Preparation and incubation of precision-cut liver and intestinal slices for application in drug metabolism and toxicity studies

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Precision-cut tissue slices (PCTS) are viable *ex vivo* explants of tissue with a reproducible, well defined thickness. They represent a mini-model of the organ under study and contain all cells of the tissue in their natural environment, leaving intercellular and cell-matrix interactions intact, and are therefore highly appropriate for studying multicellular processes. PCTS are mainly used to study the metabolism and toxicity of xenobiotics, but they are suitable for many other purposes. Here we describe the protocols to prepare and incubate rat and human liver and intestinal slices. Slices are prepared from fresh liver by making a cylindrical core using a drill with a hollow bit, from which slices are cut with a specially designed tissue slicer. Intestinal tissue is embedded in cylinders of agarose before slicing. Slices remain viable for 24 h (intestine) and up to 96 h (liver) when incubated in 6- or 12-well plates under 95% O_2/5% CO_2 atmosphere.

INTRODUCTION

Tissue slices

Precision-cut tissue slices (PCTS) are widely used by many researchers as an *in vitro* model of the organ under study[1–6]. Slices essentially are viable explants of the tissue that can be cultured *ex vivo*. By using specialized equipment, they can be prepared with a reproducible, well defined thickness. Tissue slices contain all cell types of the tissue in their natural environment, with intercellular and cell-matrix interactions remaining intact. Slices can be prepared using specially designed tissue slicers[7–9] that provide a universal method to prepare PCTS from all types of solid tissue under conditions that preserve the viability of the tissue. Non-solid organs such as the intestine[10] or the lung[3,11–13], or non-solid carcinomas (S. de Jong, I.A.M.d.G. and A. Meijer, unpublished data), can be embedded in agarose to form a solid organ-like structure, which can then be sliced in much the same way as can solid organs. PCTS have been prepared from liver (refs. 2,3,14,15 and references therein), lung[11,12,16,17], kidney[6,16–18], intestine[10,19–21], spleen[22,23], brain[24,25], heart[5,26], prostate[27] and several tumor types[28–30]. They have proven to be useful for the study of biochemical functions such as endogenous metabolism, biotransformation and its induction and transport of drugs and other xenobiotics, as well as for toxicological studies, and for assessing the efficacy of drugs in diseased tissues. Slices are also applied to study ischemia/reperfusion damage[31–34] and to evaluate the specificity of viruses as carriers for gene therapy agents[30,35–38]. In this article we describe the protocols for the preparation and incubation of rat and human liver and intestinal precision-cut slices.

Liver

Ever since the development of a method for successful isolation of rat hepatocytes by Berry and Friend in 1969 (ref. 39) and the modification by Seglen[40], isolated and cultured rat hepatocytes are the gold standard for research on liver, including physiology, pathology, endogenous metabolism and uptake, excretion, metabolism and toxicity of xenobiotics and drugs. The first publication on the isolation of human hepatocytes appeared in 1976 (ref. 41). However, in the course of time it became evident that these hepatocytes lose their differentiation in culture unless special techniques are applied, such as sandwich cultures and cocultures with other cell types[42–44]. None of these cultures, however, can provide a complete liver model. Although hepatocytes indeed comprise the largest cell fraction and represent 80% of the liver volume, they make up only about 60% of all liver cells because of their relatively large size. The other 40% consists of Kupffer cells (the resident liver macrophages), hepatic stellate cells (involved in vitamin A storage and collagen production), several types of endothelial cells lining the blood vessels (including a fenestrated type lining the sinusoids), biliary epithelial cells lining the bile ducts and several other cell types such as fibroblasts, immune cells and stem cells[45]. At present, it is well known that all these cell types communicate with each other and that their functionality depends strongly on interactions with their natural matrix. For these reasons, a model including all cell types in their natural environment is indispensable for studying complex, multicellular liver functions and reactions to toxic compounds *in vitro*. In 1980, a renaissance of an old technique described by O. Warburg[46] and H.A. Krebs[47] was initiated by C.L. Krumdieck *et al.*[7], who described a new instrument to reproducibly prepare thin, precision-cut liver slices. The thickness of these liver slices is small enough to allow sufficient oxygen and nutrient supply to the inner cell layers[48]. Hepatocytes in slices retain their membrane and intracellular polarization, in contrast to isolated hepatocytes, which lose their anatomical polarity after isolation[49]. Slicing allows a very efficient use of the tissue and is not dependent on the use of potentially damaging proteolytic enzymes, as is the case in isolation of hepatocytes.

Most studies were conducted with rat liver slices, but the technique is easily transferable to mouse, minipig, monkey or dog liver, and, most importantly, to human liver. Human liver slices can be

prepared from small pieces of human liver obtained as surgical waste after partial hepatectomy or as parts of nontransplantable donor tissue, allowing interspecies comparisons and the explanation of human-specific functions[2,50–52].

Moreover, some limitations of the liver slice system exist. Although slices can be cultured for up to 5 d when proper conditions are provided[53], this may still not be sufficient to study chronic toxicity of compounds. Besides, because preparation and incubation are relatively labor and space intensive, slices are less useful for high-throughput purposes. Moreover, as in hepatocytes, cytochrome P450 expression in slices tends to decrease during prolonged culturing, although the extent of this decrease seems to be partly dependent on culture conditions, as was recently discussed by de Graaf *et al.*[2]. Moreover, medium supplements such as insulin, dexamethasone and serum seem to slow the downregulation of cytochrome P450s during culturing (P.O. and G.M.M.G., unpublished data). Incomplete penetration of compounds is another extensively discussed limitation of liver slices. Particularly rapidly metabolized lipophilic compounds (applied under conditions that do not saturate metabolism) are rapidly taken up by the outer cell layers of the slice[54], resulting in a decreasing gradient of exposure to the substrate toward the inner cell layers; metabolism (per mg of tissue) of these compounds is thus dependent on the slice thickness. However, it has also been shown that, in principle, under optimal culture conditions, the inner cell layers of slices are fully capable of metabolizing compounds[55]. When slices are used for quantitative metabolism studies, the limitation of penetration of compounds can be easily circumvented by using thin (100 μm) slices[55].

Intestine

For the intestine, *in vitro* methods are not as widely available as for the liver and have been recently reviewed by van de Kerkhof *et al.*[56]. The Ussing chamber technique[21] accommodates intact intestinal tissue and is widely used to study permeability. A limitation of this technique is that tissue viability is generally limited to 2–4 h (ref. 56). Intestinal epithelial cells can be isolated, but recovery is low and the cells are difficult to culture. Therefore, we took the initiative to adapt the precision-cut slice technique for the preparation of intestinal slices[10]. To render the intestine suitable for slicing, we filled the pieces of intestinal tissue and/or embedded them in low-melting-temperature agarose. Cross-sectioning of these agarose-embedded tissue cores results in precision-cut intestinal slices (PCIS) that can be incubated for up to 24 h. The intestine is a very heterogeneous organ, in which structural and functional differences are prominent between duodenum, jejunum, ileum and colon. PCIS are eminently suitable for studying these regional differences. So far, PCIS have been obtained from rat, mouse and human intestine, and were used to study drug metabolism and the regulation of enzymes and transporters involved in drug disposition[17,19,20,57–61].

Comparison with other methods for slice preparation and incubation

For the preparation of precision-cut slices, three types of slicers are commercially available. The Krumdieck slicer and the Brendel-Vitron slicer are the most often used. With the Brendel-Vitron slicer, each slice is made manually, whereas the Krumdieck slicer allows automatic slicing in the continuous mode but is more expensive. Furthermore, the Brendel-Vitron slicer allows continuous oxygenation of the slicing buffer, which is not possible with the Krumdieck slicer. Previous studies, however, found no essential differences in the viability and functionality of liver slices prepared using the two slicers[8]. More recently, a new type of tissue slicer, the Leica VT1200 S vibrating blade microtome with Vibrocheck, has been developed. A recent study showed that slices prepared with this slicer have a more reproducible thickness than slices prepared with the Krumdieck slicer. However, there were no differences in the viability of slices prepared using the two slicers[9].

Various incubation systems have been described. The most widely applied incubation systems are 6- or 12-well culture plates[62] and the dynamic organ culture (DOC) system developed by Brendel and colleagues[63,64]. Other described culture systems include the 'rocker platform', on which slices are cultured on a Netwell insert in six-well plates[65], and the Erlenmeyer incubation system, in which slices are submerged in oxygenated medium[66], similar to that in the well-plate system. Slices are intermittently exposed to the ambient atmosphere and the medium in both the rocker platform and the DOC system. However, evidence that intermittent exposure to the gas phase contributes to longer viability is still lacking. Most long-term (>72 h) slice incubation studies are conducted with the DOC system[51,67–70]. Perifusion systems, in which the medium is constantly refreshed by flow, are relatively new and technically the most complicated[71–73]. The system described by van Midwoud *et al.* uses microfluidics-based perfusion of biochips[71] and is suitable for studying organ interactions by coupling and sequentially perfusing biochips containing slices from different organs (P.M. van Midwoud, M.T.M., E. Verpoorte and G.M.M.G., unpublished data). Although there are data to show that the continuous flow is beneficial[72], more research is necessary to further develop this system. In the above-mentioned biochip system, the metabolic activity of intestinal slices was shown to be comparable to that of well plates (P.M. van Midwoud, M.T.M., E. Verpoorte and G.M.M.G., unpublished data), but no studies have been published so far that have compared incubation systems for intestinal slices.

Two types of intestinal slices have been described in the literature: biopsy punches from the intestinal wall[17,74] and precision-cut slices[17,61] that are made perpendicular to an agarose-filled segment of intestine. In a comparative study, PCIS showed better viability than biopsy punches made from intestinal tissue[10].

Applications of the method

Precision-cut slices have been used for numerous applications in the past two decades. Classically, precision-cut liver and intestinal slices are used for the analysis of profiles and rate of metabolism of xenobiotics (refs. 2,15 and references therein) and physiological substrates[75], and for the study of enzyme induction and inhibition (refs. 2,15,76–79 and references therein). Recently, slices have been shown to be applicable for many more purposes: e.g., to study transporter-mediated drug uptake[80], the uptake and effect of drug-targeting preparations[52,81–83] and the regulation of drug-metabolizing enzymes and transporters[19,58–60,84,85].

Moreover, liver slices have successfully been used to investigate the mechanism of drug-induced toxicity using functional, biochemical, histomorphological and genomic read-out parameters, and for the prediction of toxicity and the discovery of early markers of toxicity (refs. 6,70,86–89 and references therein). Liver slices seem to be valuable for the detection of genotoxicity by adaptation of the Comet assay for application on liver slices[90,91] and by

detection of oxidative DNA damage by high-pressure liquid chromatography–mass spectrometry/mass spectrometry[92]. They also seem to be useful in studies on the mechanism of induction of liver fibrosis, which is a typical multicellular process, and in studies on the effect of antifibrotic drugs[51,93–97]. In addition, liver slices have been used to optimize methods for cold preservation of donor organs and to investigate the effects of brain death of organ donors and ischemia on the function of the organ after reoxygenation. In these studies, liver slices were used to simulate the events that induce ischemia-reperfusion damage during procurement, during storage and after transplantation[31–34]. Both drug-induced toxicity and ischemia-reperfusion injury are processes that do not involve only epithelial cells but are typical multicellular processes in which other cell types, particularly macrophages and endothelial cells, are important, making tissue slices a preeminently suitable *in vitro* preparation. For the same reason, they provide a good *in vitro* model to study the cell selectivity of drug-targeting preparations[98]. Finally, we and others have used liver slices as a tool to study the capability of viruses to transfect genes for gene therapy[30,35–38].

In addition to normal tissue, diseased human tissue can be used to study pathological processes and therapeutic effects of drugs. For example, slices of fibrotic liver tissue have been used to study the therapeutic efficacy of drug-targeting preparations[93] and to study the functionality of the fibrotic liver tissue[99]. Liver tumor tissue slices have been used to determine the effectiveness of anticancer therapy[38].

Cold- and cryopreservation would allow a more efficient use of the human tissue that is available. Liver slices can be preserved in University of Wisconsin organ preservation solution (UW) at 4 °C up to 24 h (ref. 100) and intestinal slices up to 5 h in Krebs buffer (data not shown). Although several methods have been reported for cryopreservation of slices (refs. 66,101–104 and references therein), survival of the slices seems to be limited to 3–4 h after thawing. New vitrification methods are now being explored to improve slice viability after thawing[101].

Development of the protocol

In our institute, we investigated several protocols to prepare and incubate tissue slices of both human and animal origin, including those from rat, mouse, minipig, dog and monkey[100,105]. Initial studies were devoted to elucidating the influence of slice thickness, preservation of the tissue (before slicing) and preservation of the slices (after slicing but before the start of the experiments)[17,61] and to developing the optimal incubation system, medium composition and oxygen supply[50,62,106]. In this article we present the optimized protocols that we are now using for rat and human liver and intestinal slices. Although the technique seems relatively simple, some hands-on experience is needed to obtain optimal and reproducible results. The incubation medium and the medium for preserving the tissue and the slices should be optimized for each type of tissue separately. In our experience, similar protocols can be used to prepare slices from tissues of different species. In addition, virtually all tissues can be sliced using the same protocol, either directly (kidney, brain) or, as described for intestine, embedded in and/or filled with agarose (lung, carcinoma) (see Experimental design), although tissue with a high collagen content (such as cirrhotic liver) is more difficult to slice.

Experimental design

In the description of the experimental procedure, the focus is on the preparation of liver and intestinal slices of rat and human organs. The methods can be easily adapted to other tissues and organs. Although we describe preparation of slices using the Krumdieck slicer, the protocol may be easily adapted for the use of other tissue slicers commercially available.

In our experience and in that of others[107], it is important to start with fresh, viable tissue. This implies that organs should be collected rapidly from the donor body and cooled as quickly as possible. Large organs such as human liver benefit from venous flushing with cold UW to limit the deleterious effects of warm ischemia. Flushing of rat liver does not seem to influence the viability of the tissue in our hands (data not shown).

It should be noted that some organs have specific requirements regarding the storage buffer composition and temperature. Liver and kidney tissue and slices can be successfully stored in ice-cold UW, but intestinal tissue and slices rapidly lose viability in UW and should preferably be stored and sliced in ice-cold Krebs-Henseleit buffer (KHB) (data not shown).

The Krumdieck slicer accommodates holders for cylinders of tissue with diameters of 3, 5, 8, 10 or 15 mm. The optimal size of the core depends on the amount of tissue available and on the requirements of the experiment to be conducted. Cylindrical cores must be prepared from solid organs such as the liver or the kidney, using a drill with a hollow bit ('coring tool'). Several options are available, such as hand-driven biopsy punches, commercially available electrical drills or specialized hand-held electrical drills. In our hands, the common commercial drill with speed regulation, fixed in a stand and equipped with a very precisely sharpened hollow bit of well defined diameter, works well. The diameter of the bit should correspond to the diameter of the tissue core holder in the slicer. Intestinal slices and other non-solid (such as lung) or very small organs are usually embedded (and filled, if applicable) in agarose using a special mold to form 16-mm cores. They do not require the use of the tissue core holder but are directly placed into the slicer.

Liver slices are routinely prepared with a thickness of 250 μm, and this allows full access of the inner cell layers to oxygen and substrates[4], but they can be made as thin as 100 μm if necessary[55].

Sheets of intestinal tissue can be embedded in cylinders of agarose, which allows the preparation of thin (about 300–400 μm) slices perpendicular to the length of the intestine. Thickness can be measured during slicing by a tissue slice thickness gauge but can also be assessed by determining the wet weight. Experienced people can judge the approximate thickness by eye.

For the incubation of PCTS, we describe the 6- and 12-well plate system. We prefer this system because it is technically the simplest and is relatively cheap, and it allows the simultaneous incubation of hundreds of slices. Moreover, taking samples of the medium is easy. Other incubation systems, however, such as the DOC system and the Erlenmeyer system, may be equally suitable, depending on the type of experiment. For any incubation system, it is vital to continuously move the medium by shaking and to refresh the medium at least once every 24 h, based on a volume to slice ratio of 0.25–0.30 ml per milligram of tissue.

The choice of medium is not completely evidence based, but most researchers use culture media such as Williams medium E, supplemented with glucose and antibiotics and saturated with 95% O_2/5% CO_2.

For long-term (>48 h) culture, some adaptations to the protocol are required to optimize viability. Particularly, the addition of insulin

(30 nM), glucagon (100 nM), corticosterone (1 mM), epidermal growth factor (1 nM) and/or fetal calf serum (5%) may be beneficial for long-term viability[53]. Furthermore, attention should be paid to preventing microbiotic contamination by sterilizing the tissue slicer and other instruments for slice preparation, handling and incubation.

We recommend monitoring the ATP content and morphology of slices routinely as a viability control in every experiment. These parameters are sensitive markers for slice integrity[104]. In addition, depending on the aim of the experiment, we recommend selecting a suitable functionality parameter (e.g., metabolism of a model compound) to monitor slice functionality. Note that viability and functionality of human tissue is strongly dependent on the quality of donor organs[50,107]. Always use untreated slices incubated in parallel with the experimental slices as controls, and incubate slices in triplicate for each experiment.

MATERIALS

REAGENTS

- Freshly isolated liver and intestinal organ material **! CAUTION** Animal and human tissue should be collected in compliance with legislative and institutional requirements
- Isoflurane (only for rat organs; Nicholas Piramal, cat. no. 45.112.110).
- Williams medium E (WME, containing L-glutamine) with supplements (Invitrogen, cat. no. 32551; see REAGENT SETUP).
- Gentamicin sulfate (50 mg ml^{-1}; Invitrogen, cat. no. 15750 037)
- Fungizone (amphotericin B) (250 µg ml^{-1}; Invitrogen, cat. no. 15290026)
- Low-gelling-temperature agarose type VII (only for intestine; Sigma-Aldrich, cat. no. A0701) **▲ CRITICAL** Use a type of agarose that has a gelling temperature just below 37 °C, as higher temperatures will induce heat shock. As this type of agarose liquefies at temperatures >60 °C, it remains a gel during incubation. Agaroses with even lower gelling temperatures may also prevent heat shock, but they usually have a lower gel strength that makes slice preparation more difficult.
- UW (ViaSpan, only for liver; Du Pont, cat. no. 1-800-474-2762)
- $CaCl_2 \cdot 2H_2O$ (Merck, cat. no. 1.02382)
- KCl (Merck, cat. no. 1.04936)
- NaCl (Merck, cat. no. 1.06404)
- $MgSO_4 \cdot 7H_2O$ (Merck, cat. no. 1.05886)
- KH_2PO_4 (Merck, cat. no. 1.04873)
- $NaHCO_3$ (Merck, cat. no. 1.06329)
- D-Glucose monohydrate (Merck, cat. no. 1.08342)
- HEPES (MP Biomedicals, cat. no. 101926)
- NaOH (Merck, cat. no. 1.06498)
- Ethanol, 96% (vol/vol) (Biosolve, cat. no. 052105)
- EDTA (Titriplex III; Merck, cat. no. 1.08418)
- Tris (VWR International Prolabo, cat. no. 28811.295)
- ATP Bioluminescence Assay Kit CLS II (Roche Diagnostics GmbH, cat. no. 1 699 695)
- Formalin 4% (formaldehyde solution, 3.8–4.2% (vol/vol); Klinipath, cat. no. 4078-9005)
- Ultrapure water
- Distilled water

EQUIPMENT

- Krumdieck tissue slicer (TSE, cat. no. 550755 A3; http://www.alspi.com/ard.htm)
- Tissue coring tool (only for liver, 5 or 8 mm; TSE, cat. no. 550755-04 or 550755-05)
- Mechanical drill with stand (only for liver; Metabo, cat. no. BSE 5010)
- Tissue embedding unit (only for intestine; TSE, cat. no. 550755-17)
- Slicing blades (TSE, for Krumdieck slicer type MD 4000, cat. no. 550755-15/01; for Krumdieck slicer type MD 1100, cat. no. 550755-15/02)
- Incubation cabinet (see EQUIPMENT SETUP) including temperature controller (Vernier, ordering code Go! Temp), electronic contact thermometer/thermostat (Ebro, cat. no. GFX 460), relay box (Ebro, cat. no. RB 1691 B-S)
- Gas washing bottles (VWR International, cat. no. 201-1420)
- Reciprocal shaker (GFL, cat. no. 3018)
- Six- or 12-well tissue culture plates (sterile, with lid; Greiner, cat. no. 657160 or 665180)
- Bottle top vacuum filter, 150 ml (0.45 µm for filtering 10× KHB; Corning, cat. no. 430627)
- Gas dispersion tube with fritted disc (13 mm; Fisher Emergo, cat. no. 101623)
- Surgical mattress (heating pillow; Inventum, cat. no. HKN20)
- Acrylic glass Petri dish with silicone insert (only for liver, 14 cm diameter; own manufacture)
- Refrigerated circulator bath for slicer cooling (Thermo Scientific, cat. no. HAAKE C10-K15)
- Surgical thread, 4/0 silk (only for intestine; Braun B Medical BV, cat. no. 1134035)
- Plastic transfer pipettes, 3.5 ml (only for intestine; Sarstedt, cat. no. 86.1171)
- Surgical scissors (only for intestine)
- Surgical blades (Swann-Morton, cat. no. 0211)
- Forceps
- Curved spatula
- Blotting paper (Macherey-Nagel, cat. no. 150047.1)
- Pins (only for rat intestine)
- Plastic syringes, Omnifix Luer lock 50 ml (only for human liver; Braun, cat. no. 4610083F)
- Cannulas, inner diameter 3 mm, about 5 cm in length (only for human liver; polyethylene), for coupling to the syringes
- Tubing, inner diameter 1.57 mm, 3 cm in length, for coupling to the cannula tubing to fit the liver portal veins (only for human liver)
- LumiCount microplate luminometer (Packard, model BL10001)
- Mini-Beadbeater (BioSpec Products)
- Sonifier, Vibra-Cell (Sonic & Materials Inc., model VC375)
- Safe-Lock micro test tubes (1.5 ml; Eppendorf, cat. no. 0030.120.086)
- Centrifuge (Eppendorf, model 5415R)

REAGENT SETUP

Preparation of KHB KHB is used for slicing and storage of intestinal slices. Prepare 10 liters of a 10× concentrated KHB stock solution (10× KHB) by first dissolving 36.7 g $CaCl_2 \cdot 2H_2O$ in 5 liters of ultrapure water (solution 1). Then dissolve 37.3 g KCl, 690 g NaCl, 27.1 g $MgSO_4 \cdot 7H_2O$ and 16.3 g KH_2PO_4 in ultrapure water to a volume of 5 liters (solution 2). Subsequently, mix solutions 1 and 2 and filter through a 0.45-µm filter. This 10× KHB can be stored at 4 °C for about 6 months. On the day of the slice experiment, prepare 5 liters KHB by dissolving 10.5 g $NaHCO_3$, 24.75 g D-glucose monohydrate and 11.9 g HEPES in about 2 liters of ultrapure water at 4 °C. Then add 500 ml 10× KHB and ultrapure water at 4 °C to yield a final volume of 5 liters. Keep this solution at 0–4 °C on melting ice. Oxygenate with 95% O_2/5% CO_2 for 30 min on ice using a gas dispersion tube with a fritted disc. Then set the pH of the solution to 7.42 by slowly adding 5 N NaOH solution. This solution can be stored for 24 h at 4 °C. Before use, it should be reoxygenated and the pH should be adjusted again. For the preparation of about 300 slices, 5 liters of KHB is sufficient. The final concentration of the components of KHB is given in **Table 1**.

Preparation of WME slice incubation medium On the day of the experiment, prepare slice incubation medium by adding 1.375 g D-glucose monohydrate and 500 µl gentamicin (50 mg ml^{-1}) to 500 ml WME (containing L-glutamine). For incubation of intestinal slices, also add 5 ml amphotericin B (250 µg ml^{-1}) to prevent the growth of fungi and yeasts. If necessary, this solution can be stored for 24 h at 4 °C. The final concentration of the components of WME slice incubation buffer is given in **Table 1**.

Preparation of 3% (wt/vol) agarose solution for tissue embedding Weigh 3 g low-melting agarose type VII and 0.9 g NaCl in a glass bottle and add distilled water until a volume of 100 ml is obtained. Heat the bottle in a water bath until the temperature reaches >60 °C to dissolve the agarose. This solution can be used after cooling to 37 °C or stored at 4 °C for about 1 month for reuse by heating again until about 60 °C. A volume of 100 ml agarose solution is sufficient for the preparation of about 300 intestinal slices.

Preparation of sonification solution for ATP determination (70% (vol/vol) ethanol, 2 mM EDTA, pH 10.9) To prepare 500 ml solution, dissolve 0.372 g EDTA in about 100 ml of ultrapure water and adjust the pH with 5 M NaOH to obtain a pH of 10.9. Add ultrapure water to the solution to obtain a volume of 130 ml and then add 370 ml ethanol (96%). This solution can be stored at 4 °C for approximately 3 months.

TABLE 1 | Composition of KHB and WME.

Components	Concentration (mM)
KHB (slicing buffer)	
NaCl	5.0
KCl	118.0
$MgSO_4 \cdot 7H_2O$	1.1
KH_2PO_4	1.2
$NaHCO_3$	25.0
$CaCl_2 \cdot 2H_2O$	2.5
D-Glucose	25.0
HEPES	9.0
WME (incubation medium)	
WME + L-glutamine	
D-Glucose	14.0
Gentamycin	50 μg ml^{-1}
Amphotericin B	2.5 μg ml^{-1}

Preparation of Tris buffer to be used for ATP determination (100 mM Tris-HCl, 2 mM EDTA, pH 7.6) To prepare 500 ml solution, dissolve 6.0 g Tris and 0.37 g EDTA in about 300 ml of ultrapure water, adjust pH to 7.6 with 6 N HCl and fill to 500 ml total volume with ultrapure water. This solution can be stored at 4 °C for approximately 3 months.

EQUIPMENT SETUP

The setup of the incubation cabinet is illustrated in **Figure 1**. The well plates containing slices will be incubated in plastic boxes with taps (**Fig. 1a**), containing an inlet for tubing for carbogen gas (95% O_2/5% CO_2) and an outlet for carbogen to prevent the buildup of pressure in the boxes. Pass the carbogen through a gas-washing bottle (**Fig. 1b**) filled with distilled water at 37 °C before it enters the boxes, to maintain a humid atmosphere in the boxes and to prevent cooling of the medium by cold gas. Place the boxes on a reciprocal shaker (**Fig. 1c**), shaking 90 times per minute. Maintain the temperature in the cabinet at 37 °C using a fan heater (**Fig. 1d**) connected to a thermostat/temperature controller (**Fig. 1e**). Place a second fan ventilator (**Fig. 1f**) opposite the fan heater to more efficiently distribute the heated air. Register the temperature continuously using a temperature controller connected to a computer.

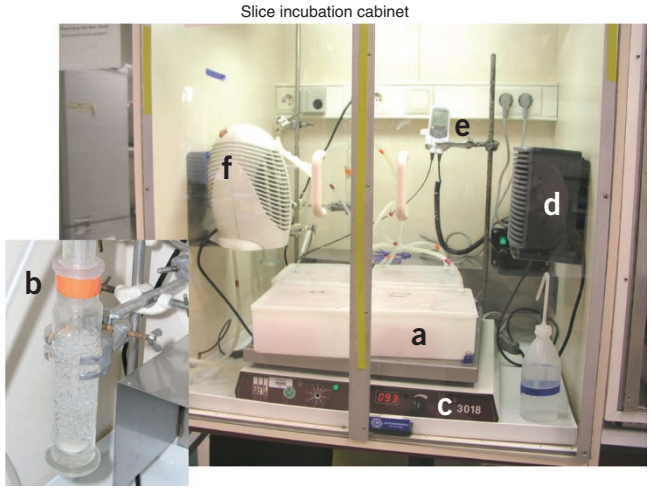

Figure 1 | Setup of the incubation cabinet. (**a–f**) Plastic boxes with well plates (**a**); gas-washing bottle (**b**); reciprocal shaker (**c**); fan heater (**d**); thermostat/temperature controller (**e**); ventilator (**f**).

PROCEDURE

Preparation for the slice experiment ● TIMING 1 h

1| Turn the heater on in the incubation cabinet and wait until 37 °C is reached.

2| Prepare KHB for slicing of liver and intestine and storage of intestinal tissue.

3| Prepare WME plus supplements for slice incubation.

4| Transfer the incubation medium into culture plates.

▲ **CRITICAL STEP** Six-well plates should be filled with 3.2 ml of culture medium per well to support 10–15 mg of tissue for 24 h. Twelve-well plates require 1.3 ml of culture medium per well to support 3–6 mg of tissue for 24 h. One slice is incubated per well. The volume-to-surface ratio of the culture medium is of great importance for optimal exchange of oxygen and CO_2 between the culture medium and the atmosphere of 95% O_2/5% CO_2, and the ratio of tissue to volume is important for sufficient supply of nutrients.

5| Prewarm and oxygenate the plates by placing them in the plastic boxes of the incubation cabinet at 37 °C under 95% O_2/5% CO_2 for at least 30 min. Place wet tissues in the boxes to maintain a humid atmosphere to prevent evaporation of the incubation medium.

6| For intestinal slices, rewarm the 3% solution of agarose in a water bath to 60 °C to liquefy the gel. Transfer the solution into the incubation cabinet or into a water bath having a temperature of 37 °C for at least 15 min to cool to 37 °C.

7| For intestinal slices, precool the tissue embedding unit by filling the reservoir with ice.

8| Assemble the Krumdieck slicer according to the manufacturer's instructions and precool it by recirculating cooled water (4 °C) through the cooling block using the refrigerated circulator bath.

Collection of tissue ● TIMING 5–15 min

9| Collect rat tissue following option A or human tissue following option B.

(A) Collection of rat tissue ● TIMING 5 min

(i) Anesthetize a rat that has had free access to food and water, using 5% isoflurane in O_2. Retrieve the organs as quickly as possible from the rat.

▲ **CRITICAL STEP** If several organs are used, first excise the intestine, as it is the most sensitive to warm ischemia.

! **CAUTION** Rat tissue should be collected in compliance with legislative and institutional requirements.

(ii) Store liver in ice-cold UW and intestine in ice-cold oxygenated KHB.

▲ **CRITICAL STEP** All further steps up to incubation need to be performed on ice (at 4 °C). Continue with Step 10, option A (liver) or B (rat intestine).

(B) Collection of human tissue ● TIMING 15 min

(i) Retrieve the organs as freshly as possible from the donor. Place tissue in ice-cold UW (liver) or ice-cold oxygenated KHB (intestine) as soon as possible after removing it from the body to prevent warm ischemia.

! **CAUTION** When working with human organ material, use protective clothing, mouth covering and protective glasses to protect against viral infections. Work in a laminar airflow cabinet where possible. At the end of the experiment, clean laboratory instruments (including the Krumdieck slicer) and bench surface with disinfectant. Human tissue should be collected in compliance with legislative and institutional requirements.

(ii) Flush pieces of human liver using UW. Introduce the UW through the open ends of portal veins, which can be identified by the accompanying bile ducts and arteries on the cut surface, using a plastic syringe with a plastic cannula that fits tightly to the opening of the vein. Flush gently until the outflow is free of blood. Flush through several portal veins until the color of the tissue changes from reddish to brown.

▲ **CRITICAL STEP** Flush immediately after organ excision to prevent the blood in the organ from clotting.

▲ **CRITICAL STEP** All further steps up to incubation need to be performed on ice (at 4 °C). Continue with Step 10, option A (liver) or C (human intestine).

Preparation of tissue ● TIMING 15–30 min

10| Prepare liver tissue (human and rat) using option A, rat intestine using option B and human intestine using option C.

(A) Preparation of human and rat liver cores ● TIMING 15 min

(i) Transfer the liver to a Petri dish that has a silicone insert. For human liver cores, cut large pieces of human liver into segments about 2 cm thick using a surgical blade. Do not use parts that are not well perfused. Keep the surface of the liver wet by pouring UW on it.

(ii) Turn on the drill at speed setting 2. Secure the tissue by holding it down loosely by hand. Prepare cores by rapidly pressing the hollow rotating tissue coring tool perpendicularly into the tissue until it touches the silicone patch at the bottom of the Petri dish (**Fig. 2a**). Cores should be cylindrical, with similar diameters at the two ends.

? TROUBLESHOOTING

(iii) Transfer the cores to UW using a spatula (not forceps) directly after core preparation. Store the cores in UW (3–4 h at most) until further use (**Fig. 2b**).

(B) Preparation of cores of rat intestine ● TIMING 30 min

(i) Carefully remove adhering fatty tissue and split the intestine into duodenum (roughly the first 10 cm distal to the stomach), ileum (roughly 10 cm proximal to the ileocecal junction), jejunum (between the duodenum and ileum) and colon (distal to the cecum). Then cut these segments into 3-cm parts, and flush by pipetting ice-cold oxygenated KHB through the lumen with a plastic transfer pipette. Remove fecal constituents gently from the lumen using a curved spatula (**Fig. 3a**). Transfer the clean pieces of intestine into ice-cold oxygenated KHB.

▲ **CRITICAL STEP** Remove fatty tissue carefully; the presence of fatty tissue hinders the slicing process by tearing the tissue out of the agarose.

▲ **CRITICAL STEP** The intestine is extremely sensitive to warm

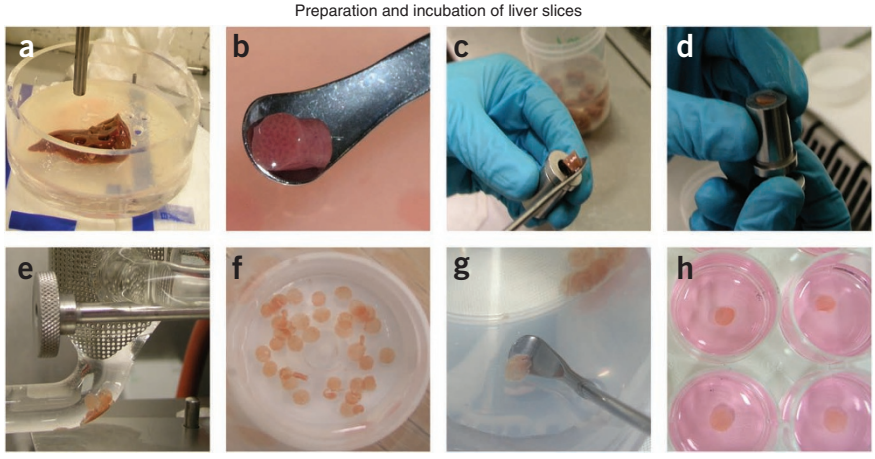

Figure 2 | Preparation and incubation of rat or human liver slices. Liver cores are prepared using a drill and a tissue coring tool (**a**), and transferred to the cylindrical core holder of the Krumdieck slicer (**b**–**d**). Thereafter, slices of 8 mm with 10–15 mg wet weight (or of other thickness and diameter) are cut (**e**). Good quality slices are round, equally thick at all sides and have smooth edges (**f**). Slices are transferred to 6- or 12-well plates using a spatula to avoid damaging the slices (**g**,**h**). Animal experiments and retrieval of human organ material were conducted in compliance with institutional and legislative regulations.

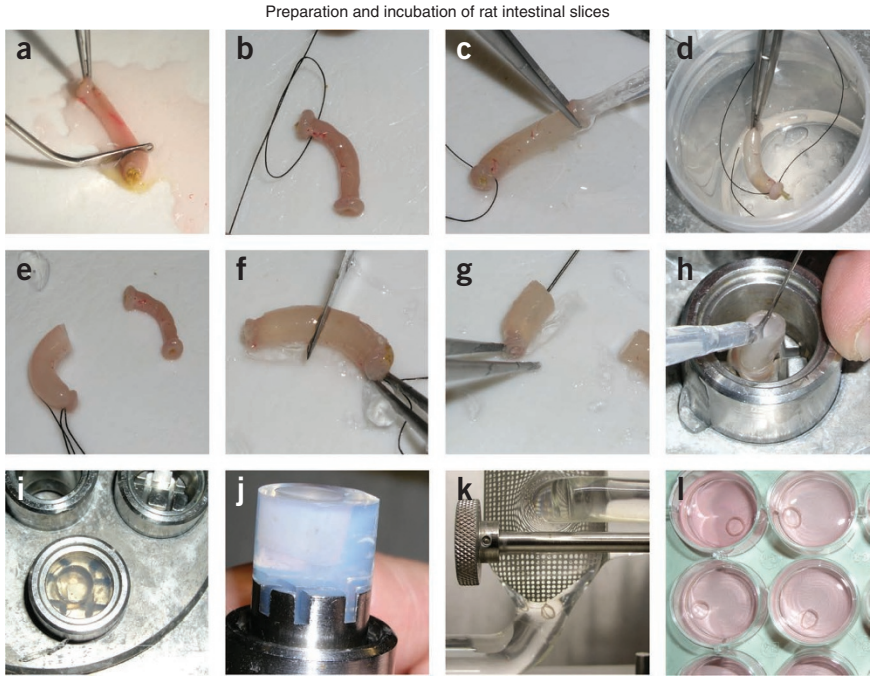

Figure 3 | Preparation and incubation of rat intestinal slices. After the fecal components are removed from the intestinal segment (**a**), one side is tied (**b**). The segment is then filled with liquid agarose solution at 37 °C (**c**) and cooled (**d**) to form a filled cylinder about 5 mm thick (**e**). After cutting the segments into two halves (**f**), a pin is placed in the filled lumen (**g**) to fix the segment in the precooled cylindrical mold plunger (**h**). The mold is then filled with agarose solution at 37 °C and cooled (**i**). The plunger is removed from the mold and transferred to the Krumdieck tissue slicer (**j**). Slices of 2–4 mg in wet weight are cut (**k**) and incubated in 12-well plates (**l**). Animal experiments were conducted in compliance with institutional and legislative regulations.

and cold ischemia. The time taken for excision of the organ and core and slice preparation until incubation should be kept as short as possible (<2 h).
▲ **CRITICAL STEP** Intestinal tissue and slices rapidly lose viability in UW and should preferably be stored and sliced in KHB. These precautions concern both human and rat intestine.

(ii) Tighten each segment at one end with surgical thread (**Fig. 3b**). Fill the intestine through the open end with the agarose solution at 37 °C using a plastic transfer pipette (**Fig. 3c**) until the intestine reaches its normal diameter (approximately 5 mm). Close the intestine using forceps and then immediately transfer the filled segment into ice-cold oxygenated KHB to allow the agarose to solidify to a flexible gel (**Fig. 3d,e**).
▲ **CRITICAL STEP** Ensure that the agarose solution has cooled to 37 °C before pipetting it into the tissue, as higher temperatures will damage the mucosal epithelium.

(iii) After about 1 min, when the agarose has solidified, cut the filled intestinal segment into two halves, without applying vertical (longitudinal) pressure to the intestine with the surgical blade (**Fig. 3f**). Place a pin in the gelled agarose in the intestinal lumen (**Fig. 3g**) without touching the tissue and transfer the segment to the precooled cylindrical mold-plunger assembly of the tissue-embedding unit, with the pin facing upward and the surgical thread, at the other end, facing downward. Secure the segment by holding the pin and fill the mold with liquid agarose at 37 °C by pipetting it slowly over the surface of the segment to prevent inclusion of air bubbles (**Fig. 3h**). Let the agarose solidify for 1 min, then remove the pin (**Fig. 3i**).

(C) Preparation of cores of human intestine ● **TIMING 30 min**

(i) Remove staples and adherent fatty tissue from the stapled piece of human intestine (**Fig. 4a**). Cut the human intestine open to form an intestinal sheet (**Fig. 4b**). Remove fecal constituents by flushing the luminal surface with ice-cold oxygenated KHB.

(ii) Fix the tissue with pins to a silicone pad in a Petri dish with the mucosal side facing downward (**Fig. 4c**). Pour ice-cold oxygenated KHB over the tissue. Gently cut the muscularis from the intestinal mucosa by using surgical scissors (**Fig. 4d,e**).

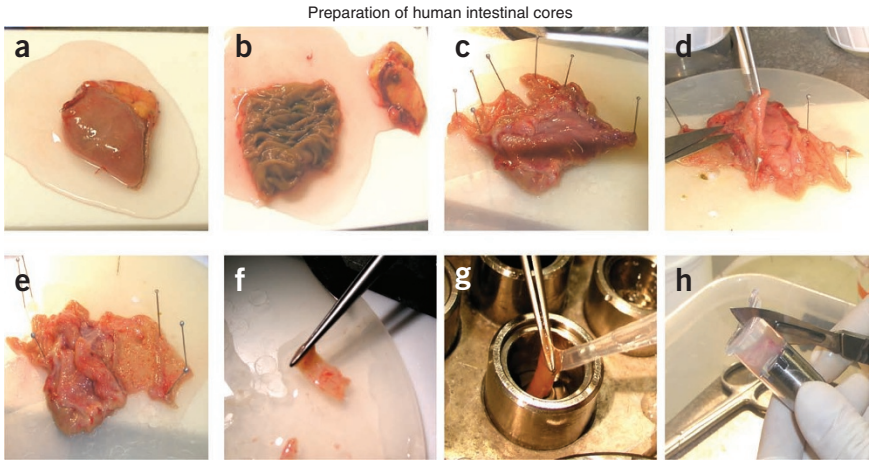

Figure 4 | Preparation of human intestinal cores. (**a**) Stapled piece of human jejunum. Staples and fat tissue are removed and the intestine is opened (here the mucosal side is facing upward) (**b**). The segment is then fixed on a silicone mattress on the precooled tissue-embedding unit with pins (**c**) and the muscularis is gently cut (stripped) away (**d**,**e**). Thereafter, the stripped intestine is cut into pieces of approximately 10 × 20 mm (**f**) and embedded with low-gelling agarose (**g**,**h**). Retrieval of human organ material was conducted in compliance with institutional and legislative regulations.

▲ CRITICAL STEP The muscularis needs to be removed from the human intestine because it is relatively tough and thick. Removal improves slice preparation and shortens the diffusion distance for nutrients and oxygen in the slices.

(iii) Cut the segment without muscularis into sheets of about 10 × 20 mm (**Fig. 4f**). Pick up the sheet while holding one of the 10-mm sides with forceps. Transfer the sheet into the precooled cylindrical mold-plunger assembly of the tissue-embedding unit. Fill the mold with liquid agarose at 37 °C by pipetting it directly on the tissue (**Fig. 4g**) while holding the tissue sheet in place with forceps until the agarose solidifies (**Fig. 4h**).

Preparation of liver and intestinal slices ● TIMING 1 h for 50–100 slices

11| Fill the slicer with ice-cold oxygenated KHB through the glass-trap assembly. Ensure that the buffer reaches the level required for proper functioning of the pump and that the tissue core is completely submerged.

▲ CRITICAL STEP The procedure described here is for the Krumdieck tissue slicer. The protocol needs to be adapted when other tissue slicers (e.g., Brendel-Vitron) are used.

12| For liver slices. use option A; for intestinal slices, use option B**.**

(A) Preparation of liver slices

(i) For liver slices, transfer the liver core from the ice-cold UW used for storage into the cylindrical core holder of the slicer (**Fig. 2c**). Preferably, use a spatula instead of forceps so as not to damage the core. Ensure that the most flat and round end of the core points upward, toward the open end of the core holder. Move the plunge downward to insert the core in the holder (**Fig. 2d**).

(B) Preparation of intestinal slices

(i) For intestinal tissue, remove the plunger with the agarose cylinder containing the tissue from the tissue-embedding unit (**Fig. 3j**) and place it directly in the slicer without using a tissue core holder.

13| Cut slices using the Krumdieck slicer at a speed setting of 30–40. Check the wet weight of the first few slices as an indication of slice thickness after carefully blotting the slices on a smooth, not too absorptive paper to remove adherent water. For intestinal slices, remove the agarose from the slice before blotting using forceps. As an indication, liver slices of 8-mm diameter and 200- to 300-μm thickness have a wet weight of 10–15 mg. Intestinal slices without agarose have a wet weight of 3–4 mg (estimated thickness 400 μm). As an alternative to the determination of wet weight as a measure of slice thickness, the Krumdieck slice thickness gauge may be used; this offers the possibility of determining the thickness of individual slices. If necessary, adjust the cutting thickness by turning the graduated thickness control knob until the required wet weight is reached. The slice thickness can be slightly increased by placing weights on top of the tissue core holder. This may also facilitate the slicing of hard (fibrotic) liver tissue.

▲ CRITICAL STEP Liver slices that are too thick (>400 μm) may develop necrosis in the center of the slice because the diffusion distance for nutrients and oxygen is too long. For intestinal slices, the diffusion distance is also determined by the thickness of the intestinal mucosa and submucosa (plus muscularis in case of rat); therefore, slice thickness is less critical. The metabolism rate seems similar in intestinal slices between 150 and 450 μm. However, both intestinal slices and liver slices should not be cut thinner than about 100 μm, as the ratio between damaged cells (cut edges) and healthy cells will be too high.

14| After slicing one core, remove the slices from the glass trap (**Figs. 2e** and **3k**) by opening the tap, collect them in a beaker and place them immediately on ice. Select the slices on the basis of appearance. Good slices have an equal thickness, uniform color and smooth edges (**Fig. 2f**). Transfer selected slices into fresh ice-cold UW (liver slices) or ice-cold oxygenated KHB (intestinal slices) and store on ice. Use a spatula, not forceps, to avoid damaging the slices (**Fig. 2g**). Every 15–30 min, replace the KHB slicing buffer by fresh, ice-cold and oxygenated KHB. Replace the knife when slice quality (judged by eye) decreases.

■ PAUSE POINT Liver slices of healthy human and rat tissue can in principle be stored in ice-cold UW for 18 h before incubation, without loss of viability and functionality[100]. This may be desirable in case of human liver, when more slices are produced than can be handled experimentally in 1 d. Intestinal slices should be incubated directly as viability rapidly declines during storage.

? TROUBLESHOOTING

Incubation of liver and intestinal slices ● TIMING dependent on experiment

15| Wash liver slices quickly by gently transferring them into a Petri dish containing ice-cold oxygenated WME to remove the UW (for intestinal slices, washing is not necessary). Transfer the slices (one slice per well) to a prewarmed culture plate that is placed on a surgical mattress to maintain the medium temperature at 37 °C (**Figs. 2h** and **3l**).

▲ CRITICAL STEP Immediately transfer the well plate back into the incubator after placing the slices, as the pH of the medium rapidly changes in normal atmosphere.

16| Incubate the slices for the desired time period in the plastic boxes that are placed on a reciprocal shaker (90 cycles per minute) in the incubation cabinet (**Fig. 1**). Regularly monitor the pH of the medium by checking the color of the pH indicator in the WME. Yellow or purple medium indicates a pH change. Continuously monitor the temperature in the boxes. It is advisable to refresh the medium after a preincubation of 1–3 h to restore the ATP content and to remove cell debris. For refreshing, fill new culture plates with medium and prewarm and oxygenate the plates. Place the incubated plates and new plates on a surgical mattress to maintain the temperature at 37 °C. Quickly transfer the slices from the incubated plates into the new plates with a spatula and place them in the incubator.
? TROUBLESHOOTING

17| Refresh the medium every 24 h.

Determination of ATP in slices ● TIMING 2–3 h, depending on number of samples

18| After an experiment, place one slice in 1 ml sonification solution in a safe-lock micro test tube and freeze immediately in liquid nitrogen. Samples can be stored at −80 °C. After thawing, homogenize the sample with a sonifier or Mini-Beadbeater.
▲ CRITICAL STEP Place the samples in melting ice with salt during homogenization. Centrifuge the homogenate for 2 min at 13,000 r.p.m. (15,000*g*) in an Eppendorf centrifuge. Perform the ATP assay according to the manufacturer's instruction (ATP Bioluminescence Assay Kit CLS II).

Preparation of tissue sections for morphology ● TIMING ≥2 d

19| After an experiment, incubate three slices in 1 ml of buffered formalin (4 °C) for 24 h. After this step, store the slices in 70% ethanol (4 °C). Embedding in paraffin, sectioning (4 µm) and staining with hematoxylin and eosin can be performed using standard histological procedures[101].

● TIMING

Steps 1–8, Preparation of the slice experiment: 1 h
Step 9A, Collection of rat tissue: 5 min
Step 9B, Collection of human tissue: 15 min
Step 10, Preparation of tissue: 15–30 min
Step 10A, Preparation of human and rat liver cores: 15 min
Step 10B, Preparation of cores of rat intestine: 30 min
Step 10C, Preparation of cores of human intestine: 30 min
Steps 11–14, Preparation of liver and intestinal slices: 1 h
Steps 15–17, Incubation of liver and intestinal slices: dependent on experiment (for liver slices, up to 96 h; for intestinal slices, up to 24 h)
Step 18, Determination of ATP in slices: 2–3 h
Step 19, Preparation of tissue sections for morphology: ≥2 d

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
10A	Frayed cores	Blunt hollow drill bit	Sharpen drill
	Conic cores	Vertical pressure on the tissue during manual fixation	Secure tissue loosely by hand without vertical pressure on tissue
14	Difficulties during slicing	See manual of the Krumdieck tissue slicer	See manual of the Krumdieck tissue slicer
16	Nonviable slices	Temperature incubation cabinet too low or too high	Adjust temperature
		Extended ischemia time	Reduce ischemia time
		Oxygen supply insufficient	Check carbogen supply
		pH change	Check carbogen supply
		Nonviable starting tissue	Not applicable
		Long time needed for preparation of tissue slices	Reduce time needed for preparation of tissue slices Change slice preparation buffer more often Collect slices from the glass trap more frequently

ANTICIPATED RESULTS

Typically 80–100 slices 8 mm thick or 200–250 slices 5 mm thick can be obtained from 10 g of rat liver. About 20–30 slices can be obtained from one core of intestinal tissue.

Immediately after slicing, the ATP content of liver slices may be low, depending on the cold preservation time. During preincubation, the ATP content of rat liver slices increases to 10–12 nmol ATP per milligram protein within 1–3 h and remains constant for at least 72 h. The ATP content of human liver slices is more variable than that of rat liver slices, and may vary from 2 to 14 nmol mg^{-1} protein. We consider an ATP content of less than 2 nmol mg^{-1} protein in human liver slices to be nonviable. The ATP content of human intestinal slices increases to 2.5–3 nmol mg^{-1} protein at 3 h and decreases slightly to about 2 nmol mg^{-1} protein after 24 h of incubation[85]. Rat intestinal slices contain 3–4 nmol ATP per milligram protein for up to 8 h, whereas after 24 h this may decline to 1 nmol mg^{-1} protein[60]. Intact RNA can be isolated from human and rat liver and intestinal slices, and glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) expression remains constant up to 24 h (refs. 60,85) for intestine and up to 72 h for liver[53]. The morphology of incubated slices is shown in **Figure 5**.

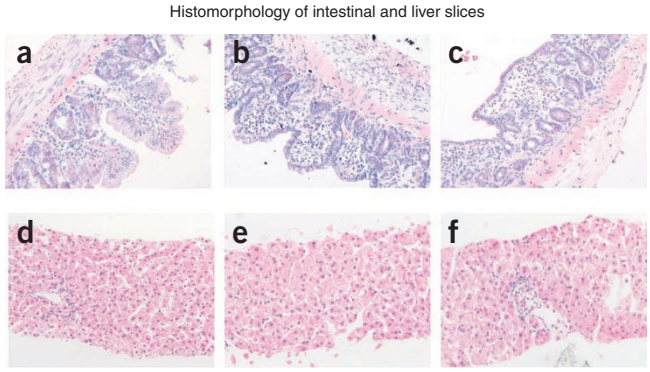

Figure 5 | Histomorphology of slices. Slices were fixed in 4% buffered formalin and embedded in paraffin, after which 4-µm sections were cut and stained with hematoxylin and eosin. (**a**) Rat intestinal slices, no incubation; (**b**) rat intestinal slices, 8-h incubation; (**c**) rat intestinal slices, 24-h incubation; (**d**) human liver slice, no incubation; (**e**) human liver slice, 3-h incubation; (**f**) human liver slice, 24-h incubation. Note that human slice morphology remained virtually unchanged during culturing, with necrotic cells only at the slice edges. The morphology of cultured intestinal slices typically changes in time. Most obvious changes are villi flattening and stretching and flattening of epithelial cells.

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