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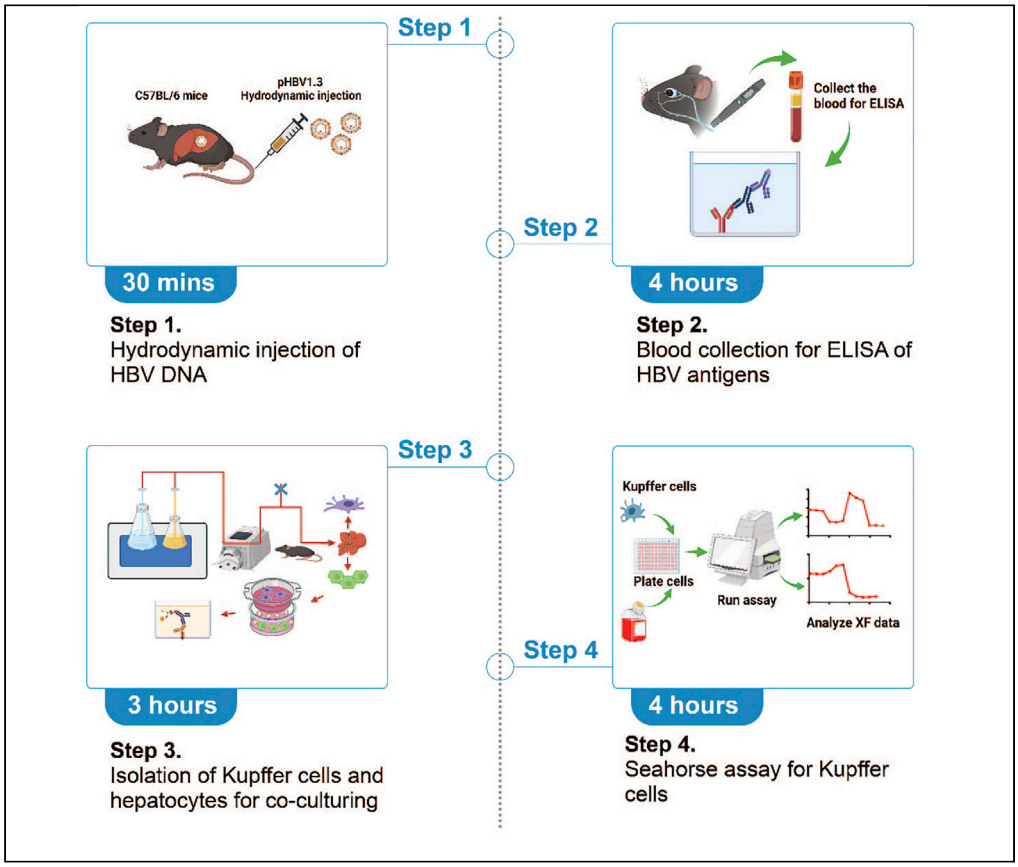
Protocol

Analysis of the interplay between hepatitis B virus-positive hepatocytes and Kupffer cells *ex vivo* using mice as a model



Kupffer cells play critical roles in both hepatitis B virus (HBV) persistence and clearance. Here, we provide a protocol for studying the interplay between Kupffer cells and HBV-positive hepatocytes *ex vivo* using mice as a model. This protocol includes hydrodynamic injection of HBV DNA into mouse hepatocytes, liver perfusion for isolating hepatocytes and Kupffer cells, and Seahorse metabolic analysis of Kupffer cells. This protocol allows the detailed analysis of how HBV-positive hepatocytes and Kupffer cells impact each other *ex vivo*.

Yumei Li, Jiyoung Lee, Doumet Georges Helou, Omid Akbari, Jing-hsiung James Ou

jamesou@usc.edu

Highlights

Optimized protocol for hydrodynamic injection of DNA into mouse hepatocytes

Efficient isolation of hepatocytes and Kupffer cells from the mouse liver

Co-culturing analysis of HBV-positive hepatocytes and Kupffer cells

Seahorse assay for studying mitochondrial metabolism of Kupffer cells

Li et al., STAR Protocols 3, 101364
June 17, 2022
https://doi.org/10.1016/j.xpro.2022.101364

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Analysis of the interplay between hepatitis B virus-positive hepatocytes and Kupffer cells *ex vivo* using mice as a model

Yumei Li,[1,2] Jiyoung Lee,[1] Doumet Georges Helou,[1] Omid Akbari,[1] and Jing-hsiung James Ou[1,3,*]

[1]Department of Molecular Microbiology and Immunology, University of Southern California, Keck School of Medicine, Los Angeles, CA 90033, USA

[2]Technical contact

[3]Lead contact

*Correspondence: jamesou@usc.edu
https://doi.org/10.1016/j.xpro.2022.101364

SUMMARY

Kupffer cells play critical roles in both hepatitis B virus (HBV) persistence and clearance. Here, we provide a protocol for studying the interplay between Kupffer cells and HBV-positive hepatocytes *ex vivo* using mice as a model. This protocol includes hydrodynamic injection of HBV DNA into mouse hepatocytes, liver perfusion for isolating hepatocytes and Kupffer cells, and Seahorse metabolic analysis of Kupffer cells. This protocol allows the detailed analysis of how HBV-positive hepatocytes and Kupffer cells impact each other *ex vivo*.

For complete details on the use and execution of this protocol, please refer to Li et al. (2022).

BEFORE YOU BEGIN

Hydrodynamic injection is a method that can be used to introduce DNA into mouse hepatocytes *in vivo* (Wu et al., 2017). This method involves the injection of a large amount of saline through tail vein within a short period of time. This sudden influx of a large amount of saline, which travels via inferior vena cava through the liver, transiently causes liver to expand and hepatocellular membranes to tear. If DNA is included in the injection, it will enter hepatocytes. Hepatitis B virus (HBV) cannot infect mice due to the lack of proper receptors on mouse hepatocytes for it to initiate infection. However, if its genomic DNA is introduced into mouse hepatocytes by hydrodynamic injection, it can direct viral gene expression and the production of progeny virus particles to stimulate host immune responses, including the activation of macrophages (i.e., Kupffer cells) (Du et al., 2021). This method, in combination with the method to efficiently isolate hepatocytes and Kupffer cells from mice, allows the study of the effect of HBV-positive hepatocytes on Kupffer cells *ex vivo* and vice versa. This protocol provides detailed procedures for hydrodynamic injection, the isolation of hepatocytes and Kupffer cells, the co-culturing of HBV-positive hepatocytes and Kupffer cells *ex vivo*, and the analysis of the effect of HBV on mitochondrial metabolism of Kupffer cells.

Institutional permissions

The animal work described in this protocol was conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health and approved by the University of Southern California *Institutional Animal Care and Use Committee* (IACUC). Readers who wish to conduct their animal work as described in this protocol will also need to obtain the permission from their IACUC following their institutional regulations.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
F4/80 Monoclonal Antibody (BM8), PE (1:100 dilution)	Invitrogen	Cat# 12-4801-82; RRID:AB 465923
FITC anti-mouse/human CD11b Antibody (1:100 dilution)	BioLegend	Cat# 101206 RRID: AB_312789
APC/Cyanine7 anti-mouse CD45, clone:30-F11 (1:300 dilution)	BioLegend	Cat# 103116 PRID:AB 312981
PE/Cyanine7 anti-mouse Tim-4, clone: RMT4-54 (1:300 dilution)	BioLegend	Cat# 130010 RRID: AB_2565719
Pacific Blue anti-mouse CD45 antibody (1:100 dilution)	BioLegend	Cat# 103126 RRID: AB_493535
Rat IgG2a kappa Isotype Control (eBR2a), PE (1:100 dilution)	Invitrogen	Cat# 12-4321-80 RRID: AB 1834380
Chemicals, peptides, and recombinant proteins		
Collagenase from Clostridium histolyticum	Sigma-Aldrich	Cat# C5138
OptiPrep™ Density Gradient Medium	Sigma-Aldrich	Cat# D1556
Deoxyribonuclease I from bovine pancreas	Sigma-Aldrich	Cat# DN25
DPBS, 1×	Corning	Cat# 21-031-CV
Zetamine-Ketamine Hydrochloride Injection	VETone	Cat# 13985-584-10
Dulbecco's Modified Eagle's Medium (DMEM)	Sigma-Aldrich	Cat# D6046
HEPES 1 M solution	Thermo Fisher Scientific	Cat# 15630-080
Penicillin-Streptomycin (10,000 U/mL)	Thermo Fisher Scientific	Cat# 15140-122
L-Proline Stock Solution (30 mg/mL)	MP Biomedicals	Cat# 219472825
Insulin Stock Solution (10 μg/mL)	Sigma-Aldrich	Cat# I1882
Dexamethasone (Dex) Stock Solution	Sigma-Aldrich	Cat# D8893
EGF Stock Solution (10 ug/mL)	Sigma-Aldrich	Cat# E9644
L-ascorbic Acid 2-Phosphate (Asc-2P) Stock Solution (0.1 M)	FUJIFILM Wako Chemicals	Cat# 013-12061
DMSO (HybriMax)	Sigma-Aldrich	Cat# D2650
Collagen I, Rat Tail, 100 mg	Corning	Cat# 354236
Percoll	Sigma-Aldrich	Cat# P1644-1L
Trypan blue	Thermo Fisher Scientific	Cat# T10282
EGTA	Sigma-Aldrich	Cat# 324626
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat# A7030-100G
Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$)	Sigma-Aldrich	Cat# C8106-500G
Collagenase type IV	Sigma-Aldrich	Cat# C5138
Sodium chloride (NaCl)	Sigma-Aldrich	Cat# S9888-500G
Potassium chloride (KCl)	Sigma-Aldrich	Cat# P3911
Magnesium chloride hexahydrate (MgCl2●6H2O)	Sigma-Aldrich	Cat# M2670-500G
Glucose	Sigma-Aldrich	Cat# D9434-250G
Amphotericin B	Sigma-Aldrich	Cat# PHR1662-500MG
Magnesium sulfate heptahydrate (MgSO4●7H2O)	Sigma-Aldrich	Cat# 63138
Sodium phosphate dibasic dihydrate (Na_2HPO_4)	Sigma-Aldrich	Cat# 71643
Potassium phosphate monobasic (KH2PO4)	Sigma-Aldrich	Cat# P0662-500G
Critical commercial assays		
Seahorse XF Glycolytic Rate Assay Kit	Agilent	Cat# 103344-100
Rotenone and Antimycin A (Rot/AA) kit 1	Agilent	Cat# 103344-100
2-deoxy-D-glucose (2-DG)	Agilent	Cat# 103344-100
Seahorse XF Cell Mito Stress Test Starter Pack	Agilent	Cat# 103708-100
Oligomycin	Agilent	Cat# 103708-100
Carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP)	Agilent	Cat# 103708-100
Rot/AA kit 2	Agilent	Cat# 103708-100
Seahorse FluxPaks	Agilent	Cat# 102601-100
Seahorse XF96 V3 PS Cell Culture Microplates	Agilent	Cat# 101085-004

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Seahorse XF Media & Calibrant	Agilent	Cat# 103575-100
Seahorse XF Cell Mito Stress Test Kit	Agilent	Cat# 103015-100
HBsAg ELISA kit	International Immuno- Diagnostics	Cat# 255
HBeAg ELISA kit	International Immuno- Diagnostics	Cat# 253
LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit	Invitrogen	Cat# L34961
Experimental models: Organisms/strains		
Mouse: C57BL/6J (8 weeks old, male)	The Jackson laboratory	Stock No: 000664
TG05 HBV transgenic mice (8 weeks old, male)	This paper	N/A
Recombinant DNA		
pHBV1.3mer	This paper	N/A
pUC19	Addgene	Cat #50005
Software and algorithms		
FlowJo v.10.5.3	TreeStar	https://www.flowjo.com; RRID: SCR_008520
GraphPad Prism 8.4.3	GraphPad	https://www.graphpad.com; RRID: SCR_002798
Other		
Mouse restraining device	Braintree Scientific Inc	Cat# TV-150-SM
Heat source (heat lamp with 120 W bulb)	This paper	N/A
Sterile Cell Strainers, 70 μm	Corning	Cat# 07-201-431
Cell culture insert, 6-well Plate with 0.4 μm Transparent PET Membrane	Corning	Cat# 353090
Sterile Cell Strainers, 40 μm	Corning	Cat# 07-201-430
6-well Plate	Corning	Cat# 353046
0.22 μm Basix™ Syringe Filters, PES, Sterile	Fisher Scientific	Cat# 13-100-106
BD PRECISIONGLIDE™ 27 G × 1/2″ HYPODERMIC NEEDLES	Becton Dickinson	Cat# 305109
3 mL BD Luer-Lok™ Syringe sterile	Becton Dickinson	Cat# 309657
Polystyrene Reservoirs	VWR	Cat# 89094-678
Masterflex™ L/S™ Platinum-Cured Silicone Precision Tubing (size 14C tube)	Fisher Scientific	Cat# 96410-14
TERUMO SURFLO IV CATHETER 24 G × 3/4″	Terumo	Cat# SR-OX2419CA
Handheld Analog Brix/Sucrose Refractometer	Fisher Scientific	Cat# 12-561-339
Deluxe Water Baths	Fisher Scientific	Cat# FSGPD20
Centrifuge 5804 R	Eppendorf	Cat# 022629981
Seahorse XFe96 Analyzer	Agilent	N/A
MasterFlex Microprosser Pump Drive Model 7524-00	Cole-Parmer	Cat# 27629
General Laboratory Scissors, straight, S/S	TED PELLA, INC	Cat# 1328
Semken Forceps	Fine Science Tools	Cat# 11009-13
LOOK 4-0 Silk Suture Spool, Black Braid (MFID: SP104)	HOSPEQ	Cat# SSSP104
Disposable Syringes with Luer-Lok™ Tips	BD	Cat# 309628
Stainless Steel Lab Scoop	Fisher Scientific	Cat# 01-189-170
Rubber Policeman	VWR	Cat# 470104-462
Straight Locking Hemostat	Fisher Scientific	Cat# 16-100-115
Curved Locking Hemostat	Fisher Scientific	Cat# 16-100-117

MATERIALS AND EQUIPMENT

Preparation of reagents

Timing: 15 min for preparation of hydrodynamic injection solution

Timing: 1 h for preparation of Hank's solutions and Solutions A–D

Prepare a solution of DPBS containing pHBV1.3mer, a 1.3mer overlength HBV genomic DNA cloned in the pUC19 vector.

Prewarm the Solution at 37°C.

Hydrodynamic injection solution	
Reagent	Amount
pHBV1.3mer	20 μg
DPBS	8% of the mouse body weight (e.g., 2 mL for a 25 g mouse)
Freshly prepare the solution each time, do not store it.	

⚠ CRITICAL: Mix pHBV1.3mer with DPBS, and filter-sterilize using a 0.2 μm filter before hydrodynamic Injection.

Optional: pHBV1.3mer may be replaced with other DNA plasmids such as pUC19 or other gene-expressing plasmids.

Prepare 10× Hank's Solution.

10× Hank's Solution		
Reagent	Final concentration	Amount
NaCl	1.37 M	80 g
KCl	54 mM	4 g
$MgSO_4 \bullet 7H_2O$	8 mM	2 g
$Na_2HPO_4 \bullet 2H_2O$	3.3 mM	0.6 g
KH_2PO_4	4.4 mM	0.6 g
Add H_2O to	n/a	1 L
The solution can be stored at 18°C–25°C for up to 6 months.		

⚠ CRITICAL: Filter-sterilize the 10× Hank's solution using a 0.2 μm filter.

Optional: As an alternative, 10× Hank's solution may be replaced with 10× HBSS from commercial vendors.

Prepare 1× Hank's Solution with HEPES buffer.

1× Hank's Solution with HEPES buffer		
Reagent	Final concentration	Amount
10× Hank's solution	1×	100 mL
1 M HEPES	12.4 mM	12.4 mL
7.5% $NaHCO_3$ (7.5 g $NaHCO_3$ in 100 mL H_2O)	0.2%	26.8 mL
Final volume with H_2O to	n/a	1 L
pH=7.4		
The solution can be stored at 18°C–25°C for up to 6 months.		

⚠ CRITICAL: Filter-sterilize the 1× Hank's solution with a 0.2 μm filter.

Prepare Solution A.

Solution A (for 2–3 mice)		
Reagent	Final concentration	Amount
EGTA (34.2 mg/mL)	0.6 mM	1 mL
1× Hank's solution	n/a	149 mL
Bovine serum albumin (BSA)	2%	3 g
	Mix to dissolve BSA and filter to sterilize the solution.	
The solution can be stored at −20°C for up to 1 month.		

⚠ CRITICAL: Filter-sterilize Solution A using a 0.2 μm filter.

Optional: As an alternative, EGTA may be replaced with EDTA from commercial vendors.

Prepare Solution B.

Solution B (for 2–3 mice)		
Reagent	Final concentration	Amount
$CaCl_2 \cdot 2H_2O$	4 mM	58.8 mg
1× Hank's solution	n/a	100 mL
Collagenase type IV (Sigma-5138)	n/a	40–42 mg
Mix to dissolve collagenase and filter to sterilize the solution		
Use the solution fresh and do not store it.		

⚠ CRITICAL: Filter-sterilize Solution B with a 0.2 μm filter.

Prepare Solution C.

Solution C (for 2–3 mice)		
Reagent	Final concentration	Amount
$CaCl_2 \cdot 2H_2O$	4 mM	58.8 mg
1× Hank's solution to	n/a	100 mL
Mix and filter to sterilize the solution		
The solution can be stored at 18°C–25°C for up to 6 months.		

⚠ CRITICAL: Filter-sterilize solution C with a 0.2 μm filter.

Optional: As an alternative, Solution C buffer may be replaced with 1× DPBS.

Prepare 10× Percoll buffer.

10× Percoll buffer		
Reagent	Final concentration	Amount
NaCl	1.4 M	81.2 g
KCl	50 mM	3.75 g
$MgCl_2 \bullet 6H_2O$	8 mM	1.624 g
Na_2HPO4	16 mM	2.272 g
KH_2PO4	4 mM	0.554 g
Add H_2O to	n/a	1 L
The solution can be stored at 18°C–25°C for up to 6 months.		

⚠ CRITICAL: Filter-sterilize 10× Percoll buffer with a 0.2 μm filter.

Prepare Percoll solution.

Percoll solution	
Reagent	Amount
10× Percoll buffer	5.2 mL
Add Percoll to	40 mL
This solution can be stored at 4°C for up to 6 months.	

⚠ CRITICAL: Filter-sterilize solution C with a 0.2 μm filter.

Prepare OptiPrep Density Gradient Solutions.

Preparation of Solution D		
Reagent	Final concentration	Amount
Na_2HPO_4	0.86 mM	1.22 g
NaH_2PO_4	0.14 mM	0.169 g
NaCl	134 mM	7.808 g
KCl	5 mM	0.373 g
$MgCl_2 \cdot 6H_2O$	0.8 mM	0.166 g
Glucose	0.2%	2 g
BSA	3%	30 g
Add ddH_2O to	n/a	1 L
pH=7.4		
Add 37 mL ddH_2O to achieve 270 mOs-280 mOs.		
The solution can be stored at −20°C for up to 3 months. mOs is milliosmolarity. 270–280 mOs is the desired osmolarity for cell separation.		

⚠ CRITICAL: Filter-sterilize Solution D with a 0.2 μm filter.

Preparation of OptiPrep Density Gradient Solutions (Specific gravity: 1.081)	
Reagent	Amount
OptiPrep	55 mL
270 mOs Solution D	200 mL
Total	255 mL
The solution can be stored at 4°C for up to 2 weeks.	

Preparation of OptiPrep Density Gradient Solutions (Specific gravity: 1.056)	
Reagent	Amount
OptiPrep	40 mL
270 mOs Solution D	200 mL
Total	240 mL
The solution can be stored at 4°C for up to 2 weeks.	

Preparation of OptiPrep Density Gradient Solutions (Specific gravity: 1.042)	
Reagent	Amount
OptiPrep	22 mL
270 mOs Solution D	200 mL
Total	222 mL
The solution can be stored at 4°C for up to 2 weeks.	

Preparation of OptiPrep Density Gradient Solutions (Specific gravity: 1.035)	
Reagent	Amount
OptiPrep	17 mL
270 mOs Solution D	200 mL
Total	217 mL
The solution can be stored at 4°C for up to 2 weeks.	

⚠ CRITICAL: Calculate Specific gravity (SG) with following formula: SG=0.0038 × read value+1.0142. The read value of the solution is obtained using the refractometer.

Prepare Hepatocytes culture medium.

Preparation of DMEM containing 10% fetal bovine serum (FBS)	
Reagent	Amount
DMEM	450 mL
1 M HEPES buffer	10 mL
Penicillin-Streptomycin (10,000 U/mL)	5 mL
Heat inactivated FBS	50 mL
Total volume	515 mL
The medium can be stored at 4°C for up to 6 months.	

Preparation of hepatocytes culture medium	
Reagent	Amount
DMEM with 10% FBS	500 mL
L-Proline Stock Solution (30 mg/mL)	250 µL
Insulin Stock Solution (10 mg/mL)	12.5 µL
Dexamethasone (Dex) Stock Solution (10 mM)	2.5 µL
EGF Stock Solution (10 µg/mL)	250 µL
L-ascorbic Acid 2-Phosphate (Asc-2P) Stock Solution (0.1 M)	500 µL
DMSO	10.2 mL
Total volume	511.2 mL
The medium can be stored at 4°C for up to 6 months.	

⚠ CRITICAL: After thawing FBS, heat at 56°C for 30 min to inactivate complement proteins.

Prepare Kupffer cells culture medium.

Kupffer cells culture medium	
Reagent	Amount
DMEM	450 mL
Amphotericin B (25 mg/mL)	0.5 mL
Penicillin-Streptomycin (10,000 U/mL)	5 mL
Heat inactivated FBS	50 mL
Total volume	505 mL
The medium can be stored at 4°C for up to 6 months.	

⚠ CRITICAL: After thawing FBS, heat at 56°C for 30 min to inactivate complement proteins.

Preparation of collagen-coated Petri dishes or 6 wells plates.

Collagen solution	
Reagent	Amount
DPBS (1×)	50 mL
Collagen I	0.5 mL

Plate coating with collagen	
Reagent	Amount
Collagen solution	3 mL/10-cm dish
Collagen solution	0.5 mL/well of 6-well dish
Incubate at 37°C for 1 h, and then remove the collagen I solution	
UV irradiation for 30 min	
Store the plates or dishes at 4°C for up to 6 months.	

⚠ CRITICAL: After collagen coating, place the plates/dishes under the UV light for 30 min in the cell culture hood to sterilize the plates/dishes.

Preparation of Solution 1		
Reagent	Final concentration	Amount
Oligomycin	100 μM	630 μL
FCCP	100 μM	720 μL
Rot/AA kit 1	50 μM	540 μL
Use the solution fresh and do not store it.		

⚠ CRITICAL: All the dissolved compounds keep on ice.

Preparation of Solution 2		
Reagent	Final concentration	Amount
Port A (Oligomycin)	2 μM	20 μL
Port B (FCCP)	0.75 μM	22 μL
Port C (Rot/AA kit 1)	0.5 μM	25 μL
Use the solution fresh and do not store it.		

⚠ CRITICAL: All the dissolved compounds keep on ice.

Preparation of Solution 3		
Reagent	Final concentration	Amount
Rot/AA kit 2	50 μM	540 μL
2-DG	500 mM	3,000 μL
Use the solution fresh and do not store it.		

⚠ CRITICAL: All the dissolved compounds keep on ice.

Preparation of Solution 4		
Reagent	Final concentration	Amount
Port A (Rot/AA kit 2)	0.5 μM	20 μL
Port B (2-DG)	50 mM	22 μL
Use the solution fresh and do not store it.		

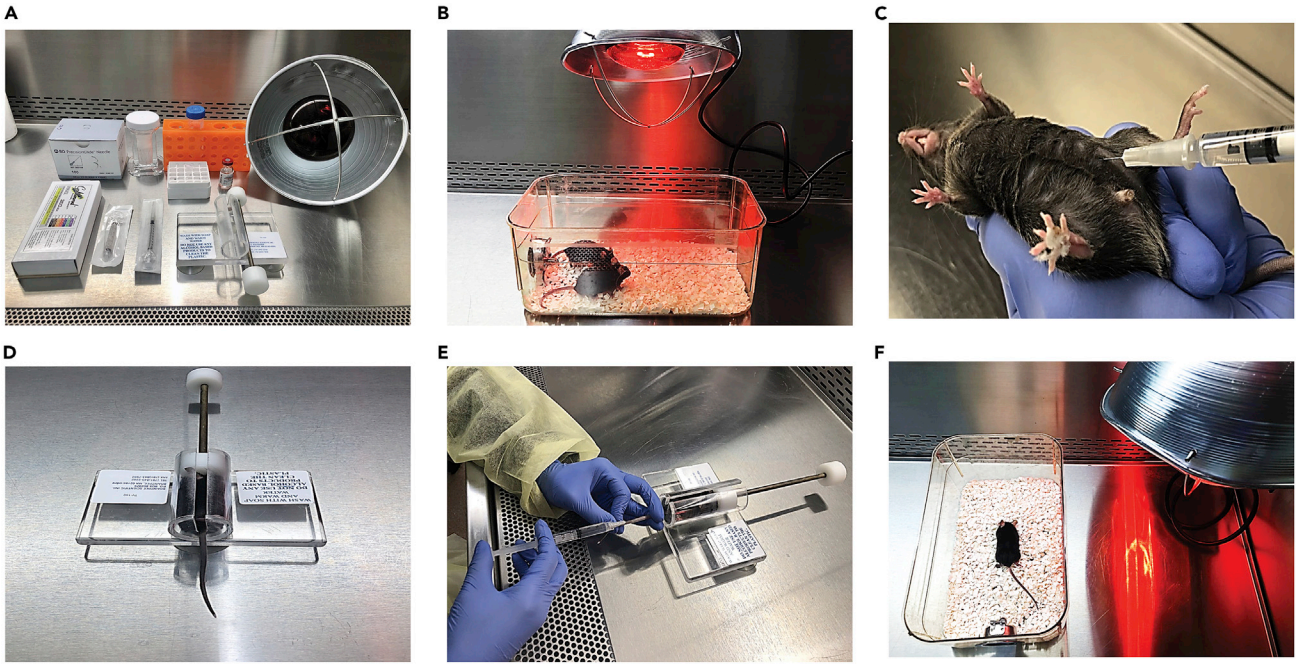

Figure 1. Hydrodynamic injection

(A) Reagents and the apparatuses needed for the injection.
(B) Dilating the tail blood vessels with a heat lamp.
(C) Anesthetizing the mouse with ketamine.
(D) Immobilizing the mouse with a restraining device.
(E) Inserting the needle in the distal portion of the tail vein and injecting the DNA solution into the tail vein.
(F) Removing the mouse from the restrainer for recovery.

⚠ CRITICAL: All the dissolved compounds keep on ice.

STEP-BY-STEP METHOD DETAILS

Hydrodynamic injection

Timing: 30 min

The use of hydrodynamic injection for gene delivery into mouse hepatocytes was first reported more than 20 years ago (Liu et al., 1999; Zhang et al., 1999). The volume of the DNA solution used for the injection is about 8% of the mouse body weight. The solution is injected rapidly into mice via the tail vein within 5–8 s.

1. Prepare reagents and instruments required for hydrodynamic injection and mouse blood collection (Figure 1A).
2. Select 8-week-old C57BL/6 mice weighing about 19–25 g.
3. Mix 20 μg HBV DNA with a volume of DPBS equivalent to 8% of the mouse body weight. For a 20-gram mouse, the volume is approximately 1.6 mL.
4. Dilate tail blood vessels by warming mice with a heat lamp (120 W bulb) for 3–5 min before performing the injection (Figure 1B).

⚠ CRITICAL: Be cautious not to overheat the mice such as by putting the lamp too close to the mice or warming the mice for too long. Excessive movements or sweating of mice is a sign of overheating. Alternatively, the tail can also be warmed in 50°C water for a couple minutes.

5. Dilute ketamine10-fold with DPBS and anesthetize the mouse by intraperitoneal (i.p.) injection of 20 μL of the diluted ketamine (Figure 1C).
6. Immobilize the mouse with a mouse restrainer (Figure 1D).
7. With the heat lamp on, locate the dilated vein on the ventral side of the mouse tail, preferably near the tip of the tail.
8. Wipe the intended injection site with an alcohol pad for disinfection and to further increase the visibility of the vein.
9. Add 8% body weight-equivalent of the DNA-saline solution to the syringe. Connect the needle to the syringe and make sure there are no air bubbles.

   Note: With the needle facing up, tap the syringe with your finger a few times to move the air bubbles to the needle, and then carefully expel the air until a small amount of solution is removed.

10. With the syringe needle beveled side up and nearly parallel to the mouse tail, insert the needle in the distal portion of the tail. This allows a better view of the needle when it enters the vein. Inject the entire volume of the DNA solution into the tail vein of the mouse with a constant speed and complete the injection within 5–8 s (Figure 1E).

   ⚠ CRITICAL: The injection speed is critical for obtaining a good DNA transfection efficiency of hepatocytes.

11. Remove the mouse from the restrainer and place it into the cage for recovery (Figure 1F).

Mouse blood collection for serologic assays

Timing: 4 h

Here is an easy method for the collection of 100–500 μL blood from the mouse facial vein. This method involves the anesthetization of the mouse, the pricking of the freckle with a lancet, and the collection of the blood. Note that HBV is a human pathogen and therefore people working with mice should be vaccinated against HBV.

12. Collect blood from the mouse facial vein on day 3 after hydrodynamic injection.
    a. To collect the blood, place the non-dominant hand on the mouse, then use the thumb and index finger to grasp the mouse. Find the hairless freckles on the side of the jaw.
    b. Pick up the lancet with the other hand. Point the lancet at the distal end of the mouse's face near the base of the ear or the base of the distal end of the mouth. Prick the freckle with a lancet (Figure 2A).
    c. Collect 100–500 μL of blood in a 1.5 mL Eppendorf tube (Figure 2B). After collecting the blood sample, put the mouse back into the cage. Bleeding should stop immediately.
13. Centrifuge the sample at 12,000 × *g* for 5 min at 18°C–25°C.
14. Transfer the supernatant (i.e., the serum) to a new Eppendorf tube and store the serum at −20°C (Figure 2C).

   ⏸ Pause point: Serum can be stored at −20°C or −80°C.

15. As an injection control, measure HBV surface antigen (HBsAg) and e antigen (HBeAg) levels using the HBsAg and HBeAg ELISA kits following the manufacturer's protocols (Figure 2D).

Isolation of hepatocytes and Kupffer cells from mouse liver

Timing: 3 h

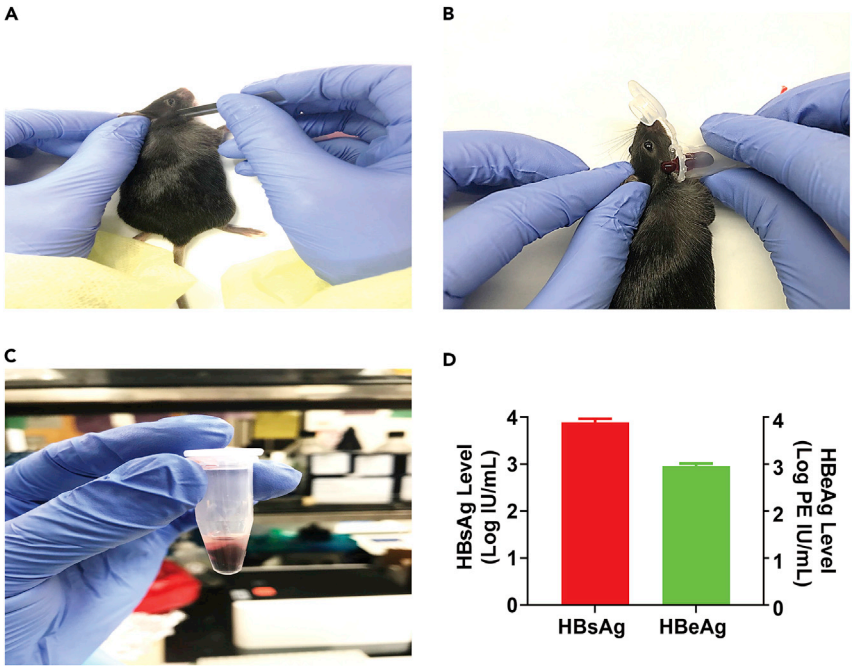

Figure 2. Mouse blood collection and measurement of HBsAg and HBeAg

(A) Pricking the freckle of the mouse with a lancet.

(B) Collecting blood from the facial vein.

(C) Centrifugation and collection of the serum.

(D) Measuring the levels of HBsAg and HBeAg in the mouse serum by ELISA. The results represent the mean ± SEM.

Here is the method to isolate hepatocytes and Kupffer cells from the mouse liver. This method yields hepatocytes and Kupffer cells with a high viability and purity. In this method, the mouse liver is perfused twice: the first one with a solution containing EGTA and BSA, and the second one with a solution containing collagenase. The purpose of the first step is to remove calcium ions (i.e., by EGTA) from epithelial cells to cause the rapid destruction of intercellular junctions and the loss of cell–cell contacts. The second step is to use collagenase to disrupt the supporting extracellular matrix of liver lobes. This is followed by the use of Percoll to isolate hepatocytes and the use of the OptiPrep density gradient to separate Kupffer cells from other nonparenchymal cells (NPCs).

Reagents

16. Pre-warm Solution A and Solution B in a 42°C water bath.
17. Prepare 70% ethanol solution (in spray and in a bottle).
18. Autoclave at least 50 mL of water (needed for washing the pump system each time).

Instruments

19. Mini-pump with size 14C tube.
20. Water bath (Figure 3A).
21. Surgical tools (scissors, forceps, sutures, syringes, and catheters (24 gauge for mouse) (Figure 3B).

Protocol (See also Methods Video S1).

22. Procedures for cleaning the pump.
    a. Pre-clean the pump with 70% ethanol solution for more than 5 min.

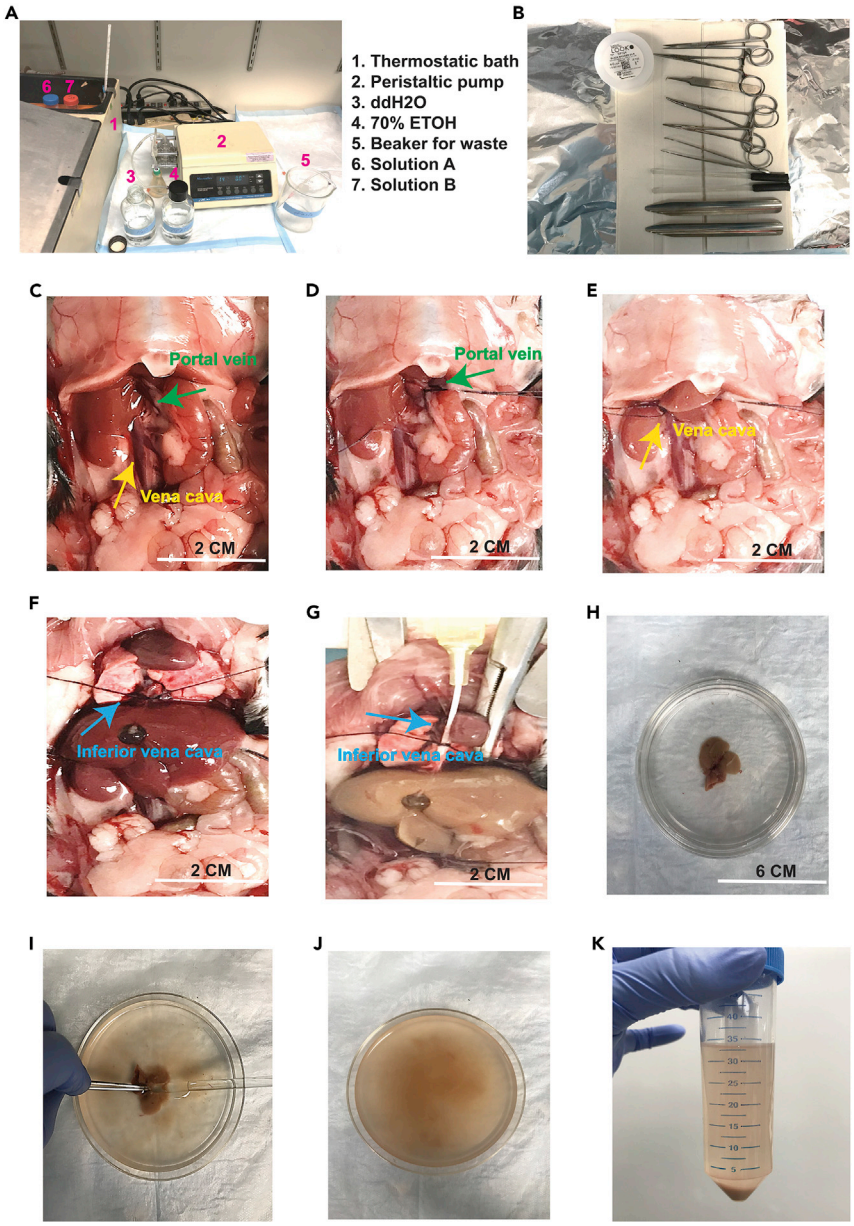

Figure 3. Procedures to isolate hepatocytes and Kupffer cells from the mouse liver

(A) Reagents and instruments.

(B) Surgical tools.

(C) Abdominal cavity opened with the intestine aside to uncover the venous system.

(D) Part of the portal vein and the suture beneath it held at both ends by a hemostat.

(E) A suture under the upper part of vena cava.

(F) An inserted 24-gauge catheter reaching the inferior vena cava downstream of the liver.

(G) The pale liver after a two-step perfusion.

(H) The liver removed from the mouse and placed in a petri dish with Solution C.

(I) Breaking the perfused liver into single cells with tools.

(J) Liver lobes dispersed into single cells.

(K) Separation of NPCs and hepatocytes by centrifugation. Scale bars in centimeters (CM) are provided for panels (C–H). See Methods Video S1 for details.

b. Flush the pump system with at least 50 mL of autoclaved water and ensure that all the water is completely removed.
c. Fill the pump system with Solution A.
d. Circulate Solution A (38°C–39°C) through the pump using a pumping rate of 7 mL/min.

⚠ CRITICAL: Pre-warming Solution A is essential for improving the cell viability.

23. Anesthetize the mouse with ketamine as described in step 7.
24. Apply 70% ethanol to the mouse abdomen for disinfection.
25. Place the sedated mouse on the operation board and fix the mouse legs with the tape to the board.
26. Open the abdominal cavity.
    a. Cut open the mouse abdomen (Figure 3C).
    b. Move the intestine to the right to uncover the venous systems (Figure 3C).
27. Insert the suture.
    a. Place a suture under the upper part of the portal vein (suture 1).
    b. Hold both ends of the suture with a hemostat (Figure 3D).
    c. Place another suture under the upper part of the vena cava (beside the right kidney) and tie a loose knot (suture 2) (Figure 3E).
28. Open the thoracic cavity.
    a. Open the thoracic cavity by cutting the diaphragm and chest bones beside the xiphisternum.
    b. Grab the xiphisternum with a tweezer and open the chest over the mouse face to unveil the heart.
    c. Turn the operation board around and puncture the heart's center using the sharp end of a 24-gauge catheter.
    d. Insert the 24-gauge catheter, which is connected to the tube in Solution A, into the right auricle and allow the catheter to reach the inferior vena cava downstream of the liver (Figure 3F).

⚠ CRITICAL: This is a critical step for a successful liver perfusion. It is essential to ensure that the catheter reaches the inferior vena cava downstream of the liver. This is important for the uniform distribution of the solution for liver digestion.

29. Perfuse the liver with Solution A.
    a. Cut the inferior vena cava.
    b. Start the perfusion of Solution A at a flow rate of 7 mL/min.
    c. Cut the portal vein with a scissor after lifting suture 1. Do not cut the suture.
    d. Lift suture 2 by grabbing both suture ends to remove remaining blood in the liver and then tie suture 2. Both steps c and d need to be carefully performed to allow smooth perfusion of Solution A.
    e. Perfuse the liver with a total volume of 50 mL of Solution A.
    f. Carefully monitor the flowing of Solution A through the liver.
    g. The color of the liver should turn pale (Figure 3G).
    h. This step should be done as quickly as possible.
30. Perfuse the liver with Solution B.
    a. After the perfusion of 50 mL Solution A, transfer the liquid inflow tube of the perfusion pump to the tube containing pre-warmed Solution B. Be careful not to trap any air bubble.
    b. Perfuse the liver with at least 25 mL but no more than 40 mL of Solution B at the flow rate of 7 mL/min.Continue the perfusion until the liver is swollen and shiny.
    c. Breakage of the liver can be seen.
    d. Stop the pump and cut the inferior vena cava in the upper region of suture 2.
31. Isolation of Liver Cells.
    a. Quickly remove the liver from the mouse abdominal cavity (Figure 3H).

b. Place the liver in a petri dish with 35 mL of Solution C (Figure 3H).
c. Hold the liver with tweezers and gently tap the perfused liver to disperse cells from the liver (Figure 3I).
d. Continue the process until all liver lobes disintegrate and separate into single cells (Figure 3J).
e. Filter liver cells with a 70 μm nylon cell strainer.
f. Centrifuge the cells in a 50 mL tube at 60 × *g* for 2 min with both acceleration and break set at 3 to separate NPCs (supernatant) and hepatocytes (pellet) (Figure 3K).

32. Purification of Hepatocytes.
a. Prepare the Percoll solution by mixing 5.2 mL of 10× Percoll buffer with the Percoll stock solution to a final volume of 40 mL. Keep the Percoll solution at 4°C.
b. Transfer the supernatant, which contains NPCs, to a new tube on ice for later purification of Kupffer cells and leave behind a little bit more than 3 mL of the solution and the pellet, which contained hepatocytes, in the tube.
c. Add 30 mL of diluted Percoll solution to the pelleted cells in the tube. Gently resuspend the cells by pipetting. Avoid vortexing.
d. Centrifuge at 1,200 × *g* in Eppendorf 5804R centrifuge for 3 min at 18°C–25°C.
e. Cells pelleted are purified hepatocytes.
f. Count cells and seed them (at a density of 0.3 × 10^6/mL) in hepatocyte culture medium in a collagen-coated plate (Figure 4A).

33. Purification of Kupffer Cells.
a. Centrifuge the supernatant isolated in step 33b at 583 × *g* for 8 min at 18°C–25°C.
b. Resuspend the cell pellet in 5.5 mL DMEM and overlay cells on a step gradient consisting of OptiPrep Density Gradient Media with densities of (from top to bottom) 1.035 (1.5 mL), 1.042 (1.5 mL), 1.056 (1.5 mL) and 1.081 (2 mL). Overlay the centrifuge tube with additional DMEM if necessary.
c. Centrifuge at 21,400 × *g* for 20 min at 18°C–25°C using a Beckman SW40Ti rotor.
d. Isolate Kupffer cells at the interface of densities 1.042 and 1.056, mix with 5 mL DMEM in a 15 mL tube, and pellet cells at 583 × *g* for 8 min at 18°C–25°C.
e. Resuspend Kupffer cells in 5 mL DMEM.
f. Pellet cells at 583 × *g* and resuspend cells in 5 mL DMEM. Repeat this step one more time to rinse Kupffer cells.
g. Remove the supernatant carefully and leave 1–2 mL medium behind depending on the pellet size.
h. Resuspend the pellet, stain dead cells with trypan blue, and count the purified Kupffer cells with a hemocytometer (i.e., Neubauer chamber) (Figure 4B).
i. Plate Kupffer cells in Kupffer cells culture medium (Figure 4B).
j. Incubate in 5% CO_2 incubator at 37°C for 3 h and remove nonadherent cells with an aspirator. Kupffer cells on the plate can be resuspended by treatment with trypsin at 37°C for 3 min if there is a need.
k. Verify the purity of Kupffer cells by flow cytometry (Figures 4C–4F).

Co-culturing of HBV-positive hepatocytes and Kupffer cells

Timing: 3 days

34. Hepatocytes isolated from mice injected with the HBV DNA or from HBV transgenic mice are seeded at a density of ~0.3 × 10^6 cells per well of a collagen-coated 6-well plate as described in step 33f.

⚠ CRITICAL: The use of collagen-coated plate is necessary for maintaining the morphology and the differentiation phenotype of hepatocytes.

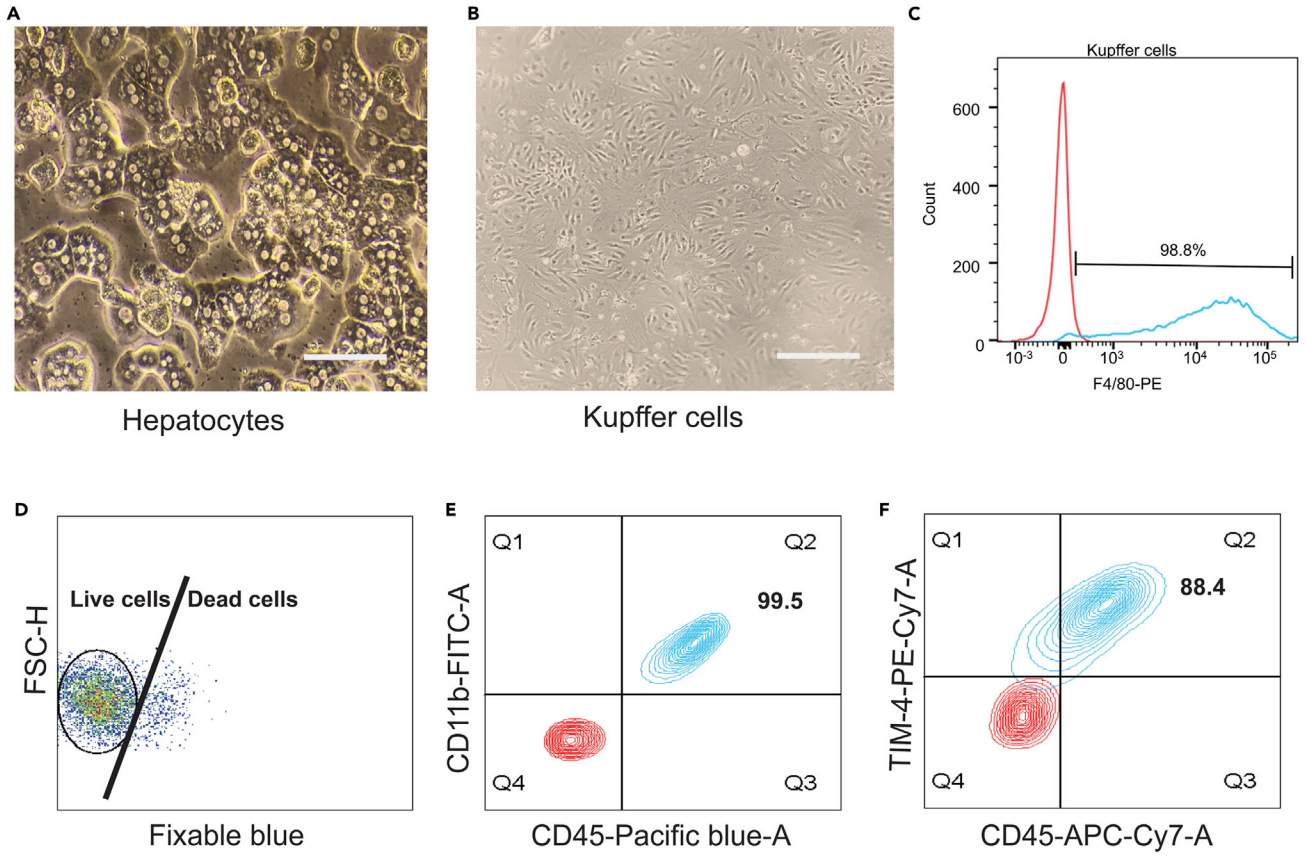

Figure 4. Hepatocytes and Kupffer cells purified from the mouse liver

(A) The morphology of hepatocytes two days after plating.

(B) The morphology of Kupffer cells. In both (A) and (B), the scale bar is 20 μm.

(C–F) Analysis of the purity of Kupffer cells by flow cytometry. Kupffer cells on petri dishes were collected by trypsinization at 37°C for five minutes, and 2×10^5 Kupffer cells were incubated with isotype control antibodies (red) or antibodies against cell markers of interest (blue) on ice for 30 min. Cells were then rinsed with DPBS three times and subjected to flow cytometry for the analysis of F4/80 (C), dead cells using Fixable Blue Dead Cell Staining Kit (D), CD11b and CD45 (E), and TIM-4 and CD45 (F). Panel (C) was adapted from Figure S1A of Li et al. (Li et al., 2022). Panels (D) and (E) were from different experiments.

35. Place hepatocytes in a 37°C CO_2 incubator for 4 h to allow the cells to attach to the plate and reach a confluency of 80%–90%.
36. Rinse cells once with DPBS and incubate them in the hepatocytes culture medium. If there is a need for long-term culturing, change the medium once every three days.

Note: Hepatocytes can be maintained for up to a week without notable change of morphology.

37. Perform the co-culturing experiment in the 6-well plate using the Transwell insert with 0.4 μm pore size for physical separation of two cell types.
38. Use sterile forceps to gently place Transwell inserts in each well above freshly attached hepatocytes.
39. Plate Kupffer cells at a density of 2×10^6 cells per Transwell insert. Co-culture Kupffer cells and HBV-positive hepatocytes in a medium containing 1:1 ratio of hepatocytes culture medium and Kupffer cells culture medium for three days.
40. By their design, Transwell inserts will be secured in place by the walls of the well. They will remain suspended in the media and not touch the bottom of the plate, allowing exchange of soluble factors between hepatocytes at the bottom and Kupffer cells in the ⊥insert.

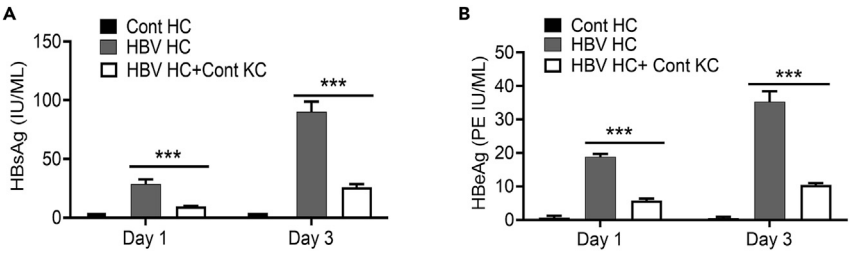

Figure 5. Effect of Kupffer cells on HBV gene expression in hepatocytes

(A and B) The incubation media of hepatocytes isolated from control mice (Cont HC), hepatocytes isolated from HBV transgenic mice (HBV HC), and HBV HC co-cultured with Kupffer cells isolated from control mice were analyzed by ELISA for the levels of HBsAg (A) or HBeAg (B) at different time points. This figure was modified from Figure S1B of (Li et al., 2022). The results represent the mean ± SEM. ***p<0.001.

41. Collect and replace the conditioned medium with fresh medium on day 1 after co-culturing at 37°C in a humidified incubator with 5% CO_2, and collect the conditioned medium again on day 3.
42. Measure the levels of HBsAg and HBeAg using commercial ELISA kits following the manufacturer's protocols (Li et al., 2022). Examples of the ELISA results are shown in Figure 5.

Metabolic analysis of Kupffer cells: Oxygen consumption rate (OCR)

Timing: Around 4 h for the OCR assay

Timing: 30 min for preparation of the Seahorse analyzer and microplate

Timing: 10 min for preparation of the assay medium

Timing: 15 min for preparation of compound stock solutions and working solutions

Timing: 25 min for loading compound solutions into the ports

Timing: 2 h for running the assay

Timing: 30 min for data analysis

Day before assay

43. Turn on the Agilent Seahorse XFe/XF Analyzer and allow it to warm up for at least 5 h.
44. Seed 10^5 Kupffer cells per well in Seahorse XF plate. Use appropriate medium for the cell culture. For Kupffer cells, use Kupffer cell culture medium for 200 μL/well.

Note: For more information, see the basic procedure, "Seeding Cells in Seahorse XF Cell Culture Microplates," available at the Agilent Cell Analysis Learning Center.

45. Add water to the Seahorse XF sensor box, and then place the calibrators in a non-CO_2 incubator16 h at 37°C.

Day of assay

46. Prepare XF medium and XF supplement in the cell culture hood. Transfer a sufficient amount of XF medium to a 50 mL tube.

Note: There is no need to warm up the medium and the supplement before this step.

47. Prepare the assay medium by supplementing Seahorse XF DMEM with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose.

Note: The medium composition can vary depending on the cell types or the desired experimental conditions.

48. Warm up the assay medium in a 37°C water bath before use.

Prepare compound stock solutions and working solutions

49. Take the foil bag and the decapper out from the Seahorse XF Cell Mito Stress Test Kit box.
50. Open the bag, remove the three test tubes containing oligomycin (blue cap), FCCP (yellow cap), and rotenone/antimycin A (red cap) and place them on a small test tube rack.
51. Insert the decapper's tines into the cap's inner lip and remove the cap from each tube.
52. Resuspend the contents of each tube with the volumes of assay medium shown in the Solution 1 table.
53. Gently pipet the medium up and down about 10 times to dissolve the compound.
54. Use the compound stock solution to prepare the working solution for loading into the injection port on the sensor cartridge. A constant compound concentration and variable loading volume approach is recommended for each compound.

Note: This method requires compound working solutions to be prepared at defined concentrations and different volumes of each compound to be loaded into the injection port (see Solution 2 table). For example, for the preparation of 20 μM oligomycin, mix 600 μL stock solution of oligomycin (100 μM) with 2,400 μL DMEM in the polystyrene reservoir. Load 20 μL diluted oligomycin (20 μM) per port using a multichannel pipette. Because the Seahorse XF cell culture microplate has 180 μL DMEM, the final concentration of oligomycin is 2 μM.

55. Prepare 2–3 mL of working solution for each compound in the analytical medium, using the volumes shown in the Solution 2 table for the XFe/XF96 analyzer.

Load compound solutions into the ports on sensor cartridge

56. The correct port loading technique can be found in the basic procedure "Loading Sensor Cartridges with Compounds" in the Agilent Cell Analysis Learning Center.Read the relevant information before loading the compound. In addition, make sure that the sensor box is adequately hydrated before use. Refer to Figure 6 for port locations.

Prepare the Agilent Seahorse XF cell culture microplate for the assay

57. Remove the Seahorse XF cell culture microplate from the 37°C CO_2 incubator and examine it under a microscope to confirm cell confluency.
58. Rinse Kupffer cells twice with 200 μL of Seahorse XF DMEM assay medium, leaving 20 μL behind after each rinse.
59. After the second rinse, add 160 μL of Seahorse XF DMEM assay medium to each well to a final volume of 180 μL/well.
60. Place the Seahorse XF cell culture microplate at 37°C without CO_2 for 45 min to 1 h before running the assay.

⚠ CRITICAL: It is essential to keep the culture microplate at 37°C without CO_2 for 45 min to 1 h, as CO_2 can interfere with the analysis.

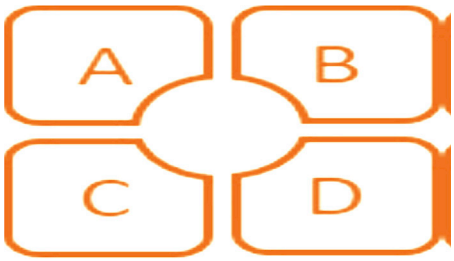

Figure 6. The location of ports in sensor cartridges

Running the assay

61. Open the Wave software and retrieve the saved assay template file. Then, follow the instructions as described below:
    a. Browse and open the saved design file, select the Review and Run tab, and click Start Run.
    b. Place the sensor box with the calibration plate into the instrument and click "I am ready" when prompted. Calibration takes about 15–30 min (remove the cartridge lid and double-check to make sure that the microplate orientation is correct).
    c. After calibrating and equilibrating the cell culture microplate, click "I am ready" when prompted.
    d. Load the Seahorse XF cell culture microplate and click "I am ready" to run the assay.

Data analysis

62. The Seahorse XF Mito Stress Test Report Generator automatically calculates Seahorse XF Cell Mito stress test parameters from Wave data and export the data to Excel or GraphPad Prism (Li et al., 2022) (Figure 7A).

Metabolic analysis of Kupffer cells: Extracellular acidification rate (ECAR)

Timing: 5 min for preparation of the Seahorse analyzer and microplate

Timing: 5 min for preparation of the assay medium

Timing: 20 min for preparation of the microplates for the assay

Timing: 15 min for preparation of stock compound solutions

Timing: 15 min for dilution of stock compound solutions

Timing: 10 min for loading sensor cartridges

Timing: 30 min for download the glycolytic rate template into the Seahorse analyzer

Timing: 2 h for running the glycolytic rate assay

Day before assay

63. Turn on the Seahorse XFe/XF96 Analyzer and let the temperature stabilize.
64. Seed 10^5 Kupffer cells per well in the Agilent Seahorse XF Cell Culture Microplate using the Kupffer cells culture medium.
65. Hydrate a sensor cartridge in Seahorse XF Calibrant medium in a non-CO_2 incubator at 37°C for 16 h.

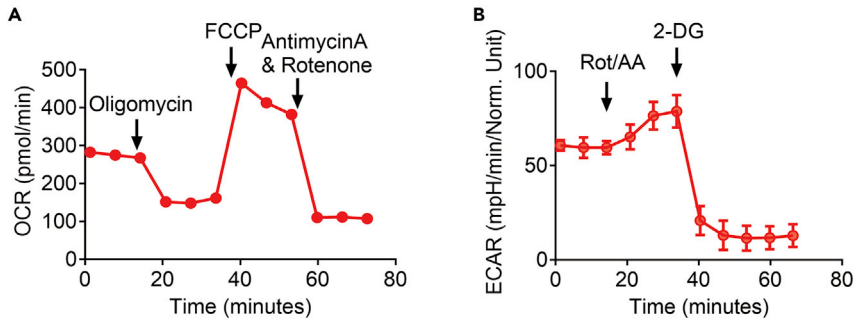

Figure 7. Metabolic analysis of Kupffer cells

(A And B) The OCR (A) and ECAR (B) of Kupffer cells determined by Seahorse assay.

66. For XFe/XF96 analyzers, select the Template for XF Glycolytic Rate Assay in Wave. Make necessary group modifications to tailor the template for the specific assay design.

Day of assay

67. Prepare Agilent Seahorse Glycolytic Rate assay medium: XF DMEM with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose. This medium contains an appropriate amount of HEPES and does not require pH adjustment.

Note: It is recommended to start with 1 mM pyruvate, 2 mM glutamine, and 10 mM glucose. However, the assay medium composition can vary depending on the cell type or the desired research conditions.

68. Prepare the XF medium and XF supplement in the cell culture hood. Transfer a sufficient amount of XF medium to a 50 mL tube.

Note: There is no need to warm up the medium and the supplement before this step.

69. Prepare the assay medium by adding XF supplement to XF medium to the desired concentration.

Note: No pH adjustment is required when using the recommended supplement concentration.

70. Warm up the assay medium in a 37°C water bath.

Prepare agilent seahorse XF cell culture microplates for assays

71. Remove the Seahorse XF cell culture microplate from the 37°C CO_2 incubators and examine it under a microscope to confirm confluency and good cell morphology.

⚠ CRITICAL: The cell quality and confluency are important for the assay.

72. Remove the cell culture medium from the Seahorse XF cell culture. Next, rinse cells twice with 200 μL warm Seahorse XF DMEM assay medium using a multichannel pipette. Leave 20 μL behind after each rinse.
73. After the second rinse, add 160 μL of Seahorse XF DMEM assay medium to each well and incubate cells in the assay medium in a non-CO_2 incubator at 37°C for 45–60 min before the assay.

74. Remove the assay medium again and add 160 μL of fresh and warm Seahorse XF DMEM assay medium to each well for a final volume of 180 μL/well assay medium. The cells are now ready for the XF assay.

Prepare stock compounds

75. Remove the foil bag from the kit box, open the bag and take out the red cap vial Rot/AA and the green cap vial 2-DG.
76. Tap the vial to make sure that the powder is at the bottom of the tube before opening the vial.
77. Prepare the relevant assay medium as shown in the Solution 3 table using a P1000 pipette. Vortex the tubes for 1 min to ensure thorough dissolution of the compounds.

Diluting the compounds

78. The dilution of compounds for loading the cartridge is shown in the Solution 4 table. It is important to note that if a different starting assay volume or port volume is used, the compound concentrations will need to be adjusted to obtain the correct final concentrations for each well.

Load sensor cartridge

79. Standard Assay - No compound injection is required before the glycolytic rate assay. Load the compound into the following ports of the hydrated sensor cartridge (Figure 6 and the Solution 4 table):
    a. Port A: For injection of Rot/AA.
    b. Port B: For injection of 2-DG.

Running the assay

80. Download the Agilent Seahorse Glycolytic Rate template into the Seahorse XFe analyzer. If the Seahorse XFe template already exists, this step can be skipped.

Run the seahorse XFe/XF96 glycolytic rate assay

81. Select Seahorse XF Glycolytic Rate Assay template from the list of available templates and click Design.
82. Confirm or modify the default groups and conditions in specific assays in the section Groups/Conditions.
83. License plate map for your assay: Confirm or modify the default groups and conditions in the specific assay.
84. Assign the distribution of the samples.
85. Place the sensor box with the calibration plate into the instrument and click "I am ready" when prompted. Calibration takes about 15–30 min.

Note: Remove the cartridge lid and double-check to ensure that the microplate orientation is correct.

86. After calibrating and equilibrating the cell culture microplate, click "I am ready" when prompted.
87. Load the Seahorse XF cell culture microplate and click "I am ready" to run the assay.

Analysis using the agilent seahorse glycolytic rate assay report generator

88. The XF Glycolytic Rate Assay Report Generator automatically calculates Seahorse XF Glycolytic Rate Assay test parameters from Wave data that are exported to Excel or GraphPad Prism (Li et al., 2022) (Figure 7B).

EXPECTED OUTCOMES

Hydrodynamic injection of mice

Mice injected with the HBV DNA by hydrodynamic injection should fully recover the next day. Their serum levels of HBsAg and HBeAg should reach approximately 4-Log IU/mL and 3-Log PE IU/mL, respectively, three days after the DNA injection (see Figure 2F).

Isolation of hepatocytes and Kupffer cells from the mouse liver

The liver perfusion of collagenase solution in combination with the mechanical dissociation of the liver will allow the isolation of all of the liver cells. In general, this protocol allows the recovery of 3–4 × 10^7 live hepatocytes and about 1 × 10^7 live KCs from the liver of one healthy 8–10-week-old mouse. The viability of recovered cells is greater than 95%, and the single cell suspensions obtained by this procedure are suitable for functional and phenotypical analyses.

OCR and ECAR of Kupffer cells

The successful seahorse assay of Kupffer cells should generate typical OCR and ECAR curves shown in Figure 7.

LIMITATIONS

The hydrodynamic injection leads to the transfection of only 15%–30% of hepatocytes by HBV DNA. Although this transfection efficiency is sufficient for many studies, it remains a challenge if a higher transfection efficiency is desired for specific studies.

TROUBLESHOOTING

Problem 1

Mice fails to be injected by hydrodynamic injection at step 10.

Potential solution

If subcutaneous bleeding occurs, the needle can be removed and reinserted in a new injection site toward the proximal end of the tail. If the needle is inserted correctly, bleeding along the needle can be observed, and there will be no local swelling or discoloration of the tail. However, if the needle is inserted incorrectly into the tail vein, there will be significant resistance during the injection, and there will be discoloration and local swelling of the tail tissue. In this case, the needle will need to be removed and repositioned correctly in a location closer to the proximal end of the tail.

Problem 2

A low HBV DNA transfection efficiency of hepatocytes is obtained after the hydrodynamic injection at step 10.

Potential solution

The duration of the hydrodynamic injection is very important for obtaining a good transfection efficiency. The completion of injections in less than 5 s will increase the mortality rate of mice. On the contrary, if the injection time exceeds 8 s, the HBV DNA transfection efficiency into hepatocytes will be reduced. A good injection requires a constant injection speed throughout the procedure.

Problem 3

Mice die after the hydrodynamic injection at step 11.

Potential solution

In general, mice are highly tolerant to the hydrodynamic injection. After the injection, they may remain motionless and exhibit difficulty breathing for about 5–10 min. Occasionally, mice will pause breathing for a few seconds, possibly due to a vasovagal response induced by a large bolus injection

of the DNA solution. The massage of the mouse chest with the index finger for 1–2 min at a rate of once per second can help to restore spontaneous breathing and promote recovery. The above steps can reduce the mouse mortality rate.

Problem 4

At step 33, the viability of Kupffer cells isolated from the mouse liver is lower than 90%.

Potential solution

After the separation of hepatocytes and NPCs, keep NPCs on ice till the isolation of Kupffer cells.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jing-hsiung James Ou (jamesou@usc.edu).

Materials availability

Materials are available upon request.

Data and code availability

This study does not generate or analyze any data sets.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101364.

ACKNOWLEDGMENTS

We wish to thank members of J.H.J.O.'s lab for their help throughout the work described in this report. This work was supported by research grants AI129540, AI145813, and AI148304 from the National Institutes of Health.

AUTHOR CONTRIBUTIONS

Conceptualization, Y.L. and J.H.J.O.; methodology, Y.L., J.L., G.D.H., and O.A.; writing – original draft, Y.L.; writing – review and editing, Y.L. and J.H.J.O.; supervision, J.H.J.O.; funding acquisition, J.H.J.O.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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