Cell isolation and Organoid Culture from Mouse Liver and Small Intestine

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Study Protocol

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Abstract

Background: Organoid culture enables disease modeling and drug screening *in vitro*. Organoids are from organs (e.g., brain, small intestine, kidney, lung, and liver). To facilitate the establishment of liver and small-intestinal organoids, we developed a protocol for collecting cholangiocytes and crypts and culturing organoids.

Methods: Cholangiocytes were collected from intrahepatic bile ducts, gallbladder, and crypts from the small intestine using gravity settling and multi-step centrifugation methods, and embedded in Matrigel to grow into three-dimensional spheroids in suitable culture medium. Passaging, cryopreservation, and thawing were performed to assess organoid cell stability. RNA and DNA extraction, as well as immunostaining procedure were optimized. For preclinical modeling, the growth rate of cholangiocyte organoids (cho-orgs) was harmonized.

Results: Large amount of Cholangiocytes and small intestine crypts were collected. Cholangiocytes developed into cyst-like structures after 3–4 days in Matrigel. After culture for 1–2 weeks, small-intestinal organoids developed buds and formed a mature structure. Cho-orgs from intrahepatic bile ducts grew more slowly but were longer lasting, expressed the cholangiocyte markers Krt19 and Krt7, and recapitulated the *in vivo* tissue organization.

Conclusions: The protocol takes 2–4 weeks to establish a stable organoid growth system. Organoids could be stably passaged, cryopreserved, and recovered. The organoids retained tissue characteristics, including marker expression.

Background

Organoids are *in vitro* three-dimensional (3D) cultures grown from primarily isolated cells or stem cells.[1] Three-dimensional culture enables organoid growth from healthy[2] or diseased[3] human or mouse primary tissue. Organoids have potential as preclinical models,[1,4,5] for drug screening,[6] and in mechanistic research.[4,7] Standardized good manufacturing practice (GMP)-compliant scalable organoids may enable replacement of damaged human organs.[8]

The liver comprises hepatocytes, cholangiocytes, and nonepithelial cells;[9] the small intestine is formed of intestinal epithelium, which contains intestinal crypt-villus units and $Lgr5^{+}$ stem cells.[10] In Matrigel, proliferation of hepatic and small-intestinal stem cells requires epidermal growth factor (EFG) and R-spondin 1 (R-SPO1) (Notch signaling components),[10] growth factors (N2, B27, and Noggin), and hepatocyte growth factor (HGF) and fibroblast growth factor (FGF).

We developed an improved method to collect cholangiocytes and intestinal crypts using gravity settling and multistep centrifugation. However, cells from different mice show different growth rates; therefore, we established an organoid hand-picking procedure (Fig. 1) to ensure that organoids had similar growth

rates, facilitating disease modeling. The organoids can be passaged, cryopreserved, and thawed, and are suitable for genetic analysis and immunochemical staining. In contrast to other methods, our procedure does not require additional equipment (e.g., flow cell sorter) and antibodies, does not subject cells to mechanical damage or chemical injury, does not require physical exertion, is easy to perform, and unifies the cell growth rate.

Organoids grown from the bile ducts and small intestine may have the characteristics of the originating tissue, which makes them suitable for disease modeling. They may also have therapeutic investigation potential for various diseases, such as primary sclerosing cholangitis (PSC),[11] intestinal cancer[12], and inflammatory bowel diseases (IBD).[13]

Experimental Design

Cell isolation

To isolate bile ducts (cholangiocytes) from mouse liver, the ducts and gallbladder can be digested. The liver was perfused with PBS 1× through the inferior vena cava before removing liver tissue. If a small number of cholangiocytes is needed, or if cho-orgs must be cultured rapidly, cholangiocytes can be obtained from the mouse gallbladder. The differences are discussed by Rimland.[14] After digestion, ducts and crypts from the small intestine can be collected by gravity settling, followed by multi-step centrifugation. If cholangiocytes are to be enumerated, bile ducts can be digested using a single-cell TrypLE Express medium.

Organoid culture and establishment of stable organoid lines

To establish a long-term organoid culture, we picked and collected larger organoids to passage, to unify the whole growth speed of organoids in the same culture-well. Organoid size, proliferation rate, physical morphology, formation rate, and expression of markers/genes need to be considered for culturing.[15] Methods of home-made Wnat3a and R-SPO1 were descripted by Broutier[16]. When a stable culture line is established, organoids can be maintained long term.

Organoid analysis

The extraction of DNA or RNA of organoids is similar with normal cells. Matrigel is first removed using pre-cold basal medium. It is suggestable for the usage of cell recovery solution to remove the Matrigel without disrupting organoids structures for immunofluorescent and immunohistochemical staining analysis. Whole-mount immunofluorescence staining enables 3D structure observation and characterization, such as detecting aimed antibody signal distribution. When to observe cellular morphology and store organoids for further staining analysis after some time, paraffin sectioning and immunohistochemical staining is preferred.

Materials

Animals

Male or female mice of any genetic background, ranging in age from 6 weeks to 1.5 years (weighing 20–28 g), can be used for cho-org culture; for in-org culture, < 6-week-old C57BL/6 mice should be used.

Reagents

- Collagenase D (Roche, cat. no. 1108866001)
- Dispase II (Life Technologies, cat. no. 17105-041)
- DNase I (Sigma-Aldrich, cat. no. DN25)
- TrypLE Express (Life Technologies, cat. no. 12605-028)
- EDTA (Sangon Biotech inc.Shanghai CAS: 60-00-4)
- Matrigel matrix, phenol-red-free (BD, cat. no. 356231)
- Advanced DMEM/F-12 (Life Technologies, cat. no. 12634-010)
- DMEM, high glucose, GlutaMAX, pyruvate (Life Technologies, cat. no. 11995-065)
- GlutaMAX (100×; Life Technologies, cat. no. 35050-068)
- HEPES (Life Science Products & Services, cat.no. HB0264)
- Penicillin/streptomycin (Corning, cat.no.30002297)
- B27 Supplement 50×, minus vitamin A (Life Technologies, cat. no. 12587-010)
- N2 Supplement 100× (Gibco, Life Technologies, cat. no. 17502-048)
- L-Glutamine (Gibco, Life Technologies, cat. no.25-005-CI)
- N-acetylcysteine (Sigma-Aldrich, cat. no. A0737-5MG)
- Nicotinamide (Sigma-Aldrich, cat. no. N0636)
- Recombinant mouse (Rm) FGF10 (Peprotech, cat. no. 100 – 26)
- Rm EGF (Life Technologies, cat. no. PMG8043)
- Rm HGF (Peprotech, cat. no. 100 – 39)
- [Leu15] - Gastrin I human (PL Laboratories, cat. no. P2000646)
- Rho kinase inhibitor Y-27632 dihydrochloride (Sigma-Aldrich, cat. no. Y0503)
- Recombinant human (Rh) Noggin (Peprotech, cat. no. 120-10C)
- Wnt3a-conditioned medium (home-made)
- Rspo1-conditioned medium (home-made)
- Freezing solution (Life Technologies, cat. no. 12648-010)
- Cell recovery solution (Corning, cat. no. 354253)

Additional reagents (Table S1), instruments (Table S2) and antibodies (Table S3) are listed in Supplementary Materials.

Reagent Setup

Mouse liver digestion medium

Digestive medium should be prepared fresh and used immediately. Collagenase D and II were dissolved in a sterile washing medium (see below) at a concentration of 0.125 mg/ mL, respectively. Supplement 0.1 mg /mL DNase I (dissolved in sterile H_2O).

Mouse liver basal medium

Ad DMEM/F-12 was added with 1% penicillin / streptomycin, 1% GlutaMAX and 10 mM HEPES. It can be stored at 4°C for 1 month.

Mouse liver isolation medium

The mouse liver isolation medium is the mouse liver expansion medium supplemented with 25 ng/mL recombinant human Noggin, 30% (vol/vol) Wnt3a-conditioned medium and 10 μM Rho kinase (ROCK) inhibitor (Y-27632; if single cells were cultured). The medium was stored at 4°C for up to 2 weeks.

Mouse liver wash medium

DMEM (high glucose, GlutaMAX and pyruvate) supplemented with 1% FBS and 1% penicillin/streptomycin. The medium was stored at 4°C for up to 1 month.

Mouse liver expansion medium

Mouse liver Basal medium supplemented with B27 50×, 1 mM N-acetylcysteine, 5% (vol/vol) Rspo1, 10 mM Nicotinamide, 10 nM Gastrin I, 50 ng/mL EGF, 100 ng/mL FGF10, 50 ng/mL HGF. Store the medium at 4°C for up to 2 weeks.

Mouse intestine expansion medium

Ad DMEM/F-12 supplemented with 10mM HEPES,2 mM L-Glutamine□N2 50×□B27×□50 ng/mL EGF, 100 ng/mL Noggin□10% (vol/vol) R-spondin1. Store it at 4°C for up to 1 month.

Mouse intestine digestion medium

PBS 1× supplemented with 10mM HEPES, 1%(vol/vol) L- Glutamine, 1mM EDTA, 1% penicillin/ streptomycin and 5□FBS.

Regent preparation for Immunofluorescence (IF):

1% (vol /vol) PBS-BSA: 1 g BSA per 100 mL PBS 1×. Store at 4℃ for 2 weeks.

0.1% (vol /vol) PBT: 1 mL Tween 20 per 1000 mL PBS 1×. Store at 4℃ for 4 weeks.

Washing buffer: 1 mL Triton X-100 and 2 g BSA per 1 L PBS 1×. Store at 4℃ for 2 weeks.

F-G clearing solution: 2.5 M fructose and 60% glycerin. Store at 4°C in dark for up to 1 month.

Regent preparation for Immunohistochemistry (IHC):

96% (vol/vol) alcohol: dilute 100% alcohol with purified water.

0.5% (wt/vol) Eosin solution: 0.5 g Eosin dissolve in 100 mL 96% alcohol.

PROCEDURE

I. Culture of mouse liver and small-intestinal organoids: Cell isolation

Mouse liver duct cells may be collected from the liver or gallbladder.

I.i Collection of bile ducts from the mouse liver

1. To collect liver tissue, anesthetize the mouse and expose the liver. Find the inferior vena cava using a sterile swab, place a sterile cotton ball beside the liver, inject PBS 1× using a 10 mL injector, and cut off the portal vein immediately when the liver swells. By standard surgical procedures, remove the liver as an entire organ and transfer to a 10 cm Petri dish.
2. Transfer the Petri dish to a biological safety cabinet and place on ice. Preheat the digestion solution to 37°C.
3. Cut the liver tissue into small pieces (< 1 mm^3) using fine scissors. Transfer the small pieces to a 50 mL sterile centrifuge tube, add up to 5 mL precooled washing medium, and pipette up and down several times using a 10 mL pipette to wash the minced tissue. Repeat the washing procedure.
4. Transfer the tissue to a new 50 mL sterile centrifuge tube. Add 5 mL prewarmed digestion medium. Shake the tube at 120–160 rpm and 37°C for ~ 2 h.
5. During incubation, check the appearance of bile ducts using a light microscope. Pipette up and down the supernatant in a biological safety cabinet using a 1 mL pipette, and transfer ~ 200 µL solution to a glass slide for observation (Fig. 2b, upper row). If none are present, return the solution to the shaker. Perform the check at 20–30 min intervals. Ducts usually appear after 60 min.
6. When bile ducts appear, transfer the digestion supernatant to a fresh 50 mL centrifuge tube, add the same volume of precooled washing medium, and centrifuge at 80 g for 4 min at 4°C. Discard the supernatant and add 15 mL precooled washing medium, and repeat the centrifugations to remove the remaining digestion solution.
7.

Add precooled washing medium to the pellet and pipette up and down to mix. Place the 50 mL centrifuge tube upright on ice for 30 min.

8. Remove the supernatant without disrupting the pellet. Add 5 mL precooled washing medium, transfer the mixture to a fresh 15 mL centrifuge tube, and centrifuge at 60 g for 2 min at 4°C.

9. Remove the supernatant carefully. Add 1 mL precooled basal medium, transfer the mixture to a 1.5 mL microcentrifuge tube, and centrifuge at 500 rpm for 2 min at 4°C.

10. The pellet can be directly cultured. If there needs to collect single bile cells, following the next procedure.

II. i Enrichment of single cholangiocytes

1. Resuspend bile ducts in 5 mL prewarmed TrypLE solution supplemented with 5 µL DNase I (10 mg/mL). Using narrow 1000 µL tips, pipette up and down to mix and incubate at 37°C for 2–10 min.
2. Check the solution every 2 min using a bright-field microscope. Stop the digestion when the majority (85–95%) of the mixture consists of single cells by adding precooled wash medium (Fig. 2a, upper row).
3. Add 10 mL cold washing medium to stop the digestion. Transfer the mixture to a 50 mL centrifuge tube through a 70 µm filter and centrifuge at 300–350 g for 5 min at 4°C. Remove the supernatant and repeat the centrifugation to wash out any remaining TrypLE. Add 1–5 mL washing medium to resuspend the pellet.
4. Enumerate the cells using a standard cell-counting chamber.

I.iii Collection of cholangiocytes from the gallbladder

Prewarm the mouse liver digestion medium and TrypLE solution (supplemented with 0.1% [vol/vol] DNase I [10 mg/mL]) to 37°C.

1. By standard surgical procedures, anesthetize the mouse and expose the liver. Strip the gallbladder using two ophthalmic forceps and transfer it to a 6 mm dish containing precooled mouse liver washing medium.
2. Cut the gallbladder into small pieces using ophthalmic scissors, transfer to a 15 mL sterile centrifuge tube using a 1000 µL Eppendorf, add ~ 5 mL precooled washing medium, gently pipette up and down, and centrifuge at 4°C, 200–250 g for 4 min to remove bile.
3. Discard the supernatant and add 5 mL mouse liver digestion medium. Shake the tube at 120–180 rpm and 37°C for 1 h.

4. Add 5 mL precooled washing medium to stop the digestion, centrifuge at 4°C and 200–250 g for 4 min and discard the supernatant.
5. Resuspend gallbladder tissue in 1 mL prewarmed TrypLE solution and pipette up and down 30 times to isolate single cholangiocytes.
6. Incubate the tube for 2–4 min in a 37°C culture bath and pipette up and down 30 times.
7. Add precooled wash medium to ~ 5 mL and filter the mixture through a 70 μm mesh into a 50 mL centrifuge tube using a 1000 μL Eppendorf. Pipette the fluid into a 15 mL centrifuge tube, and centrifuge at 4°C and 300–350 g for 4 min.
8. Discard the supernatant and enumerate the cells. The plates are ready for Matrigel embedding and 3D organoid culture.

I.iv. Collection of crypts from mouse small intestine

1. Anesthetize the mouse, and by standard surgical procedures expose and remove the intestine using ophthalmic forceps. Cut the optional intestine located in 1 cm upper the terminal ileum and 2–3 cm below the stomach, and transfer them to 10 cm dishes containing PBS 1×.
2. Put the 10 cm dish on ice. Squeeze out the intestinal contents using forceps and cut the tissue longitudinally. Use a blunt instrument (e.g., the curved part of curved dissecting forceps) to scrape the intestinal villi, wash two or three times, and cut the tissue into 1–2 cm pieces.
3. Transfer the pieces to a 50 mL centrifuge tube and add 20 mL mouse intestine digestion medium. Put the tube on a shaker, at 120–160 rpm, 4°C incubate for about 30 min.
4. Carefully remove the supernatant and resuspend the tissue in a new 50 mL centrifuge tube, add ~ 25 mL PBS 1×, and vigorously pipette up and down 30–50 times to isolate crypts from tissues.
5. Filter the supernatant through 100 and 70 μm meshes into a new 50 mL centrifuge tube.
6. Allow the tube to stand for 8 min, discard the supernatant or transfer them to a new tube for another 8 min-standing circulation. Gain the palates together and check the proportion of crypts. Add 1–5 mL PBS 1× to the white sediment and pipette up and down gently. Observe the mixture under a light microscope.
7. If the proportion of single cells is too high, add ~ 25mL pre-cold PBS 1×, and repeat 8 min-standing circulation procedure in step (D-6) again to collect higher proportion of crypts.
8. Count the crypts using a counting board.

II. Seeding and culture: Cholangiocytes and crypts

1. Prewarm 24-well (or 48-well) culture plates at 37°C for at least 30 min. Pre-dissolved the Matrigel and keep them on ice.
2. Resuspend the appropriate number of cholangiocytes, duct structures, or crypts (e.g., 5,000 cells or 250 duct/crypt structures per well of a 24-well plate) in Matrigel for seeding. For example, use a volume of 40 μL per 24-well plate or 20 μL per 48-well plate.

3. Mix the plates and Matrigel gently. Add a droplet of the mixture (basement matrix and cultures) to the center of each well, preventing the drop from touching the edges. Incubate for 15–20 min at 37°C until Matrigel solidifies.
4. Add the appropriate medium to each well (500 µL per well for a 24-well plate or 250 µL per well for a 48-well plate)—liver isolation medium for cho-org culture; intestine expansion medium for in-org culture.
5. Incubate the plate at 37°C in a 5% CO_2 atmosphere. For cho-orgs, after 3 days, exchange isolation medium for expansion medium and incubate for ~ 14 days. For in-orgs, retain intestine expansion medium. Change the medium every 3–4 days. Organoids will start to develop on days 3–5.

III. Organoid passage

1. Prewarm culture plates for 1 h–overnight. Place Matrigel on ice and thaw before use. Prewarm TrypLE Express solution (~ 2 mL per tube) in a water bath at 37°C.
2. To disrupt the basal matrix, add 500–1000 µL precooled basal medium (for cho-orgs) or PBS 1× (in-orgs) and pipette up and down using a 1000 µL pipette. Transfer the organoid suspension (three wells for 24-well culture plates or six wells for 48-well culture plates) to a 15 mL centrifuge tube, add ~ 10 mL precooled basal medium (cho-orgs) or PBS 1× (in-orgs) to the top, and mix by pipetting vigorously 5–10 times to wash away Matrigel.
3. Centrifuge the tube at 200–250 g for 5 min at 4°C.
4. Discard the supernatant, leaving 100 µL mixture.
5. Add prewarmed TrypLE Express solution and mix by vigorously pipetting up and down using a 1000 µL pipette. Transfer the tube to a water bath at 37°C for 1–4 min, checking every 2 min under a light microscope. When the majority (85–95%) of the material is single cells, add ~ 10 mL precooled basal medium to stop digestion.
6. Centrifuge the tube at 300–350 g for 4 min at 4°C and carefully aspirate the supernatant.
7. Organoids can be mechanically dissociated and split at a 1:3–1:6 ratio. Resuspend the cells in Matrigel (40–50 µL per well for 24-well plates or 20–25 µL per well for 48-well plates). Pipette the mixture up and down gently to resuspend the cells. Add a droplet of the mixture to the center of each well. Incubate for 15–20 min to allow Matrigel polymerization.
8. Overlay the cultures with expansion medium (500 µL per well for 24-well plates or 250 µL per well for 48-well plates).
9. Replace the medium every 2–3 days.

IV. Harmonizing the growth rates of cho-orgs

The growing speeds of cho-orgs various when develop from different primary culture cells, for some experimental needs, organoids in different growing state are hard to perform experiments. To resolve this problem, we purified cho-orgs with similar growth rates using the procedure below, which can be repeated one to three times according to the growth situation.

1. When cho-orgs have budded for 2–3 days (5–7 days after seeding), check their growth state daily under a light microscope.
2. Before cells aggregate inside the lager cho-orgs, prepare to selectively passage then, as referred in (III-1)): Prewarm culture plates and TrypLE Express solution, and thaw the Matrigel before use.
3. Move the plate to a sterile environment. Under a light microscope, transfer larger cho-orgs to precooled basal medium using a 10 µL pipette.
4. Subsequent steps are the same as organoids passage, please follow the steps (III-3) ~ 8)).

V. Cryopreservation and thawing of organoids

V.i Freezing organoids

1. At least one confluent well (24-well plates) or two confluent wells (48-well plates) of organoids are needed per cryovial tube.
   Proceed as in steps (III-1) ~ 5)) to digest organoids into single cells, and resuspend the cells in 500 µL precooled freezing medium per well (24-well plates) or two wells (48-well plates). Transfer the mixture to cryovials (500 µL each) and immediately place them on ice. Transfer the cryovials to −80°C and then to liquid nitrogen after 24 h. Organoids can be stored for years.
   At least one confluent well (24-well plates) or two confluent wells (48-well plates) of organoids are needed per cryovial tube.

VI. II Thawing of organoids

1. Prewarm a 15 mL tube with 10 mL basal medium (for cho-orgs or in-orgs) to 37°C. Prewarm a 24- or 48-well plate according to the needs.
2. Incubate the cryovial in a 37°C water bath and remove when the frozen cell mass is almost completely thawed. Transfer the thawed cell aggregates to prewarmed basal medium and pipette up and down gently.
3. Centrifuge the tube at 250–300 g for 4 min at 4°C.
4. Remove the supernatant without disturbing the pellet.
5. Proceed as in steps (II-1) ~ 3)) to seed cells in Matrigel, and add the appropriate expansion medium (500 µL per well for 24-well plates or 250 µL per well for 48-well plates) to each well.
6. Replace the medium every 2–3 days.

VI. Analysis of organoids

To characterize organoids, use option VI A-B for isolation of DNA (A) and RNA (B), VI C-D for organoid staining, VI C for immunofluorescence analysis, and VI D for immunohistochemical analysis.

VI. i Immunofluorescence staining

1. To collect organoids completely, remove expansion medium and add 500–1000 µL precooled Cell Recovery Solution to each well.

2. Gently shake the plate horizontally at 4°C for 30–60 min to disrupt the Matrigel.
3. Cut off the tops of 1000 μL tips. Blow the tips twice in precooled 1% (v/v) PBS-BSA, and wash 15 mL centrifuge tubes using 1% (v/v) PBS-BSA to precoat tips and tubes.
4. Transfer the mixture to precoated tubes using precoated tips, add PBS 1× to ~ 10 mL and mix gently. Centrifuge at 70 g and 4°C for 4 min and carefully remove the supernatant.
5. Repeat the PBS 1× wash steps once or twice to wash out Matrigel completely.
6. Fix the organoids by resuspending in 4% (w/v) paraformaldehyde at 4°C for 45 min and mix once or twice.
7. Add precooled 0.1% (vol /vol) PBT to ~ 10 mL, mix and centrifuge at 70 g and 4°C for 4 min.
8. Remove the supernatant and resuspend organoids in precooled washing buffer. Transfer the mixture to a 24-well plate (> 200 μL per well) and incubate at 4°C for 15 min.
9. When organoids sink to the bottom, tilt the culture plate to 45° and remove washing buffer to leave about 200 μL liquid.
10. Add a twofold concentration of primary antibody (diluted in 0.5% PBS-BSA) (200 μL) to each well. Incubate overnight at 60 rpm and 4°C.
11. Add 1 mL washing buffer to each well, and pipette gently.
12. When organoids sink to the bottom (3 min), remove 1 mL washing buffer, add 1 mL washing buffer, and incubate for 30 min.
13. Repeat step (11) at least twice.
14. When organoids sink to the bottom, remove washing buffer to leave to 200 μL liquid.
15. Add a twofold concentration of secondary antibody (diluted in 0.5% PBS-BSA) (200 μL) to each well. Incubate overnight at 60 rpm and 4°C.
16. Repeat 10–13). Transfer the organoids to 1.5 mL EP tubes, and centrifuge at 70 g and 4°C for 4 min. Organoids can be stored in washing buffer at 4°C for 2 days.
17. Remove washing buffer without touching the plates.
18. Add F-G clearing solution (> 50 μL, RT) to the EP tube using top-cut 200 μL tips.
19. Add an appropriate volume of DAPI and incubate for 20 min at room temperature. Organoids can be stored in F-G clearing solution for 1 week at 4°C or 6 months at − 20°C.
20. Transfer the organoids to a 24-well plate using 200 μL top-cut tips.
21. Organoids can be subjected to immunofluorescence imaging.

VI.ii Immunohistochemical staining of organoids

1. To obtain organoids completely perform steps VI-C 1) ~ 6).
2. Remove 4% (w/v) paraformaldehyde, add ~ 10 mL precooled PBS 1×, and centrifuge at 70 g and 4°C for 4 min.
3. Repeat the washing step once or twice.

4. Remove the supernatant and add ~ 10 mL 70% alcohol. Organoids can be stored at 4°C in 70% alcohol for 1 week.
5. Adjust a water bath to 65°C and prewarm Histowax (~ 3 mL per sample), plastic straws, soft EP tubes, and a metal mold. Be careful to keep the materials dry.
6. Place a centrifuge tube on ice upright until organoids sink to the bottom. Remove the supernatant, resuspend organoids in 0.5% (w/v) Eosin solution to dehydrate, and stain for > 30 min.
7. When organoids sink to the bottom, discard the supernatant, and resuspend in 100% alcohol to wash for at least 30 min, repeat this wash steps for 3 times.
8. Resuspend organoids in dimethylbenzene and wash three times for ~ 30 min each.
9. Remove the dimethylbenzene.
10. Place the centrifuge tube containing organoids mentioned above in a 65°C water bath, absorb propriate volume of prewarmed liquid Histowax (≤ 500 μL) to organoids using prewarmed plastic straws, quickly pipette two or three times and transfer the mixture to prewarmed soft EP tubes.
11. Incubate the soft EP tubes at 65°C overnight.
12. Transfer the soft EP tubes to RT or cold area to solidify Histowax.
13. Peel off the tube carefully and cut the Histowax to a smaller size suitable for embedding. This step concentrates organoids, facilitating their staining and visualization.
14. Re-embedding the small wax block to a new Histowax in metal mold.
15. Cool the mold and take out Histowax, which can be stored at RT for years.
16. Cut the Histowax into pieces for further staining.

The steps of organoids DNA and RNA extraction are in the Supplemental Materials.

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1
Troubleshooting.

Step	Problem	Possible reason	Solution
I.i-5	Low yield after digestion	Over- or under-digestion of liver tissue	Check the duct cells every 20 min during digestion, (Fig. 2a upper row)
I.i-7 I.ii-2	Large amount of cell debris	Over-digestion of the liver tissue or the gravity sinking time was too long	When large flakes of ducts appear, stop the digestion; shorten the gravity sinking time
II-2	Air bubbles in the Matrigel	The Matrigel was pipetted too quickly	Pipetted slowly when seeding; if bubbles are formed, centrifuged tube at 4°C to push the bubbles to the top of the mixture
II-3	Matrigel solidified in the tube or in the tip	Over-temperature of Matrigel when seeding	Keep the Matrigel mixture on ice and pre-cold the tips; perform seeding procedure as soon as possible
III-5	Low formation rate of passaged organoids	Over-digestion of organoids or mechanical separation too fierce.	Check the single cells under a microscope every 2 min; slow down the pipetting speed.
VI.ii-10	Wax blocks touch softly	Not enough incubation of wax blocks in cold area or too much vapor mixed in the wax.	Prolong the incubation time of wax in cold area and keep care of the vapor
VI.ii-13	Wax blocks crack easily	The wax block has not set completely; peel off the soft EP tube too fiercely.	Prolong the incubation time of wax in cold area; Cut the soft EP tube by fine scissor before peeling off the tube

Step times

Step I.i-ii, collection of mouse liver bile-duct cells: 4 h

Step I.iii, collection of mouse gallbladder cholangiocytes: 2 h

Step I.iv, isolation of mouse intestinal crypts: 2 h

Step II, seeding of mouse cholangiocytes or crypts: 30 min

Step III, passaging mouse organoids: 30–45 min

Step IV, harmonizing organoid growth rate: 40–60 min

Step V, cryopreservation and thawing of mouse organoids: 30 min each

Step VI, analysis of organoids: 2 days for isolation of DNA (Supplemental method I), 40–60 min for isolation of RNA (Supplemental method II), 3 days for immunofluorescence staining and 2 days for

immunohistochemical staining.

Results

Establishment of organoid culture

The bile ducts, cholangiocytes, and crypts embedded in Matrigel developed into 3D structures (Fig. 2). Cho-orgs from cholangiocytes were cystic spheres, whereas primary cho-orgs from bile ducts comprised cystic sphere-like organoids, which can separate after passage. In-orgs were bud-shaped with cells aggregated inside them.

Growth of organoids

After seeding for 2–4 days, cells started to develop into a cyst-like structure (Fig. 3a). After 10–14 days, the organoids increased in size and matured (Fig. 3b) and could be passaged at a 1:3–1:4 ratio for further expansion.

Characteristics of cho-orgs

Cho-orgs expressed the cholangiocyte markers Krt19 (Fig. 4a) and Krt7 (Fig. 4b), showed high proliferative activity, and consisted of more than one cell layer (Fig. 4c).

Digestion in TrypLE Express solution is essential for organoid viability. Prolonged incubation time leads to over-digestion and lower cell viability; insufficient digestion results in a small number of organoids. Digestion should be monitored under a light microscope to verify that 80–90% of the cells are single cells and determine the optimum digestion time.

The growth rate of cholangioids from intrahepatic bile ducts was slower than that of cholangioids from gallbladders, but the former could be passaged for months (Fig. 4d).

Stable growth of cho-orgs after cryopreservation and recovery

Organoids grew stably after thawing, as observed by light microscopy (Fig. 5). After stable culture and harmonizing of growth rates, the organoids were ready for RNA/DNA isolation, immunochemical staining, or cryopreservation.

Conclusions

Our protocols have high repeatability if tissue dissociation steps performed correctly and the composition of medium is precise. Cholangiocyte/bile duct and crypt collection from mouse liver or small intestine was achieved by gravity settling and multistep centrifugation. The organoids were stable after passaging,

cryopreservation, and thawing and suitable for mechanistic analysis, preclinical research, and drug screening.

Abbreviations

3D
Three-dimensional; PSC:primary sclerosing cholangitis; IBD:inflammatory bowel diseases; EGF:Epidermal growth factor; FGH:Fibroblast growth factor; HGF:hepatocyte growth factor; ROCK:Rho kinase; IF:Immunofluorescence; IHC:Immunohistochemistry

Declarations

Acknowledgment

Not applicable.

Authors' contributions

WC and HC contributed to the study design and method optimize. WC, QY, and RW contributed to organoids culture and experiments. WC, YX, JY, and JW contributed to methodological optimization. WC, and QY contributed to the production of figures. WC and HC contributed to the manuscript writing. LL conducted the study supervision. All authors have read and approved the final manuscript.

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Availability of date and materials

All data generated or analyzed during this study are included in this article.

Declarations

Ethics approval and consent to participate

All animal experimental procedures were conducted according to a protocol approved by the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University (reference number 2020-1088).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interest.

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Figures

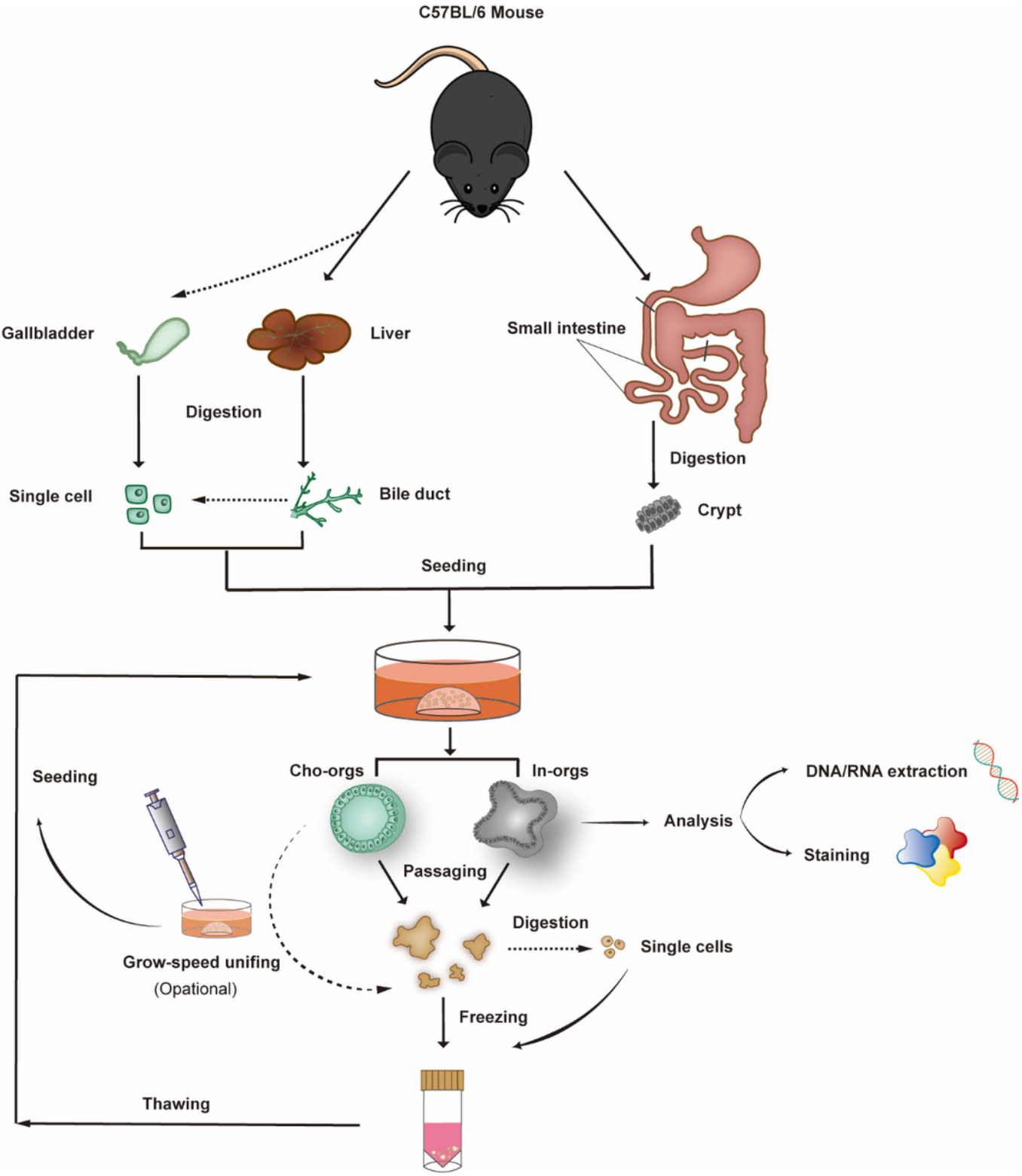

Figure 1

Organoid isolation, culture, growth-rate harmonization, passaging, and freezing. Bile ducts, single bile duct (or gallbladder) cells, and crypts from the small intestine derived from C57BL/6 mice form 3D structures after 7–14 days of culture (5–7 days for gallbladder-derived organoids) in Matrigel. After expansion, organoids can be characterized or frozen in liquid nitrogen.

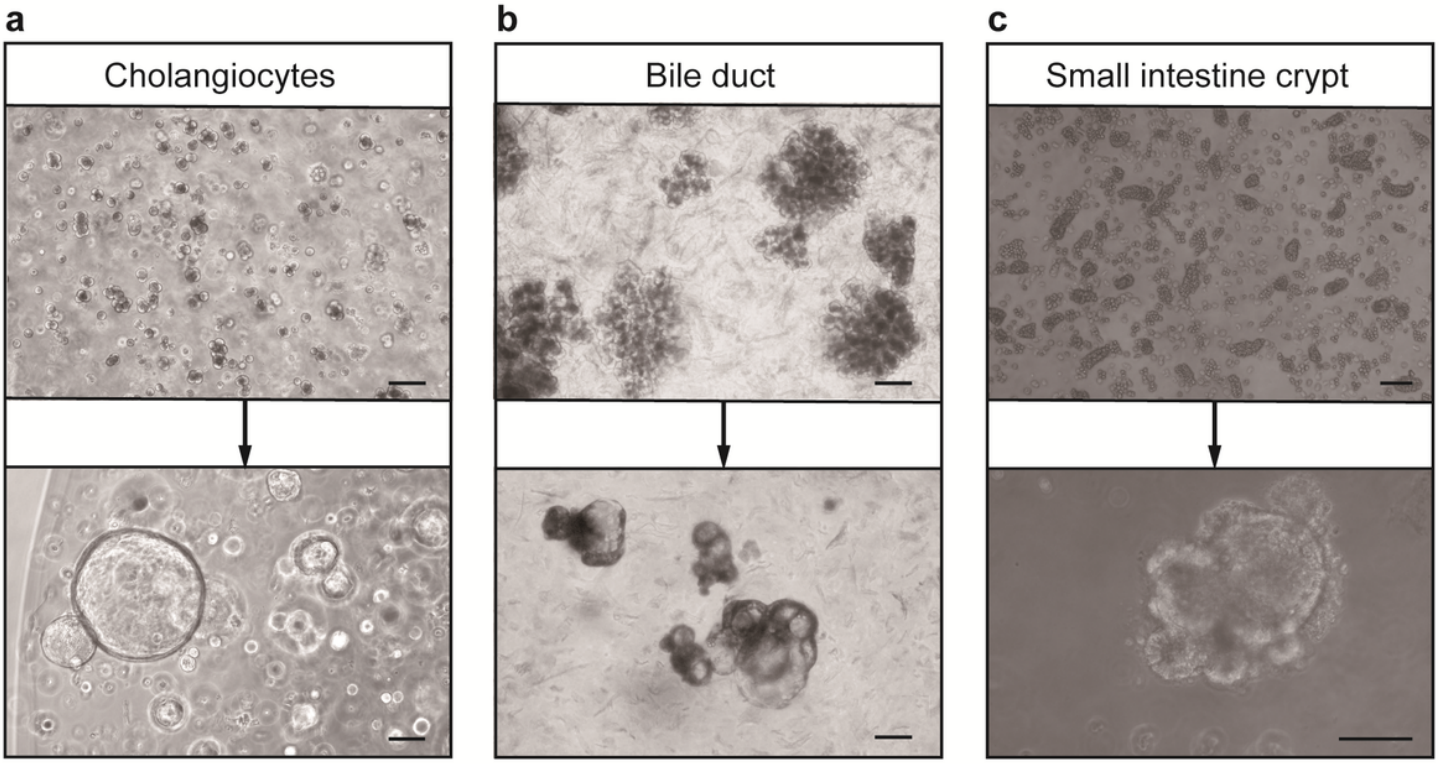

Figure 2

Organoid culture from bile ducts and small intestines of mice. After gravity settling and multi-step centrifugation, cholangiocytes (derived from bile ducts), bile ducts, and crypts from the small intestine formed organoids after ~14 days in culture. Cholangiocytes (day 0) from (a) bile ducts formed cho-orgs (day 3, passage 2) (bar, 100 μm); (b) intrahepatic bile ducts (day 0) formed cho-orgs (day 3) (bar, 100 μm); (c) crypts from the small intestine (day 0) (bar, 200 μm) formed in-orgs (day 13) (bar, 100 μm).

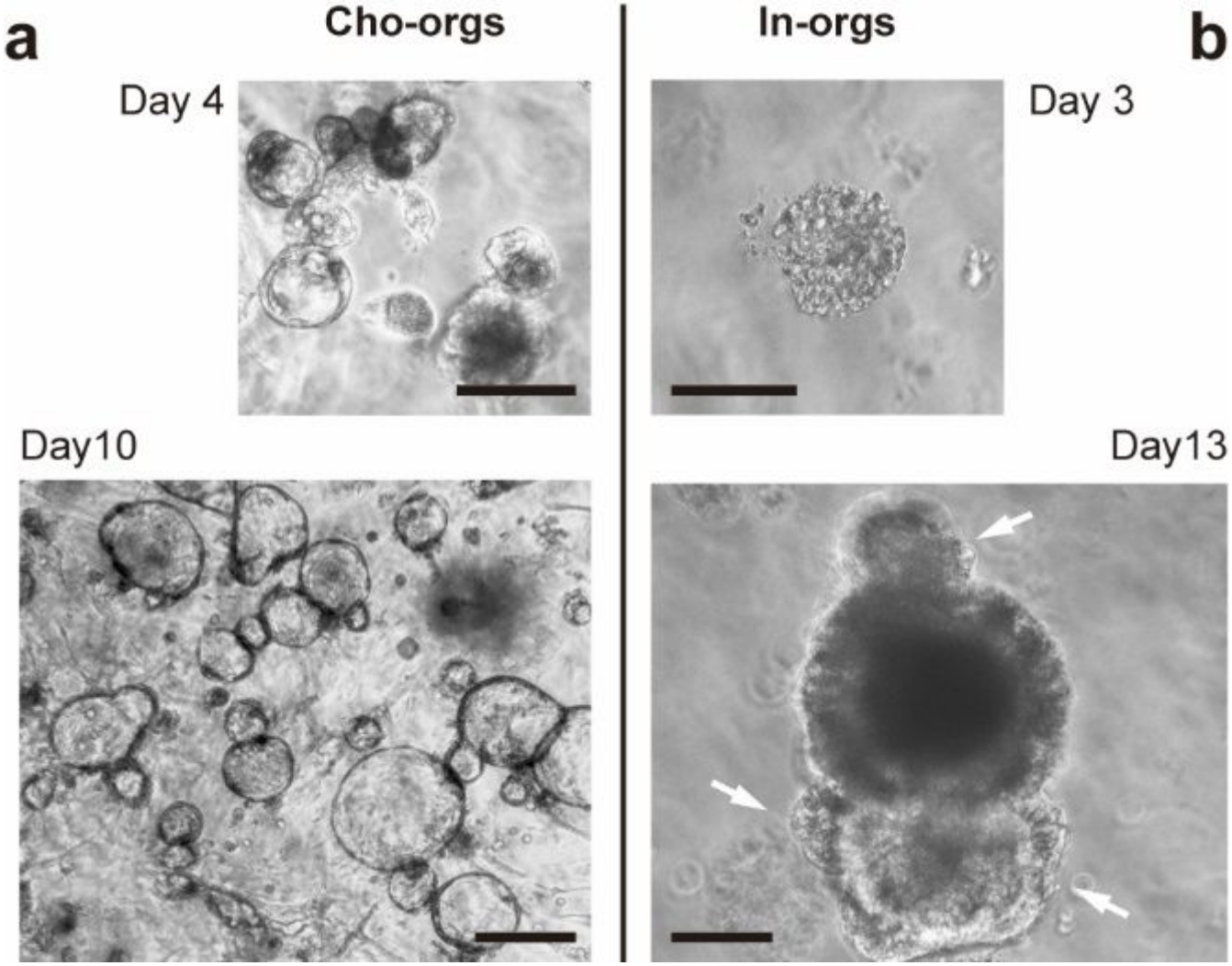

Figure 3

Development of cho-orgs and in-orgs. Cholangiocytes (bile ducts) and crypts from the small intestine embedded in Matrigel developed into cyst-like structures. Cells congregated in the cysts of in-orgs (day 3) and were outside of cho-orgs (day 4). After culturing for 10–13 days, cho-orgs (day 10) and in-orgs (day 13) increased in size, and in-orgs developed three buds (white arrow) to form a mature structure (bar, 100 μm).

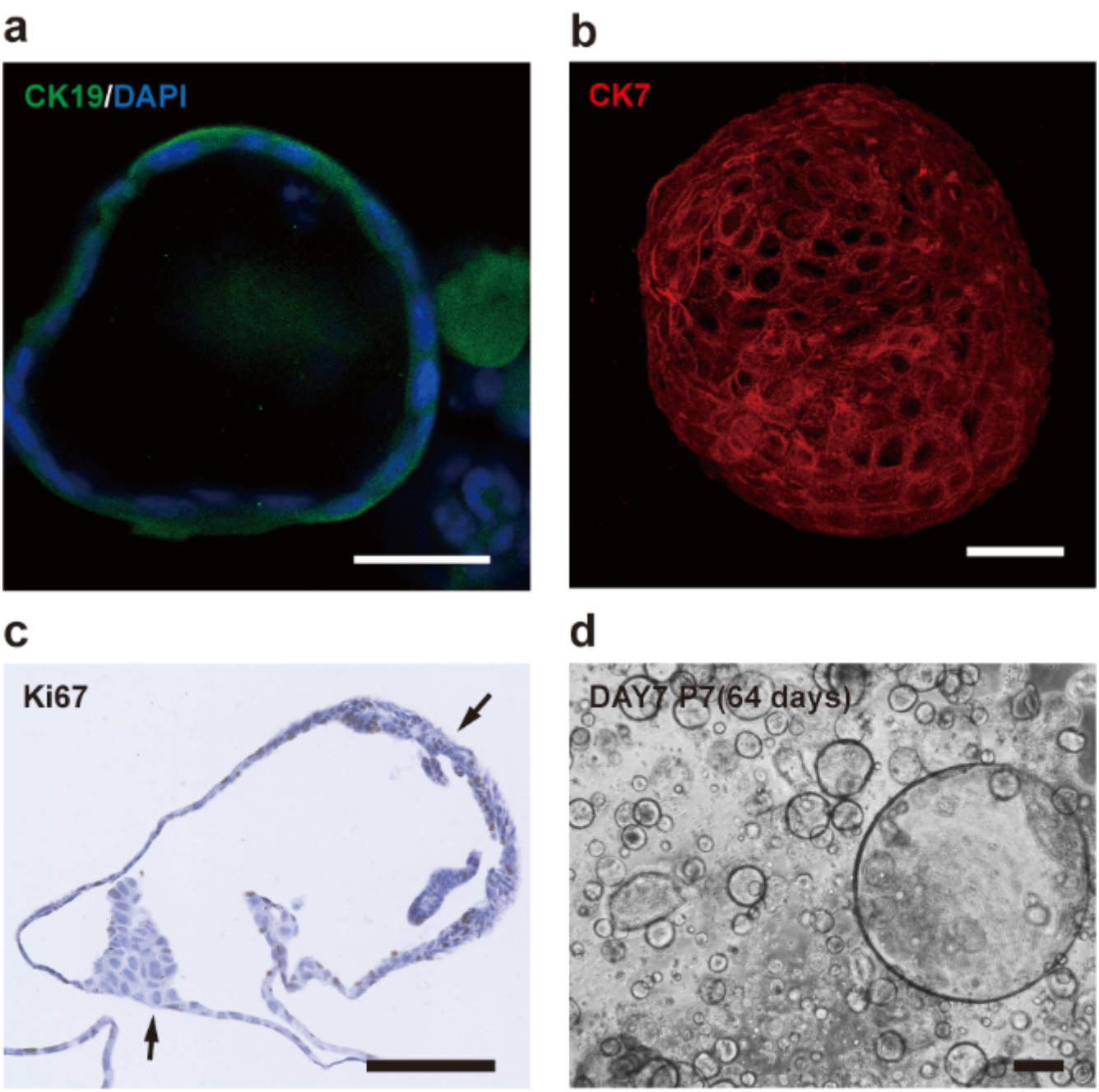

Figure 4

Characteristics of cho-orgs. (a) Immunofluorescence staining for the cholangiocyte marker CK19 (green) (bar, 100 μm). (b) Immunofluorescence staining for CK7 (red) in a 3D structure (bar, 100 μm). (c) Cho-orgs consisted of more than one cell layer and express the proliferation marker Ki67 (bar, 100 μm). (d) Cho-orgs from intrahepatic bile ducts were cultured for 7 days after seeding, and passaged seven times, for a total culture duration of > 64 days (bar, 200 μm).

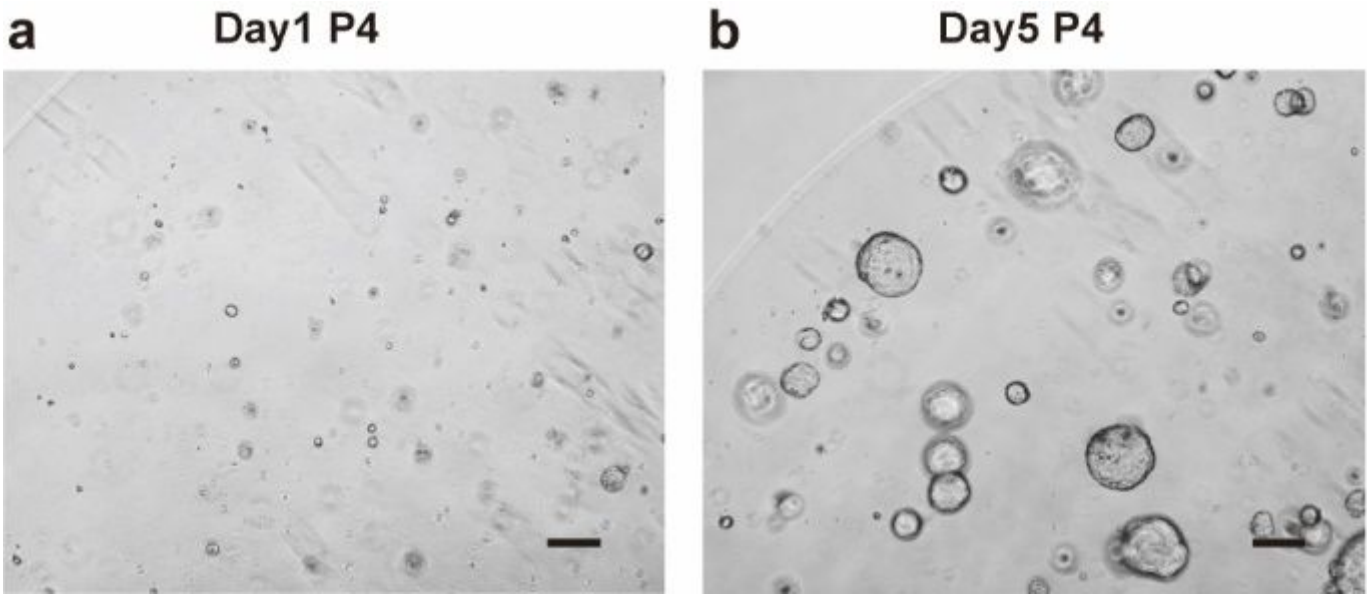

Figure 5

Organoid culture after thawing. (a) Cho-orgs cultured for 1 day after thawing (bar, 200 μm) (P4, passage 4). (b) Cho-orgs cultured for 5 days after thawing (bar, 200 μm).

Supplementary Files

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