Stains & Staining Techniques: Bacterial Staining
Source: General microbiology by Stanier, Bacteriology by Salle, Prescott et al Microbiology
Three Lineages of Life: Domain Bacteria

All living organisms are classified into 1 of 3 domains:

- **Domain Bacteria**
  - No true nucleus
  - No membrane-bound organelles
  - Cell Wall composed of peptidoglycan
  - Reproduce asexually by budding and fission
  - Very small (1 - 10 µm)

- **Domain Archaea**

- **Domain Eukarya (Eukaryotes)**
Identification of Bacteria

Bacteria:
- Small in size
- Lack discernable internal features

Methods of identifying bacteria:
- Macroscopic examination:
  - Colony color, shape, and odor
- Microscopic examination:
  - Cell shape
  - Cell surface
Microscopic Examination: Cell Shape

Spherical (cocci)  Rod-shaped (bacilli)  Spiral
Microscopic Examination: Cell Shape: Coccus

- Single Coccus
- Diplococcus (paired)
- Staphylococcus
- Streptococcus (chain)
Microscopic Examination: Cell Shape: Bacillus

- Bacillus
- Single Bacillus
- Single Bacillus (fusiform)
- Streptobacillus (chain)
Microscopic Examination: Cell Shape: Spirillum

- Spirillum
- Single Spirillum
- Multiple Spirillum
Purpose of Staining

- **Bacteria cells are almost colorless and transparent**
  - To render microscopic and semitransparent objects visible.
  - To reveal their shape and size.
  - To show the presence of various internal and external structures.
  - To produce specific physical or chemical reactions.
Dye and Stain

A coloring agent that is to be used for general purposes » **Dye**

**One that is used as biological** » **Stain**

- Biological coloring agents are manufactured with greater care under more rigid specifications so that they will be satisfactory for the procedure in which they are employed.

- Textile coloring agents which are not so exacting in their characteristics are called dyes.

✓ Stains also may be used for textile dyes, although less purified preparations are satisfactory for such purposes.
Chemical Makeup of Stains

• Benzene = organic compound
• Chromophore (Gk. chroma = colour; phoros = to bear) = colour
• Auxochrome (Gk. auxein = to increase, chroma = colour) = ionization properties
• Benzene + Chromophore = Chromogen
  – Chromogen is a colored compound only
• Auxochrome with Chromogen allows the dye to form salt compounds that adhere to cells.
Stain may be defined as an organic compound containing a benzene ring plus a chromophore and auxochrome group (Figure III.1).

Benzene

Organic colorless solvent

Chromophore: Chemical group that imparts color to benzene

Chromogen:
Colored compound, not a stain

Auxochrome: Chemical group that conveys the property of ionization to the chromogen, enabling it to form salts and bind to fibers or tissues
**Bacterial staining**

**STAIN** – an organic compound composed of a benzene ring, a Chromophore and auxochrome group.

Benzene is a organic colorless solvent. Chromophore is the molecule that gives color to benzene.

(A CHROMOGEN – isn’t a stain, just a colored molecule. It is made up of the benzene and the chromophore).

Auxochrome ionizes the chromogen, gives it a charge. This helps the chromogen form salts and bind to substances like tissues or fibers.

All tissue cells as well as stains exhibit some type of charge.

**ACIDIC STAINS** – are Anionic. Their chromogen exhibits a Negative charge. These type stains have an affinity for the Positive components of a cell. Example – Picric Acid.

**BASIC STAINS** – are Cationic. Their chromogen exhibits a Positive charge. These type stains have a strong affinity for The negative components of a cell. Example – Methylene Blue.
The stain picric acid may be used to illustrate this definition:

\[
\begin{align*}
\text{Benzene} & \quad \text{Nitro groups} & \quad \text{Trinitrobenzene} & \quad \text{Auxochrome} \\
\text{colorless} & \quad \text{chromophore} & \quad \text{chromogen, yellow} & \quad \text{(picric acid) yellow stain}
\end{align*}
\]

The ability of a stain to bind to macromolecular cellular components such as proteins or nucleic acids depends on the electrical charge found on the chromogen portion, as well as on the cellular component to be stained.

**Acidic stains** are anionic, which means that, on ionization of the stain, the chromogen portion exhibits a negative charge and therefore has a strong affinity for the positive constituents of the cell. Proteins, positively charged cellular components, will readily bind to and accept the color of the negatively charged, anionic chromogen of an acidic stain. Structurally, picric acid is an example of an acidic stain that produces an anionic chromogen as illustrated:

\[
\begin{align*}
\text{Picric acid} & \quad \text{Ionization} & \quad \text{Anionic chromogen}
\end{align*}
\]

**Basic stains** are cationic, because on ionization the chromogen portion exhibits a positive charge and therefore has a strong affinity for the negative constituents of the cell. Nucleic acids, negatively charged cellular components, will readily bind to and accept the color of the positively charged, cationic chromogen of a basic stain. Structurally, methylene blue is a basic stain that produces a cationic chromogen as illustrated:

\[
\begin{align*}
\text{Methylene blue} & \quad \text{Ionization} & \quad \text{Cationic chromogen}
\end{align*}
\]
Acidic and basic stains

Bacterial cells are slightly negatively charged (rich in nucleic acids bearing negative charges as phosphate groups) → combine with positively charged Basic stains

Acidic stains do not stain the bacterial cell → can stain the background material with a contrasting color.
Basic Dyes

- Work best in basic pH
- Ionizes (Cl-, SO4-)
- Creates (+) Cationic chromogen
- Attracted to (-) acidic cell components [DNA, proteins]
- Examples
  - Methylene Blue
  - Crystal Violet
  - Carbol Fuchsin
  - Safranin
  - Malachite Green
Acidic Dyes

- Works best in acidic pH
- Ionizes (Na+, K+, Ca++)
- Creates Anionic (-) chromogen
- Attracted to (+) cell components [AA]
- Examples
  - Picric Acid
  - Nigrosin
  - India Ink
  - Eosin
Theories of staining

• Physical theories

1. Simple solubility e.g. Fat stains are effective because the stain is more soluble in fat than in 70% alcohol.
2. Absorption: This is a property by which a large body attracts to itself minute particles from a surrounding medium.

• Chemical theories

1. It is generally true that acid dyes stain basic elements (Cytoplasm) and basic dyes stain acidophilic material (nucleus) however this far from being complete truth, Indeed hematoxylin, which is an acid dye, does not stain the cytoplasm, but (in the presence of mordant) is one of the most widely used nuclear stains.
Mordant and Its Function

- Mordant is a substance that forms an insoluble compound with a stain and helps to fix the colour to the cell components. Some stains never stain the cells or its components unless treated with a mordant. The mordant becomes attached to the cell or its components and then combines with the stain to form an insoluble colour complex. This complex is called a lake.
- Commonly used mordants are the oxides of aluminium, iron, and chromium. Alizarin is an example of a stain that imparts colour only in collaboration with a mordant. It gives different colours when used with different mordents. It gives red colour with aluminium and tin salts, brownish red colour with a chromium mordant, and black-violets with an iron mordant.
Classification (Types) of Stains

Classification Based on Origin

(i) Natural stains
These stains are obtained from natural resources directly as natural products. Haematoxylin and carmine are good examples. Haematoxylin is obtained from the heart wood of a tree (*Haematoxylon campechianum*), whereas carmine is obtained from a cochineal female insect. The natural dyes are used mainly for histological purposes.

(ii) Synthetic stains
Synthetic stains are artificially produced mainly from fractionation and recombination of coal-tar products hence popularly are called coal-tar dyes. The latter are used mainly for the bacterial stain preparations. Important synthetic stains are safranin, fast green, aniline blue, methylene blue, crystal violet, eosine, acid fuchsin, orange-G, etc.
Classification Based on Purpose of Use

(i) Direct or general stains
Aniline dyes are able to stain bacteria directly. Exceptions are bacterial spores, e.g., of *Bacillus* spp. and the bacteria that have waxy coaling on their cell wall, e.g., *Mycobacterium* spp.

(ii) Indirect stains
These are the dyes which stain only the background, e.g., nigrosin or India ink used either for observing mucilaginous covering enveloping bacteria (capsules) or certain spores of fungi or cells of unicellular animals.

(iii) Selective stains
These stains are used for special purposes, to stain particular parts of the organism such as spores, metachromatin granules, flagella, nuclei, etc.

(iv) Differential stains
These stains are those which enable one to differentiate two different groups of bacteria in a mixture, for instance, gram-positive and gram-negative.
(i) Nuclear stains
Nuclear stains are acidic in nature and stain the chromatin materials only. Examples are carmine, haematoxylin, etc.

(ii) Cytoplasmic stains
These stains are basic in nature and stain the cytoplasm and its inclusions. Fast green, aniline blue, erythrosine, eosin, orange-G, etc. are the examples.

(iii) Histological stains
Histological stains are those that specifically stain some particular tissues in the sections. Safranin stains the lignified and suberized cell walls.
Classification based on Charge

(i) Acidic stains (dyes)
Acidic stains (dyes) are acidic in nature because they possess negative (anionic) charge on their surface on ionization. Acid fuchsin, eosin, and picric acid are examples.

(ii) Basic stains (dyes)
Basic stains (dyes) are basic in nature because they possess positive (cationic) charge on their surface on ionization. Fast green, aniline blue, methylene blue, crystal violet, safranin, etc. are examples.

(iii) Neutral stains (dyes)
Neutral stains (dyes) are formed by the combination of acidic and basic stains in aqueous form. The colouring matter in neutral stains is present in both the anionic and the cationic groups. Therefore, these dyes are neither acidic nor basic. Neutral red is an example.
Staining Methods

- Negative Staining
- Simple Staining
- Differential Staining
  - Group
    - Gram Staining
    - Acid Fast Staining
  - Special Structures
    - Capsule Staining
    - Endospore Staining
    - Flagellar Staining
Slide Preparation

- Clean slide
- LABEL !!!
- Smear in circle
  - Broth
  - Solid + H₂O
- Air dry first
- Heat fix (usually)
  - Kill organism
  - Adhere to slide
  - Accepts dye
- Problems
  - Too thick
  - Wash off specimen
Negative Staining

- Acid Dye
- (-) chromogen
- Repelled by (-) cell wall
- Cells
  - Colorless
  - Seen against dark background

No heat fixation or strong chemicals are used → the bacteria are less distorted than in other staining procedure
Simple Staining

- One reagent used
- Soak smear 30-60 seconds
- Rinse with H2O
- Background stained
- Bacteria stained
- Basic dye
  - (+) chromogen
  - (-) cell wall
  - Shows morphology
    - Size
    - Shape
    - Arrangement
- Examples
  - MB
  - CF
  - CV
Differential Staining

• Two or more reagents
• Distinguish
  – Bacterial groups
  – Specific Structures
• Example
  – Gram staining
  – Acid Fast Staining
Gram Staining General Theory

Gram-positive

Gram-negative

- cell membrane
- cytoplasm
- periplasmic space
- peptidoglycan
- outer membrane
Time Frame
1) 1 minute
2) 1 minute
3) 15 seconds
4) 1 minute

Rinse with water between each step
Proteus

Staphylococcus aureus

Bacillus cereus
1. Dirty slides
2. Uneven smears
3. Overheating smears
4. Faulty reagents
5. Excessive decolorizing
6. Excessive rinsing
7. Excessive counterstaining
8. Old cultures
9. Broth cultures
10. Non-conforming bacteria
Acid-fast Staining
(Ziehl-Neelsen stain)
Acid fast Staining

- It is a special bacteriological staining used to identify acid-fast organisms, mainly *Mycobacteria*. *Mycobacterium tuberculosis* is the most important of this group because it is responsible for tuberculosis (TB) and other important *Mycobacterium* species.

- Acid fast organisms like *Mycobacterium* contain large amounts of waxy lipid substances within their cell walls called *mycolic acids*. These acids resist staining by ordinary methods such as a Gram stain. It can also be used to stain a few other bacteria, such as *Nocardia*.

- The reagents used are Ziehl–Neelsen carbol fuchsin, acid alcohol, and methylene blue. Acid-fast bacilli will be bright red after staining.
Principle of acid fast staining

- Cell wall of *M. tuberculosis* is impermeable to stains and dyes. But *M. tuberculosis* can be stained by acid-fast stain with long time heating, this mean that carbol fuchsin which is a phenolic stain is soluble in the lipids of mycobacterial cell wall and the heating process increase the penetration of the carbol fuchsin.

- Intense decolorization does not release primary stain from the cell wall of AFB, since the carbol fuchsin is more soluble in mycolic acid than in the decolorizer.

- Bacteria except *M. tuberculosis* can be decolorized by 3% acid alcohol.

- So the color of *M. tuberculosis* is red and that of other Mycolic acid negative bacteria is blue after Counter staining with methylene blue.
Preparation of AFB Smears

1. Make a smear of the sputum, dry and fix it.

2. Ziehl-Neelsen acid-fast staining:
   (1) Flood the slide with carbol fuchsin. Heat the slide to steaming for 5 mins. Do not boil or allow the smear to dry. As stain evaporated from the slide, replenish with additional carbol fuchsin. Allow the slide to cool and rinse it thoroughly with water.

   (2) Decolorize the slide with acid alcohol until the red color no longer comes off in the decolorizer. It takes about 30 secs. Rinse the slide with water.

   (3) Counterstain with methylene blue, allow the stain to react for 1 min, rinse as above.

   (4) Bolt the slide carefully. Examine under microscope.
Acid fast bacteria
Endospore

- The name "endospore" is suggestive of a spore or seed-like form (endo means within).
- Endospore formation is usually triggered by a lack of nutrients, and usually occurs in Gram-positive bacteria.
- Endospores enable bacteria to lie dormant for extended periods, even centuries. Revival of spores millions of years old has been claimed. When the environment becomes more favorable, the endospore can reactivate itself to the vegetative state.
- Most types of bacteria cannot change to the endospore form. Examples of bacteria that can form endospores include Bacillus and Clostridium.
- The endospore consists of the bacterium's DNA and part of its cytoplasm, surrounded by a very tough outer coating.
- Endospores can survive without nutrients. They are resistant to ultraviolet radiation, desiccation, high temperature, extreme freezing and chemical disinfectants.
- According to scientist Dr. Steinn Sigurdsson, "There are viable bacterial spores that have been found that are 40 million years old on Earth - and we know they're very hardened to radiation." Common anti-bacterial agents that work by destroying vegetative cell walls do not affect endospores.
Endospore Location

• The position of the endospore differs among bacterial species and is useful in identification.

• The main types within the cell are terminal, subterminal, and centrally placed endospores.

• Terminal endospores are seen at the poles of cells, whereas central endospores are more or less in the middle.

• Subterminal endospores are those between these two extremes, usually seen far enough towards the poles but close enough to the center so as not to be considered either terminal or central.

• Lateral endospores are seen occasionally.
Endospore staining
(Schaeffer-fulton Method)

- Prepare a smear of the bacteria *Bacillus megatarium* (a spore-producing organism)
- Flood the smear with malachite green
- Do not allow the stain to evaporate or completely evaporate
- Remove from heat and allow slides to cool
- Once the slides are cool (important) → rinse with water
- Flood the sample with safranin (30-60 seconds)
- Rinse the slide (with water) → blot dry → observe under microscope [10x, 40x, 100x (oil immersion)].
Endospore staining
Capsule

- Capsules are structures that lay outside of an organism's cell wall and thus are in direct contact with the environment. Many, perhaps most, bacteria produce capsules under the right conditions.
  - Protect the cell from desiccation (drying)
  - Protect the cell from phagocytes (being engulfed by white blood cells)
  - Provide a food reserve when certain organic compounds are in excess
  - A virulence determinant of pathogenic microbes
  - They serve as binding or adhesion agents for sticking cells together and/or to a surface such as a rock in flowing stream

- Heat fixation cause capsule shrinkage
- Because most capsular materials are water soluble, simple stains will not adhere to them
Capsule Staining

• Older cultures are more likely to exhibit capsule production. When performing a capsule stain on your unknown, be sure the culture you take your sample from is at least five days old.

• Acidic dye as India Ink and Nigrosen are used to stain the background of the slide but basic dye as methylene blue and crystal violet are used to stain the cell.
Capsule Staining
Flagella Staining

1. Prepare a smear on a slide
2. Flood the slide with Leifson’s stain
3. After appearance of shiny film give a stream of water wash
4. Treat the slide with 1% Methylene blue for 1 minute
5. Wash the slide with water, air dry and observe under oil immersion
Mechanism of Flagella Staining

- The Leifson’s stain is made up of tannic acid, basic fuschin stain prepared in alcohol base.
- When we treat cell with Leifson’s stain, the tannic acid get attach to the flagella and alcohol get evaporated. After evaporation of alcohol the thickness of flagella is increased due to deposition of tannic acid. Where as Basic fuschin stain the Flagella.
- After Leifson’s stain treatment cells are treated with Methylene blue stain which stains the cell.