Appendix i: Using a Compound Microscope

1. A Little Optics

A light microscope is perhaps the most important instrument in all biology. Whether you love genetics, botany, zoology, or paleontology, sooner or later you will find yourself using a light microscope.

To be specific, a light microscope is a coordinated system of lenses that magnifies an object at high resolution. There are 4 general optical considerations that define how well a microscope magnifies an image.

Resolution- *the ability to distinguish two points as being separate.*
The resolution of the human eye, for example, is around 0.1 mm (1 inch = 25.4 mm). The higher the resolution, the sharper the image will be. Note that resolution is not the same as magnification. You can buy hand held lenses that purport to magnify objects 1200X and cost $50! All you will see, however, is a blur. The compound scopes you will be using magnify objects up to 1000X and cost around $2000. The difference in cost has to do with resolving power, not magnification.

Depth of Field- *the range of depth that an object is in focus*
Think about an old photograph of a family vacation. The people appear in focus (hopefully) while the background will be blurry. In a microscopy, the more you magnify the image, the shallower the depth of field becomes.

Contrast- *the ratio between dark and light in an image*
Most microscopes use absorption contrast, that is, the specimen is subjected to stains in order to be seen. This is called **bright field microscopy**. Other types of microscopy use more exotic means to generate contrast, such as phase contrast, dark field and differential interference contrast.

Illumination Source
The higher the magnification, the more light is needed to form an image. In addition, the more light there is (brightness), the more leeway you have to make adjustments in resolution, depth of field, and contrast.

These four factors trade off against one another. You cannot simultaneously have maximum contrast and maximum resolution. Resolution/Brightness are antagonistic to Contrast/Depth of Field.

2. Compound Microscope Parts

Adhere to the following guidelines when using the scopes:

- Carry the scope with TWO hands close to your body.
- NEVER clean lenses on the microscope with anything other than lens paper.

There are two basic systems that allow the user to control the tradeoffs discussed above: the illuminating system and the imaging system.

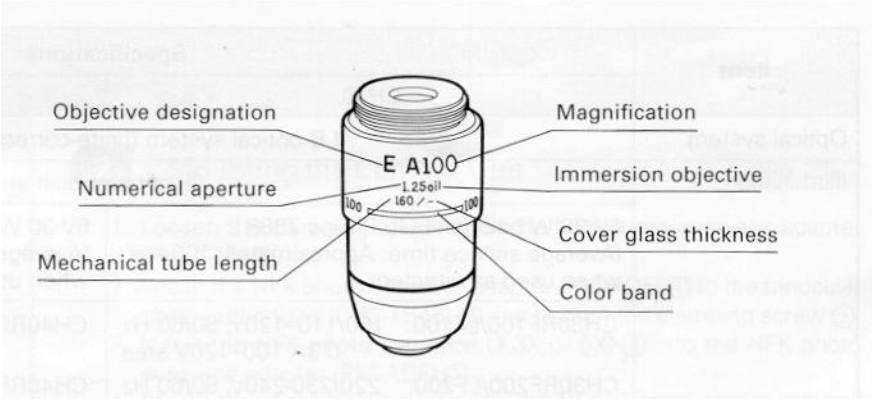
The Illuminating System
The illuminating system concentrates light on the specimen and usually consists of:

- **light source** – the brightness of the light source is controlled by the light intensity knob and the field iris diaphragm dial.
- **condenser lens** – focuses light on the specimen
- **iris diaphragm –** regulates how much light reaches the specimen.
  The numbers on the side indicate how large the diaphragm opening is in millimeters. Changing the diaphragm setting affects the depth of field of the image: the smaller the opening, the greater the depth of field. Usually, you should adjust the diaphragm opening so that it is about 70-80% of the numerical aperture of the objective lens you are using (discussed below).

Imaging System
The imaging system magnifies the specimen and improves the resolution of the resulting image and consists of:

- **objective lens** – these are the 3-4 lenses mounted on the nosepiece. An objective is actually a series of lenses that magnify the image, improve resolution and correct for aberrations (distortions) in the image. The magnifying power of each objective is shown on the side (see Figure 1). Other information includes the type of objective it is (objective designation), the length of the objective tube, whether or not it is an oil immersion objective, the size cover slip to be used, the wavelength of light that should be used with the objective, and the numerical aperture (NA) in millimeters of the lens. NA is a measure of the resolving power of the lens and is a function of lens size. *The smaller the NA, the greater the resolving power*.

Figure 1: Objective Lens



- **ocular lens** – These are the lenses through which you view the image formed by the objective lens. Ocular lenses magnify this image. The magnification is written on the side and is 10X for our scopes. The final magnification at which the specimen is viewed is calculated as follows:

$$MAG_{total} = MAG_{objective} \ X \ MAG_{ocular}$$

The microscopes you are using are binocular; they have two ocular lenses.

Setting up a Compound Microscope

We use two different models of Olympus microscopes. The accompanying diagrams outline the parts of each compound microscope.

1. Carry your scope using the "**carrying points**". ALWAYS USE TWO HANDS!! Hold the scope close to your body. When putting your scope away, make sure to return it with the 4X objective in place and with the dust cover on.

2. Clean the ocular and objective lenses using lens paper.

3. Place your slide very carefully in the **specimen holder.** You can then use the **stage controls** to move your specimen around.

4. This is a binocular scope. You must first adjust the distance between the lenses for how wide your eyes are apart. (**interpupillary distance**). You then must adjust for vision differences between your eyes using the **diopter adjustment ring** on the left ocular lens.

5. Find the specimen you are interested using the 4X objective first. Focus on the image using the **course focus adjustment knob**.

6. These are **parafocal** (image remains in focus when switching between objectives). When switching to a higher power objective, you should only have to make minor focus adjustments as long as you do not "skip" objectives (i.e., go from 4X to 40X). Do this by using the **fine focus adjustment knob**. DO NOT SKIP OBJECTIVES ON YOUR WAY TO HIGHER MAGNIFICATIONS!!

7. Only focus using the **fine focus adjustment knob** when looking through 10X, 40X or 100X objectives.

There are two methods to adjust the lighting on these scopes:

1. You can adjust the intensity by using the **light intensity knob**.

2. You can control the amount of light reaching the sample using the **aperture iris diaphragm knob** located on the **condenser** and the **field iris diaphragm knob**. In general, you want to view your specimens using the lowest intensity of light possible (this helps prevent the sample from drying out too quickly). Start with the aperture iris diaphragm leer set to 70-80% of the NA of the objective lens you are using. Adjust the diaphragm to a setting that you find gives you a good balance between image resolution and image contrast.

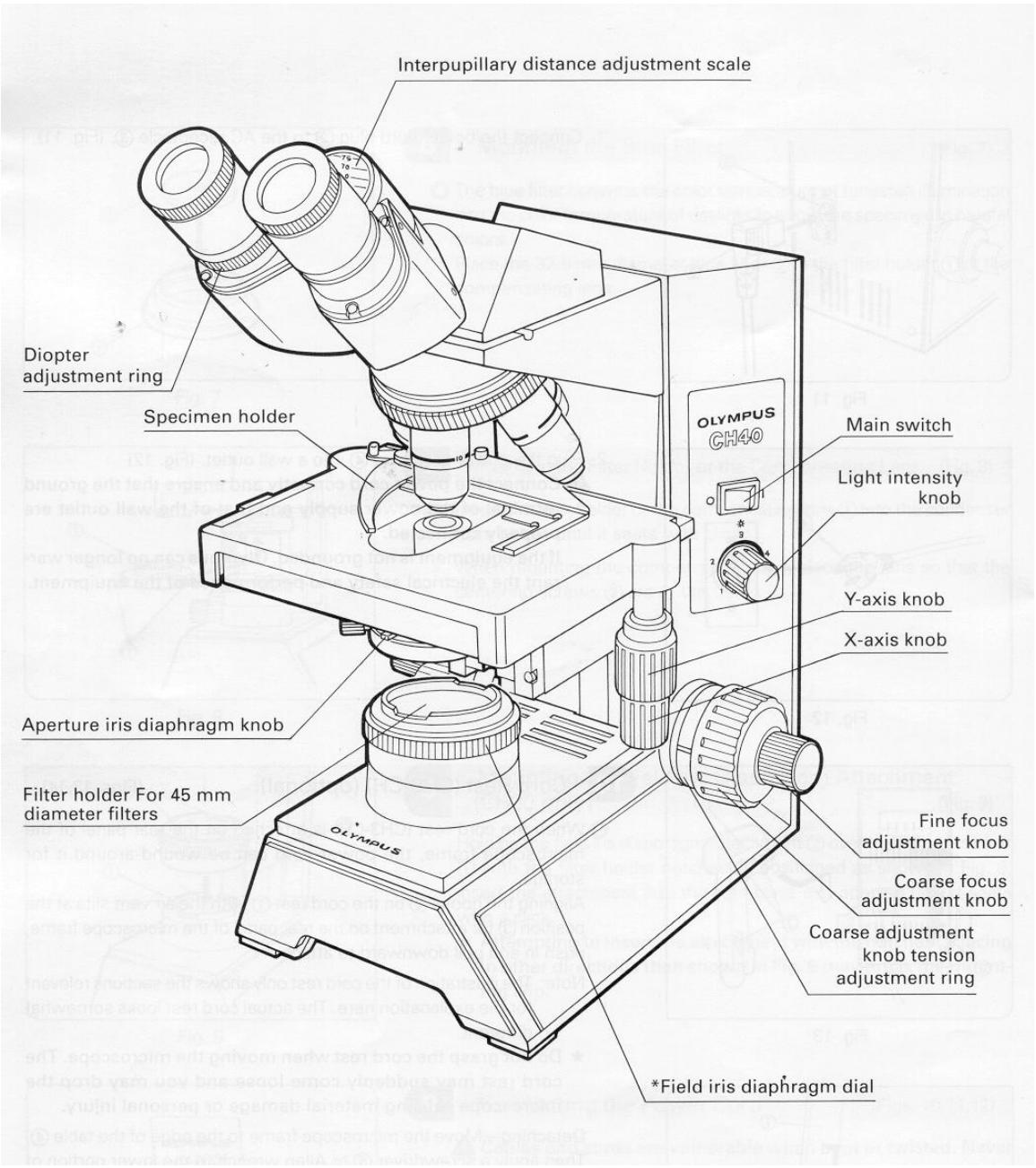
Estimating Image Size

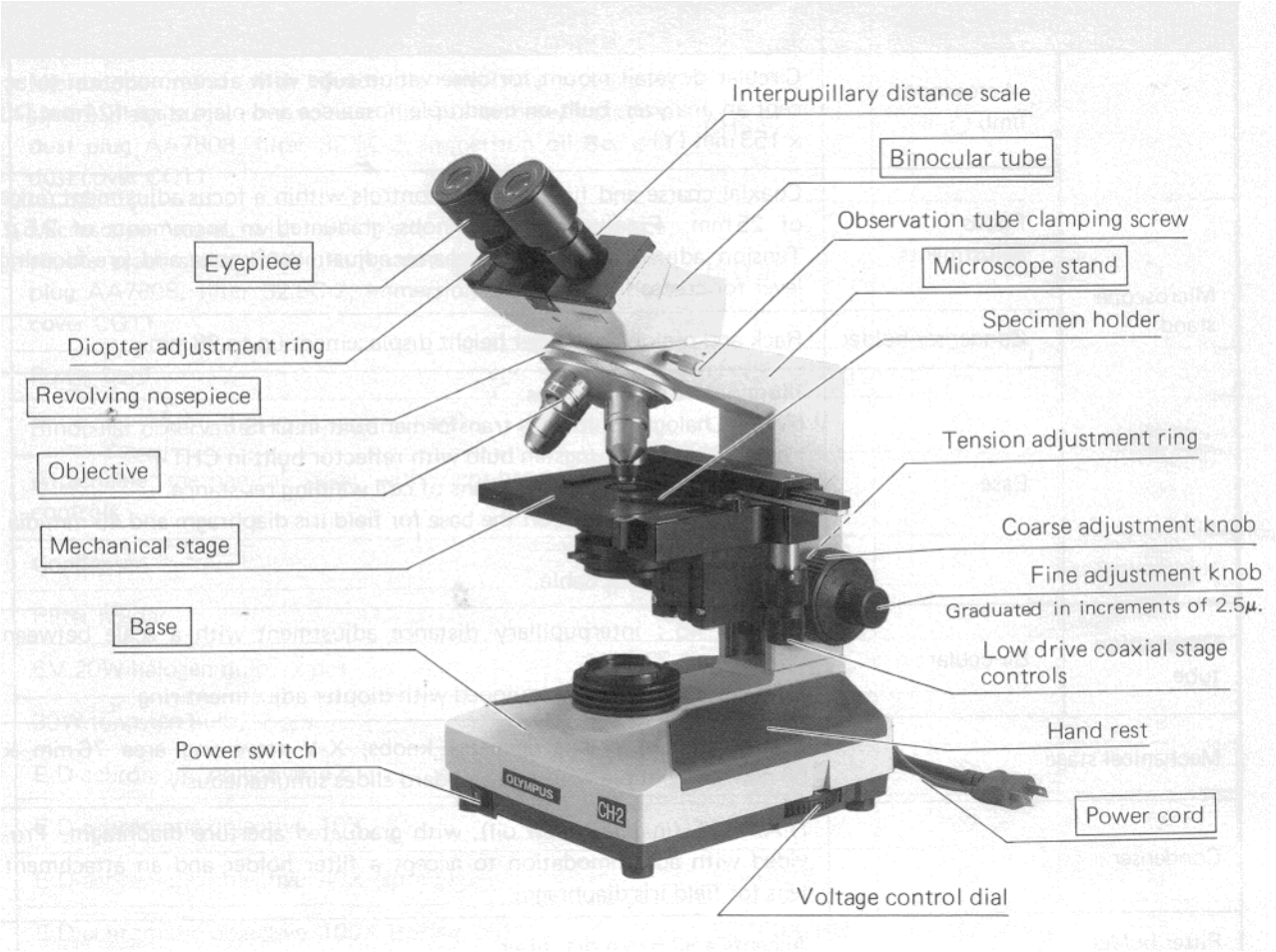
It is important to be able to estimate the size of a specimen. An easy way of doing this is to compare the object in the image with the size of the **field of view**. The size of the field of view for the objectives we use is given in Table 1.

Table 1: Diameter, area and Pointer Length for the Field of View of Different Objectives

Objective Power	Diameter (µm)	Area (mm^2)	Pointer Length (µm)
4X	4500	15.9	2250
10X	1800	2.50	900
40X	440	0.16	220
100X	180	0.02	90

Acknowledgements: Andrew Davis, PhD


Interpupillary distance adjustment scale
Diopter adjustment ring
Specimen holder
OLYMPUS CH40
Main switch
Light intensity knob
Y-axis knob
X-axis knob
Aperture iris diaphragm knob
Filter holder For 45 mm diameter filters
Fine focus adjustment knob
Coarse focus adjustment knob
Coarse adjustment knob tension adjustment ring
*Field iris diaphragm dial


Interpupillary distance scale
Binocular tube
Observation tube clamping screw
Eyepiece
Microscope stand
Specimen holder
Diopter adjustment ring
Revolving nosepiece
Tension adjustment ring
Objective
Coarse adjustment knob
Mechanical stage
Fine adjustment knob
Graduated in increments of 2.5μ.
Base
Low drive coaxial stage controls
Hand rest
Power switch
Power cord
Voltage control dial
OLYMPUS
CH2

Appendix ii: **Chi-Square Test**

The chi-square test is a statistical test used to compare observed data with data we would expect to obtain based on a specific scientific hypothesis. This method of analysis tests a **null hypothesis**, which is hypothesis that makes a statement of no relationship. Basically, a null hypothesis states that the variable being manipulated in an experiment will have no effect. If this is true, then no significant difference between expected data and observed data should be found and slight differences observed would be due to chance or sampling error. The chi-square test helps determine whether or not the null hypothesis is supported by the data.

The formula for calculating chi-square (X^2) is:

$$X^2 = \Sigma[(o-e)^2 / e]$$

This translates into chi-square equaling the sum of the squared differences between observed and expected data (or the deviation, d) divided by the expected data in all categories. Once X^2 is calculated, it is compared to critical values in a chi-square distribution table. If the calculated value is greater than the value in the table, then it means that the difference between the values is significant and the null hypothesis is rejected. On the other hand, if the calculated value is less than the value in the table, the null hypothesis is not rejected because there is no significant difference between the values and any differences are most likely due to chance.

In order to use the table, we need to know two things. First, we must calculate a value called **degrees of freedom** (df). Degrees of freedom is always one less than the number of categories. When calculating X^2 for Hardy-Weinberg, we are working with the frequencies for the genotypes, RR, Rr and rr. What is our degrees of freedom?

Second, we must decide a probability value, or ***p* value**, to use. This provides a standard that serves as a basis for accepting or rejecting the null hypothesis. Biologists generally chose a p value of 0.05 which means that our chances of drawing a wrong conclusion based on our sample data is 5 chances out of 100 (1 in 20).

Once you calculate X^2 and determine your degrees of freedom, consult the chi-square distribution table and compare your value to the value in the table under the $p = 0.05$ column. If your calculated X^2 value is larger than that in the table, you reject your null hypothesis, which means the difference in expected and observed values is significant and not just due to chance. If your calculated X^2 value is smaller, the null hypothesis is not rejected and the difference between expected and observed values can be attributed to chance or sampling error.

(Note: It is not considered acceptable to say you "accept" the null hypothesis; you either reject or fail to reject the null hypothesis.)

Chi Square Distribution

Degrees of Freedom (df)	Probability (p)										
	0.95	0.90	0.80	0.70	0.50	0.30	0.20	0.10	0.05	0.01	0.00
1	0.00	0.02	0.06	0.15	0.46	1.07	1.64	2.71	3.84	6.64	10.83
2	0.10	0.21	0.45	0.71	1.39	2.41	3.22	4.60	5.99	9.21	13.82
3	0.35	0.58	1.01	1.42	2.37	3.66	4.64	6.25	7.82	11.34	16.27
4	0.71	1.06	1.65	2.20	3.36	4.88	5.99	7.78	9.49	13.28	18.47
5	1.14	1.16	2.34	3.00	4.35	6.06	7.29	9.24	11.07	15.09	20.52
6	1.63	2.20	3.07	3.83	5.35	7.23	8.56	10.64	12.59	16.81	22.46
7	2.17	2.83	3.82	4.67	6.35	8.38	9.80	12.02	14.07	18.48	24.32
8	2.73	3.49	4.59	5.53	7.34	9.52	11.03	13.36	15.51	20.09	26.12
9	3.32	4.17	5.38	6.39	8.34	10.66	12.24	14.68	16.92	21.67	27.88
10	3.94	4.86	6.18	7.27	9.34	11.78	13.44	15.99	18.31	23.21	29.59
	Nonsignificant								Significant		

Source: R.A. Fisher and F. Yates. *Statistical Tables for Biological, Agricultural and Medical Research*. 6th ed. Table IV. Longman Group UK Ltd, 1974