

## **Topic 2: Microscopy and Staining**

### **Measurement of Microorganisms**

- Micrometre ( $\mu\text{m}$ ) =  $10^{-3}\text{mm}$  or  $10^{-6}\text{m}$
- Nanometre (nm) =  $10^{-3}\mu\text{m}$  or  $10^{-9}\text{m}$
- Angstrom ( $\text{\AA}$ ) =  $10^{-1}\text{nm}$  or  $10^{-10}\text{m}$  (Angstroms are units for measuring light wavelengths)

Most bacterial cells are between 0.5-1.0  $\mu\text{m}$  in diameter. A light microscope can't resolve objects smaller than  $\sim 0.2\text{ }\mu\text{m}$ .

In order to magnify an object using the human eye, we bring the subject closer to the eye. The closer the object, the larger the image appears on the retina. Maximum enlargement depends on how close the object can be brought to the eye and remain in focus (usually around 25cm).

Microscopes effectively increase retinal surface area occupied by the image, using specific lenses to magnify the image.

*Resolution:* Is the ability to distinguish between two adjacent points as separate. Resolution depends on the physical properties of light, so resolution defines the limit of what can be seen with a light microscope.

- 'Unresolved' – Where the two points just looked like one big blur or blob. Cannot even tell there is two points.
- 'Poorly resolved' – Can see there are two points, but they are not focused or completely separated.
- 'Resolved' – Complete and clear separation of the two points.

### **Compound Microscope**

Zacharias Janssen invented the compound microscope (but Leeuwenhoek's simple scope was better than the compounds available at the time.)

Bright-field, or light microscopes (the most basic type of compound scope) use visible light to view objects. Specimens are made visible by differences in contrast between them and the surrounding medium. Contrast is due to absorption or scattering of light by the specimen. This forms a bright image against a darker background.

In compound microscopes, there is a light source located in the base. There are two focusing knobs also: fine and coarse, and the viewer can move either the stage (which hold the specimen) or the nosepiece to focus the image.

Underneath the stage is a sub-stage condenser which focuses a cone of light onto the slide.

The nosepiece holds 3-5 objective lenses. The microscope itself should be parfocal, which means that the image should remain in focus when the objective lenses are changed. The eyepieces, or oculars for viewing the specimen are attached to the top end of the arm.

Two lenses separate the object from the eye. The objective lens is place next to the object, and the ocular lens is located next to the eye. Focus (resolve image) by moving the objective and ocular lenses relative to the object.

Note: When working out magnification multiply the strength of the ocular lens by the strength of the objective lens.

- Objective magnifications are usually: 4x, 10x, 20x, 40x, or 100x ← use 100x for oil.
- Oculars are usually 10x, sometimes 15x

1500 fold magnification is about the upper limit, due to resolution and also the lack of brightness of the object becomes limiting as the object is enlarged- the condenser's job is to concentrate light on the subject.

Resolving power is a function of light wavelength and objective lens is numerical aperture. NA is made of refractive index of the medium (air or oil) and the angle of the light entering the objective lens.

### How does a lens bend light?

When a light ray passes through one substance such as air, into another substance such as glass, the ray slows as it enters the glass and bends towards the normal. This bending is called refraction. As the ray leaves the glass and enters air, it accelerates and bends away from the normal.

Refractive index: Measure of ability of medium (air, water, glass) to bend light rays.

Lenses act as a series of prisms, bending or focusing light on a point called the focal point. Focal length: distance between centre of lens and focal point. Microscope lens effectively decreases the focal length. The shorter the effective focal length, the larger the object appears. Microscope magnifies image by decreasing focal length.

Resolution: The minimum distance (d) between two objects that can be distinguished as separate can be determined from formula:

$$D = \frac{0.5 \times \text{wavelength}}{\text{Refractive index} \times \sin \theta}$$

Theta for air is 60 and for oil is 90. The light microscope cannot be used with wavelengths <500 nm. Human eye cannot see wavelengths shorter than violet.

At 500nm (0.5um), resolution is increased by increasing numerical aperture. You want the answer in um, because there are less zeros. The numerical aperture for air = 0.87. So, at 500nm:

$$D = (0.5 \times 0.5) / 0.87 = 0.29 \text{ um}$$

In order to increase resolution, you must increase numerical aperture. Immersing the object in immersion oil effectively changes numerical aperture to 1.5. So maximum resolution occurs if the shortest possible wavelength is used (500nm = 0.5 um), maximum numerical aperture = 1.5.

$$D = 0.5 (0.5) / 1.5 = 0.17 \text{ um}$$

Anything smaller than 0.17 um will not be resolved.

An oil lens increases numerical aperture because it allows the lens to be moved closer to the sample, thereby decreasing focal length and increasing magnification. A greater refractive index results in a greater angle over which light is spread before entering the lens.

Maximum resolution occurs if you use the shortest possible wavelength of light (0.5um), and the maximum aperture value = 1 (sin 90= 1), refractive index of 1.5 (oil).

$$D = (0.5 \times 0.5) / 1.5 = 0.17\mu\text{m}$$

That is, we can only just see a spec of approximately 0.2 um in diameter. A useful magnification limit is about 1000x but magnification can be increased to about 10 000x

### Fixing and Staining

Small size and poor contrast with surrounding medium makes microorganisms hard to see under the light microscope. Fix and stain to:

1. Increase visibility
2. Accentuate specific morphological features
3. Preserve specimens for future study

### Fixation

Internal and external structures preserved and fixed in positions.

1. *Heat fixation*: Gently flame-heat an air dried film of bacteria (doesn't preserve internal structure).
2. *Chemical fixation*: Required to protect internal structure (chemicals penetrate cells and react with cellular components to make them insoluble and immobilise them, eg. Ethanol, acetic acid, formaldehyde, glutaraldehyde).

### Staining

Dye used to stain cells have two common features:

- They have a chromophore group (gives dye its colour)
- They bind by ionic, covalent or hydrophobic binding

Dye stains cells, increases contrast with background. (Gram negatives= red and gram positive= purple).

### Positive Staining

(Basic and acidic dyes, and fat soluble stains)

1. *Cationic (basic dye)*: Have positively-charged groups (eg.  $\text{NH}_4$ ) that bind to negatively charged materials (eg. Nucleic acids, acid polysaccharides). Bacterial surfaces are usually negatively charged, so these are good simple dyes for staining them. Dye examples include: Methylene blue, crystal violet, basic fuchsin, safranin, malachite green.
2. *Anionic (acidic dye)*: Have negatively-charged groups (eg.  $\text{COOH}$ ,  $\text{OH}$ ) that bind to positively-charge materials (eg. Some proteins). Some examples are congo red, acidic fuchsin, eosin.

pH can alter the staining effectiveness of both types of dyes, because nature and degree of charge on cell components changes with pH.

Anionic (acidic) dyes work best at low pH (acid) where many molecules carry a positive charge.

Cationic (basic) dyes work best at high pH (base) where many molecules carry a negative charge.

3. *Fat soluble (lipophilic)*: Stains dissolve in and combine with lipid inclusions, will not dissolve in aqueous portions of cell (eg. Sudan black). These stains are not ionic.

### Negative Staining

Cells left unstained, background is dark. Use opaque material, spread in thin layer, must not dry out.

Used for showing presence of capsules around cells eg. India ink. So everything is stained except what we are trying to visualise. Good for looking at capsules.

### Differential Staining

Not all bacteria or bacterial component are stained equally – this is the basis for differential staining. There are several types of differential stain:

1. *Endospore stain*: Bacillus and clostridium species form endospores. Morphology and intracellular location vary with species and endospores are not stained well by most dyes. Once stained, spores strongly resist decolourisation, basis for most spore staining methods. Stains the spore, not cell body (cell washed free of dye).
2. *Acid-fast staining*: Some species, eg. Mycobacterium, do not readily bind simple stains. Due to high lipid content of a wall, we must use harsher treatment. Instead they are heated with basic fuchsin and phenol (Ziehl-Neelson method). They retain the red colour of the dye when rinsed in 3% solution of HCl in ethanol. Non-acid fast bacteria are decolourised and are stained blue by methylene blue counterstain.
3. *The GRAM stain*: Now the most widely used of all the stains because it chemically distinguishes two kinds of cell wall and divides bacteria into two classes: Gram-positive (stains purple) and gram-negative (stains red). Remember: 'Purple and positive start with P.'

Gram positive cell walls have a lots peptidoglycan layers, where gram negative cells have a fewer layers.

### Steps in differential staining

Heat fix cells to slide → stain with crystal violet for 30 seconds, and water rinse for 2 seconds → Gram's iodine for 1 minute, water rinse → wash with 95% ethanol or acetone for 10-30 seconds, water rinse → safranin for 30-60 seconds, water rinse and blot.

### Mechanisms behind gram staining reaction

1. *Crystal violet (CV)*- All cells stain violet
2. *Iodine solution (I)*- Water insoluble CV-I complex forms within cells. Cells remain violet. Iodine functions as mordant: increases the interaction between cell and dye; mordant=cement.
3. *Alcohol*- Gram positive bacteria: cell wall is dehydrated, pores shrink, CV-I complex can't pass out of cells, cells remain violet. Gram negative bacteria: Lipid leached from cell wall increasing porosity, CV-I escapes from the cell, cell becomes colourless.
4. *Safranin*- Gram positive bacteria not affected and remain violet but gram negative bacteria take up stain and become pink and red.