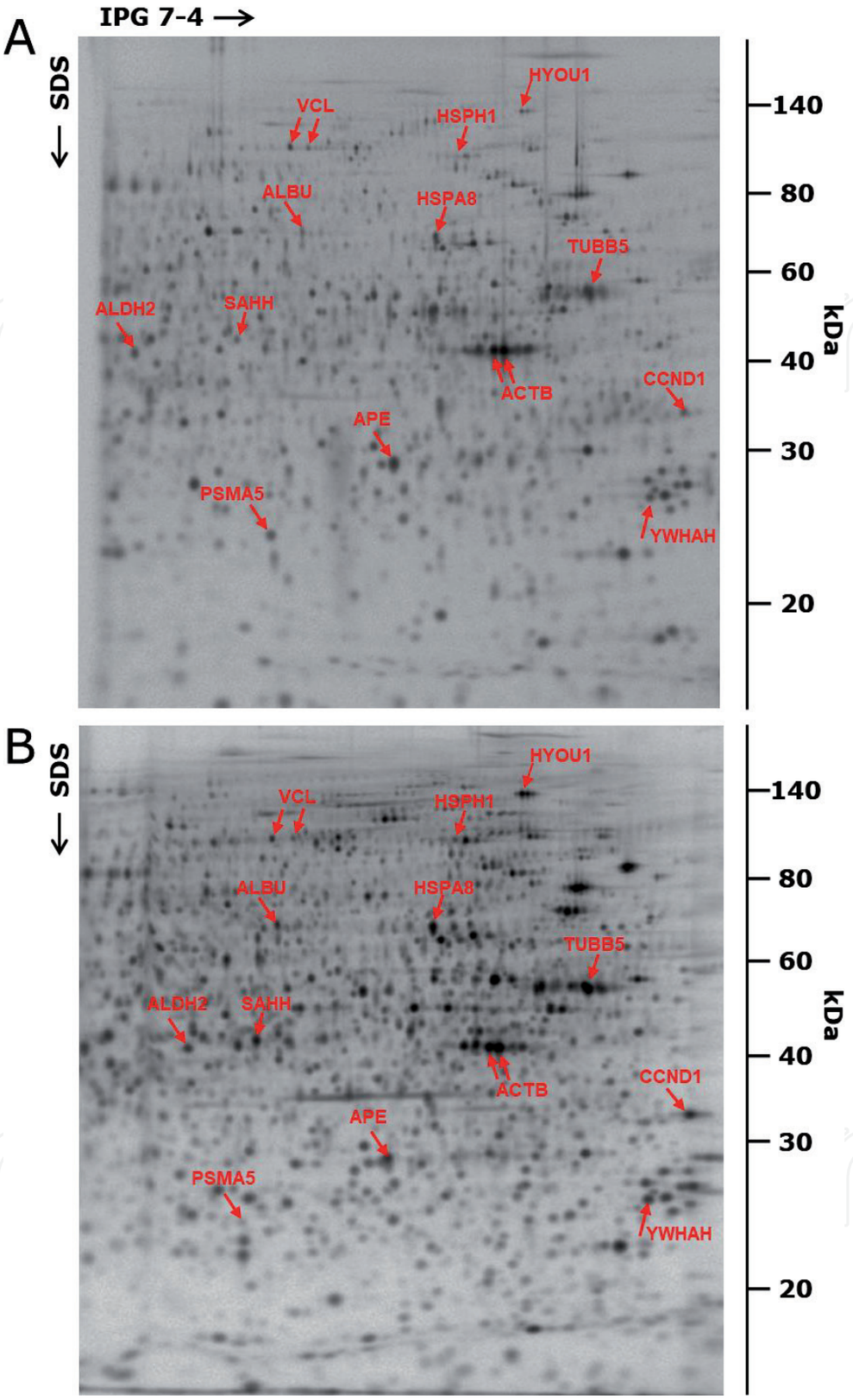

Figure 1.
Human biopsy tissue (ca. 0.5 mm³) and spheroids (29 day-old, ca. 1 mm³) were biosynthetically labelled for 20 hrs with [³⁵S]-methionine. Some of the proteins are named for reference: ACTB, Actin beta; ALBU, Albumin; ALDH2, Aldehyde dehydrogenase 2; APE, Apolipoprotein E; CCND1, Cyclin D; HSPA8, Heat shock protein 8; HSPH1, Heat shock protein H1; HYOU1, Hypoxia up-regulated protein 1; PSMA5, Proteasome subunit alpha type-5; SAHH, S-adenosyl-L-homocysteine hydrolase; TUBB5, Tubulin beta chain 5; VCL, Vinculin; YWHAH, 14–3-3 protein eta. The tissue was homogenised, freeze dried, redissolved in lysis buffer and analysed by 2DGE according to [2]. Images were collected using AGFA phosphorimager plates and reader. [³⁵S]-methionine labelled (A) human liver and (B) C3A spheroids.

2. Allow the cells to do what they want. Do not provide unnecessary compounds and do not stress them in any unphysiological way.

3. Keep the culture conditions as close to that seen *in vivo* as is practical.

4. Keep it simple (both for the cells and the user).

The main requirements that we addressed were: diffusion gradients, shear stress and time.

2.1 Diffusion gradients

Atmospheric oxygen (21%) provides a partial pressure (pO_2) of about 145 mm Hg (ca. 190 μM) to cells grown in 2D cultures. This is considerably more than the partial pressure of oxygen measured in tissues (11% to 0.1%) which should be considered as normoxic for cell culture [3]. Thus, cells grown in 2D cultures are exposed to unphysiological, hyperoxic conditions.

In the human body cells are usually located within 200 μm of a capillary [4] (corresponding to only 10 to 40 cell layers thick). Because cells are actively consuming oxygen, there will be a diffusion gradient into the cell. This is not problematic in 2D because the cultures are typically only one cell layer thick, but it becomes a challenge in 3D because there the spheroids can become tens or hundreds of cell layers thick. Despite that there may be a preferential transport of oxygen through cells and tissues by hydrophobic channelling within membranes, suggesting that oxygen diffusion within cells and tissues may be faster than through water, there will clearly be a limit [5].

In seminal work, Sutherlands group clearly demonstrated that the pO_2 in the centre of a spheroid fell to 0% when the radius was greater than 250 μm, corresponding quite well with the *in vivo* measurements [6]. The oxygen diffusion gradient started considerably outside of spheroids (ca. 100–200 μM) and continued to fall to the centre of the spheroid. They showed that this was an oxygen diffusion-depletion zone surrounding the spheroid and that in it the partial oxygen pressure fell by one third.

A flow of media past a spheroid significantly reduced this zone and had a very significant effect on the oxygenation of the spheroid. This allowed the spheroids to become larger before their cores reached anoxia. The beneficial effect of flow was almost completely negated if the spheroid was resting on a gas impermeable surface (e.g. glass or a gas-impermeable membrane) [6]. Spheroids appear to have a large capacity to adapt and significantly reduce their consumption when the supply of either O_2 or glucose or both is restricted [7–9].

Interestingly, hepatocytes, (which express haemoglobin *in vivo*), when grown as spheroids, massively overexpress (x30) haemoglobin, presumably to assist with oxygen transport into the oxygen depleted core [1]. Based on the changes in protein expression seen as cells recover their tissue mimetic metabolic equilibrium, it has been proposed that one of the strongest forces driving this recovery process which re-establishes the *in vivo* phenotype is hypoxia (affecting proteins like HIF 1 alpha (the main hypoxia sensor) and HYOU1 (a stress-responsive protein - see **Figure 1**)) [9]. The oxygen gradient formed has also been shown to have a marked effect on the formation of hepatic zonation during the differentiation of human embryonic stem cells and thus there will be a gradient of differences in metabolic enzymes into the spheroid [10].

Exactly the same arguments apply to CO_2: it has been demonstrated that CO_2 (as HCO_3^-) diffuses through spheroids of many cell types essentially as if the cells are not there [11]. Cells in clusters increase their aerobic respiration and decrease

oxidative phosphorylation as they reprogram to a more anabolic based metabolism. This reduces their need for O_2 and their production of CO_2. Although this was first noted by Warburg in relation to cancer [12], it probably more strongly reflects the effects of 'mis-culture' of tumour cells in 2D rather than a metabolic style reflective of tumours *in vivo*.

Interestingly the rate of glucose diffusivity through spheroids of different cell types has been shown to differ by a factor of up to 4. The diffusion into a spheroid is quite rapid and an equilibrium is established after about 1 hour (single cells reach this equilibrium within a minute) [13]. These differences may be correlated to how tightly connected the cells become. C3A spheroids have been shown to rapidly deplete normoglycaemic media (5.5 mM) of glucose within 8 hours, converting much of it to glycogen. The cells were then able to reconvert the glycogen to glucose and survive for the next 40 (or 64 hrs) hrs until the next media exchange [9].

Diffusion gradients will also apply to nutrients and waste products. For example, NH_3 is produced by transamination followed by deamination, from biogenic amines and purine and pyrimidine bases. NH_3 (as NH_4OH) is a smaller and less lipophilic molecule and thus its diffusivity five times slower than CO_2 through cells than through pure water at 37 °C making it more difficult to 'escape' [11].

The conclusions are clear for 3D cell culture: without a vasculature, cell clusters should not be too big in order to avoid anoxia (and ensuing necrosis) and should be irrigated on all sides to diminish the depletion zone and accelerate gas, substrate and metabolite exchange.

2.2 Shear stress

There is a growing appreciation that the mechanical properties and cell mechanics, play an important role in gene expression and cell development. The concept that is emerging is that cell types which experience shear stress *in vivo*, (usually fluid movement induced) actually need the stress to differentiate correctly (and retain their differentiation) and that shear stress is detrimental to cells that are not naturally exposed *in vivo* [14].

Thus, in some cases shear stress is positive: see-saw shaking of induced pluripotent stem cell (iPSC) constructs for 17 days promoted cell aggregation, and induced significantly higher expression of chondrogenic-related marker genes than observed in static cultures [15]. A platform rocking at 7 ° with a 3 second cycle results in an average shear stress of about 0.01 Pascal [16]. These shear forces are however distributed unevenly – both spatially and temporally during the motion of the container (bag, or flask) [17]. A similar, ultra-low shear stress is also seen in clinostat cultures. In this case though, the shear stress is distributed essentially homogeneously spatially and temporarily throughout the culture [9].

Fluid-induced shear stress (ca. 0.02–0.06 Pa) in microfluidic devices *in vitro* increased the mechanical properties of neocartilage [18] and have been shown to be beneficial for several epithelial or endothelial cell layers (as seen in ducts, blood vessels and the kidney) [19, 20].

Stirred tank suspension bioreactors and orbital shakers are used widely [21–23] but both result in significantly higher shear forces (0.3–0.66 Pa and 0.6–1.6 Pa respectively) and are considered to be in the critical/lethal range for mammalian cells [24].

Since most cells are found in tissues which experience very little shear stress, equipment that is designed to cultivate cells from these tissues should expose cell clusters to as little shear stress as possible. It is easier to reintroduce shear stress if needed, than to struggle to remove it from a system not designed to be shear stress free.

2.3 Time

As mentioned above, enzymatic treatment of tissues or cells in 2D damages both the ECM and surface located proteins (including their modifications). This raises a number of questions.

The first is as to whether this damage can be repaired, i.e. can the 3D cultures recover the metabolic or physiological properties that they exhibited *in vivo* in the living organism. *In vivo* performance thus becomes an important benchmark – even though it may be very difficult to measure.

If the damage cannot be repaired, then the question raised is whether this failure is due to a limitation of the cells used, whether several cell types are needed, whether the procedures used prevent the recovery, or whether it is a true limitation that the performance cannot be replicated *in vitro*. Answering this question could therefore require a great deal of research. Fortunately, it appears that often the damage can be repaired.

If it can, the next question is how long do cells need to repair this damage?

A final question is that if the damage can be repaired, then once the cells have recovered, how stable is the 3D culture, i.e. for how long can the 3D culture be used?

The answer to these questions depends very much on the origin of the cells. For immortal cells, there are now numerous publications suggesting that these cells need to grow as spheroids for at least 2 weeks to recover their *in vivo* functionality [25–27]. Stem cells, including induced pluripotent stem cells appear to need a wide variety of times – some need only a few days while others never fully recover *in vivo* functionality [27, 28]. As a general rule primary cells seem to retain (most of) their functionality but use some of their mortal time to re-establish partial tissue organisation [29–31].

One observation that appears to be true for the majority of cells, irrespective of their origin, is that they grow considerably much slower as spheroids or organoids as they do in 2D cultures. Thus, doubling times in 3D may be as long as every 50 or 100 days rather than the 1 to 2 days seen in 2D [32, 33]. This makes sense: tissues and tumours *in vivo* do not double in volume every day or even every week. In other words, cells grown in 2D *in vitro* do not represent either their parental tissue or the tumour from which they were isolated. For example, the HepG2 cell line has a doubling time of 1.4 days [8] compared to 135 days for an average hepatocellular carcinoma [34] and approximately 327 days for liver hepatocytes [35].

Starting with isolated cells in culture, it is necessary therefore to anticipate that the cells need to re-adapt to being in clusters once again. There is a lot that needs to occur in such a readaptation process: for example, isolated cells do not have tight junctions and so their import and export pumps will have mixed: and need to be 're-deployed' to different regions of the plasma membrane once tight junction has been re-established [36]. While this is critically important for most cells, the specialisation of pumps in the liver cells is exquisite and intricate [37]. Redeployment of pumps might not be an active process – the cell after all was not designed to work in 2D cultures. Hence, the cell may have to rely on protein turnover to degrade the misplaced pumps and on membrane delivery processes to establish the pumps on the correct sides of the tight junctions. And there are innumerable other redeployments (changes to the cytoskeleton [1], organelle organisation, gene and protein expression, epigenetic marking [38], post-translational modifications and metabolic reprogramming [8, 9]) to complete.

The take home message here is that researchers have to be much more patient and expect that they will need to maintain 3D cultures for extended periods of time

(weeks or months). With this in mind, these extended periods will place a premium on cultures that are highly reproducible and preferably also high yield. Cultures that reach and maintain a dynamic equilibrium for weeks or months will be advantageous in that they will mimic the homeostasis seen in tissues and will provide a large window of utility. Within this window, it will be possible to for example collect multiple samples from the same culture (like collecting biopsies from the same animal at different times) or perform repeated treatment studies [39]. These samples could be used for the same assay at different times or multiple different assays at the same time, or both if there is sufficient biomass available. But the stability of the biological process needs to be documented throughout this window before perturbation experiments can be initiated [32, 40].

Maintaining cultures for extended times greatly increases the risk of infections, and thus requires great care. This can be facilitated if all aspects of a 3D culture system have to be considered so that the culture is well protected from external contamination.

3. A clinostat incubator

So – what does a cell culture system that addresses all of these issues look like?

First it is a CO_2 incubator. The incubator constructed can maintain a steady temperature both over time and within its volume. The inclusion of a powerful fan to mix the air within the incubator ensures no differences in temperature or gas partial pressures (and speed temperature recovery after the door has been opened). Measurements show that the difference in temperature between the top of the chamber and the bottom are less than 0.2 °C.

Figure 2 illustrates that the incubator quickly reaches running conditions after about 15 minutes when it is first switched on and that it can maintain a steady temperature and CO_2 % (in the figure, over 12 hrs).

When the door is opened, the controlling software switches off the fan, heaters and the UV-C lamp (if active) and closes the CO_2 valve. Closing the door reactivates these functions. **Figure 3** illustrates the effect of opening the door wide open (90 °) for respectively either 30 seconds or two minutes showing that running conditions are re-established by about 4 or 6 minutes.

Note that the internal temperature in the culture vessel falls by a maximum of only 0.1 °C while the door is open illustrating that the cultures are not exposed to any cold shock which would affect their gene expression.

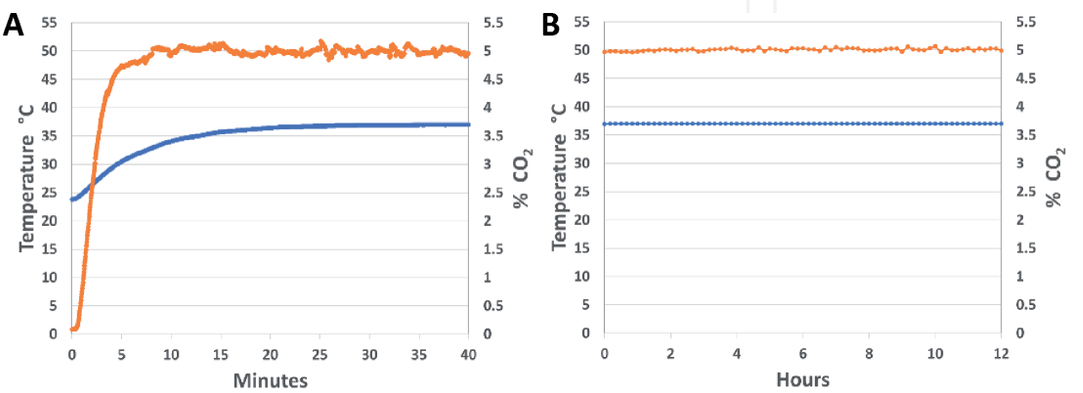

Figure 2.
% CO_2 and temperature levels at (A) initial start up and (B) during a 12 hour period. Measurements were collected using the internal sensors.

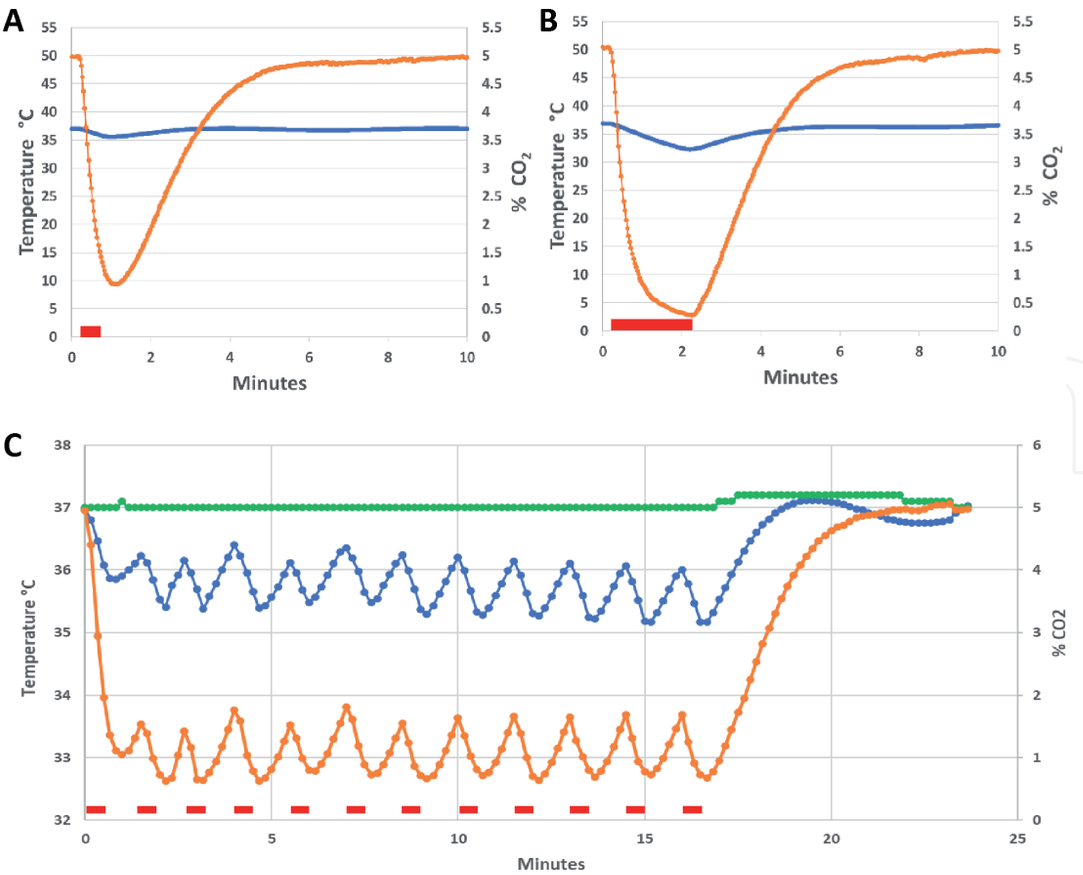

Figure 3.
The effect on CO_2 % and temperature levels of opening the door to 90 ° for either 30 seconds or 2 minutes (as indicated by the red bars). C) Illustrates the effect on the temperature inside a culture vessel of changing the media in the 6 cultures (open door for 30 seconds to take out the culture vessel, 1 minute to change the media, 30 seconds to return the vessel, 1 minute to prepare the sterile bench for the next vessel – All repeated 6 times) Measurements were collected using the internal sensors and an external thermocouple inside a culture vessel.

4. Clinostat technology

In order to reduce the diffusion depleted zone to a minimum, we have adopted the clinostat technology. Introduced for cell culture more than 20 years ago, this technology has been used to culture hundreds of different types of cells and tissues (cell lines, stem cells, primary cells) as well as bacteria and viruses and produced some excellent research. The major limitation of the initial equipment was that it was difficult to use (for example the culture chamber could not be opened but had to be accessed via luer lock ports).

Basically, a clinostat keeps cell clusters in suspension by rotating the culture vessel in a vertical direction (like a wheel). At the right speed, the uplift caused by the effect of liquid viscosity between the vessel and the clusters is balanced by the effect of gravity (these systems are often referred to as 'simulated microgravity' because the clusters appear to float or be maintained in a 'stationary orbit' (relative to the culture vessel)). Thus, the incubator that has been built has been equipped with clinostat motors. These have to run very smoothly so that there is no vibration (which would otherwise shake the clusters apart). **Figure 4** A) would reveal 50/60 Hz mains 'noise' effects while B) would reveal high frequency noise.

To retain 'continuity' with previously published data, we have maintained the basic geometry of the culture chamber.

The next requirement is to be able to open the culture vessel easily for the purpose of introducing or removing cell clusters, for changing the media, or adding compounds or collecting samples.

Figure 5 illustrates a culture vessel with a top access port for media exchange, a front access port for collecting individual cell clusters, and front window that can be removed to reveal a petri-dish like 10 mL culture chamber.

Changing the growth media is illustrated in the video (https://bit.ly/2PkiE9m). The culture chamber is removed from the incubator and the spheroids are allowed to sink to the bottom. The top port plug is removed and 90–95% of the media is sucked away using a sterile syringe and long needle. Fresh media is introduced in the same way, making sure to overfill the growth chamber so that any bubbles are driven into the 'drip-cup' around the top port. The plug is then replaced, the drip cup emptied,

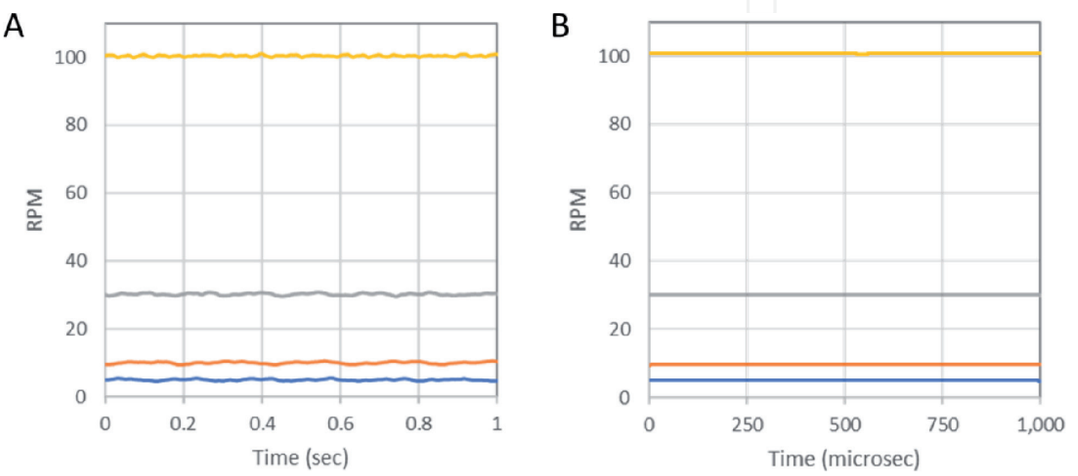

Figure 4.
Variations in the rotational speed of a clinostat motor. (A) Long term variations during 1 second: RPM (± STD) 5.03 ± 0.210; 10.03 ± 0.277; 30.21 ± 0.278; 100.38 ± 0.224. (B) Sort term variations during one thousandth of a second. RPM (± STD) 5.12 ± 0.002; 9.82 ± 0.004; 30.13 ± 0.008; 100.66 ± 0.007. The Permanent Magnet Synchronous Motor (PMSM) used for the clinostat was loaded with a dummy inertia block to mimic the weight of the full culture vessel and run with an Odrive V3.6 motor drive and controller software with anticogging feature. A TLE5012B encoder and digital oscilloscope (Picoscope 2200) was used to measure rpm, Measurements were made each microsecond.

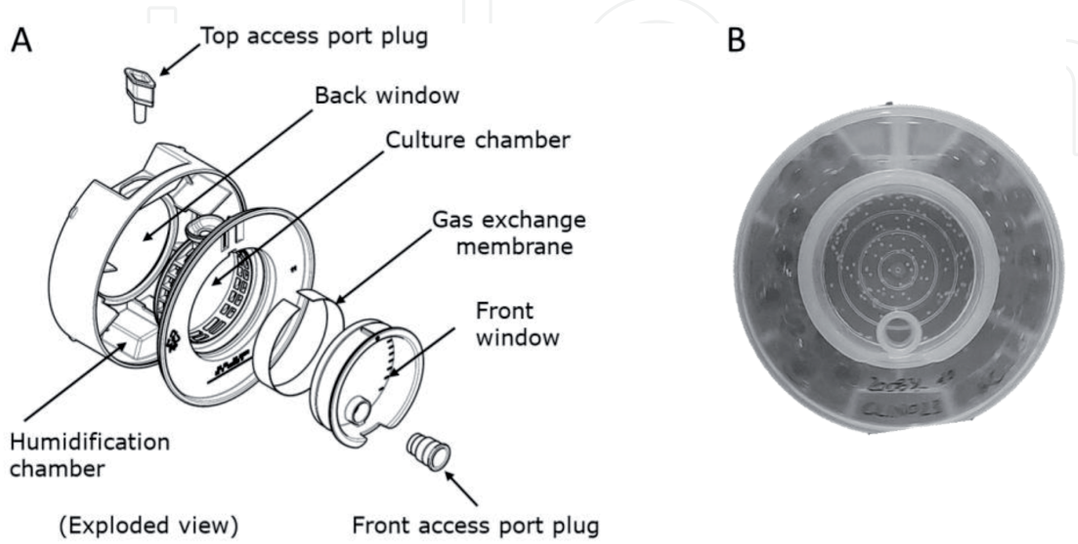

Figure 5.
A culture vessel designed to provide easy access to the culture chamber. (A) Exploded view of parts. (B) Front view showing spheroids in the culture chamber (white spots).

washed with 70% ethanol for sterility and the culture vessel replaced in the clinostat incubator. The whole procedure takes less than 40 seconds, resulting in minimal stress for the cell clusters (a video of this can be seen on CelVivo's website). Small samples of the media can be collected at any time using the same approach.

In this design (**Figure 5**), the gas exchange membrane has been moved from its 'cylinder end' position to a circumferential 'side' position whilst still retaining essentially the same area to allow rapid gas exchange between the culture chamber and the humidification chamber. Relocating the gas exchange membrane allows the culture chamber to be illuminated from the back and observed from the front (using the cameras), facilitating inspection of the clusters without disturbing the culture, or even opening the incubator door. This also helps to minimise infection risks.

5. Humidification

Most incubators are humidified with water trays or tanks, which usually increase the relative humidity close to 100% to prevent cultures from losing water and concentrating the media. This is fortunate because CO_2 normally does not transverse dry membranes very readily. By hydrating the atmosphere, CO_2 can dissolve into the water vapour (giving H_2CO_3) and then diffuse easily across the membrane [41].

Unfortunately, the combination of high humidity and the warm temperatures inside an incubator are strongly conducive to infections and so regular thorough cleaning is necessary to prevent contamination of the cultures.

Microbial infection can be mitigated if the humidification of the incubator chamber can be reduced. For that reason, a culture vessel has been designed that can humidified itself. This permits the incubator to be run in a 'dry state' (i.e. with only ambient humidity). Assuming that the air around the incubator contains 40% relative humidity, (at 20 °C and normal pressure), then when this air enters the incubator and is warmed up to 37 °C, its relative humidity will fall to 13.9% making microorganism growth more difficult.

The culture vessel is humidified by placing water beads in a circumferential chamber around the gas exchange membrane and allowing air exchange into this

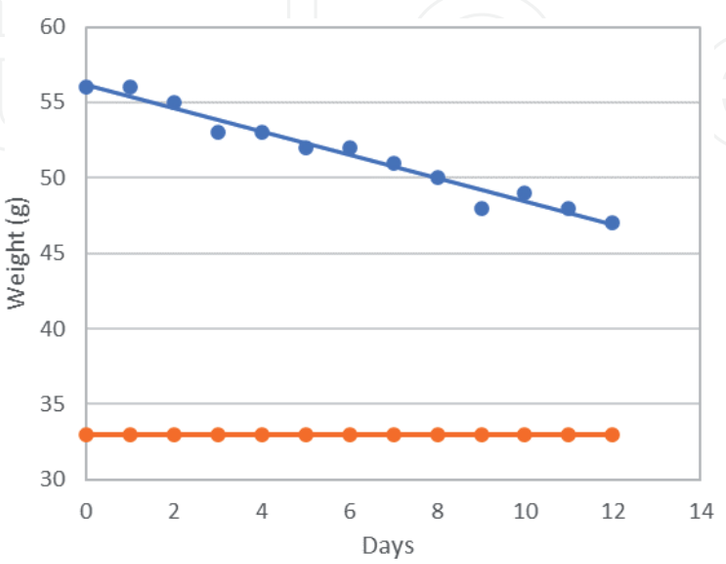

Figure 6.
Loss of water from water beads in a culture vessel in a clinostat incubator at 37 °C. Two culture vessels, one containing hydrated water beads (blue line) and the other containing unhydrated beads (orange line) were rotated at 20 rpm in a clinostat incubator at 37 °C and weighed on a daily basis.

chamber. In use, the hydrated beads release water linearly with time to the atmosphere (1 mL water gives about 1.67 L water vapour) and maintain a very high humidity close to the membrane and facilitating gas exchange (**Figure 6**).

6. Illumination and visualisation

The circumferential position of the gas exchange membrane facilitates another feature of the culture chamber. Because there is nothing on either planar side of the vessel, it is possible to provide uniform illumination from one side and observe the culture from the other side. Fey *et al.* demonstrated that photographs of spheroids in culture could be used to determine the amount of soluble protein (or DNA or number of cells) by measuring their shadow area and using a look-up table [39, 42]. Thus, if the culture chamber is uniformly illuminated from one side and a camera is placed on the other side (**Figure 7**), it is possible to measure the sizes of the clusters without removing them from the incubator.

Even though most modern incubators have a double door, with the inner door made of glass, it is difficult to see the cell clusters clearly often because of poor illumination. The use of an integrated back-light and a camera to inspect the cultures brings yet another advantage: it is not necessary to open the incubator to see the cultures. If these shadow area measurements are repeated over time it becomes possible to follow the growth curve of the clusters. The only manipulation required is to change the media (typically this would be every 2–3 days). If the media is changed for example on a 2, 2 and 3 day (weekly) cycle, then the incubator will need to be opened only 10 times during a 21 day culture. Since a practiced person can change the media within 1 minute each time, this means that the cells need to be out of the incubator for less than 10 minutes in the 21 day period.

Thus, the construction using illumination and a camera for each cell culture vessel minimises the number of times that the incubator needs to be opened. This in turn further reduces the risk of infection, in addition to the effect gained by running the incubator 'dry'.

An extra source of illumination has been included on the same side as the camera so that it is possible to see the clusters by direct inspection and not just their silhouettes.

All of the images are displayed on (and can be captured from) a tablet that also serves to regulate the temperature, CO_2 and rpm of the culture vessels (**Figure 8**).

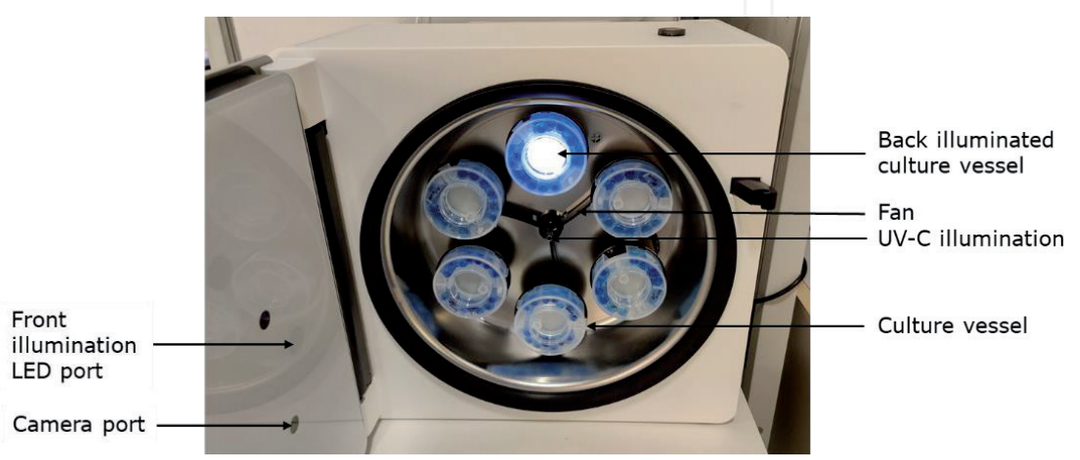

Figure 7.
A clinostat incubator, containing 6 culture vessels (one back illuminated).

Figure 8.
The tablet display showing regulation of the rpm and set value (left panel), culture vessel (main panel), clinostat incubator number (A-1, main panel top left), actual rpm (top right), actual temperature and CO_2 (bottom left). The front access port plug can be seen at the bottom right of the culture vessel.

7. Decontamination

One final step has been taken to reduce the risk of microorganism contamination even further. A UV-C light has been built into the incubator. The UV-C LED source itself is behind the bowl of the incubator but the UV-C light is led out through a fused silica light guide which passes along the shaft of the fan. UV-C capture from the LED was calculated to be 88%. By terminating the light guide in an arrow shape (with angles of 52 and 56 °), the emitted UV-C sweeps the incubator as the fan rotates. Normally, UV-C light reflectance from stainless steel is usually about 5% but this has been increased to about 75% by using a special ePTFE coating. Thus, the UV-C irradiation is reflected in all directions and will reach all surfaces. All clinostat motors are activate during decontamination so that all sides of the culture vessel holder will also be irradiated. The inner surface of the glass door is assumed to be totally absorbing for the UV-C. According to the specifications of the LED lamp, the UV-C light emitted will provide a dose of at least 12 mJ/cm^2 everywhere in the incubator after 2 hours. This results in a log4 reduction (99.99% reduction) in viable bacteria. A log6 reduction is normally defined as sterile for medical facilities (and therefore this is classified only as a decontamination).

8. Yield and variability

One easy way to initiate spheroid cultures is to use embryoid body plates (**Figure 9**).

Here, cells are centrifuged into the bottom of inverted square-based pyramid micro-indentations in microtitre plate wells. Not surprisingly, the spheroids are somewhat squarish immediately after their release from the embryoid body plate and there is quite a lot of loose cells (seen most clearly in the 'Day 0 and 2' image in **Figure 9**).

These loose cells do not sediment down as quickly as the spheroids and thus are lost during successive media changes. The remaining spheroids become steadily

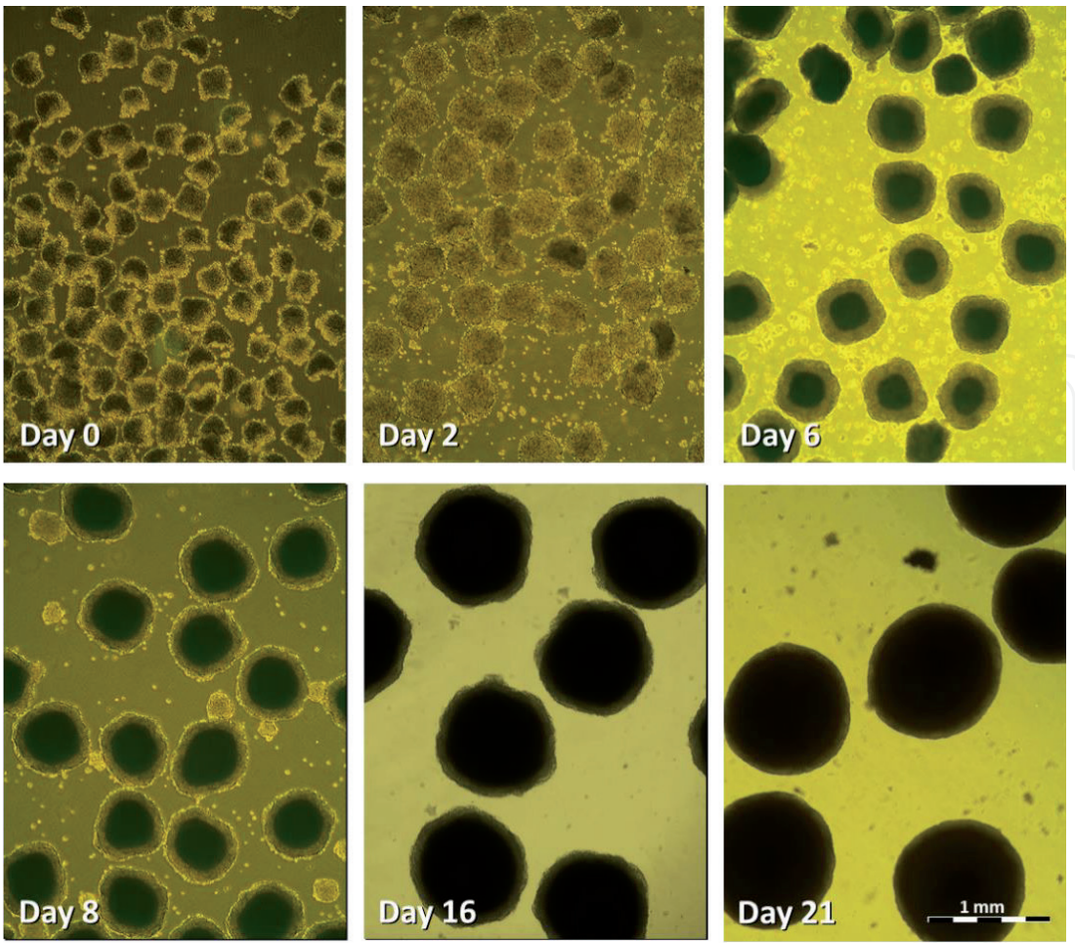

Figure 9.
C3A spheroids at various times. C3A cells (800 cells per well) are left overnight in an embryoid body plate to form a cluster and are released on day 0 and then cultured in DMEM containing 5% foetal bovine serum in a non-humidified clinostat incubator at 37 °C and 5% CO_2/95% air for the number of days shown. The same magnification has been used for all images and the scale bar in the bottom corner illustrates 1 mm.

rounder and more robust as they grow. Starting from a single embryoid body plate well, these procedures have been shown to produce large numbers of spheroids (about 1200) similar to those shown in **Figure 9** after 21 days (these would normally be cultured in four culture vessels). At this stage, each spheroid contains about 82,300 cells and contains 12.31 μg protein (C3A spheroids). The standard deviation of their diameters is less than 21% (after 21 days in culture) and this approach thus provides large numbers of very reproducible spheroids for further experimentation [39, 43].

Once C3A spheroids have recovered, they reach a metabolic equilibrium, characterised by a constant production of ATP, cholesterol and urea for at least 24 days [32]. During this period, treatment of these spheroids with for example any one of six commonly used drugs (acetaminophen (APAP), amiodarone, diclofenac, metformin, phenformin and valproic acid) causes them to respond (as shown by the increase in ATP production) and then recover to the pre-treatment conditions [43]. This can be repeated multiple times and has been proposed to be useful for assessing repeated-dose drug toxicity [39].

The liver is the primary source of many of the proteins found in the blood. To illustrate just how stable C3A spheroids are, they have been kept alive for 302 days. Even after this length of time C3A spheroids are quite capable of synthesising and post translationally modifying these blood proteins (**Figure 10**).

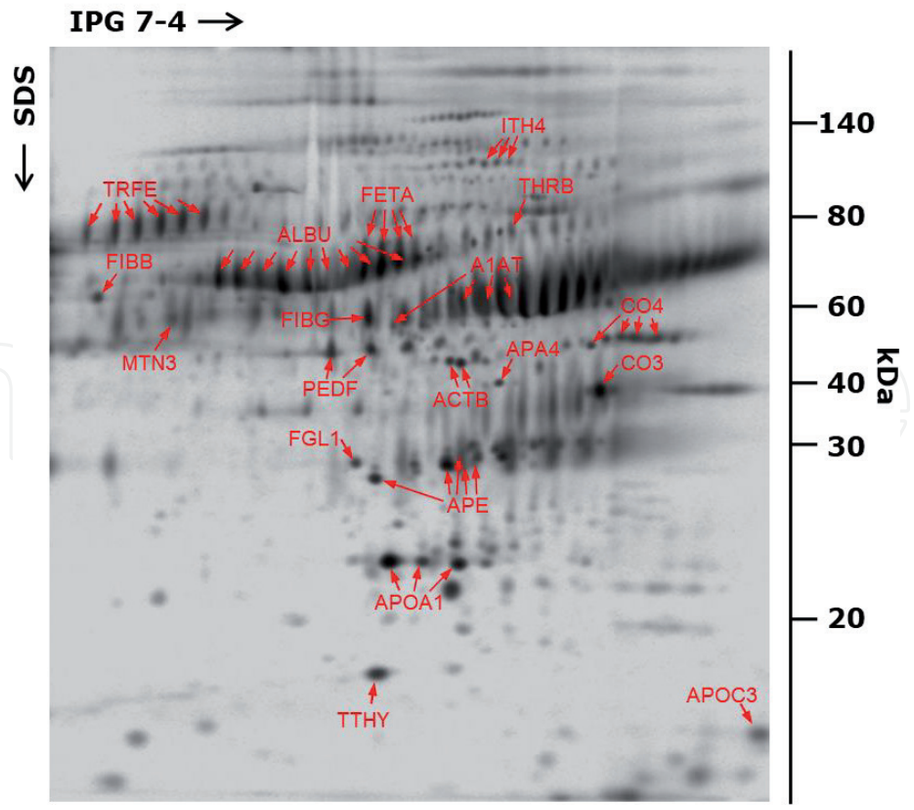

Figure 10.
Proteins secreted from 302 day old C3A spheroids. Some of the proteins are named for reference: ACTB, actin beta; ALBU, albumen; APA4, apolipoprotein a I; APA4, apolipoprotein a IV; APOC3, apolipoprotein C III; APOE, apolipoprotein E, CO3 complement C3 alpha; CO4 complement C4; FETA Foetal albumen; FGL1, fibrinogen-like protein 1; FIBB, fibrinogen beta; FIBG, fibrinogen gamma; ITH4, inter alpha trypsin inhibitor heavy chain 4; MTN3, Matrilin3; PEDF, pigment epithelium derived factor; THRB, prothrombin; TRFE, Serotransferrin; TTHY, transthyretin. 302 day old C3A spheroids were biosynthetically labelled for 20 hrs with [^{35}S]-methionine in order to be able to distinguish newly synthesised proteins from proteins present in the media. The whitish vertical streaks above ALBU indicate the presence of unlabelled BSA. Proteins in the growth media were collected by precipitation, washed, freeze dried, redissolved in lysis buffer and analysed by 2DGE according to [2]. Images were collected using AGFA phosphorimager plates and reader.

9. Applications

Already 8 years ago, clinostat spheroids constructed from C3A cells were shown to be more effective of predicting drug toxicity than primary human hepatocytes *in vitro* [43] and much of the data obtained since has been summarised from a biotransformation and toxicity perspective [44]. 3D spheroid cultures of primary human hepatocytes in chemically defined conditions have been used to evaluate the hepatotoxicity of 123 drugs in clinical drug induced liver injury (DILI) [45]. This has been followed by a demonstration that the system is a good candidate for determining repeated-dose toxicity (i.e. to detect accumulative toxicity) [39, 40].

C3A spheroids have also been used to reveal novel signalling pathways involved in drug treatment (acetaminophen) [46].

Currently one of the major weakness of testing for genotoxicity is the inability of indicator cells to express metabolic enzymes needed for the activation and detoxification of genotoxic compounds *in vitro*. C3A spheroids, cultivated in a clinostat system have been shown to express higher levels of these key metabolic enzymes from phase I and II, as well as DNA damage responsive genes. This suggests that this system can contribute significantly to a more reliable assessment of the genotoxic activities of both pure chemicals, and complex environmental samples. The system has been shown to be sensitive enough to detect genotoxicity even at the very low concentrations relevant to typical environmental exposure situations [47].

Herbal medicines are often assumed to be safe because they are 'natural products', despite the lack of data. To reduce the cost and accelerate their testing, a C3A 3D spheroid model has been developed and benchmarked against Sprague Dawley rats to test one of the most widely used extracts, (*Xysmalobium undulatum*, commonly known as Uzara). The results showed comparable toxicological data [48].

Heteromeric proteins from spheroids even been used as an internal quality control for proteomic data [49].

Epigenetic marking and histone clipping have been shown to be recovered in spheroids [38] and this has been shown to occur during intestinal differentiation *in vivo* [50]. Metabolic reprogramming,which may be a key change during cancer development is also demonstrated in 3D spheroids [9]. The cell line LS180 is a very appropriate cell line for studying colorectal cancer, having been very gently developed but its application suffers from the fact that the cells do not readily form spheroids or organoids. This has recently been overcome by encapsulating the LS180 cells in sodium alginate. These spheroids were shown to respond to standard chemotherapeutic drug, paclitaxel at expected concentrations and even show the development of resistance often seen *in vivo* during paclitaxel treatment. The LS180 alginate spheroids are now being used to screen for novel chemotherapeutic compounds for colorectal cancer [51]. Similar studies have shown that pitavastatin can inhibit stem cell proliferation in colon carcinoma [52].

Spheroids and organoids are being used to investigate the self-organisation of multicellular tissues [53]. Human induced pluripotent stem cells (hiPS cells) have been used to generate neural spheroids that contain oligodendrocytes, neurons and astrocytes [28] and to mimic the blood brain barrier [54]. Primary and stem cells have been used to recapitulate the intricate pattern and functionality of pancreatic islets, working towards regenerative medicine for diabetes [55]. Progress is also being made towards producing transplantable photoreceptor precursors using pluripotent stem cell-derived retinal organoids to treat retinal degeneration diseases [56].

Bacterial-host interactions during Salmonella infections are being studied using iPSCs organoids and stem cell enteroids to mimic the intestinal villus and crypt [57].

Multicellular, physiologically active organotypic cultures are being use to study a wide variety of human viral pathogens with a view to pre-clinical evaluation of vaccines, antivirals and therapeutics [58].

Clinostat cultures are also being used in bone research. Low dietary intake of both vitamin D and K is negatively associated with fracture risk, often seen in persons suffering from osteoporosis. Treatment of primary human osteoblasts (hOBs) 3D multicellular spheroids with a combination of vitamin D and K, enhanced gene expression of periostin and collagen (COL-1), as well as inducing extended osteoid formation. The two vitamins apparently affected bone mechanical properties differently: vitamin D enhancing stiffness and K2 conveying flexibility to bone. It is anticipated that the combination of these effects may translate to increased fracture resistance *in vivo* [59].

10. Conclusions

Clinostat bioreactors systems clearly provide readily controllable 3D cell culture conditions, needing small amounts of cells, media or other compounds and provide sufficient cellular material for a wide variety of assays. The culture vessels and clinostat incubator described here, would be beneficial for many *in vitro* cellular models.

The advantages of culture stability for months, its reproducibility and the possibility to treat and then see the response and recovery (if necessary, for multiple times on the same culture) offer a great potential for future research. The novel equipment described here, will facilitate this research.

Furthermore, the fact that the clinostat system does not require changes in media, the use of scaffolds or special growth factors will not only facilitate the transition from other systems to this clinostat approach but will also allow the cells to respond in their own natural pre-programmed manner.

Thanks to 3D cell culture, the border between basic research and clinical applications is dissolving – and this new era of self-assembling tissue mimetic structures requires a new range of purpose-built equipment.

Conflict of interest

The authors are all employees of CelVivo ApS.

Notes

The culture vessel and clinostat incubator illustrated in this chapter are sold under the ClinoReactor® and ClinoStar® trade names by CelVivo ApS.

Video materials

Video materials referenced in the text are available at: https://bit.ly/2PkiE9m.

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References

[1] Wrzesinski K, Rogowska-Wrzesinska A, Kanlaya R, Borkowski K, Schwammle V, Dai J, Joensen KE, Wojdyla K, Carvalho VB, Fey SJ (2014) The Cultural Divide: Exponential Growth in Classical 2D and Metabolic Equilibrium in 3D Environments. PLoS One 9 (9):e106973. doi:10.1371/journal.pone.0106973

[2] Fey SJ, Larsen PM (2001) 2D or not 2D. Two-dimensional gel electrophoresis. Curr Opin Chem Biol 5 (1):26-33. doi:10.1016/s1367-5931(00)00167-8

[3] Carreau A, El Hafny-Rahbi B, Matejuk A, Grillon C, Kieda C (2011) Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. J Cell Mol Med 15 (6):1239-1253. doi:10.1111/j.1582-4934.2011.01258.x

[4] Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407 (6801):249-257. doi:10.1038/35025220

[5] Dotson RJ, Smith CR, Bueche K, Angles G, Pias SC (2017) Influence of Cholesterol on the Oxygen Permeability of Membranes: Insight from Atomistic Simulations. Biophysical journal 112 (11):2336-2347. doi:10.1016/j.bpj.2017.04.046

[6] Mueller-Klieser WF, Sutherland RM (1982) Influence of convection in the growth medium on oxygen tensions in multicellular tumor spheroids. Cancer research 42 (1):237-242

[7] Mueller-Klieser W, Freyer JP, Sutherland RM (1986) Influence of glucose and oxygen supply conditions on the oxygenation of multicellular spheroids. British journal of cancer 53 (3):345-353. doi:10.1038/bjc.1986.58

[8] Cassim S, Raymond VA, Dehbidi-Assadzadeh L, Lapierre P, Bilodeau M (2018) Metabolic reprogramming enables hepatocarcinoma cells to efficiently adapt and survive to a nutrient-restricted microenvironment. Cell cycle 17 (7):903-916. doi:10.1080/15384101.2018.1460023

[9] Wrzesinski K, Fey SJ (2018) Metabolic Reprogramming and the Recovery of Physiological Functionality in 3D Cultures in Micro-Bioreactors. Bioengineering (Basel) 5 (1). doi:10.3390/bioengineering5010022

[10] Tonon F, Giobbe GG, Zambon A, Luni C, Gagliano O, Floreani A, Grassi G, Elvassore N (2019) In vitro metabolic zonation through oxygen gradient on a chip. Sci Rep 9 (1):13557. doi:10.1038/s41598-019-49412-6

[11] Hulikova A, Swietach P (2014) Rapid CO2 permeation across biological membranes: implications for CO2 venting from tissue. Faseb J 28 (7):2762-2774. doi:10.1096/fj.13-241752

[12] Warburg O (1956) On the origin of cancer cells. Science 123 (3191):309-314. doi:10.1126/science.123.3191.309

[13] Casciari JJ, Sotirchos SV, Sutherland RM (1988) Glucose diffusivity in multicellular tumor spheroids. Cancer research 48 (14):3905-3909

[14] Li W, Li P, Li N, Du Y, Lu S, Elad D, Long M (2020) Matrix stiffness and shear stresses modulate hepatocyte functions in a fibrotic liver sinusoidal model. Am J Physiol Gastrointest Liver Physiol. doi:10.1152/ajpgi.00379.2019

[15] Limraksasin P, Kosaka Y, Zhang M, Horie N, Kondo T, Okawa H, Yamada M, Egusa H (2020) Shaking culture enhances chondrogenic differentiation of mouse induced pluripotent stem cell constructs. Sci Rep 10 (1):14996. doi:10.1038/s41598-020-72038-y

[16] Tsai AC, Liu Y, Yuan X, Chella R, Ma T (2017) Aggregation kinetics of human mesenchymal stem cells under wave motion. Biotechnology journal 12 (5). doi:10.1002/biot.201600448

[17] Kalmbach A, Bordas R, Oncul AA, Thevenin D, Genzel Y, Reichl U (2011) Experimental characterization of flow conditions in 2- and 20-L bioreactors with wave-induced motion. Biotechnology progress 27 (2):402-409. doi:10.1002/btpr.516

[18] Salinas EY, Aryaei A, Paschos N, Berson E, Kwon H, Hu JC, Athanasiou KA (2020) Shear stress induced by fluid flow produces improvements in tissue-engineered cartilage. Biofabrication 12 (4):045010. doi:10.1088/1758-5090/aba412

[19] Thuenauer R, Rodriguez-Boulan E, Romer W (2014) Microfluidic approaches for epithelial cell layer culture and characterisation. Analyst 139 (13):3206-3218. doi:10.1039/c4an00056k

[20] Arora S, Lam AJY, Cheung C, Yim EKF, Toh YC (2019) Determination of critical shear stress for maturation of human pluripotent stem cell-derived endothelial cells towards an arterial subtype. Biotechnol Bioeng 116 (5):1164-1175. doi:10.1002/bit.26910

[21] Naing MW, Williams DJ (2011) Three-dimensional culture and bioreactors for cellular therapies. Cytotherapy 13 (4):391-399. doi:10.3109/14653249.2011.556352

[22] van Putten S, Shafieyan Y, Hinz B (2016) Mechanical control of cardiac myofibroblasts. J Mol Cell Cardiol 93:133-142. doi:10.1016/j.yjmcc.2015.11.025

[23] Melke J, Zhao F, van Rietbergen B, Ito K, Hofmann S (2018) Localisation of mineralised tissue in a complex spinner flask environment correlates with predicted wall shear stress level localisation. European cells & materials 36:57-68. doi:10.22203/eCM.v036a05

[24] Croughan MS, Wang DI (1991) Hydrodynamic effects on animal cells in microcarrier bioreactors. Biotechnology 17:213-249. doi:10.1016/b978-0-409-90123-8.50015-x

[25] Ferruzza S, Rossi C, Scarino ML, Sambuy Y (2012) A protocol for differentiation of human intestinal Caco-2 cells in asymmetric serum-containing medium. Toxicol In Vitro 26 (8):1252-1255. doi:10.1016/j.tiv.2012.01.008

[26] Wrzesinski K, Fey SJ (2013) After trypsinisation, 3D spheroids of C3A hepatocytes need 18 days to re-establish similar levels of key physiological functions to those seen in the liver. Toxicology Research 2 (2):123-135. doi:10.1039/c2tx20060k

[27] Costantini D, Overi D, Casadei L, Cardinale V, Nevi L, Carpino G, Di Matteo S, Safarikia S, Valerio M, Melandro F, Bizzarri M, Manetti C, Berloco PB, Gaudio E, Alvaro D (2019) Simulated microgravity promotes the formation of tridimensional cultures and stimulates pluripotency and a glycolytic metabolism in human hepatic and biliary tree stem/progenitor cells. Sci Rep 9 (1):5559. doi:10.1038/s41598-019-41908-5

[28] Marton RM, Miura Y, Sloan SA, Li Q, Revah O, Levy RJ, Huguenard JR, Pasca SP (2019) Differentiation and maturation of oligodendrocytes in human three-dimensional neural cultures. Nat Neurosci 22 (3):484-491. doi:10.1038/s41593-018-0316-9

[29] Wang Q, Wang K, Solorzano-Vargas RS, Lin PY, Walthers CM, Thomas AL, Martin MG, Dunn JCY (2018) Bioengineered intestinal muscularis complexes with long-term spontaneous and periodic

contractions. PLoS One 13 (5):e0195315. doi:10.1371/journal.pone.0195315

[30] Haigh CL (2017) Cellular Analysis of Adult Neural Stem Cells for Investigating Prion Biology. Methods Mol Biol 1658:133-145. doi:10.1007/978-1-4939-7244-9_11

[31] Park TI, Schweder P, Lee K, DieriksBV, Jung Y, Smyth L, Rustenhoven J, Mee E, Heppner P, Turner C, Curtis MA, Faull RLM, Montgomery JM, Dragunow M (2020) Isolation and culture of functional adult human neurons from neurosurgical brain specimens. Brain Commun 2 (2):fcaa171. doi:10.1093/braincomms/fcaa171

[32] Wrzesinski K, Magnone MC, Visby Hansen L, Kruse ME, Bergauer T, Bobadilla M, Gubler M, Mizrahi J, Zhang K, Andreasen CM, Joensen KE, Andersen SM, Olesen JB, O.B. SdM, Fey SJ (2013) HepG2/C3A 3D spheroids exhibit stable physiological functionality for at least 24 days after recovering from trypsinisation. Toxicology Research 2 (3):163-172. doi:10.1039/c3tx20086h

[33] Bressan E, Ferroni L, Gardin C, Pinton P, Stellini E, Botticelli D, Sivolella S, Zavan B (2012) Donor age-related biological properties of human dental pulp stem cells change in nanostructured scaffolds. PLoS One 7 (11):e49146. doi:10.1371/journal.pone.0049146

[34] Nathani P, Gopal P, Rich N, Yopp A, Yokoo T, John B, Marrero J, Parikh N, Singal AG (2020) Hepatocellular carcinoma tumour volume doubling time: a systemic review and meta-analysis. Gut. doi:10.1136/gutjnl-2020-321040

[35] Seim I, Ma S, Gladyshev VN (2016) Gene expression signatures of human cell and tissue longevity. NPJ Aging Mech Dis 2:16014. doi:10.1038/npjamd.2016.14

[36] Alrefai WA, Gill RK (2007) Bile acid transporters: structure, function, regulation and pathophysiological implications. Pharm Res 24 (10):1803-1823. doi:10.1007/s11095-007-9289-1

[37] Walsh DR, Nolin TD, Friedman PA (2015) Drug Transporters and Na+/H+ Exchange Regulatory Factor PSD-95/Drosophila Discs Large/ZO-1 Proteins. Pharmacol Rev 67 (3):656-680. doi:10.1124/pr.115.010728

[38] Tvardovskiy A, Wrzesinski K, Sidoli S, Fey SJ, Rogowska-Wrzesinska A, Jensen ON (2015) Top-down and middle-down protein analysis reveals that intact and clipped human histones differ in post-translational modification patterns. Molecular & cellular proteomics : MCP. doi:10.1074/mcp.M115.048975

[39] Fey SJ, Korzeniowska B, Wrzesinski K (2020) Response to and recovery from treatment in human liver-mimetic clinostat spheroids: a model for assessing repeated-dose drug toxicity. Toxicol Res (Camb) 9 (4):379-389. doi:10.1093/toxres/tfaa033

[40] Fey SJ, Wrzesinski K (2013) Determination of Acute Lethal and Chronic Lethal Dose Thresholds of Valproic Acid Using 3D Spheroids Constructed from the Immortal Human Hepatocyte Cell Line HepG2/C3A. Valproic Acid. Nova Science Publishers, Inc, New York

[41] Pinnau I, Toy LG (1996) Gas and vapor transport properties of amorphous perfluorinated copolymer membranes based on 2,2-bistrifluoromethyl-4,5-difluoro-1,3-dioxole/tetrafluoroethylene. Journal of Membrane Science 109:125-133. doi:10.1016/0376-7388(95)00193-X

[42] Fey SJ, Wrzesinski K (2013) Microgravity spheroids as a reliable, long-term tool for predictive toxicology.

Toxicol Lett 221S:S153. doi:/10.1016/j.toxlet.2013.05.318

[43] Fey SJ, Wrzesinski K (2012) Determination of drug toxicity using 3D spheroids constructed from an immortal human hepatocyte cell line. Toxicol Sci 127 (2):403-411. doi:10.1093/toxsci/kfs122

[44] Calitz C, Hamman JH, Fey SJ, Wrzesinski K, Gouws C (2018) Recent advances in three-dimensional cell culturing to assess liver function and dysfunction: from a drug biotransformation and toxicity perspective. Toxicology mechanisms and methods 28 (5):369-385. doi:10.1080/15376516.2017.1422580

[45] Vorrink SU, Zhou Y, Ingelman-Sundberg M, Lauschke VM (2018) Prediction of Drug-Induced Hepatotoxicity Using Long-Term Stable Primary Hepatic 3D Spheroid Cultures in Chemically Defined Conditions. Toxicol Sci 163 (2):655-665. doi:10.1093/toxsci/kfy058

[46] Wojdyla K, Wrzesinski K, WilliamsonJ,FeySJ,Rogowska-Wrzesinska A (2016) Acetaminophen-induced S-nitrosylation and S-sulfenylation changes in 3D cultured hepatocarcinoma cell spheroids. Toxicology Research 5:905-920. doi:10.1039/c5tx00469a

[47] Stampar M, Sedighi Frandsen H, Rogowska-Wrzesinska A, Wrzesinski K, Filipic M, Zegura B (2020) Hepatocellular carcinoma (HepG2/C3A) cell-based 3D model for genotoxicity testing of chemicals. Sci Total Environ:143255. doi:10.1016/j.scitotenv.2020.143255

[48] Calitz C, Hamman JH, Fey SJ, Viljoen AM, Gouws C, Wrzesinski K (2019) A sub-chronic Xysmalobium undulatum hepatotoxicity investigation in HepG2/C3A spheroid cultures compared to an in vivo model. J Ethnopharmacol 239:111897. doi:10.1016/j.jep.2019.111897

[49] Rogowska-Wrzesinska A, Wrzesinski K, Fey SJ (2014) Heteromer score-using internal standards to assess the quality of proteomic data. Proteomics 14 (9):1042-1047. doi:10.1002/pmic.201300457

[50] Ferrari KJ, Amato S, Noberini R, Toscani C, Fernandez-Perez D, Rossi A, Conforti P, Zanotti M, Bonaldi T, Tamburri S, Pasini D (2021) Intestinal differentiation involves cleavage of histone H3 N-terminal tails by multiple proteases. Nucleic acids research. doi:10.1093/nar/gkaa1228

[51] Smit T, Calitz C, Willers C, Svitina H, Hamman J, Fey SJ, Gouws C, Wrzesinski K (2020) Characterization of an Alginate Encapsulated LS180 Spheroid Model for Anti-colorectal Cancer Compound Screening. ACS medicinal chemistry letters 11 (5):1014-1021. doi:10.1021/acsmedchemlett.0c00076

[52] Zhang ZY, Zheng SH, Yang WG, Yang C, Yuan WT (2017) Targeting colon cancer stem cells with novel blood cholesterol drug pitavastatin. Eur Rev Med Pharmacol Sci 21 (6):1226-1233

[53] Toda S, Blauch LR, Tang SKY, Morsut L, Lim WA (2018) Programming self-organizing multicellular structures with synthetic cell-cell signaling. Science 361 (6398):156-162. doi:10.1126/science.aat0271

[54] Bergmann S, Lawler SE, Qu Y, Fadzen CM, Wolfe JM, Regan MS, Pentelute BL, Agar NYR, Cho CF (2018) Blood-brain-barrier organoids for investigating the permeability of CNS therapeutics. Nature protocols 13 (12):2827-2843. doi:10.1038/s41596-018-0066-x

[55] Dayem AA, Lee SB, Kim K, Lim KM, Jeon TI, Cho SG (2019) Recent advances in organoid culture for insulin production and diabetes therapy: methods and challenges. BMB Rep 52 (5):295-303

[56] Llonch S, Carido M, Ader M (2018) Organoid technology for retinal repair. Dev Biol 433 (2):132-143. doi:10.1016/j.ydbio.2017.09.028

[57] Yin Y, Zhou D (2018) Organoid and Enteroid Modeling of Salmonella Infection. Front Cell Infect Microbiol 8:102. doi:10.3389/fcimb.2018.00102

[58] Ramani S, Crawford SE, Blutt SE, Estes MK (2018) Human organoid cultures: transformative new tools for human virus studies. Curr Opin Virol 29:79-86. doi:10.1016/j.coviro.2018.04.001

[59] Schroder M, Riksen EA, He J, Skallerud BH, Moller ME, Lian AM, Syversen U, Reseland JE (2020) Vitamin K2 Modulates Vitamin D-Induced Mechanical Properties of Human 3D Bone Spheroids In Vitro. JBMR Plus 4 (9):e10394. doi:10.1002/jbm4.10394

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