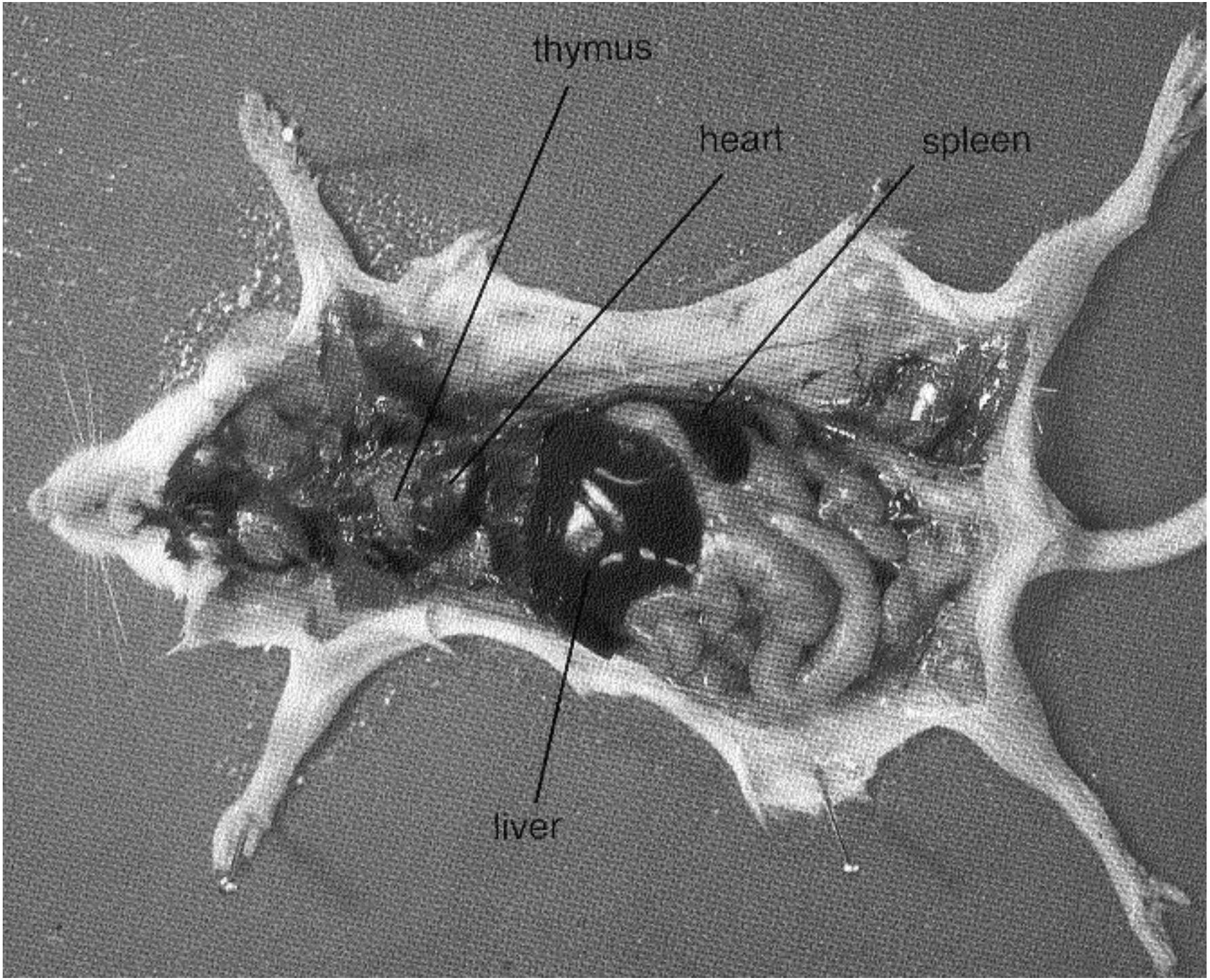
# *IMMUNOLOGY LAB: # 1*
## *Cells and Organs of Immune System*

Purpose:

1. To examine the cells that comprise the immune system (counts and morphology)
2. To examine location or organs and tissues of immune system (primary and secondary)
3. To examine morphology of immune organs and tissues


thymus
heart
spleen
liver

## Materials needed:

1. Mouse (1 per 4 students)
2. Jar with anesthetic and cotton balls
3. Dissecting tools
4. 70% ethanol, cotton balls
5. Microscope slides and cover slips
6. Wright's stain
7. Prepared slides (histology of tissues)
8. Hemocytometer
9. Isotonic saline
10. 1cc syringe with 26 gauge needle

Primary Lymphoid Organs

1. Bone marrow-
    1. Stem cells, hemotopoiesis, CSFs
2. Thymus
    1. Lies above heart
    2. Cortex (outer) and medulla (inner)

# Secondary Lymphoid Organs

1. **Spleen**
    1. located on left side of abdomen
2. **Lymph nodes**
    1. neck, armpits, groin, mesenteric
3. **primary follicles** and **germinal centers**
4. **mucosal associated lymphoid tissue**
    1. (MALT) [tonsils and peyers patches]

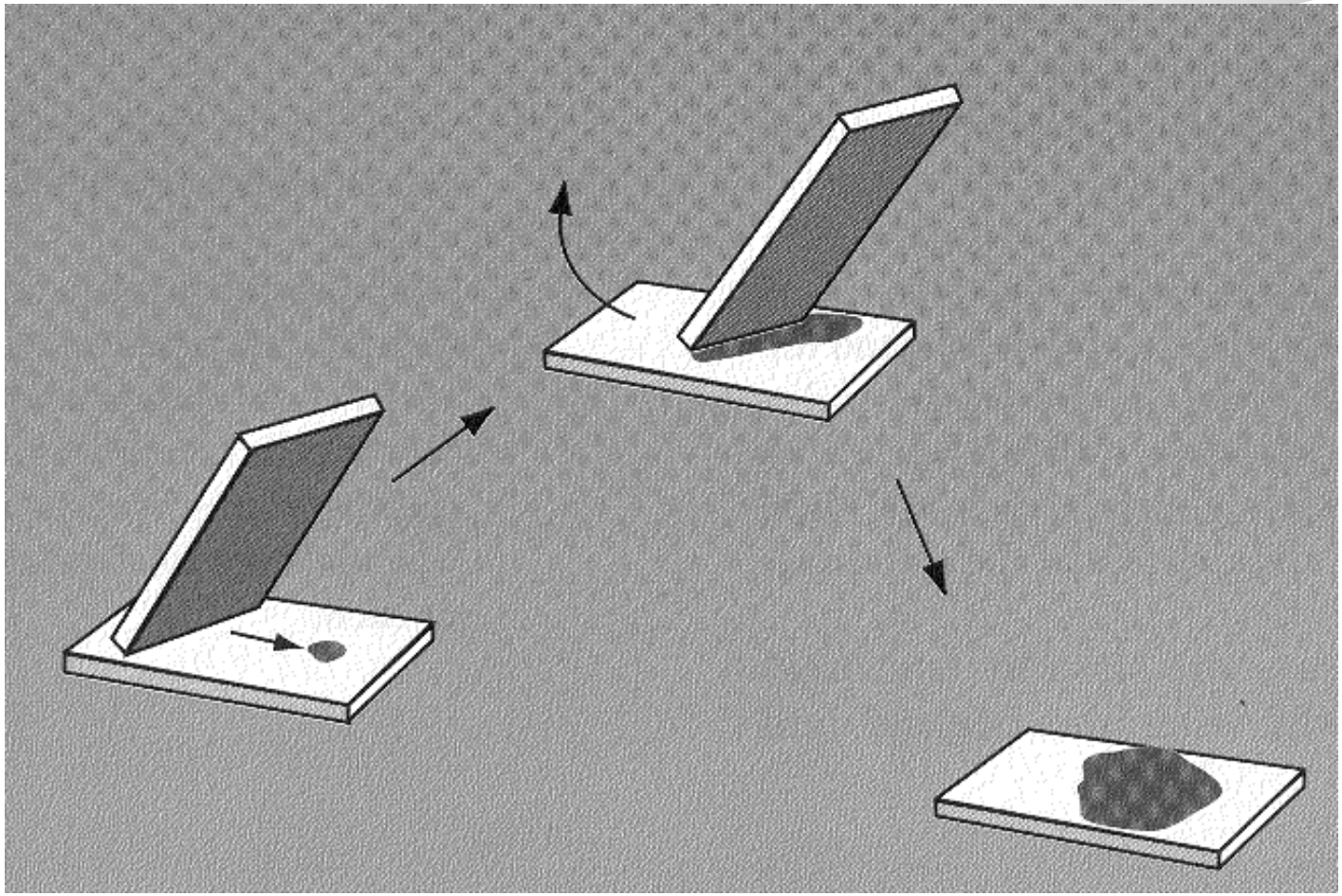
Cells of Immune system

1. **Red blood cells**
2. **White blood cells**
    1. granulocytes (neutrophil, basophil, eosinophil), NK cells
3. **Monocyte-**Macrophage(APCs), dendritic (internal), langerhans (skin)
4. **T cells & B cells-**
    1. Cellular and humoral responses

# *Mouse Dissection Video* *:(click when ready)*

- Lab:
  1. Euthanize mouse
  2. remove long bone of hind leg
     1. clean and “blow out” bone marrow using saline and syringe
        1. count and make smear on slide and stain with Wright’s
  3. collect a blood sample and smear onto slide
     1. stain with Wright’s stain
  4. find and remove spleen and thymus
     1. make single cells suspension
        1. count cells using hemocytometer
        2. make smear on slide and stain with Wright’s stain

*When making a blood smear, place a drop of blood onto clean slide, pull another slide into drop and then pull (never push) drop to spread*



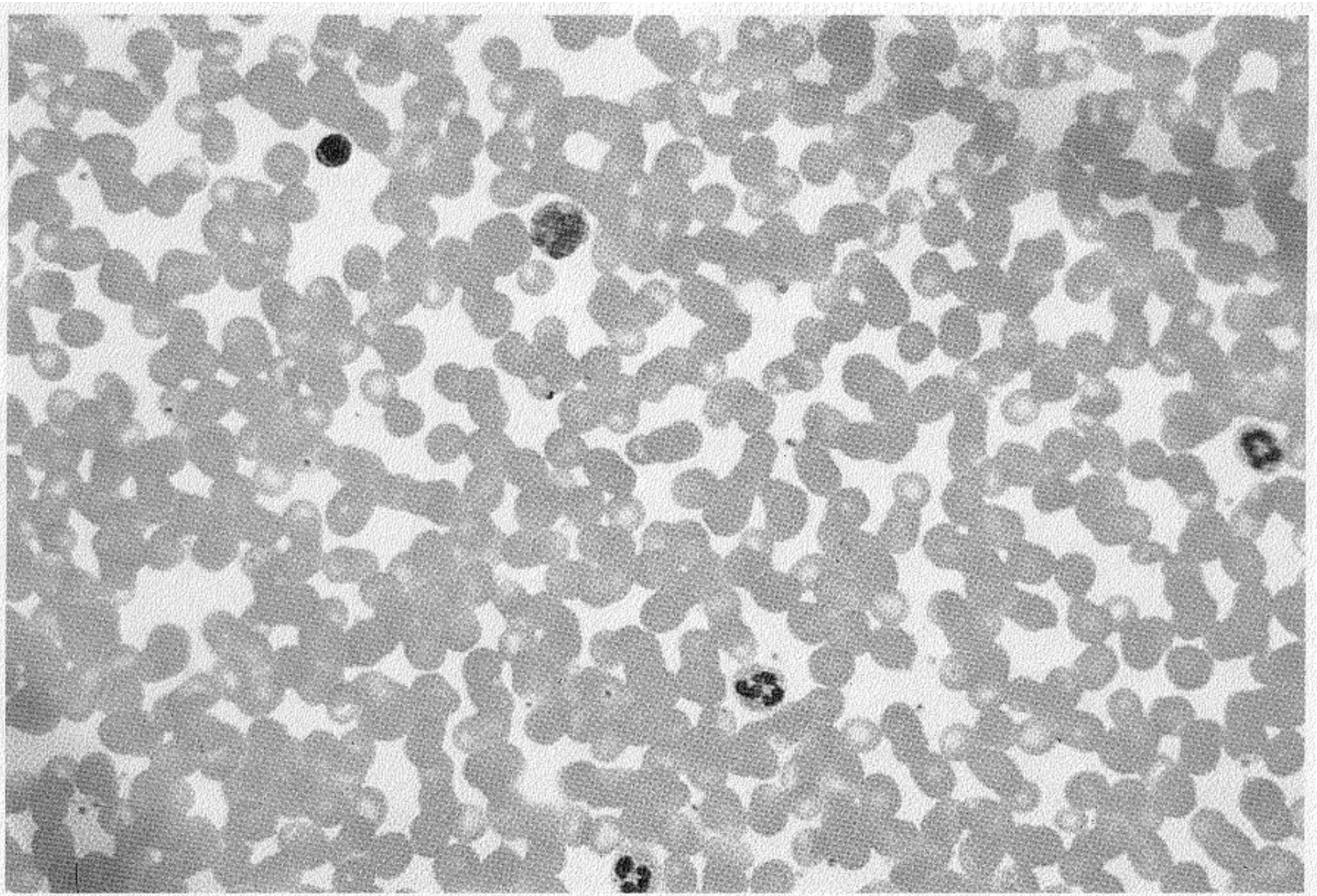
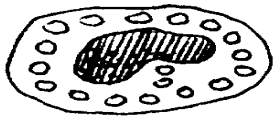
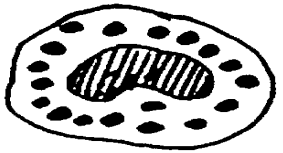


Figure 1.4 Smear of human peripheral blood stained with Wright's stain. Many erythrocytes can be seen, and also a lymphocyte, a monocyte, and neutrophils.

| | Cell Type | Cells/μl Blood | Function | Morphology |
|---|---|---|---|---|
|  | erythrocytes | $6 \times 10^6$ | $O_2$ transport | concave discs, no nuclei, pink |
|  | platelets | $3 \times 10^5$ | blood clotting and blood vessel repair | small, irregular, red granules with blue cytoplasm |
|  | neutrophils | 5,000 (50–70%) | phagocytosis | purple granules, 4-lobe nuclei, size: 9–12 μ |
|  | lymphocytes | 3,000 (20–30%) | specific immunity | large round nucleus, blue cytoplasm, size: 7–13 μ |
|  | monocytes | 500 (2–6%) | phagocytosis, present antigens | convoluted nucleus, granules, size: 14–18 μ |
|  | eosinophils | 300 (1–5%) | destroy antibody-antigen complexes, fight parasites | bright reddish-orange granules, size: 9–12 μ |
|  | basophils | 30 (<1%) | may prevent clotting in inflammation | deep purple granules, size: 9–12 μ |

# *Making "Smear": Blood, Cells*

* Clean slide with ETOH
* Place drop of blood or cells onto slide on one end (the cells should be somewhat concentrated)
* Place 2nd slide onto edge of drop (start from center of slide and back slide into drop) and once fluid is pulled across 2nd slide push the 2nd slide across the 1st slide
* Let cells dry, heat fix, and stain with Wright's stain

# *Wright's Stain Procedure*

* Flood smears with Wright's stain for 2-3 minutes (do not let slides dry out)
* After 2-3 minutes add an equal amount of Wright's buffer and let stand 3 minutes (mix solutions together by blowing over slide)
* gently rinse slide by washing with water from one end until water runs clear
* Let dry and examine under high (oil) power

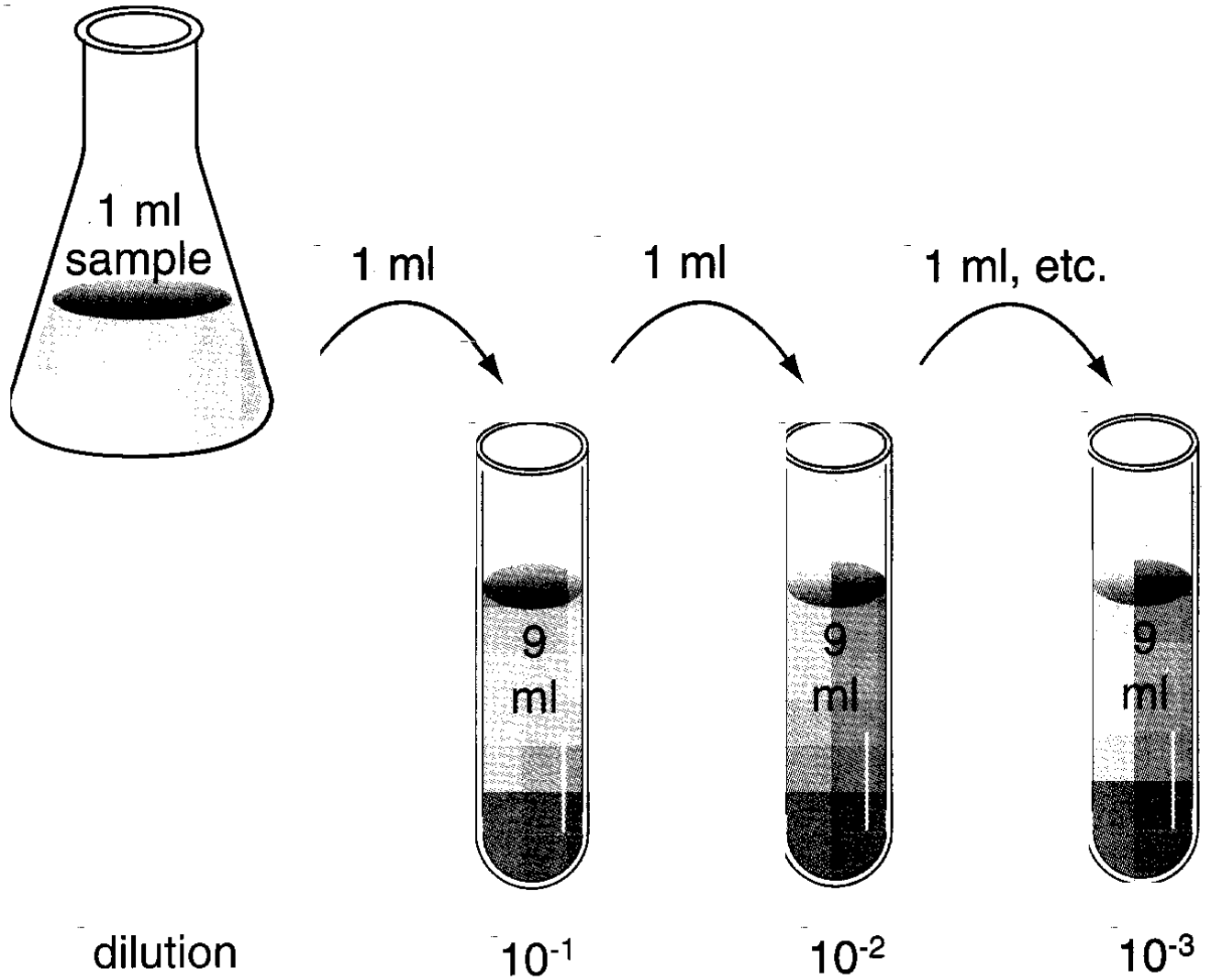
***Draw the cells in each category (blood, thymus, spleen, LN, BM)/calculate the number of cells of each type/give percentage of cells (this should be done by counting at least 100 cells from smears)***

| Neutrophils | Lymphocytes | Monocytes | Eosinophils | Basophils |
|---|---|---|---|---|
| | | | | |
| Total cells | | | | |
| Percent | | | | |

# *Lab # 2a- Cell Dilutions*

- Purpose- To learn serial dilution techniques and to calculate cell concentration in order to set up various types of assay's (proliferation, CTL, $^{51}$Cr-release, etc…)
  - perform 2 fold and 10 fold dilutions
  - count cells in each dilution using hemocytometer (determine accuracy of dilution technique)

# *Serial Dilutions*



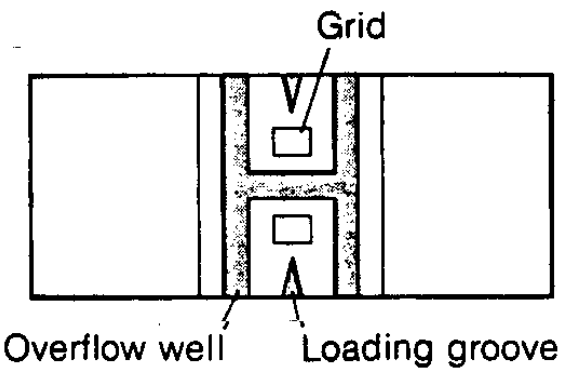
# *Materials Needed:*

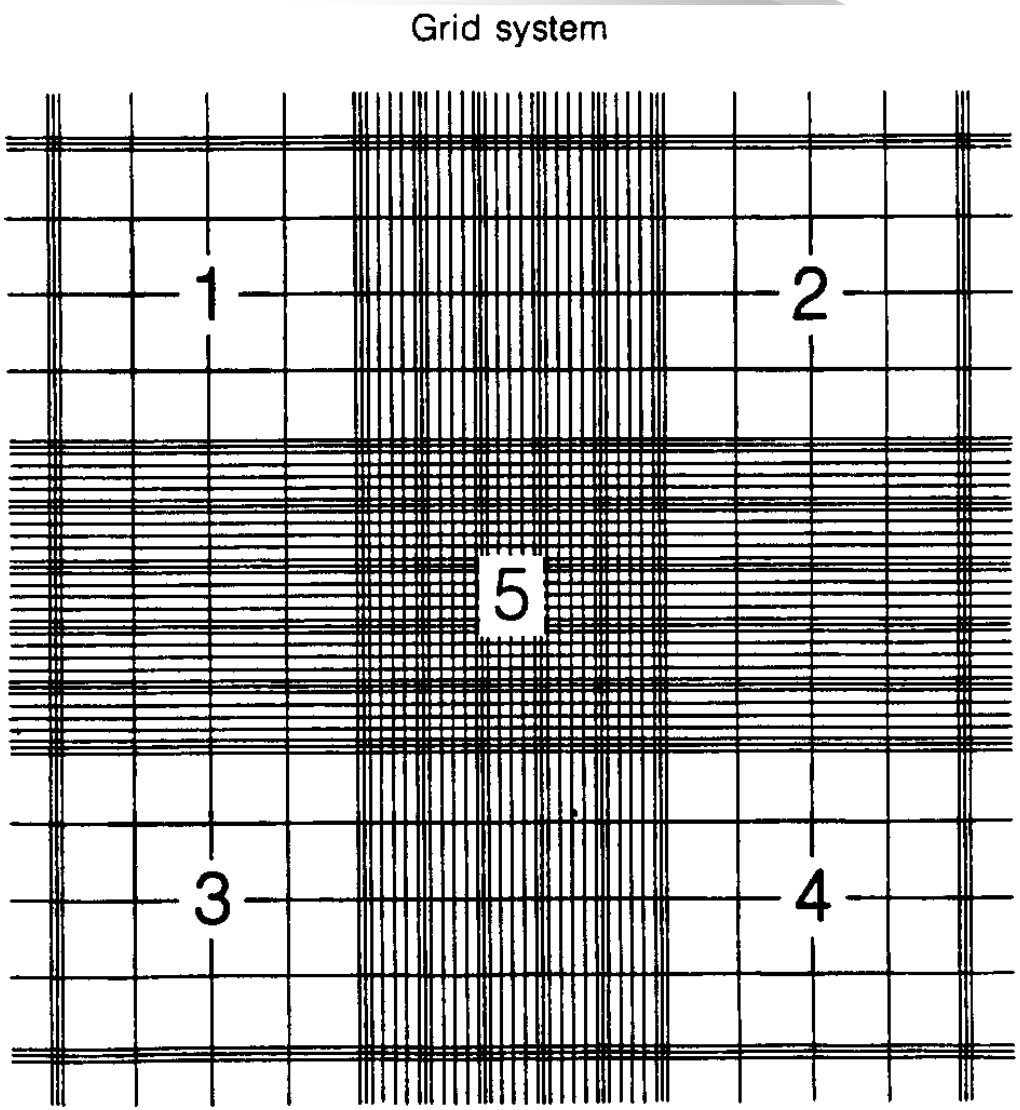
- **One mouse per 4 groups of 3 students each:**
  - **the mouse should be dissected and the following organs removed:**
    - **spleen, thymus, bone marrow, and lymph nodes: one group will examine ONE of the organs for cell counts**
    - **remove brain and prepare for NEXT experiment (brain must be homogenized rather than sonicated as are cells)**
- **Isotonic buffer, lyse buffer, trypan blue**
- **hemocytometer, pipettes, pipettemen (women)**

# *Procedure for hemocytometer*

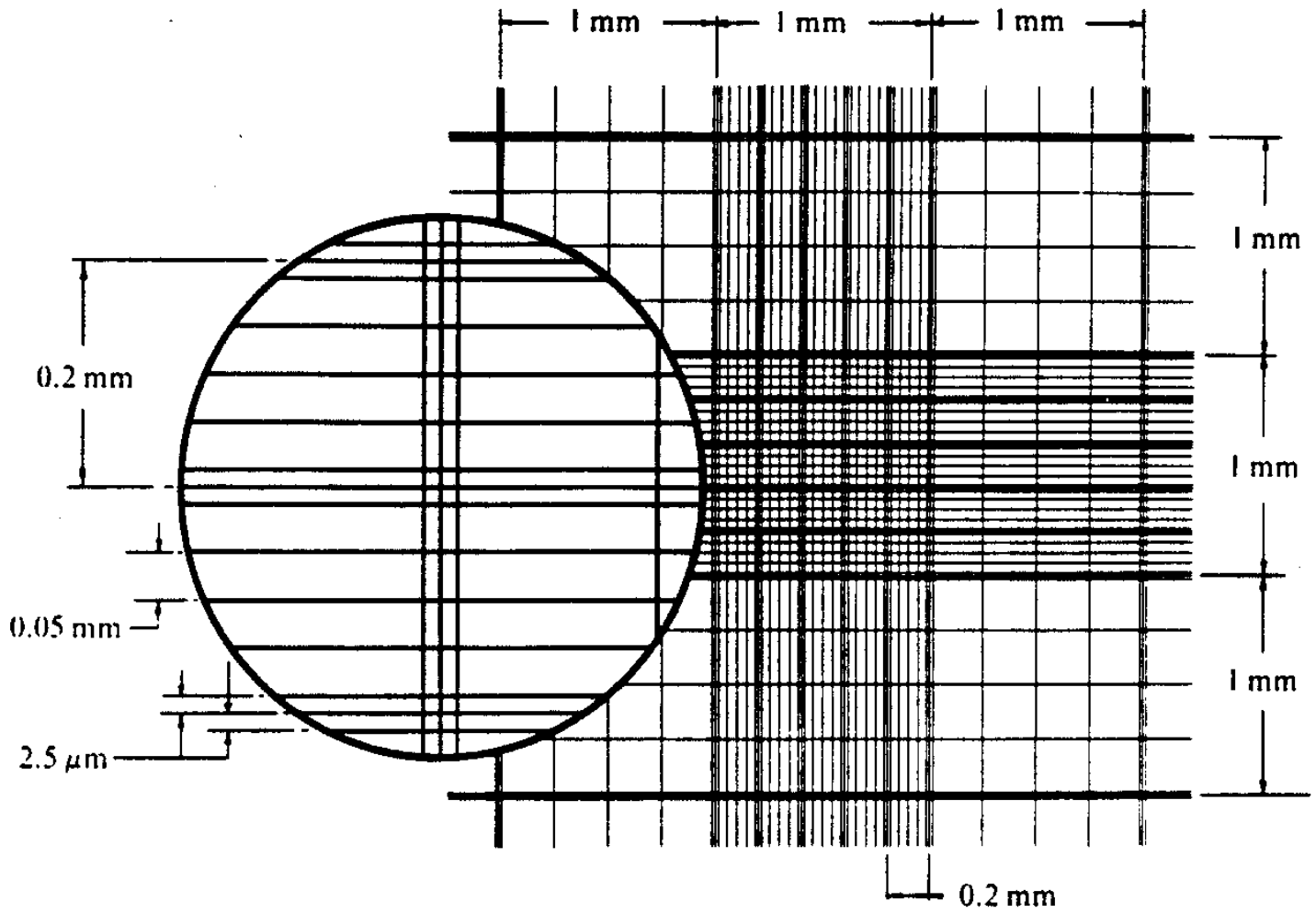
- In the figure (next slide) there are 5 numbered squares
  - each corner square has 16 squares (each 1mm square)
  - **the center square has 25 smaller squares (each 0.2 mm)**
  - **If you dilute your sample by 1:100, and count the number of cells in each of the 16 squares in one corner, the number of cells that you have is # of cells counted X $10^6$.**
  - **OR USE METHOD DESCRIBED 3 SLIDES AHEAD**

# *Hemocytometer*



# *Hemocytometer*



# *Using Hemocytometer*

## APPENDIX 1: COUNTING LYMPHOID CELLS

(There are additional notes on the following page.)

1. The optimal cell concentration for counting by this method is 50 x $10^6$ cells/ml. Cells from a single mouse spleen are usually resuspended in 2 ml of fluid to count.

2. Withdraw 25 µl of cell suspension from the tube with a 20-200 µl Pipetman, and place the cells in a 12x75 mm glass tube. Add 25 µl of 0.04% trypan blue (in PBS) to the cells in the glass tube, mix gently, and wait ~30 seconds. Then add 50 µl of 4% acetic acid (in water).

3. Add the stained sample into one chamber of a hemacytometer with a cover glass via the groove at the top or bottom of the chamber (see figure below). Add sample only until the chamber is full (~10 µl), do not overfill the chamber. Let the cells settle in the chamber for about 1 min.

4. View the hemacytometer under the 10X objective. Locate the central large square ("5" in the figure below) containing 25 smaller squares each circumscribed by 3 lines. The average number of cells in a smaller square is the average number of cells x $10^6$ per ml of original cell suspension.

5. To determine the cell concentration, count the cells in a series of smaller squares bounded by three lines: record the number of live cells (colorless), dead cells (blue), and the number of squares counted. Divide the total number of live cells by the total number of squares counted; multiply by $10^6$. This value is the cell concentration in the original suspension. Cell viability is determined by dividing the total number of live cells by the sum of live and dead cells, and is expressed as percent viability (*i.e.*, x 100).

# *Lab # 2b: Protein Determination*

- **Purpose- To determine protein concentration using the BIORAD assay**
- **WHY?**
  - **To be able to equilibrate protein concentration for enzyme assays**
  - **to equilibrate protein concentration on PAGE and Western blots**

# *Methods*

- **Use brain sample prepared previously (work in groups of 3)**
  - **thaw**
  - **Dilute Biorad buffer 1:4**
  - **prepare Albumin for standard (1mg/ml stock)**
  - **set up assay as described in 96 well plate**
  - **measure absorbance using plate reader**
  - **Set up Sigma Plot and Sigma Stat for data**

# *Lab # 3a: Separation of Peripheral Blood Cell Components*

- **Use Ficoll-Hypaque solution to separate white blood cells from red blood cells and mononuclear cells from polymorpho-nuclear cells (PMNs= neutrophiles)**
- **Inject mice intraperitoneally (ip) with thioglycolate to induce macrophage infiltration and activation in abdomen (for next lab)**

# *Lab # 4: Phagocytosis and Microbial killing by Macrophages*

- **Phagocytes- ingest foreign material (bacteria), debris and irritants like asbestos particles), and dead host tissue.**
  - **Phagocytic defect results in Chediak-Higashi syndrome or chronic granulomatous disease (susceptible to infections by organisms that have low virulence)**
    - **Neutrophil- main phagocytic cell of blood**
    - **Macrophages- main phagocytic cell of tissues (arise from circulating monocytes that migrate (diapedesis) into tissues)**

# *Materials for Lab # 4*

- **Thioglycollate injected mice**
  - **24 hour post injection (IP) gives primarily neutrophil migration into abdomen**
  - **48-96 hours post injection (IP) gives macrophage infiltration**
- **Dissecting equipment**
- **Anesthetic jar**
- **Microscope slides**
- **Sterile test tubes**
- **Overnight culture of *Staphylococcus aureus***
- **Syringes and needles**
- **Sterile Ringers media**
- **Sterile pasteur pipette**
- **Agar plates**
- **Normal rabbit serum**
- **Water bath**
- **Centrifuge**

# *Procedure*

- **On day of experiment prepare bacterial culture by centrifuging at 1800 X g for 20 minutes**
  - **Discard supernatant and wash bacteria in HBSS**
  - **Centrifuge and resuspend again**
  - **Dilute to concentration of $10^7$ cells/ml in a total of 5 ml**
- **Euthanize mouse**
  - **Wet fur with 70% ETOH and cut open outer skin and muscle layers of abdomen WITHOUT cutting through abdomen**
  - **Inject 10 ml of cold HBSS into peritoneal cavity and gently shake and massage lower abdomen to loosen peritoneal cells (3 minutes)**
  - **Pull up peritoneal membrane and snip small hole. Place sterile pasteur pipette into cavity and aspirate cells**

- **Label 4 test tubes “A”, “B”, “C”, and “D” and place the mixtures below into the tubes and incubate for 1 hour {tube “A” is test for phagocytosis and B,C, and D are experimental)**

| Tube | **A** | **B** | **C** | **D** |
|---|---|---|---|---|
| **serum** | 0.2ml | 0.2ml | **----** | **----** |
| **Cells** | 1ml | **-----** | 1ml | **----** |
| **Bact.** | 0.5ml | 0.5ml | 0.5ml | 0.5ml |
| **HBSS** | 0.3ml | 1.3ml | 0.5ml | 1.5ml |

- **During the one hour incubation, prepare 8 dilution blanks, each containing 0.9 ml saline, for each test tube A-D. You should have 32 dilution blanks and 32 plates total**
- **After the one hour incubation, spread a drop from each tube A-D on each of 4 slides to make smears. Air Dry**
- **Make 10 fold serial dilutions of each of the tubes A-D by transferring 0.1 ml from tube A to the 1st of the 8 dilution tubes. Mix, then transfer 0.1 ml to the next tube (etc…)**
  - **Continue for B-D**
- **Transfer 0.1 ml to each agar plate from each dilution, and spread using sterile bent glass rod (flame in ETOH)**
- **Stain slides with Wright's stain**
- **Invert plates (top side down) and incubate 24-48 hrs**
  - **Count "countable" plates (30-100 colonies) next day**

# *Complement Fixation*

- **Important nonspecific factor (C')**
  - **Guinea pig serum usually used as source**
    - **Concentration more constant and amount of complement produced is greatest in this species**
  - **Complement combines with an antibody only after the antibody has been "sensitized" by binding to antigen.**
    - **Antigen:antibody complex (can lodge in tissue to cause damage)**
    - **C' binds to Fc region in IgG and IgM**

- **Complement Fixation-**
  - **Binding of C' to antibody**
    - **This reaction removes reactive C' from serum**
    - **Can measure C' levels in serum to determine whether there are Ag:Ab complexes or complex disease**
  - **C' components**
    - **Labile to heat, prolonged storage, shaking, acids, bases and bile salts**
    - **Heating at 56$^{o}$C sufficient to destroy C'**
  - **Action of C' is by lysis or opsonization**
    - **Lysis of cellular Ag's (bacteria, RBCs, ….)**
    - **C' fixed if Ab binds to soluble Ags, such as virus**

- **Protocol— to determine whether Ab is present in subjects' serum**
  - **Put Ag in test tube**
  - **Put in small amount of C'**
  - **Add subjects serum (Ab present?)**
    - **If Ab present– will bind Ag and then fix C', thereby lowering amount of C' in test serum**
  - **Take aliquot of fluid in test tube and add to a RBC + anti-RBC antibody mixture**
    - **Without C' no lysis**
    - **If no C' fixation in above, then aliquot containing C' will cause lysis of RBCs**
    - **If fixation of C' in above due to Ag:Ab complex then no lysis of RBCs occurs**

- **For each sample of complement, set up a series of 11 tubes**
- **Follow dilution protocol in table:**
  - **Add complement 1st**
  - **Add anti-sRBC antibody 2nd**
  - **Add the magnesium-saline solution 3rd**
  - **Add the sRBCs 4th**
- **Mix contents and incubate in a 37oC water bath for 30 minutes**
- **Determine the smallest amount (highest dilution) of complement giving complete lysis**

| Tube # | C’ (ml) | Anti-RBC | Mag-Sal | 2% sRBC | +/- Rx |
|---|---|---|---|---|---|
| 1 | 0.1 | 0.5 | 1.4 | 0.5 | |
| 2 | 0.15 | 0.5 | 1.35 | 0.5 | |
| 3 | 0.2 | 0.5 | 1.3 | 0.5 | |
| 4 | 0.25 | 0.5 | 1.25 | 0.5 | |
| 5 | 0.3 | 0.5 | 1.2 | 0.5 | |
| 6 | 0.35 | 0.5 | 1.15 | 0.5 | |
| 7 | 0.4 | 0.5 | 1.1 | 0.5 | |
| 8 | 0.45 | 0.5 | 1.05 | 0.5 | |
| 9 | 0.5 | 0.5 | 1.0 | 0.5 | |
| 10 | 0.5 | --- | 1.5 | 0.5 | |
| 11 | --- | 0.5 | 1.5 | 0.5 | |

- **Student Questions:**
  - **Explain which tube is the control tube and why**
    - **What was left out of the control tube and why?**
  - **What does this experiment show?**
  - **What can this type of assay be used for?**

# *VIDEO Presentations:*

- Lab safety
- Wright's stain & smear
- dilutions
- hemocytometer
- mouse handling & dissection (organ location)
- Biorad assay and plate reader

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