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**Deliverable Report D8.1:**  
**Validated protocol for the assessment of mitochondrial dysfunction, and apoptotic and inflammatory markers in 3D liver and heart models and in patients samples**

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## PUBLISHABLE SUMMARY

SOPs and supporting data presented for the measurement of oxygen consumption, glycolytic flux, reactive oxygen species, apoptotic markers and inflammation markers.

## OBJECTIVES

Develop and test a SOP for measuring mitochondrial and inflammatory markers in samples of *in vitro* 3D assays and in samples from patients.

## INTRODUCTION

Recent years have seen a growing appreciation for the importance of the mitochondrion as a site for off-target effects of drug therapy [1]. This is unsurprising when one considers both the multitude of sites where mitochondrial function can be perturbed and the deleterious consequences of such perturbation. Such drug-induced mitochondrial dysfunction has been shown to be a significant cause of drug-induced toxicity but detection of this toxicity in *in vitro* models has been hampered by the lack of suitable screening technologies. There has also been an increasing awareness of the limitations of standard tissue culture models due to their ability to circumvent mitochondrial insult, absence of critical metabolic pathways and an absence of cell-cell interactions contributing to a significant gap between *in vitro* model and the *in vivo* condition. The biological relevance of the *in vitro* observation therefore is heavily influenced by the model used. For liver studies, the gold standard for such measurements is primary human hepatocytes and additional biological relevance can be realised by culturing in 3D. This also offers the opportunity to co-culture primary human hepatocytes with non-parenchymal cells, including Kupffer cells, thereby better reflecting the cell composition of the human liver. Here we use scaffold-free 3-dimensional microtissues (spheroids) grown using the hanging drop method on GravityPLUS™ plates and transferred in GravityTRAP™ 96-well format for subsequent testing. The hepatocytes used are cryopreserved to address donor variability and access.

The same measurement protocols will be used for testing spheroids generated from stem cell derived cardiomyocytes.

Oxygen is one of the most sensitive and direct indicators of mitochondrial dysfunction as it allows direct measurement of electron transport chain (ETC) activity. Using a water-soluble oxygen probe (MitoXpress®-Xtra), mitochondrial function can be assessed in a high throughput fashion on standard fluorescence plate readers. This can be done on both mitochondria isolated from tissue or cultured cells [2,3]. Measuring whole cells addresses the perceived risk of over-predictivity due to the free compound access while transporter activity and CYP activity can also be incorporated. An additional layer of information can also be added by measuring extracellular acidification which provides information on glycolytic flux [4] using pH-Xtra™. Using both MitoXpress®-Xtra and pH-Xtra™ allows the simultaneous assessment of the cells main ATP generating pathways; OxPhos and Glycolysis. Using this dual parameter assay, true mitochondrial toxicity would be expected to result in a decrease in oxygen consumption and a resultant increase in acidification due to glycolytic compensation while non-specific mitochondrial insult would lead to a decrease in oxygen consumption without subsequent acidification [4]. Here we present data describing how such measurements have been adapted to meet the technical challenges of measuring spheroid metabolism resulting in an SOP facilitating the interrogation of mitochondrial function in spheroids.

Extending these observations to *ex vivo* samples is also a significant technical challenge but one that must be met if functional measurement of 3D tissue models and *ex vivo* material is to be achieved. The oxygen consumption of such samples is a key indicator of mitochondrial function and the challenge when testing such material is to maximise the sensitivity of the measurement such that data can be generated from small amounts of material. We have developed an SOP using mouse liver as a model such that data from 0.3-0.1 mg of sample can be measured. This is achieved using hand homogenised crude homogenate generated from snap frozen liver samples. Concentration dependent ETC activity is observed as is ETC inhibition.

As the mitochondria is the main producer of reactive oxygen species (ROS) within the cell, changes in the formation of ROS, as a consequence of mitochondrial dysfunction is also an important mechanism of toxicity [5]. Enhanced ROS formation can induce different signalling pathways and can cause oxidative damage to cellular constituents such as lipids, proteins and DNA. Electron Spin Resonance (ESR/EPR) spectroscopy is deployed here as it is the only technique available that can directly identify and quantify radical formation and is the most effective and direct method for detecting ROS free radicals. ESR can be used to assess ETC-related radicals directly or in combination with the so-called spin trapping technique for detection of short-lived free radicals, thereby allowing direct analysis of superoxide anion radical formation. To facilitate the use of this technique to assess drug induced mitochondrial dysfunction we have developed an SOP to detect enhanced mitochondrial ROS formation after cellular exposure to xenobiotics.

Microtitre plate measurements of apoptosis induction and inflammation in 3D models are also feasible and are described here. Caspase 3/7 activities will be assessed using the commercially available Caspase-Glo® 3/7 assay (Promega). The assay has been adapted for use with spheroids. IL-6 and TNF- $\alpha$  secretion from the microtissues is assessed using commercially available ELISA kits (Life Technologies™). These assays have also been adapted for use with spheroids.

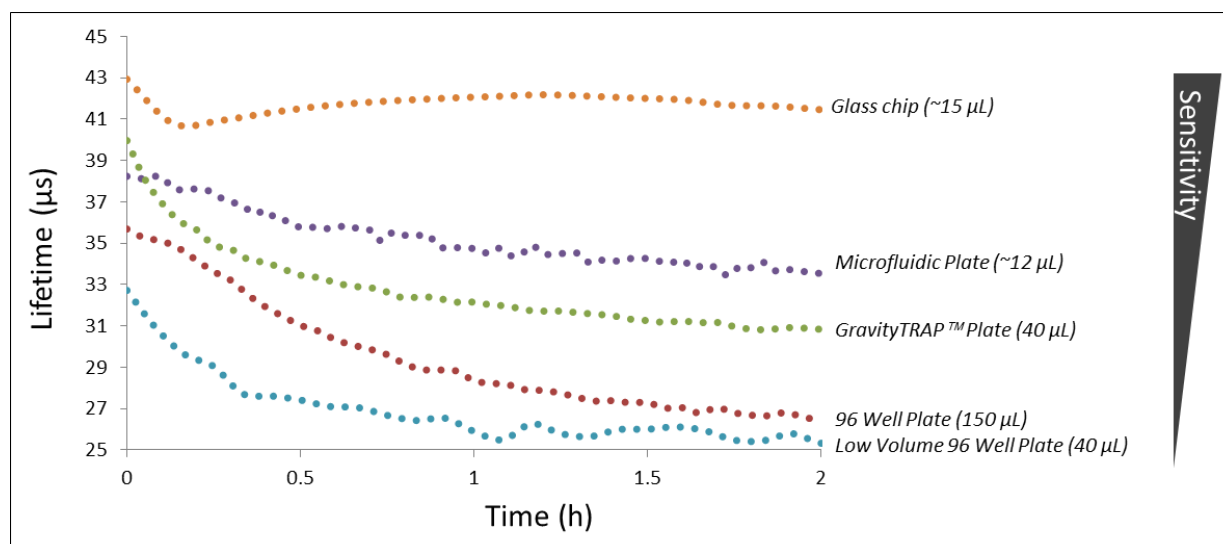
## RESULTS

### Measuring Mitochondrial Function

#### Assay Development and Optimisation

When measuring oxygen depletion, a key parameter of the performance of an assay is the barrier offered by the plate being used to ambient oxygen. The greater the barrier the more sensitive the oxygen depletion assay. This is particularly relevant to measurements of the spheroids (and biopsy material) where the amount of biomaterial is limited. The assay however must also be sufficiently robust to allow routine measurements and must be compatible with conventional plate reader technology to allow oxygen probe interrogation. Therefore, as a first step towards optimising SOPs for spheroids and biopsy material a comprehensive assessment of the oxygen barrier properties of a range of plate types was performed. This included standard microtitre plates as well as novel microfluidic plates and microfluidic chips. To assess barrier properties, measurement buffer is equilibrated at 5% oxygen and is then added to the test well/channel and sealed as appropriate. The

rate of oxygen back diffusion is then assessed kinetically by monitoring oxygen probe signal over time. The plate with the greater barrier properties is the slowest to return to ambient oxygen. This plate by extension is the most sensitive for oxygen depletion measurements. A summary of the data generated is presented in **figure 1**.



**Figure 1:** Measuring degree of sample re-oxygenation across a variety of plate types to determine assay sensitivity of oxygen depletion analysis of spheroids and biopsy samples.

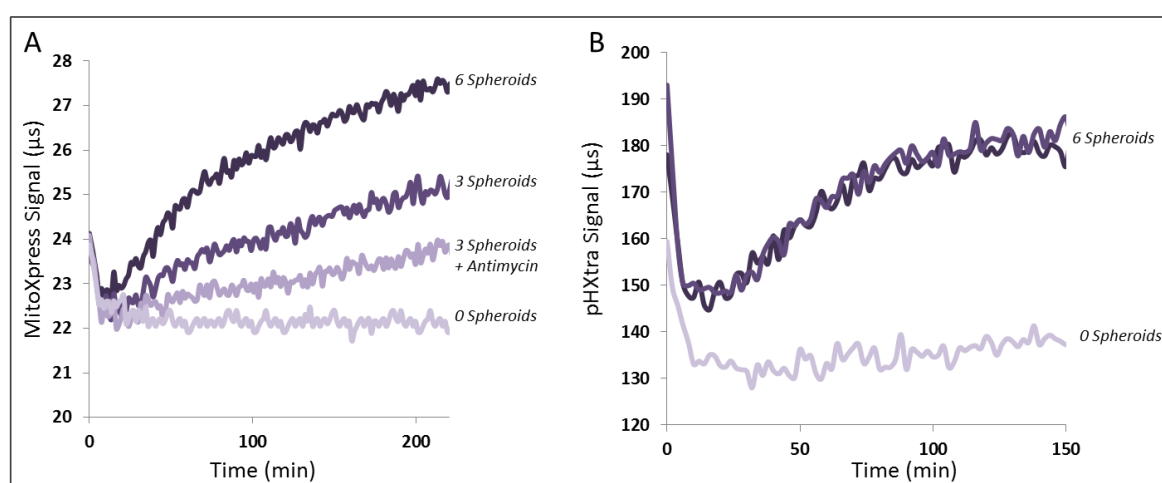
These data demonstrate that the glass chip is the most sensitive in that it maintains low oxygen for an extended period of time, this is then followed by the Microfluidic plate, GravityTRAP™ plate, 96 well plate and low volume 96 well plate. This in turn facilitates rational assay design allowing plate types to be chosen in an informed manner. For measurement of spheroids, the second major factor affecting sensitivity is assay volume, whereby lower volume offer greater sensitivity due to an increase in the cell to volume ratio of the test well.

### Spheroid Measurement

While it is clear from the data presented above that glass chips provide the greatest sensitivity, they are the least amenable to high throughput measurements as only 4 chips can be run in parallel on a conventional plate reader. The most convenient plate type to use for such measurements is the GravityTRAP™ plate in which the spheroids are supplied. To assess the performance of spheroid measurement in such plates, oxygen depletion was monitored using increasing numbers of spheroids per well. These measurements were performed using spheroids derived from primary human hepatocytes (Human Liver Microtissues: HuLiMTs). The data presented in **figure 2A** and demonstrate that there is clear concentration dependence with 3 spheroids per well showing measurable levels of oxygen depletion while 6 per well show significantly stronger depletion. These measurements are performed as per **SOP 1** and **SOP 2**. The effect of treatment with the ETC inhibitor Antimycin is seen to cause a decrease in oxygen consumption indicating that, using this protocol, mitochondrial dysfunction can be detected in spheroids. Also, as was demonstrated in **figure 1** the opportunity also exists to measure lower numbers of spheroids. While these measurements are not on standard microtitre plates, the approach may be useful tool later in the

project depending on the exposure times required for testing and the levels of inhibition observed upon drug treatment.

Optimization was also conducted to develop an SOP for measuring extracellular acidification of spheroids. This protocol uses a pH sensitive probe (pHXtra™) and can be run in the presence or absence of an oil seal. When measuring unsealed the vast majority of the observed acidification is as a result of glycolytic flux while in a sealed system CO<sub>2</sub> production also contributes significantly. The difference between sealed and unsealed acidification rates is there an indication of Krebs cycle activity. During protocols development, measurement in GravityTRAP™ plates shows a drift in pH in control wells possibly due to plate coatings. This is under continued investigation. An assay protocol has however been developed using low volume 96 well plates and sample data is presented in figure 2A.

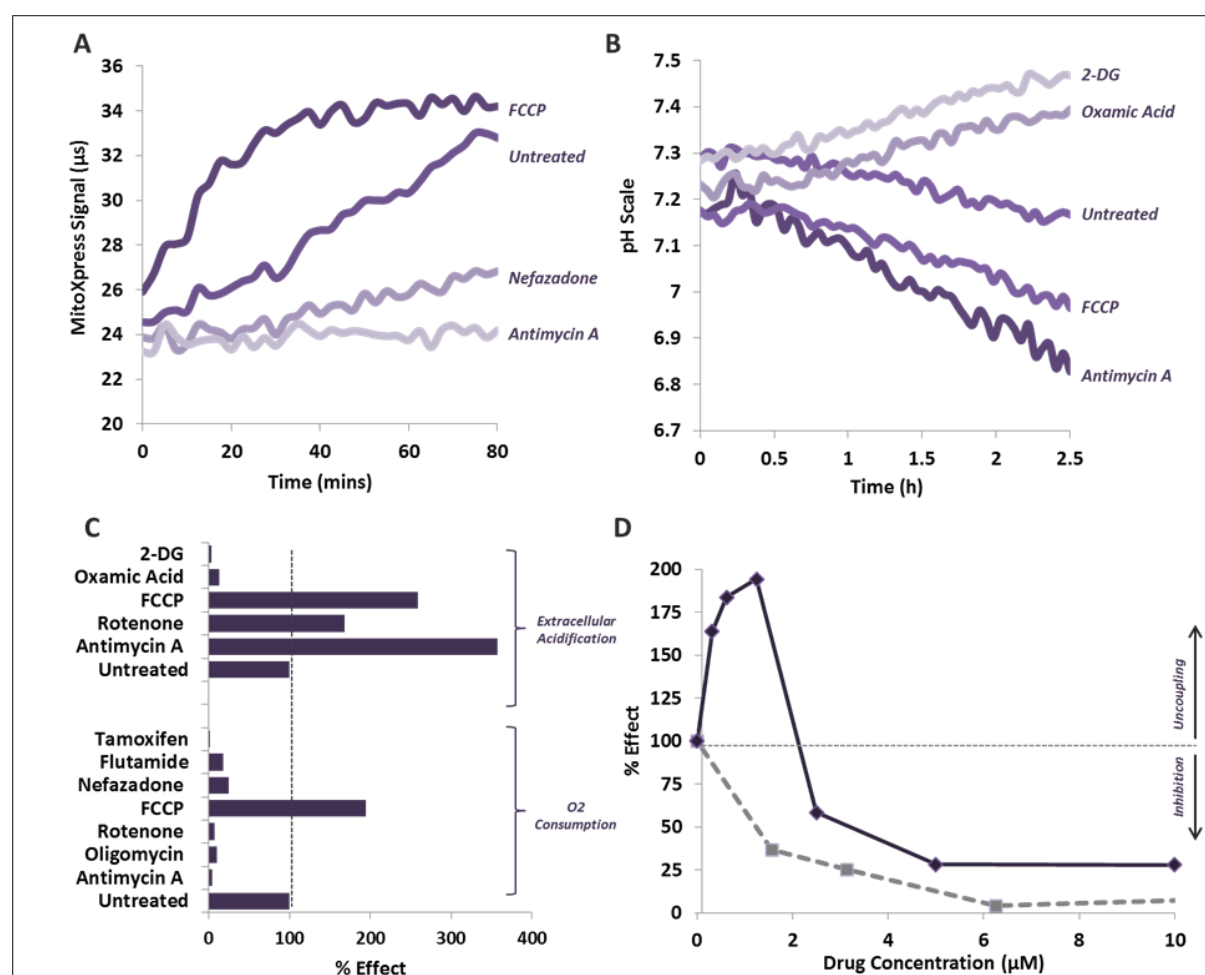


**Figure 2:** **A:** Sample data showing oxygen depletion by HuLiMT spheroids and demonstrating the inhibitory effect of the ETC inhibitor antimycin. **B:** Extracellular acidification profiles of HuLiMT spheroids.

### 2D Cardiomyocyte measurements Measurement

While the majority of the functional mitochondrial assessments of *in vitro* samples within the project is intended to be carried out on 3D models, for some endpoints, spherical tissues are not compatible with the assay technologies to be deployed. This is the case for the measurement of Cardiomyocyte electrophysiology using the xCELLigence system (D8.8) where contact is required between the cell and the surface of the assay plates. To facilitate any necessary parallel mitochondrial assessments of cells assayed in this manner, an SOP was developed for the assessment of the mitochondrial function of human stem cell derived cardiomyocytes. Measurements were performed as outlined in **SOP 1** and **SOP 2** and sample data is presented in **figure 3**. Again, oxygen consumption informs specifically on the activity of the electron transport chain with FCCP treatment with causing a more rapid signal increase due to an increased rate of oxygen consumption, while antimycin treatment results in no signal change due the inhibition of mitochondrial function and oxygen consumption. These effects are evident immediately post treatment and uncouplers can distinguished from inhibitors based on the effect of the compound on oxygen consumption. Extracellular acidification measurement shows that both FCCP and Antimycin cause increased glycolytic flux as the cardiomyocytes attempt to increase glycolytic ATP supply in the face of mitochondrial impairment. In contrast acidification is impaired in the presence of 2 deoxy-glucose as it competitively inhibits

glycolysis, and oxamic acid as it blocks the production of lactate. Additional validation was performed by treating with compounds including the antiandrogen, flutamide, a known Complex I inhibitor and the antiestrogen Tamoxifen, also a known mitochondrial modulator. These data again show that detailed information on the implications of drug treatment on cardiomyocyte mitochondrial function can be generated immediately post treatment. Also the incorporation of glycolytic flux measurements provides additional confidence in the identification of mitochondrial toxicity as can be seen for Antimycin, Rotenone and FCCP [5].

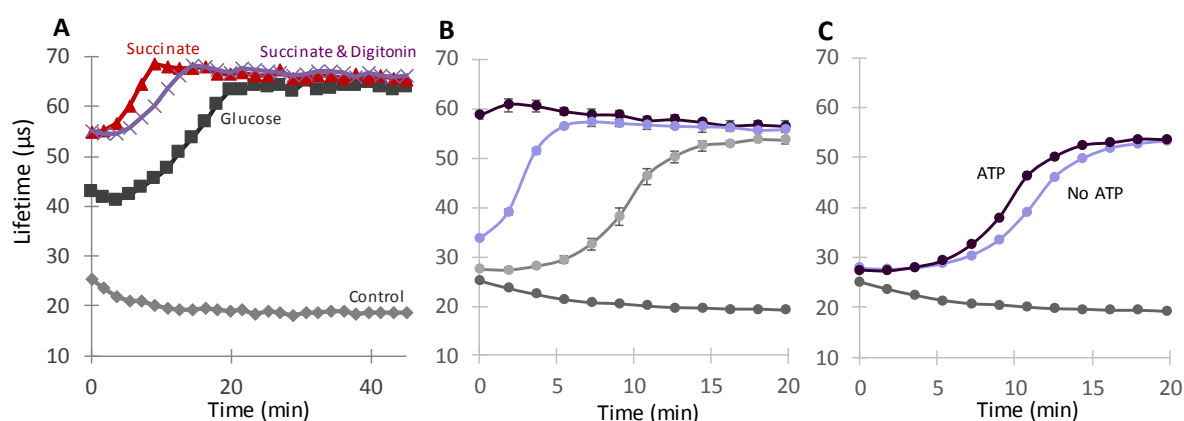


**Figure 3** A: Kinetic oxygen consumption profiles of Cor.4U cardiomyocytes treated with FCCP (1.25 μM), Antimycin A (1 μM) and Nefazadone (1.56 μM) B: Extracellular acidification profiles of Cor.4U cardiomyocytes detected using pH-Xtra® treated with Antimycin (1 μM) and uncoupler FCCP (2.5 μM) Oxamic acid (25 mM) and 2-DG (25 mM) C: Single concentration treatment of multiple drugs across both the MitoXpress®-Xtra oxygen consumption and the pH-Xtra® glycolysis assay. D: Sample Dose Response Graph for the MitoXpress®-Xtra oxygen consumption assay showing treatment of FCCP (uncoupler) and Nefazadone (inhibitor).

### Ex vivo material

A measurement protocol to assess mitochondrial function in Biopsy material was developed using snap frozen mouse liver as a model looking at both quartz cuvette measurements of collagenase digested liver and crude liver homogenate measured on low volume 96 well plates. All measurements are plate reader based. Measurements on liver fragments in quartz cuvettes demonstrate the impact of the substrate provided on oxygen consumption, with glucose showing lower oxygen consumption than succinate. The fact the succinate, which is cell impermeable,

demonstrates such high levels of oxygen consumption suggests considerable cell membrane damage allowing succinate access to the cellular mitochondrial network. This is supported by the observation that the addition of digitonin does not increase cell respiration (Fig 4A). While this method has been shown to be capable of assessing mitochondrial dysfunction, limited through-put and a requirement for biomaterial normalization could present difficulties in applying such measurement to the analysis of subtle changes between *ex vivo* samples. Measurements of crude liver homogenate were therefore assessed and optimized using lower volume hand homogenisation. While this approach does not allow the assessment of a pure mitochondrial fraction as available when using cell fractionation, it does facilitate the use of approximately ten fold less material and, as the homogenate can be easily pipetted, it overcomes some of the throughput and normalisation issues associated with measurement of tissue pieces. Data shows clear concentration dependence with higher homogenate concentrations showing higher levels of oxygen depletion (Fig4B). In addition, antimycin inhibition is observed illustrating that ETC activity is being measured specifically. It should also be noted that the addition of ADP has little effect on oxygen depletion (Fig 4C) indicating that again, these mitochondria have been uncoupled by freezing and thawing process. The method of measuring snap frozen samples is therefore well suited to assessing ETC inhibition but not the assessment of uncoupling of mitochondria or membrane potential mediated changes in ETC activity. This can be overcome by performing the measurement on fresh samples.



**Figure 4 A:** Measurements of collagen digested snap frozen mouse liver slimes in quartz cuvettes demonstrating the effect of substrate and cell permeabilisation on oxygen consumption. **B:** Concentration dependence of crude liver homogenate prepared from snap frozen liver samples measuring state 2 respiration on succinate. **C:** Effect of ADP addition to succinate-driven oxygen consumption of crude homogenate.

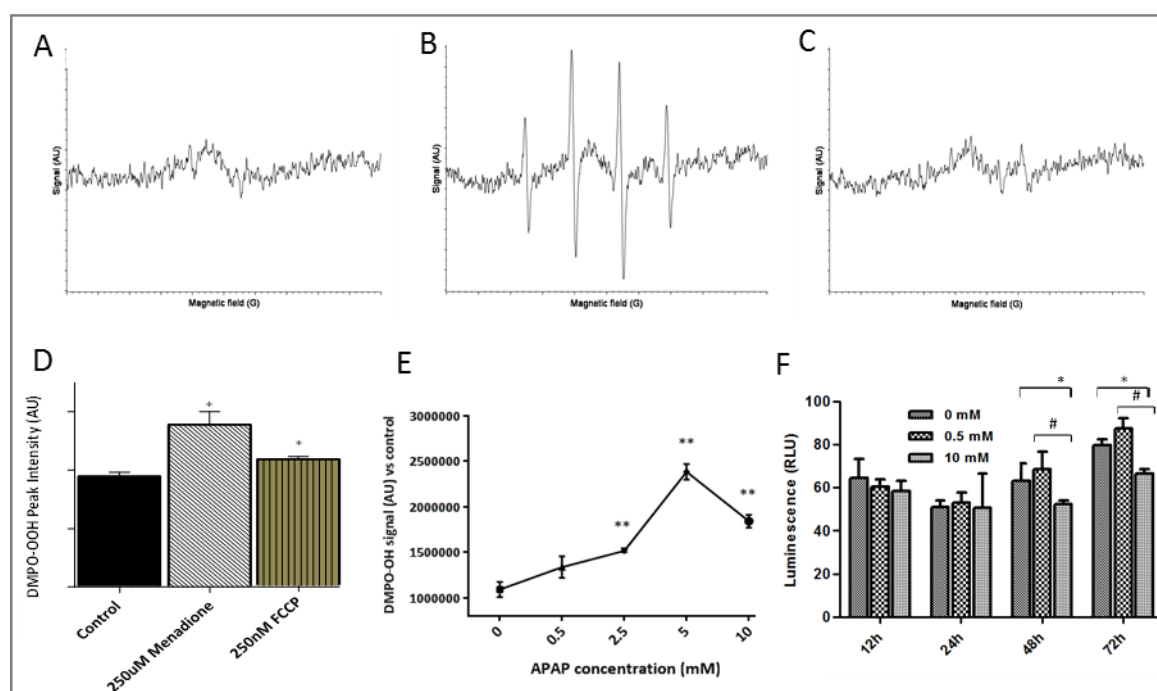
## ESR Measurements of ROS

### Measurement in cells

An SOP was developed to detect enhanced mitochondrial ROS formation after cellular incubations with compounds/drugs (**SOP 4**). Measurements were performed as described in SOP X. This SOP was initially developed on a 2D cell model (HepG2) and is expected to be applicable on spheroids, with only minor modifications. To investigate mitochondrial ROS formation, basal mitochondrial ROS formation was first identified in isolated mitochondria by ESR in combination with the spin trap DMPO (Fig 5A-C). This showed that ROS production was only detected in the presence of mitochondrial substrates and could be completely inhibited by superoxide dismutase, which further support that mitochondrial superoxide formation is measured by ESR. Control experiments were



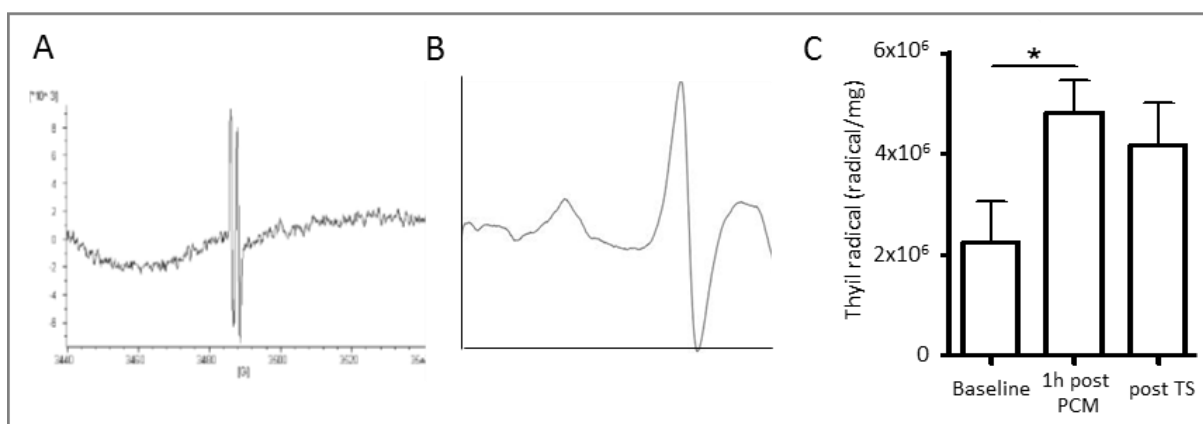
then run to investigate how cellular incubations with compounds known to uncouple mitochondria (FCCP) or induce cellular ROS formation (menadione) enhance mitochondrial ROS formation. As shown in Fig 5D, both compounds significantly increase the ESR detectable mitochondrial superoxide formation. Enhanced mitochondrial ROS formation could imply damage to or dysfunctioning of proteins involved in the ETC of mitochondria. To support that these proteins, involved in the production of ATP via the ATP synthase, are affected, mitochondrial production of ATP was investigated after cellular incubations with acetaminophen. Both a time-dependent as well as dose-dependent decrease in mitochondrial ATP formation was detected in line with the enhanced mitochondrial ROS production (Fig 5E). Drug-induced mitochondrial damage in resulting in enhanced mitochondrial ROS formation was studied by cellular incubations with acetaminophen, showing a dose-dependent increase in mitochondrial ROS formation (Fig 5E). Enhanced mitochondrial ROS formation could imply damage to or dysfunctioning of proteins involved in the ETC of mitochondria. To support that these proteins, involved in the production of ATP via the ATP synthase, are affected, mitochondrial production of ATP was investigated after cellular incubations with acetaminophen. Both a time-dependent as well as dose-dependent decrease in mitochondrial ATP formation was detected in line with the enhanced mitochondrial ROS production (Fig 5F).



**Figure 5:** ESR spectra of basal ROS formation in isolated mitochondria: Mitochondria were isolated from HepG2 cells and supplied with the spin trap DMPO: **A:** isolated mitochondria without substrates. **B:** Isolated mitochondria supplied with 120 mM malate, 400 mM Glutamate and 400 mM succinate. A typical hydroxyl and/or superoxide derived DMPO-OH signal is detected. **C:** same as B, but now also 100 U/ml superoxide dismutase (SOD) added. **D:** HepG2 cells incubated with menadione and FCCP. Mitochondrial superoxide formation in isolated mitochondria detected by ESR in combination with the spin trap DMPO. **E:** Increase in dose-dependent mitochondrial ROS formation by HepG2 liver cells exposed to non-cytotoxic (MTT-test) concentrations of acetaminophen. Stars indicate significant ( $p < 0.05$ ) increased ROS formation compared to solvent control. At higher doses (10 mM) a decline in increased mitochondrial ROS formation was detected, probably caused by severe damage to ETC proteins. **F:** HepG2 cells were incubated with acetaminophen for the time indicated and at the indicated concentrations. A significant decrease in ATP formation was detected at the highest dose applied.

### Measurement in patient samples

Different methods are available to assess radical/ROS formation in biopsy materials by ESR. As described above, for mitochondrial ROS formation, mitochondria can be isolated and analysed by ESR. Direct tissue ROS levels can be measured indirectly by detection of vitamin C/ascorbic acid radicals in the supernatant of homogenized tissue samples show (Fig 6A) which can be interpreted as derived from ROS formation. Vitamin C reacts rapidly with superoxide, hydroxyl, alkyl, peroxy, and alkoxy radicals resulting in a one electron oxidized ascorbyl radical. Therefore the steady-state concentration of the resonance-stabilized ascorbyl radical serves as a marker for the degree of oxidative stress. This however required a significant amount of material and as such can be applied to larger samples of material but is not applicable to small biopsies. Therefore biopsy material will be investigated directly at low temperature with ESR on (mitochondrial) radical formation as described in SOP 4 after placement into liquid nitrogen in a finger dewar. In human liver biopsies, radicals like thyl radicals derived from GSH, and related to ROS formation can be directly observed (Fig 6B). Thyl radical formation was further investigated in liver biopsies of 7 patients that were treated with acetaminophen during a pylorus preserving pancreaticoduodenectomy (modified Whipple's procedure). During the operation three liver biopsies were taken and were snap frozen in liquid nitrogen. These were further analysed by ESR as described in SOP 4. There was a significant increase in the level of thyl radicals in liver tissue 1 h after acetaminophen challenge in these patients (Fig 6C).

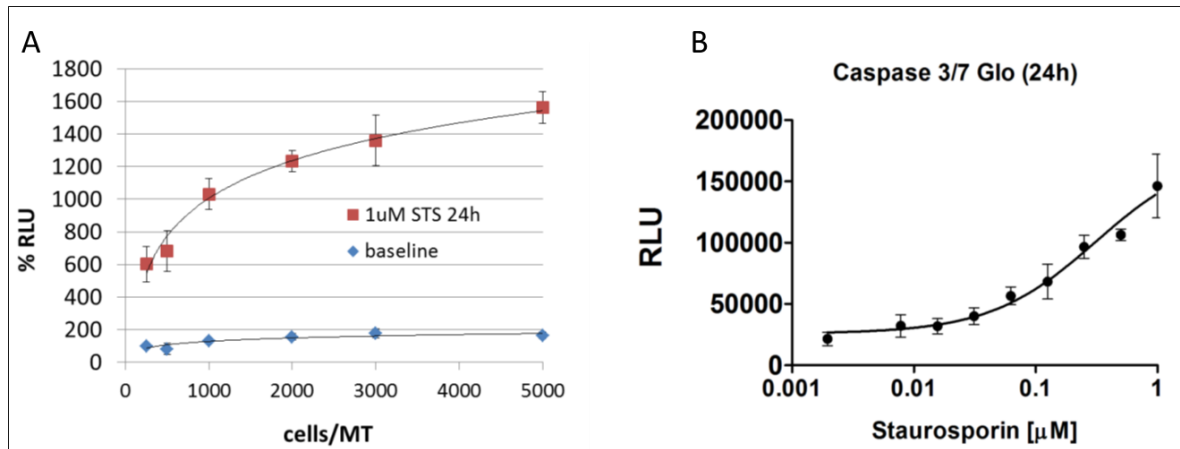


**Figure 6.** **A:** Vitamin C radical detected in homogenized human liver sample. **B:** Typical ESR spectrum of a thyl radical observed as broad signals (45G) with  $g = 2.02$  detected in a human liver biopsy at low temperature in liquid nitrogen. **C:** Level of thyl radicals measured in liver biopsies of patients that underwent a Whipple procedure: there was a significant increase 1 hour after acetaminophen (PCM) compared to baseline (\* $p=0.04$ ).

### Measuring Apoptotic Markers

Apoptosis induction is measured using the Caspase-Glo® 3/7 assay where a Z-DEVD-aminoluciferin substrate is cleaved making the aminoluciferin substrate available for the luciferase. The assay was developed for 2D-monolayer or single-cell-suspensions but has recently been adapted for the evaluation of caspase activity in scaffold-free 3-dimensional spheroids. Data shows signal linearity is maintained up to a 3D microtissue size of approx. 3000 cells, which roughly corresponds to a

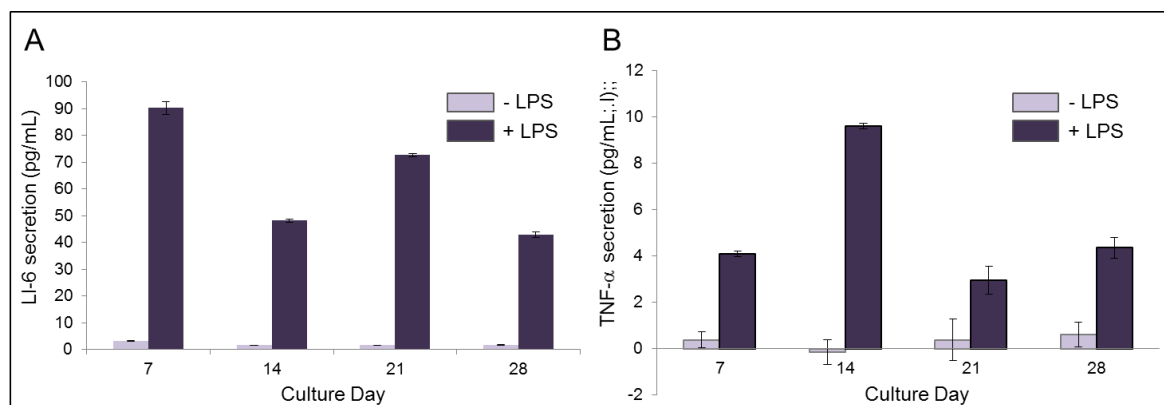
spherical volume of 0.04 mm<sup>3</sup> or a diameter of 300-400 µm. Sample data is presented in figure 7 and measurement are performed as outlined in **SOP 5**.



**Figure 7:** **A:** Linearity of caspase signal with spheroid size corresponding to 250, 500, 1000, 2000, 3000, and 5000 cells were treated with 1µM staurosporin for 24 hours and assayed for caspase-3/7 activity as per SOP 5 (n=4) **B:** Spheroids (approx. 300 µm in diameter) treated with Staurosporine (0-1µM) for 24 hours and assayed for caspase-3/7 activity as per SOP 5 (n=4)

### Inflammatory Markers

IL6 and/or TNF-α will be used as inflammation markers. To this end, SOPs were developed to assess both makers using commercially available ELISA kits. These kits use a capture antibody and a biotinylated secondary antibody, which is detected in turn by a Streptavidin-HRP conjugate. The data presented in Figure 8 demonstrates that these kits, originally developed for 2D cell cultures, are also applicable to scaffold-free 3-dimensional microtissues. Measurements performed as outlined in **SOP 6** and **7**. Where applicable the methods for IL6, Caspase 3/7 and TNF will also be applied to patient material, where the basis testing protocols will be the same.



**Figure 8.** 3D human liver microtissues cultured for increasing periods and incubated with LPS for 24 hours prior to measurement for IL-3 (**A, SOP 6**) and TNF-α secretion (**B, SOP 7**). Medium from 6 wells are pooled for testing.

## DIFFICULTIES

Protocols have been developed for the measurement of oxygen consumption of *ex vivo* samples. As the process of freezing and thawing uncouples the mitochondria, coupled measurements will require that measurement be performed on fresh material.

Patient samples may be limited in size and frequency, particularly for cardiac samples. If supply becomes limiting, rational decisions will need to be made as to which of the listed endpoints provide the most value to the modelling effort.

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## ANNEX

### **SOP 1: Oxygen Consumption Measurements of 2D and 3D *in vitro* Samples**

#### **1.1: Measuring 2D *in vitro* Models (Cor. 4U<sup>®</sup> human stem cell derived cardiomyocytes)**

1. Standard TC<sup>+</sup> 96-well plate(s) are coated with Fibronectin (50µl/well), as per protocol.
2. After thawing cells Cor.4U<sup>®</sup> cryo vial, or typrsinising fresh Cor4U from T75 flasks, count viable cells and adjust the cell concentration to ~2x10<sup>5</sup> cells/ml.
3. Plate the cells at 200µl per well to yield 4x10<sup>4</sup> cells/well final concentration, ensuring a uniform monolayer in each well [Fig 2], then place in CO<sub>2</sub> incubator (manufacturers suggest not using edge wells).
4. Prepare a **MitoXpress<sup>®</sup>-Xtra** stock in 15ml of pre-warmed Cor.4U<sup>®</sup> medium
5. Replace culture media in each well with 150µl of this solution.
6. Add 1µl of compound stock (150X) to each well and ensure to include untreated samples.
7. Seal the plate by overlaying with pre-warmed HS mineral oil, 100µl per well (to inhibit oxygen back diffusion into the sample). This is best done using a repeater pipette.
8. Measure 96-well plate kinetically for 90-120mins with ~2 minute interval exciting the probe at 380nm and measuring emission at 650nm.

#### **1.2: Measuring 2D *in vitro* Models (HuLiMT spheroids)**

Measuring in GravityTRAP™ plates:

1. Prepare a **MitoXpress<sup>®</sup>-Xtra** stock in 8ml of pre-warmed spheroid maturation medium (2X).

2. Combine spheroids into a single well to achieve desired number of spheroids per well, typically 3/well or 6/well.
3. Transfer these combined spheroids using 50µl volume of media as described in guidelines.
4. Allow time for spheroids to settle to bottom of GravityTRAP™ well and observe under magnification to ensure samples are in place. Ensure no air bubbles are trapped at the bottom of the well. Remove bubbles using P200 tip by gently pressing air bubble until it releases.
5. Add 10µl of media to each well of a new sterile GravityTRAP™ plate in preparation for spheroid addition and measurement. Tap plate gently on bench to ensure wells are wetted and ready for spheroid additions.
6. Transfer samples (3 spheroids) in a 50µl volume to the prepared GravityTRAP™ plate. Inspect loaded pipette tip to ensure correct number of spheroids are present.
7. Remove transfer media and replace culture media in each well with 50µl of **MitoXpress®-Xtra** stock.
8. Add 1µl of compound stock (50X) to each well and ensure to include untreated samples.
9. Seal the plate by overlaying with pre-warmed HS mineral oil, 70µl per well (to inhibit oxygen back diffusion into the sample). This is best done using a repeater pipette.
10. Measure 96-well plate kinetically for 90-120mins with ~2 minute interval exciting the probe at 380nm and measuring emission at 650nm.

Measuring on low volume plates:

1. Prepare a **MitoXpress®-Xtra** stock in 0.2ml of pre-warmed spheroid maturation medium (75X).
2. Remove Media from samples in GravityTRAP™ plate and replace with 20ul of **MitoXpress®-Xtra** stock solution. Ensure no air bubbles are trapped at the bottom of the well. Remove bubbles using P200 tip by gently pressing air bubble until it releases.
3. Transfer a single spheroid in 15ul volume and Add to channel of Microfluidic plate, with pipette held vertically when filling. Ensure sample is added across entire channel and into both filling chambers.
4. Seal both filling chambers with ~10ul of pre-warmed mineral oil, (to inhibit oxygen back diffusion into the sample).
5. Measure Microfluidic plate kinetically for 90-120mins with ~2 minute interval exciting the probe at 380nm and measuring emission at 650nm. Using designated wells selected of 384-well SBS layout.

## **SOP 2: ECA Measurements of 2D and 3D *in vitro* Samples**

### **2.1: Measuring 2D *in vitro* Models (Cor. 4U<sup>®</sup> human stem cell derived cardiomyocytes)**

1. Standard TC<sup>+</sup> 96-well plate(s) are coated with Fibronectin (50µl/well), as per protocol.
2. After thawing cells (Cor.4U<sup>®</sup> cryo vial, or typrsinising fresh Cor4U from T75 flasks, count viable cells and adjust the cell concentration to  $\sim 1.5 \times 10^5$  cells/ml.
3. Plate the cells at 200µl per well to yield  $4 \times 10^4$  cells/well final concentration, ensuring a uniform monolayer in each well [Fig 2], then place in CO<sub>2</sub> incubator (manufacturers suggest not using edge wells).
4. Place seeded 96-well plate in CO<sub>2</sub> free incubator for 3-hours.
5. Wash plate using Respiration Buffer 3 times (0.5 mM KH<sub>2</sub>PO<sub>4</sub>, 0.5 mM K<sub>2</sub>HPO<sub>4</sub>, 20 mM Glucose, 4.5 g/L NaCl, 4.0 g/L KCl, 0.097 g/L MgSO<sub>4</sub>, 0.265 g/L CaCl<sub>2</sub>).
6. Prepare a **pH-Xtra™** stock in 15ml of pre-warmed Respiration Buffer
7. Replace culture media in each well with 150µl of this solution.
8. Add 1µl of compound stock (150X) to each well and ensure to include untreated samples.
9. Measure 96-well plate kinetically for 90-120mins with  $\sim 2$  minute interval exciting the probe at 380nm and measuring emission at 615nm.

### **2.2: Measuring 2D *in vitro* Models (HLiMT spheroids)**

1. Place GravityTRAP™ plate with spheroids in CO<sub>2</sub> free incubator for 3-hours.
2. Wash plate using Respiration buffer 3 times. Remove spent media from each well and replace with 100µl of Respiration buffer.
3. Combine spheroids into a single well to achieve desired number of spheroids per well, typically 3/well or 6/well.
4. Transfer these combined spheroids using 50µl volume of buffer as described in guidelines.
5. Allow time for spheroids to settle to bottom of GravityTRAP™ well and observe under magnification to ensure samples are in place. Ensure no air bubbles are trapped at the bottom of the well. Remove bubbles using P200 tip by gently pressing air bubble until it releases.
6. Prepare a **pH-Xtra™** stock in 8ml of pre-warmed Respiration Buffer (2X).
7. Remove Respiration buffer from each well and replace with 75µl of **pH-Xtra™** stock.
8. Transfer samples (3 spheroids) in a 50µl volume of this **pH-Xtra™** stock to the prepared Half-Volume 96-well plate. Inspect loaded pipette tip to ensure correct number of spheroids are present.
9. Add 1µl of compound stock (50X) to each well and ensure to include untreated samples.
10. Measure 96-well plate kinetically for 90-120mins with  $\sim 2$  minute interval exciting the probe at 380nm and measuring emission at 615nm. Thaw liver sample at 37 °C for approximately 1 min. Weigh the sample (wet weight).

### **SOP 3: Measuring Oxygen Consumption in crude tissue homogenate using snap frozen samples**

1. Thaw liver sample at 37 °C for approximately 1 min. Weigh the sample (wet weight).
2. Gently wash the liver (0.1-0.3 g) with 3mL ice-cold Hank Balance Salt Solution (HBSS). Remove HBSS and repeat wash twice.
3. Homogenise liver in ice-cold HBSS (1 ml) with hand held glass pestle and mortar. (approx. 10 passes)
4. Retrieve liver into a 50 ml conical tube and rinse the mortar with ice-cold HBSS to ensure all the homogenate has been retrieved.
5. Centrifuge homogenate at 200 x g, RT for 5 min. Discard supernatant and re-suspend pellet in ice-cold 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (~3 mL) to give a crude homogenate stock [Concentration can be assessed photometrically or using original wet weight].
6. Dilute the crude homogenate stock to desired concentration in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer using a dilution of 1:3 or greater in 2 mL tubes.
7. Add substrates at the desired concentration from concentrated stocks (typically 20-50X). Typical final concentrations are as follows; Glucose 25 mM, Succinate 10 mM, Glutamate/malate 12.5 mM
8. Add 50 µL of each preparation to the well of a low-volume 96 well plate (n=4).
9. Re-suspend MitoXpress Xtra in 1mL of measurement buffer and add 5 µL to each well.
10. Add compounds being tested including control compounds
11. Add 70 µL pre-warmed high Sensitivity Oil to each well. This is best done using a repeater pipette.
12. Measure 96-well plate kinetically for 90-120mins with ~2 minute interval exciting at 380nm and measuring emission at 650nm.

## **SOP 4: ESR measurement of ROS**

### **4.1: Measuring In vitro Models**

1. Cells are typically cultures to  $1.5 \times 10^6$  cells cultured in T25 flask.
2. Cell collection:
  - 5ml trypsin was added into each 150cm<sup>2</sup> flask and incubated at 37°C for 5 min.
  - Add 11 ml of 10%FCS HBSS to stop trypsinization.
  - Transfer the cells in to 50ml tubes and centrifuge the cell suspension at **600g** for 5 min at 4°C and discard the supernatant.
  - Wash cells once by resuspending cell pellet in **6ml** ice-cold PBS (or HBSS).
  - For each group, separate the cell suspension into 2 15ml conical tubes.
  - Centrifuge the cell suspension at **600g** for 5 min at 4°C and discard the supernatant.
3. Detergent lysis:
  - For each tube, re-suspend the cell pellet in **2ml** ice-cold CHAPS buffer and gently pipet up and down for a few times.
  - Incubate the cells for **15 min** on ice and gently pipet the mixture up and down **20 times**.
  - Subsequently, **2ml** mitochondrial isolation medium (MiB01) is added to the tube to protect the mitochondria integrity. The tube was inverted several times to mix the contents.
4. Centrifugation:
  - Centrifuge the lysate at **600g** for 10min at 4°C.
  - Carefully decant the **supernatant** (2 tubes) to one fresh 10ml ultracentrifuge tube (8ml supernatant per tube). The pellet containing cell debris, nuclei and unbroken cells were discarded. Ensure that centrifuged is pre-cooled (Pro: 21.0, Tem: 4°C, Time: 15 min)
  - Centrifuge the supernatant at 3,000-12,000 g, at 4°C for 15min. (lower speed centrifuge can increase the mitochondria purity but sacrifice the yield)
  - According to the pellet size resuspend the pellet with **10-20ul** MiB01 and transfer the mitochondria into a precooled 1.5ml tube and keep it on ice.
5. Determination of the concentration
  - Determination of total protein content of the mitochondrial pellet is done by the Fluorescamine method (SOP: O2k-01), with bovine serum albumin as a standard. For this purpose, dilute 3.5 µl of the mitochondrial suspension in 31.5 µl of milliQ for triplicate (2.5ul mito:22.5ul milliQ for duplicate) and Vortex.
  - Measure the protein content in triplicate. Thus, add 1.4 ml (700ul x 2 times) 0.2 M potassium borate (K3BO3, pH 9.0) in each of 3 wells of a 12-well plate and add **10 µl** of the diluted mitochondrial solution to each of the three wells and mix the solutions on a plate-shaker.
  - Add, under continuous stirring on the plate-shaker, 700 µl stock solution to each of the three wells, cover the plate with aluminum foil and mix on the plate-shaker.
  - Wait 5 min up to an hour before measuring the fluorescence on a spectrofluorophotometer (Ex/Em 390nm/475nm)
6. ESR Measurement
  - Diluted isolated mitochondria in MIB01 (180 ul) final conc. should be **0.05mg/ml** with the CPH and the complex of substrates (200 ul) and reactive the mitochondria with **5min** incubation in 37°C water bath.
  - At the same time, dilute the stock glutamate, succinate, malate in MIB01 to working solution. Table 1.
  - Add 5 ul CPH (25mM), 5ul glutamate (400mM), 5 ul succinate (400mM), 5ul malate (120mM) and heat the diluted mitochondria of each group for another **20 minutes** with substrates and DMPO at 37°C in the water bath (drill some holes on the lid)
  - Vortex carefully, and briefly, Fill capillary till above the black line, close capillary with wax and place into ESR.



- ESR spectra were recorded at room temperature in glass capillaries on a Bruker EMX 1273 spectrometer equipped with an ER 4119HS high sensitivity cavity and 12 kW power supply operating at X band frequencies. The modulation frequency of the spectrometer was 100 kHz. Instrumental conditions for the recorded spectra were as follows: magnetic field, 3490 G; scan range, 60 G; modulation amplitude, 1 G; receiver gain,  $1 \cdot 10^5$ ; microwave frequency, 9.85 GHz; power, 50 mW; time constant, 40.96 ms; scan time, 20.97 s; number of scans, 35. Quantitation of the spectra (in arbitrary units) was performed by peak height measurements using the WIN-EPR spectrum manipulation program.

**Table 1 substrate dilution**

compounds	stock conc. (mM)	NV (ul/well)	MIB 01 (ul)	FV/well (ml)	working conc. (mM)
Malate	800	15	85	100	120
Glutamate	2000	20	80	100	400
Succinate	1000	40	60	100	400

#### 4.2: Measuring Frozen human liver biopsies

1. Frozen biopsies were placed in liquid nitrogen in a quartz liquid finger dewar at the center of the 1273 ER4119HS high sensitivity cavity.
2. ESR spectra were recorded on an X-band spectrometer (Bruker EMX 1273, Biospin, Rheinstetten, Germany) operating at 9.50 GHz.
3. Instrumental conditions for the recorded spectra were: magnetic field: 3325 G; scan range: 150 G; modulation frequency 100 kHz modulation amplitude: 5 G; receiver gain:  $1 \times 10^5$ ; power: 20 mW; time constant: 20.84 ms; scan time: 40.96 ms; number of scans: 20.
4. Radicals were quantified by peak surface measurements using the WIN-EPR spectrum manipulation program (Version 2.11, Bruker, Rheinstetten, Germany).

## **SOP 5: Measuring Caspase Activity in Spheroids using Caspase-Glo® 3/7 (Promega, cat# G8092)**

### Culture and preparation

1. Pre-warm microtissue culture medium to 37°C in a water bath and carefully open the bag to remove the Gravity-TRAP™ plate containing the microtissues.
2. Place the GravityTRAP™ plate into the incubator for 1 hour to liquefy the shipping medium, then centrifuge the plate in a microtiter plate centrifuge at 250x RCF for 1 min at room temperature.
3. Open the lid under a sterile working bench and gently remove the sealing membrane processing only one plate at a time (This keeps the shipping media above 30°C and in liquid form).
4. Carefully aspirate the shipping medium from each well using the ledge near the bottom of the well to position your pipette tip. The ledge can be localized by touch. this ensures maximum liquid withdrawal without risk of aspirating the microtissue. The medium which is left behind will have a volume of approx. 5 µl.
5. Add 70 µl of pre-warmed medium, again by positioning the pipette tip at the ledge, replace the lid and incubate the plate for one hour.
6. Repeat the aspiration step and again add 70 µl of pre-warmed fresh medium.
7. Remove the MicroClima™ lid from the bag and apply 4 ml of sterile distilled H2O into the filling area on each short side of the lid.
8. Cover plate with the MicroClima™ lid to reduce edge effects during incubation of the microtissues.

### Caspase Assay

1. Treat microtissues with test compound for the desired time, including a blank control without microtissues (if required on a separate plate), a negative control (microtissues treated with vehicle only) and ideally a positive control (e.g. Staurosporine or Taxol)
2. Prepare sufficient Caspase-Glo® 3/7 Reagent (cat #: G8092, mix 1:1 with medium) and remove medium from each well of the Gravity-TRAP™ plate by carefully placing the pipette tip at the ledge of the well and aspirate at low speed.
3. Add 50 µl of diluted reagent mix to each well, mix and transfer the entire well content into a white walled multiwell plate. Then shake the plate for 30 min on an orbital plate shaker at room temperature.
4. Read luminescence in a plate reading luminometer.

### **SOP 6: Measuring Human IL-6 using ELISA kit (Invitrogen, cat # KHC0061)**

1. Reconstitute standard to 2500 pg/mL with Standard Diluent Buffer and mix gently and allow to sit for 10 minutes to ensure complete reconstitution and use within 1 hour.
2. Add 0.200 mL of the reconstituted standard to a tube containing 0.800 mL Standard Diluent Buffer and label as 500 pg/mL Hu IL-6. Mix. Then add 0.300 mL of Standard Diluent Buffer to each of 6 tubes making them up to 250, 125, 62.5, 31.2, 15.6, and 7.8 pg/mL Hu IL-6.
3. Dilute 10 µL of a 100x concentrated solution with 1 mL of Streptavidin-HRP Diluent for each 8-well strip used in the assay. Label as Streptavidin-HRP Working Solution. The Streptavidin-HRP 100x concentrate is in 50% glycerol. To ensure accurate dilution, allow it to reach room temperature. Store remaining Streptavidin-HRP concentrate at 4°C.
4. Remove the required number of 8-well strips for use and add 100 µL of the Standard Diluent Buffer to zero wells. Well(s) reserved for chromogen blank should be left empty.
5. Add 100µL of standards, samples or controls to the appropriate microtiter wells. For spheroid measurements, 6 wells are pooled.
6. Add 50 µL of biotinylated anti-IL-6 (Biotin Conjugate) solution into each well except the chromogen blank(s). Tap gently on the side of the plate to mix.
7. Cover plate with plate cover and incubate for 2 hours at room temperature, then thoroughly aspirate or decant solution from wells and discard the liquid and wash wells 4 times. (Allow the Wash Buffer Concentrate (25X) to reach room temperature and mix to ensure that any precipitated salts have redissolved before diluting for use)
8. Add 100µL Streptavidin-HRP Working Solution to each well except the chromogen blank(s). Then cover plate with the plate cover and incubate for 30 minutes at room temperature. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times.
9. Add 100 µL of Stabilized Chromogen to each well. The liquid in the wells will begin to turn blue. Incubate for 30 minutes at room temperature and in the dark (Note: Do not cover the plate with aluminum foil or metalized mylar)
10. Add 100 µ L of Stop Solution to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow. Read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 µL each of Stabilized Chromogen and Stop Solution.
11. Read the Hu IL-6 concentrations for unknown samples and controls from the standard curve.

### **SOP 7: Measuring Human TNF- $\alpha$ using ELISA kit (Invitrogen cat # KHC3014)**

1. Reconstitute standard to 380 pg/mL with Standard Diluent Buffer and mix gently and allow to sit for 10 minutes to ensure complete reconstitution and use within 1 hour.
2. Add 0.050 mL of the reconstituted standard to a tube containing 0.550 mL Standard Diluent Buffer and label as 32 pg/mL Hu TNF- $\alpha$  Mix. Then add 0.300 mL of Standard Diluent Buffer to each of 6 tubes making them up to 16, 8, 4, 2, 1 and 0.5 pg/mL Hu TNF- $\alpha$ .
3. Dilute 10  $\mu$ L of a 100x concentrated solution with 1 mL of Streptavidin-HRP Diluent for each 8-well strip used in the assay. Label as Streptavidin-HRP Working Solution. The Streptavidin-HRP 100x concentrate is in 50% glycerol. To ensure accurate dilution, allow it to reach room temperature. Store remaining Streptavidin-HRP concentrate at 4°C.
4. Remove the required number of 8-well strips for use and add 50 $\mu$ L of the Incubation Buffer to all wells. Well(s) reserved for chromogen blank should be left empty. Add 100  $\mu$ L of the Standard Diluent Buffer to zero wells. Well(s) reserved for chromogen blank should be left empty.
5. Add 100  $\mu$ L of standards or controls to the appropriate microtiter wells. For all samples (serum, plasma, buffered solution and cell culture medium), add 50  $\mu$ L of Standard Diluent Buffer to each well followed by 50  $\mu$ L of sample. Tap gently on the side of the plate to mix. For spheroid testing 6 wells are typically pooled.
6. Pipette 50  $\mu$ L of biotinylated Hu TNF-  $\alpha$  Biotin Conjugate solution into each well except the chromogen blank(s). Tap on the side of the plate to thoroughly mix and cover plate with plate cover and incubate for 2 hours at 37 °C .
7. Thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times. (Allow the Wash Buffer Concentrate (25X) to reach room temperature and mix to ensure that any precipitated salts have redissolved before diluting for use)
8. Add 100  $\mu$ L Streptavidin-HRP Working Solution to each well except the chromogen blank(s) and cover plate with plate cover and incubate for 30 minutes at room temperature. Then thoroughly aspirate or decant solution from wells and discard the liquid. Wash wells 4 times.
9. Add 100  $\mu$ L of Stabilized Chromogen to each well. The liquid in the wells will begin to turn blue. 12. Incubate for 30 minutes at room temperature and in the dark (Note: Do not cover the plate with aluminum foil or metalized mylar)
10. Add 100  $\mu$ L of Stop Solution to each well. Tap side of plate gently to mix. The solution in the wells should change from blue to yellow. Then read the absorbance of each well at 450 nm having blanked the plate reader against a chromogen blank composed of 100 $\mu$ L each of Stabilized Chromogen and Stop Solution. Then read the Hu TNF- $\alpha$  concentrations for unknown samples and controls from the standard curve taking account of dilution factors.