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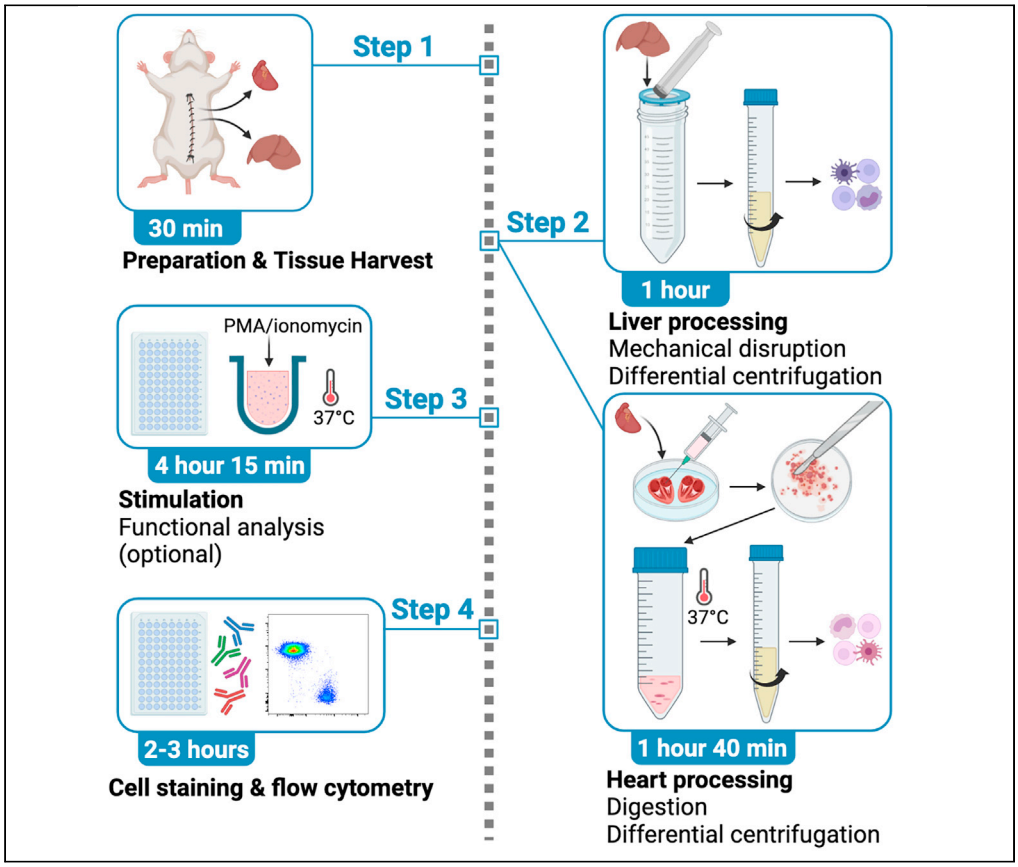
Protocol

Flow cytometric characterization of tissue-resident lymphocytes after murine liver and heart transplantation



Alterations to organ biology caused by transplantation can have major impacts on the outcome. Tissue-resident lymphocytes normally maintain an organ's immunity and function and are transferred during transplantation. Here, we provide a detailed protocol for the isolation of leukocytes, including tissue-resident lymphocytes, from transplanted livers and hearts in mice. Phenotypic and functional analysis of conventional and unconventional T cells by flow cytometry is included. This protocol can also be used for the effective isolation of leukocytes from non-transplanted livers and hearts.

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Highlights

Protocol for isolation of murine tissue leukocytes before and after transplantation

Efficient leukocyte isolation from the liver and heart

Detailed phenotyping of tissue-resident lymphocytes by flow cytometry

Functional analysis of donor- and recipient-derived lymphocytes

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Flow cytometric characterization of tissue-resident lymphocytes after murine liver and heart transplantation

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SUMMARY

Alterations to organ biology caused by transplantation can have major impacts on the outcome. Tissue-resident lymphocytes normally maintain an organ's immunity and function and are transferred during transplantation. Here, we provide a detailed protocol for the isolation of leukocytes, including tissue-resident lymphocytes, from transplanted livers and hearts in mice. Phenotypic and functional analysis of conventional and unconventional T cells by flow cytometry is included. This protocol can also be used for the effective isolation of leukocytes from non-transplanted livers and hearts.

For complete details on the use and execution of this protocol, please refer to Prosser et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the isolation and characterization of leukocytes, including tissue-resident lymphocytes, from livers and hearts transplanted between congenic mice. We have also used this protocol for isolation and analysis of leukocytes from healthy, non-transplanted organs from mice of various genetic backgrounds. All experiments for the establishment of the protocols were conducted with approval of the University of Western Australia Animal Ethics Committee under protocol numbers RA/3/100/1364 and RA/3/100/1568 and conformed to the Australian Code for the Care and Use of Animals for Scientific Purposes.

The described protocol focusses on lymphocyte identification and characterization, however myeloid cells (e.g., dendritic cells and macrophages, though not Kupffer cells) and granulocytes (e.g., neutrophils and eosinophils) can also be isolated using this method. An appropriate animal ethics protocol must be approved according to institutional guidelines. Experience with high parameter (up to 16 color) flow cytometry is also necessary. Key reagents and equipment should be readied before starting the experiment, as efficient cell isolation will reduce cell death. The given time required for each major step is for processing of one heart or one liver, though it is feasible to process multiple samples at the same time. Each additional heart or liver adds approximately 15 min to the time required. Care must be taken particularly when processing multiple hearts to ensure incubation times are accurate for each sample, staggered start times are recommended in this instance.

Prepare required reagents

Timing: 30 min

Prepare the following required reagents as described in the materials and equipment section:

1. FACS buffer (PBS with 2% heat-inactivated newborn calf serum (HI-NCS))
2. Heart digestion cocktail (DMEM with 400 U/mL collagenase II and 48 U/mL DNase I)
3. DMEM (high glucose + glutamine + pyruvate) with 15% HI-NCS
4. 42% isotonic Percoll
5. Red blood cell (RBC) lysis solution
6. Zombie UV
7. Surface and intracellular antibody cocktails
8. FoxP3/Transcription Factor Staining Buffer Set for intracellular staining
9. BD Stabilizing Fixative

Optional: Cell activation cocktail and DMEM with 10% HI-NCS.

Prepare for tissue harvest

Timing: 10 min

Assemble the following equipment:

10. Anesthetic, dissection board, surgical tape, 70% ethanol, forceps, surgical scissors, clamp forceps, cold saline (sodium chloride 0.9% for irrigation), cotton tip applicators, cotton balls, ice, institution approved bags for waste and carcass disposal.

Prepare the following equipment and reagents per mouse:

11. Liver harvest:

    a. 10 mL syringe with 23 G needle filled with 10 mL cold saline
    b. 1× 5 mL tube filled with 4 mL DMEM

12. Heart harvest:

    a. 3 mL syringe with 30 G needle filled with 3 mL cold saline
    b. 2× 5 mL tubes filled with 4 mL DMEM

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD127 PE (A7R34) 1:100	BioLegend	Cat#135009 RRID:AB_1937252
CD4 Brilliant Violet 786 (GK1.5) 1:1000	BD Biosciences	Cat#563331 RRID:AB_2738140
CD4 SuperBright600 (RM4-5) 1:700	Thermo Fisher	Cat#63-0042-80 RRID:AB_2637461
CD45.1 APC/eFluor 780 (A20) 1:200	Thermo Fisher	Cat#47-0453-82 RRID:AB_1582228
CD45.2 FITC (104) 1:100	BD Biosciences	Cat#561874 RRID:AB_10894189
CD49a Brilliant Blue 700 (Ha31/8) 1:100	BD Biosciences	Cat#742164 RRID:AB_2861198
CD49a Brilliant Violet 510 (Ha31/8) 1:50	BD Biosciences	Cat#740144 RRID:AB_2739900
CD49d Brilliant Violet 711 (R1-2) 1:20	BD Biosciences	Cat#740661 RRID:AB_2740350
CD69 Brilliant Violet 711 (H1.2F3) 1:20	BD Biosciences	Cat#740664 RRID:AB_2740352

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
CD69 PE/Cy7 (H1.2F3) 1:50	BD Biosciences	Cat#552879 RRID:AB_394508
CD8α Brilliant UV 395 (53–6.7) 1:400/1:500	BD Biosciences	Cat#563786 RRID:AB_2732919
CD8β Brilliant Violet 480 (H35–17.2) 1:1000	BD Biosciences	Cat#746835 RRID:AB_2744086
CD8β Brilliant Violet 650 (H35–17.2) 1:1500	BD Biosciences	Cat#740552 RRID:AB_2740253
CX3CR1 Brilliant Violet 421 (SA011F11) 1:2000	BioLegend	Cat#149023 RRID:AB_2565706
CXCR3 Brilliant Violet 650 (CXCR3-173) 1:100	BioLegend	Cat#126531 RRID:AB_2563160
CXCR6 PE (SA051D1) 1:1000	BioLegend	Cat#151103 RRID:AB_2566545
Eomes PE/Cy7 (Dan11mag) 1:70	Thermo Fisher	Cat#25-4875-80 RRID:AB_2573453
FoxP3 PE/CF594 (MF223) 1:100	BD Biosciences	Cat#562466 RRID:AB_11151905
GATA3 Brilliant Violet 421 (L50-823) 1:20	BD Biosciences	Cat#563349 RRID:AB_2738152
Granzyme B PerCPCy5.5 (QA16A02) 1:20	BioLegend	Cat#372211 RRID:AB_2728378
IFNγ Brilliant Violet 785 (XMG1.2) 1:50	BioLegend	Cat#505837 RRID:AB_11219004
IL-10 PE (JES5-16E3) 1:100	BioLegend	Cat#505007 RRID:AB_315361
KLRG1 Brilliant Violet 711 (2F1) 1:200	BD Biosciences	Cat#564014 RRID:AB_2738542
LAG3 Brilliant Violet 650 (C9B7W) 1:50	BioLegend	Cat#125227 RRID:AB_2687209
Ly6C PE/Cy7 (AL-21) 1:1000	BD Biosciences	Cat#560593 RRID:AB_1727557
PD1 Brilliant Violet 421 (J43) 1:50	BD Biosciences	Cat#565942 RRID:AB_2739406
RORγt Brilliant Violet 650 (Q31-378) 1:20	BD Biosciences	Cat#564722 RRID:AB_2738915
T-bet Brilliant Violet 605 (4B10) 1:20	BioLegend	Cat#644817 RRID:AB_11219388
TCRβ AlexaFluor 700 (H57-597) 1:50	BioLegend	Cat#109223 RRID:AB_1027654
TIM3 PE (5D12) 1:300	BD Biosciences	Cat#566346 RRID:AB_2739702
Chemicals, peptides, and recombinant proteins		
Sodium chloride 0.9% for irrigation	Baxter	Cat#AHF7123
DMEM (high glucose + glutamine + pyruvate)	Thermo Fisher	Cat#10569010
HBSS with Ca^{2+} and Mg^{2+}	Gibco	Cat#14025-134
Collagenase II	Worthington Biochemicals	Cat#CLS-2
DNase I	Thermo Fisher	Cat#AM2224
Distilled H_2O for irrigation	Baxter	Cat#AHF7113
PBS tablets	Thermo Fisher	Cat#18912014
Percoll	Cytiva Life Sciences	Cat#17–0891-01
10× PBS	Thermo Fisher	Cat#70013-032
Heat-inactivated newborn calf serum (HI-NCS)	Thermo Fisher	Cat#26010-074
Red blood cell lysis solution	Miltenyi Biotec	Cat#130-094-183
CD1d Tetramer APC (αGalCer loaded) 1:150	ProImmune	Cat#E001-4A-G
Critical commercial assays		
Zombie UV Fixable Viability Kit 1:1000	BioLegend	Cat#423107
Brilliant Stain Buffer	BD Biosciences	Cat# 566349
FoxP3/Transcription Factor Staining Buffer Set	Thermo Fisher	Cat#00-5523-00
BD stabilizing fixative	BD Biosciences	Cat#338036
Optional: Cell Activation Cocktail (with Brefeldin A)	BioLegend	Cat#423304
Experimental models: Organisms/strains		
Mouse B6.SJL-Ptprc^{a}Pepc^b/BoyJArc CD45.1$^+$ H2^b Liver transplant: male, 12–14 weeks, 25–30 g Heart transplant: female, 6–8 weeks	Animal Resources Centre	PTP
Mouse C57BL/6JArc CD45.2$^+$ H2^b Liver transplant: male, 12–14 weeks, 25–30 g Heart transplant: female, 6–8 weeks	Animal Resources Centre	B6
Mouse BALB/cArc CD45.2$^+$ H2^d Liver transplant: male, 12–14 weeks, 25–30 g Heart transplant: female, 6–8 weeks	Animal Resources Centre	BC
Software and algorithms		
FlowJo v10	BD Biosciences	RRID: SCR_008520

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
Isoflurane anesthetic (Isothesia)	Henry Schein	Cat#988–3244
Dissection board	DispoCut	Cat#M630-1
Surgical tape	3M	Cat#1530-0
Straight forceps	INKA Surgical Instruments	Cat#25547.15
Surgical scissors	INKA Surgical Instruments	Cat#1550.11
Clamp forceps	INKA Surgical Instruments	Cat#16305.01
Cotton tip applicators	N/A	N/A
Cotton balls	N/A	N/A
23 G Needle	BD	Cat#301810
30 G Needle	BD	Cat#305106
10 mL Syringe (without needle)	Terumo	Cat#SS+10L
3 mL Syringe (without needle)	Terumo	Cat#SS+03S
0.5 mL Insulin syringe	BD	Cat#326769
Scalpel (disposable, 23 blade, No. 4 handle)	Livingstone	Cat#SCP23L
5 mL Polycarbonate tubes	Techno Plas	Cat#C5016UU
15 mL Tubes	Greiner Bio-One	Cat#188271
50 mL Tubes	Greiner Bio-One	Cat#227261
70 µm Cell strainer	Greiner Bio-One	Cat#542070
1 mL Transfer pipettes	Sarstedt	Cat#86.1172
96-Well U bottom plates	Corning	Cat#353077
1.5 mL Graduated microtubes	Quality Scientific Plastics	Cat#509-GRD-Q
UltraComp eBeads Compensation Beads	Thermo Fisher	Cat#01–2222-42
Ultra Rainbow Calibration Kit	Spherotech	Cat#URCP-38-2K
Cytometer Setup and Tracking (CST) Beads	BD Biosciences	Cat#655051
Countess II Automated Cell Counter	Thermo Fisher	Cat#AMQAX1000
BD LSRFortessa Flow Cytometer	BD Biosciences	N/A

Alternative resources table

Reagent or resource	Alternative	Source	Identifier
Surgical scissors, forceps, and clamp forceps	Any similar instruments that enable dissection of the required tissue	Various	Various
DMEM	RPMI-1640 (ATCC modification)	Thermo Fisher	Cat#A1049101
Collagenase II	Collagenase II	Thermo Fisher	Cat#17101015
	Collagenase II	Stem Cell Technologies	Cat#07419
	Collagenase II	Merck	Cat#C2-22
DNase I	DNase I	Thermo Fisher	Cat#EN0521
	DNase I	Sigma-Aldrich	Cat#DN25
Red Blood Cell Lysis Solution	Red Blood Cell Lysis Solution	Tonbo	Cat#TNB-4300
	Red Blood Cell Lysis Solution	BioLegend	Cat#420301
42% Isotonic Percoll	Liver – 44% Isotonic Percoll		
	Heart – 38%–42% Isotonic Percoll		
Zombie UV fixable viability kit	Fixable Viability dye eFluor 455 UV	Thermo Fisher	Cat#65–0868
	Blue Live/Dead Fixable Dead Cell Stain	Thermo Fisher	Cat#L23105
	Fixable Viability Stain 450	BD Biosciences	Cat#562247
	GloCell Fixable Viability Dye UV450	Stem Cell Technologies	Cat#75008.1
FoxP3/Transcription factor staining buffer set	Transcription factor staining buffer set	Miltenyi	Cat#130-122-981
	Transcription factor buffer set	BD Biosciences	Cat#562574
	True-Nuclear Transcription Factor Buffer Set	BioLegend	Cat#424401

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Reagent or resource	Alternative	Source	Identifier
Cell Activation Cocktail	Leukocyte Activation Cocktail with BD GolgiPlug	BD Biosciences	Cat#550583
	PMA Ionomycin Brefeldin-A	Sigma-Aldrich Sigma-Aldrich Sigma-Aldrich	Cat#P1585 Cat#I3909 Cat#B6542
Manual or automated cell counting	CountBright Absolute Counting Beads	Thermo Fisher	Cat#C36950
	Precision Count Beads	BioLegend	Cat#424902
Flowjo	Kaluza	Beckman Coulter	RRID:SCR_016182
	FCS Express	De Novo Software	RRID:SCR_016431
	CytoBank	N/A	RRID:SCR_014043

This protocol has been optimized for use with the resources and reagents listed in the key resources table. The table above is not an exhaustive list of available alternatives, though the suggested resources are expected to produce similar results. Any alternative resource or reagent requires validation before experimental use.

MATERIALS AND EQUIPMENT

FACS buffer

Reagent	Final concentration	Amount
PBS	n/a	490 mL
HI-NCS	2%	10 mL
Total	**n/a**	**500 mL**

Prepare in a sterile environment and store at 4°C for up to six months.

Heart digestion cocktail

Collagenase II stock solution

Prepare a stock solution of 10,000 U/mL. The activity per weight of enzyme differs between lots, thus the required volume must be calculated for each lot. In a sterile environment, add the required volume of HBSS with Ca^{2+} and Mg^{2+} to the enzyme vial and dissolve the enzyme powder by mixing gently. Filter-sterilize the HBSS/enzyme solution through a 0.22 μm filter attached to a syringe. Aliquot the filter-sterilized enzyme stock solution into 200 μL volumes and store at −20°C.

Heart digestion cocktail

Reagent	Final concentration	Amount
Collagenase II (10,000 U/mL)	400 U/mL	200 μL
DNase I (2U/ μL)	48 U/mL	120 μL
DMEM	n/a	4.68 mL
Total	**n/a**	**5 mL**

Prepare fresh immediately before use.

Alternatives: DNase I and collagenase II enzymes can be sourced from a number of manufacturers and may be used in this protocol after validation, so long as the final concentrations in the cocktail are as described. The DNase I listed in the key resources table is supplied as a pre-made solution. If a different DNase I is used, prepare a stock solution as per the manufacturer's instructions. Refer to the Alternative Resources Table for further information.

DMEM buffers

Prepare DMEM (high glucose + glutamine + pyruvate) with 15% v/v HI-NCS for processing of heart samples and DMEM with 10% v/v HI-NCS for cell stimulation by adding 15% or 10% v/v HI-NCS,

respectively, to the required volume of DMEM. For each heart, 10 mL of DMEM with 15% HI-NCS is required. Prepare in a sterile environment and store at 4°C for up to six months.

Alternatives: RPMI with a similar composition to the DMEM described may be used in this protocol after validation. Refer to the Alternative Resources Table for further information.

Forty-two percent isotonic Percoll

Differential centrifugation with Percoll is often used to enhance leukocyte purification from murine liver and heart tissue (Deng et al., 2020; Dong et al., 2004; Mackay et al., 2016; Pinto et al., 2012). In this protocol, a Percoll solution of 42% successfully separates leukocytes from parenchymal cells in both hearts and livers (Prosser et al., 2021). From the liver, we have found that solutions of a concentration lower than 42% can affect isolation of myeloid cell populations, though a 44% Percoll solution may be used. In the heart, a solution between 38% and 42% can be used in this protocol successfully.

Reagent	Final concentration	Amount
10× PBS	n/a	2.1 mL
1× PBS	n/a	29 mL
Percoll	42%	18.9 mL
Total	**n/a**	**50 mL**

Prepare in a sterile environment, aliquot to polycarbonate tubes, and store at 4°C for up to six months.

⚠ CRITICAL: Ensure Percoll is stored in polycarbonate tubes to avoid adherence of the silica particles to the walls of the tubes.

RBC lysis solution

Prepare according to the manufacturer's instructions by diluting 1:10 with distilled H_2O. (https://www.miltenyibiotec.com/AU-en/products/red-blood-cell-lysis-solution-10x-1628.html#gref). Prepare fresh on the day of harvest and store at 21°C–23°C.

Alternatives: RBC lysis solutions from other manufacturers may be used in this protocol after validation. Please refer to the Alternative Resources Table for further information.

Zombie UV

Stock solution

Prepare according to the manufacturer's instructions (https://www.biolegend.com/en-us/products/zombie-uv-fixable-viability-kit-9336). Reconstitute 1 vial of lyophilized reagent with 100 µL DMSO (included in kit). Vortex to mix and spin briefly. Store as 5 µL aliquots at −20°C protected from light and avoid freeze/thaw cycles.

Working solution		
Reagent	Final concentration	Amount
PBS	n/a	As required
Zombie UV	1:1000	As required
Total	**n/a**	**40 µL per stain**

Alternatives: Fixable viability stains from other manufacturers may be used in this protocol after validation. Please refer to the Alternative Resources Table for further information.

Antibody cocktails

Prepare each of the following fresh for staining. Protect from light and store on ice.

Surface cocktail		
Reagent	Final concentration	Amount
Brilliant Stain Buffer	n/a	Up to 40 µL per sample
Conjugated antibodies	1:20–1:2000	As required
Total	**n/a**	**40 µL per stain**

Intracellular cocktail		
Reagent	Final concentration	Amount
Brilliant Stain Buffer	n/a	Up to 20 µL per sample
Conjugated antibodies	1:20–1:100	As required
Total	**n/a**	**20 µL per sample**

⚠ CRITICAL: Centrifuge antibodies at 16,000 × *g* for 5 min at 4°C prior to making cocktail to remove dye aggregates from solution.

Note: Brilliant Stain Buffer should be used whenever two or more Brilliant Violet dyes are present in a cocktail to reduce staining artifacts caused by fluorescent dye interactions. The final staining volumes described allow optimal staining whilst minimising the use of costly reagents.

FoxP3/Transcription Factor Staining Buffer Set

Prepare according to the manufacturer's instructions (https://www.thermofisher.com/order/catalog/product/00-5523-00#/00-5523-00). Dilute the Fixation/Permeabilization Concentrate 1:4 with the included kit diluent. Dilute the Permeabilization Buffer 1:10 with distilled H_2O. Prepare fresh on the day of staining and store at 21°C–23°C.

Alternatives: Intracellular staining buffers from other manufacturers may be used in this protocol after validation. Please refer to the Alternative Resources Table for further information.

BD Stabilizing Fixative buffer

Prepare according to the manufacturer's instructions by diluting 1:3 with distilled H_2O (https://www.bdbiosciences.com/us/applications/clinical/blood-cell-disorders/other-reagents/sample-prep-reagents/stabilizing-fixative-3x-concentrate/p/338036). Prepare fresh on the day of staining and store at 21°C–23°C. BD stabilizing fixative is recommended as it decreases the rate of tandem dye breakdown caused by prolonged storage in formaldehyde-containing buffers after staining.

Cell Activation Cocktail (with Brefeldin A) (optional)

Prepare only if proceeding with the stimulation step of the protocol. This reagent is supplied as a 500× pre-mixed cocktail of phorbol-12-myristate 13-acetate (PMA; 2.5 mg/mL), ionomycin (669.3 µM) and Brefeldin A (2.5 mg/mL) in DMSO (https://www.biolegend.com/en-us/products/cell-activation-cocktail-with-brefeldin-a-9407?GroupID=GROUP22). At 1×, these concentrations are: PMA 5 µg/mL, ionomycin 1.34 µM and Brefeldin A 5 µg/mL. Aliquot desired volumes and store at −80°C, avoiding repeated freeze/thaw cycles. Prepare immediately before use by diluting 1:500 in DMEM with 10% HI-NCS.

Alternatives: Cell activation cocktails and reagents from other manufacturers may be used in this protocol after validation. Please refer to the Alternative Resources Table for further information.

Table 1. BD LSRFortessa configuration for example antibody panels

Laser	Filter
355	379/28
355	450/50
405	525/50
405	610/20
405	670/30
405	780/60
488	530/30
488	695/40
561	582/15
561	610/20
561	780/60
640	670/30
640	730/45
640	780/60

BD LSRFortessa Flow Cytometer

The provided example antibody panels have been designed and optimized for a 5-laser BD LSRFortessa configured as per Table 1. Flow cytometer type and configuration will determine if the provided panels can be used successfully; adjustments to the choice of antibody and fluorochromes may be necessary. Following the leukocyte isolation procedure, any panel appropriate for the available flow cytometer can be used for individualized characterization of the cell populations of interest.

STEP-BY-STEP METHOD DETAILS

Tissue harvest

Timing: 20 min per mouse

For non-arterialized orthotopic liver transplantation and intra-abdominal heterotopic heart transplantation surgical procedures please refer to (Yokota et al., 2016) and (Corry et al., 1973). Here, we describe the harvesting of transplanted and native hearts and livers from mice. Harvesting of tissues is ideally performed under terminal anesthesia to reduce the risk of thrombosis, though may be done immediately after euthanasia. The transplanted heart is located in the abdomen, thus harvesting of the transplant and native hearts require separate procedures. The transplanted liver replaces the native liver, thus the procedure for harvesting either a transplanted or native liver is the same.

1. Induce anesthesia and place the mouse in a supine position with continuous anesthetic delivery. Secure the limbs with tape to provide access to the abdomen.
2. Sterilize the abdomen with 70% ethanol.
3. Open the mouse:
   a. With forceps, lift the skin at the pelvis and make an incision with scissors.
   b. Continue the incision to the sternum, exposing the peritoneal cavity membrane.
   c. Carefully cut open the peritoneum from the pelvis to the sternum.
   d. Further open the abdomen by incising along the flanks.
   e. Move the abdominal organs aside with cotton tip applicators to expose the transplanted organ.
4. Proceed with step 5 for liver transplant mice, and steps 6 and 7 for heart transplant mice.

5. Perfuse and collect the transplanted liver (Methods videos S1, S2, and S3):
   a. Locate and incompletely sever the inferior vena cava (IVC) at the top of the liver (Methods video S1).
   b. Perfuse the liver with 10 mL cold saline by inserting a 23 G needle attached to a 10 mL syringe into the IVC. The liver will blanch and appear pale with correct perfusion.
   c. Remove and discard the gallbladder (Methods video S2).
   d. Resect the liver, taking care to cut any adhesions to the surrounding tissue (Methods video S3), place in DMEM and keep on ice.
6. Perfuse the mouse and collect the transplanted heart (Methods video S4):
   a. Perfuse the mouse by injecting 3 mL cold saline into the abdominal aorta using a 30 G needle below the heart graft. The transplanted heart will become pale with correct perfusion.
   b. Cut the aorta and soak up the perfusate with a cotton ball.
   c. Resect the heart graft from the abdomen, place in DMEM and keep on ice.
7. Collect the native heart (Methods video S5):
   a. Cut both lateral sides of the rib cage and the diaphragm.
   b. Clamp the sternum with clamp forceps and invert to expose the chest cavity.
   c. Isolate and resect the heart from the chest cavity, place in DMEM and keep on ice.

⚠ CRITICAL: Ensure the entire liver is well perfused to limit collection of blood-borne cells that will contaminate the tissue-resident leukocyte populations of interest.

Note: Other tissues such as blood (prior to euthanasia), spleen, lymph nodes and bone marrow can also be harvested for analysis and should be collected into DMEM and placed on ice prior to processing.

Preparation of single-cell suspension from liver tissue

Timing: 1 h

This step describes the process of mechanical disruption and differential centrifugation of liver tissue to prepare a single-cell suspension of leukocytes for flow cytometry (Figure 1). We have found that compared to enzymatic digestion, mechanical disruption has reduced impact on the expression of markers of interest, but does not allow for isolation of Kupffer cells.

8. Record the weight of liver tissue to be used for flow cytometry if taking a section for histology.
9. Place the liver into a 70 μm cell strainer atop a 50 mL tube.
10. Mash the liver through the strainer with the rubber end of a 3 mL syringe plunger, rinsing with FACS buffer (Troubleshooting 1).
11. Centrifuge the cell suspension at 300 × *g* with swinging buckets for 3 min at 21°C–23°C then carefully remove and discard the supernatant.
12. Resuspend the pellet in 1 mL 42% isotonic Percoll and transfer to a 15 mL tube. Wash the original tube with 4 mL 42% isotonic Percoll and combine in the 15 mL tube.

⚠ CRITICAL: Ensure Percoll is at 21°C–23°C before use.

13. Centrifuge at 800 × *g* for 20 min at 21°C–23°C with no brake to separate hepatocytes (top cellular layer) and leukocytes (pellet).

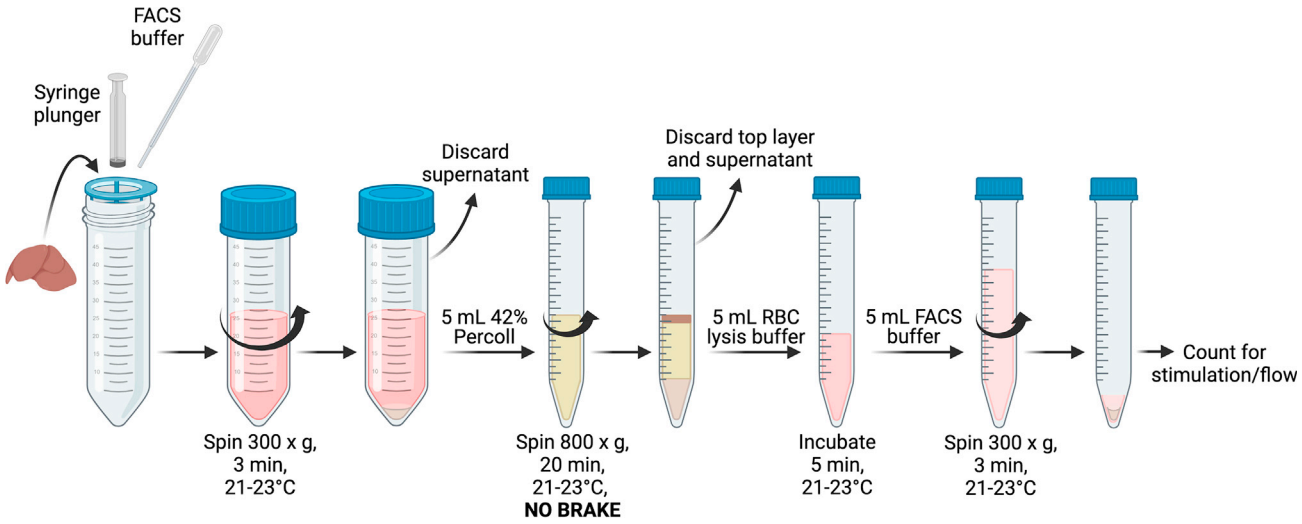

Figure 1. Illustration of liver leukocyte isolation procedure

⚠ CRITICAL: Ensure brake is off during centrifugation.

14. Carefully remove and discard the top cellular layer and Percoll from the pellet (Troubleshooting 2).
15. Resuspend the pellet in 100 μL of RBC Lysis buffer and transfer to a new 15 mL tube containing 4.8 mL of RBC Lysis buffer (Troubleshooting 3).

⚠ CRITICAL: Transfer pellet to new tube to avoid contamination with hepatocytes.

16. Wash the bottom of the original 15 mL tube with another 100 μL of RBC Lysis buffer, combine in the new 15 mL tube and mix well.
17. Incubate for 5 min at 21°C–23°C with occasional shaking.
18. Add 5 mL FACS buffer and centrifuge at 300 × *g* for 3 min at 21°C–23°C to re-pellet the leukocytes. At this point the pellet should be pale and free of RBCs.
19. Resuspend the pellet in 400 μL of FACS buffer and count with 5–10 μL of the cell suspension (Troubleshooting 4).

Pause point: Samples can be kept on ice for 1–2 h until ready to proceed to the next step.

Preparation of single-cell suspension from heart tissue

Timing: 1 h 40 min

This step describes the process of enzymatic digestion and differential centrifugation of heart tissue to prepare a single-cell suspension of leukocytes for flow cytometry (Figure 2).

20. Record the weight of heart tissue to be used for flow cytometry if taking a section for histology.
21. Transfer the heart to a 60 mm petri dish and rinse with PBS.
22. Dissect the heart in half lengthways, rinse again with PBS and transfer to a clean 60 mm petri dish.
23. Inject 500 μL of the heart digestion cocktail into the heart tissue. Disperse the cocktail evenly throughout the tissue by injecting at multiple points.

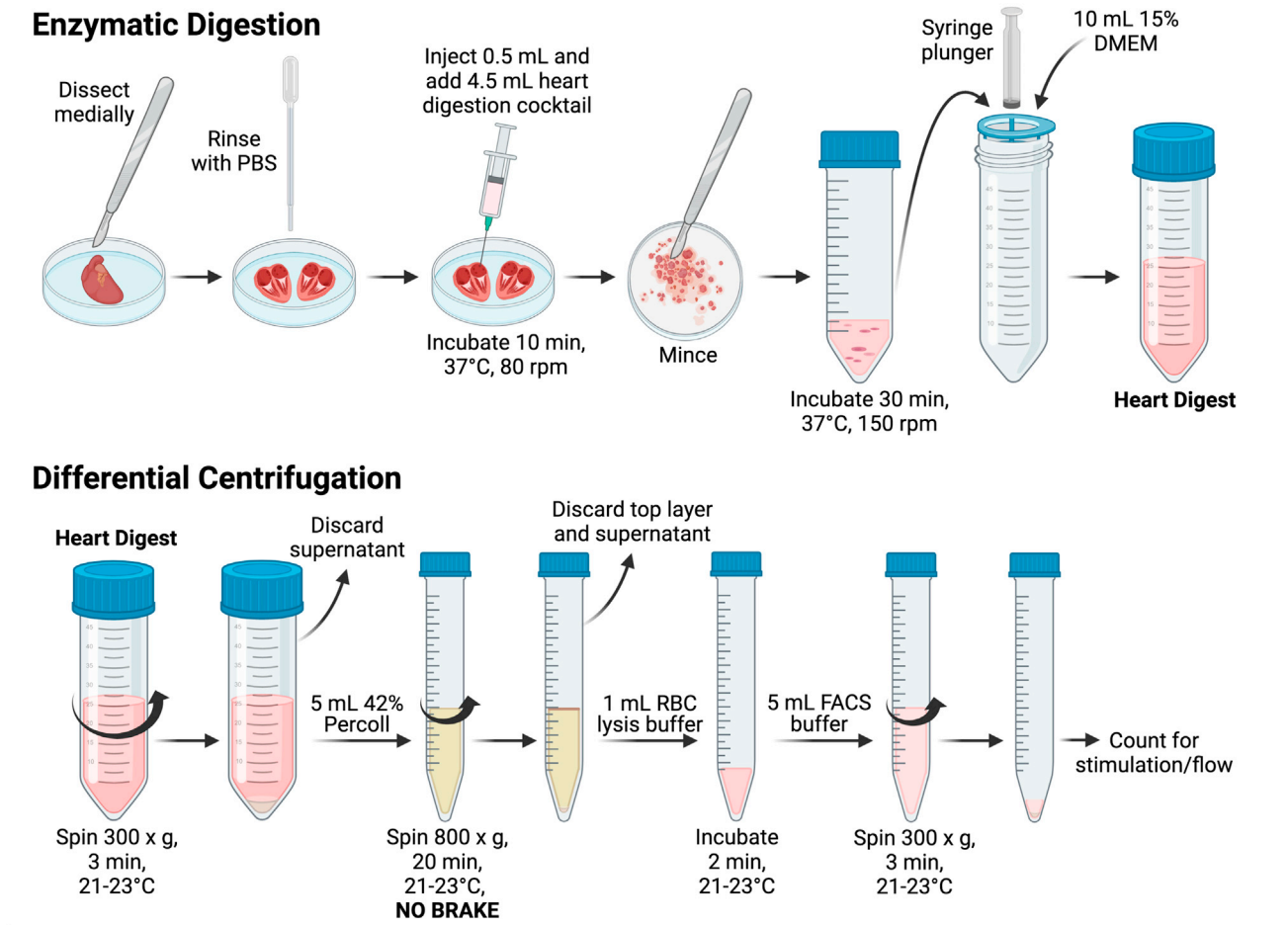

Figure 2. Illustration of heart leukocyte enzymatic digestion and differential centrifugation isolation procedure

24. Decant the remaining 4.5 mL of the digestion cocktail into the petri dish with the heart.
25. Incubate for 10 min at 37°C with gentle shaking at 80 rpm.
26. Remove the heart from the digestion cocktail (e.g., to the petri dish lid) and mince with a scalpel until pieces are approximately 0.5–1 mm^3 in size.
27. Resuspend the minced heart tissue in the digestion cocktail and transfer to a 50 mL tube.
28. Incubate for 30 min at 37°C with shaking at 150 rpm to prevent the tissue from settling.
29. Pass through a 70 μm strainer, mashing any remaining clumps gently through with the rubber end of a 3 mL syringe plunger.
30. Rinse the cell strainer with 10 mL DMEM with 15% HI-NCS.
31. Centrifuge at 300 × *g* for 3 min at 21°C–23°C to pellet cells then carefully remove and discard the supernatant.
32. Resuspend the pellet in 1 mL 42% isotonic Percoll, transfer to a new 15 mL tube, wash the tube with a further 4 mL 42% isotonic Percoll and combine in the new tube.
33. Centrifuge at 800 × *g* for 20 min at 21°C–23°C with no brake to separate parenchymal cells (top layer) and leukocytes (pellet).

⚠ CRITICAL: Ensure brake is off during centrifugation.

34. Carefully and completely remove and discard the top cellular layer and supernatant.
35. Resuspend the pellet in 100 μL RBC lysis buffer and transfer to new 15 mL tube containing 900 μL RBC lysis buffer (Troubleshooting 3).
36. Rinse the bottom of the tube with a further 100 μL RBC lysis buffer and combine in new tube.
37. Incubate for 2 min at 21°C–23°C until RBCs are lysed.
38. Add 5 mL FACS buffer and centrifuge at 300 × *g* for 3 min at 21°C–23°C to re-pellet the leukocytes. At this point the pellet should be pale and free of RBCs.
39. Resuspend the pellet in 100 μL of FACS buffer and count with 5–10 μL of the cell suspension (Troubleshooting 4).

Stimulation for functional analysis

Timing: 4 h 15 min

Here we describe the *in vitro* stimulation of isolated leukocytes for the analysis of cytokine and cytotoxic granule production by T cells (CD8αβ T, CD4 T, regulatory T (Treg), natural killer T (NKT), CD8αα T, and $CD8^{-}CD4^{-}$ double-negative (DN) T cells) (Figure 3). Stimulation reveals the capacity of these cells to produce these cytokines and granules, while unstimulated controls show the existing activity of the cells. Both stimulated and unstimulated samples should contain the same number of cells per well and simultaneously be subjected to identical incubation times and conditions.

40. Transfer 0.1–4 × 10^6 cells per well to a 96 well U bottom plate.
41. Pellet the cells by centrifugation at 200 × *g* for 3 min at 21°C–23°C and flick the plate firmly over a waste receptacle to discard the supernatant.
42. In unstimulated wells, resuspend the cells in 200 μL DMEM with 10% HI-NCS. In stimulated wells, resuspend the cells in 200 μL Cell Activation Cocktail diluted 1:500 in DMEM with 10% HI-NCS.
43. Incubate for 4 h at 37°C with 5% CO_2.
44. Pellet the cells by centrifugation at 200 × *g* for 3 min at 21°C–23°C and discard the supernatant.
45. Continue with the 'staining for flow cytometry' step below using a panel such as that detailed in Table 2.

Table 2. Example staining panel for functional analysis of lymphocytes after stimulation on a 5 laser BD LSRFortessa flow cytometer

Laser	Detector	Fluorophore	Antigen	Dilution
355	379/28	BUV395	CD8α	1:400
355	450/50	UV	Live/Dead	1:1000
405	525/50	BV510	CD49a	1:50
405	610/20	SuperBright600	CD4	1:700
405	670/30	BV650	CD8β	1:1500
405	780/60	BV785	IFNγ	1:50
488	530/30	FITC	CD45.2	1:100
488	695/40	PerCPCy5.5	Granzyme B	1:20
561	582/15	PE	IL-10	1:100
561	610/20	PECF594	FoxP3	1:100
561	780/60	PECy7	CD69	1:50
640	670/30	APC	$CD1d^{tet}$	1:150
640	730/45	AF700	TCRβ	1:50
640	780/60	APCeF780	CD45.1	1:200

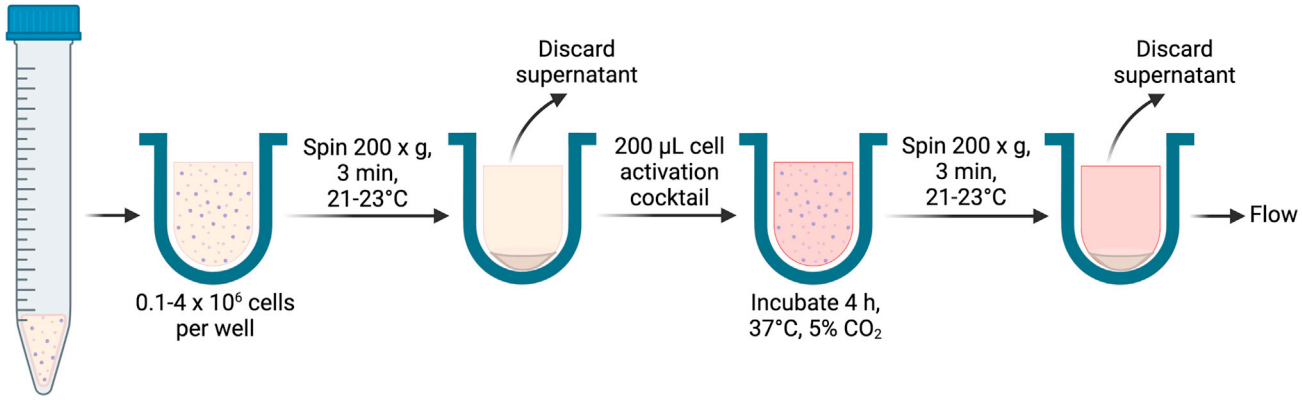

Figure 3. Illustration of leukocyte stimulation procedure

Note: Intracellular antibodies are: IFNγ, granzyme B, IL-10 and FoxP3. All remaining antibodies are for surface staining use. For exact antibodies, see key resources table.

Staining for flow cytometry

Timing: 2 h

This major step details the method of staining isolated leukocytes for identifying and characterizing lymphocyte populations (CD8αβ T, CD4 T, Treg, NKT, CD8αα T, and DN T cells). We have performed staining in 96 well U-bottom plates, however this can also be done in Eppendorf or FACS tubes.

46. Transfer 0.1–4 × 10^6 cells per well for staining.
47. Pellet the cells by centrifugation at 200 × *g* for 3 min at 21°C–23°C and discard supernatant.

Note: This protocol uses Zombie UV for assessing the viability of cells prior to surface marker staining; therefore cells must be in a protein-free buffer such as PBS during this step.

48. Wash the cells by resuspending in 150 µL PBS then pellet the cells by centrifugation at 200 × *g* for 3 min at 21°C–23°C and discard supernatant.
49. Resuspend the cells in 40 µL Zombie UV solution diluted 1:1000 in PBS.
50. Incubate for 20 min at 21°C–23°C in the dark.
51. Wash the cells with 150 µL FACS buffer.

Pause point: Samples can be kept on ice for 1–2 h until ready to proceed to the next step.

52. Pellet the cells by centrifugation at 200 × *g* for 3 min at 21°C–23°C and discard supernatant.

Note: CD69 and CD49a markers should be included for analysis of tissue-resident populations as the majority express a $CD69^+CD49a^+$ phenotype (Mackay et al., 2016; Prosser et al., 2021). In the liver, CXCR6 is also a useful inclusion as many tissue-resident lymphocytes express this molecule (Prosser et al., 2021).

53. Resuspend the cells in 40 µL surface antibody cocktail as per Tables 2 or 3.

Table 3. Example staining panel with options for phenotypic analysis of lymphocyte populations on a 5 laser BD LSRFortessa flow cytometer

Laser	Detector	Fluorophore	Antigen	Dilution
355	379/28	BUV395	CD8α	1:500
355	450/50	UV	Live/Dead	1:1000
405	450/50	BV421	CX3CR1 or GATA3 or PD1	1:2000 or 1:20 or 1:50
405	525/50	BV480	CD8β	1:1000
405	610/20	BV605	T-bet	1:20
405	670/30	BV650	CXCR3 or LAG3 or RORγt	1:100 or 1:50 or 1:20
405	710/50	BV711	CD69 or KLRG1 or CD49d	1:20 or 1:200 or 1:20
405	780/60	BV786	CD4	1:1000
488	530/30	FITC	CD45.2	1:100
488	695/40	BB700	CD49a	1:100
561	582/15	PE	CXCR6 or CD127 or TIM3	1:1000 or 1:100 or 1:300
561	610/20	PECF594	FoxP3	1:100
561	780/60	PECy7	CD69 or Ly6C or Eomes	1:50 or 1:1000 or 1:70
640	670/30	APC	$CD1d^{tet}$	1:200
640	730/45	AF700	TCRβ	1:50
640	780/60	APCeF780	CD45.1	1:200

Note: Intracellular antibodies are: GATA3, T-bet, RORγt, FoxP3 and Eomes. All remaining antibodies are used for surface staining. For exact antibodies, see key resources table.

54. Incubate for 20 min at 21°C–23°C in the dark.
55. Wash the cells with 150 μL FACS buffer then pellet the cells by centrifugation at 200 × *g* for 3 min at 21°C–23°C and discard the supernatant.
56. Resuspend in 100 μL of Fixation/Permeabilization buffer.
57. Incubate for 10 min at 21°C–23°C in the dark.
58. Add 100 μL of Permeabilization buffer and mix well.
59. Pellet cells by centrifugation at 800 × *g* for 3 min at 21°C–23°C and discard supernatant.

Note: Centrifugation speed is higher after permeabilization to ensure adequate pelleting of cells.

60. Wash the cells by adding 150 μL of Permeabilization buffer then pellet the cells by centrifugation at 500 × *g* for 3 min at 21°C–23°C and discard supernatant.
61. Add 20 μL of the intracellular antibody cocktail as per Tables 2 or 3 and incubate for 20 min at 21°C–23°C in the dark.
62. Wash the cells by adding 150 μL of Permeabilization buffer then pellet the cells by centrifugation at 500 × *g* for 3 min at 21°C–23°C and discard supernatant.
63. Wash the cells once more by adding 150 μL of FACS buffer then pellet the cells by centrifugation at 500 × *g* for 3 min at 21°C–23°C and discard the supernatant.
64. Resuspend the cells in 100 μL 1× BD Stabilizing Fixative and incubate for 15 min at 21°C–23°C in the dark.
65. Add 150 μL FACS buffer then pellet the cells by centrifugation at 500 × *g* for 3 min at 21°C–23°C and discard supernatant.
66. Resuspend in 150 μL FACS buffer.
67. Cells are now ready for analysis on a flow cytometer (Troubleshooting 5). Samples may be transferred to appropriate tubes or plates to suit individual cytometer requirements.

Pause point: Cells may be stored at 4°C in the dark for up to 4 days prior to analysis.

Table 4. Expected leukocyte numbers isolated from livers before and after transplantation

		$\times 10^6$			
Transplant		Day 0	Day 1	Day 7	Day 28
$H2^b$ to $H2^b$	MHC match	2–3	1–7	3–10	1–6
$H2^b$ to $H2^d$	MHC mismatch	2–3	2–6	13–50	11–25

⚠ CRITICAL: The suggested flow cytometry panels require the following appropriate controls. For detailed information for each of the recommended resources please refer to the key resources table.

Compensation Controls

UltraComp eBeads compensation beads are recommended to achieve reliable and robust positive and negative populations for the fluorochromes listed in each suggested antibody panel.

Flow cytometer setup and quality control

BD Cytometer Setup and Tracking (CST) Beads consist of bright, mid and dim beads dyed with a mixture of fluorochromes. Using CST beads, the BD acquisition software measures changes in median fluorescence intensity and robust CV for each bead intensity in all fluorescence detectors and reports on changes from baseline. It is recommended to run CST each time the cytometer is switched on to confirm consistency of data being acquired.

Ultra Rainbow Calibration Particle (URCP) Kits

Similar to CST beads, URCP allow for rapid routine calibration and long-term performance tracking of flow cytometers. URCP produce standardized multiple peaks in each fluorescence detector, which can be easily tracked for changes in cytometer performance. It is recommended to run URCP at the start of each data acquisition experiment.

Fluorescence Minus One (FMO) Controls

FMO controls are recommended for gating accuracy and confirmation of fluorochrome spread. For each panel, it is recommended to prepare FMO control samples for each non-lineage phenotypic or functional marker.

EXPECTED OUTCOMES

Liver

The expected total leukocyte yield from a whole liver before and after MHC-matched and MHC-mismatched liver transplantation is shown in Table 4. It is expected that 90% of leukocytes will be viable as determined by flow cytometry when using the described isolation protocol.

Heart

The expected total leukocyte yield from a whole heart before and after MHC-matched and MHC-mismatched heart transplantation is shown in Table 5. Our $H2^b$ to $H2^d$ MHC-mismatched heart

Table 5. Expected leukocyte numbers isolated from hearts before and after transplantation

		$\times 10^5$			
Transplant		Day 0	Day 1	Day 7	Day 28
$H2^b$ to $H2^b$	MHC match	1–4	5–12	1–5	1–13
$H2^b$ to $H2^d$	MHC mismatch	1–4	5–24	10–32	n/a

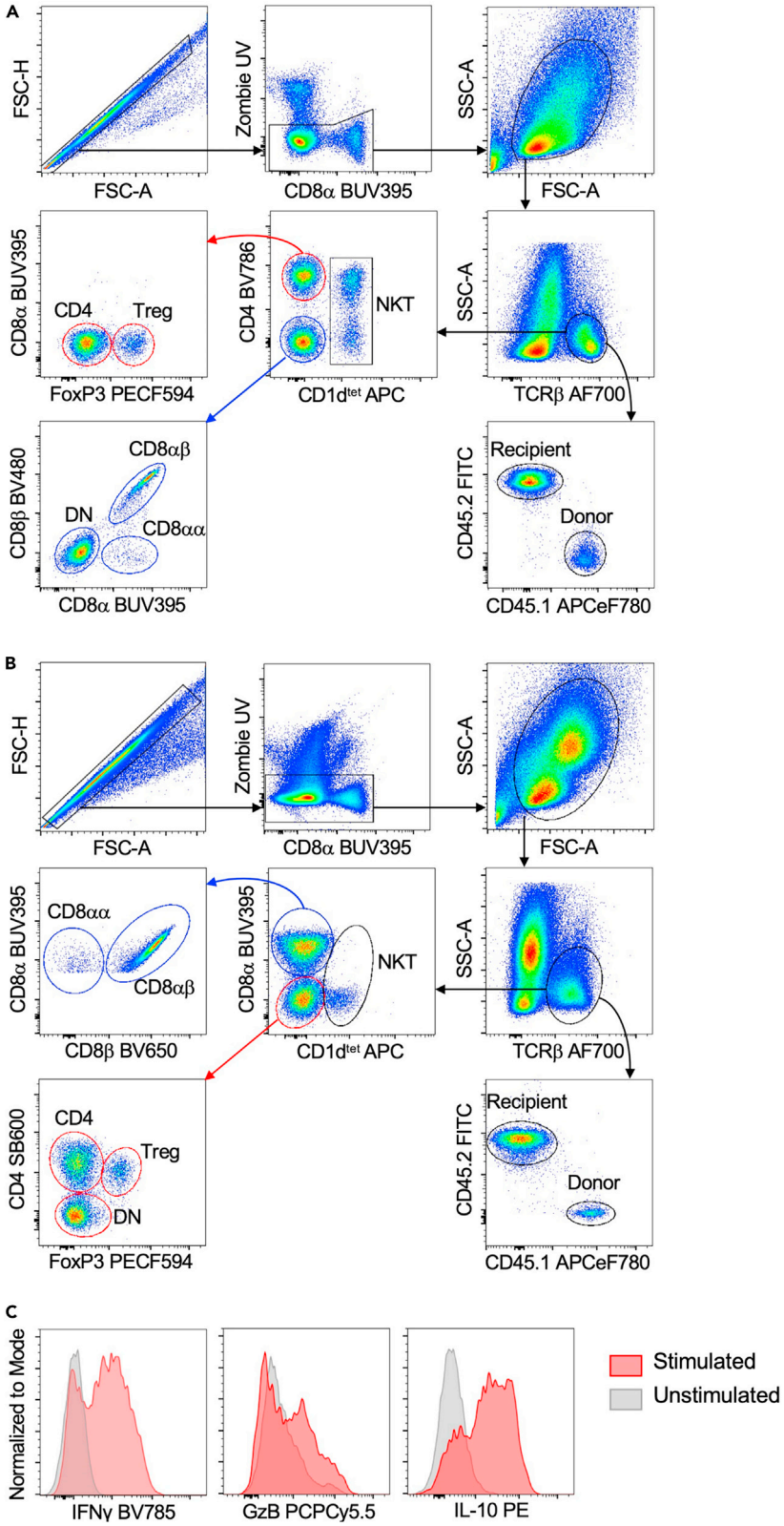
A
FSC-H
FSC-A
Zombie UV
CD8α BUV395
SSC-A
FSC-A
CD8α BUV395
CD4
Treg
FoxP3 PECF594
CD4 BV786
NKT
CD1dtet APC
SSC-A
TCRβ AF700
CD8αβ
DN
CD8αα
CD8β BV480
CD8α BUV395
Recipient
Donor
CD45.2 FITC
CD45.1 APCeF780
B
FSC-H
FSC-A
Zombie UV
CD8α BUV395
SSC-A
FSC-A
CD8αα
CD8αβ
CD8α BUV395
CD8β BV650
CD8α BUV395
NKT
CD1dtet APC
SSC-A
TCRβ AF700
CD4
Treg
DN
CD4 SB600
FoxP3 PECF594
Recipient
Donor
CD45.2 FITC
CD45.1 APCeF780
C
Normalized to Mode
IFNγ BV785
GzB PCPCy5.5
IL-10 PE
Stimulated
Unstimulated

Figure 4. Flow cytometry gating strategy

Gating strategy to identify donor and recipient lymphocyte populations from the suggested flow cytometry panels for (A) phenotyping and (B) functional analysis.

(C) Representative histograms of IFNγ and granzyme B expression by total T cells, and IL-10 expression by Treg cells after 4 h of stimulation, or not, with PMA/ionomycin in the presence of brefeldin A.

transplants typically fail by day 12 post-transplant (Prosser et al., 2021). It is expected that 80% of leukocytes from hearts will be viable when using the described isolation protocol.

From the total leukocytes isolated, the suggested panels in this protocol allow the identification of donor and recipient CD8αβ T, CD4 T, Treg, NKT, CD8αα T and DN T cell subsets. These example panels focus on T cell characterization; however, this isolation protocol can be used to examine cell types including innate lymphoid cells (ILC), dendritic cells, macrophages (excluding Kupffer cells), monocytes and granulocytes using other panels. For expected numbers, frequencies, phenotypic marker expression and functional capacity of lymphoid cell subsets refer to Prosser et al, 2021.

Tissue-resident lymphocytes can be identified in the described systems as donor lymphocytes maintained long-term after transplantation. Robust populations of tissue-resident ILC, unconventional T cells characterized by expression of CD69 and CD49a, and small populations of tissue-resident conventional T cells, which are phenotypically heterogeneous, are present in the liver. The heart does not contain significant numbers of tissue-resident lymphocytes that persist long-term after transplantation. In addition to tissue-resident lymphocytes, other leukocyte subsets are typically present in abundance, particularly recipient cells in post-transplant organs (Prosser et al., 2021).

QUANTIFICATION AND STATISTICAL ANALYSIS

When quantifying cell numbers per organ, the amount of tissue initially processed must be accounted for, particularly if sections have been taken for other purposes such as histology. Cell isolation efficiency can vary from day to day, so same day processing of experimental groups that will be used for comparison is recommended where possible. Cells may be counted manually prior to staining, or by using counting beads during sample acquisition. Manual or automated counting is recommended to ensure optimal staining; we have found that population resolution decreases when more than 4×10^6 cells are stained per well. Counting beads also do not account for the unknown number of cells that are lost during the staining procedure. Gating strategies for lymphocyte subset identification using the suggested panels are included in Figure 4 and Table 6. Functional analysis of cells after stimulation can be performed by gating positively stained cells of interest and assessing the frequency of expression and median fluorescence intensity of the functional markers measured (Figure 4C).

LIMITATIONS

Heart

The heart contains relatively few leukocytes, particularly native hearts from non-transplanted mice. Thus, one heart will generally yield enough leukocytes for analysis with one flow cytometry panel. This is in contrast to the liver, which generally yields enough leukocytes for staining with four separate panels. However, this is dependent on the cell subsets of interest, as rare populations will require a greater number of starting cells to ensure adequate events of interest are acquired.

Liver

Compared to enzymatic digestion, mechanical disruption and Percoll density centrifugation of the liver enables better purification of macrophages, though with reduced yield (Lynch et al., 2018). If macrophages are the leukocyte subset of interest, enzymatic digestion may be a better isolation procedure. In particular, the protocol described herein does not isolate Kupffer cells.

Table 6. Cell subset identification markers

Subset	Identifying markers
Donor[a]	$CD45.1^+$ $CD45.2^-$
Recipient[a]	$CD45.2^+$ $CD45.1^-$
NKT	$TCR\beta^+$ $CD1d^{tet+}$
CD4 T	$TCR\beta^+$ $CD1d^{tet-}$ $CD4^+$ $FoxP3^-$
Treg	$TCR\beta^+$ $CD1d^{tet-}$ $CD4^+$ $FoxP3^+$
CD8αβ T	$TCR\beta^+$ $CD1d^{tet-}$ $CD4^-$ $CD8\alpha^+$ $CD8\beta^+$
CD8αα T	$TCR\beta^+$ $CD1d^{tet-}$ $CD4^-$ $CD8\alpha^+$ $CD8\beta^-$
DN T	$TCR\beta^+$ $CD1d^{tet-}$ $CD4^-$ $CD8\alpha^-$ $CD8\beta^-$

[a]Dependent on strain combination used for transplantation. Not applicable for non-transplanted samples.

TROUBLESHOOTING

Problem 1

Unable to pass liver through cell strainer (step 10)

Potential solution

Transplanted organs, particularly those that are MHC-mismatched, are more difficult to process than non-transplanted organs. This is possibly due to the increased number of cells per gram of tissue, fibrosis, or some other mechanism. The tissue becomes very firm and difficulty may be encountered in passing the liver through the cell strainer. This can be mitigated by mincing the liver with a scalpel and applying small amounts of this minced preparation to the strainer while rinsing with FACS buffer when passing through with a syringe plunger.

Problem 2

Incomplete separation after Percoll differential centrifugation (step 14)

Potential solution

Single cell preparations from livers occasionally do not separate completely after Percoll differential centrifugation, with some hepatocytes loosely associating with the leukocyte and red blood cell pellet (Figure 5). One of two options may be used to rectify this problem. First, carefully remove the hepatocyte debris using either a transfer pipette or 200 μL pipette and tip. This debris is often easily completely removed without disturbing the pellet. Second, a repeat of the Percoll centrifugation step will separate the remaining hepatocytes from the cell pellet.

Problem 3

Contamination of leukocytes with parenchymal cells (steps 15 and 35)

Potential solution

After Percoll centrifugation, the top layer of parenchymal cells must be removed. For the heart, an absorbent material such as a tissue held with a pair of forceps may assist in removing the thin layer of parenchymal cells. For both liver and heart samples, be sure to transfer the cell pellet after Percoll centrifugation to a clean tube for RBC lysis as parenchymal cells may be stuck to the sides of the tube, as indicated in Figure 6. The liver preparation may contain excess fat, depending on the age and diet of the mouse, so an additional wash step prior to red blood cell lysis may be helpful in removing hepatocyte and fat contamination.

Problem 4

Low leukocyte yield or poor viability (steps 19 and 39)

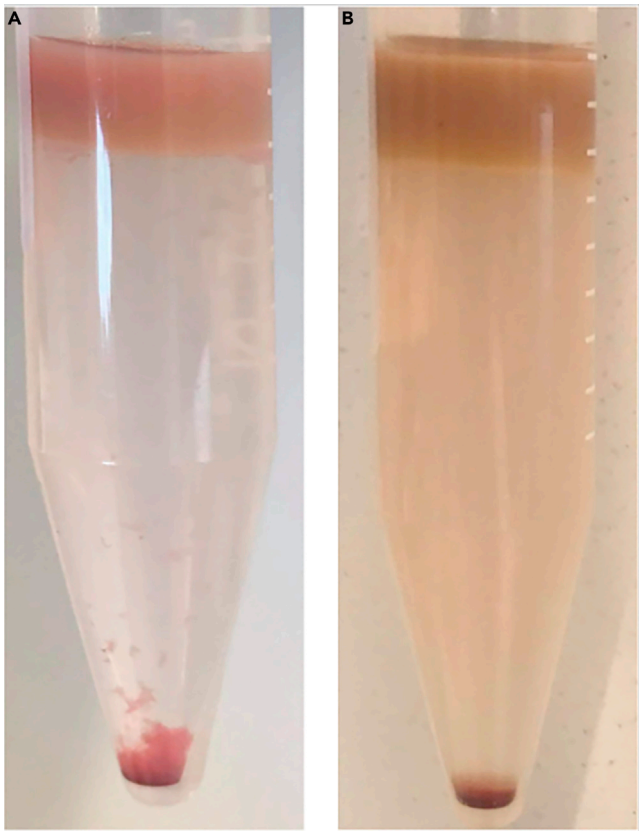

Figure 5. Liver Percoll differential centrifugation
Incomplete (A) and complete (B) Percoll differential centrifugation of liver single cell preparations.

Potential solution

Low yield and/or poor viability can result from inefficient cell isolation or over-incubation with digestion enzymes or RBC lysis buffer. Ensure all required reagents and equipment are prepared prior to starting so that the protocol is completed rapidly and to the incubation times specified.

It is advisable to stagger the protocol when processing multiple heart samples. Accurate timing of digestion is critical to avoiding cell death and maximizing yield. Digestion enzyme function is affected by freeze and thaw cycles so ensure stocks are aliquoted before freezing and thawed immediately before use.

After passing either liver or digested heart through the cell strainer, be sure to rinse the strainer well and scrape off any residue from the underside and add to the single cell suspension. When passing samples through strainers, ensure the sample is kept moist with either FACS buffer or DMEM with 15% HI-NCS, as indicated in the protocol. Rinse tubes when transferring cells to new tubes and add to the cell suspension.

Problem 5

Poor staining and population resolution (step 67)

Potential solution

Poor flow cytometry results can be caused by several factors including damaged reagents, inadequate panel optimization, and incorrect flow cytometer setup. Fluorescent reagents, including antibodies, are at risk of photo-bleaching when exposed to light. These reagents must be kept in the

Figure 6. Example contamination of tube with hepatocytes after Percoll differential centrifugation

dark as much as is practicable to avoid damage. All antibody storage, cocktail preparation, and staining must be done in the dark or low light conditions for optimal results.

Each antibody panel and flow cytometer require optimization for staining and population resolution. The provided antibody dilutions are a starting point for the specific antibodies listed, however these should be titrated to maximize negative and positive population separation (Figure 7A), whilst minimizing spread of the positive signal into off-target detectors (Figure 7B).

Flow cytometer gain settings (voltages) for each detector should be set such that the negative population in each detector is 2.5× the electronic noise, which can be sourced from CST reports of BD cytometers. If using a non-BD cytometer, negative populations should be clearly visible on a logicle

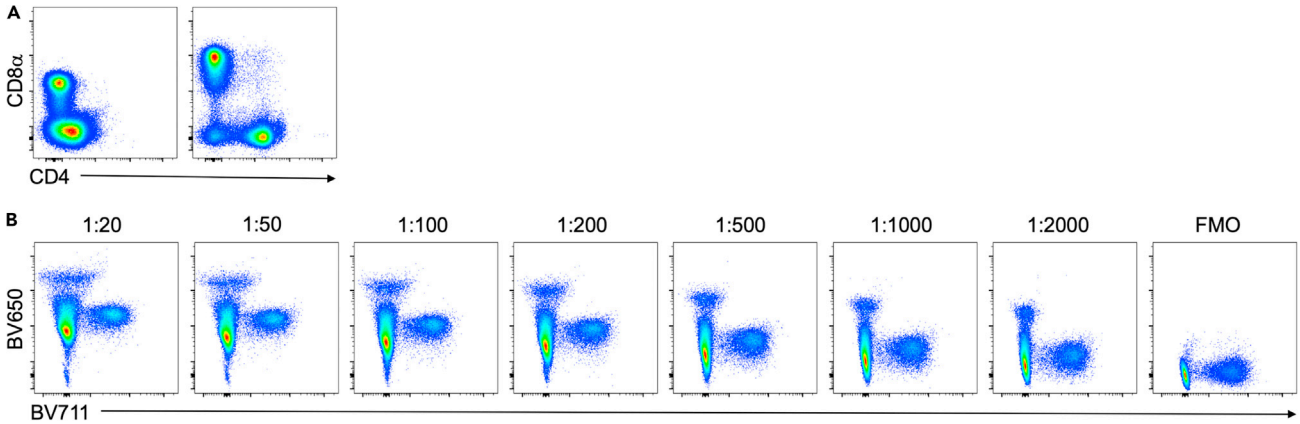

Figure 7. Flow cytometry staining and population resolution optimization

(A) Example of poor $CD4^+$ and $CD8^+$ population separation caused by inadequate panel optimization (left), and good separation after optimization (right).

(B) Example antibody titration showing reduction of spread from a BV650 antibody into the BV711 detector with increasing dilution.

scale, generally at just below 10^2. The positive population must sit within the linear scale of each detector (10^2–10^5), which should be achieved by adjustment of the staining concentration of antibodies (Figures 4 and 7).

The use of CST bead and URCP controls as listed in the major step Staining for Flow Cytometry will also provide quality control for the acquisition of data.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Michaela Lucas; Michaela.lucas@uwa.edu.au.

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100810.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.P. and M.L.; methodology, A.P., I.L.-C., and S.D.; investigation, A.P. and S.D.; data curation, A.P. and S.D.; writing – original draft, A.P.; writing – review and editing, A.P., S.D., and M.L.; funding acquisition, A.P. and M.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Corry, R.J., Winn, H.J., and Russell, P.S. (1973). Heart transplantation in congenic strains of mice. Transpl. Proc. *5*, 733–735.

Deng, Y., Wu, S., Yang, Y., Meng, M., Chen, X., Chen, S., Li, L., Gao, Y., Cai, Y., Imani, S., et al. (2020). Unique phenotypes of heart resident type 2 innate lymphoid cells. Front. Immunol. *11*, 802.

Dong, Z.J., Wei, H.M., Sun, R., Tian, Z.G., and Gao, B. (2004). Isolation of murine hepatic lymphocytes using mechanical dissection for phenotypic and functional analysis of NK1.1+ cells. World J. Gastroenterol. *10*, 1928–1933.

Lynch, R.W., Hawley, C.A., Pellicoro, A., Bain, C.C., Iredale, J.P., and Jenkins, S.J. (2018). An efficient method to isolate Kupffer cells eliminating endothelial cell contamination and selective bias. J. Leukoc. Biol, *104*, 579–586.

Mackay, L.K., Minnich, M., Kragten, N.A., Liao, Y., Nota, B., Seillet, C., Zaid, A., Man, K., Preston, S., Freestone, D., et al. (2016). Hobit and Blimp1 instruct a universal transcriptional program of tissue residency in lymphocytes. Science *352*, 459–463.

Pinto, A.R., Paolicelli, R., Salimova, E., Gospocic, J., Slonimsky, E., Bilbao-Cortes, D., Godwin, J.W., and Rosenthal, N.A. (2012). An abundant tissue macrophage population in the adult murine heart with a distinct alternatively-activated macrophage profile. PLoS One *7*, e36814.

Prosser, A., Huang, W.H., Liu, L., Dart, S., Watson, M., de Boer, B., Kendrew, P., Lucas, A., Larma-Cornwall, I., Gaudieri, S., et al. (2021). Dynamic changes to tissue-resident immunity after MHC-matched and MHC-mismatched solid organ transplantation. Cell Rep. *35*, 109141.

Yokota, S., Ueki, S., Ono, Y., Kasahara, N., Perez-Gutierrez, A., Kimura, S., Yoshida, O., Murase, N., Yasuda, Y., Geller, D.A., et al. (2016). Orthotopic mouse liver transplantation to study liver biology and allograft tolerance. Nat. Protoc. *11*, 1163–1174.