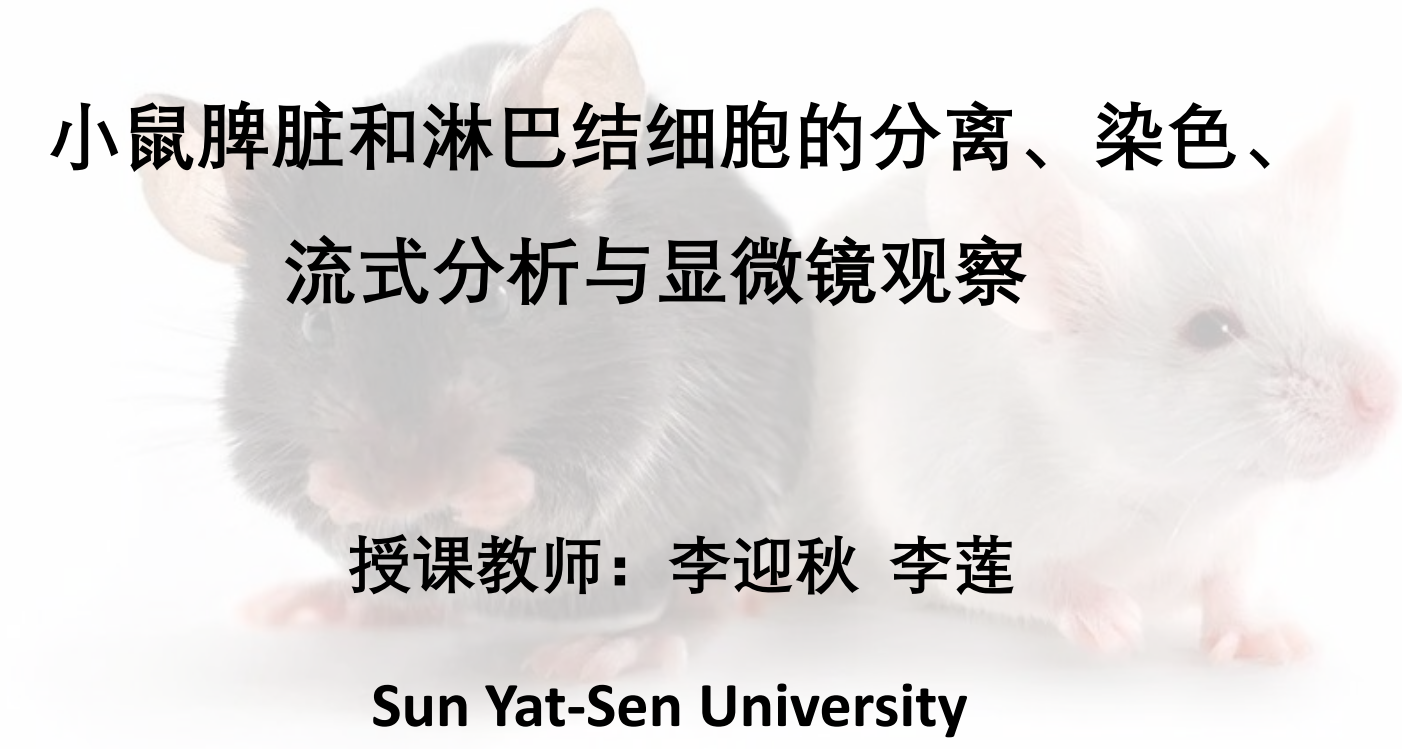


小鼠脾脏和淋巴结细胞的分离、染色、流式分析与显微镜观察

授课教师：李迎秋 李莲

Sun Yat-Sen University

2023.11.18

课程时间安排

09:00-10:00	课程相关理论、设备及操作讲解 解剖示范(赵晨思博士)
10:00-12:00	分离小鼠脾脏和淋巴结免疫细胞
13:30-	免疫荧光染色—流式分析 台盼蓝染色– 显微镜观察
15:00-	流式分析 (杜芸婷助教)

【目的和要求】

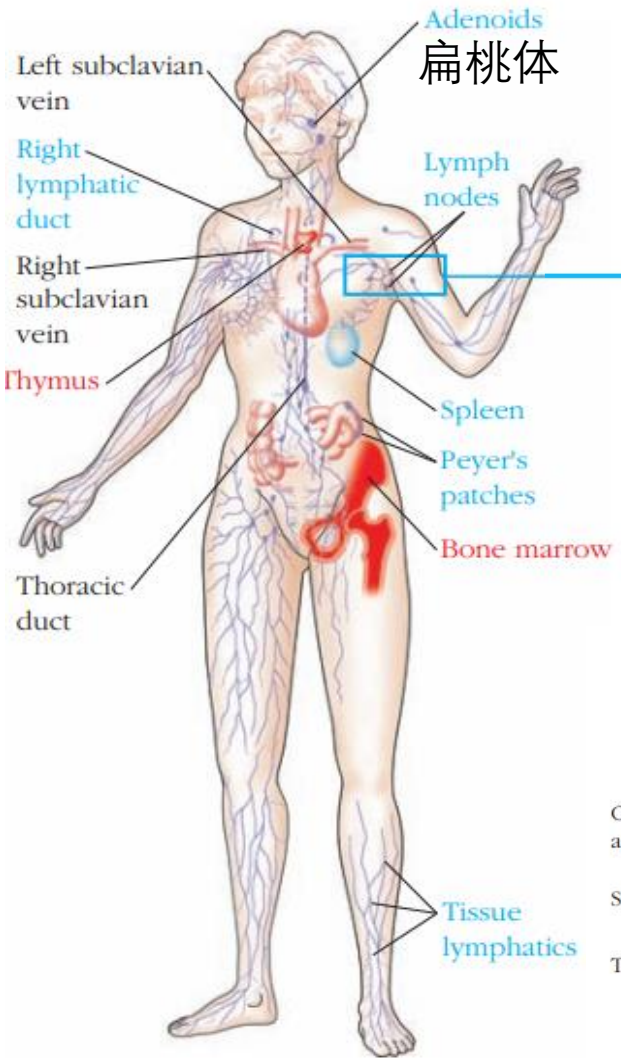
1. 掌握小鼠脾脏及淋巴结免疫细胞（主要是淋巴细胞）分离及细胞表面免疫染色的基本原理和实验方法。

2. 了解流式细胞仪的基本工作原理、使用方法及简单的数据分析。

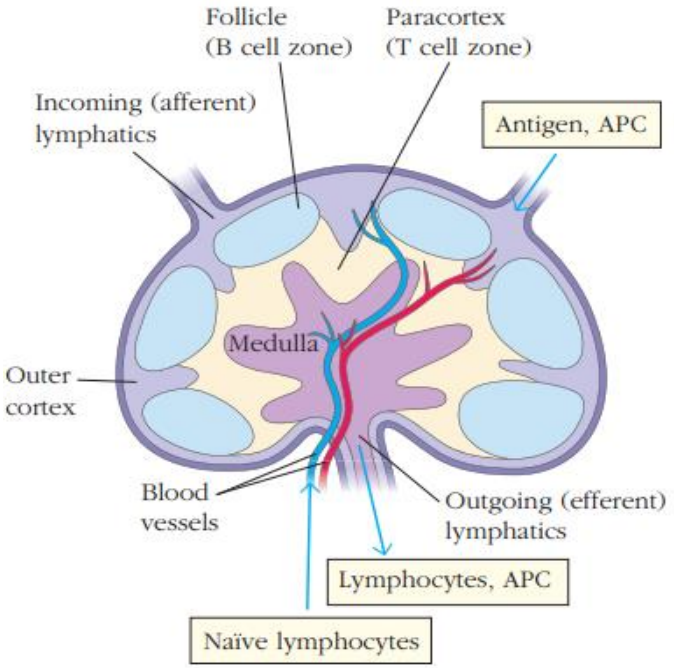
3. 熟悉小鼠脾脏及淋巴结的分布特点及淋巴细胞的形态。

Background

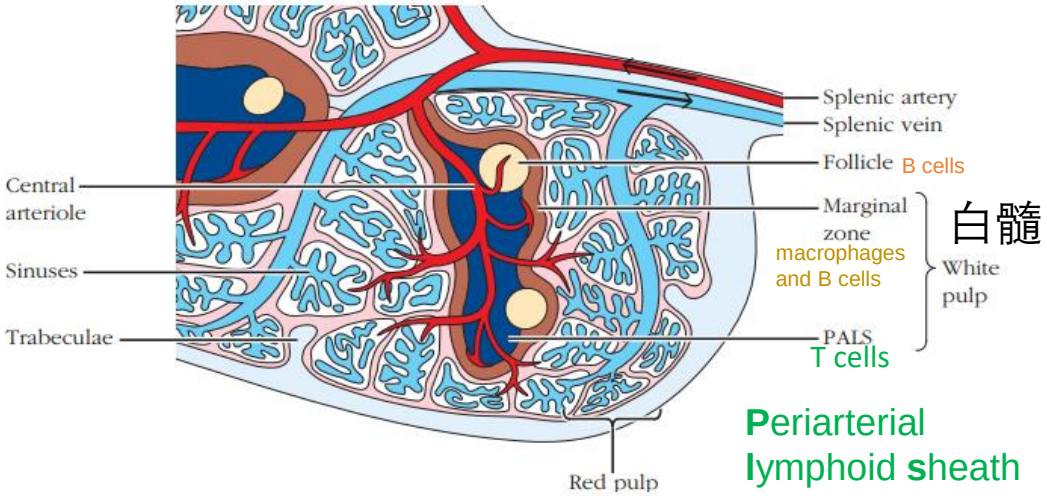
I. Secondary Lymphoid Organs 次级淋巴器官



Lymph node

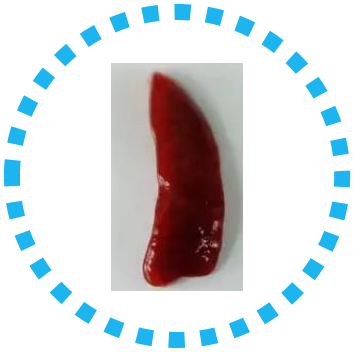


Spleen

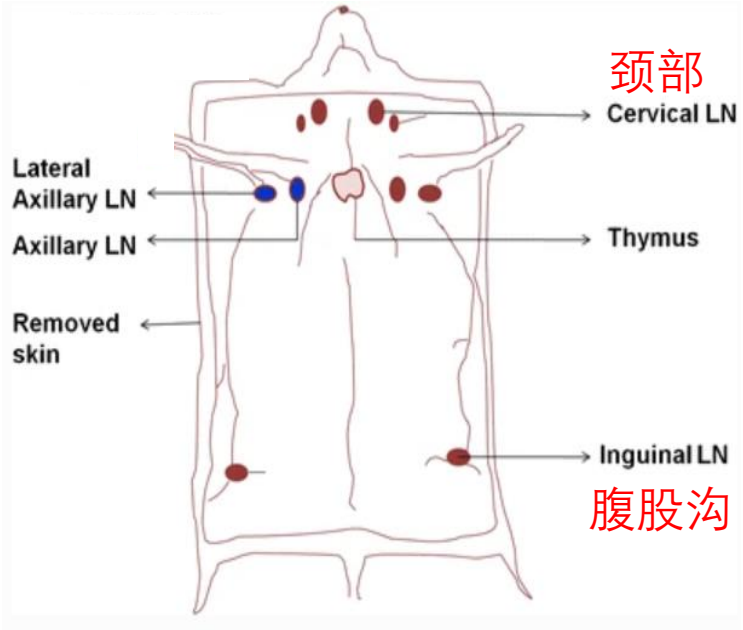


I. Secondary Lymphoid Organs
小鼠次级淋巴器官

C57BL/6

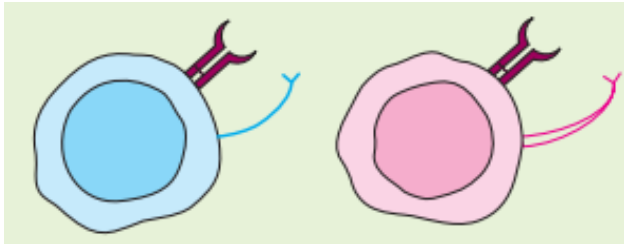


spleen

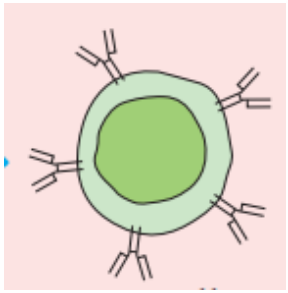


lymph node

Lymphocytes (peripheral)



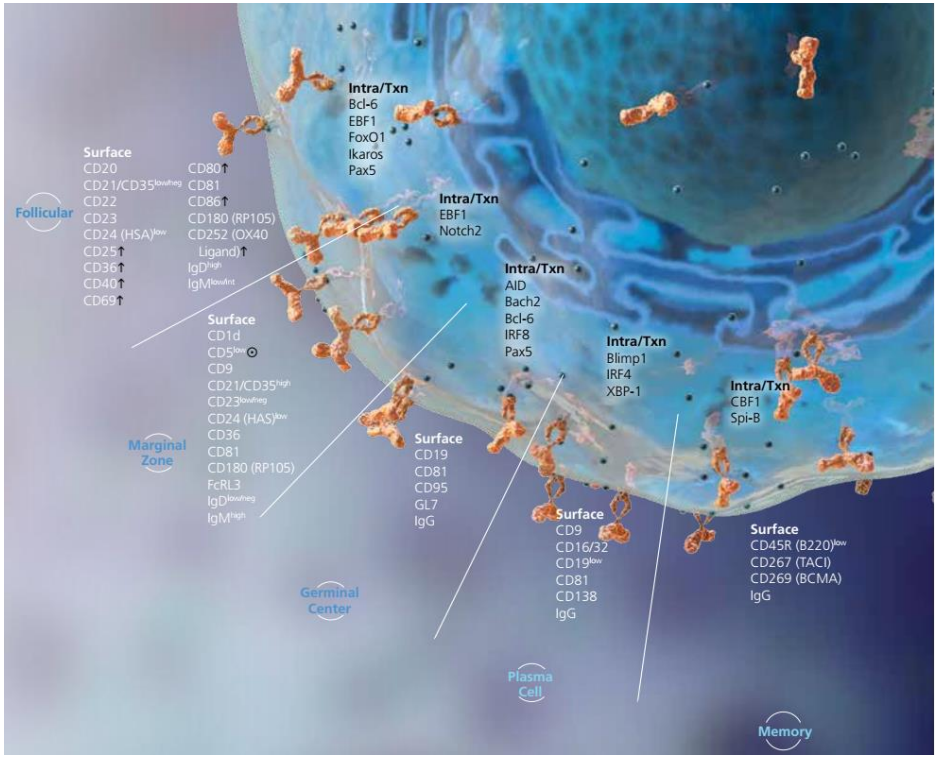
T cell (30-40% in spleen)

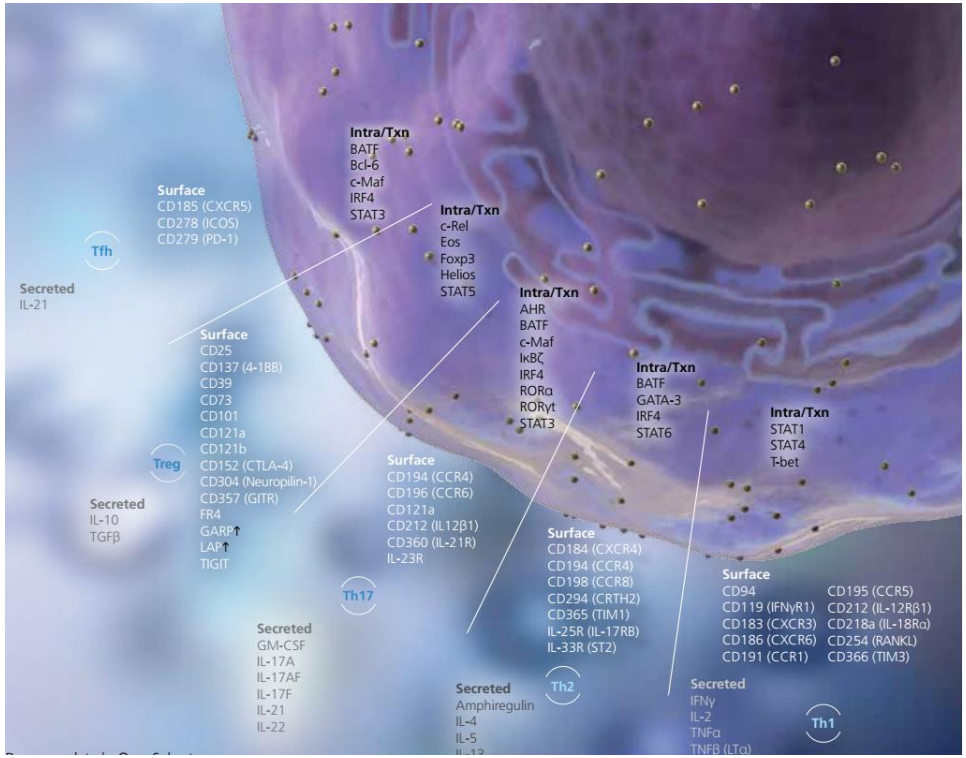


B cell (50-60% in spleen)

others (Macrophage, Monocyte, Erythrocyte, etc.)

II. Cell Surface Markers细胞表面标志物CD





Mouse B cell markers

Pan: CD19, CD20, CD21/CD35$^{low/neg}$, CD22, CD23, CD24 (HSA)^{low}, CD25^{h}, CD36^{h}, CD40^{h}, CD69^{h}, CD80^{h}, CD81, CD86^{h}, CD180 (RP105), CD252 (OX40 Ligand)^{h}, IgD^{high}, IgM$^{low/int}$

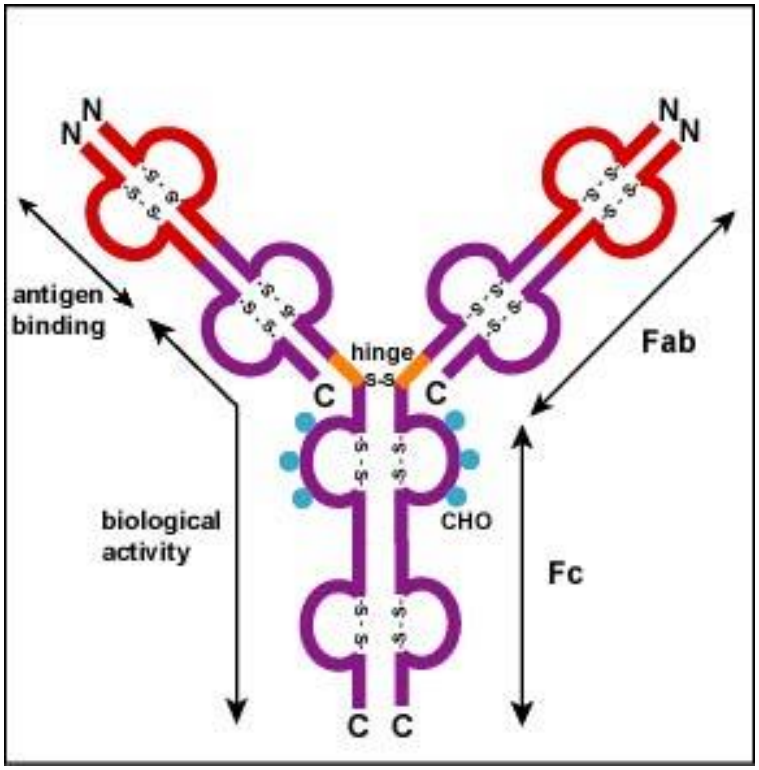
http://www.liankebio.com/article-information_Newsletter-968.html

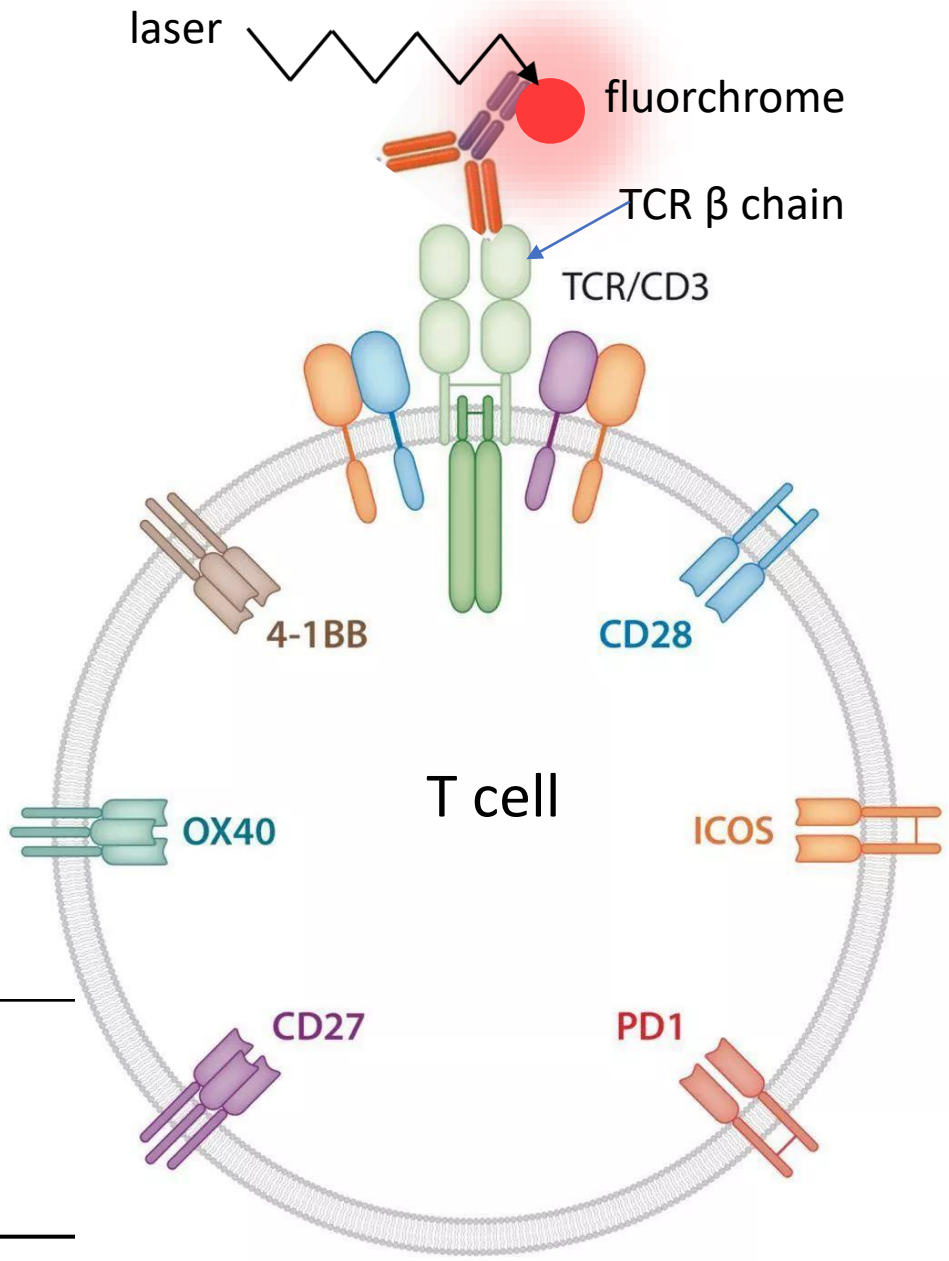
Mouse CD4^{+} T cell markers

Pan: CD3, CD4, CD5, CD7, CD25^{h}, CD27, CD28, CD44^{h}, CD62L (naïve:high, effector: low, memory: high), CD69^{h}, CD127 (IL7Rα), CD134 (OX40)^{h}, CD137 (4-1BB)^{h}, CD152 (CTLA-4)^{h}, CD154 (CD40L)^{h}, CD272 (BTLA)^{h}, CD278 (ICOS)^{h}, CD279 (PD-1)^{h}

II. Immuno-Staining Cell Surface Markers

荧光素标记抗体-抗原





PerCP-Cy5.5 Anti-MouseTCRβ Ab

FITC Anti-Mouse CD19 Ab

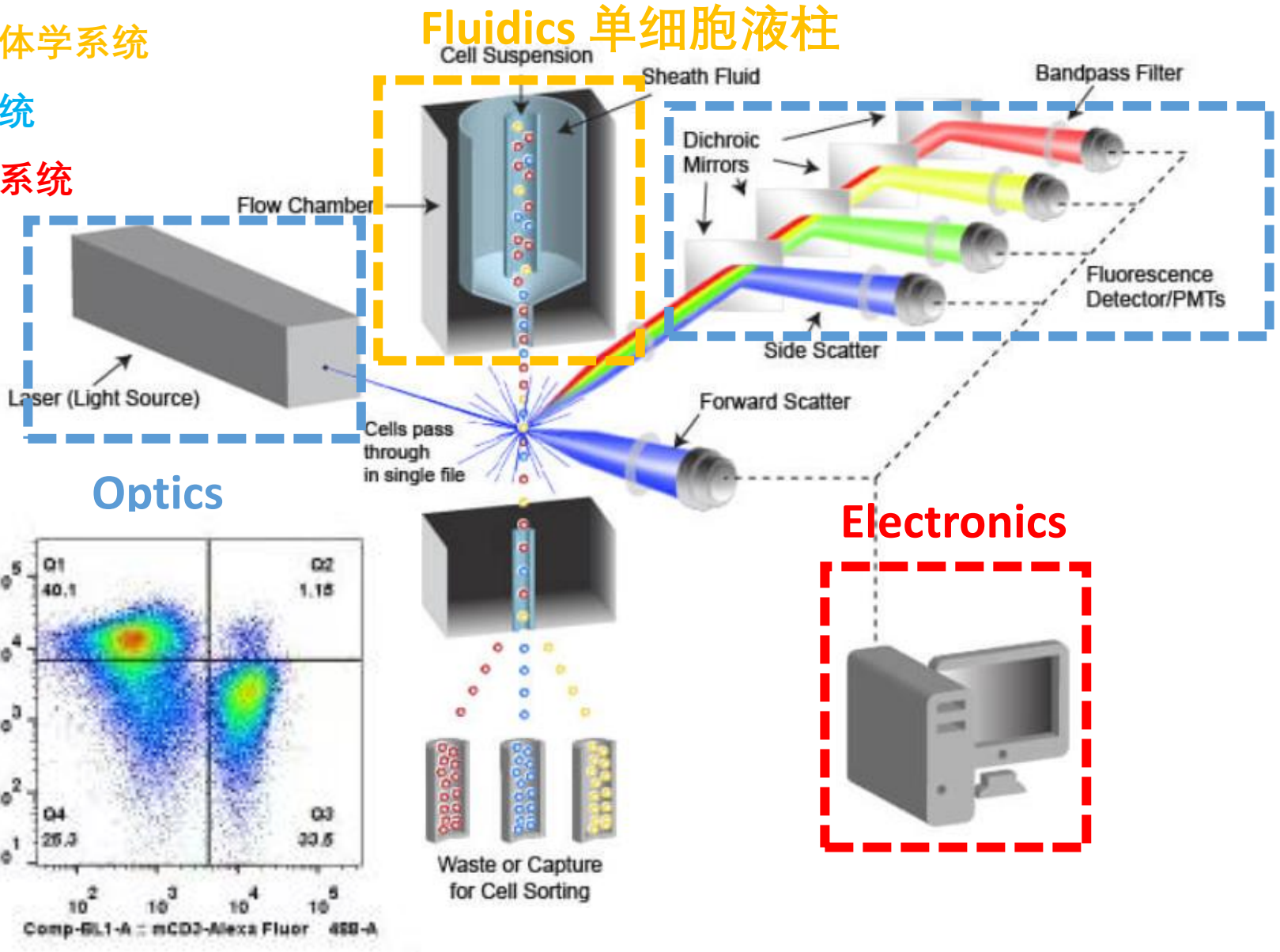
III. Flow Cytometry 流式细胞术

单细胞、快速、高通量、多参数、准确、灵敏

液体流体学系统

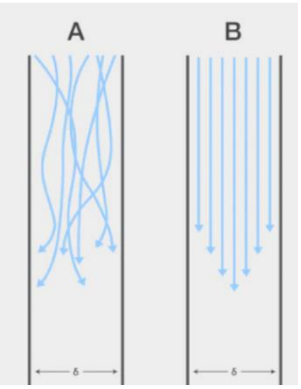
光学系统

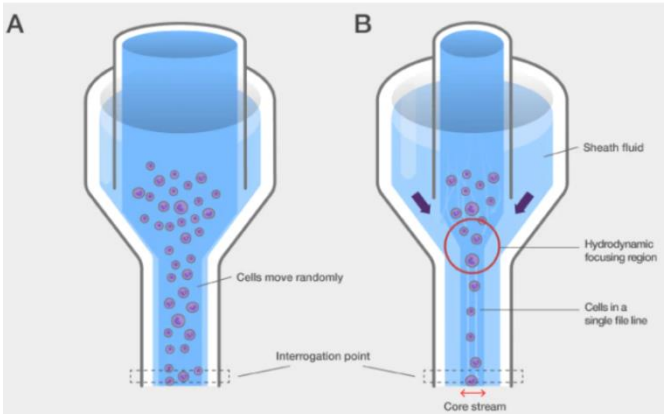
电子学系统



流式细胞分析仪：　液流系统

单细胞流：鞘液迫使样品进入较小的核心流





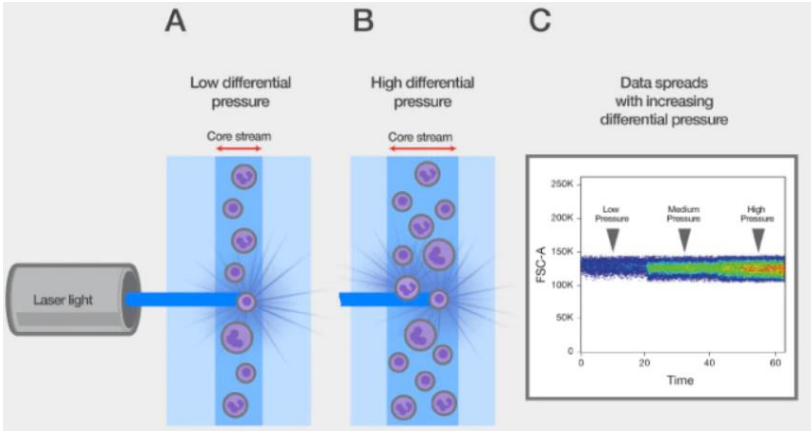


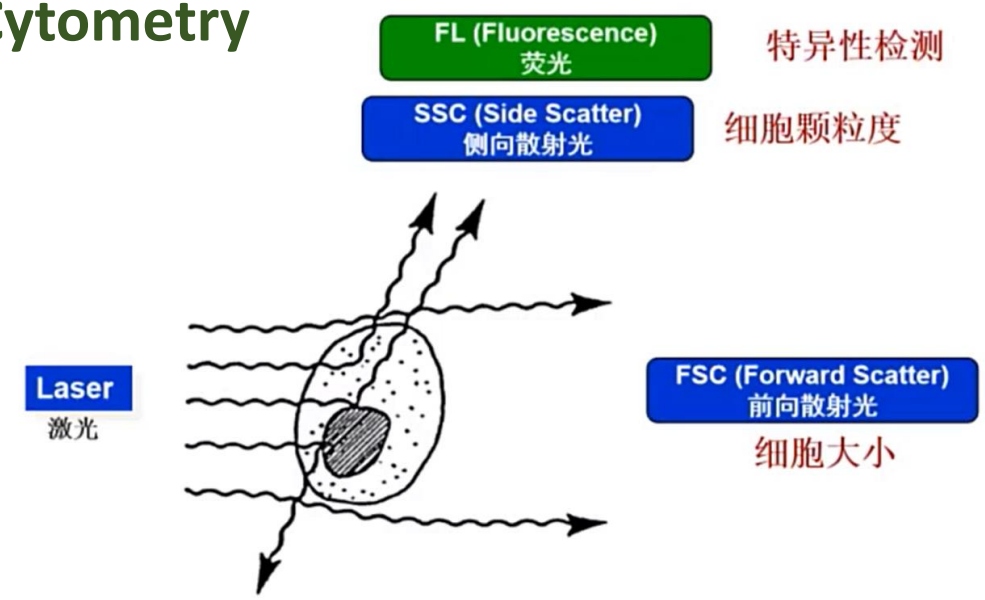
图1 微粒运动示意图

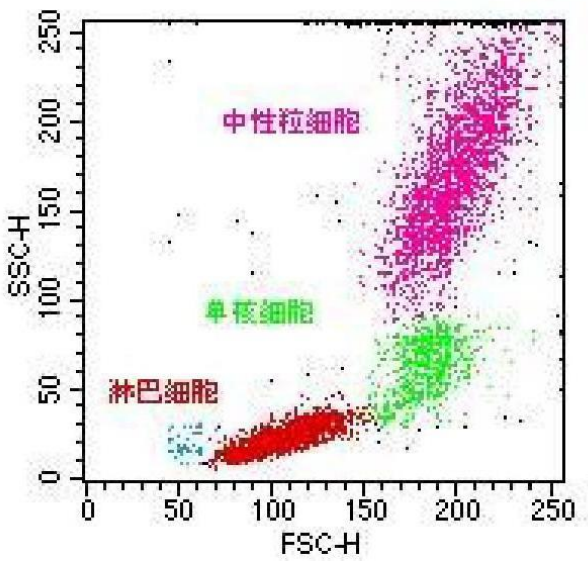
图 2.流体动力学聚焦

图 3.压力增加对流式细胞仪数据的影响 （改变样品与鞘液流之间的压差，细胞少时，加上上样速度）

III. Flow Cytometry

- 散射光

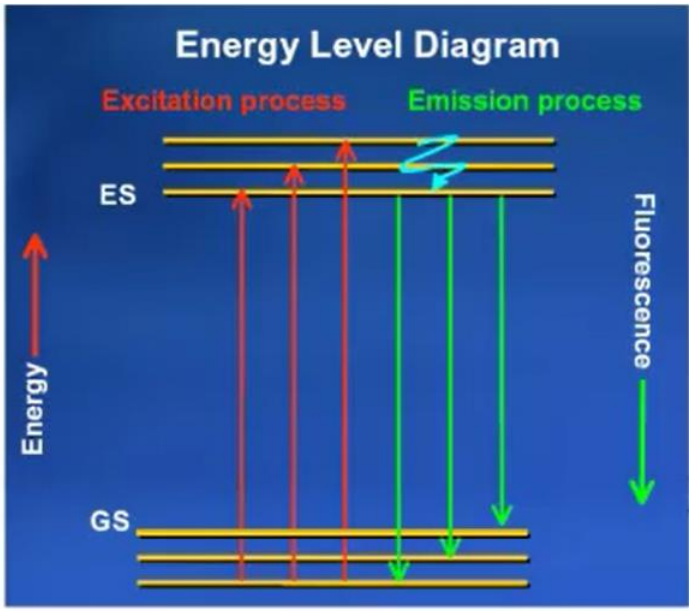




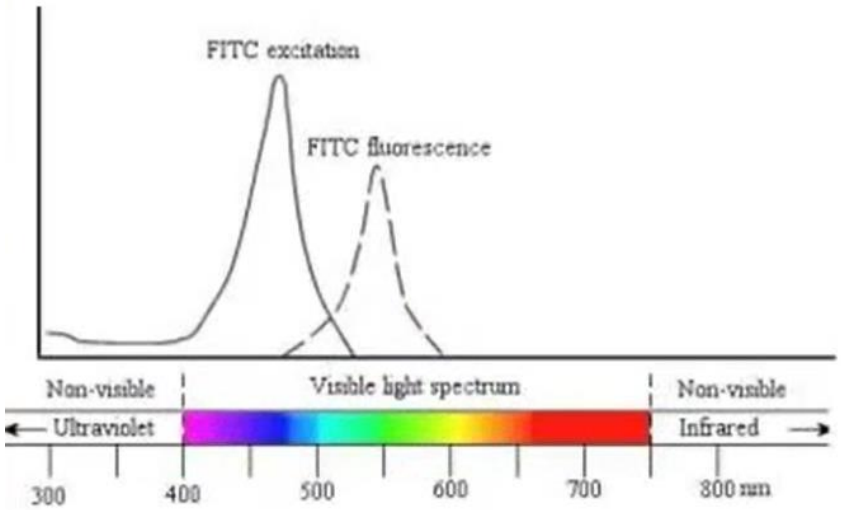
- Fluorchrome荧光素信号

激发态

基态



激发波长



发射波长

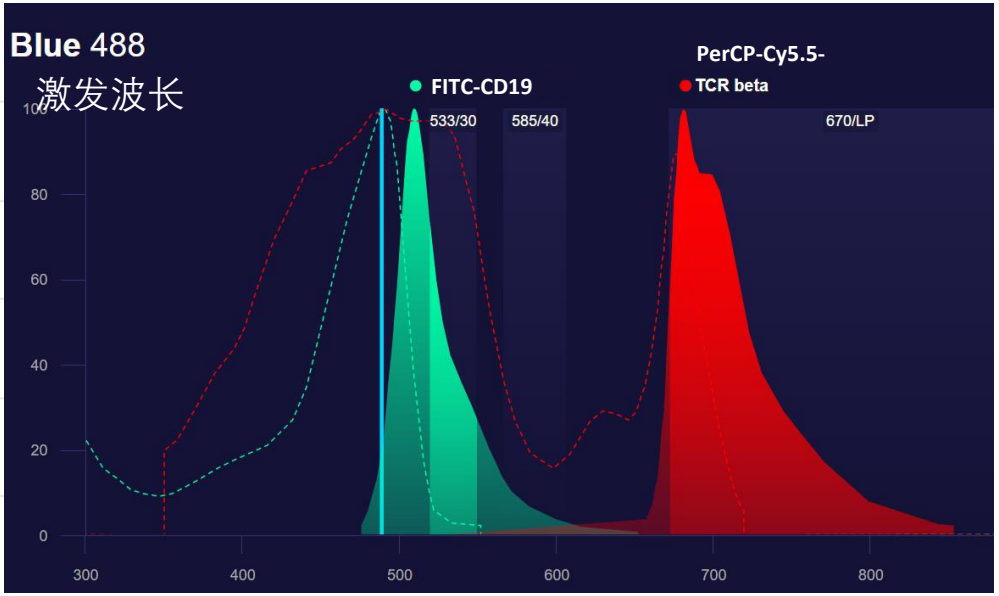
III. Flow Cytometry

PerCP-Cy5.5 Anti-Mouse TCR β Ab

FITC Anti-Mouse CD19 Ab

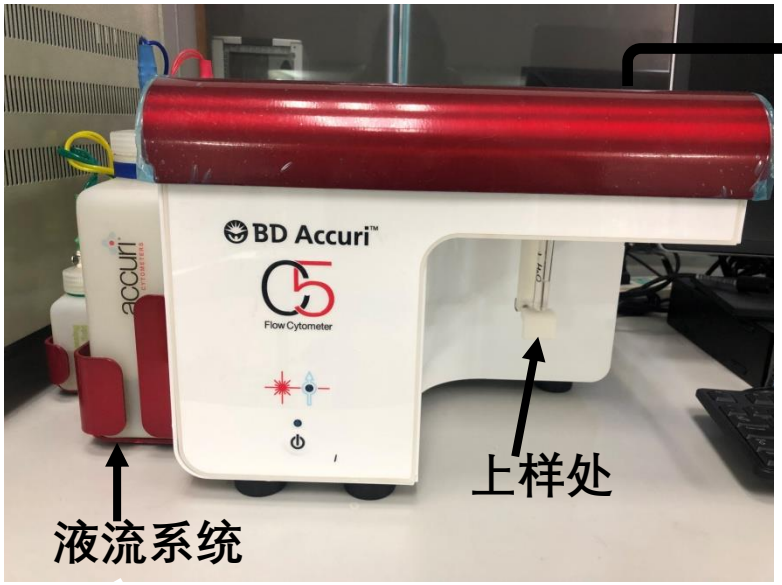
编号	荧光	激发波长 Ex/nm	Laser激光器 nm	发射波长 Ex/nm	滤光片	相对荧光亮度	相同通道的荧光素
FL1	FITC	494	488	520	530/30	2	BB515/AF488
FL2	PE	491,563	488,532,561	575	575/26	5	
FL3	APC	650	633	660	660/20	5	eFluor660
FL4	APC-Cy7	650	633	785	780/60	1	APC-H7,APC-Alexa Fluor750,APC-VIO770

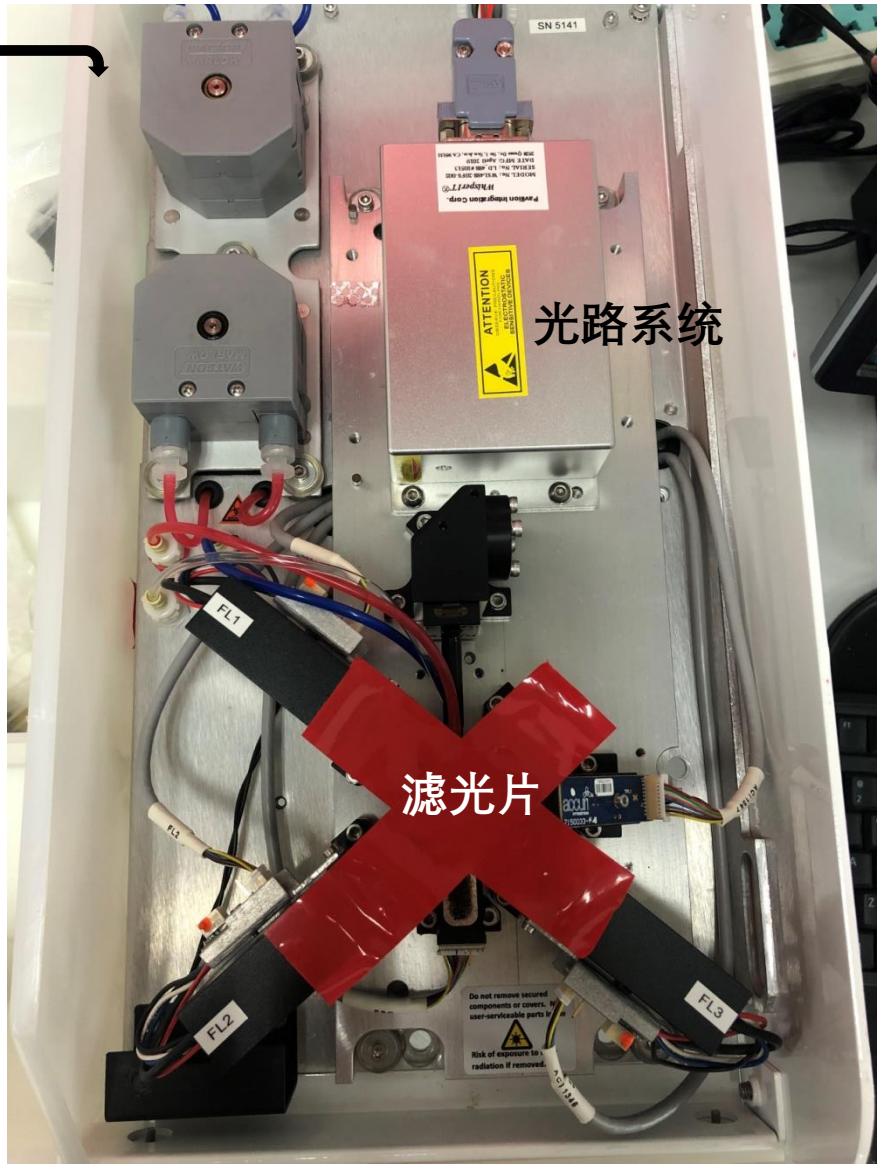
荧光图谱：	FITC	PerCP-Cy5.5
激发波长Ex/nm：	494	482
Laser激光器/nm：	488	488
发射波长Em/nm：	520	695
滤光片：	530/30	695/40
相对荧光亮度：	2	2



III. Flow Cytometry

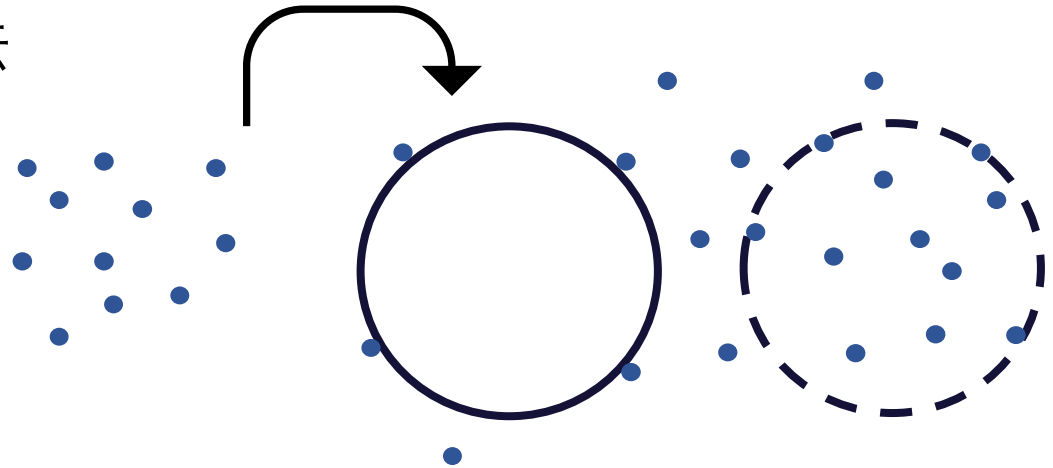
型号：BD Accuri C5





IV. Trypan Blue Staining

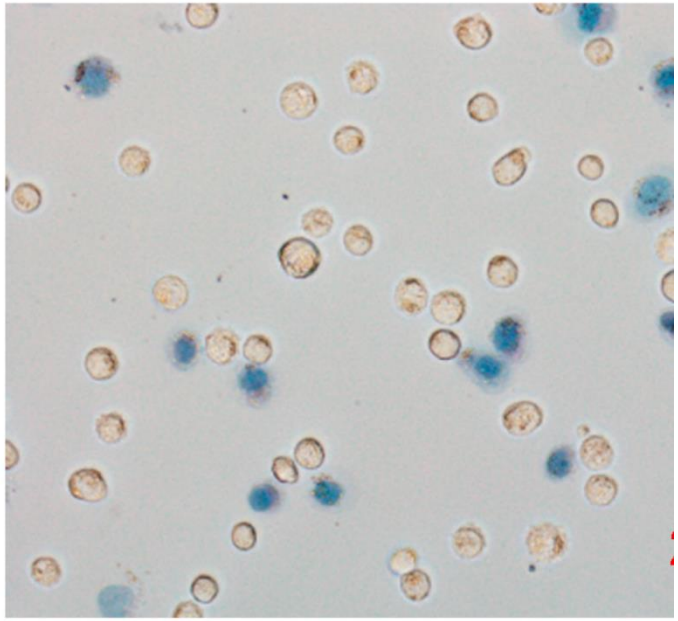
台盼蓝活细胞拒染法



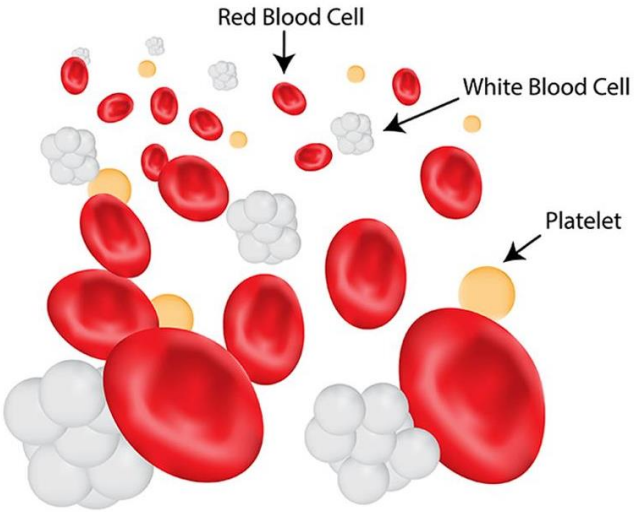
viable cells % = 1.00 – (Number of blue cells / Number of total cells)

实验准备母液：0.2%
trypan blue in PBS

staining for 5 min
not more than 10 min

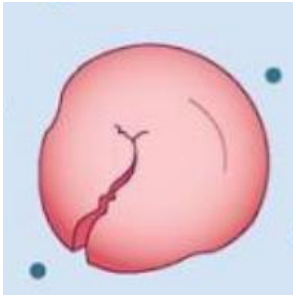


V. Red Blood Cell Lysis



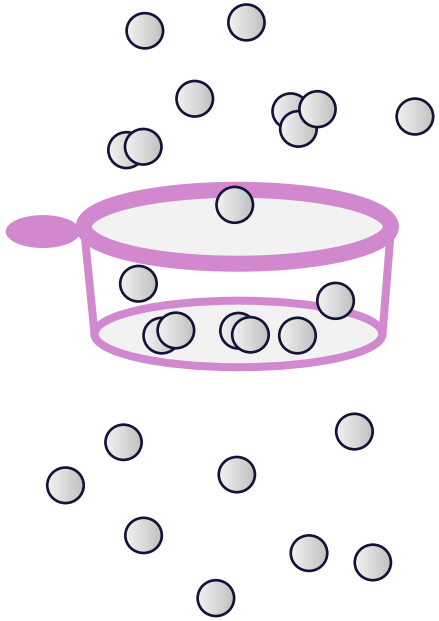
Red Blood Lysis
(hypotonic solution)

红细胞裂解液（Tris-NH4Cl）



基于红细胞淋巴细胞细胞膜的离子转运通道不同，改变其渗透压，涨破

VI. Cell Strainer 细胞筛网

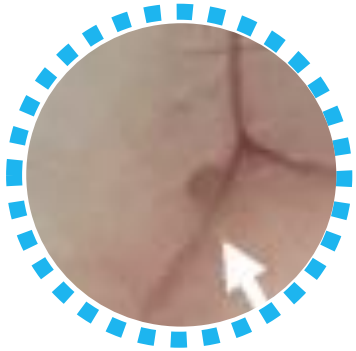


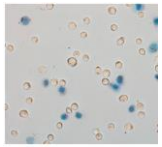
single cell suspension

实验流程

C57BL/6

spleen

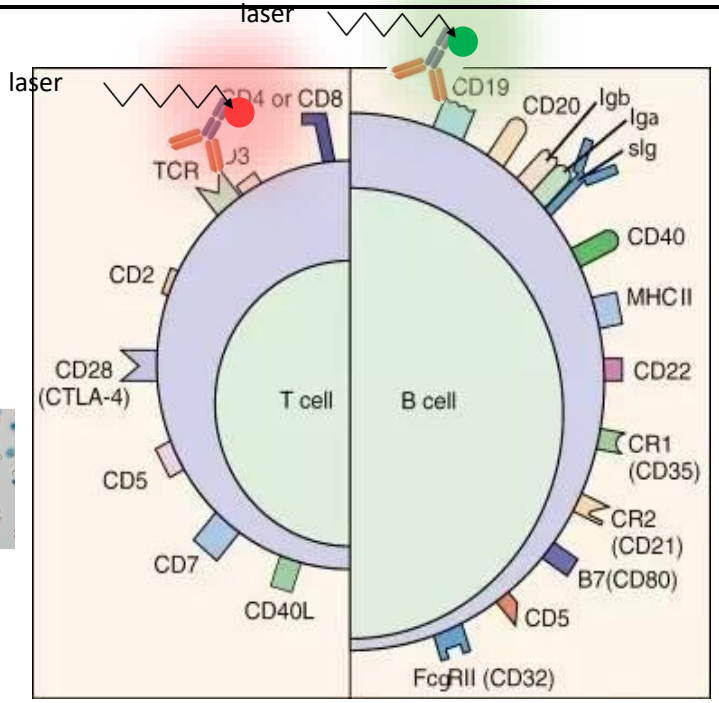




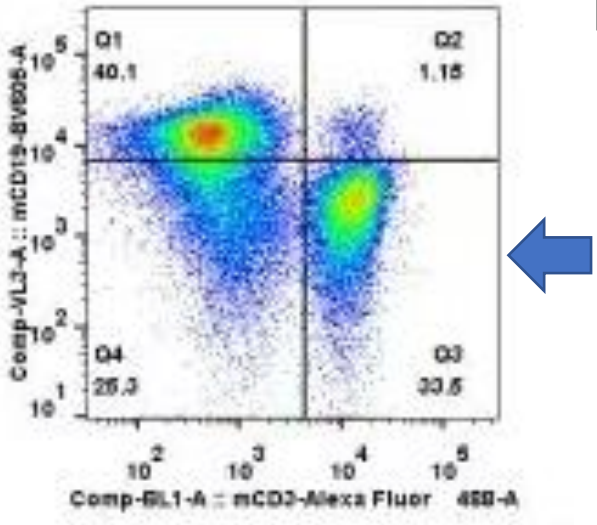
lymph node

PerCP-Cy5.5 Anti-Mouse TCR β

FITC Anti-Mouse CD19



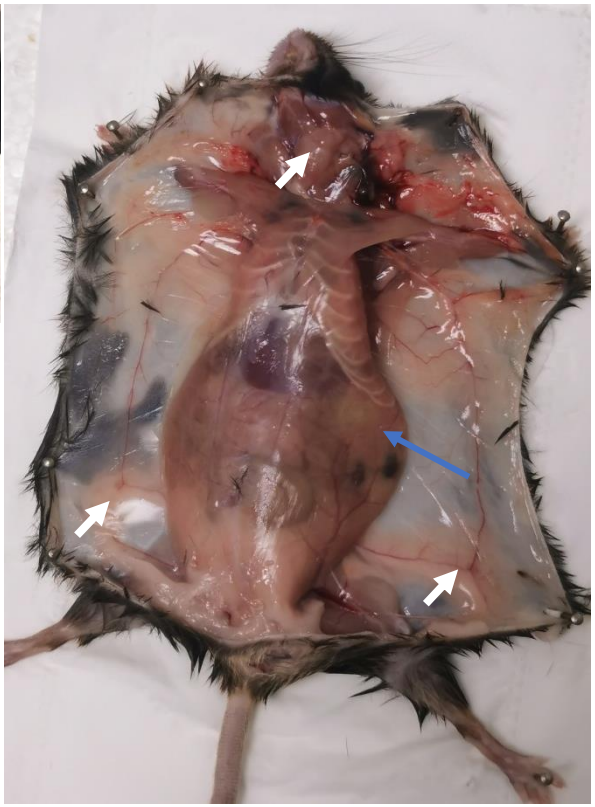
Lymphocytes

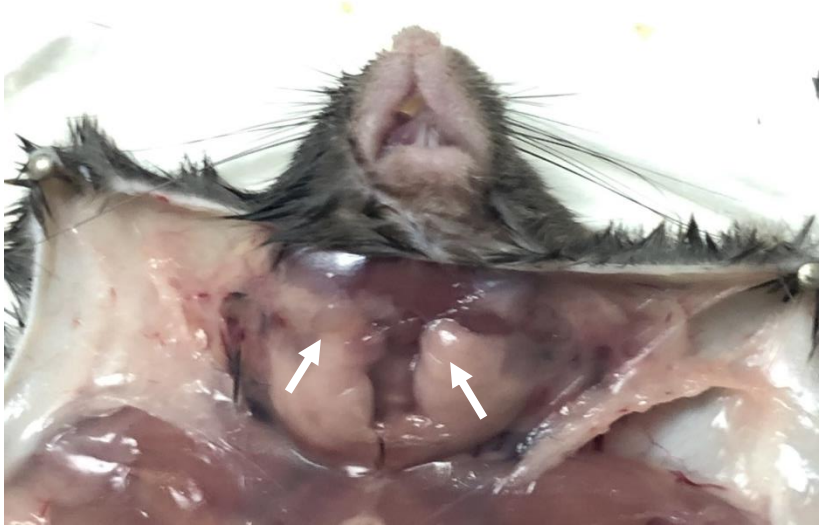


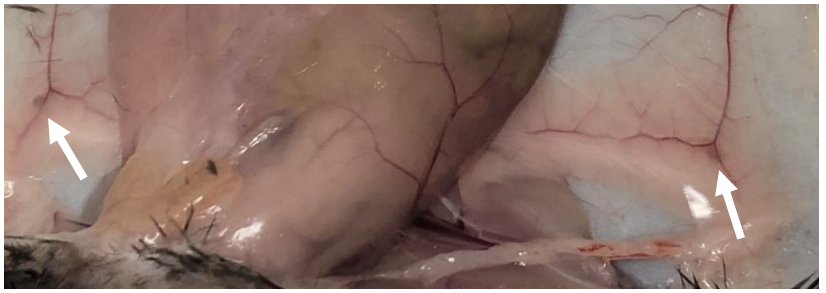
Experiments

I. Isolation of Lymphocytes from mouse spleen and lymph nodes

颈椎脱臼法



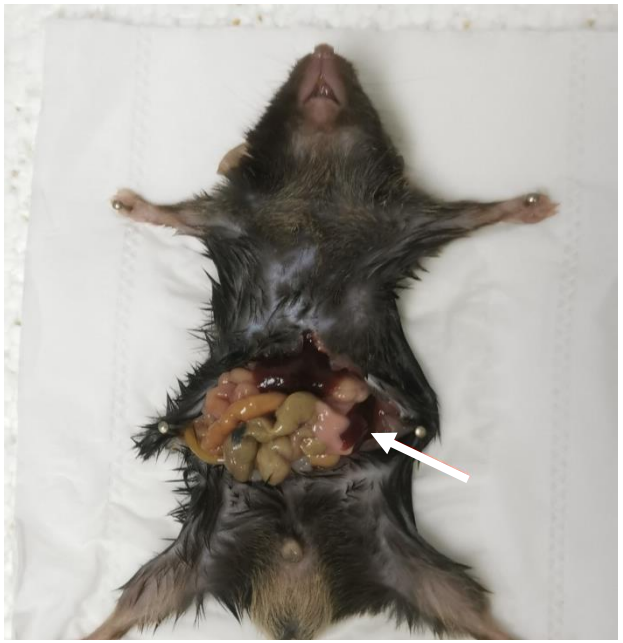


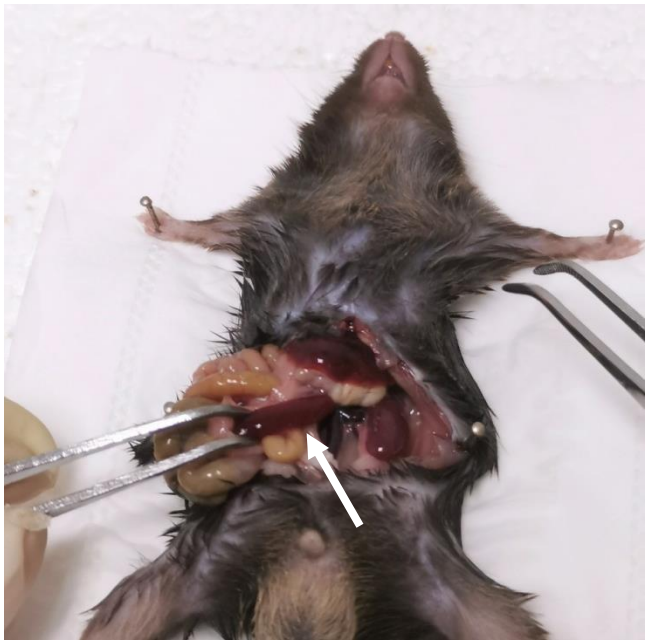


lymph nodes

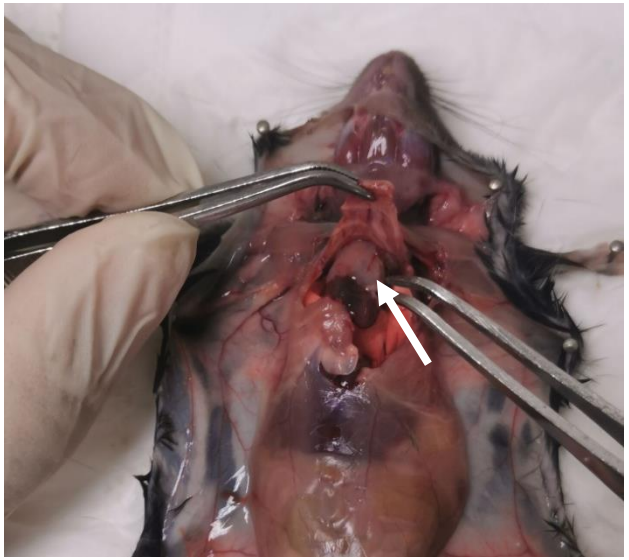
Experiments

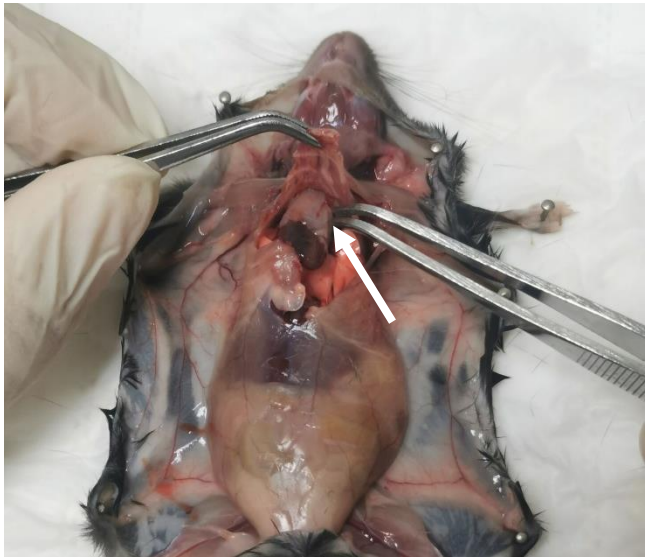
I. Isolation of Lymphocytes from mouse spleen and lymph nodes





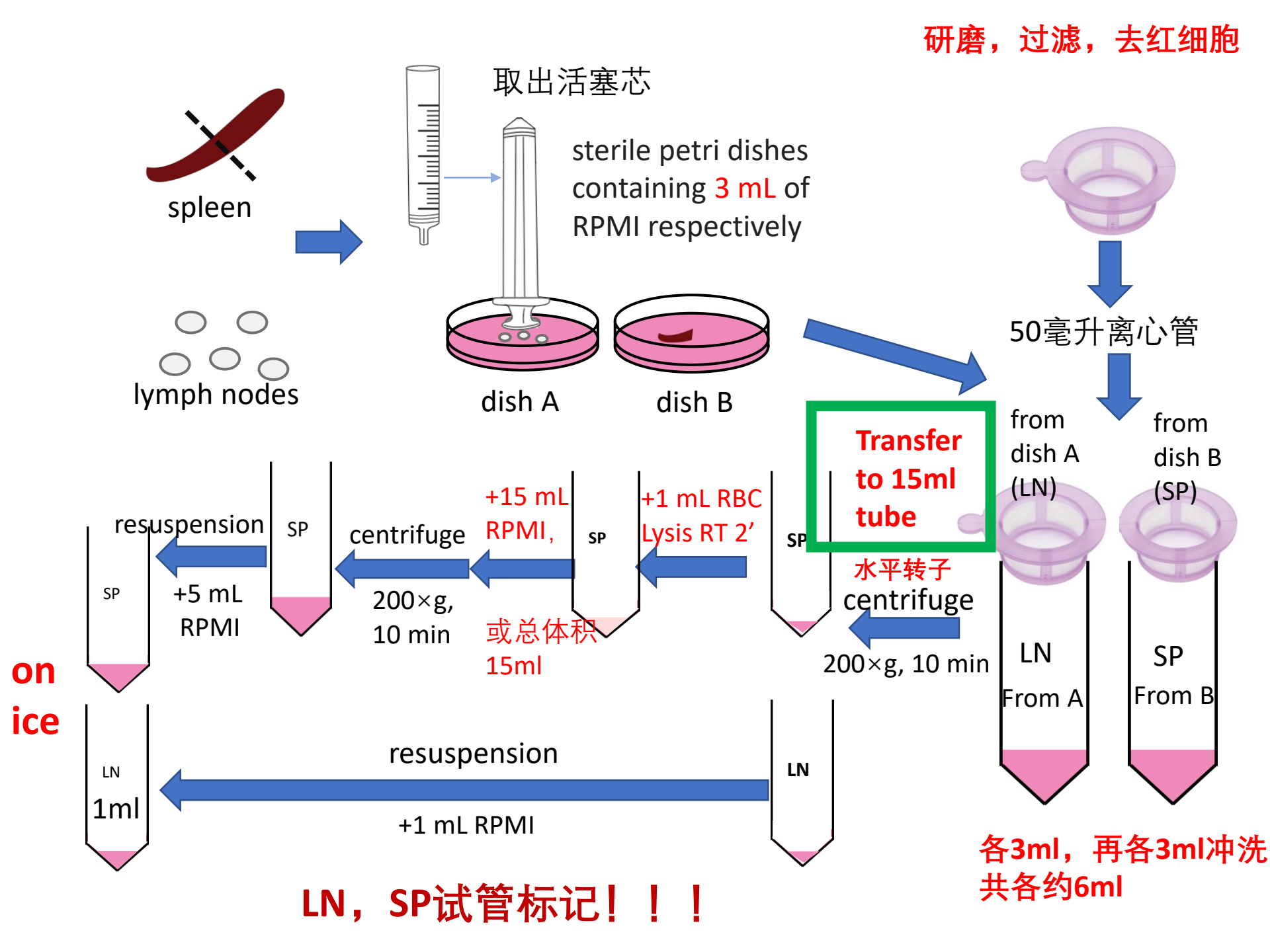
spleen





thymus

研磨，过滤，去红细胞



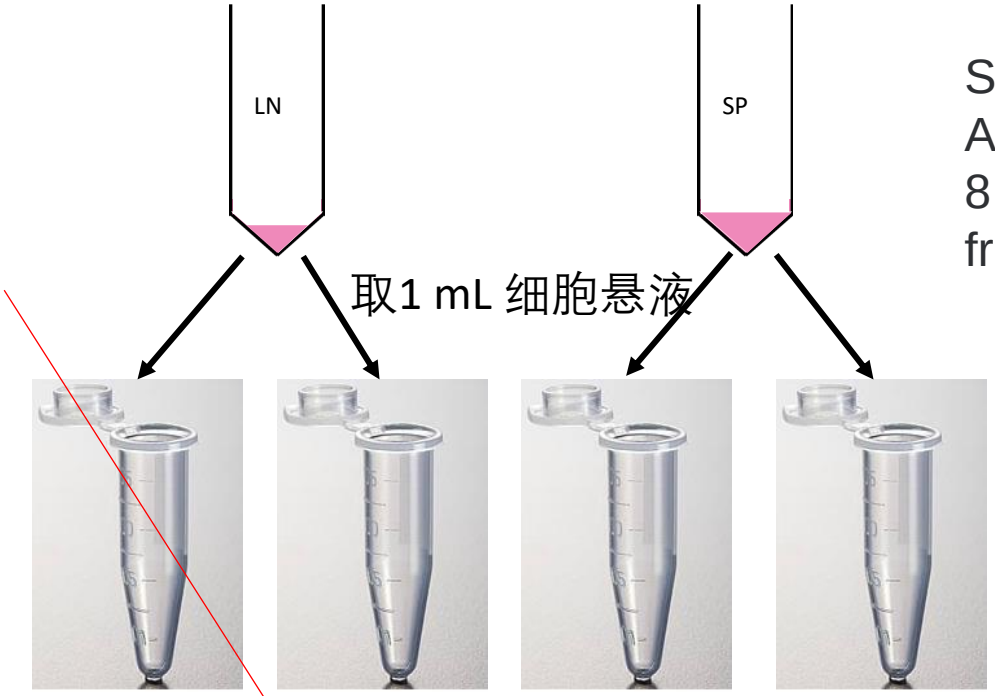
各3ml，再各3ml冲洗
共各约6ml

LN，SP试管标记！！！

Experiments

流式缓冲液重悬洗

III. Cell immunostaining and flow cytometry analysis



Spleen cells were about 3-5×10^6 cells /mL. About 5-8×10^7 spleen lymphocytes and 8×10^6 lymph node cells can be obtained from one mouse.

以第二组为例，试管标记：

2L, 2Sc, 2SS

离心 200 × g for 10 min.

各用1 mL Flow Cytometry Buffer流式缓冲液重悬

离心 200 × g for 10 min. → 弃上清，获得细胞沉淀 →

III. Cell immunostaining and flow cytometry analysis

用流式缓冲液46.5 μL 重悬，准备染色，染色体积 50 μL

试剂	使用量（染色组）	对照
PerCP-Cy5.5 Anti-Mouse TCR β (0.2 mg/mL)	2.5μL(终浓度10 μg/mL)	0
FITC Anti-Mouse CD19 (0.5 mg/mL)	1 μL(终浓度10 μg/mL)	0
流式缓冲液	46.5 μL	50 μL
终体积	50 μL	50 μL

Incubate the cells for at least 30 min on ice in dark.

Resuspend the cells in 1 mL of Flow Cytometry Buffer.

Centrifuge the cells at 200 × g for 10 min.

0.5 mL流式缓冲液重悬，移入流式管，准备流式分析

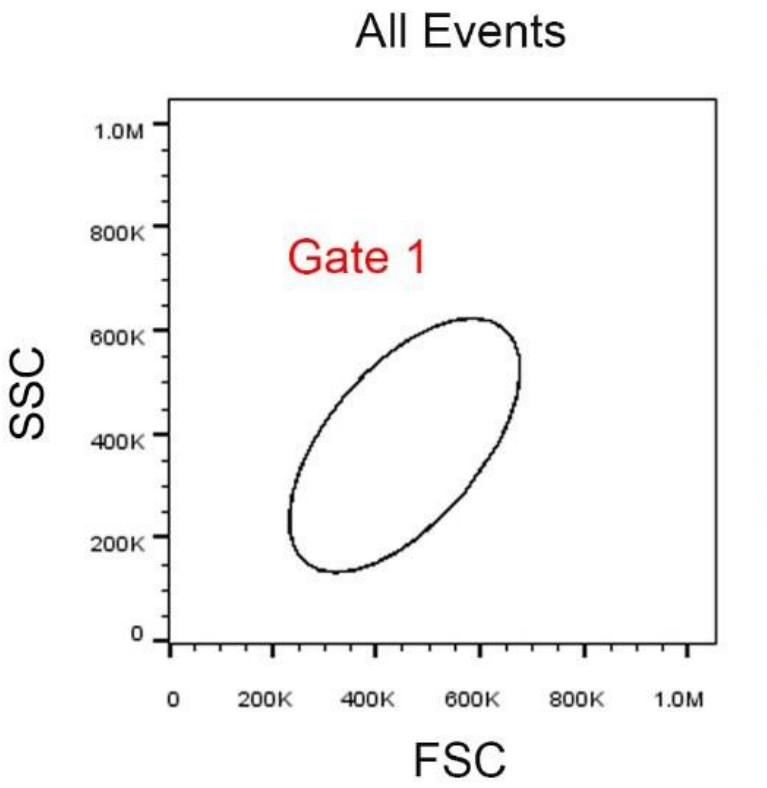
流式管标记！

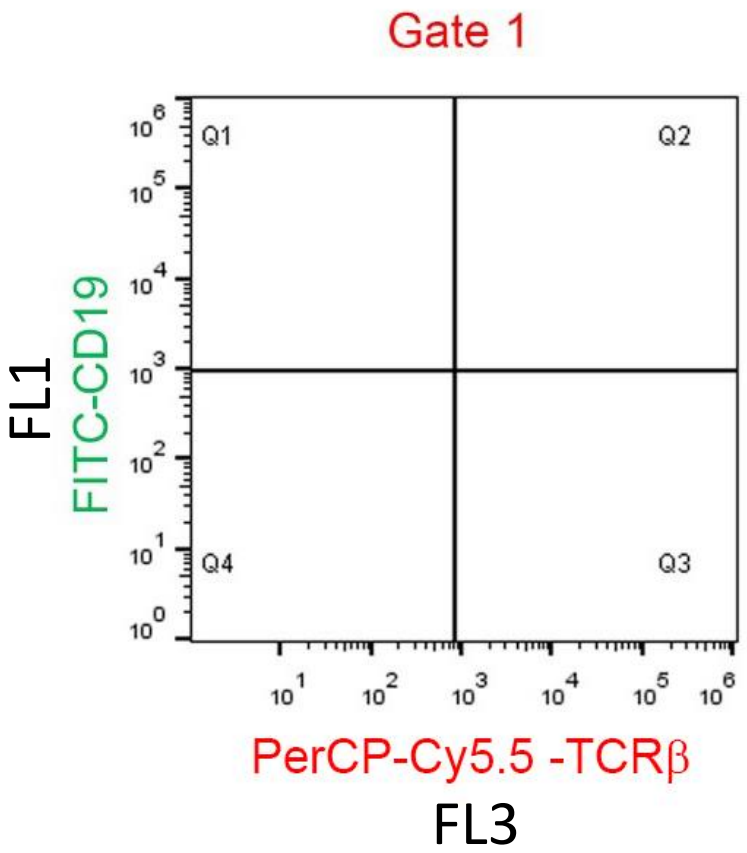
以第二组为例，试管标记：

2L, 2Sc, 2SS

Experiments

III. Cell immunostaining and flow cytometry analysis





Record 10,000 cells from Gate 1.

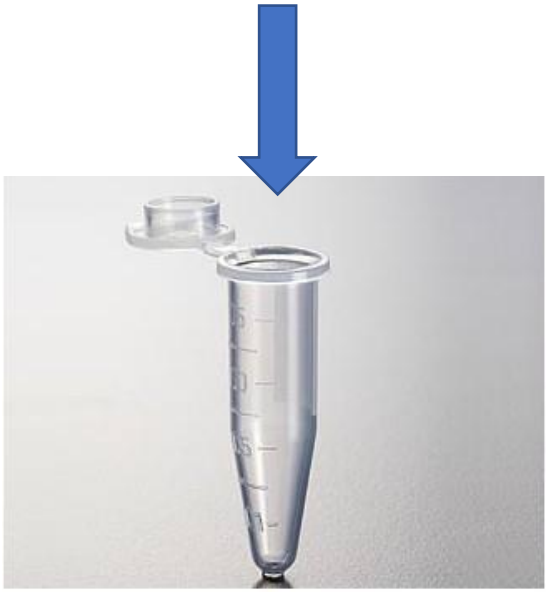
III. Cell immunostaining and flow cytometry analysis

IV. Microscopic observation of cell morphology

Experiments

IV. Microscopic observation of cell morphology

45 μL 细胞悬液
5 μL 0.2% 台盼蓝染液



混匀!

染色3-5分钟

Wait for 30s –1 min then transfer 30μL of the sample onto a slide, observe it under a microscope within 5 minutes, and take pictures.

拍照!

注意事项：

1. 小鼠原代细胞容易死亡，分离脾脏细胞时尽量保证每个步骤不要超时，特别是裂解红细胞的步骤，应防止过度裂解。
2. 流式染色应设置对照组，对照组的染色体系中不加入抗体，其余操作不变。
3. 需注意每个实验步骤操作所处的温度，对于室温操作需要将冷的试剂（如分离缓冲液）提前预热；对于冰上操作的步骤（如流式染色），相应用到的离心机需提前预冷。
4. 小鼠原代淋巴细胞脆弱，台盼蓝染色时间不易过长。

用完的活塞芯重新推入针筒，放在实验台上，陈老师统一收集

Study Questions

- 1. 绘制典型的淋巴结切面图。
- 2. 流式细胞术可以进行单细胞分析的原理？如何借助流式细胞仪得出分离的脾脏或淋巴结免疫细胞的总数？其中淋巴细胞及T与B细胞的比例？可能影响这些数据的原因有哪些？

T-B细胞相遇牵手

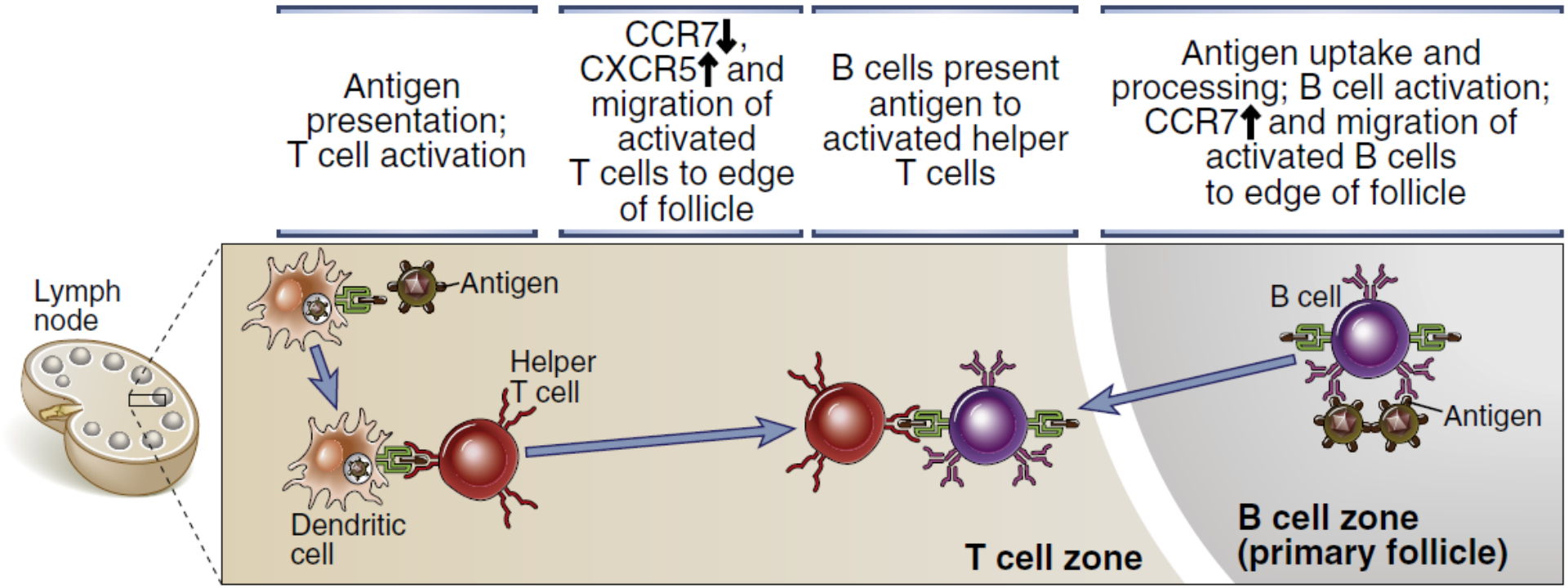


FIGURE 12-8 Migration of B cells and helper T cells and T-B interaction. Antigen-activated helper T cells and B cells move toward one another in response to chemokine signals and make contact adjacent to the edge of primary follicles.

The B cell still also **expresses CXCR5**, which binds CXCL13, expressed **by FDCs** in the B-cell follicles