# Isolation and propagation of primary human cholangiocyte organoids for the generation of bio-engineered biliary tissue

Olivia C Tysoe[1,2]*, Alexander W Justin[3]*, Teresa Brevini[1,4]*, Si Emma Chen[1], Krishnaa T. Mahbubani[2], Anna Frank[5, 6, 7], Espen Melum[5,8,9], Hajer Zedira[1], Kourosh Saeb-Parsy[2]†, Athina Markaki[3]†, Ludovic Vallier[1,2,†] and Fotios Sampaziotis[1,2,10,11]†.

[1]Wellcome Trust-Medical Research Council Stem Cell Institute, Cambridge Stem Cell Institute, Anne McLaren Laboratory, Department of Surgery, University of Cambridge, Cambridge, UK

[2]Department of Surgery, University of Cambridge and NIHR Cambridge Biomedical Research Centre, Cambridge, UK

[3]Department of Engineering, University of Cambridge, Trumpington Street, Cambridge, UK.

[4]Department of Medical Biotechnology and Traslational Medicine (BIOMETRA), Università degli Studi di Milano, Milan, Italy

[5]Norwegian PSC Research Center, Department of Transplantation Medicine, Division of Surgery, Inflammatory Diseases and Transplantation, Oslo University Hospital, Rikshospitalet, Oslo, Norway.

[6]Research Institute of Internal Medicine, Division of Surgery, Inflammatory Diseases and Transplantation, Oslo University Hospital, Rikshospitalet, Oslo, Norway,

[7]Department of Medicine III, University Hospital Aachen, Aachen, Germany,

[8]Section for Gastroenterology, Department of Transplantation Medicine, Division of Surgery, Inflammatory Diseases and Transplantation, Oslo University Hospital Rikshospitalet, Oslo, Norway,

[9]Institute of Clinical Medicine, Faculty of Medicine, University of Oslo, Oslo, Norway

[10]Department of Hepatology, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK

[11]Department of Medicine, University of Cambridge, Cambridge, UK

Authorship note: * Olivia Tysoe, Alexander Justin and Teresa Brevini contributed equally to this manuscript. † Fotios Sampaziotis, Ludovic Vallier, Athina Markaki and Kourosh Saeb-Parsy share senior authorship for this manuscript.

**Correspondence:** Fotios Sampaziotis, Laboratory for Regenerative Medicine, West Forvie Building, Robinson Way, University of Cambridge. Cambridge CB2 0SZ, United Kingdom. Telephone: 44.1223.747489; Fax: 44.1223.763.350; E-mail: fs347@cam.ac.uk

Ludovic Vallier, Laboratory for Regenerative Medicine, West Forvie Building, Robinson Way, University of Cambridge. Cambridge CB2 0SZ, United Kingdom. Telephone: 44.1223.747489; Fax: 44.1223.763.350; E-mail: lv225@cam.ac.uk

## Abstract

Biliary disorders are the leading indication for pediatric liver transplantation due to the lack of alternative treatments for repairing or replacing damaged bile ducts. To address this challenge, we developed a protocol for generating bioengineered biliary tissue suitable for biliary reconstruction. Our platform allows the derivation of cholangiocyte-organoids (COs) expressing key biliary markers and function from primary extra- or intrahepatic duct cholangiocytes, within 2 weeks of isolation. COs are subsequently seeded on Poly-Glycolic Acid scaffolds or densified collagen constructs for 4 weeks to generate bioengineered tissue retaining biliary characteristics. Therefore, expertise in organoid culture and tissue-engineering are desirable for optimal results. Importantly, COs correspond to mature functional cholangiocytes, differentiating our method from alternative organoid systems propagating adult stem cells. Consequently, COs provide a unique platform for studies in biliary physiology and pathophysiology; while the resulting bioengineered tissue has broad applications for regenerative medicine and cholangiopathies.

Keywords: Cholangiocytes, organoids, cholangiopathies, biliary atresia, scaffolds, tissue engineering, bioengineering

# INTRODUCTION

Cholangiopathies comprise a diverse group of disorders characterized by damage to the biliary tree and loss of bile ducts resulting in cholestasis, hepatic injury and ultimately liver failure[1,2]. However, treatment options are limited to liver transplantation. Indeed, biliary disease remains the leading indication for this intervention in children, with more than 70% of paediatric liver grafts being used to treat biliary atresia[3]. The generation of healthy biliary tissue suitable for replacing or reconstructing damaged bile ducts could address the clinical need for alternative therapeutic approaches and reduce pressure on the transplant waiting list. However, progress in this area has been hampered by challenges in long-term culture and large-scale expansion of primary cholangiocytes. Here, we describe a protocol for the fabrication of functional bioengineered biliary tissue, using biocompatible and biodegradable scaffolds and a novel method for the isolation and propagation of primary cholangiocytes in organoid format.

## Development of the protocol

To generate bioengineered biliary tissue suitable for surgical manipulation and biliary reconstruction, we decided to combine primary biliary epithelium with appropriate matrices.

### Development of the protocol for cholangiocyte organoid culture

First, we developed a culture protocol for the propagation and large-scale expansion of primary adult, functional cholangiocytes[4]. Because biliary reconstruction is predominantly performed on the common bile duct (CBD), we initially focused on the isolation of CBD cholangiocytes using excised bile ducts from deceased organ donors. To achieve expansion of the isolated cholangiocytes, the cells were embedded in Matrigel and treated with a combination of EGF, R-Spondin-1 and DKK-1. We have previously demonstrated that the combination of EGF and 3D culture can promote limited growth of partially mature, foetal CLC organoids derived from iPS[5,6], while primary adult stem cell organoids have been isolated from murine biliary tissue[7]. To support the long-term expansion of adult cholangiocytes we used R-spondin-1, a WNT agonist reported to stimulate organoid derivation from multiple adult epithelia [8-13]. However, R-spondin promotes the propagation of adult stem cells rather than mature epithelial populations by enhancing canonical Wnt signalling [14]. To avoid the amplification of adult stem

cells, we introduced DKK-1[15], a canonical WNT/β-catenin pathway antagonist [16,17]. When used in combination with R-spondin, DKK-1 inhibits canonical and enhances non-canonical WNT signalling though the planar cell polarity (PCP) pathway [4], which has been reported to play a role in cholangiocyte maturation [18,19]. The combination of EGF, R-Spondin-1 and DKK-1 allowed for long-term, large-scale expansion of functional, genetically stable, primary adult cholangiocyte organoids (COs).

Our system was developed and optimized for the culture of CBD cholangiocyte organoids, isolated from fresh excised bile ducts. However, this isolation method depends on a major complex operation, thereby posing significant limitations related with access to biliary tissue. To overcome this issue, we subsequently validated the capacity of our culture system for the derivation of cholangiocyte organoids from multiple sources such as excised gallbladders, endoscopic retrograde cholangiopancreatography (ERCP) brushings and liver biopsy samples. Using this platform cholangiocyte organoids can be isolated and expanded using samples from any area of the biliary tree acquired by any of these isolation methods.

## Choice of scaffolds

Our next goal was to identify appropriate scaffolds that could be combined with the cells to generate tissue-like constructs. Important considerations for the choice of scaffolds were their potential to support growth of the cells, their capacity to be integrated to the host tissue following transplantation with minimal inflammatory response and the use of biodegradable materials allowing for tissue remodelling which is crucial for neo-vascularization. Furthermore, to support future clinical translation, the materials used should be compatible with human transplantation.

Importantly, both synthetic and biological polymer scaffolds with these specifications are available. Since there are advantages and disadvantages associated with each approach, we decided to explore both. Synthetic polymer scaffolds are widely used as they can be easily processed using a wide range of techniques and adapted for multiple tissue engineering applications [20]. They have tuneable and reproducible physicochemical properties, mechanical strength, and degradation rates, making them highly customisable as a scaffold material [20,21]. Biological polymer scaffolds are more challenging to tailor to a particular application owing to purity issues, immunogenicity, and scaffold homogeneity and reproducibility [22]. However biological polymer scaffolds have superior bioactive properties (e.g. cell attachment, migration, cell scaffold remodelling) and thus better cell and tissue interactions [22,23].

## Generation of bioengineered tissue using synthetic scaffolds

First, we focused on synthetic scaffolds commercially available 'off the shelf', to optimize cell seeding, attachment and culture of the resulting tissue. We decided to use a Poly-Glycolic Acid (PGA) matrix due to its biodegradability, flexibility and lack of inflammatory response *in vivo*[24]. Additionally, synthetic PGA scaffolds can be easily processed into tailored architectures [22,23]. For these reasons, PGA is one of the most commonly used synthetic polymers in tissue engineering[21] and has been approved by the FDA for use in human studies[25].

We subsequently identified the optimal method for seeding COs on the scaffolds. Our results demonstrated the use of cell clumps concentrated in small volumes to be the optimal seeding method. Indeed, the dissociation of organoids into single cells requires aggressive enzymatic digestion. This approach reduces cell viability and attachment due to the cleavage of multiple cell-to-matrix adhesion molecules[26]. Furthermore, small volumes of cell suspension are absorbed by the scaffold, maximizing cell to scaffold contact. This technique resulted in the generation of confluent PGA-scaffolds seeded with COs, which were used to successfully repair biliary tree wall defects in immunocompromised mice, following transplantation.

## Generation of bioengineered tissue using biological scaffolds

We subsequently used the same seeding methodology to populate biological scaffolds. Here we chose densified collagen hydrogel due to its biocompatibility, low immunogenicity, ability to favour cell attachment and growth, biodegradability, and ability to be naturally remodelled by cells [27]. Importantly, unlike standard collagen gel, densified collagen demonstrates superior mechanical properties for surgical applications[27,28]. Densification expels the majority of the water content of the collagen gel, vastly reducing its volume, resulting in a polymer scaffold of higher concentration and fibrillar alignment [28]. Through the use of appropriate moulds, the densification process can be customized to generate constructs of more complex geometries, which can be successfully seeded with COs.

## Generation of bioengineered mouse bile ducts

One of the most relevant clinical applications of bioengineered biliary tissue is the generation of tubular constructs suitable for biliary reconstruction. However, bile duct transplantation can be associated with significant inflammatory response, epithelial damage and formation of anastomotic strictures in humans

[29,30]. Consequently, in vivo validation of the function, biocompatibility and patency of bioengineered ducts following reconstruction is required. To achieve this goal, we decided to generate bioengineered constructs populated with human COs, transplant them in immunocompromised mice and characterise them.

A key challenge was the fabrication of tubular structures with dimensions comparable to the mouse CBD (250 μm inner diameter and 30 μm wall thickness). Indeed, commercially-available fibrous PGA scaffolds[31] were incompatible with these requirements due to a minimum thickness (300 μm) significantly larger than the required wall thickness. Appropriate thickness pre-densified collagen gel sheets could be fashioned into a tube using sutures; however, interruption of the collagen fibrils along the seam led to partial collapse of the lumen under the weight of the wall. To overcome these challenges, a method was developed in which collagen was cast around a central rod and densified afterwards, to yield a seamless tube with a thin and robust wall. Furthermore, a conical funnel mould was used which allowed a proportionately large volume of collagen to densify into a short length of tube and enabled efficient water removal via multiple routes (through on-axis absorption and radial evaporation). These techniques yielded bioengineered tubes with a patent lumen and dimensions similar to the mouse CBD.

We subsequently used the approach we developed for seeding COs on flat collagen scaffolds to seed the luminar surface of the construct with cells and generate mouse-sized bioengineered bile ducts populated with human cells. Importantly, the potential of these constructs for biliary reconstruction *in vivo* was illustrated through their successful transplantation in immunocompromised mice[4].

## Applications and target audience

The propagation of cholangiocyte organoids and generation of bioengineered biliary tissue is likely to be of interest to a broad scientific audience including clinician scientists focusing on translation of new therapies to clinic, bioengineers working on whole organ reconstruction, groups focusing on biliary physiology and disease and the pharmaceutical industry. Currently the only therapeutic option for biliary disorders is liver transplantation. The generation of bioengineered biliary tissue could provide one of the first alternative treatments and pioneer the use of regenerative medicine for cholangiopathies [32,33].

Furthermore, a limitation of complex liver co-culture systems is the lack of a biliary system. The capacity of cholangiocyte organoids to grow in a variety of matrices and scaffolds makes them an ideal addition for complex tissue engineering applications that focus on recapitulating the microanatomy of the liver and the development of artificial whole-organ systems.

Cholangiocytes constitute a rare liver cell type and access to primary tissue has limited large scale analyses in the past. Cholangiocyte organoids resemble primary biliary epithelium very closely in terms of transcriptional profile and function [4]. Consequently, extrahepatic COs (ECOs) and intrahepatic (ICOs) could serve as a surrogate for primary cholangiocytes from any region of the biliary tree, enabling in depth, large scale studies of biliary physiology and pathophysiology. Similarly, COs recapitulate the effects of compounds such as verapamil or somatostatin, rendering them suitable for drug screening applications.

## Comparison to other methods

A unique feature of our culture system is that it enables the long-term culture of mature cholangiocytes through the inhibition of canonical WNT signalling by DKK-1, maintaining adult characteristics and functionality with no need for additional differentiation. Therefore, our system is distinct to alternative primary organoid platforms based on canonical WNT signalling which propagate adult stem cells [8,11,13,34] rather than bona-fide biliary epithelium. Consequently, COs may be better suited for studies on biliary physiology and disease requiring high fidelity cholangiocytes, while adult stem cells are optimal for studies on liver repair and regeneration.

While methods for short-term culture of murine[35] and human primary cholangiocytes have been reported [36,37], these systems are technically challenging, only allow for limited expansion restricting large scale analyses and the function of the resulting cells has not been extensively characterized. COs combine high proliferative capacity, increased functionality and the potential for large scale expansion, which is crucial for regenerative medicine or for high-throughput applications.

Importantly, cholangiocytes can be derived from induced pluripotent stem cells [5,6,38,39]. However, these cells correspond to fetal intrahepatic cholangiocytes, whereas our method can generate both intrahepatic and extrahepatic adult cholangiocytes. Additionally, COs can be rapidly isolated and

expanded, unlike iPSC systems which require a lengthy differentiation process and cannot be further propagated once terminally differentiated. Consequently, stem cell derived cholangiocytes are optimal for studies on intrahepatic bile duct development and its disorders, while COs are better suited for studies of adult intra- and extra-hepatic cholangiocyte physiology or regenerative medicine applications requiring large numbers of highly functional cells in little time.

Our system provides multiple advantages for tissue engineering and regenerative medicine. The generation of bioengineered tissue is versatile, allowing the use of synthetic (PGA) or biological scaffolds (collagen). Furthermore, it is compatible with GMP-compliant materials, such as collagen which has an excellent *in vivo* profile and is used extensively in clinical applications [24]. The method by which the densified collagen tubes are formed allows their generation at customisable length-scales compatible with small animal studies. Further, the densification process does not impart significant stress upon the cells, which can be mixed in the gel if required. The resulting constructs can be maintained more than 2 months in culture enabling the generation of large batches of bioengineered tissue with prolonged 'shelf-life'. Importantly, our bioengineered biliary tissue provides the first proof-of-principle for organ reconstruction using primary epithelial organoids, where the resulting bioengineered construct was used to fully replace the native organ [4].

## Limitations

A limitation of our system is that COs, as adult primary cholangiocytes, are not suitable for studying biliary development, an application for which stem cell derived systems are more appropriate. Additionally, as COs represent a pure epithelial population, this system does not currently allow for the study of epithelial and mesenchymal interactions, although the potential exists within our system to generate bioengineered tissue with additional cell types. A further, technical, limitation is that COs currently rely on the use of Matrigel, a non-GMP-compliant extracellular matrix. However, multiple chemically defined hydrogel matrices, which could replace Matrigel, are currently in development [40].

For research groups without access to a hospital with hepatobiliary services access to primary tissue may present a challenge, as primary samples need to be processed promptly following isolation. We note that samples can be obtained in any hospital that offers a cholecystectomy, ERCP or liver biopsy

service and organoid derivation is still feasible after small delays associated with tissue transfer over short distances. Nevertheless, to compensate for transport associated delays when multiple samples from distal centres are processed, a team of trained technicians working in parallel may be required.

Finally, although mouse-sized (submillimetre) constructs serve as proof-of-principle for the generation of bioengineered ducts populated with human cells, their mechanical properties do not translate to those of the human bile duct (7mm diameter, 1mm wall thickness). Therefore, optimisation of our technique for the generation of human sized constructs with appropriate physical attributes will be required prior to clinical translation.

## Experimental design

Our method describes a system for isolating primary extrahepatic and intrahepatic cholangiocytes from primary tissue, culturing these primary cholangiocytes in a 3D organoid system and generating bioengineered biliary tissue using artificial scaffolds (Figure 1). In this section we describe infrastructure and experimental setup considerations that need to be taken into account prior utilising this protocol.

## Isolation of cholangiocytes from primary tissue

Our protocol requires the isolation of cholangiocytes from primary human tissue, either from living patients or deceased organ donors. As such, appropriate ethical approval from the relevant regulatory bodies is required, while informed consent is essential prior to acquiring any human tissue samples.

Obtaining fresh human tissue samples will require access to a hospital with one of the following services: hepatology, advanced endoscopy, hepatobiliary surgery, transplant organ procurement or liver transplantation. Importantly, donor tissue viability decreases proportionally to ex-vivo storage and bile exposure. Therefore, tissue must be flushed from bile and stored immediately in cold preservation solution (such as University of Wisconsin (UW) solution) or supplemented William's E+ media) at 4 °C until it can be processed [41].

When multiple tissue samples are obtained simultaneously (such as from a deceased organ donor), the tissue should be processed in order of sensitivity to cold storage (supplemental figure 1). Liver biopsies require immediate processing, while extrahepatic tissue can be stored at 4 °C for several

hours, provided it is appropriately flushed of bile. Furthermore, all tissue handling should be performed under aseptic conditions to avoid contamination. Consequently, good communication with the clinicians obtaining the sample is crucial. Long-distance transport of samples is likely to impact the viability of explanted tissue, so samples must ideally be processed in a facility close to the site of collection. Tissue must be processed in a category 2 tissue culture hood under aseptic conditions and sterile surgical equipment must be used.

CO lines can be generated using multiple approaches, depending on the source of available tissue and the method of sample collection (Fig. 1). Surgically excised tissue samples such as gallbladders and bile ducts can be used for the generation of COs following isolation of the luminal layer of cholangiocytes through mechanical scraping (Fig. 2a-e). Endoscopic Retrograde Cholangiopancreatography (ERCP) brushings provide an alternative source of tissue for patients having endoscopy (Fig. 3a-d). Isolation of COs from liver biopsy tissue can be performed by dividing a liver biopsy core into small pieces (approximately 1 $mm^3$) and plating them directly in CO organoid culture conditions (Fig. 4a-e). Alternatively, CO lines can be derived from a population of EpCAM+ sorted single cells (Supplementary Fig. 2a-c). Tissue can be enzymatically digested to a single-cell suspension and then EpCAM+ sorted through either FACS or MACS.

Provided the tissue has been appropriately and promptly processed, the methods described should produce CO lines with almost 100% efficiency, apart from EpCAM+ sorting, which yields viable lines with an efficacy of 66% (supplementary table 1) due to the impact of single cell dissociation on cell viability. Flushing the tissue of bile is crucial to achieving these results. Indeed, derivation efficiency is reduced from 95% to to 40% if flushing is not performed (supplementary table 1). The optimal technique for CO line derivation should be decided based on sample availability and access to tissue.

## Establishment and maintenance of cholangiocyte organoids

Once plated, primary cholangiocytes should form organoids in 3-10 days. Clump size and seeding density can affect the speed and efficiency of derivation. Single cells tend to require longer culture and yield lower numbers of organoids for the same number of starting cells. Large clumps of >50-100 cells and high seeding density may result in cell attachment and limit organoid growth.

CO lines should be passaged approximately every five days (Fig. 5a-b), although the cells should be monitored daily for reaching confluency (Fig. 5b, Supplementary Fig. 3). Delays in passaging confluent wells can result in organoid collapse and affect the long-term health of the organoid line (Supplementary Fig. 4b). The same considerations as for organoid derivation apply with regards to clump size and seeding density during passaging (Supplementary Fig. 4b). Importantly, our protocol relies on Matrigel as an extracellular matrix which has a profound impact on the quality of the resulting organoid lines. Therefore, Matrigel must be batch tested before use. It is also important to ensure that all reagents and media used in the maintenance of CO lines are not used for longer than three months as this can impact on organoid quality.

Organoids can be analysed through a variety of methods such as qPCR, immunofluorescence and flow cytometry, as we have previously described [5,6]. COs should show robust expression of key biliary markers such as cytokeratin 19, cytokeratin 7, Sox9 and gamma-glutamyl transferase (GGT), as well as key cholangiocyte functions such as alkaline phosphatase (ALP) and GGT activity (Fig. 6a-e), which can be used to assess the quality of CO derivation and culture.

## Scaffold preparation

Bioengineered tissue can be generated using both synthetic (PGA) and biological (densified collagen gel) polymeric scaffolds, each of which may be suited to different applications. PGA scaffolds can be commercially sourced, are cheap, and require minimal processing to yield positive results. The mechanical properties of the scaffold (e.g. Young's modulus, strength) are tuneable and can be customized by adjusting density and pore size. Furthermore PGA constructs can be easily fabricated into custom architectures [22,23].

Collagen constitutes a physiological component of the extracellular matrix [42] with high bioactivity [43] which can interact with cells and present multiple cues that enhance attachment, survival, proliferation and tissue remodelling [42,44]. Furthermore, unlike PGA, cells may be mixed directly into the collagen gel precursor solution prior to gelation and densification, generating a uniform network of cells throughout the scaffold, which is useful for applications such as complex co-culture systems. However, it is more expensive than synthetic alternatives and scaffolds with adequate mechanical strength for surgical manipulation [45,46] are not commercially available and need to be custom made. Indeed, densified collagen sheets need to be fabricated from collagen gels through water absorption (figure 7a-b); while

densified collagen tubes, need to be formed through moulding of collagen gel around a cylindrical template and water removal by evaporation from the gel surface (figure 8a-d). Consequently, densified collagen scaffolds are more physiological but require a higher level of expertise and pose a greater number of potential pitfalls, while PGA scaffolds may be appropriate for settings where the expertise and equipment for the generation of custom-made scaffolds are not available.

## Scaffold seeding

PGA and densified collagen scaffolds (both flat and tubular) can be seeded with COs to generate bioengineered biliary tissue within 2-4 weeks. The cells are seeded as clumps in small volumes to maximize contact with the scaffold and incubated for 1 hour to allow attachment prior to the addition of media. For tubular scaffolds cannulation with a small gauge (< 30G) needle is necessary for seeding. This procedure is technically challenging and may require the assistance of a surgeon to avoid damaging the scaffold.

The efficiency and quality of cell attachment following seeding (Fig. 9d-e and 10c-d) depends on clump size, cell number, seeding cell suspension volume and drying time for the scaffold. Single cells and low cell numbers are associated with reduced seeding efficiency, while large clumps may attach only partially. Importantly, parts of a clump which have not attached may remodel to form organoid-like structures connected to the scaffold or overlap with neighbouring clumps which have attached generating a pseudostratified epithelium. Consequently, the use of 30-50 cell clumps is crucial to achieve optimal seeding results.

Low cell suspension volumes result in poor scaffold coverage and therefore suboptimal seeding, while cell viability may be reduced due to media evaporation during the incubation period. High volumes may lead to overflow and ‘spillage’ of the cell suspension from the scaffold to the plate resulting in a reduced cell-to-surface ratio and poor seeding efficiency. Consequently, the seeding volume must be optimized based on the scaffold surface as described in the procedure section. This is particularly important when seeding on the luminal surface of a tubular scaffold, where the cell suspension must be optimised for minimal volume and maximal density, to be contained within the tube lumen.

Reduced incubation time does not allow an adequate period for the cells to attach. Consequently, the addition of media at the end of the incubation phase results in washing the poorly attached cells off the scaffold. Prolonged incubation can result in scaffold drying and reduced cell viability.

Importantly, the scaffold is rarely confluent following seeding. Indeed, in most cases only a proportion of the scaffold is covered with cells. However, these expand to generate a confluent layer within weeks. The time to confluency can be reduced by seeding higher number of cells or performing repeated rounds of seeding. Importantly, if an additional round of seeding is performed the scaffold should not be allowed to dry before the new cells are added to preserve the viability of the cells already attached.

Bioengineered biliary tissue can be analysed through immunofluorescence or functional assays as previously described [4] and should show robust expression of key biliary markers such as cytokeratin 19 and cytokeratin 7 (Fig. 11a, 11c and 11e) as well as key cholangiocyte functions such as GGT activity (Fig. 11b, 11d and 11f). Once confluent, the scaffolds can be transplanted *in vivo* or maintained in culture for several months.

## Scaffold specific considerations

Pore size is an important consideration for PGA scaffolds. The scaffold pore size will determine the optimal cell clump size for seeding- scaffold with large pores would, in principle, allow deeper cell penetration, provided there is good interconnectivity between pores. However, constructs with larger pores are weaker, and seeding with large clumps is required to ensure the cells do not ‘fall through’ the spaces between fibres. Furthermore, preparation of the scaffolds for seeding includes treatment with ethanol and high concentration NaOH. It is crucial that at the point of seeding the scaffold is completely free of ethanol or NaOH remnants which could result in cell death and poor attachment.

For collagen scaffolds, the quality of collagen plays a key role for the attachment and growth of cells. As collagen solutions are not 100% pure, testing of each new collagen batch for cell attachment, proliferation, expression of biliary markers and function is recommended.

For the generation of tubular densified collagen scaffolds, assistance of an experienced engineer and access to engineering facilities, including a 3D printer, is essential. Furthermore, transplantation of bioengineered ducts into small animal models requires significant skill due to construct size and the procedure must be performed by an experienced surgeon.

## Appropriate Controls

Freshly isolated primary biliary tissue should be used as a positive control for the expression of biliary markers. For histology or immunofluorescence (IF) analyses, whole tissue can be frozen in OCT or fixed in 10% formalin and embedded in paraffin for sectioning. Cells can be isolated as described in step 2, sections A and D and dissociated to a single cell suspension for flow cytometry or resuspended in RNA lysis buffer for RNA extraction and quantitative PCR (QPCR), as described in steps 41, section C. Alternatively, RNA can be extracted from snap frozen tissue. While primary tissue represents the ideal control for CO function and marker expression, it is also possible to compare COs established cholangiocyte cell lines could be used as alternatives if access to primary tissue is not possible.

## Starting population considerations

We have demonstrated that our protocol for cholangiocyte organoid derivation is reproducible with >70 CO lines (Supplementary Table 1). Additionally, CO lines can be generated from very low numbers (<20,000) of viable primary cells (Supplementary Fig. 5). CO lines can be maintained in culture for >20 passages or 6 months. COs represent primary cells rather than immortalized cell lines, therefore it is possible that a reduction in the growth potential of function of the line is observed after this time. The 'incubator life' of each line varies and therefore the expansion potential, expression of biliary markers and function should be regularly tested after passage 20 to periodically validate the quality of the line.

For the generation of bioengineered tissue, some variability in cell attachment and expansion potential following seeding and after transplantation is expected. Therefore, minor optimization may be required for certain CO lines as described in the following sections.

# MATERIALS

## REAGENTS

- Human bile duct, gall bladder and liver tissue samples or cholangiocytes from ERCP brushings.

CAUTION: All human tissue samples must be collected with the appropriate ethical approval in place and with full informed consent. Donors should be tested to exclude HIV, hepatitis B and hepatitis C.

- William's E basal medium, no phenol red (Invitrogen; cat. no. A12176-01)
- Nicotinamide (Sigma Life science, cat. no. N0636-100G)
- Sodium Bicarbonate (Sigma Life Science, cat. no. S6014-500G)
- Sodium Pyruvate (Invitrogen, cat. no. 11360-070)
- D- Glucose (Gibco, cat. no. 15023-021)
- HEPES (Sigma, H0887-20ml)
- ITS+ universal cell culture premix (20ml) (SLS, cat. no. 354352)
- Dexamethasone (R&D Systems, cat. no. 1126/100)
- L- Glutamine (Life Technologies, cat. no. 25030)
- Penicillin-streptomycin (Life Technologies, cat. no. 15140122)
- L-phospho-ascorbic acid (Sigma Life Sciences, cat. no. 49752-10G)
- Matrigel (BD Biosciences, cat. no. 356237)
- Cell recovery solution (SLS, cat. no. 354253)
- Cell Banker 2 (Amsbio, cat. no. 11891)
- Recombinant Human Epidermal Growth Factor (EGF) (R&D Systems, cat. no. 236-EG)
- Recombinant Human DKK-1 protein (R&D Systems, cat. no. 5439-DK-01M/CF)
- Recombinant Human Rspondin-1 (R&D Systems, 4645-RS)
- Recombinant Human HGF (Peprotech, cat. no. 100-39)
- Recombinant Human Forskolin (FSK) (Sigma Aldrich, cat. no. F6886-10MG)
- Y27632 (Stratech Scientific, cat. no. S1049-SEL)
- Liberase™ DL Research Grade (Sigma Aldrich, cat. no. 5466202001)
- Deoxyribonuclease I from bovine pancreas (Sigma Aldrich, cat. no. D5025-150KU)
- UW cold storage solution (Belzer; cat. no. BTLBUW-1000)
- Dulbecco's PBS (DPBS; Life Technologies, cat. no. 14190)
- Red Blood Cell Lysis Solution (10×) (MACS Miltenyi Biotech, cat. no. 130-094-183)
- Bovine Serum Albumin (Sigma Life Sciences, cat. no. A3059)
- PolyGlycolic Acid BIOFELT scaffold (1 mm thickness, 50 mg/cm$^3$ PGA density) (Biomedical structures LLC, custom order- enquire with the manufacturer)
- CD326 (EpCAM) MicroBeads, human (MACS Miltenyi Biotech, cat. no. 130-061-101)

- FcR Blocking Reagent (MACS Miltenyi Biotech, cat. no. 130-059-90)
- MACS BSA Stock Solution (MACS Miltenyi Biotech, cat. no. 130-091-376)
- AutoMACS™ Rinsing Solution (MACS Miltenyi Biotech, cat. no. 130-091-222)
- AutoMACS® running buffer (MACS Miltenyi Biotech, cat. no. 130-091-221)
- AutoMACS® pro washing solution (MACS Miltenyi Biotech, cat. no. 130-092-987)
- Collagen I, High concentration rat tail collagen solution, 100 mg (Scientific Laboratory Supplies, 354249)
- 10x M199 (Sigma, cat. no. M0650)
- 1M Sodium Hydroxide (Sigma, cat. no. 2770)

  CAUTION: Sodium hydroxide can cause inflammation, irritation or corrosion upon contact with skin, eyes or when ingested or inhaled. It should be handled while wearing appropriate safety equipment
- DI water
- Water for embryo transfer (Sigma, cat. no. W1503)
- Trigene (Distel concentrate; Starlab, cat. no. TM309)
- Absolute ethanol (Fisher Scientific, cat. no. 10041814)
- Trypan Blue solution (Thermo Fisher Scientific, cat. no. 15250061)

  CAUTION: Trypan Blue is a potential carcinogen and can potentially cause damage to fertility
- Donkey serum (AbD Serotec, cat. no. c06sb)
- Triton-X100 solution (Sigma, cat. no. X100-500ML)
- Paraformaldehyde 16% (wt/vol) (PFA; Alfa Aesar, cat. no. 30525-89-4)

  CAUTION: Paraformaldehyde contains formaldehyde, which is carcinogenic. Paraformaldehyde can cause tissue damage if inhaled, ingested or exposed to skin and should be handled using appropriate safety measures
- Cytokeratin 7 antibody (Abcam, cat. no. ab68459; Table 1)
- Cytokeratin 19 antibody (Abcam, cat. no. ab7754; Table 1)
- Goat anti-human Sox9 (R&D Systems, cat. no. AF3075; Table 1)
- Rabbit anti-human albumin (Abcam, cat. no. ab137885; Table 1)
- Mouse anti-human GGT-1 (Abcam, cat. no. ab55138; Table 1)
- Donkey anti-mouse Alexa Fluor 488 (Life Technologies, cat. no. A2102; Table 1)

- Donkey anti-rabbit Alexa Fluor 568 (Life Technologies, cat. no. A10042; Table 1)
- Donkey anti-goat Alexa Fluor 488 (Life Technologies, cat. no. A21447; Table 1)

## EQUIPMENT

- Plate heater (TAP Biosystem, cat. no. 016-0R10)
- Inverted microscope (Olympus, cat. no. CKX41)
- 100-mm TC-Treated Culture Dish (Corning, cat. no. 430167)
- Costar 24-Well Clear TC-Treated Multiple-Well Plates (Corning, cat. no. 3526)
- Surgical Scalpel Blade No.22 (sterile) (Swann Morton Ltd, cat. no. 0508)
- Dumont #5 - Fine Forceps (F.S.T., cat. no. 11254-20)
- 15 ml and 50 ml Centrifuge tubes (Corning, cat. nos. 430791 and 430291)
- 500-ml Vacuum Filter/Storage Bottle System, 0.22-µm pore (Corning, cat. no. 431097)
- $CO_2$ incubator (Sanyo, cat. no. MCO-18AC)
- Centrifuge (Eppendorf, cat. no. 5804)
- Orbital shaking incubator (New Brunswick Scientific, cat. no. M1299-0082)
- Disposable serological pipettes (5, 10 and 25 ml) (Corning, cat. nos. 4487, 4488 and 4489)
- Graduated filter tips (1000, 200, 20 and 10 µl) (Starlab, cat. nos. S1122-1830, S1120-8810, S1120-1810, S1120-3810)
- Cryotube vials (2 ml) (Thermo Scientific, cat. no. 368632)
- AutoMACS® Pro Separator (MACS Miltenyi Biotech, cat. no. 130-092-545)
- 40 µm cell strainers (Corning, cat. no. 352340)
- Countess™ II Automated Cell Counter (Thermo Fischer Scientific, cat. no. AMQAX1000)
- Countess™ cell counting chamber slides (Thermo Fischer Scientific, cat. no. C10283)
- Insulin syringes, 1 ml (VWR International, cat. no. 613-4892)
- Syringes, 20 ml (Fisher Scientific, cat. no. 15829152)
- Needles, 18G, 23G (Camlab, cat. no. 305180, 300700)
- Stainless steel fine tweezers (Onecall, cat. no. 1779183)
- Dissecting scissors, straight (Fisher Scientific, cat. no. 15207266)
- Precision wipes, Kimtech (Fisher Scientific, cat. no. 12660543)
- Self-seal sterilisation pouches (Fisher Scientific, cat. no. 15428782)

- 0.2 µm syringe filters (Fisher Scientific, cat. no. 10268401)
- 6 well plates (Fisher Scientific, cat. no. 10396482)
- Autoclave tape (Greiner Bio-One, cat. no. TAP02)
- Micro-spatula, 21 mm length (VWR International, cat. no. 231-0446)
- Specimen tubes, flat bottom, 10 mm (Samco, cat. no. G05017)
- Nylon membrane, 10 µm pore size, hydrophilic (Millipore, cat. no. NY1002500 )
- 25 µl Model 1702 Hamilton syringe (Hamilton, cat. no. 80265)
- 34 gauge small hub removable needle (Hamilton, cat. no. 207434)

Critical: While the exact make and model of syringe and removable needle can vary according to the researcher's preference, it is essential that small volume syringes and removable needles with no dead space are used for seeding the tubular scaffolds (step 40, section B), due to the very small (25 µl) volumes needed for seeding.

## REAGENT SET UP

- **Human biliary and liver tissue** Primary tissue can be obtained from surgically excised gallbladder or bile duct tissue, ERCP brushings or liver biopsies. Once collected, tissue should be stored immediately at 4°C in Williams E+ media with 50 ng/ml of EGF and 10 µM of Y27632 or in University of Wisconsin (UW) cold storage solution.

CAUTION: Leaving primary tissue longer than 8 hours before processing will negatively affect the viability of the isolated cells

- **Nicotinamide 0.4 M stock solution** Dissolve 24.4 g of nicotinamide powder in 500 ml of embryo transfer water.

  *CRITICAL: Sterilize nicotinamide stock solution using a vacuum filter/storage bottle system. Mix it well before filtration. Store the solution at 4 °C for up to 3 months.*

- **Sodium bicarbonate 1 M stock solution preparation** Dissolve 42 g of sodium bicarbonate powder in 500 ml of embryo transfer water.

*CRITICAL: Sterilize sodium bicarbonate stock solution using a vacuum filter/storage bottle system. Mix it well before filtration. Store the solution at 4 °C for up to 3 months.*

- **Ascorbic acid trisodium salt 100 mM stock solution preparation** Dissolve 16.1 g of ascorbic acid trisodium salt powder in 500 ml of embryo transfer water.
  *CRITICAL: Sterilize ascorbic acid trisodium salt stock solution using a vacuum filter/storage bottle system. Mix the solution well before filtration. Store it at 4 °C for up to 3 months. Protect it from light.*

- **D-Glucose 1 M stock solution preparation** Dissolve 90.1 g of D-glucose powder in 500 ml of embryo transfer water. Warm the mixture to 50 °C to facilitate dissolution.
  *CRITICAL: Sterilize D-glucose stock solution using a vacuum filter/storage bottle system. Mix the solution well before filtration. Store it at 4 °C for up to 3 months.*

- **Dexamethasone 10 mM stock solution** Dissolve 100 mg of dexamethasone in 25.4797 ml of DMSO. Prepare 50- to 100-µl aliquots. Store them at −80 °C for up to 12 months.

- **Supplemented William's E medium (Willliam's E+)** Combine 443 ml of William's E medium with 12.5 ml of nicotinamide stock solution, 8.5 ml of sodium bicarbonate stock solution, 1 ml of ascorbic acid trisodium salt stock solution, 7 ml of glucose stock solution, 3.15 ml of sodium pyruvate, 10 ml of HEPES solution, 5 ml of ITS+ premix, 5 µl of dexamethasone (R&D Systems), 5.3 ml of glutamine and 5 ml of pen/strep.
  *CRITICAL: Sterilize William's E+ medium using a vacuum filter/storage bottle system. Mix the medium well before filtration. Store it at 4 °C for up to 1 month. Warm it to 37 °C before use.*

- **Sodium bicarbonate 7.5 % (wt/vol) stock solution** Dissolve 3.75 g of sodium bicarbonate powder in 46.25 ml of deionised water.
  *CRITICAL: Sterilize sodium bicarbonate stock solution using a 50 ml syringe and 0.2* µm syringe filter*. Mix it well before filtration. Store the solution at 4 °C for up to 3 months.*

- **70% (wt/vol) ethanol.** Combine 700 ml of absolute ethanol and 300 ml deionised water.

- **Matrigel preparation.** 10 ml Matrigel vials should be thawed slowly in a refrigerator placed at 4 °C overnight. Thawed Matrigel should be mixed well and then divided into 1 ml aliquots. Aliquotting of Matrigel should always be performed in a tissue culture hood to avoid bacterial contamination. Matrigel should be kept constantly on ice to avoid solidification. All equipment coming into contact with Matrigel should be precooled to 4 °C. This includes pipette tips and media for diluting Matrigel. Tubes for aliquotting should be kept on ice. Store Matrigel aliquots at −20 °C or −80 °C for up to 3 months.
  *CRITICAL: Each aliquot should undergo a maximum of two freeze–thaw cycles. This can be achieved by adjusting aliquot volumes accordingly.*

- **Preparation of a 3X supplemented William's E+ solution.** William's E+ media is supplemented with 1.5 µg/ml RSPO, 300 ng/ml DKK, 150 ng/ml EGF and 3 µM Y27632 (3X the typical concentration of all Supplementary cytokines).

COs are cultured in 3D conditions, suspended in 50 µl droplets composed of a 66.7% (vol/vol) Matrigel and 33.3% (vol/vol) 3X supplemented William's E+ solution, which form a dome after plating (Fig. 4b, Image 6A(i)). Prior to plating, a master mix is prepared with a volume corresponding to the number of droplets that will be plated as described in steps 3-10. Importantly, for generating this master mix COs or primary cholangiocytes are first resuspended in the 3X supplemented William's E+ solution (step 3) and Matrigel is subsequently added (step 5). To calculate the volume of supplemented William's E+ needed for resuspension of the CO pellet, the following formula can be used:

*Volume of supplemented William's E+ media= [(number of CO wells) x 50 µl]/3*

*CRITICAL: As the volume of media needed to resuspend the CO pellet will typically be very small, it is advisable to instead prepare a larger volume of 3X concentrated William's E+ solution. This can then be diluted to 1X and added to the wells after plating as their plating*

*medium. This volume corresponds to 1/3 the total number of wells to be plated (e.g. if plating 12 wells, prepare 4 ml of 3X William's E+ solution) assuming 1ml of media / well is used. A small aliquot of the resulting 3X solution can then be used to resuspend the pellet and the remaining solution can be diluted to 1X with William's E+ devoid of additional cytokines and used as plating medium.*

*E.g. If plating 12 wells, resuspend the pellet in 200 µl of 3X supplemented William's E+ solution, taken from the initial 4 ml of 3X supplemented William's E+ solution. After CO plating (steps 3-10), add 8 ml of non-supplemented William's E+ media to the 4 ml of supplemented William's E+ solution to make a 1X supplemented William's E+ solution*

**Preparation of a 66.7% Matrigel (vol/vol) solution** To obtain the volume of Matrigel needed when plating, multiply the volume of 3X supplemented William's E+ solution by two. E.g. if plating 12 wells, 200 µl of supplemented William's E+ solution should be added to the pellet followed by 400 µl of Matrigel.

*CRITICAL: The 3X supplemented William's E+ solution should be precooled to 4 °C*

*CRITICAL: Both Matrigel and the 3X supplemented William's E+ solution should be kept on ice for the duration of the procedure to avoid Matrigel solidification*

- **Liberase preparation** Reconstitute the lyophilised liberase enzyme with 10 ml of sterile injection-quality water, as per the manufacturer's instructions, to obtain a stock solution.

  *CRITICAL: Aliquot the reconstituted liberase stock solution into 1 ml aliquots to avoid repeated freeze-thaw cycles and store at -15 to -25 °C. Reconstitution and aliquotting should take place in a tissue culture hood to avoid contamination.*

- **DNase I preparation** Reconstitute the lyophilised DNase I to a 4 mg/ml stock solution using sterile PBS.

  *CRITICAL: Aliquot the reconstituted DNase I stock solution into 100 µl aliquots to avoid repeated freeze-thaw cycles and store at -80 °C. Reconstitution and aliquotting should take place in a tissue culture hood to avoid contamination.*

- **PBS 1% BSA (wt/vol) preparation** Weigh out 5 g of BSA powder and dissolve in 500 ml of PBS to obtain a 1% solution.
  *CRITICAL: Sterilize PBS 1% BSA solution using a vacuum filter/storage bottle system. Mix the solution well before filtration. Aliquot in 50 ml centrifuge tubes and store at -20 °C to avoid contamination.*

- **MACS buffer preparation** Prepare a 1 in 20 dilution of MACS BSA stock solution in AutoMACS™ Rinsing Solution
  *CRITICAL: MACS buffer should be freshly prepared at point of use and should be kept at 4 °C. Buffer preparation should take place in a tissue culture hood to avoid contamination*

- **Preparation of a 0.1% Triton X-100 solution** Add 50 µl of Triton X-100 to 50 ml of PBS in a 50 ml centrifuge tube. Gently shake the tube by inversion until the Triton X-100 is fully dissolved

## EQUIPMENT SET UP

### Plate heater setup

- Clean the plate heater with trigene and 70% (vol/vol) ethanol and place it in a tissue culture hood. Set the temperature to 37 °C and place a 24-well plate on the heating surface.

*CRITICAL: Allow a minimum of 30 min for the plate to warm up, before plating Matrigel with cells. If you are using multiple plates, these can be prewarmed in an incubator for a minimum of 30 min, with each plate placed on the plate heater immediately before plating.*

### AUTOMACS pro separator set up

- Run column exchange before use. Check that the columns are secured and there are no leaks anywhere in the system. Check that there is sufficient running buffer, washing solution and 70% (vol/vol) ethanol and that the waste bottle is not too full. Run an additional “Qrinse” before starting cell sorting. This can be done while the cells are incubating with the MACS beads to

save time. To shut down the machine after EpCAM+ sorting, select the “sleep” programme. Turn off the machine once the “sleep” programme has finished

**Fabrication and assembly of densification chamber for densified collagen tubes.**

Critical step: The densification chamber, used to form the densified collagen tubes, requires fabrication and assembly ahead of the collagen gel preparation. This involves 3D printing the base and funnel pieces, mounting a rigid metal wire in the base, and fixing paper towels between the base and funnel. This procedure requires access to basic engineering facilities.

I. Design chamber geometry in computer-aided design (CAD) package (e.g. Autodesk Inventor). See Supplementary Software 1 and 2 for reference.
II. Export as .stl file and prepare file for 3D printing using 3D printer software (e.g. Doraware).
III. 3D print chamber model using poly-lactic acid (PLA) filament and assemble components shown in Fig. 8a, Image 1.
IV. Mount straight rigid wire in hole of base piece of chamber by supergluing it into place (Fig. 8a, Image 2).

Critical step: Let superglue cure before continuing (1 hour).

V. Fold 2 sheets of absorbent precision wipe paper towels four times and using scissors, cut to size of base plate. Autoclave these paper towels in an autoclave pouch.

Critical step: Let towels fully dry before continuing.

VI. Sterilize base plate and funnel piece by immersion in 70% (vol/vol) ethanol.
VII. Place 3D printed components under a sterile biological cabinet until dry.
VIII. Autoclave 4x M4 screws and nuts.
IX. Using a sterile 23G needle, punch a hole through the centre of the sheets and feed the mounted metallic wire through the needle (Fig. 8a, Image 3).
X. Pull out 23G needle and push down paper towels.
XI. Feed metallic wire through funnel piece (Fig. 8a, Image 4) and fix funnel piece to base piece using 4x M4 screws and nuts (Fig. 8a, Image 5).
XII. Tighten using screwdriver. Chamber is now ready for densification process.

Critical Step: Screws must be tight in order to prevent leaking of water to the towels prior to a collagen gel forming.

Critical Step: The top of the metallic wire must not extrude above the top of the funnel. This is a necessary condition for successful collagen densification for tube formation.

# PROCEDURE

**Derivation of cholangiocyte organoids from primary human tissue**

1. Primary tissue can be obtained from surgically excised gallbladder or bile duct tissue, ERCP brushings or liver biopsies (Fig. 1b). Once collected, tissue should be stored immediately as described in “reagent setup”.

Pause point: Tissue can be kept in media/cold storage solution for up to 8 hours although for optimal viability, tissue should be processed as soon as possible after collection. Guidance on the maximum length of storage before processing for each tissue type is shown in Supplementary Fig. 1.

CAUTION: Dissection of tissue should take place in a category 2 cell culture hood under aseptic conditions

2. For derivation of COs from excised bile ducts or gall bladders, see section A. For derivation of COs from ERCP brushings, see section B. For derivation of COs from liver tissue, see section C and for derivation of COs from an EpCAM+ sorted single cell suspension, see section D.

**Step 2, Section A: Derivation of extrahepatic cholangiocyte organoids from deceased organ donors**

**Timing: 1-2 hours (including plating)**

Critical step: All equipment used should be sterile and all work must be done under aseptic conditions in a category 2 tissue culture hood.

I. Transfer the tissue from the storage container onto an empty 10 cm plate.

II. Using a scalpel and forceps, make a longitudinal incision along the length of the excised bile duct or from the fundus to the neck of the excised gallbladder. (Fig. 2b, Images 1A(ii) and 2B(ii)) to expose the lumen.

This step should result in a flat sheet of biliary tissue with the biliary epithelium on the luminal surface, usually pigmented yellow by bile and an 'exterior' surface corresponding to the outer wall of the bile duct or gallbladder.

CAUTION: Ensure the luminal surface is facing upwards to avoid loss of cholangiocytes

III. Wash the tissue by transferring in a 50 ml centrifuge tubes containing PBS to remove excess bile (Fig. 2b and c, Image 2). Repeat twice using a fresh tube each time [Troubleshooting]

Critical step: PBS washes must be performed cautiously to prevent detachment of the biliary epithelial layer. This is particularly important if the bile duct or gallbladder tissue has been kept on ice for <2-4 hours after surgical excision

IV. Transfer the tissue to an empty plate

V. Add Williams' E+ media to the plate until the tissue is fully submerged in media. It is not necessary to supplement the media with additional cytokines.

CAUTION: The tissue must be submerged in media quickly to prevent it from drying

VI. Gently scrape the luminal surface of the tissue with a scalpel to release the cholangiocytes into the media (Fig. 2b and c, Image 3)

Critical step: Examine the cell suspension under the microscope after scraping the tissue and before collecting the cells into a centrifuge tube to ensure the mechanical dissociation has been successful. The epithelial cells should be released from the tissue as small clumps and should display columnar morphology (Fig. 2b and c, Image 9).

VII. Collect the media and cells into a 50 ml centrifuge tube using a 10 ml pipette

VIII. Wash the tissue again by adding approximately 10 ml of fresh media directly on the luminal surface of the tissue in the plate with a 10 ml pipette

IX. Repeat the process of scraping and washing until the entire epithelial layer is collected. By the end of this stage, the luminal side of the biliary tissue will appear smooth, losing the

characteristic velvet-like appearance of the biliary epithelium (Fig. 2b and c, Images 1A(iii) and 1B(iii)).

Critical step: Scrape cautiously to avoid releasing fibrous tissue and debris into the cell suspension [Troubleshooting]

X. Centrifuge the cells at 444 g for 4 minutes at room temperature (23 °C)

XI. Aspirate the supernatant

XII. Resuspend the pellet in 10 ml of WE+ media (regardless of pellet size) to wash the cells. If there is bile or debris remaining in the suspension, repeat this wash step.

Critical step: Remove large pieces of debris and fibrous tissue using a p1000 pipette as these may not be easily removed by washing [Troubleshooting]

XIII. Centrifuge the cells at 444 g for 4 minutes at room temperature

XIV. Optional step: Red cell lysis to avoid erythrocyte contamination

XV. Optional step: Resuspend in 10 ml of ice cold red cell lysis buffer

XVI. Optional step: Incubate the cell suspension on ice for up to ten minutes.

XVII. Optional step: Wash twice in William's E+ media as described in steps XI – XII before continuing with step XXI

XVIII. Aspirate the supernatant

XIX. Wash the pellet in William's E media.

XX. Plate the cells as described in steps 5-14 [Troubleshooting]

Critical step: typical morphology for COs plated from GB or BD tissue after one week and >20 weeks after plating is displayed in Fig. 2d Image 1 and 2d Image 2 respectively. COs should begin to form approximately 3 days after plating and debris should disappear from the CO culture after the first two passages

**Step 2, Section B: Derivation of extrahepatic cholangiocyte organoids through Endoscopic retrograde cholangiopancreatography (ERCP) brushings**

**Timing: 30 minutes**

I. Prepare a 50 ml centrifuge tube of William's E+ media with 50 ng/ml of EGF and 10 μM of Y27632. Provide this tube to the clinicians before the start of the ERCP procedure

II. Following brushing, wash the ERCP brush in the tube of media to dislodge the collected cells (Fig. 3a, panel 2)

III. Transport the centrifuge tube at 4 °C to a category 2 tissue culture hood under aseptic conditions

IV. Centrifuge the 50 ml tube at 444 g for 4 minutes at room temperature

V. Plate the resulting pellet of cells as described in the "plating of cholangiocyte organoids" section below (steps 3-10) [Troubleshooting]

Critical step: typical morphology of COs derived from ERCP brushings immediately after plating, 24 hours after plating and one week after plating are displayed in Fig. 3b Image 5, 3c Image 1 and 3c Image 2 respectively. Organoids should begin to form within 24 hours of plating and debris should disappear from the organoid culture after the first two passages.

**Step 2, Section C: Derivation of intrahepatic cholangiocyte organoids from liver tissue**

**Timings: 30-40 minutes (including plating)**

I. Place a liver biopsy core into a well of a 6 well tissue culture plate. Alternatively, place a small piece of liver tissue (approximately 1 $cm^3$) on a sterile 10 cm tissue culture dish and cut into small pieces (approximately 3-4 $mm^3$) before transferring into a well of a 6 well plate for further dissection

II. Using a scalpel, carefully dissect the tissue into as small pieces as possible, approximately <1 $mm^3$ (Fig. 4b and c, Image 1) [Troubleshooting]

Critical step: Care should be taken to ensure the tissue is cut as small as possible so it can fit into a p1000 pipette tip (Fig. 4b and c, Image 2). Tissue dissection should be done as quickly as possible to prevent the tissue from drying out.

III. Add 1 ml of William's E+ media with 50 ng/ml EGF, 10 μM (1 μl/ml) Y27632 to the dissected liver tissue

IV. Transfer the dissected tissue and media into a 15 ml centrifuge tube using a p1000 pipette (Fig. 4b and c, Image 3) and centrifuge at 300 g for 2 minutes at room temperature (Fig. 4b and c, Image 4)

V. Carefully aspirate the supernatant

VI. Wash the pellet in Williams E+ media

VII. Centrifuge at 300 g for 2 minutes at room temperature

VIII. Plate the dissected tissue pieces following the instructions in the "plating of cholangiocyte organoids" section below (steps 3-10) (Fig. 4b and c, Image 6(i)) [Troubleshooting]

Critical step: Typical morphology for COs plated from diced liver tissue < one week after plating and >20 weeks after plating is displayed in Fig. 4d Image 1 and 4d Image 2 respectively.

**Step 2, Section D: Derivation of intrahepatic cholangiocyte organoids through EpCAM+ MACS sorting**

**Timing: 2-3 hours**

Critical step: the liberase and DNase I solutions should be pre-warmed to 37 °C before starting the isolation process

I. Place a liver biopsy core into a well of a 6 well tissue culture plate. Alternatively, place a small piece of liver tissue (approximately 1 $cm^3$) on a sterile 10 cm tissue culture dish and cut into small pieces (approximately 3-4 $mm^3$) before transferring into a well of a 6 well plate for further dissection

II. Using a sterile scalpel, dissect the tissue into very small pieces < 1 $mm^3$ (Fig. 4b and c, Image 2)

III. Add 1.5 ml of pre-warmed liberase digestion solution (0.5 U) with 4 mg/ml of DNase I (Supplementary Fig. 2b, Image 3)

Critical step: DNase I must be added during the dissociation to prevent the cells from forming clumps.

IV. Place the plate on a heated orbital shaker at 170 rpm and 37 °C for 30 minutes. Ensure the plate is secured to the orbital shaker.

V. Every ten minutes, remove the tube from the rocker and gently pipette up and down with a p1000 pipette.

Critical step: Examine the cells under the microscope at these ten minute intervals to check the progress of the dissociation. The dissociation will be finished once all the tissue has been dissociated to a single-cell suspension, with only the collagen scaffold of the tissue remaining (Supplementary Fig. 2b, Image 4). [Troubleshooting]

VI. After 30 minutes, stop the reaction by adding an equivalent volume of cold PBS 1% BSA (wt/vol).

VII. Filter through a 40 µm filter into a 15 ml centrifuge tube [Troubleshooting]

Critical step: filter gently and do not force any material through the filter as this will reduce viability and result in fibrous material in the final cell suspension

VIII. Centrifuge at 444 g for 4 minutes at room temperature

IX. Aspirate the supernatant using a p1000 pipette

X. Resuspend the pellet in 10 ml of red cell lysis buffer with 4 mg/ml of DNase I and 10 µM of Y27632

Critical step: Aspiration of the supernatant should not be done using a vacuum pump aspirator from this stage onwards as this presents a risk of losing the pellet

XI. Incubate the cells on ice in red cell lysis buffer for up to ten minutes.

XII. Add an equivalent volume of PBS 1% BSA (wt/vol) to the red cell lysis buffer and centrifuge at 444g for 4 minutes at room temperature

XIII. Aspirate the supernatant using a p1000 pipette and resuspend in 1 ml of cold PBS 1% BSA (wt/vol) with 4 mg/ml of DNase I and 10 µM of Y27632.

XIV. Repeat this wash step one more time to ensure the complete removal of the red cell lysis buffer, and resuspend the pellet once more in in 1ml of cold PBS 1% BSA (wt/vol) with 4 mg/ml of DNase I and 10 µM of Y27632

XV. Take a 10 µl aliquot of the cell suspension and mix with an equivalent volume of Trypan Blue. Count the cells using a haemocytometer or an automated cell counter. [Troubleshooting]

XVI. Centrifuge the pellet at 444 g for 4 minutes at room temperature.

XVII. Resuspend the pellet in the appropriate volume of MACS buffer, FcR blocking reagent and CD326 (EpCAM) MicroBeads according to the manufacturer's instructions

Critical step: Use the cell count obtained in step XV to determine the appropriate volume of each reagent to add for the number of cells according to the manufacturer's instructions (300 µl MACS buffer, 100 µl FcR blocking reagent and 100 µl CD326 (EpCAM) MicroBeads for every 5 x $10^7$ total cells)

XVIII. Incubate the cells for 30 minutes at 4 °C

XIX. While the cells are incubating, prepare the AutoMACS® Pro Separator for cell sorting. See "AUTOMACS® pro separator set up" in "Equipment Setup" for instructions.

XX. Wash the cells with 5 ml of PBS 1% BSA (wt/vol)

XXI. Centrifuge at 300 g for 10 minutes at room temperature

XXII. Aspirate the supernatant and resuspend in 5 ml of PBS 1% BSA (wt/vol) with 4 mg/ml of DNase I and 10 µM of Y27632

XXIII. Filter the sample through a 40 µm filter immediately before sorting (Supplementary Fig. 2b, Image 6)

XXIV. Run the sample through the AutoMACS® Pro Separator on a "POSSELD" programme. This will select for the EpCAM+ fraction. Collect the EpCAM+ fraction in a 15 ml centrifuge tube. [Troubleshooting]

Critical step: Ensure this takes place under aseptic conditions- the AutoMACS® Pro Separator should be in a tissue culture hood and all buffers and running solutions should be kept sterile.

Critical step: The MACS centrifuge tube rack should be pre-cooled in the fridge for at least an hour before use

XXV. Top up the 15 ml centrifuge tube with PBS 1% BSA (wt/vol)

XXVI. Centrifuge at 444 g for 4 minutes at room temperature

XXVII. Plate the cell suspension following the instructions in the "plating of cholangiocyte organoids" section (steps 3-10) below. [Troubleshooting]

XXVIII. Critical step: Typical morphology for COs plated from EpCAM+ sorted liver tissue < one week after plating is displayed in (Supplementary Fig. 2c). Organoids will grow from single cells over

the course of 5-15 days after plating. After the first passage, EpCAM+ sorted organoids will display typical CO morphology, as demonstrated in Supplementary Fig. 4a, Image 5).

**Plating of primary cholangiocytes in organoid format**

**Timing: 10-40 minutes**

Critical step: Pre-heat an adequate number of 24-well tissue culture plates at 37 °C for at least one hour prior to use (pre-heating overnight is preferable)

Critical step: Thaw Matrigel on ice for two hours to overnight prior to starting the isolation procedure. Matrigel will solidify at room temperature and so should always be kept on ice when in use. It is important to work quickly to prevent the Matrigel solidifying during the plating procedure.

Critical step: COs should be plated in 3D Matrigel domes composed of a 2:1 ratio (33.3% to 66.7% vol/vol solution) of Matrigel and William's E+ media. The CO pellet must first be resuspended in a 3X supplemented William's E+ solution before the separate addition of Matrigel. See "preparation of a 3X supplemented William's E+ solution" in Reagent Setup for instructions on how to calculate the volumes of 3X William's E+ and Matrigel required to prepare this solution.

3. Resuspend the CO pellet in a volume of the 3X supplemented William's E+ solution appropriate for the number of wells being plated. E.g. if plating 9 wells, resuspend in 150 µl of WE+ media with 1.5 µg/ml R-spondin, 150 ng/ml EGF, 30 µM (3 µl/ml) Y27632 and 300 ng/ml DKK-1.

Critical step: Y27632 should always be freshly added at this stage and kept in the culture media for 48 hours to ensure maximal survival of the cholangiocyte cells

4. Mix the Matrigel stock thoroughly with a p1000 pipette.

Critical step: Matrigel should be kept on ice throughout the entire procedure and must be mixed with care to avoid bubbles

5. Add Matrigel to the cell suspension in a 2:1 ratio (66.7% vol/vol) and mix well. See "preparation of a 66.7% Matrigel (vol/vol) solution" for instructions on how to calculate the amount of Matrigel required.

6. Plate the organoids in 50 µl Matrigel/media domes using a p1000 pipette, each in a well of a 24 well plate. To plate the dome, hold the tip of the p1000 pipette very close to the surface of the well, in the centre, and slowly start pipetting. Move the pipette upwards as the droplet forms. [Troubleshooting]

Critical step: Do not go down to the second stop of the pipette as this will form bubbles in the Matrigel dome.

Critical step: Mix thoroughly before plating each dome

7. Allow the Matrigel to solidify for 1-2 minutes in the plate heater. Gently tilt the plate to test that the Matrigel has solidified.
8. Invert the plate and keep in the 37 °C incubator for 30 minutes. This step should be omitted when plating dissected liver tissue (Section C) or EpCAM+ sorted single cells (Section D) [Troubleshooting]

Critical step: Without the plate inversion step, cell clumps are likely to migrate to the bottom of the well and attach to the plate. If this happens, the cells will not be able to form organoids.

9. Make up the 3X William's E+ media solution to a final concentration of 500 ng/ml Rspondin, 50 ng/ml EGF, 10 µM (1 µl/ml) Y27632 and 100 ng/ml DKK-1 (+/- 50 ng/ml HGF) by following the formula below

*Final volume of media = initial volume of media prepared in step 5 + [initial volume of media prepared in step 5 x 2]*

E.g. If 1 ml of media was initially prepared with 1.5 µg/ml Rspondin, 150 ng/ml EGF, 30 µM (3 µl/ml) Y27632 and 300 ng/ml DKK-1 (+/- 50 ng/ml HGF and 6 µM FSK), add a further 2 ml of William's E+ media without cytokines, for a total of 3 ml of media with 500 ng/ml Rspondin, 50 ng/ml EGF, 10 µM (1 µl/ml) Y27632 and 100 ng/ml DKK-1 (+/- 50 ng/ml HGF and 2 µM FSK)

10. Add 1 ml of this supplemented media per organoid well using a 5 ml or 10 ml pipette.

Critical step: Add the media slowly to the side of each well to avoid disrupting the Matrigel domes

Critical step: When plating from primary cells, organoids from the common bile duct and gallbladder should begin to form within two days of plating and should be ready for the first passage within 5-7 days of initial plating (Fig. 2d, Image 2 and Fig. 3c, Image 2). Organoids from liver biopsies or excised

liver tissue should take about 5-10 days to develop (Fig. 4d, Image 2) and should be passaged when the plate is approaching 80% confluency [Troubleshooting].

Critical step: CO lines should always express the biliary markers CK19, CK7, Sox9 and GGT (Fig. 6a and 6b) and display ALP and GGT activity (Fig. 6c and 6d, respectively). Established CO lines should comprise a <99% pure population of CK19+/CK7+ cells (Fig. 6e). After approximately passage 2, CO lines derived from different tissues of origin or through different derivation methods will appear morphologically identical (Supplementary Fig. 4a).

**Cell culture: Changing media for CO lines for maintenance**

**Timing 20 minutes**

Critical step: Once primary cholangiocytes have been isolated and plated as described above, culture conditions for all CO lines are the same and the following procedural steps for CO maintenance (steps 15-30) apply equally to all CO lines, regardless of tissue of origin or derivation method. Equally, the characterisation data shown in Fig. 6 is representative of CO lines derived from all tissue types or derivation methods

11. Media should be changed approximately every 48 hours.
12. Prepare 1ml of William's E+ media per well supplemented with 500 ng/ml Rspondin, 50 ng/ml EGF and 100 ng/ml DKK-1 to each well of a 24 well plate. Additionally, 50 ng/ml of HGF and 2 μM of FSK can be added optionally for slow growing lines requiring > 5-7 days between passages. [Troubleshooting]
13. Carefully aspirate the old media from the well

Critical step: tilt the plate when aspirating and aspirate from the edge of the well to avoid disrupting the Matrigel dome

14. Add the new media to all the wells using a 5 ml or 10 ml pipette.

Critical step: When adding media, tilt the plate and hold the pipette against the side of the well to avoid disrupting the Matrigel dome. Add the media slowly.

**Passaging of CO lines for maintenance**

**Timing: 90 minutes**

15. CO lines should be passaged approximately once every five days. However, the optimal time for passaging should be decided based on the confluency of the organoids, therefore the cells should be examined daily. (Fig. 5b, Image 1)

Critical step: Allowing the wells to become too confluent may lead to collapse of organoids and subsequent cell death (Supplementary Fig. 4b, Image 2)

16. Remove the media and add 500 µl of cell recovery solution to each well. The pipette should be aimed at the centre of the Matrigel dome and the cell recovery solution should be ejected forcefully to disrupt the surface of the dome.
17. Mechanically dissociate the remaining Matrigel dome by scraping with a p1000 pipette (Fig. 5a, step 1)

Critical step: Ensure that the whole surface of the well has been scraped to remove as many cells as possible

18. Transfer the cells from each well to a 15 ml centrifuge tube
19. Wash each well with 500 µl of cell recovery solution

    Optional step: The same 500 µl can be carried across to each of the wells at this step to minimize the volume of cell recovery solution required for the washes
20. Incubate the cells on ice at 4 °C for 30 minutes to fully dissolve the Matrigel
21. Centrifuge at 444 g for 4 minutes at room temperature [Troubleshooting]
22. Aspirate the supernatant
23. Optional: If appropriate, use this wash step to split the cell pellet in multiple fractions.

    Critical step: We recommend the splitting the pellet appropriately to allow the generation of a single plate of organoids from a single pellet fraction. This is important to avoid prolonged use of the Matrigel master mix in following steps. This can lead to Matrigel solidification and gravitation of larger cell clumps to the bottom of the mix, compromising uniform distribution of cells and seeding density.

24. Resuspend in WE+ media mixing well using the following formula to calculate the minimum volume of media required:

*Volume of media to add (ml) = number of pellets to be made after splitting*

Example: For a 1:4 split, resuspend in 4 ml of media

25. Aliquot the cell suspension into multiple 15 ml centrifuge tubes adding 1ml per tube (e.g. for a 1:4 split, share the 4 ml volume equally between four 15 ml centrifuge tubes)

Critical step: CO pellets can typically be split between 1:4-1:6, depending on the number of wells in the initial plate and their confluency (Fig. 5a). For reference, Fig. 5b, Image 4 depicts a representative pellet for plating 9 CO wells (approximately 4.0 x $10^5$ cells), obtained after a 1:4 split

26. Centrifuge at 444 g for 4 minutes at room temperature
27. Optional: CO pellets can be cryopreserved at this point if necessary by resuspending in 1 ml of CellBanker2, transferring to a 2 ml cryovial and freezing immediately at -80 °C.

Critical step: For long-term cryopreservation, COs should be stored in a liquid nitrogen cryobank. Cryopreserved COs can be stored short-term at -80 °C but long-term storage at this temperature will affect the viability and overall health of the line

28. Resuspend the pellet in a volume of 3X supplemented Williams E+ medium (supplemented with 1.5 µg/ml Rspondin, 150 ng/ml EGF, 30 µM (3 µl/ml) Y27632 and 300 ng/ml DKK-1) as described in Step 5. See the “preparation of a 3X supplemented William’s E+ solution” in Reagent Setup for instructions on how to prepare this solution and how to determine the appropriate volume to add.

Critical step: The number of plated wells of COs depends on the size of the resulting cell pellet. E.g. a pellet of approximately 4.0 x $10^5$ cells should be plated in 9 wells (Fig. 5b, Image 4).

29. Mechanically dissociate the pellet to break up the organoids, using a p200 pipette. This should typically be done around 30-50 times although the exact number will vary according to the starting size of the organoids. [Troubleshooting]

Critical step: Organoids must be dissociated into small clumps of approximately 10-20 cells to allow cysts to reform after splitting (Fig. 5b, Image 5). Mechanical dissociation should be done slowly to avoid damaging the cells

Critical step: Organoid breaking must be carried out with a p200 pipette, even if the volume of 3X William's E+ media required exceeds 200 µl. In that case, add the initial 200 µl and break the organoids as described in step 29 before adding the remaining volume of media.

30. Plate the organoids onto a pre-heated 24 well tissue culture plate as described in steps 3-10 [Troubleshooting]

Critical step: Organoids should reform from small clumps into organoids 24-48 hours hours after plating (Fig. 5b, Image 7(ii), enlarged Image in Supplementary Fig. 2, Image 2). Organoids should proliferate rapidly and should reach approximately 80% confluence 5 days after plating.

**Generation of densified collagen scaffolds**

Densified collagen scaffolds can be prepared to yield a sheet or tubular form. The sheet form produces a structure which has well a defined thickness and density, and yields highly reproducible scaffolds owing to the controlled nature of the process, while the collagen tube method is more technically challenging.

**Preparation of 5 mg/ml Collagen Gel**

**Timing: 10 minutes**

Critical step: A collagen gel is produced by first mixing a collagen gel precursor solution. This neutralizes the pH of the stock collagen solution, and raises the ionic content, which induces the collagen fibrils to form a gel. Here we produce two collagen sheets by preparing 2.5 ml of collagen precursor solution, which is prepared to a final collagen concentration of 5 mg/ml. 1 ml of this volume is sufficient to produce a collagen scaffolds of a reproducible thickness (approximately 750 µm), using the recommended specimen tubes. The quantities in brackets represent the volumes required for a final volume of 2.5 ml; enough for two collagen sheets.

Critical step: All equipment and reagents need to be sterilized before use and the following steps need to take place in a tissue culture hood under aseptic conditions.

31. Transfer 10x M199 at 10 %(vol/vol) final volume to a sterile 50 ml tube (0.25 ml).

32. Calculate the volume of stock collagen solution required to yield a final collagen concentration of 5 mg/ml (1.25 ml). This is calculated by:

$$\text{Volume stock collagen [ml]} = \frac{5\ [mg/mL]}{\text{stock collagen concentration}\ [mg/mL]} \times \text{precursor solution final volume [ml]}$$

33. Add sterile 1 M NaOH solution to the precursor solution at 2.5 % (vol/vol) the volume of stock collagen solution to be added (31 µl).

34. Add sterile 7.5 % (wt/vol) sodium bicarbonate solution to the precursor solution at 3 %(vol/vol) the final volume (75 µl).

Critical step: This volume makes up a constituent volume of the collagen gel precursor solution and so must be accurate or the concentration of collagen after gelation will vary.

35. Using a 1 ml syringe, transfer stock collagen solution, at pre-calculated volume, to precursor solution (1.25 ml). [Troubleshooting]

Critical step: Stock collagen solution will be viscous due to high concentration; use a syringe rather than a pipette to transfer the collagen accurately.

36. Shake the collagen precursor solution vigorously until of a uniform colour. [Troubleshooting]

37. Calculate the remaining volume of cell medium to be added to the precursor solution to reach the required final volume (0.894 ml) and transfer to Falcon tube.

38. Centrifuge collagen precursor solution at 200 g for 1 min at 4 ºC in order to remove air bubbles and return the liquid to the bottom of Falcon tube.

**39. Generation of densified collagen scaffolds**

Critical step: Collagen scaffolds can be generated either as flat sheets or as tubes (Fig. 1c). For fabrication of flat collagen sheets, see Step 39, Section A. For Fabrication of collagen tubes, see Step 39, section B

**Step 39, Section A: Fabrication of Densified Collagen Sheets**

**Timing: 1 hour 30 minutes- 2 hours**

Critical step: Larger-sized densified collagen sheets are possible with larger (flat-bottomed) containers (e.g. a 24 well plate). In order to produce collagen sheets of the same thickness, the collagen precursor solution should be poured into the container such that the height of the solution is 25 mm.

I. Using a 1 ml syringe and 18G needle, transfer the 5 mg/ml collagen precursor solution to a specimen tube until the height of the solution is 25 mm (Fig. 7b, Image 1). Repeat as necessary.

Critical step: Avoid inserting air bubbles into the mixture. Add collagen solution slowly.

II. Gel the collagen solution by placing the specimen tube in a 37 °C incubator for 30 min. [Troubleshooting]

Critical step: To maintain sterility transfer the specimen tube within an upside down 15 ml falcon tube

III. Tightly roll three sheets of absorbent paper towels into a cylinder, roughly 5 mm in diameter and secure with autoclave tape.

IV. Using scissors, cut the paper cylinder to 25 mm in length. Flatten end by pushing cylinder against a sterile surface (i.e. a 10cm plate). Autoclave both paper cylinder and nylon membrane.

Critical step: The paper cylinder must have a flat edge in order to suitably contact and thus dry the collagen gel evenly.

V. Using scissors, cut a piece of nylon membrane into a round piece of a slightly lower diameter than the specimen tube.

VI. Return the specimen tube to the tissue culture hood. Carefully place the nylon membrane on top of the gel and then place the paper towel wadding into the specimen tube, on top of the membrane (Fig. 7b, Image 2(i)).

VII. Apply very light finger pressure on the top of the wadding so as to make good contact with the gel and then leave to densify for approximately 1 hour (Fig. 7b, Images 1-3(i)). [Troubleshooting]

VIII. Monitor the densification process every 5-10 minutes to ensure the wadding is in contact with the gel and occasionally apply light pressure to keep the wadding in place. [Troubleshooting]

Critical step: Applying too much pressure will rip the top surface of the gel.

IX. The densification is terminated when the paper towel has almost reached the bottom of the specimen tube and water cannot be removed any further (Fig. 7b, Image 3(ii)). Once this has occurred, remove the paper wadding; the collagen gel and nylon membrane should be adhered to the wadding. [Troubleshooting]

X. Using tweezers, carefully grip the edge of the collagen sheet and peel it away from the paper wadding.

XI. The nylon membrane is likely to remain attached to the collagen sheet. By gripping the nylon sheet with tweezers, this can also be peeled away from the collagen sheet (Fig. 7b, Image 4) [Troubleshooting]

Critical step: Avoid ripping the collagen sheet by gently peeling it away from the nylon membrane.

XII. Transfer 4 ml of William's E+ media or PBS to a 6 well plate, and then transfer the densified collagen sheet to the 6 well plate (Fig. 7b, Image 5). For larger collagen sheet preparations, transfer to an appropriately-sized container.

XIII. For storage, place the plate containing the densified collagen sheet at 4 °C. Warm to 37 °C prior to cell seeding, by placing it in the incubator.

XIV. Cells can be seeded onto the surface of the collagen sheet at this point and cultured for several months.

**Step 39, Section B: Fabrication of Densified Collagen Tubes**

**Timings: 5 – 25 hours**

I. Using a 1 mL pipette, transfer the precursor solution to the densification chamber until the chamber is full (approximately 1.5 mL) (Fig. 8c, Image 1).

Critical step: Avoid inserting air bubbles into the mixture. Add collagen solution slowly.

II. Gel the collagen solution by placing the densification chamber in a 37 °C incubator for 30-60 minutes. [Troubleshooting]

III. In order to form a collagen tube, it is necessary to dislodge the collagen gel from the top of the funnel to encourage the gel surface to drop. Thus, using a sterile pair of tweezers under

a sterile hood, gently peel the collagen gel away from the walls of the chamber (Fig. 8c, Image 2).

IV. Loosen the screws attaching the base to the funnel.

V. Return the densification chamber to the incubator. Monitor the chamber regularly for 30 min until the level of the collagen gel drops to the top of the metallic wire (Fig. 8c, Image 3).

Critical step: The top end of the metallic wire, embedded in the collagen, will prevent the gel surface dropping further. Water will continue to be removed through evaporation around the sides of the funnel to yield a tubular structure. [Troubleshooting]

Critical step: There should be a visible gap between the collagen gel and the edges of the funnel.

VI. Monitor the densification chamber in the incubator for a further 4-24 hours to allow for evaporation of the water phase of the gel (Fig. 8b, panel 4).

Critical step: It is important to monitor the collagen gel and prevent the tube from over-drying, based on the humidity conditions of the incubator.

VII. Continue the process until the vast majority of water has evaporated. The collagen scaffold should consist of a dense and thin cylindrical component around the wire core, with a larger region near the top of the funnel. (Fig. 8c, Image 4). [Troubleshooting]

VIII. Transfer 4 mL of William's E+ media to a 6 well plate.

Taking a sterile pair of tweezers, grip the top of the collagen tube and slowly pull it upwards over the end of the metallic wire (Fig. 8c, Image 5). [Troubleshooting]

Critical step: Be careful not to damage the collagen tube with the tweezers or the end of the metallic wire.

IX. Place collagen tube in 6 well plate for storage. The pink colouration will disappear from the collagen tube over several hours (Fig. 8d, Images 1 and 2).

X. Prior to surgical implantation, it is necessary to trim away the excess collagen sheet and cut a suitable length of tube for the experiment. Place the tube under a dissecting microscope and, using a surgical scalpel, cleanly trim any excess collagen (Fig. 8d, Image 3).

XI. Determine a suitable length of collagen tube along the scaffold. Pick a region which is cylindrical in nature (likely the middle section). Using a surgical scalpel, cleanly cut across the tube to yield the required length for the particular application. (Fig. 8d, Image 4).

Critical step: Trimming the tube is a particularly difficult step, likely requiring the assistance of an experienced surgeon. Be extremely careful not to cut into the lumen of the tube. Doing so will result in leakages when the tube is perfused.

XII. Observe patent lumen under phase contrast using inverted microscope (Fig. 8d, Image 5)

XIII. Transfer the densified collagen tube to the 6 well plate.

XIV. Store the collagen tube in William's E+ medium in the incubator until ready. The surface of the tubes can be further seeded with cells at this point.

**40. Seeding COs onto scaffolds**

Critical step: The use of an optimal CO line is essential for scaffold seeding. Suboptimal lines will result in reduced cell attachment, proliferation and long-term viability. All CO lines should express the biliary markers CK19, CK7, Sox9 and GGT (Fig. 6a and 6b) and display ALP and GGT activity (Fig. 6c and 6d). Healthy CO lines should require passaging every five days (Fig. 5b, Image 1). CO lines that fail to meet these criteria should not be considered for scaffold seeding. See Troubleshooting for steps 10, 12, 29 and 30 for guidance on how to improve the quality of a suboptimal CO line.

Critical step: For seeding COs onto densified collagen sheets, see Step 40, Section A. For seeding COs onto densified collagen tubes, see Step 40, Section B. For seeding COs onto PGA scaffolds, see Step 40, Section C (Fig. 1d).

**Step 40, Section A: Seeding on densified collagen sheets**

**Timing: 2 hours 30 minutes - 4 hours**

Critical step: Collagen scaffolds must be of an optimal quality to be used for seeding. Batch test the stock collagen solution used for collagen densification for cell attachment and proliferation and ensure that the densification process has completed fully (see Troubleshooting for Step 39, Section A) and the scaffold has not dried out during storage to ensure the quality of the collagen scaffolds

Critical step: For long term storage, the collagen scaffolds are maintained in PBS to prevent them from drying out.

I. Remove the PBS and place the collagen scaffold in a 24 well tissue culture plate, then place it in the incubator for 30 minutes to 1 hour, or until the PBS is evaporated and residues on the surface are no longer visible.

Critical step: The excess presence of liquid on the scaffold's surface may prevent cell attachment

Critical step: Allowing the scaffold to dry for a prolonged period following evaporation of the PBS may affect the collagen fibre micro-architecture.

II. Remove the organoids from the Matrigel domes using cell recovery solution and incubate on ice for 30 minutes as described in steps 16-26. An optimal seeding density of $1.5x10^6$ cells/$cm^2$ has been observed to give confluent scaffolds over a short period of time; the number of wells required for a certain surface area can be calculated with the given formula:

III.

$$no.of\ cells = scaffold\ surface\ (cm^2) \times\ 1.5 \times 10^6 cells/cm^2$$

IV. Aspirate the supernatant and resuspend the pellet in an adequate volume of William's E+ media supplemented with 500 ng/ml Rspondin, 50 ng/ml EGF, 100 ng/ml DKK-1 and 10 µM Y27632.

Critical step: For the seeding solution a cell density of 5 x $10^4$ cells/µl is recommended. E.g. a pellet of $1x10^6$ cells, equivalent to approximately 10 confluent wells, is resuspended in a volume of 20 µL. Once seeded on a scaffold surface of 0.65 $cm^2$ this should allow to reach a confluent layer of cells in approximately 2 weeks' time, with slight variations depending on the organoid line.

V. Gently pipette up and down with a p20 pipette 30-40 times to mechanically dissociate the organoids in small clumps (approximately 10-20 cells per clump) and obtain a homogeneous solution. [Troubleshooting]

VI. Seed the cells by directly pipetting this solution onto the scaffold's surface with a p10 pipette (Fig. 9b, Image 3(i) and 3(ii)) [Troubleshooting]

Critical step: In order to achieve a homogeneous attachment, seeding multiple aliquot of 5 µl in different positions all over the scaffold surface is recommended.

VII. After seeding on collagen scaffolds, keep the plate in a 37 °C incubator for 1-2 hours to allow the cells to attach to the scaffold

Critical step: monitor the scaffolds every 30 minutes to avoid drying the cells out [Troubleshooting]

VIII. Prepare 2 ml of William's E+ media supplemented with 500 ng/ml Rspondin, 50 ng/ml EGF, 100 ng/ml DKK-1 and 10 µM Y27632 for each scaffold

IX. To add the media, tilt the plate and slowly add from the bottom of the well (Fig. 9b, Image 5) [Troubleshooting]

Critical step: Media must be added as slowly as possible, using a P1000 pipette. Care must be taken to avoid disrupting the scaffold when adding media

X. The seeded scaffold should be kept in culture for 4 days before the first media change, in order to maximize cell attachment. After this period, 500 µl of William's E+ media supplemented with 500 ng/ml Rspondin, 50 ng/ml EGF and 100 ng/ml DKK-1 are changed every other day. [Troubleshooting]

Critical step: When changing the media, all previous media must first be aspirated. Care must be taken to avoid disrupting the scaffold during media aspiration

Critical step: COs will start growing and expanding on the scaffold after a first lag phase (lasting approximately 4-5 days after seeding), then confluency should be reached in approximately 2 weeks, according to the age of the cell line.

**Step 40, Section B: Seeding onto the lumen of densified collagen tubes**

**Timings: 2 hours 30 minutes- 3 hours 30 minutes**

I. Working under aseptic conditions, transfer the densified collagen tube onto a dry 10 cm plate

II. leave to dry under a tissue culture hood for approximately half an hour, or until almost all the residual PBS has evaporated

III. Passage the organoids as described in steps 16-26.

Critical step: Preparation of the CO cell suspension (steps III-IV) can be done during the waiting step described in step II

IV. Resuspend the cells in 25 µl of William's E+ media with 50 ng/ml EGF and 10 µM Y27632

V. Using a p20 pipette, carefully break the COs into small clumps (10 – 20 cells) [Troubleshooting]

Critical step: failure to break the COs into small enough clumps (Supplementary Fig. 2, Image 2) will result in the cells failing to form a confluent monolayer on the luminal surface of the tube

VI. Using a 34 G Hamilton removable needle and a pair of fine forceps, cannulate the tube with the 34 G needle. For best results, this should be done under a dissecting microscope (Fig. 10b, Image 2)

Critical step: Cannulation of the tube should ideally be performed by an experienced surgeon. Improper attempts at cannulation can result in disruption of the construct wall beyond repair.

VII. Using a p200 pipette, transfer the cell suspension into a 25 µl or 50 µl Hamilton syringe

Critical step: it is highly advisable to use Hamilton syringes, as recommended in the equipment list, or some other model of small volume syringes with removable needles and no dead space. This is due to the very low volume of cell suspension required for seeding, which is smaller than the dead space in standard needles

VIII. Slowly depress the plunger on the syringe to deposit the cell suspension in the lumen of the tube while simultaneously removing the needle from the tube in order to distribute the cells evenly along the tube lumen (Fig. 10b, Image 3) [Troubleshooting]

IX. Incubate the freshly seeded tube in a covered 10 cm plate at 37 °C without media for up to an hour to allow cell attachment [Troubleshooting]

X. Transfer the tube to a 6 well plate

XI. Prepare 2 ml of William's E+ media supplemented with 500 ng/ml Rspondin, 50 ng/ml EGF, 100 ng/ml DKK-1 and 10 µM Y27632

XII. Using a P1000 pipette, tilt the plate and add the media slowly to the side of the well, until the tube is covered [Troubleshooting]

Critical step: Media must be added as slowly and carefully as possible, to avoid disruption of the newly attached cells. Care must be taken to avoid touching the tube with the pipette

XIII. The seeded tube should be kept in culture for 4 days before the first media change, in order to maximize cells attachment. After this period, 2 ml of William's E+ media supplemented with 500 ng/ml Rspondin, 50 ng/ml EGF and 100 ng/ml DKK-1 are changed every 4 days in order to reduce the disruption caused by media change. [Troubleshooting]

Critical step: When changing the media, all previous media must first be aspirated. Care must be taken to avoid disrupting the tube during media aspiration.

Critical step: The construct is kept in culture and a confluent layer of cells should be reached in approximately 4 weeks' time, depending on the age of the cell line (Fig. 10c and 10d).

**Step 40, Section C: Seeding on PGA scaffolds**

**Timings: 3 hours 30 minutes - 4 hours**

I. PGA scaffolds are available commercially (see Materials list) and can be stored until needed.

II. Place the PGA scaffold, with a thickness of 1 mm and density of 50 mg/cm$^3$, in 1 M NaOH for 10-30 seconds

III. Sterilize the scaffold by immersion in 70% ethanol for 30 minutes.

IV. Air-dry the PGA scaffold in a 6 well tissue culture plate under a sterile tissue culture hood for a further 30 minutes (Fig. 9c, Image 1).

Critical step: Ensure that all traces of NaOH and ethanol are gone from the scaffold before beginning seeding. Improper drying can lead to cell death and failure to attach to the scaffold

V. Remove the COs from organoid culture and prepare a suspension of small clumps as described in step 40, Section A II-V [Troubleshooting]

Critical step: Given the pores size of the electrospun PGA it is important that the CO clumps are no smaller than 40-60 cells per clump, as small cell clumps will fall through the pores of the scaffold and not be retained.

VI. Gently pipette up and down with a p20 pipette 10-20 times in order to have a homogeneous cells suspension of with CO clumps of approximately 40-60 cells per clump.

VII. Seed the cells onto the PGA scaffold as described in step 40, section A VI-X (Fig. 9c, Images 3(i) and 3(ii)). [Troubleshooting]

**41. Characterisation of cholangiocyte organoids**

41. For analysis of COs through immunofluorescence, see Step 41, Section A. For analysis through flow cytometry, see Step 41 Section B. For RNA extraction from COs for qPCR, see Step 41, Section C.

**Step 41, Section A: Immunofluorescence**

I. Day 1: Aspirate William's E+ culture medium

II. Add 1 ml of 4 % PFA (vol/vol) per well

Critical step: PFA should be added gently to the side of the well to not disrupt the Matrigel dome

III. Incubate at 4 °C for 20 minutes to fix the cells [Troubleshooting]

IV. Aspirate the PFA

Critical step: PFA should be aspirated with a p1000 pipette to avoid disruption to the Matrigel dome

V. Wash twice in PBS. Each wash should take 10 minutes

Pause Point: The CO plate can be sealed and kept at 4 °C for up to four weeks

VI. Prepare a solution of 10% (vol/vol) donkey serum and 0.1% (vol/vol) TritonX-100 in PBS. [Troubleshooting]

VII. Add 1 ml of this solution to each organoid well and incubate at room temperature for one hour to block and permeabilise the COs [Troubleshooting]

VIII. Dilute the primary antibodies in a solution of 1% (vol/vol) donkey serum and 0.1% (vol/vol) TritonX-100 in PBS.

IX. Add 500 µl of primary antibody solution per CO well

X. Stain the COs overnight at 4 °C

XI. Day 2: wash the COs three times with 1% (vol/vol) donkey serum and 0.1% (vol/vol) TritonX-100 in PBS. Each wash should take 45 minutes

XII. Dilute the secondary antibodies in a solution of 1% (vol/vol) donkey serum and 0.1% (vol/vol) TritonX-100 in PBS.

XIII. Add 500 µl of secondary antibody solution per CO well

XIV. Stain the COs overnight at 4 °C [Troubleshooting]

XV. Critical step: CO plates should be wrapped in foil to prevent exposure of the secondary antibody to light

XVI. Day 3: aspirate the secondary antibody solution

XVII. Prepare a solution of Hoechst 33258 1:10,000 (vol/vol) in PBS

XVIII. Incubate the COs in this Hoecscht 33258 solution for 10 minutes at room temperature [Troubleshooting]

XIX. Aspirate the Hoecscht 33258 solution

XX. Wash the COs three times with PBS. Each wash should take 45 minutes

XXI. Add a final 1 ml of PBS per CO well and Image the COs immediately or store the plate at 4 °C until ready for analysis

XXII. Image using a confocal microscope. All IF images (Figs. 6a and Fig. 11a, 11c and 11e) were acquired using a Zeiss LSM 700 confocal microscope. Imagej 1.51h software (Wayne Rasband, NIHR, USA, http://Imagej.nih.gov/ij) was used for Image processing such as merging of different channels.

**Step 41, Section B: Flow Cytometry**

**Timings: 3-5 hours**

**Preparation of a single-cell suspension**

I. Passage organoids as described in steps 16-24

II. Resuspend the pellet in 1 ml of William's E+ media and centrifuge at 444g for 4 minutes

III. Prepare a solution of Accutase (pre-warmed to 37 °C) with 4 mg/ml of DNase I and 10 µM Y27632

Critical step: Omission of DNase I and Y27632 can lead to cell clumping and greatly reduce viability

IV. Aspirate the supernatant and resuspend the pellet in 1 ml of Accutase solution

V. Incubate the cells at 37 °C for up to five minutes to produce a single-cell suspension [Troubleshooting]

Critical step: Examine the cells under a microscope halfway through to check the progress of the dissociation

VI. Add 1 ml of William's E+ media or PBS 1% BSA (wt/vol) with 4 mg/ml of DNase I and 10 μM Y27632 to the cell suspension

VII. Centrifuge the cells at 444 g for 4 minutes at room temperature

VIII. Resuspend the pellet in 1 ml of William's E+ media or PBS 1% BSA (wt/vol) with 4 mg/ml of DNase I and 10 μM Y27632

IX. Filter through a 40 μm filter [Troubleshooting]

Critical step: If performing flow cytometry on a live cell population, skip steps X-XV and go directly to steps XVI-XXIX (staining of a single cell suspension for flow cytometry)

X. Centrifuge the cells at 444 g for 4 minutes at room temperature

XI. Resuspend the pellet in 1 ml of 4% PFA (vol/vol)

XII. Incubate the cells at 4 °C for 15 minutes to fix the cells

XIII. Add 1 ml of PBS 1% BSA (wt/vol)

XIV. Centrifuge the cells at 444 g for 4 minutes at room temperature

XV. Resuspend the pellet in 1 ml of PBS 1% BSA (wt/vol)

Critical step: Cells should be handled very carefully after fixation to avoid damage. Pellets should be resuspended by flicking the centrifuge tube (as opposed to pipetting) and all pipetting should be done slowly

**Staining a single cell suspension for flow cytometry**

XVI. If staining for cell-surface markers only, prepare a 1 in 20 dilution of FcR block in PBS 1% BSA (wt/vol)

XVII. If staining for intracellular markers, prepare a 1 in 20 dilution of FcR block in PBS 1% BSA (wt/vol) with 0.1% Triton X (vol/vol)

XVIII. Centrifuge the cells at 444 g for 4 minutes at room temperature and resuspend the pellet in 200 μl ml of diluted FcR block

XIX. Incubate at room temperature for 30 minutes

Critical step: An aliquot of the cell suspension (at least 1 x $10^5$ cells) should be used as an unstained control. If using separate primary and secondary antibodies, a further aliquot must be used for a secondary-only control. Both aliquots should be kept at 4 °C until required

XX. Prepare a master mix of all antibodies (if using conjugated antibodies) or all primary antibodies (if using separate primary and secondary antibodies) in PBS 1% BSA (wt/vol)

XXI. Centrifuge the cells at 444 g for 4 minutes at room temperature

XXII. Resuspend the pellet in 200 µl of master mix solution

XXIII. If using conjugated antibodies, incubate at 4 °C for half an hour. If using primary antibodies, incubate at room temperature for an hour

XXIV. If using conjugated antibodies, wash the cells three times in PBS 1% BSA (wt/vol) and filter through a 40 µm filter before analysing the sample on the flow cytometer

XXV. If using unconjugated antibodies, wash the cells three times in PBS 1% BSA (wt/vol). Each wash should take 5 minutes

XXVI. Prepare a solution of all secondary antibodies in PBS 1% BSA (wt/vol)

XXVII. Resuspend the cells in 200 µl of secondary antibody solution and incubate at room temperature for one hour.

Critical step: The secondary-only control should also be stained at this point

Critical step: The cells should be kept in the dark to prevent exposure of the secondary antibody to light

XXVIII. Wash the cells three times in PBS 1% BSA (wt/vol). Each wash should take 5 minutes

XXIX. Resuspend the cells in 200 µl of PBS 1% BSA (wt/vol) and filter through a 40 µm filter before analysing the sample on the flow cytometer. All flow cytometric analyses were performed on a FACS Cyan flow cytometer and analysed using FlowJo version 10.4.2.

**Step 41, Section C: RNA extraction**

**Timings: 2 hours**

I. If isolating RNA from an established CO line, first remove COs from organoid culture as described in steps 16-25. If isolating RNA from a suspension of primary cells, go straight to step II

II. Centrifuge the cells at 444 g for 4 minutes

III. Aspirate the supernatant and resuspend the pellet in 350 µl of RNA lysis buffer.

Critical step: Ensure the lysed cell suspension is fully homogenised

IV. Transfer the lysed cell suspension into a pre-labelled 1.5 ml Eppendorf tube and store the tube immediately at -80 °C [Troubleshooting]

Critical step: Delay in transferring the lysed sample to -80 °C storage can result in degradation of RNA quality

Pause Point: Samples in RNA lysis buffer can be stored at -80 °C for > 1 year until needed

V. Thaw samples on ice

VI. Use the Sigma Aldrich "GenElute™ Mammalian Total RNA Miniprep Kit" to extract RNA from the samples according to the manufacturer's instructions

## Timings

These timings are estimated based on the approximate time required for a researcher with experience in this protocol to complete each of the steps. When attempting this protocol for the first time, researchers may find that aspects of the procedure take longer to complete.

**Step 1, tissue collection: 1-8 hours**

**Step 2 section A, derivation of extrahepatic cholangiocyte organoids from deceased organ donors: 1-2 hours (**Washing, dissecting and scraping the tissue (steps I-VIII): 20-30 minutes**; w**ash steps and optional red blood cell lysis (steps IX-XII): 20-40 minutes**; o**rganoid plating (steps XIII-XX): 40 minutes)

**Step 2 section B, derivation of extrahepatic cholangiocyte organoids through Endoscopic retrograde cholangiopancreatography (ERCP) brushings: 30 minutes**

**Step 2 section C, derivation of intrahepatic cholangiocyte organoids from liver tissue: 30-40 minutes** (Tissue dissection (steps I-II): 10-20 minutes; washing and plating preparation (steps III-VI): 10-15 minutes; plating (steps VII-VIII): 10 minutes)

**Step 2 section D, derivation of intrahepatic cholangiocyte organoids through EpCAM+ MACS sorting: 2-3 hours** (Preparing the tissue for digestion (steps I-III): 10 minutes; digestion and filtering (steps IV-VII): approximately 1 hour; red cell lysis, washing and cell counting (steps VIII-XVI): 20-30 minutes; MACS sorting and plating (steps XVII-XXVII): approximately 1 hour)

**Steps 3-10, plating of primary cholangiocytes in organoid format: 10-40 minutes**

**Steps 11-14, changing media for CO lines for maintenance: 20 minutes**

**Steps 15-30, passaging of CO lines for maintenance: 90 minutes** (Removing organoids from Matrigel (steps 15-21): 40 minutes; washing and splitting the pellet (steps 22-26): 10 minutes; plating the cells (steps 28-30): 45 minutes)

**Steps 31-38, preparation of 5 mg/ml collagen gel: 20 minutes**

**Section 39A: Fabrication of Densified Collagen Sheets: 1 hour 30 minutes-2 hours (**Preparation of the densification chamber (steps I-VI): approximately 45 minutes; collagen densification (steps VII-IX): 1 hour; **r**emoval and storage of the densified collagen scaffold (steps X-XIV): approximately 10 minutes)

**Section 39B: Fabrication of Densified Collagen Tubes: 5-27 hours (**Loading chamber with collagen and gelation (steps I-II): 40 minutes-1 hour; collagen densification (steps III-VII): 4-24 hours; removal and storage of the densified collagen tube (steps VIII-XIV): approximately 30 minutes)

**Section 40A: Seeding on flat densified collagen scaffolds: 2 hours 30 minutes-4 hours (d**rying of the collagen scaffold (step I): 30 minutes-1 hour; preparation of the COs for seeding (steps II-V): 45 minutes**; s**caffold seeding (step V): 15 minutes**;** waiting step before media addition (step VII): 1-2 hours; media addition (steps VII-IX): 5 minutes)

**Section 40B: Seeding on densified collagen tubes: 2 hours 30 minutes-3 hours 30 minutes** (drying of collagen tube (steps I-II): 30 minutes-1 hour; preparation of the COs for seeding (steps III-

V): 45 minutes; tube cannulation and cell seeding (steps VI- VII): 30 minutes; cell attachment (Step IX): 30 minutes- 1 hour; Addition of media (steps IX- XII): 15 minutes

**Section 40 C: Seeding on PGA scaffolds: 3 hours 30 minutes - 4 hours (**Preparation of the PGA scaffold (steps I-III): Approximately 1 hour**; p**reparation of CO suspension (steps IV-V): 40 minutes; seeding of the PGA scaffold (step VI): 15 minutes**; w**aiting step before media addition (step VI): 1-2 hours; media addition (step VI): 5-10 minutes)

**Section 41 A: Immunofluorescence: 3 days** (Organoid fixation (steps I-V): 40 minutes; blocking and primary antibody addition (steps VI-IX): approximately 90 minutes; primary antibody staining (step X): overnight; washing and secondary antibody addition (steps XI-XIII): approximately 2 hours and 30 minutes; secondary antibody staining (steps XIV-XV): overnight; washing and nuclear staining (steps XVI-XXI): approximately 2 hours and 40 minutes)

**Section 41 B: Flow cytometry: 3 hours – 5 hours** (Preparation of a single-cell suspension (steps I-X): 60 minutes; fixation (steps XI-XV): 30 minutes; blocking (steps XVI-XIX): 45 minutes; staining with conjugated antibodies (steps XX- XXIV): 45 minutes**; s**taining with separate primary and secondary antibodies (steps XXV- XXIX): 2 hours 30 minutes)

**Section 41 C: RNA extraction: 2 hours (c**ell lifting (steps I-II): 40 minutes**;** RNA lysis (steps III-IV): 10 minutes**;** RNA extraction (steps V-VI): 1 hour)

# TROUBLESHOOTING

| Step | Problem | Explanation | Solution |
|---|---|---|---|
| Step 2, Section A III | Very few or no cells are collected after mechanical dissociation | Cell detachment during PBS washes | 1. Reduce the number of washes<br><br>2. If the tissue has been in cold storage solution for longer than 2-4 hours, consider omitting the wash |

| | | | |
|---|---|---|---|
| | | | step. Aspirate any excess bile with a p1000 pipette instead<br><br>3. Centrifuge the PBS to collect the cells detached during the washes |
| Step 2, Section A IX, XII | Pellet contains too much debris | Forceful scraping of the tissue can mechanically dissociate part of the fibrous tissue as well as biliary epithelia | 1. Scrape the tissue very gently and avoid scraping the same area of tissue more than twice<br><br>2. Where possible, remove large debris with a p1000 pipette before centrifugation |
| Step 2, Section A X | Cells do not form a pellet in step X after mechanical dissociation | 1. Inadequate wash<br><br>2. Remnants of bile and/or debris in the cell suspension | 1. Increase the number of PBS washes prior to mechanical dissociation<br><br>2. Ensure that the tissue is adequately washed before starting mechanical dissociation<br><br>2. Remove large pieces of debris using a p1000 pipette before transferring the cells to a 50 ml centrifuge tube |
| Step 2, Section A X | Cells are lost during the wash steps | Washing was done in PBS or media with inadequate protein content | Use only complete Williams E+ media during the wash steps or PBS supplemented with 1%BSA (wt/vol) |
| Step 2, Section A XVIII | Organoids do not form following plating | Poor cell viability due to: | 1. Dissect the tissue as quickly as possible and submerge in media as soon as dissection is complete |

| | | | |
|---|---|---|---|
| Step 2, Section B V | | 1. Tissue drying during the dissection steps<br><br>2. Stress of mechanical dissociation<br><br>3. Prolonged cold storage<br><br>4. Lack of necessary cytokines in the media<br><br>5. Poor quality of tissue culture media and reagents (Matrigel or cytokines) | 2. Avoid vigorous scraping of the tissue resulting in cell death<br><br>2. Avoid vigorous washes and pipetting resulting in increased cell stress and/or death<br><br>3. Process the tissue as soon as possible after surgical excision<br><br>4. Ensure that the media contains 150 ng/ml of EGF and 10 µM Y27632 at every stage<br><br>5. Batch test Matrigel and media components<br><br>Always store Matrigel and cytokines at -80 °C<br><br>Avoid repeat freeze-thaw cycles of cytokines and do not use Matrigel that has undergone more than two freeze-thaw cycles |
| Step 2, Section C VIII | Organoids do not form once the tissue is plated | Poor cell viability due to:<br>1. Tissue drying during the dissection steps | 1. Complete the dissection step as quickly as possible |

| | | | |
|---|---|---|---|
| Step 2, Section D XXVII | | 2. Stress of mechanical dissociation<br><br>3. Prolonged cold storage<br><br>4. Lack of necessary cytokines in the media<br><br>5. Poor quality of tissue culture media and reagents (Matrigel or cytokines) | 2. Ensure the dissected liver pieces are as small as possible so they can fit into the tip of a p1000 pipette without difficulty<br><br>3. Process the tissue as soon as possible after surgical excision<br><br>4. Ensure that the media contains 150 ng/ml of EGF and 10 μM Y27632 at every stage<br><br>5. Batch test Matrigel and media components |
| Step 2, Section D V | Poor viability of cells before EpCAM+ sorting | 1. Prolonged enzymatic dissociation<br><br>2. Vigorous pipetting to dissociate cell clumps resulting in cell death<br><br>3. Prolonged cold storage | 1. Examine the cells every ten minutes during the dissociation. Ensure the dissociation is stopped as soon as a single cell suspension is obtained and the majority of liver cells have been released from the extracellular matrix (Supplementary Fig. 2b, Image 4)<br><br>2. Dissociate cell clumps gently. Prolong enzymatic digestion if vigorous pipetting is required |

| | | | |
|---|---|---|---|
| | | | 3. Process the tissue as soon as possible after surgical excision |
| Step 2, Section D V, VII | Cells clumping during or after dissociation to a single cell suspension | DNA fragments in the suspension originating from non-viable cells cause cholangiocytes to clump | 1. Monitor the cells closely during dissociation. If cells show signs of clumping, increase the concentration of DNase I by 30%<br><br>2. Following filtering the cells should be resuspended in PBS containing 1% BSA (wt/vol) supplemented with 4 mg/ml of DNase I and 10 µM Y27632 |
| Step 2, Section D XV | Significant loss of cells after single cell dissociation | Cell adhesion to the surfaces of the centrifuge tube during centrifugation or MACS sorting | 1. Resuspend cells in sterile PBS 1% BSA (wt/vol) with 4 mg/ml of DNase I and 10 µM Y27632<br><br>2. Prime centrifuge tubes with PBS 1% BSA (wt/vol) before use |
| Step 2, Section D XXIV | Blockages during the MACS sorting | Cell clumps remain in the cell suspension | Filter the sample immediately before running on the MACS cell sorter |
| 6 | Matrigel does not form a dome but attaches to the side of the well during plating | Remnants of solidified Matrigel blocking the pipette tip and preventing uniform dispensation of the gel | Change pipette tip |
| 8<br><br>30 | Cells attach to the bottom of the plate | 1. Delay in inverting the plate after plating of the cells | 1. Ensure the plates are adequately pre-warmed |

| | | | |
|---|---|---|---|
| | | 2. Delay in Matrigel solidification due to low plate starting temperature allowing cell clumps to gravitate to the bottom of the plate | 2. Ensure plating occurs on a plate heater to allow the Matrigel to solidify quickly as possible<br><br>3. Keep the plates inverted for 30 minutes in a 37 °C incubator |
| 10<br><br>11 | Cells form small spheres lacking a lumen after plating | Overly dense initial plating, preventing organoid expansion | 1. Passage the cells earlier than 5 days and plate at a reduced density<br><br>2. Add 2 μM FSK to the media |
| 21 | Matrigel remnants are present in the pellet following the incubation with cell recovery solution | Incubation time was too short | Ensure the cells have been incubated for the full 30 minutes in cell recovery solution and that the volume of ice is adequate to keep the cells at 4 °C |
| 10<br><br>11<br><br>29 | Cells demonstrate signs of stress such as thickening of organoid walls, organoid collapse, and reduced organoid proliferation.<br>Cell death is observed for several days after passaging/consist | 1. Events during passaging could be causing stress to the cells:<br>a. Dissociating the organoids too vigorously during step 29<br>b. Prolonged duration of passaging, stressing the cells by delaying return to optimal culture conditions<br><br>2. Suboptimal batch of Matrigel | 1a. Avoid vigorous dissociation of during passaging<br><br>1b. Minimize delays during passaging<br><br>2. Batch-test new lots of Matrigel<br><br>3. Store all stock solutions at 4 °C and do not use stock solutions made more than 3 months previously Where applicable, do not use stock |

| | | | |
|---|---|---|---|
| | ently over several passages | 3. Suboptimal quality of the cytokines media components used<br><br>4. Mycoplasma contamination<br><br>5. Delay in passaging the cells (Supplementary Fig. 4b)<br><br>6. Overly dense plating preventing organoid proliferation (Supplementary Fig. 4b) | solutions beyond their expiry date. Store all cytokines at -80 °C<br><br>4. Test organoid cultures for Mycoplasma contamination<br><br>5. Passage COs when 80% confluent with no signs of organoid collapse, typically every five days. Plates should be observed daily to determine the optimum time for passaging<br><br>6. Passage the cells earlier than the typical five days and ensure the cells are plated more sparsely next passage<br>7. Increase the working concentration of EGF used during maintenance to up to 100 ng/ml<br><br>8. Add FSK at 2 µM working concentration (0.2 µl/ml of a 10 mM stock)<br><br>9. Add HGF at 50 ng/ml working concentration |
| 35 | Stock collagen solution arrives highly viscous | If the stock collagen solution is stored at temperatures below 2 °C, | Warm the stock collagen solution to room temperature until the collagen |

| | | | |
|---|---|---|---|
| | | it can appear to have gelled | solution is liquid. Once liquid, store at 4 °C |
| 36<br><br>Step 39, Section A II | Collagen precursor solution solidifies in the mixing tube. | 1. Solution mixing occurs too slowly<br><br>2. The room temperature is too high. | 1. Before starting cool down all the reagents to 4 °C<br><br>2. Mix the solution in an ice bath |
| Step 39, Section A VII, VIII | Collagen gel densification stops prematurely | 1. The absorbent paper wadding has poor contact with the collagen gel and so cannot remove water effectively<br><br>2. As the paper wadding absorbs water, it expands and gets wedged in specimen tube, preventing further densification | 1. Remove the paper wadding from the specimen tube and flatten the end in contact with the collagen gel Once sufficiently flat, return to densification chamber<br><br>2. Gently remove the paper wadding from the specimen tube and replace with a fresh roll, with a slightly reduced diameter |
| Step 39, Section A IX | Following densification steps and removal of paper wadding, specimen tube is empty | Collagen solution has been prematurely absorbed into the paper wadding before gelation | 1. Repeat process and extend time for collagen gelation from 30 min to 1 h<br><br>2. Test gelation by carefully pressing the metal spatula against the top surface. Only add paper wadding once sure that collagen has gelled |
| Step 39, Section A XI | Collagen sheet not attached to paper wadding on its final removal | After the removal of the wadding, the collagen sheet remains at the | The scaffold can be removed carefully using a pair of tweezers or by flushing some PBS into the specimen tube |

| | | | |
|---|---|---|---|
| | | bottom of the specimen tube | |
| Step 39, Section A XI | Once removed from the densification chamber the lower part of the scaffold retains a gel-like structure | Densification has been stopped prematurely, causing the lower part of the collagen sheet to retain a higher water content | Return the scaffold to the densification chamber and continue the densification process with fresh paper wadding |
| Step 39, Section B II | Prior to collagen gelation, the level of the collagen solution has dropped. | The collagen precursor solution is leaking out of the bottom of the funnel of the densification chamber. | 1. Keeping the chamber upright, carefully tighten the screws such that the base is firmly attached the funnel.<br><br>2. Transfer an extra volume of collagen precursor solution until funnel is once again full. |
| Step 39, Section B II | The level of the collagen gel has dropped below the top of the metallic wire. | The volume of collagen above the wire was unable to stop the collagen gel from dropping below the wire. | 1. Using scissors, remove a couple of millimetres of length from the top of the metallic wire.<br><br>2. Repeat collagen gelling process. |
| Step 39, Section B V | The level of the collagen gel has not dropped to the top of the metallic wire. | The paper towels at the base are not in sufficient contact with the gel and so water is not being drawn out. | Repeat the process. When loosening the screws, do not displace the base away from the funnel, thereby keeping the paper towels in contact with the bottom of the collagen gel. |
| Step 39, Section B V, VII, VIII | Collagen gel has not densified after 24 hours. | There has been insufficient evaporation of water from the collagen gel to form a tube. | 1. Using sterile tweezers, repeat the process of peeling the collagen gel from the chamber walls. One should observe a 1-2 mm spacing between |

| | | | |
|---|---|---|---|
| | | | the collagen gel and funnel walls. Continue densifying until tube forms.<br><br>2. The humidity of the incubator may be high and so an insufficient volume of water has evaporated from the collagen gel. Continue Step VII, monitoring the evaporative process, until a collagen tube forms. |
| Step 40, Section A V<br><br>Step 40, Section C VI | Immediately after seeding, the cell suspension overspills from the scaffold | 1. The seeding volume is too big<br><br>2. The seeding has occurred too close to the edge | 1. Immediately retrieve the spilled solution and seed it again onto the scaffold<br><br>2. Reduce the total volume of cell suspension used during seeding |
| Step 40, Section A IV, V, IX<br><br>Step 40, Section B V, VIII, IX<br><br>Step 40, Section C V, VI | Low cell attachment immediately after seeding | 1. Reduced incubation time preventing cell attachment<br><br>2. Cell detachment during media change<br><br>3. Low cell number. Optimal cell number and attachment potential may vary between lines<br><br>4. Prolonged incubation leading to cell death and drying | 1. After seeding, increase the duration of the incubation step a further 30 minutes<br><br>2. Add media very carefully. Tilt the plate and hold the pipette against the side of the well to avoid disrupting the Matrigel dome. Add the media slowly using a p1000 pipette<br><br>3. Optimise the seeding density for each CO line<br><br>4. Monitor the scaffolds closely after seeding and before addition of |

| | | | |
|---|---|---|---|
| | | 5. Small clump size leading to loss of cells through the pores of the PGA scaffold<br><br>6. Single cells or very small clumps floating in the seeding solution and preventing attachment<br><br>7. Remnants of EtOH or NaOH in PGA scaffolds leading to cell death in PGA scaffolds<br><br>8. Excessive breaking of the CO clumps reducing cell viability | media. Check the scaffolds every 30 minutes to ensure the seeding solution has not evaporated<br><br>5. Optimise the clump size when seeding PGA scaffolds<br><br>6. Pipette more slowly and fewer times when breaking the COs into small clumps<br><br>7. Increase the drying time to ensure all traces of NaOH and EtOH are gone (PGA scaffolds only)<br><br>8. Use a P200 pipette instead of a p20 to break the CO clumps<br><br>Pipette more slowly when breaking the CO clumps |
| Step 40, Section B V | CO clumps form organoid-like 3D structures rather than a monolayer | CO clumps were not dissociated to a sufficiently small size | Break clumps further, using a p10 pipette if necessary |
| Step 40, section B VIII | Cell suspension overflows from the construct lumen during seeding | Rapid addition of the cell suspension with inadequate needle withdrawal | Dispense cells slowly, simultaneously withdrawing the needle from the tube lumen |
| Step 40, Section A X | Low cell density on the scaffold | The cells may have been seeded too sparsely | 1. Increase the cell density |

| | | | |
|---|---|---|---|
| Step 40, Section B XIII<br><br>Step 40, Section C VI | | | 2. Allow a longer time for the cells to reach confluency |
| Step 40, Section A X<br><br>Step 40, Section B XII<br><br>Step 40, Section C VII | Poor cell survival and/or expansion on the scaffold | 1. Cells are washed away during media change<br><br>2. Y27632 not added to the media<br><br>3. Suboptimal CO line<br><br>2. Suboptimal collagen batch for collagen scaffolds<br><br>5. Suboptimal quality of cytokines, media components or collagen stock<br><br>6. Remnants of EtOH or NaOH in PGA scaffolds leading to cell death in PGA scaffolds | 1. Increase the media to 1 ml and change it very carefully every 4 days<br><br>2. Ensure fresh Y27632 is added to the media when seeding<br><br>3. All CO lines should express the biliary markers CK19, CK7, Sox9 and GGT (Fig. 6a and b) and display ALP and GGT activity (Fig. 6c and 6d). Healthy CO lines should be proliferating at a rate requiring passaging every five days (Fig. 5b, Image 1). Do not attempt scaffold seeding with a CO line that does not meet these criteria. See the Troubleshooting for steps 10, 12 and 29 for recommendations on how to improve the quality of a suboptimal CO line |

| | | | |
|---|---|---|---|
| | | 7. Prolonged incubation leading to cell death and drying | 4. Batch-test all new lots of collagen solution. (collagen scaffolds only)<br><br>5. Store all stock solutions at 4 °C and do not use stock solutions made more than 3 months previously. Where applicable, do not use stock solutions beyond their expiry date. Store all cytokines at -80 °C<br><br>6. Increase the drying time (70) to ensure all traces of NaOH and EtOH are gone (PGA scaffolds only)<br><br>7. Monitor the scaffolds closely after seeding and before addition of media- check the scaffolds every 30 minutes to ensure they have not dried out |
| Step 41, Section A III | Staining appears weak or non-specific | 1. The 4% (vol/vol) PFA may have degraded<br><br>2. Samples may have been fixed for too long | 1. Store 4% (vol/vol) PFA at 4 °C and do not use for longer than one month<br><br>2. Reduce fixation time. Do not attempt to fix too many samples at one time, to avoid accidental over-fixation |
| Step 41, Section A VI | Nuclear and intracellular staining is poor | The cells are not properly permeabilised, preventing adequate penetration of the antibody | Increase the concentration of Triton X-100, e.g 0.3% (vol/vol) Triton X-100 or 0.5% (vol/vol) Triton X-100 |

| | | | |
|---|---|---|---|
| Step 41, Section A VII, XIV | High levels of non-specific and background staining | 1. Blocking time may have been too short<br><br>2. Secondary antibody incubation may have been too long | 1. Increase blocking time e.g. to one hour<br><br>2. Incubate secondary antibodies for 1 hour at room temperature instead of overnight at 4 °C |
| Step 41, Section A XVII | DAPI staining is unclear | Incubation with DAPI may not have been long enough | Increase incubation with DAPI to 20 or 30 minutes |
| Step 41, Section B V | COs do not dissociate into a single-cell suspension | 1. Cell clumping and/or poor viability<br><br>2. CO clumps are not breaking up into single cells during the 5 minute incubation period | 1. Increase the concentration of DNase I in the accutase solution<br>Ensure that all pipetting is done as slowly and carefully as possible<br>2. Ensure the Accutase solution is pre-warmed<br>Prolong incubation for longer (e.g. 7-10 minutes) |
| Step 41, Section B IX | Single cell suspension forms clumps | Concentration of DNase I is too low | Increase the concentration of DNase I, e.g. to 6 mg/ml |

# ANTICIPATED RESULTS

We describe a protocol for the isolation of primary biliary epithelial cells from a variety of clinical samples, their propagation as cholangiocyte organoids and the generation of bioengineered biliary tissue by seeding these organoids on PGA or densified collagen scaffolds. Our cholangiocyte isolation methods (ERCP, mechanical scraping, EPCAM sorting) result in a >95% pure (CK7+/CK19+) population of isolated cholangiocytes (Fig. 2e, 3d and 4e, respectively). Furthermore, 95% of plated samples derived from excised bile ducts (when appropriately flushed of bile after excision) and 100% of samples derived from gallbladders, liver biopsies and ERCP brushings yield robust CO lines within

5 days of initial plating (Supplementary Table 1). CO lines can be derived from EpCAM+ sorted liver samples with an approximate efficiency of 66% (Supplementary Table 1). While it is important to derive lines from fresh, highly viable primary tissue to ensure the most reliable results, robust, highly-proliferative CO lines can also be derived from poor-quality samples with low cell numbers (supplemental Fig. 5). CO lines can subsequently be passaged approximately every 5 days and can typically be split in a 1:4-1:6 ratio for further expansion, analysis or cryopreservation.

The resulting organoids consist of a near homogenous (>99%) population of cholangiocytes (Fig. 6e). They express biliary markers such as CK19, CK7, Sox9 and GGT (Fig. 6a) at levels comparable to primary tissue (Fig. 6b) in the absence of hepatic markers such as albumin (Fig. 6a). Furthermore, CO lines exhibit key cholangiocyte functions, such as alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) activity (Fig. 6c and 6d). COs also display functional secretory capacity, as measured by the luminal secretion of rhodamine 123 (Sampaziotis et al; Nat. Med. (2017)- Fig. 2a-c) and bile acid transport, as measured by export of the fluorescently-labelled bile acid CLF (Sampaziotis et al; Nat. Med. (2017)- Fig. 2d-f). Additionally, COs respond appropriately to hormonal signals such as secretin and somatostatin ((Sampaziotis et al; Nat. Med. (2017)- Fig. 2i and 2j). For further validation, COs can be transplanted under the kidney capsule of immunocompromised mice where they form tubular structures retaining expression of biliary markers, such as CK7 and CK19 and are capable of surviving long-term [4] (Sampaziotis et al; Nat. Med. (2017) - Supplementary Fig. 7).

COs can be seeded onto both densified collagen and PGA scaffolds to form bioengineered biliary tissue. Additionally, COs can be seeded onto the lumen of tubular densified collagen scaffolds (Fig. 11e). The efficiency of cell attachment following seeding is variable between CO lines. However, following attachment the cells demonstrate a unique potential to expand and fully populate the scaffold. Therefore, the generation of confluent constructs is always possible by varying the time the scaffolds are maintained in culture. The resulting tissue expresses key cholangiocyte markers such as CK19 and CK7 (Fig. 11a, 11c and 11e) and exhibits biliary function such as GGT activity (Fig. 11b, 11d and 11f). These constructs can be used to successfully reconstruct or repair the biliary tree of immunocompromised mice[4] (Sampaziotis et al; Nat. Med. (2017) - Fig. 4 and 6) while retaining the expression of biliary markers and function following transplantation (Sampaziotis et al; Nat. Med. (2017) - Fig. 4 and 6).

## Data Availability

The authors declare that the main data supporting this study are available within the article. Extra data are available from the corresponding authors upon request.


## Acknowledgements

The authors would like to acknowledge the Cambridge Biorepository for Translational Medicine for the provision of human tissue used in the study and to thank K. Mahbubani, N. Georgakopoulos and B. Bareham for their role in collecting and distributing this tissue. The authors would additionally like to thank William G. Bernard and Laure Gambardella for their help and advice with regards to the fixation and staining of tubular bioengineered biliary constructs.

OCT was supported by an MRC-Sackler Doctoral Training Partnership. FS has been supported by an Addenbrooke's Charitable Trust Grant, the Academy of Medical Sciences Clinical Lecturer Starter Grant and an NIHR Clinical Lectureship. AWJ and AEM acknowledge support from EPSRC (EP/R511675/1 & EP/N509620/1) and the Isaac Newton Trust. The LV lab is funded by the ERC Proof of Concept grant Relieve-Chol, by the ERC advanced grant New-Chol and the core support grant from the Wellcome Trust and Medical Research Council to the Wellcome–Medical Research Council Cambridge Stem Cell Institute. AWJ, FS, LV, AEM and KSP gratefully acknowledge support from the Rosetrees Trust (REAG/240 & NMZG/233). AKF and EM were supported by funds from the Norwegian PSC research center


## Author contributions

OCT: manuscript writing and editing, coordination of study, execution of experiments and data acquisition, validation of the CO culture protocol, design and production of figure, final approval of the manuscript. AWJ: manuscript writing and editing, design and concept of the collagen densification protocol, development and validation of the collagen densification protocol, final approval of the manuscript. TB: manuscript writing and editing, collection of data, validation of the CO culture, collagen

densification and scaffold seeding protocols. SEC: production of schematics for figures 1, 2, 3, 4, 6 and 7 and validation of the collagen densification protocol. KTAM: execution of experiments and data acquisition. AF: development and validation of the CO and ERCP brushing collection protocols. EM: critical revision of the manuscript, validation of the CO protocols. HZ: validation of CO culture and data acquisition. KSP: design and concept of the study, development of the protocol, critical revision and final approval of the manuscript. AEM: design and concept of the collagen densification protocol, critical revision and final approval of the manuscript. LV: design and concept of the study, critical revision and final approval of the manuscript. FS: design and concept of the study, development and validation of the protocol, manuscript writing and editing, critical revision and final approval of the manuscript. OCT, AWJ and TB contributed equally to this work.

## List of Supplementary Information

**Supplementary Fig. 1: Flowchart showing recommended order of primary tissue processing for CO line derivation**

**Supplementary figure 2: Derivation of intrahepatic organoids through EpCAM+ sorting**

**(a)** Schematic representation of the optional EpCAM+ sorting step (procedure steps D III-DXXVI) for the derivation of intrahepatic COs. (**b**) Representative brightfield images of key EpCAM+ sorting steps. Numbers correspond to schematic stages in (a). 3: Liver tissue before enzymatic dissociation. 4: Liver tissue after enzymatic dissociation demonstrating release of cells in the medium and remnants of the extracellular matrix. 6: Single-cell suspension after filtration and before EpCAM+ sorting. Scale bars, 100 µm. (**c**) Representative brightfield image of an organoid derived from a single EpCAM+ cell, 48 hours after plating. Scale bar, 50 µm.

**Supplementary Figure 3:** Enlarged images of COs before and after organoid breaking (from Figure 5)

Enlarged images of COs before and after organoid breaking (from Figure 5). Enlarged brightfield images of figures 5b, image 1 and 5b, image 5. Scale bars, 100 µm.

**Supplementary figure 4:** Representative images of cholangiocyte organoids

Additional characterisation and troubleshooting of CO lines

(**a**) Representative brightfield images of healthy CO lines derived from all tissue types: 1: bile duct (BD), gallbladder (GB), Endoscopic Retrograde Cholangio-Pancreatography (ERCP), liver biopsy (biopsy) and EpCAM+ sorted cells (EpCAM). Scale bars- 200 µm. (**b**) Representative brightfield images of CO lines showing typical CO culture issues contrasted with optimal CO lines. Scale bars, 200 µm

**Supplementary figure 5:** Derivation of a CO line from low cell numbers

(a) Representative brightfield images of a CBD CO line derived from ~3.0 x $10^3$ viable cells/well (total: ~2.0 x $10^4$ viable cells). D0- D12: days after plating. P1: passage 1. (**b**) Graph illustrating cell number over time for the CBD line derived in (a) demonstrating appropriate expansion.

**Supplementary Figure 6:** Gating strategy for flow cytometric analyses

Representative flow cytometry plots showing gating strategy for all flow cytometric analyses. (**a**) Exclusion of debris. (**b**) Exclusion of doublets. (**c**) Secondary-only control to exclude negative population. (**d**) Representative C19+/CK7+ population. A minimum of 2 × $10^4$ gated events were used for analysis.

**Supplementary Table 1: List of all CO lines derived since September 2016**

Table showing key details of all CO lines derived since September 2016: anonymised donor ID number; tissue type (bile duct (BD), gallbladder (GB) or intrahepatic ducts (IHD)); age; sex; donor type (if applicable) (donation after brain death (DBD) or donation after cardiac death (DCD); blood group; and the success of the line derivation

**Supplementary Table 2: List of antibodies used for flow cytometry and immunofluorescence**

**Supplementary Software 1: CAD file for the base of the 3D-printed densification chamber used in Step 39, Section B**

**Supplementary Software 2: CAD file for the funnel of the 3D-printed densification chamber used in Step 39, Section B**

## REFERENCES

1 Park, S. M. The Crucial Role of Cholangiocytes in Cholangiopathies. *Gut and Liver* **6**, 295-304, doi:10.5009/gnl.2012.6.3.295 (2012).

2 Lazaridis, K. N. & LaRusso, N. F. The Cholangiopathies. *Mayo Clinic proceedings* **90**, 791-800, doi:10.1016/j.mayocp.2015.03.017 (2015).
3 Murray, K. F. & Carithers, R. L. AASLD practice guidelines: Evaluation of the patient for liver transplantation. *Hepatology* **41**, 1407-1432, doi:10.1002/hep.20704 (2005).
4 Sampaziotis, F. *et al.* Reconstruction of the mouse extrahepatic biliary tree using primary human extrahepatic cholangiocyte organoids. *Nature Medicine* **23**, 954, doi:10.1038/nm.4360

https://www.nature.com/articles/nm.4360#supplementary-information (2017).
5 Sampaziotis, F. *et al.* Cholangiocytes derived from human induced pluripotent stem cells for disease modeling and drug validation. *Nature Biotechnology* **33**, 845, doi:10.1038/nbt.3275

https://www.nature.com/articles/nbt.3275#supplementary-information (2015).
6 Sampaziotis, F. *et al.* Directed differentiation of human induced pluripotent stem cells into functional cholangiocyte-like cells. *Nature Protocols* **12**, 814, doi:10.1038/nprot.2017.011

https://www.nature.com/articles/nprot.2017.011#supplementary-information (2017).
7 Lugli, N. *et al.* R-spondin 1 and noggin facilitate expansion of resident stem cells from non-damaged gallbladders. *EMBO reports* **17**, 769-779, doi:10.15252/embr.201642169 (2016).
8 Sato, T. *et al.* Long-term Expansion of Epithelial Organoids From Human Colon, Adenoma, Adenocarcinoma, and Barrett's Epithelium. *Gastroenterology* **141**, 1762-1772, doi:https://doi.org/10.1053/j.gastro.2011.07.050 (2011).
9 Huch, M. *et al.* In vitro expansion of single Lgr5+ liver stem cells induced by Wnt-driven regeneration. *Nature* **494**, 247, doi:10.1038/nature11826

https://www.nature.com/articles/nature11826#supplementary-information (2013).
10 Kessler, M. *et al.* The Notch and Wnt pathways regulate stemness and differentiation in human fallopian tube organoids. *Nature Communications* **6**, 8989, doi:10.1038/ncomms9989

https://www.nature.com/articles/ncomms9989#supplementary-information (2015).
11 Huch, M. *et al.* Long-Term Culture of Genome-Stable Bipotent Stem Cells from Adult Human Liver. *Cell* **160**, 299-312, doi:10.1016/j.cell.2014.11.050 (2015).
12 Karthaus, Wouter R. *et al.* Identification of Multipotent Luminal Progenitor Cells in Human Prostate Organoid Cultures. *Cell* **159**, 163-175, doi:https://doi.org/10.1016/j.cell.2014.08.017 (2014).
13 Barker, N. *et al.* Lgr5+ve Stem Cells Drive Self-Renewal in the Stomach and Build Long-Lived Gastric Units In Vitro. *Cell Stem Cell* **6**, 25-36, doi:https://doi.org/10.1016/j.stem.2009.11.013 (2010).
14 Koo, B.-K. & Clevers, H. Stem Cells Marked by the R-Spondin Receptor LGR5. *Gastroenterology* **147**, 289-302, doi:https://doi.org/10.1053/j.gastro.2014.05.007 (2014).
15 Glinka, A. *et al.* Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* **391**, 357, doi:10.1038/34848 (1998).
16 Fedi, P. *et al.* Isolation and Biochemical Characterization of the Human Dkk-1 Homologue, a Novel Inhibitor of Mammalian Wnt Signaling. *Journal of Biological Chemistry* **274**, 19465-19472, doi:10.1074/jbc.274.27.19465 (1999).
17 Bafico, A., Liu, G., Yaniv, A., Gazit, A. & Aaronson, S. A. Novel mechanism of Wnt signalling inhibition mediated by Dickkopf-1 interaction with LRP6/Arrow. *Nature Cell Biology* **3**, 683, doi:10.1038/35083081 (2001).
18 Cui, S., Capecci, L. M. & Matthews, R. P. Disruption of planar cell polarity activity leads to developmental biliary defects. *Developmental Biology* **351**, 229-241, doi:https://doi.org/10.1016/j.ydbio.2010.12.041 (2011).

19 Strazzabosco, M. & Fabris, L. Development of the bile ducts: Essentials for the clinical hepatologist. *Journal of Hepatology* **56**, 1159-1170, doi:https://doi.org/10.1016/j.jhep.2011.09.022 (2012).
20 Place, E. S., George, J. H., Williams, C. K. & Stevens, M. M. Synthetic polymer scaffolds for tissue engineering. *Chemical Society Reviews* **38**, 1139-1151, doi:10.1039/B811392K (2009).
21 Polymeric Scaffolds in Tissue Engineering Application: A Review. *International Journal of Polymer Science* **2011**, doi:10.1155/2011/290602 (2011).
22 O'Brien, F. J. Biomaterials & scaffolds for tissue engineering. *Materials Today* **14**, 88-95, doi:https://doi.org/10.1016/S1369-7021(11)70058-X (2011).
23 Lutolf, M. P. & Hubbell, J. A. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. *Nature Biotechnology* **23**, 47, doi:10.1038/nbt1055 (2005).
24 Cheung, H.-Y., Lau, K.-T., Lu, T.-P. & Hui, D. A critical review on polymer-based bio-engineered materials for scaffold development. *Composites Part B: Engineering* **38**, 291-300, doi:https://doi.org/10.1016/j.compositesb.2006.06.014 (2007).
25 Kehoe, S., Zhang, X. F. & Boyd, D. FDA approved guidance conduits and wraps for peripheral nerve injury: A review of materials and efficacy. *Injury* **43**, 553-572, doi:https://doi.org/10.1016/j.injury.2010.12.030 (2012).
26 Frisch, S. & Francis, H. Disruption of epithelial cell-matrix interactions induces apoptosis. *The Journal of Cell Biology* **124**, 619-626, doi:10.1083/jcb.124.4.619 (1994).
27 Zeugolis, D. I., Paul, R. G. & Attenburrow, G. Engineering extruded collagen fibers for biomedical applications. *Journal of Applied Polymer Science* **108**, 2886-2894, doi:doi:10.1002/app.27208 (2008).
28 Abou Neel, E. A., Cheema, U., Knowles, J. C., Brown, R. A. & Nazhat, S. N. Use of multiple unconfined compression for control of collagen gel scaffold density and mechanical properties. *Soft Matter* **2**, 986-992, doi:10.1039/B609784G (2006).
29 Landi, F. *et al.* Endoscopic treatment of anastomotic biliary stricture after adult deceased donor liver transplantation with multiple plastic stents versus self-expandable metal stents: a systematic review and meta-analysis. *Transplant International* **31**, 131-151, doi:doi:10.1111/tri.13089 (2018).
30 Seehofer, D., Eurich, D., Veltzke-Schlieker, W. & Neuhaus, P. Biliary Complications After Liver Transplantation: Old Problems and New Challenges. *American Journal of Transplantation* **13**, 253-265, doi:doi:10.1111/ajt.12034 (2013).
31 Cellon. *Revolutions in Cell & Tissue Culture. BioFELT scaffold*, <http://www.cellon.lu/biofelt-scaffold.html > (2019).
32 Zhang, L. & Hui, L. Bile ducts regenerated. *Nature* **547**, 171, doi:10.1038/547171a (2017).
33 Thomas, H. Bioengineering the common bile duct. *Nature Reviews Gastroenterology &Amp; Hepatology* **14**, 504, doi:10.1038/nrgastro.2017.99 (2017).
34 Huch, M. *et al.* Unlimited in vitro expansion of adult bi-potent pancreas progenitors through the Lgr5/R-spondin axis. *The EMBO Journal* **32**, 2708-2721, doi:10.1038/emboj.2013.204 (2013).
35 Cho, W. K., Mennone, A. & Boyer, J. L. Isolation of functional polarized bile duct units from mouse liver. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **280**, G241-G246, doi:10.1152/ajpgi.2001.280.2.G241 (2001).
36 Demetris, A. J. *et al.* Isolation and primary cultures of human intrahepatic bile ductular epithelium. *In Vitro Cellular & Developmental Biology* **24**, 464-470, doi:10.1007/BF02628499 (1988).
37 H., A. M. K. *et al.* Morphogenesis of primary human biliary epithelial cells: Induction in high-density culture or by coculture with autologous human hepatocytes. *Hepatology* **33**, 519-529, doi:doi:10.1053/jhep.2001.22703 (2001).

38 Ogawa, M. *et al.* Directed differentiation of cholangiocytes from human pluripotent stem cells. *Nature Biotechnology* **33**, 853, doi:10.1038/nbt.3294 https://www.nature.com/articles/nbt.3294#supplementary-information (2015).
39 De Assuncao, T. M. *et al.* Development and Characterization of Human Induced Pluripotent Stem Cell-Derived Cholangiocytes. *Laboratory investigation; a journal of technical methods and pathology* **95**, 684-696, doi:10.1038/labinvest.2015.51 (2015).
40 Gjorevski, N. *et al.* Designer matrices for intestinal stem cell and organoid culture. *Nature* **539**, 560, doi:10.1038/nature20168 (2016).
41 Petrowsky, H. & Clavien, P.-A. in *Transplantation of the Liver (Third Edition)* (eds Ronald W. Busuttil & Göran B. G. Klintmalm) 582-599 (W.B. Saunders, 2015).
42 Badylak, S. F. The extracellular matrix as a scaffold for tissue reconstruction. *Seminars in Cell & Developmental Biology* **13**, 377-383, doi:https://doi.org/10.1016/S1084952102000940 (2002).
43 Dong, C. & Lv, Y. Application of Collagen Scaffold in Tissue Engineering: Recent Advances and New Perspectives. *Polymers* **8**, 42 (2016).
44 Glowacki, J. & Mizuno, S. Collagen scaffolds for tissue engineering. *Biopolymers* **89**, 338-344, doi:doi:10.1002/bip.20871 (2008).
45 Drury, J. & J Mooney, D. *Hydrogels for Tissue Engineering: Scaffold Design Variables and Applications*. Vol. 24 (2003).
46 Brown, R. A., Wiseman, M., Chuo, C.-B., Cheema, U. & Nazhat, S. N. Ultrarapid Engineering of Biomimetic Materials and Tissues: Fabrication of Nano- and Microstructures by Plastic Compression. *Advanced Functional Materials* **15**, 1762-1770, doi:doi:10.1002/adfm.200500042 (2005).

## Figure. Legends

**Figure 1:** Flowchart of key steps for the generation of bioengineered biliary tissue

**(a)** Overview flowchart showing the 3 major protocol steps: cell isolation, scaffold preparation and generation of bioengineered biliary tissue. (**b**) Flowchart summarising the process of cell isolation from different biliary tissue samples with reference to the relevant protocol sections. (**c**) Flowchart illustrating the different types of biological or synthetic scaffolds used, with reference to the relevant protocol sections. (**d**) Flowchart summarising the seeding of acellular scaffolds with cholangiocyte organoid (CO) cells, with reference to the relevant protocol sections.

**Figure 2:** Derivation of extrahepatic cholangiocyte organoids from extrahepatic biliary tissue

(**a**) Schematic representation of key stages of the derivation of extrahepatic cholangiocyte organoids (ECO) from primary bile duct (BD) and gallbladder (GB) tissue (Procedure step 2, section A). (**b**, **c**) Representative images of key stages of the derivation of cholangiocytes from (**b**) primary BD and GB

**(c)** tissue. Numbers in (b) and (c) correspond to the schematic stages illustrated in (a): **1A(i), 1B(i)**: Resected biliary tissue prior to dissection. **1A(ii), 1B(ii):** Tissue dissection. **1A(iii),1B(iii)**: Exposed luminal surface following dissection. **2:** PBS wash. **3:** Mechanical dissociation of the biliary epithelium. **4**: Primary cholangiocytes in suspension following dissociation. Scale bars: **(b)** Images **1(i) – 3**, 1 cm. **(c)** Images **1(i) – 3**:, 2 cm. **(b**) Image **4, (c)** Image **4:** 100 µm. (**d**) Representative brightfield images demonstrating key time points of ECO derivation. Scale bars, 200 µm. **1:** Primary cholangiocytes 24 hours after plating demonstrating the formation of early organoid structures. **2**: ECOs following long term culture (passage 20). (**e**) Flow cytometry analysis of the primary cell suspension, demonstrating >90% cholangiocyte isolation efficiency (Steps 2 A I-A VIII; gating strategy demonstrated in supplementary figure 6).

**Figure 3:** Derivation of extrahepatic cholangiocyte organoids through ERCP brushings

(**a**) Schematic representation of ECO derivation from ERCP brushings (procedure step 2, section B). (**b**) Representative images of key stages of the derivation procedure. Numbers correspond to schematic stages in (a). **1**: ERCP brush. **2**: Media wash to dislodge the collected cholangiocytes from the brush. **3**: Representative cell pellet after isolation. **4**: Brightfield image of ERCP isolated cholangiocytes following plating.**1-3**: Scale bars, 1 cm. Scale bar, 200 µm. (**c**) Brightfield images demonstrating representative time points in the derivation of organoids from cholangiocytes obtained through ERCP brushings. **1**: Primary cholangiocytes 24 hours after plating demonstrating the formation of early organoid structures. **2**: Cholangiocyte organoids one week after plating. Scale bars, 200 µm. (**d**) Flow cytometry analysis of the cell suspension obtained with through ERCP, demonstrating >90% cholangiocyte isolation efficiency (Steps 2 B I- BV; gating strategy demonstrated in supplementary figure 6).

**Figure 4:** Derivation of intrahepatic organoids

(**a**) Schematic representation of intrahepatic cholangiocyte organoid (ICOs) derivation (procedure step C). (**b** and **c**) Representative images of key stages of ICO derivation for (b) liver biopsies and (c) surgically resected liver tissue. Numbers correspond to schematic stages in (a). **1**: Dissection of liver tissue. **2**: Collection of dissected tissue. **3-4**: Dissected tissue before **(3)** and after **(4)** centrifugation.

**6(i)**: Representative image of liver tissue after embedding in Matrigel, prior to media addition. **1-6(i)**: Scale bars, 1 cm. **6(ii)**: Representative brightfield images of liver tissue after plating. Scale bar, 200 µm. (**d**) Representative brightfield images demonstrating key time points of ICO derivation **1**: ICO culture 5 days after plating demonstrating the emergence of an organoid from a segment of liver tissue. **2**: Established ICO line (passage 20). Scale bars: 200 µm. (**e**) Flow cytometry analysis of ICO cells, demonstrating >95% cholangiocyte isolation efficiency (gating strategy in supplementary figure 6).

**Figure 5:** Passaging of cholangiocyte organoids

(**a**) Schematic representation of the CO passaging procedure (procedure steps 15-30). (**b**) Representative images of key steps of the CO passaging procedure. Numbers correspond to schematic stages in (a). **1**: Confluent COs prior to passaging. **4**: Representative organoid pellet yielding 9 organoid wells following plating (approximately 4.0 x $10^5$ cells). **5**: Suspension of COs after manual dissociation demonstrating representative clump size for passaging (approximately 30-100 cells per clump) (procedure step 28). **7(i)**: COs immediately after plating (procedure steps 9-13) demonstrating that the majority of cells remain in small clumps and have not yet formed organoids at this stage. **7(ii)** COs 24 hours after passaging, demonstrating that the majority of CO clumps have remodelled into organoids at this point. **1, 5, 7(i)** and **7(ii)**: Scale bars, 100 µm. **4**: Scale bar, 5 mm.

**Figure 6:** Characterisation of cholangiocyte organoids

(**a**) Immunofluorescence images demonstrating expression of key biliary markers in cholangiocyte organoids. Scale bar, 50 µm. (See table 1 for a detailed list of antibodies and concentrations used). (**b**) qRT–PCR confirming the expression of key biliary markers in ECOs compared to freshly isolated primary cholangiocytes (PC); n = 4 biological replicates. Centre line, median; box, interquartile range (IQR); whiskers, range (minimum to maximum). Values relative to the housekeeping gene HMBS (HydroxyMethylBilane Synthase). # P > 0.05 (two-tailed Student's t-test). (**c**) Cholangiocyte organoids demonstrate ALP activity. Scale bars, 100 µm. (**d**) GGT activity of cholangiocyte organoids measured in absorbance units (a.u.); n = 3; MEFs: mouse embryonic feeders, used as a negative

control. Error bars, s.d.; individual data points are demonstrated; ****P < 0.0001, two-tailed Student's t-test. GGT activity were assessed using a commercially available (MaxDiscovery gamma-Glutamyl Transferase (GGT) Enzymatic Assay) according to the manufacturer's instructions. (**e**) Flow cytometric analyses performed on COs after long term culture (20 passages) demonstrating >99% CK7+/CK19+ expression (gating strategy demonstrated in supplementary figure 6).

**Figure 7:** Generation of densified collagen sheets

(**a**) Schematic representation of the procedure for generating densified collagen sheets (procedure step 39, Section A). (**b**) Representative images of key stages of the collagen densification process. Numbers correspond to schematic stages in (a). **1**: Specimen tube containing 5% collagen gel before densification (25 mm height). **2**-**3(i)**: Representative gel height following water absorption for 0 (**2(i)**), 30 (**2(ii)**) and 60 (**3(i)**) minutes. **3(ii)**: Specimen tube containing fully densified collagen scaffold (indicated by the black arrow). **4**: Removal of nylon mesh. **5**: Representative image of the resulting densified collagen sheet. 1-4: Scale bars, 1 cm. 5: Scale bars, 5 mm.

**Figure 8:** Generation of densified collagen tubes

**(a)** Representative images of densification chamber assembly (Equipment Setup). **1:** Densification chamber components: (i) Funnel, (ii) Base, (iii) wire, (iv) 25 G needle, (v) paper towels, (vi) M4 screws and (vii) nuts. **2:** Mounting of the rigid metal wire in the chamber base. **3:** Addition of paper towels. **4:** Addition of funnel. **5.** Chamber assembly. Scale bars: 20 mm. **(b)** Schematic representation of collagen tube densification (step 39B I-IX). **(c)** Representative images of tube densification. Numbers correspond to schematic stages in (b). **1:** Addition of collagen precursor solution. **2.** Peeling of collagen from funnel walls. **3:** Gel optimally positioned for evaporation. **4:** Completion of densification. **5.** Removal of densified tube from wire. Scale bars, 15 mm. **(d)** Tube trimming following densification (step 39B, X-XV). **1:** Collagen tubes immediately post-densification. **2.** Washed tube. **3, 4:** Tube trimming. **5**. Trimmed tube with patent lumen (white dashed lines). Scale bars: (1)-(3), 2 cm; (4), 2mm; (5) 1 mm.

**Figure 9:** Seeding of flat densified collagen or PGA scaffolds

(**a**) Schematic representation of the procedure for seeding densified collagen sheets and polyglycolic acid (PGA) scaffolds (procedure step 40, sections A and C, respectively). (**b** and **c**) Representative images of key stages of the seeding procedure for (b) densified collagen and (c) flat PGA scaffolds. Numbers correspond to schematic stages in (a). **1**: Dried scaffold before seeding. **3(i)**: Addition of cell suspension on the scaffold at the start of seeding process. William's E+ media with phenol red used for illustrative purposes. The use of phenol red in the media is optional. **3(ii)**: Scaffold after completion of seeding and prior to incubation. **5**: Seeded scaffold following media addition. Scale bars, 1 cm. (**d**) Representative fluorescent image of a flat densified collagen scaffold confluently seeded with RFP+ COs. Scale bar, 100 μm (**e**) Representative fluorescent image of a PGA scaffold confluently seeded with GFP+ COs. Scale bar, 50 μm.

**Figure 10**: Seeding of densified collagen tubular scaffolds

**(a)** Schematic representation of densified collagen tube seeding (procedure step 40B). **(b)** Representative images of the seeding process. Image numbers refer to the corresponding stages illustrated in the schematic in (a). 2: Cannulation of tube lumen with 34 G needle. 3: Seeding of tube lumen with COs. Note the change in the tube colour as the lumen fills with Phenol Red containing media. Scale bars: (2), 1 mm; (3), 5 mm. (**c**) Representative brightfield image of a CO-seeded densified collagen tube. Scale bar, 200 μm. (**d**) Representative fluorescent image of a densified collagen tube seeded with RFP+ COs. Scale bar, 200 μm.

**Figure 11**: Characterisation of bioengineered biliary tissue

(**a**) Immunofluorescence images demonstrating expression of key biliary markers in CO-seeded PGA scaffolds. Scale bar, 100 μm. (**b**) CO-seeded PGA scaffolds demonstrate GGT activity. A.U: absorbance units; n = 3; mouse embryonic feeders (MEFs) used as a negative control. Error bars, s.d.; individual data points are demonstrated; ****$P < 0.0001$, two-tailed Student's t-test. (**c**) Immunofluorescence images showing expression of key biliary markers in CO-seeded densified collagen sheets. Scale bar, 100 μm. (**d**) CO-seeded collagen scaffolds demonstrate GGT activity.

A.U.: absorbance units; n = 3; mouse embryonic feeders (MEFs) used as a negative control. Error bars, s.d.; individual data points are demonstrated; **$P$= 0.0055, two-tailed Student's t-test. (**e**) Immunofluorescence images showing expression of key biliary markers in CO-seeded densified collagen tubes. Scale bar, 100 μm. (**d**) CO-seeded collagen tubes demonstrate GGT activity. A.U.: absorbance units; n = 3; mouse embryonic feeders (MEFs) used as a negative control. Error bars, s.d.; individual data points are demonstrated; ****$P$= 0.0001, two-tailed Student's t-test. GGT activity was assessed using a commercially available kit (MaxDiscovery gamma-Glutamyl Transferase (GGT) Enzymatic Assay Kit) according to the manufacturer's instructions.