MTh Microscope Theory

Learning Objectives

The student will

- Follow oral and written instructions and manage time in the lab efficiently.
- Apply correct terminology regarding microbiological techniques, instruments when making observations.
- Use the bright field light microscope to view microbes under oil immersion, make accurate observations and appropriate interpretations and store the microscope according to lab procedures.

Background/Theory

Over the past several centuries, we have learned to manipulate light to peer into previously invisible worlds—those too small or too far away to be seen by the naked eye. Through a microscope, we can examine microbial cells and colonies, using various techniques to manipulate color, size, and contrast in ways that help us identify species and diagnose disease. (OpenStax CNX, 2018)

Microscopes magnify images and use the properties of light to create useful images of small objects. **Magnification** is defined as the ability of a lens to enlarge the image of an object when compared to the real object. For example, a magnification of 10× means that the image appears 10 times the size of the object as viewed with the naked eye. (OpenStax CNX, 2018) Two or more lenses can be combined to magnify the image sequentially. The total magnification is then the product of the magnifications of the two lenses.

Greater magnification typically improves our ability to see details of small objects, but magnification alone is not sufficient. One could keep compounding the magnification with additional lenses making the image larger and larger. At some point, however, all you will see is a big blurry image. Increasing the magnification alone will not give any additional useful information because it fails to add detail to the image. To see additional detail, one must also enhance the **resolution** or clarity of the image. You can think of resolution as the image sharpness or the amount of detail one can see. A low-resolution image appears fuzzy, whereas a high-resolution image appears sharp.

Two factors affect resolution. The first is the **wavelength** of light used. Shorter wavelengths are able to resolve smaller objects. An electron microscope has a much higher resolution than a light microscope, since it uses an electron beam with a very short wavelength, as opposed to the longer-wavelength visible light used by a light microscope. The second factor that affects resolution is **numerical aperture**, which is a measure of a lens's ability to gather light. The higher the numerical aperture the more light captured and the better the resolution. (OpenStax CNX, 2018) For more on the properties of light as they pertain to microscopy click here.

Resolution can be quantified using this modification of the Abbe equation.

$$D = \frac{\lambda}{NA(\text{obj}) + NA(cond)}$$

In the Abbé equation the **limit of resolution, D** is the minimal distance where two objects next to one another can be resolved or distinguished as individual objects. (Bruslind, 2017)The sharper the image, the closer the objects can be while appearing as separate images. The smaller the limit of resolution, the greater the clarity or image sharpness.

The Abbé equation shows the relationship between wavelength and numerical aperture. A shorter wavelength, the numerator, will result in a smaller D and thus, better resolution. Conversely,

the more light captured, a higher total numerical aperture in the denominator, the smaller D and thus better resolution. Both the objective and the condenser lenses play a role in gathering light. The numerical aperture is a quality of each lens and, like magnification is stamped on the side. See Figure 1.

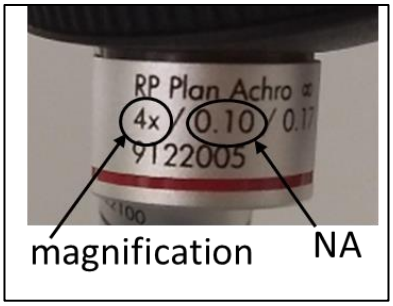

Figure 1 Scanning Objective. Both the magnification and the numerical aperture (NA) are stamped on the side.

At very high magnifications, resolution may be compromised when light passes through the small amount of air between the specimen and the lens. This is due to the large difference between the refractive indices of air and glass; the air scatters the light rays before they can be captured by the objective lens. To solve this problem, a drop of oil can be used to fill the space between the specimen and an **oil immersion lens**, a special lens designed to be used with immersion oils. Since the oil has a refractive index very similar to that of glass, there is less refraction (the bending of light rays). This increases the light collected and, thus, the resolution of the image. See Figure 2. (OpenStax CNX, 2018)

The maximum total magnification for a microscope using visual light for illumination is around 1500X, where the microscope has 15x oculars and a 100x **oil immersion objective**. The highest resolution possible is around 0.2 µm. If objects or cells are closer together than this, they cannot be distinguished as separate entities. (Bruslind, 2017)

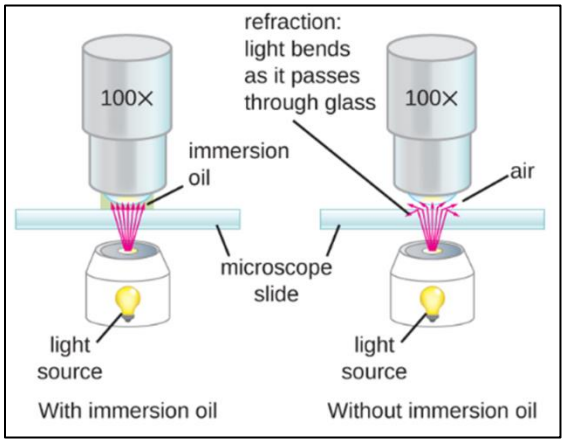

Figure 2 Oil Immersion. Because immersion oil and glass have very similar refractive indices, there is a minimal amount of refraction before the light reaches the lens. Without immersion oil, light scatters as it passes through the air above the slide, degrading the resolution of the image. (OpenStax CNX, 2018)

Many types of microscopes fall under the category of **light microscopes**, which use light to visualize images. Examples of light microscopes include brightfield microscopes, dark-field microscopes, phase-contrast microscopes, differential interference contrast microscopes, fluorescence microscopes, confocal scanning laser microscopes, and two-photon microscopes. These various types of light microscopes can be used to complement each other in diagnostics and research. (OpenStax CNX, 2018) Our discussion will center on brightfield microscopy. More information about other types of microscopy can be found here.

Your Student Microscope

Your student microscope is **brightfield** and **compound,** perhaps the most commonly used type of microscope. Refer to Figures 3-6. Brightfield means that the image produced is dark against a bright (or light) background. Compound refers to the fact that light passes through two lens systems sequentially so that the image is magnified twice. The magnification is "compounded" by a second lens. Our brightfield microscopes are **binocular** allowing one to view the image with both eyes. Each eyepiece contains a lens called an **ocular lens**. The ocular lenses typically magnify images 10 times (10×). At the other end of the body tube are a set of **objective lenses** on a rotating nosepiece. The magnification of these objective lenses typically ranges from 4× to 100×, with the magnification for each lens designated on the metal casing of the lens. The ocular and objective lenses work together to create a magnified image. The **total magnification** is the product of the ocular magnification and the

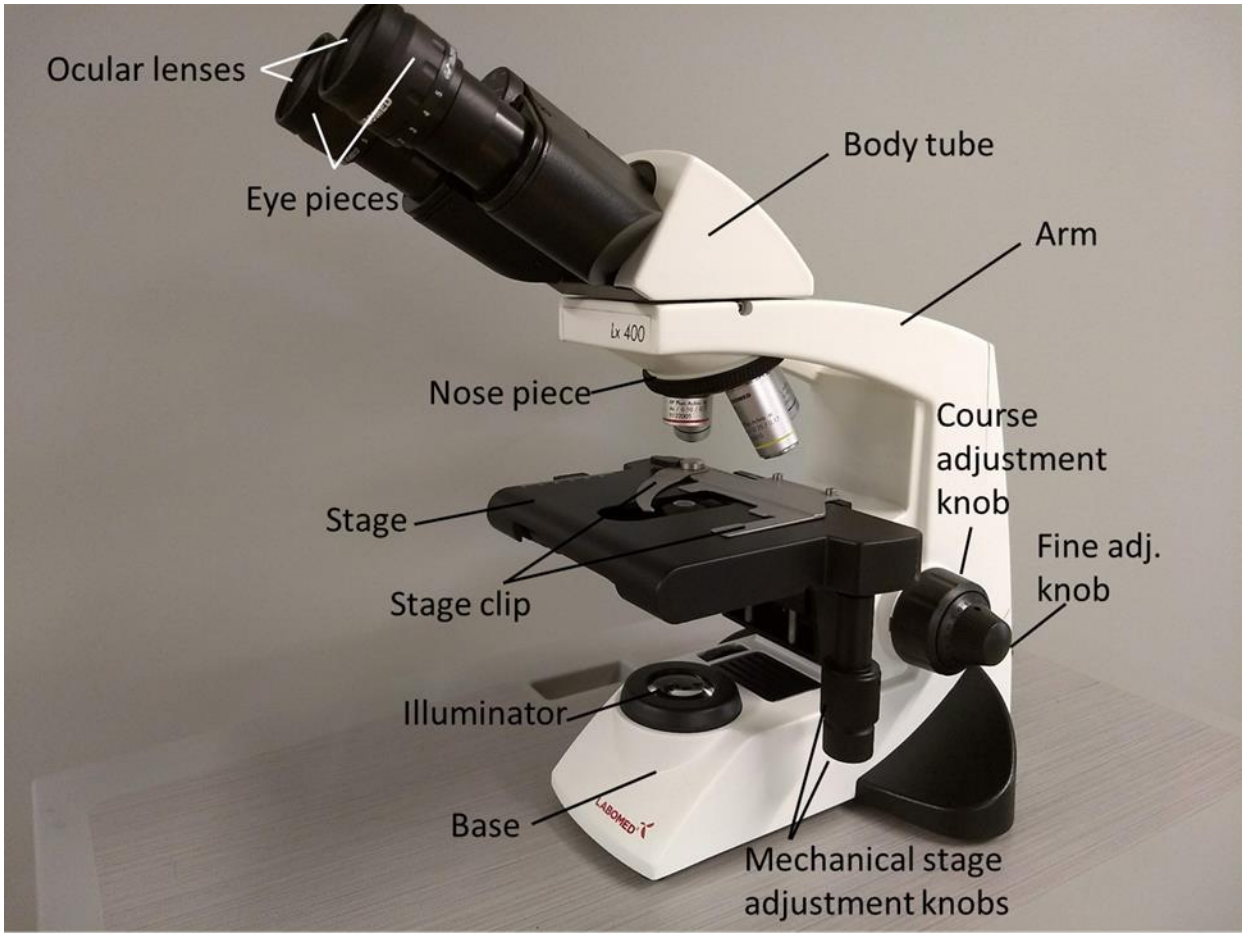

Figure 3 Labomed Compound Microscope used in LSMCRB 121L

objective magnification. For example, if a 40× objective lens is selected and the ocular lens is 10×, the total magnification would be 40 x 10 = 400×. (OpenStax CNX, 2018)

Your microscope has four objectives. The scanning objective magnifies the image to four times the original size (4x) and is designated with a red band. See Figure 2. The lower power objective has a 10x magnification and a yellow band. The high power or high dry objective magnifies 40x and has a blue band. The oil immersion objective magnifies 100x and is banded in grey. The magnifications and numerical apertures for each are stamped on the sides.

The item being viewed is called a **specimen**. The specimen is placed on a glass slide, which is then clipped into place on the **stage** (a platform) of the microscope. Once the slide is secured, the specimen on the slide is positioned over the light using the **x-y mechanical stage knobs (mechanical stage adjustment knobs)**. These knobs move the slide on the surface of the stage, so that different areas of the slide can be viewed. Once the specimen is

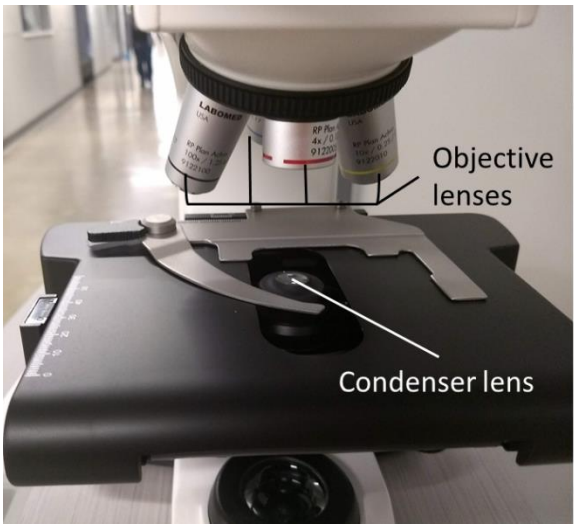

Figure 4. The condenser lens is mounted in the condenser and is seen from above the stage.

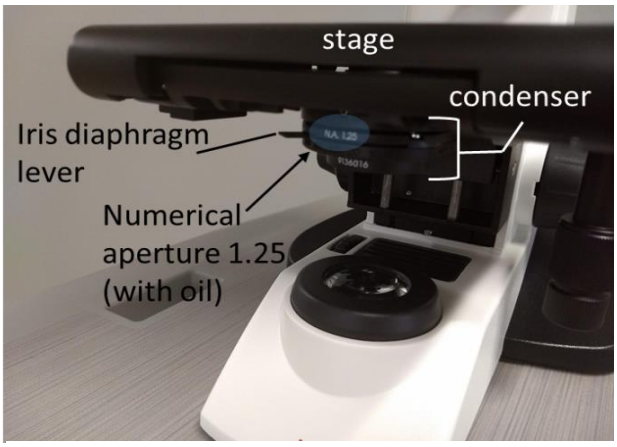

Figure 5 The condenser as seen from below the stage. The iris diaphragm is controlled by the lever. The numerical aperture is stamped on the side.

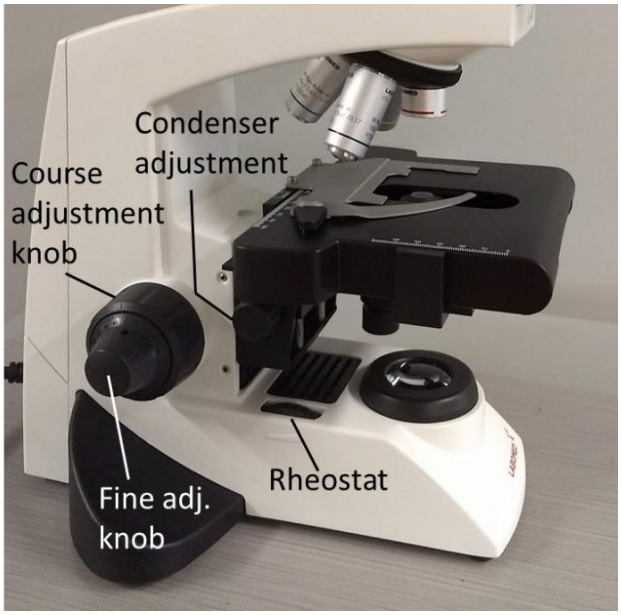

Figure 6 Labomed microscope base. From this perspective one can view the rheostat and the condenser height adjustment.

centered over the light, the stage position can be raised or lowered to focus the image. The **coarse adjustment knob** is used for large-scale movements with 4× and 10× objective lenses; the **fine adjustment knob** is used for small-scale movements, especially with 40× or 100× objective lenses. (OpenStax CNX, 2018)

When images are magnified, they become dimmer because there is less light per unit area of image. Highly magnified images produced by microscopes, therefore, require intense lighting. In a brightfield microscope, this light is provided by an **illuminator**, which is typically a high-intensity bulb below the stage. Light from the illuminator passes up through a **condenser lens** (located within the condenser, below the stage, see Figures 4 and 5), which focuses all of the light rays on the specimen to maximize illumination. The position of the condenser can be optimized using the attached condenser adjustment knob; once the optimal distance is established, the condenser should not be moved. If less-than-maximal light levels are needed, the amount of light striking the specimen can be easily adjusted by opening or closing a **diaphragm** within the condenser. In some cases, brightness can also be adjusted using the **rheostat**, a dimmer switch that controls the intensity of the illuminator. (OpenStax CNX, 2018) See Figure 6.

For our purposes, when the condenser is used with the oil immersion lens, you will use the NA of 1.25. When one of the other objectives is in place, you will use an NA of 0.90 for the condenser.

More Terms

When using the scanning or low power objectives the **working distance** (the distance between the lens and the slide) is large enough so that the slide will never make contact with the lens. This is not the case when using the high-dry and oil immersion lenses, where the working distance is significantly less. This is why the coarse adjustment knob can only be used with the two low-power lenses. (Petersen, 2016)

When using the scanning and low power objectives, you will not be able to make out much detail—the purpose is to find where your specimen is on the slide so that it is easier to locate when you switch to high power. The scanning objective has a large **field of view/visual field** (the circular area seen when looking through the microscope) and a large **depth of field** (the thickness of a specimen that is in sharp focus). This also explains why you start with a small opening in the iris diaphragm. A large field of

view allows a large amount of light to enter the lens system. (As you might experience if you are driving with the sun in your eyes in the summer time, if there is too much light, you may not be able to see anything at all.) As magnification increases when moving to the high dry and oil immersion objectives, both the field of view and depth of field decrease. Because you are viewing a smaller area, there is less cross sectional area for the light and the brightness diminishes. This is why you will need to increase the amount of light coming from the condenser by opening the iris diaphragm as you increase magnification. (Petersen, 2016)

Since your microscope is **parcentric** (par=same; "same center"), when you increase magnification you will be zooming in at the center of the field of view. Objects that are not centered at low power may be out of the field of view at high power. This is why it is important to move the object you want to look at to the center before moving to the next objective. (Petersen, 2016)

The microscope you are using is **parfocal** ("same focal length")—this means that when it is in focus with one objective lens in place the same stage position will be in focus (or close) with all other lenses. Therefore when switching objectives, DO NOT change the position of the stage with the course adjustment—just click the next objective into place. Once focused with the scanning or low power objective, you should only need to touch the fine adjustment when using the high dry and oil immersion objective. (Petersen, 2016)

Step-by-step Guide to Use of the Microscope

1. Carry the microscope from its storage location to your work area with two hands supporting the base. Refrain from sliding the microscope on the surface of the bench. Set it down gently.
2. Always start by cleaning the microscope lenses with **lens paper** and lens cleaning solution
3. Start on **lowest power,** the **scanning objective** with the **iris diaphragm** closed to allow only a pinhole of light**.**
4. Hold the slide up and locate the smear with the naked eye. You may want to circle it with a wax pencil. Place a wax pencil mark on the top of the slide at the edge.
5. Place the slide on the microscope stage and secure it with the stage clips.
6. Use the mechanical stage knobs to move the slide so that the wax pencil mark is in the light path. Focus sharply on the edge of the mark (the thinnest part) first. This is my trick for making sure you are focused on the top of the slide and will make the specimen easier to find on the slide.
7. Once you focus sharply on the mark's edge, then scan the slide in the area of the smear looking for cells. Remember, you are using low magnification, so the cells will be very, very tiny. You will not see individual cells. Instead, it will look like a haze or "dirt".
8. Once you find an area of the slide containing something (we hope cells), then you are ready to rotate the next higher power objective into place. Watch from the side as you move the revolving nose piece so that the new objective clicks into place.
9. Focus here using the course adjustment if you are on low power. You may need to increase the light a little (why?). You will still not be able to make out individual cells.
10. Choose an area where the smear is NOT thick. Place this area in the center of the visual field.
11. Rotate the high power objective (40x) into place. Again, watch from the side (never through the eye piece) while rotating the nosepiece. Use **only the fine adjustment when the high power objective is in place**. You may need to open the iris diaphragm a little more for better light and contrast. You may begin to distinguish bacterial cell shapes at this magnification.
12. Move the slide again so that the area of interest is in the center. If you lose the cells, go back to step 5---do **not** proceed to the oil objective until you have found the cells and focused on them under high dry! Check with the instructor before proceeding.

13. Once cells have been located and brought into focus under high dry, you are ready to use the oil immersion lens**. Without moving the adjustment (focusing) knobs**, rotate the nosepiece toward the oil immersion objective so that the objective is halfway between the oil immersion objective and the high power objective. Place one small drop of immersion oil on the slide in the light path.
14. Then, while watching from the side, rotate the oil immersion objective into place. The lens should make contact with the oil but NOT touch the slide. If the objective begins to touch the slide, do not force it! See Problems and Causes/Solutions/Suggestions #6 below or consult with your instructor.
15. You may need to increase the light again. **Focus using the fine adjustment only**. You should only need to move the adjustment knob one half turn, at most, in either direction. It may help to keep your fingers "glued" the knob so that you can easily come back to the original position. If you see things floating by, you have probably focused above the slide and will need to start over. See Problems and Causes/Solutions/Suggestions #5 below.
16. After you have viewed your specimen and made observations, follow these steps to store the microscope properly.
    a. Before removing the slide, turn the scanning objective into place and lower the stage.
    b. Dispose of the slide properly.
    c. **Always** wipe the objective lenses off with lens cleaning solution and **lens paper** and clean off the stage when you are finished.
    d. Store the scope 1) with the 4x objective clicked into place 2) with clean lenses 3) with the cord wrapped around the base 4) in the correct location.

Problems and Causes/Solutions/Suggestions

1. If you can focus using one objective, but can't find anything on the next higher power, your lenses may need a good cleaning.
2. If, when you move the slide, nothing in the field moves, you are not focused on the slide. You need to go back to step 4 above.
3. If you only see white while on low power or scanning lens, you need to lower the light level.
4. If you find cells under oil immersion, but cannot quite get them into sharp focus, try adjusting the condenser lens slightly.
5. If you have already put oil on the slide and find that you need to go back to a lower power, do **not** attempt to wipe the oil off. Find a different area of the smear to use or focus through the oil.
6. If you cannot rotate the oil immersion objective into place without hitting the slide, you have probably placed the slide upside down on the stage. See steps 2 and 4 above.

DON'Ts of Microscope Use

- DO NOT use a cotton swab or Kim wipes on any lenses.
- NEVER use the course adjustment knob while looking into the microscope unless you are using the scanning or low power objective.
- Never touch the lenses with anything but lens paper. Know the differences between bibulous paper, Kim wipes and lens paper.
- Never allow an objective lens to touch a cover glass or slide.
- Never remove a slide while the oil-immersion objective lens is the light path.

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Dos of Microscope Use

- Carry the microscope carefully with both hands.
- When rotating the nosepiece, always watch from the side of the microscope.
- Clean the microscope after use and store with the scanning objective in place. Get most of the oil with plain lens paper. Finish with a new sheet of lens paper and a drop of lens cleaning solution to make sure all the oil is removed from all objectives.
- When storing the microscope: rotate the scanning objective into place, clean oil off of all lenses using lens paper, clean other parts of the scope with a Kim wipe, wrap cord around the base, (replace the cover, if necessary) and place the scope in the correct location.

Experiment/Exercise

Materials per student pair

None

Cultures

None

Procedure Lab 1

1. Carefully carry your assigned microscope from the cabinet to your work area and set it down gently.
2. Identify all the parts described above and listed in the diagram.
3. Find the magnification and numerical aperture of each lens and fill in the data table.
4. Clean and store your microscope as described above.

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Lab Report: Microscope Theory

Name _____
Lab Section _____

Data and Observations

Lens name	Location and color band	Magnification	Numerical Aperture	Role: Light gathering and/or magnification	Total magnification when in place
Ocular lens					n/a
Scanning objective					
Low power objective					
High power (High dry) objective					
Oil immersion objective					
Condenser lens					n/a

Post Lab Questions

1. Which type of microscope are you working with in this course?

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2. What is magnification? How is it calculated?

3. As the wavelength of light decreases, what happens to the limit of resolution? What happens to the resolving power or clarity?

4. What is the purpose of the blue filter located below the condenser lens of your microscope? (HINT: How does the wavelength of blue light compare to the rest of the visible spectrum? How does this affect the resolving power?)

5. Another factor that affects resolving power is the amount of light that enters the objective lens. Why must you use immersion oil when viewing a specimen with the 100x objective?

6. Should the lens be allowed to touch the oil? Explain.

7. For the following scenarios, assume you are using your microscope. Show how you arrived at your answer.
   a. If the blue filter on your microscope produces a wavelength of 450 nm, what is the limit of resolution on low power?

   b. What is the limit of resolution on high dry?

   c. On high dry, if there are two cells 200 nm apart, would they blur into one or can you distinguish them as separate cells?

   d. What is the limit of resolution on oil immersion?

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e. On oil immersion, if there are two cells 200 nm apart, would they blur into one or can you distinguish them as separate cells?

f. If the cells were 100 nm apart would they blur into one or can you distinguish them as separate cells?

8. Using our light microscopes, what wavelength will allow you to resolve cells or other objects only 100 nm apart? What keeps us from doing this?

9. Which of these correlates to <u>better</u> resolving power:
   Higher wavelength of light
   Higher limit of resolution
   Higher numerical aperture
   Higher power objective using our microscopes
   Higher D

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