

Isolation Procedures

1. Mouse hepatic stellate cell (HSC) and hepatic macrophage (HM) isolation

Mouse liver is perfused via superior vena cava first with EMEM at 5 ml/minute for 10 minutes, next with 0.5-0.7% (w/v) pronase for 18~20 minutes, and finally with 0.044% (w/v) collagenase for 6~10 minutes. After agitation of the digested liver tissue with 10 µg/ml DNase I in a rotary shaker for 10-15 minutes, the cell suspension is subjected to 50xg centrifugation for 30 seconds. The supernatant is centrifuged at 150xg for 5 minutes. After washing the non-parenchymal liver cells by 150xg centrifugation, they are laid on the top of four OptiPrep™ gradients (1.085, 1.058, 1.043, 1.034) and centrifuged in the SW-41Ti rotor at 20,000 rpm for only 15 minutes at 25°C. Collection of HSC and HM enriched interfaces and subsequent washing procedures are same as rat HSC and HM isolation. HM are further purified by the adherence method as described for rat HM. Yield of HSC and crude HM from a mouse are $2-5 \times 10^6$ and $2-3.5 \times 10^7$ cells, respectively. The purity of HSC exceeds 96% as determined by UV-excited autofluorescence microscopy and that of crude and purified HM exceeds 80% and 97% as assessed by latex bead uptake, respectively. HSC and HM viability are >96% as judged by trypan blue exclusion. The adherence method will increase the HM purity to above 94%.

2. Mouse HSC isolation from Col1a1^{GFP} transgenic mice by fluorescence-activated cell sorting (FACS)

Autofluorescence of vitamin A (VitA) lipids stored in HSC can be detected by FACS (1, 2). Combining the detection of GFP in the collagen1a1 promoter-GFP (Col1a1-GFP) transgenic mouse livers, we isolate heterogeneous HSC. Col1a1-GFP mice have been used for detection of activated HSC (2-5). After perfusion of the Col1a1-GFP mouse liver, 5-10 % of nonparenchymal cells show VitA+ HSC in normal livers. Negligible number (0.1%) of VitA-GFP+ cells, which might represent fully activated HSCs or other mesenchymal cells, are present in the nonparenchymal cells. FACS detects around 10-70% of GFP+ HSC in all VitA+ HSCs. After sorting, VitA+GFP+ express more Col1a1 mRNA compared to VitA+GFP- cells. The yield is $1-5 \times 10^5$ cells for 1 hour sorting. This technique will be useful to separate heterogeneous HSC populations or different liver mesenchymal cells.

3. Simultaneous isolation of mouse hepatocyte and HM

Mouse liver is perfused through superior vena cava with EMEM at 5 ml/minute for 10 minutes, and then with digestion buffer (DMEM containing 0.044% (w/v) collagenase) for 6-10 minutes. The liver is removed and agitated in a rotary shaker for 10-15 minutes to further digest and dissociate the cells in DMEM medium containing 10µg/ml DNase I. The cell suspension is centrifuged at 700rpm (50xg) for 1min at 4°C. The resulting cell pellet is used for hepatocyte purification as described above. The supernatant containing non-parenchymal cells is centrifuged at 150xg for 5 minutes, and the pellet is wash twice with DMEM medium. The final cell suspension is laid on the top of four OptiPrep™ gradients (1.085, 1.058, 1.043, 1.034) and centrifuged in the SW-41Ti rotor at 20,000 rpm for only 15 minutes at 25°C. The viability of both cell types is checked by trypan blue exclusion. Isolation from a normal male mouse (BW=25-30g) yields $1.5-4.5 \times 10^7$ hepatocytes with the purity and viability exceeding 90%, and $0.5-1.5 \times 10^7$ HM with the purity > 80% and the viability >95%. The adherence method will increase HM purity to 94% with the yield of 3.9×10^6 cells.

4. Purification of mouse HM by magnetic-activated cell sorting (MACS)

In mouse, the CD11b antigen (Integrin α M) is expressed on monocytes/macrophages and to a lower extent on granulocytes and NK cells (6). Based on the expression of CD11b, CD11b+ HM subset can be isolated by MACS. HM are isolated by the digestion of livers with pronase and collagenase digestion followed by discontinuous gradient ultracentrifugation as described above. The HM-enrich fraction (10^7 cells) obtained from the 1.043/1.058 interface is suspended in 90 µl

MACS buffer (1xPBS, 0.5% BSA) containing 0.6% citrate-dextrose solution (Sigma). Then, 10 μ l of CD11b MicroBeads (Miltenyi Biotech, #130-049-601) to the cell suspension and incubate 20 minutes at 4°C. The cell suspension is applied to the column and CD11b+ HM is separated by autoMACS separator. The yield is 1-4x10⁶ HM and the purity is greater than 98%.

5. Monocyte tracking

We have recently used *in vivo* tracking method to study peripheral blood monocytes (PBMC) transmigration into the liver tissue and subsequent differentiation into proinflammatory HM (8, 9). PBMC from the donor mice are isolated by negative selection using Mouse Monocyte EasySep Kit (Stemcell #19761). Briefly, intravenous blood (0.8-1.0 ml) is collected and lysed (1 part blood with 9 parts ammonium chloride). Unwanted cells are targeted for removal with biotinylated antibodies directed against non-monocytes and tetrameric antibody complexes recognizing biotin and dextran-coated magnetic particles. The magnetically labeled unwanted non-monocyte cells will remain bound inside the reaction tube, held by magnetic field of the EasySep magnet (negative selection) (Stemcell #18000). The desired PBMC in the reaction buffer are collected. The purified PBMC are fluorescently labeled using the PKH26 Red Fluorescent Cell Linker Kit (Sigma #PKH26GL). A total of ~2x10⁷ PBMC in 1 ml Diluent C buffer is mixed with 1 ml of PKH26 dye (4mM) and incubated for 2-5 minutes. The staining reaction is stopped by adding an equal volume of 1% BSA. Centrifuge the cells at 1000xg for 5 minutes to remove cells from staining solution, and wash the cells for 3 times in PBS. Resuspend cells in PBS and examine under immunofluorescent microscope to determine the labeling efficiency, which is usually >95-99%. The PKH26 labeled monocytes are injected to recipient mice through tail-vein. PBMC transmigration into the liver is examined by fluorescent microscopy and quantitated by FACS analysis of PKH26⁺ PBMC/HM.

6. FACS separation of migrating vs. resident HM

Using aforementioned monocyte tracking and FACS analysis, we have developed a method to separate transmigrated vs. resident HM (9). Briefly, five days after injection of the PKH26 labeled PBMC, HM (including migrated PBMC and resident Kupffer cells) are isolated from the recipient mice. HM are incubated with anti-mouse CD16/CD32 for 10 minutes on ice to block unspecific binding, and then with following antibodies: CD45-V450 (BD Biosciences), F4/80-FITC (eBioscience), and CX3CR1-APC (R&D systems) for 30 minutes, and washed 3x with cold PBS. For FACS analysis, after gating on the CD45, HM are separated into PKH26⁺ and PKH26⁻ groups. The PKH26⁺ cells are transmigrated donor PBMC and are characterized by F4/80⁺CX3CR1^{High}. The PKH26⁻ cells were further sorted into F4/80⁺CX3CR1^{High}- and F4/80⁺CX3CR1^{Low}- subsets, which represent transmigrated recipient PBMC and resident Kupffer cells, respectively.

7. Mouse mesothelial cell (MC) isolation

Liver lobes are incubated with 1 mg/ml pronase in DMEM/F-12 medium for 20 minutes at 37°C with gentle shaking (130 rpm). Cells detached from the liver are collected by centrifugation at 1,700xg for 5 minutes and the pellet is re-suspended in DMEM containing 10% FBS. After washing 3 times, the cells are incubated with anti-Gpm6a antibody (MBL International, D0553) at 1,500-fold dilution (1 μ l per 1.5 ml) in DMEM for 15 minutes at 4°C(7). After centrifugation, the cells are incubated with anti-rat IgG MicroBeads (20 μ l per 1 ml) and are purified by autoMACS (Miltenyi Biotech) according to their instructions(8). MC are plated on a collagen-coated dish in DMEM with low glucose containing 10% FBS, ITS (Gibco), and 50 ng/ml hydrocortisone. MC form epithelial colonies in culture and immunocytochemistry of podoplanin, a MC marker, shows its expression >95% MCs in culture. The yield is 2x10⁴ MC from 1 mouse liver.

8. Liver CD133+ progenitor isolation by FACS

Liver lobes are perfused EMEM, cut into small pieces and digested in 50 ml of 25 mg/ml of collagenase IV at 37°C for 10 minutes with gentle shaking. Undigested tissues are further broken up by passing through 70 µm cell strainer and incubated again at 37°C for 10 minutes with gentle shaking. After washing the cells with DMEM/F-12 containing 10% FBS one time, the pellet was incubated with 5 ml of Red Blood Cell lysis buffer for 5 minutes at room temperature. The cells are washed one time with DMEM/F-12 containing 10% FBS, one time with PBS and one last time with serum free DMEM/F-12. The cells are then counted with Countess Counter. The yield ranges from 5-9x10⁷ cells and the viability ranges from 60-90%. The cells are then depleted with CD45 microbeads (Miltenyi Biotec, 130-052-301) according to the manufacturer's protocol. Briefly, the cells are resuspended in serum free DMEM/F12 and incubated with CD45 microbeads on ice for 15 minutes. They are passed through LS magnetic column to collect CD45 negative liver cells. The resulting cells are counted with Countess Cell Counter and resuspended in PBS with 1%BSA and blocked with FcR blocking reagent (Miltenyi Biotec) according to the manufacturer's protocol. The cells are then stained with 1 µl of antibody per 1x10⁶ cells in 100 µl on ice for 20 minutes. The cells are then washed with PBS and resuspended in PBS and sorted for CD133+ cells (9, 10). The following antibodies are used depending on the experimental question: Anti-mouse CD133-APC (eBioscience, 17-1331), Rat IgG1 K isotype control APC (17-4301), Anti-human/mouse CD49f-PE (12-0495), CD45-eFluor450 (9048-9459). The yield ranges from 0.1-1x10⁴ cells.

9. Mouse/rat sinusoidal endothelial cell (SEC) isolation by MACS

Rat SEC are isolated using SE-1 antibody by the method previously described (11). The livers are digested by the two-step collagenase perfusion technique. After removing undigested tissues, cells are suspended in Hanks' buffer and are centrifuged at 70xg for 1 minute. Then, the supernatant was recovered and centrifuged at 620xg for 7 minutes. The pellet is suspended in MACS buffer containing 0.6% ACD (1x10⁷ cells/100 µl) and incubated with SE-1 antibody (IBL; 0.5 µg per 1x10⁷ cells) for 30 minutes at 4°C. Then, the cells are further incubated with rat anti-mouse IgG2a+2b microbeads (Miltenyi Biotech; 20 µl microbeads per 1x10⁷ cells) for 10 minutes at 4°C. After centrifugation, the cells were resuspended in ACD buffer and SE-1+ SEC are separated by autoMACS pro Separator (Miltenyi Biotech) according to the manufacturer's instruction. Similarly, mouse SEC are isolated using anti-mouse CD146 (LSEC) microbeads (20 µl per 1x10⁷ cells) (12). Using this method, approximately 1.2 x 10⁷ SEC are isolated from a rat with the purity exceeding 97% using 5µl SE-1 antibody. The viability is >97%.

10. Simultaneous isolation of rat hepatocytes and HM

Simultaneous isolation of rat hepatocytes and HM is carried out using modified protocol of collagenase digestion (13, 14). In brief, the rat liver is perfused through portal vein with 100 ml of Ca²⁺ free Hanks buffer containing 1ml of EGTA (34mg/ml, Sigma) and 1g of bovine serum albumin (BSA) at 37°C for 10 minutes. This is followed by enzymatic digestion with 60 ml of Hanks buffer containing 0.05% (w/v) collagenase type IV (Sigma) and CaCl₂ (58.8mg/ml) for 4 to 7 minutes via recirculation. The liver is then removed and cut into small pieces. Liver cells are further disassociated by gentle pipetting in the Hanks buffer, and filtered through a Nylon filter (BD #9240501) to remove undigested tissues and debris. The resulting cell suspension is centrifuged at 700 rpm for 1 minute. The supernatant containing non-parenchymal cells is collected for HM purification (see below). The cell pellet is re-suspended in 10ml of Hanks buffer mixed with 5 ml Percoll (Sigma #P1644), and centrifuge at 2,500 rpm (150xg) for 5 minutes at 4°C (15). The cell pellet is washed 2 times with DMEM medium and centrifuged at 700rpm (50xg) for 1minute at 4°C.

The non-parenchymal liver cell fraction is separated by centrifugation at 50xg for 1-2 minutes. After two washes of the fraction by 150xg centrifugation, it is laid onto the top of four layers of OptiPrep™ gradients (1.085, 1.058, 1.043, 1.034) in Beckman ultracentrifuge tubes (16-18). The tubes are centrifuged in the SW-41Ti rotor at 21,400 rpm for 35 minutes at 25°C. Relatively pure

(>83%) HM are collected from the 1.043/1.058 interface and less pure HM from the 1.058/1.085 interface. The viability of both cell types is checked by trypan blue exclusion. Isolation from a normal male rat (BW=350-550g) yields $2-3 \times 10^8$ hepatocytes with the purity and viability exceeding 92%, and $1.8-2.5 \times 10^8$ HM with the purity > 83% and the viability >95%. HM are further purified by the adherence method and cultured in PRMI containing 5% FCS and antibiotics at the density of $3-4 \times 10^6$ cells per 100 mm dish. After adherence method, the HM purity will exceed 95% with the yield of 2.7×10^7 cells from a rat.

11. Rat HSC and HM isolation

HSC and HM are isolated by sequential digestion of rat liver with pronase and collagenase followed by low centrifugation and discontinuous gradient ultracentrifugation. The procedure is a modified version of the method originally described by Friedman and Roll (16). The Core Director has used the procedure since 1985 for isolation of the cells from rat or mouse livers (19-21). Briefly, for a normal rat, the liver is sequentially perfused *in situ* with 150 ml of MEM without calcium, 95 ml DMEM/F-12 containing 0.74-0.89% (W/V) pronase, and 180-200 ml DMEM/F-12 with 0.025% (w/v) type IV collagenase at 10 ml/minute. After agitation of the digested liver in a siliconized 250 ml bottle containing 100 ml DMEM/F-12 and 10 μ g/ml DNase I, a non-parenchymal liver cell fraction is separated by centrifugation at 50xg for 1-2 minutes. After two washes of the fraction by 150xg centrifugation, it is laid onto the top of four layers of OptiPrep™ gradients (1.085, 1.058, 1.043, 1.034) in Beckman ultracentrifuge tubes. The tubes are centrifuged in the SW-41Ti rotor at 21,400 rpm for 35 minutes at 25°C. A pure HSC fraction is collected from the medium/1.034/1.043 interface. Relatively pure (>83%) HM are collected from the 1.043/1.058 interface and less pure HM from the 1.058/1.085 interface. The purity of HSC is determined by observation of the characteristic lipid-containing morphology under phase-contrast microscopy; identification of intracellular vitamin A by UV-excited fluorescence microscopy; and immunohistochemistry of desmin or GFAP if necessary. The purity of HM is assessed by phagocytosis of latex beads (1 μ m) and/or peroxidase staining. The viability of both cell types is checked by trypan blue exclusion. Isolation from a normal male rat (BW=350-550g) yields $1.8-3.3 \times 10^7$ freshly isolated HSC with the purity and viability exceeding 97% and $1.8-2.5 \times 10^8$ freshly isolated HM with the purity > 83% and the viability >95%. HM are further purified by the adherence method and cultured in PRMI containing 5% FCS and antibiotics at the density of $3-4 \times 10^6$ cells per 100 mm dish. The purity after this method always exceeds 95% with the yield of 2.7×10^7 cells per rat. HSC are cultured in RPMI containing 10% FCS and antibiotics at the similar density.

12. Pancreatic stellate cell (PSC) isolation

Pancreas tissues from wild type or *Pdx1*^{Cre}; LSL-*Kras*^{G12D} mice are digested with 125 μ g/ml collagenase type XI (Sigma) and 125 μ g/ml Dispase II (Thermo Fisher Scientific) at 37°C for 30 min (22). Digested cells are collected by centrifugation and are plated on dishes. One hour after plating, floating cells are removed.

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