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Isolation of brain infiltrating lymphocytes V.2

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We use this protocol and it's working

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Abstract

This protocol details the isolation of brain infiltrating lymphocytes. Splenic lymphocytes are screened to verify the presence/phenotype of CD8 in the periphery.

Materials

Materials and reagents:

- gentleMACS Octo Dissociator with Heaters **Miltenyi Biotec Catalog #** 130-096-427
- Collagenase D **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**11088882001
- Corning® RPMI 1640, Corning **VWR International Catalog #**45000-416
- Deoxyribonuclease I from bovine pancreas, type IV **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**D5025
- Dulbeccos phosphate-buffered saline (DPBS) **Gibco - Thermo Fischer Catalog #**14190144
- L(+)-Glutamine solution 200 mM in water (29.20 mg/ml) cell culture reagent, Corning® **VWR International Catalog #**45000-676
- Corning™ HEPES, Liquid **Fisher Scientific Catalog #**MT25060CI
- Sodium pyruvate solution 100 mM with 8.5 g/L NaCl cell culture reagent, Corning® **VWR International Catalog #**45000-710
- 2-Mercaptoethanol **Thermo Fisher Catalog #**21985023
- Corning® MEM (Minimum Essential Medium) Nonessential Amino Acids, Corning **VWR International Catalog #**45000-700
- Fetal Bovine Serum, qualified, Canada **Thermo Fisher Catalog #**12483020
- ACK Lysing Buffer **Thermo Fisher Scientific Catalog #**A1049201
- Micro test plate, 96 well, slip lid, flat bottom, PS, transparent **Sarstedt Catalog #**82.1581.001
- Falcon™ Cell Strainers **Fisher Scientific Catalog #**08-771-2
- Percoll **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P1644-500ML
- Phorbol 12-myristate 13-acetate (PMA) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P1585
- Ionomycin from Streptomyces conglobatus **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**I9657
- Brefeldin A **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**B6542-5MG

RPMIc:

RPMI (500 mL)
FBS 10 % (50 mL decomplemented)
L-Glutamine (5 mL)
Sodium pyruvate (5 mL)
HEPES (5 mL)
Antibiotic (Pen-Strep) (5 mL)
Non-essential amino acids (5 mL)
HEPES buffer (5ml)
b-mercaptoethanol 50 mmol/L final (Very important, essential growth factor for mouse T-lymphocytes).

- 4% formaldehyde:

	A	B
	PBS 10X	14 ml
	Formaldehyde 37.5%	10.8 ml
	Distilled H2O	75.2 ml
	Filter through 0.2 µm	
	Keep at 4°C.	

$FACS_{WASH}$:

- For 1 L volume Mix:

	A	B
	DMEM without phenol-red in powder	10 g
	Horse serum	30 mL
	HEPES 1M	30 mL
	sodium azide 10%	10 mL

1. Dissolve the mix in H_2O mQ and complete to 1 L.
2. Filter sterile in 250 ml bottle.
3. Keep at 4 °C.

List of Antibodies:

	A	B	C	D
	Antibody		Supplier	Catalogue #
	rat anti-mouse CD8 (1B2)	Biotin	Custom made	F23.1, 53-6.72
	CD101 (Moushi101)	PE-Cy7	ThermoFisher	25-1011-80
	CD11b (M1/70)	BV711	Biolegend	101242
	CD127 (A7R34)	BV421	Biolegend	135027
	CD25 (PC61)	APC	Biolegend	102012
	CD4 (RM4-5)	BV605	Biolegend	100548
	CD44 (IM7)	APC-cy7	Biolegend	103028
	CD45.2 (104)	FITC	Biolegend	110706
	CD45.2 (104)	Alexa flour 700	Biolegend	109822
	CD62L (MEL)	PercP	Biolegend	104430
	CD69 (H1.2F3)	APC	Biolegend	104513

	A	B	C	D
	CD8 (53-6.7)	BV785	Biolegend	100750
	CXCR3 (CXCR3-173)	PE	Biolegend	126505
	CXCR6 (SA051D1)	PEdazzle594	Biolegend	151116
	KLRG1 (1MAFA)	APC	Biolegend	138412
	P2XR7 (1F11)	PercP-Cy5.5	Biolegend	148710

- CD101 Monoclonal Antibody (Moushi101), PE-Cyanine7, eBioscience™ **Thermofisher Catalog #**25-1011-80
- Brilliant Violet 711™ anti-mouse/human CD11b Antibody **BioLegend Catalog #**101242
- Brilliant Violet 421™ anti-mouse CD127 (IL-7Rα) Antibody **BioLegend Catalog #**135027
- APC anti-mouse CD25 Antibody **BioLegend Catalog #**102012
- Brilliant Violet 605™ anti-mouse CD4 Antibody **BioLegend Catalog #**100548
- APC/Cyanine7 anti-mouse/human CD44 Antibody **BioLegend Catalog #**103028
- FITC anti-mouse CD45.1 Antibody **BioLegend Catalog #**110706
- Alexa Fluor® 700 anti-mouse CD45.2 Antibody **BioLegend Catalog #**109822
- PerCP anti-mouse CD62L Antibody **BioLegend Catalog #**104430
- APC anti-mouse CD69 Antibody **BioLegend Catalog #**104513
- Brilliant Violet 785™ anti-mouse CD8a Antibody **BioLegend Catalog #**100750
- PE anti-mouse CD183 (CXCR3) Antibody **BioLegend Catalog #**126505
- PE/Dazzle™ 594 anti-mouse CD186 (CXCR6) Antibody **BioLegend Catalog #**151116
- APC anti-mouse/human KLRG1 (MAFA) Antibody **BioLegend Catalog #**138412
- PerCP/Cyanine5.5 anti-mouse P2X7R Antibody **BioLegend Catalog #**108710

Brain infiltrating T cells staining panel:

- Z_{NIR}-APC/Cy7
- CD8a-BV785
- CD44-BV650
- 1B2 -PE

Or Tet_OVA

- CD69-APC
- CD62L-BV421
- P2X7-PercP-Cy5.5
- CD101-PE-Cy7

- CXCR3-BV510
- CXCR6-PE-dazzle
- CD11-BV711
- CD45.2-APC-eF780
- CD4-BV605

Infiltration procedure

1h 20m

1 Inject mice intravenously with CD45-FITC 3ug/mice (6 µL from vial/mice) for 00:03:00 - 00:05:00 , then euthanized directly by decapitating head to stop blood circulation. 5m

1.1 CD45 is prepared as 180 µL CD45-FITC + 4320 µL PBS → to inject 150 ul/mice.

1.2 Keep one mouse non injected for Live/Dead staining.

2 Collect the brain for each experimental condition vs control.

3 Dissociate brain in RPMIc plus collagenase-D 1 undetermined , DNase 150 ug/ml as follow:

3.1 Place the brain in 15mm petri dish and add 2ml/brain of the collagenase-DNase mix.

3.2 Cut the brain into 8 longitudinal pieces and collect in one tube using 1ml tip (cut the tip to allow pieces to go through).

3.3 Transfer to tube (gentleMACS TM C tubes, cat 130096334) and make sure to lock it.

3.4 Keep the brain pieces on the downside of the spiral bend.

3.5 Fix the tube to the machine and select the brain dissociation (preset the machine as shown below in the brain dissociation machine setup section)→OK, will turn light and start homogenization.

Tissue dissociation machine setup:

1. Temp. ON
2. Spin 200 rpm 1”
3. Spin 300 rpm 1”

4. Spin 400 rpm 3”
5. Spin -400 rpm 3”
6. Spin 400 rpm 3”
7. Spin -400 rpm 1”
8. Spin 200 rpm 4”
9. Spin -400 rpm 2”
10. Spin 400 rpm 3”
11. Spin 200 rpm 6”
12. Spin -200 rpm 2”
13. Spin 200 rpm 8”
14. Spin 0 rpm 45’ 0”

Temp OFF

3.6 When dissociation finish (00:45:00), homogenize with the end of the 5ml syringe over 70 um strainer on a 50 ml falcon tube. Wash the strainer with PBS to ensure the collection of all cells. 45m

4 Centrifuge 2000 rpm, Room temperature, 00:05:00 . 5m

5 During that time prepare 37% and 70% percoll from 90% percoll in 10X PBS.

5.1 For 100 mL of 90% Percoll:

- 90 mL percoll and 10 mL PBS 10X.

5.2 For 45 mL of 70% Percoll:

- 35.7 mL from 90% Percoll + 10.3 mL PBS 1X.

5.3 For 43.75 mL of 37% Percoll:

- 18 mL from 90% Percoll + 25.75 mL PBS 1X.

6 Add 3 mL of 70% Percoll in a 15 ml tubes. Resuspend the brain pellet in 3 mL of 37% Percoll and overlay gently on top of 70% Percoll (drop on the wall to prevent disruption).

7 Centrifuge 2000 rpm, 00:20:00, brake-off. 20m

8 After centrifugation, with the help of vacuum suction clear the upper lipid layer on the top of the tubes.

9 Transfer the intermediate layer containing the cells to a new labelled 15ml tube.

10 Complete the volume to 10 mL with 1X PBS, gently invert the tube up and down at least one time.

11 Centrifuge at 2000 rpm, 00:05:00. 5m

12 Stain 1x10^6 cells from the brain or 3x10^6 cells from the spleen with the live/dead stain and after with extracellular staining panel.

> **Note**
>
> Do not forget to add a compensation for FITC in the compensation beads.

For splenic cell induction: 5h 38m

13 Collect the spleen from CD45-FITC injected mice in 3 mL of sterile complete RPMI (RPMIc).

14 Harvest the spleen in the culture hood with frosted microscope slide.

15 Centrifuge at 1300 rpm, Room temperature, 00:05:00. 5m

16 Perform red blood cell lysis: 5 mL of NH_4Cl 0.83%, 00:05:00 at Room temperature . 5m

17 Add 5 mL of sterile RPMIc and centrifuge at 1300 rpm, Room temperature, 00:05:00 . 5m

18 Reconstitute in 2.5 mL of sterile RPMIc.

19 Count the cells on hemocytometer (1/100 dilution).

20 Add volume to reach $30x10^6$ cells/ml.

21 Add 100 µL of cells (3 x 10^6 cells) per well in 96-Well Round-Bottom plate (Fisher # 07200760).

22 Add 100 µL of the following restimulation mix (at 2X concentration).

22.1 Don't forget to add one well of PMA-IONO control (2 µL of stock PMA 5 undetermined and Ionomycine 50 undetermined) in 200 µL final. The final concentration is 50 undetermined of PMA and 500 undetermined Ionomycin.

22.2 No-Stim (2X): RPMIc + 20 undetermined Brefeldine A (BFA).

22.3 OVA Re-stimulation (2X): RPMIc + 20 undetermined BFA final (stock solution at 1 undetermined) + 4 undetermined OVA peptide.

22.4 SYRGL Restimulation (2X): RPMIc + 20 undetermined BFA final (stock solution at 1 undetermined) + 4 undetermined SYRGL peptide.

22.5 **For example:**

- for 4 mL of RPMIc, we add 1.6 µL of OVA peptide (stock at 10 undetermined) and 80 µL of BFA.
- for 4 mL of RPMIc, we add 1.6 µL of SYRGL peptide (stock at 10 undetermined) and 80 µL of BFA (stock at 1 undetermined).

23 Incubate the cells in presence of the re-stimulation mix for 05:00:00 at 37 °C and 5% CO_2. 5h

24 Centrifuge at 1300 rpm, 12°C, 00:03:00 and remove supernatant. 3m

25 Wash in cold 1X PBS then repeat step 24.

26 Add 100 µL cold 1X PBS per well.

27 Add 100 µL of formaldehyde 4% per well (up and down).

28 Incubate 00:20:00 at Room temperature . 20m

29 Add 100 µL of 1X PBS and repeat step 24.

30 Wash in cold 1X PBS (300 µL) and repeat step 24.

31 Resuspend the cells in 200 µL of $FACS_{WASH}$.

32 Keep at 4 °C in parafilm and perform the intracellular staining within 5 days (it is better to do it the day after).

Plate layout_2C (2 plates; Intracellular staining and Isotypic control):

	A	B	C	D	E	F	G	H	I	J	K
	2C_WT_male		PMA/IONO/BFA		SYRGL		SIINFEKL		BFA		Non-treated
	2C_WT_male		PMA/IONO/BFA		SYRGL		SIINFEKL		BFA		Non-treated
	2C_WT_female		PMA/IONO/BFA		SYRGL		SIINFEKL		BFA		Non-treated
	2C_WT_female		PMA/IONO/BFA		SYRGL		SIINFEKL		BFA		Non-treated
	PTx_CTL		PMA/IONO/BFA		SYRGL		SIINFEKL		BFA		Non-treated
	Plate layout_OT1(2 plates; Intracellular staining and Isotypic control):										
	OT1_WT		PMA/IONO/BFA		SYRGL		SIINFEKL		BFA		Non-treated
	OT1_KO		PMA/IONO/BFA		SYRGL		SIINFEKL		BFA		Non-treated
	PTx_CTL		PMA/IONO/BFA		SYRGL		SIINFEKL		BFA		Non-treated