# EXPERIMENTAL ANIMALS

# INOCULATION METHODS

# STAINS, STAINING METHODS

# What is an experimental animal?

- Animals, which are used in experiments and researches with hypothesis based on scientific rules to obtain the results of drug, active substance, treatment etc. in living organisms, are called “experimental animals”.

**BUT!**

**Each study can not be done in any animal species or genus.**

So before starting to our research, it is necessary to determine the spesific animal strain or line which is the most suitable for the study, and then the project should be planned and presented, after it can be seen that you can obtain the animal line.

# EXPERIMENTAL ANIMALS

- The effects of microorganisms on experimental animals are different. Some microorganisms are not effective on any experimental animal whereas some of them cause pathological changes that are not specific for this animal.

- **To prove the pathogenity of a microorganism to an experimental animal**, the microorganism should be always showed the same clinical and pathological changes in animal when it is given in significant and suitable ways.

**Therefore the experimental animal and the inoculation site have great importance.**

Because in microbology laboratory, we are studying with microorganisms and their infections.

When we use experimental animals, we try to evaluate an infection that is spesific for microorganism that we tested.
Then we try to find some new antimicrobial agent against them.

So every microorganism has the spesific infection site, settlement areas like; nerves for meningitidis or rabies, stomach for *H. pylori*, lungs for tuberculosis etc.

So every microorganism has the spesific infection site, so the area where we inject microorganisms into the experimental animal is very important.

# WHEN WE USE EXPERIMENTAL ANIMALS IN MICROBIOLOGY LABORATORY?

- To produce microorganisms that can survive only in living cells.
- To produce hard, slow or weakly growing microorganisms
- To obtain pure culture from hard culture
- To determine pathogenicity and toxicity
- To give immunity – by using vaccines
- To obtain antiserum or immunserum (for diagnostic and therapeutic purposes)
- To increase the virulance of microorganisms

**Virulance: Microorganism's ability to make disease, to infect a host.**


**But we can not use experimental animals in every study and every time we want.**

**Animal Rights Law:**

The aim of the law is ”**to provide the comfort of animals and the best and suitable treatment of animals, to prevent any kind of victimization of animals and ensure the best protection against pain, suffering and torture**.”

Article 9 of the Law on animal experiments. According to this article, "**the institutions and organizations that conduct animal experiments should have consent through ethics committees already established and will be established in their own organizations**".

- When we want to study with experimental animals, firstly we consult to animal experiment ethics commitee. If the ethics commitee confirms our experiment then we can start to our study.

- Especially universities have their own animal experiment ethics committee, so before animal experiment studies, we must consult to this committee.

# ANKARA UNIVERSITY
# Animal experiment ethics committee

**ANKARA ÜNİVERSİTESİ HAYVAN DENEYLERİ YEREL ETİK KURULU YÖNERGESİ**

**AMAÇ**

**MADDE 1 –** Bu Yönerge, Ankara Üniversitesinde deney hayvanları ile gerçekleştirilecek olan bilimsel araştırma, deneysel çalışma, sağlık hizmetleri uygulamaları, eğitim-öğretim ve yayın gibi temel etkinliklerde kullanılan yöntem ve materyaller ile ilgili minimum etik standartları saptamak, gerektiğinde yeni ilkeler oluşturmak, bunların uygulamalarına ilişkin düzenlemeleri yapmak, etik ilkeler doğrultusunda görüş bildirmek ve araştırma önerilerini bu açıdan değerlendirmek üzere oluşturulan "Ankara Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu"nun (AÜHADYEK) kuruluş ve çalışma esaslarını belirlemek amacıyla düzenlenmiştir.

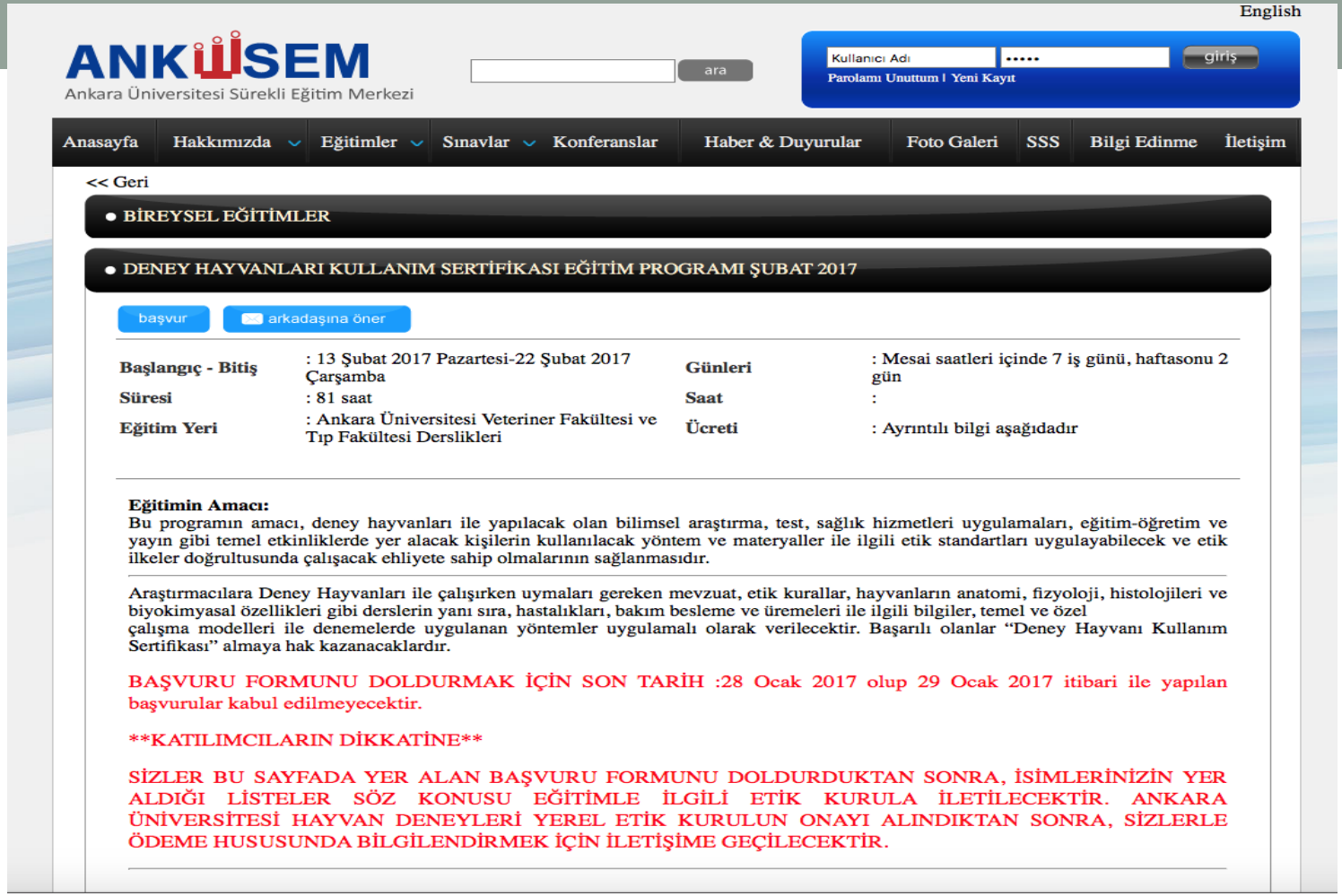
**KAPSAM**

**MADDE 2 –** Bu Yönerge, Ankara Üniversitesinde Madde 1'de yer alan etkinliklerde kullanılacak hayvanların kullanımından önce alınması gereken izinleri, bu amaçla AÜHADYEK'in oluşturulmasını, çalışma usul ve esaslarını, görevlerini, eğitim, denetim ve yükümlülüklerini kapsar.

HADYEK is our university's animal experiment ethics committe.

And we must have an animal use certificate to study with animals in our experiments. After 81 hours of training and after the exam, you can get "the animal use certificate" from the ethics committee.

- http://hadyek.ankara.edu.tr/?page_id=90




Experimental studies on live animals should be carried out by people with a **Certificate of Experimental Animal Use**, if required by a veterinary consultant.

# THE THREE Rs (RULES) IN SCIENTIFIC STUDIES ON ANIMALS

These rules take their name from the first letter of three words.

1-**Replacement:** The use of animals with alternative techniques, or avoid the use of animals altogether when possible. (Cell culture, tissue culture, in vitro methods etc.)

2-**Reduction**: To keep the number of animals used to a minimum, to obtain information from fewer animals or more information from the same number of animals.

3-**Refinement:** While performing the experience, to make sure animals suffer as little as possible. This includes better housing and improvements to procedures which minimize pain and suffering and/or improve animal welfare.

The welfare of animals used in our studies/researches is very important. There are good ethical, scientific, legal and economic reasons for making sure that animals are looked after properly and used in minimum numbers.

- And experimental animal units should have special conditions like;

– Stable heat

– Stable moisture

– Regular photoperiod (day/night)

– Ventilation systems

– Cage volume, height, ideal number (for mice 180 $cm^2$ ve 12 cm)

– Filters for operation room

# Most used experimental animals in microbiology laboratory are:

- Mice
- Guinea pig
- Rabbit

Less common:

- Rat
- Hamster

# Before starting with experimental animals..

We need to hold the animal in proper way during the first phase of the study.

We should be grip the loose skin in the rear of the neck properly with one hand's thump and forefinger, in order to prevent the animal turning its head and biting into the handler's fingers. The other hand should be used to support the lower parts of the body.

!!!Holding method is the same for all animals that used in our lab, only animal size can change

# Holding of experimental animals

- Holding of mice:

The tail of the mouse is gripped at its base and the mouse is laid onto the grid cage top.

By gently pulling the tail backward, the animal tends to move forward and to hold on to the grid with its forelegs.

At this moment, the other hand approaches the rear of the neck and a skin fold, quite close to the ears, is grasped with the thumb and the forefinger, while the loose skin extending over the back is gripped with the other fingers.

It is important to grip the loose skin in the rear of the neck properly, in order to prevent the animal from turning its head and biting into the handler’s fingers.

- Holding of Guinea pigs:

Place a hand on the back of the animal near the head area; The head and the toe are surrounded by a finger grip. During this time, the other fingers catch the body and the palm rests on the back of the animal.

When the guinea pig is held and lifted, the other hand should be used to support the lower parts of the body.

The back legs are held and extended with the free hand for injection.

- Holding of rats:

  The hand must be on the back of the animal and behind the head.

  The rough skin behind the animal's head is firmly gripped by the index finger and thumb.

  The index finger must be in the lower jaw line to prevent the animal from moving or biting.

  The thorax should be depressed very little.

- Holding of rabbits:

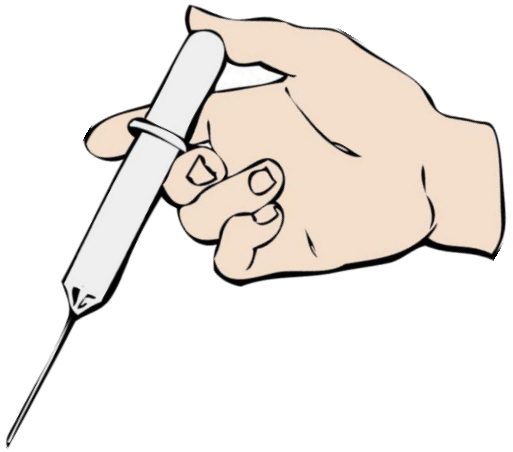
  While left hand holding the skin of nape of rabbit, the other hand should hold the back legs. The hind legs are moved toward the front legs to prevent the animal from moving.

- Holding of hamsters:

  It is immobilized with one hand covering the body, allowing the head to come out from under the hand. Then loose skin covering the neck and torso should be pulled with the thumb and index fingers.

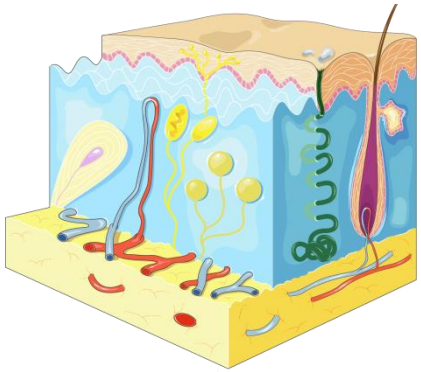
  The animal is removed with one hand similar to the rat. If the thumb and index finger are placed at the bottom of the jaw, good control is achieved with one hand.

How we inoculate microorganisms to experimental animals?



# INOCULATION METHODS

# Cutaneous (Epiderm) inoculation



The **abdomen** and **chest** skin are usually used as the inoculation site. Skin in the area to be inoculated is firstly shaved and cleaned with antiseptic.

Then the skin surface is drawn or treated with sandpaper in a non-bleeding form for sensitization.

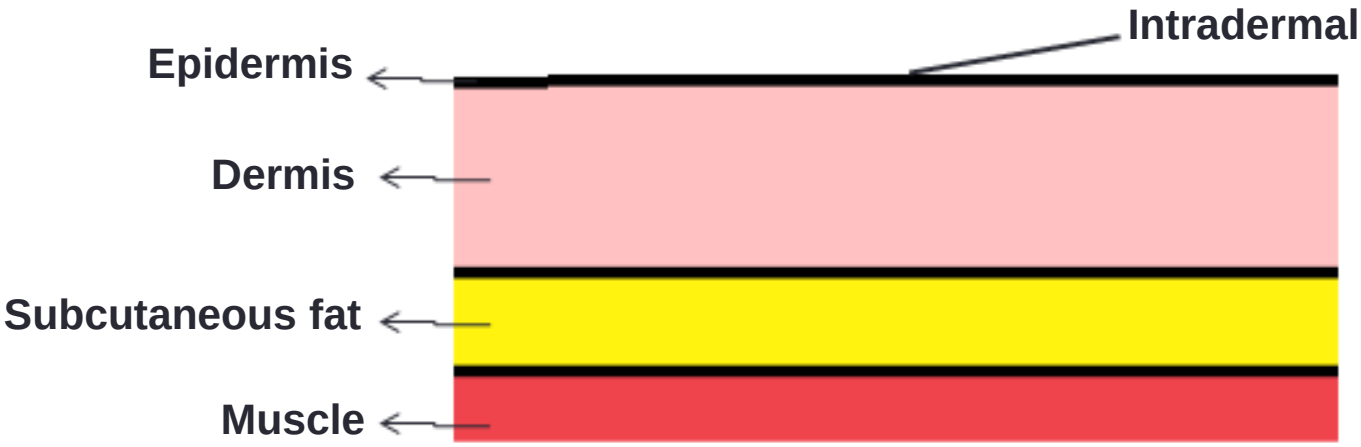
Solid material is applied to the surface of the skin and the liquid material is inoculated by dripping to the sensitized region. For the animal not to be infected, the inoculated place is wrapped with sterile cheesecloth.

Isolation and identification of microorganisms such as *B.anthracis*, *Yersinia pestis*, and *Leptospira* in sewage waters.

# Intradermal (into the skin) inoculation

Inoculation is usually done to the back skin. After skin is shaved and cleaned with antiseptic, 0.1 $cm^3$ of microorganism solution is injected into the skin with a fine tipped needle.

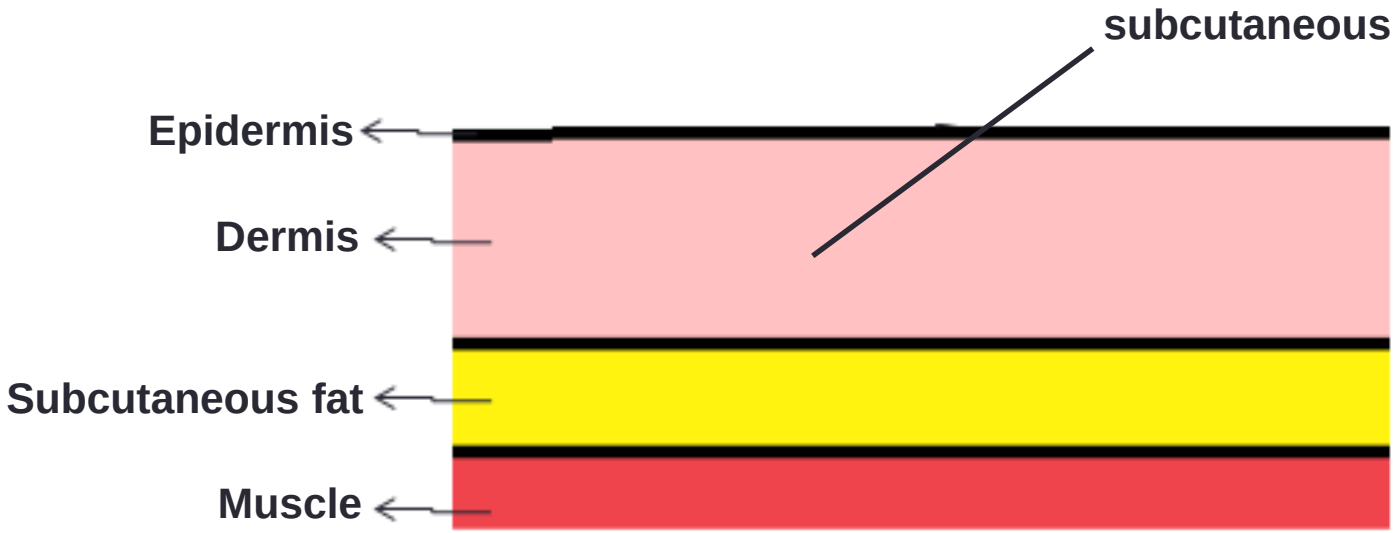
It is mostly used for experiments such as allergic reaction and toxin titration experiments.



# Subcutaneous (under the skin) inoculation

The skin in the back or abdomen is shaved, wiped with antiseptic, held between two fingers and lifted, inoculation is done by implanting the needle under the skin.

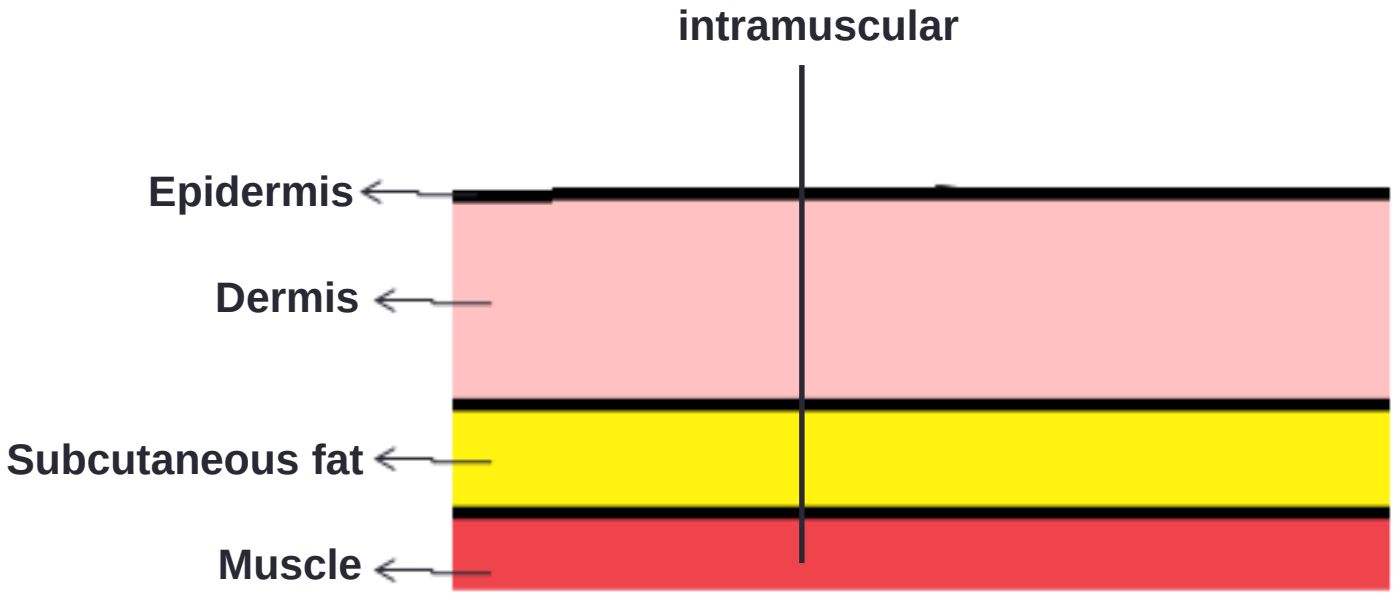
It is used to examine the pathogenicity of various microorganisms.



# Intramuscular (into the muscle) inoculation

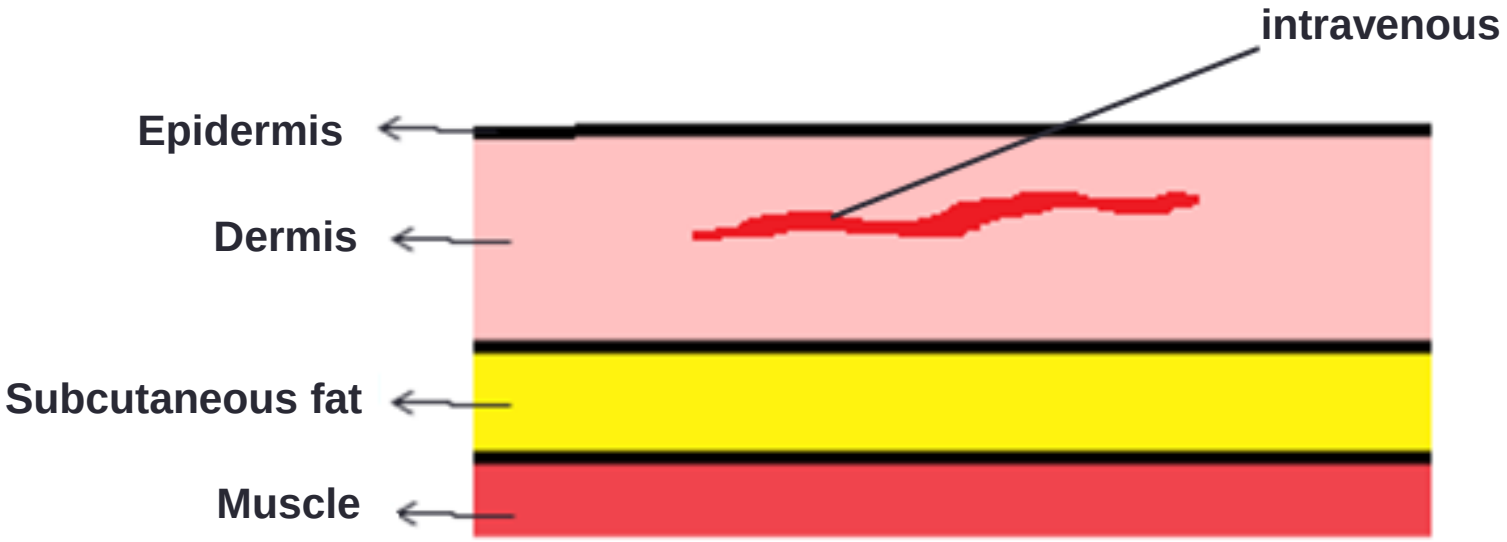
Method is used for diagnosis of tetanus, gas gangrene, etc.

Microorganism inoculation is done to shaved and cleaned (with antiseptic) nates site on the hip by holding the injector with right angle to the injection site.



# Intravenous (into the ven) inoculation

Inoculation is done to ear vein on the rabbit, tail vein on mouse, wing venine on chicken. Needle is inserted in the direction of the blood flow and inoculum is injected.



# Intraperitoneal (into the peritoneum) inoculation

The head of the animal is held down and the bottom of the abdomen is implanted with the injector perpendicular to the right or left of the middle line.

The injector is inserted under the skin firstly, then the needle is slightly advanced under the skin, then the abdominal muscles are punctured vertically to reach the peritoneum and the microorganism suspension in the injector is injected.

## Intracardiac (into the heart) inoculation

The animal is lied down to its right side and the place where the heartbeat is most felt is determined. A sharp-tipped needle is carefully inserted into the heart.

## Intraocular (into the eye) inoculation

The cornea is first scratched with a sterile needle. The cornea sensitized in this way and microorganism suspension is inoculated by dropping with a pasteurized pipette or drip cap.

## Intracranial (into the skull) inoculation

Often baby animals are preferred for this method, because their skulls are not hardened yet. If inoculation will be done to adult animals; first a slim hole is bored in animal's skull with a drill. An injection is done by inserting the injection needle through this hole.

## Intranasal (into the nose) inoculation

The animal's head is held upwards and the microbial suspension is inoculated by dripping into the nose by pasteur pipette, dropper or injector.

- Per rectum (rectally) inoculation: It is a method based on the inoculation of microbial suspension by rectal route.
- Inhalation (bronchially) inoculation: The material to be inoculated is sprayed at the place where the animal is located and it is provided to take the material during respiration.
- Intratracheal (into the breath tube) inoculation: The microorganism is injected through two cartilages.
- Peros (oral) inoculation: The material to be inoculated is mixed into the water and poured into the animal's mouth, or put into the appropriate nutrient and fed into the animal, or entered into the stomach with a thin rubber probe and given directly to the stomach.

- Intrathorasic (into the chest) inoculation
- Intrapulmoner (into the lung) inoculation
- Intramammar (into the breast) inoculation
- Intratesticular (into the testis) inoculation

# STAINS

- Examination of microorganisms under the microscope gives information about their size, shape, spore formation, capsule, flagella, intracellular granules and staining conditions of microorganisms.

- The biological molecules in microorganisms do not absorb visible light well. For this reason microorganisms should be examined by staining. The dye molecules absorb the light in distinct ways, allowing the microorganisms to appear differently.

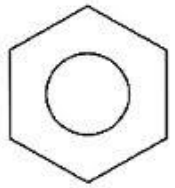
# STAINS

**Natural or native dyes**

**Synthetic or artificial dyes**

- First, Antonie van Leeuwenhoek dyed microorganisms with beet juice, making them visible at 1719.
- Natural dyes obtained from plant roots are not currently used other than hematoxylin.
- Hematoxylin is derived from the Greek words Haimato (blood) and Xylon (wood). *Haematoxylon campechianum L.* grown in Mexico is obtained from the shells of the named tree.
- Hematoxylin-eosin dye, which is used in the differentiation of nucleus and cytoplasm, which differs in different regions of the tissue, has the widest use in histological staining as a general tissue dye .

- Synthetic dyes obtained from aniline in advance but today there are benzene derivatives that are obtained from distillation of coal.

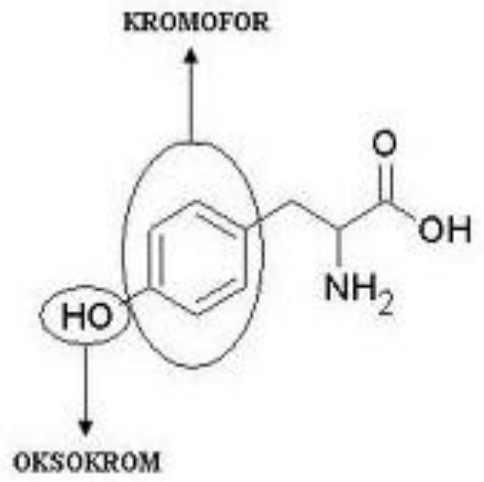


Benzene
$C_6H_6$

- Most of the dyes are organic compounds and contain **chromophore** and **auxochrome** groups united with the benzene ring.

- The benzene ring containing the chromophore group is called the chromogen ring. The chromogen group gives the combination dye property.

- However, although this compound is colored, it is not yet a dye character due to not having the ability to bind to bacteria, tissue or fiber. The resulting color can easily be removed by mechanical impacts.



- It is necessary that the auxochrome group and the chromophore to be included together so that the dye can be described as a real dye.
- Synthetic dyes are usually found in salt form. The auxochrome group determines the basic (cationic) or acidic (anionic) structure of the dye.
- They act on the pH property of the medium during staining and stain the oppositely charged tissue.
- For example; Bacterial organelles, proteins in the tissues, glycoproteins, lipoproteins contain negatively charged ions (phosphate groups), so they form salt compounds by binding to positive ions.

# DYES ACCORDING TO ELECTRICAL CHARGE OF THE DYE MOLECULE

- Acidic Dyes

  If color matter (chromophore group with negative electrical charge) is in acid base, colorless substance at alkaline base.

  For example; acid fucsin, acid picric, eosin, nigrosine, congo red, etc.

- Alkaline Dyes

  If color matter (the chromophore group has a positive electrical charge) is in alkaline base, colorless substance at acid base.

  For example; Methylene blue, crystal violet, basic fucsin, malachite green, etc.

- Neutral Dyes

  Neutral dyes are obtained from appropriate mixtures of acid and alkaline dyes.

  For example; Giemsa, Wright, Leishman, etc.

# STAINING METHODS

1.Simple Staining: The simple stain can be used as a quick and easy way to determine cell shape, size and arrangements of bacteria (morphology of bacteria). True to its name, the simple stain is a very simple staining procedure involving single solution of stain. Any basic/alkaline dye such as methylene blue, safranin, or crystal violet can be used to color the bacterial cells.

2.Differential Staining: The process of using two or more dyes, solvents, mordant in a certain time and alignment.

Objective: To determine the structure differences between various bacterial species and specific structures such as spores, capsules, flagellas.

# *Mordant

In microbiology, a mordant is a compound used to hold down molecules of a stain onto a microorganism. Classically defined, mordants are usually ions such as metal ions or halide ions, but can be any molecule that serves the purpose of holding down a dye.

Mordant is a substance that makes the dye fix to the bacterial cell wall, thus forming compounds which do not lose color in water and in alcohol. Can be used before or after staining.

For example; $NH_4$, oxalate, KOH, tannic, oxalic, picric, phenic acids, Al, Fe, Zn, Cu salts.

3. Panoptic Staining: The method that neutral dyes are used for staining such as Giemza, Wright, May Grünwald.

Objective: To determine the nuclei and cytoplasm of tissues and cells of microorganisms (protozoa) outside bacteria in different colors.

4. Negative Staining: The floor coloring is done with the contrast agent. A dark bacterial suspension is prepared by adding india ink or nigrosine in it and spread to the slides. The floor on which the present bacterium is stained, the dyes should have no affinity for the bacterial cell.

Objective: Determination of capsules of bacteria

5. Vital Staining: The method used for staining living cells.

Intra vital – a stain which is taken up by living cells after parenteral administration for example intravenously or subcutaneously. (live staining on the body)

Supra vital – relating to or capable of staining living cells after their removal from a living or recently dead organism. (live staining outside the body)

6. Impregnation: Causing dye agglutination after exposure of metallic salt solutions (heavy metals) to microorganism surfaces.

The surface of the microorganisms is treated with mineral salt solutions (heavy metals) and the resultant dye is precipitated.

Objective: to stain of tissues and spirochetes.

# How to prepare a preparation?

1. Slides are cleaned with an ether-alcohol mixture.

2. Slides are drawn with a glass pencil to determine the area where we will place the sample of microorganism.

3. The sample of the microorganism is placed on the slide and allowed to dry.

4. The preparation is fixed.

# What is fixation?

- Fixation is a process that based upon to kill bacteria without destroying the cell structures and to prevent the removal of the bacteria from slides during the staining.

- Fixation also facilitates easy staining of the preparation by increasing the permeability of the cell membrane and cell wall of the bacterium.

- A microorganism is usually killed and firmly attached to microscope slide during fixation.

# Fixation

!!Kill the bacteria without destroying cell structure

!!Fix bacteria to slide

!!Prevent the bacterium from being removed from the slide during the staining process

!!Easy to dye by increasing the permeability of the cell membrane and the wall

We can use two types of fixation;

## Physical fixation:

Air drying: 2 - 18h in air

Heat drying: licking slide at a 45 degree angle to the gasburner

## Chemical fixation:

Includes waiting in organic solvents such as;

methyl alcohol 3 min,

95% ethyl alcohol 15-20 min

# SIMPLE STAINING

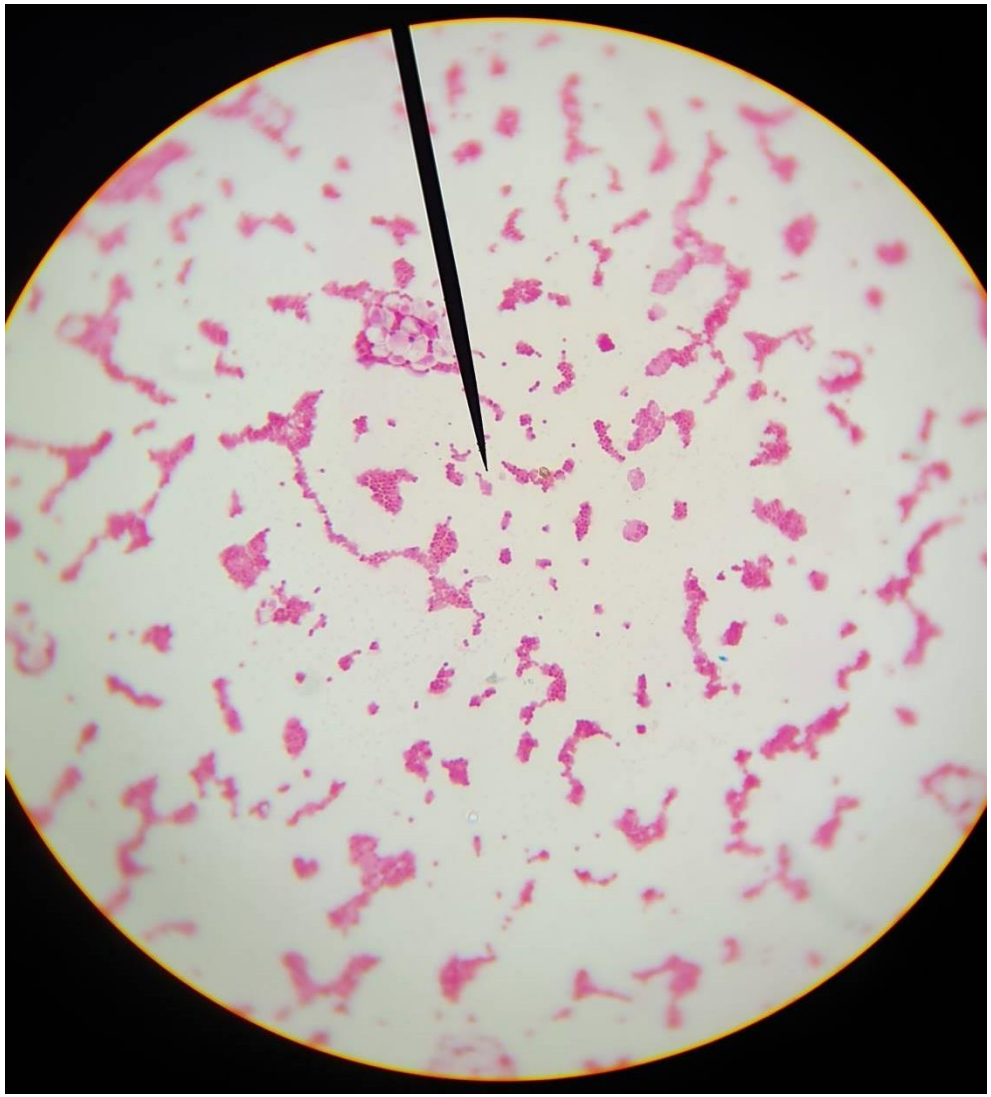
-Fix the preparation

-Drop the methylen blue or aqueous fucsin (wait 1 min)

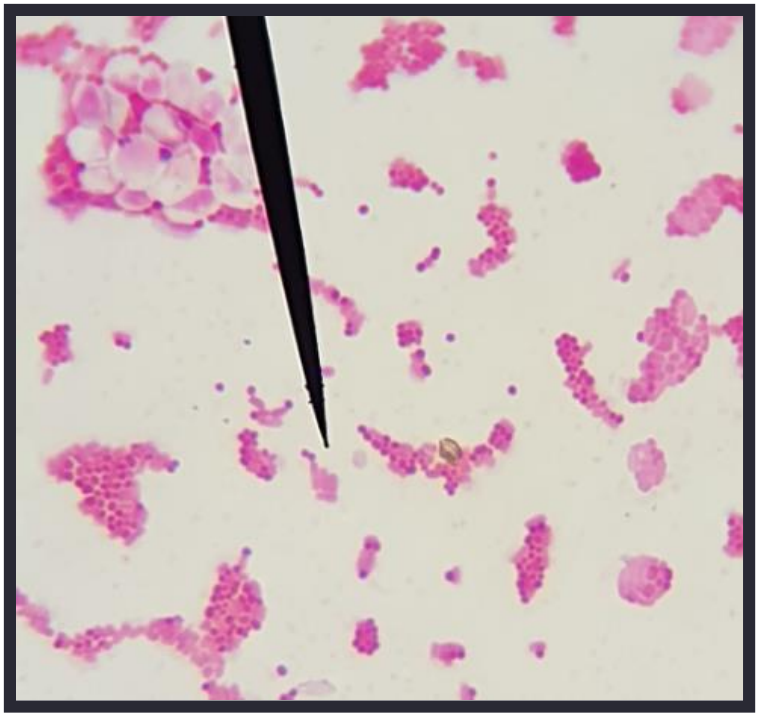
-Wash with water, dry

-Drop the immersion oil and examine in 100X objective.

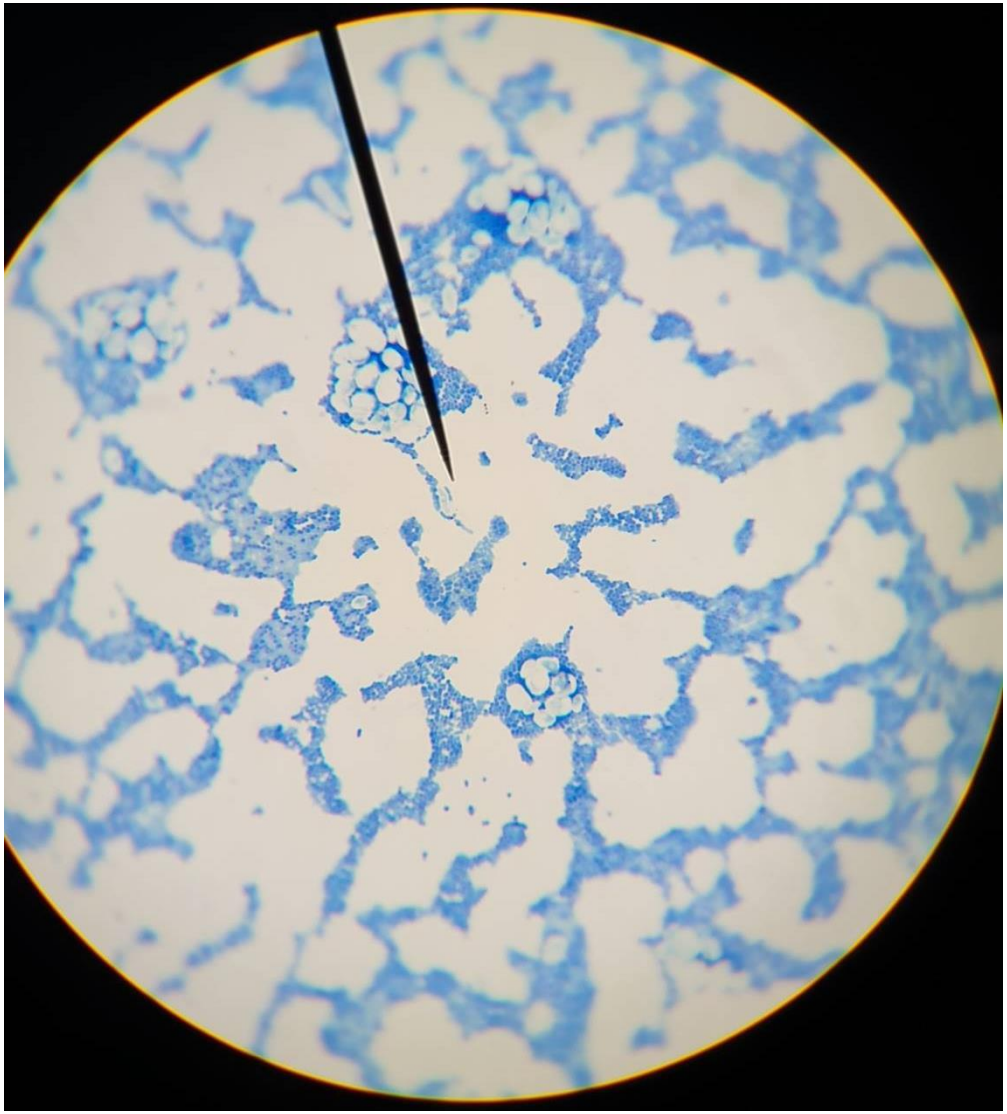
# Simple staining Microscope Image



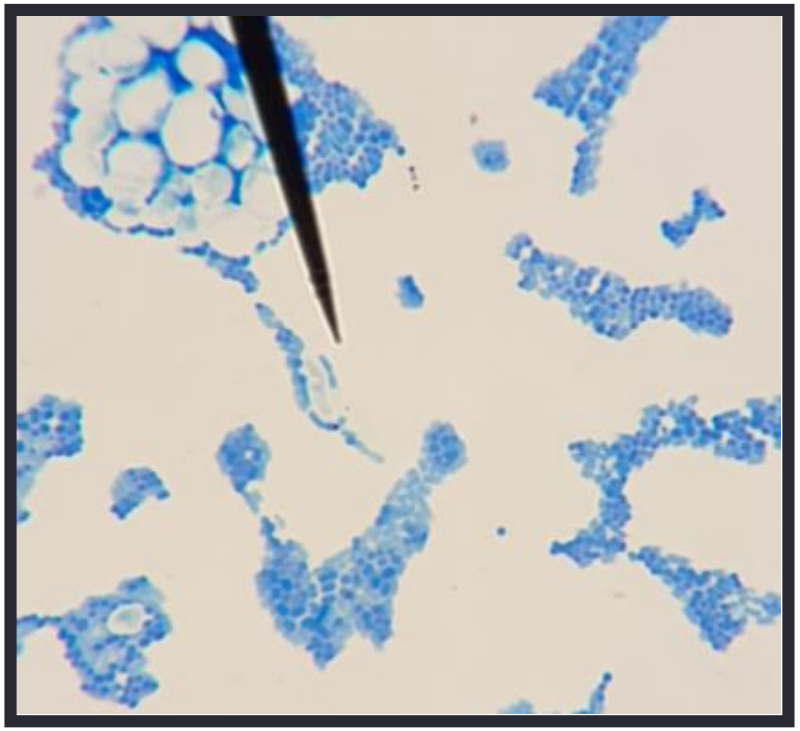
**Simple staining-Aqueous fucsin**



# Simple staining Microscope Image



**Simple staining- methylen blue**



# GRAM STAINING

-Fix the preparation

-Drop crystal violet (wait 3 min)

-Wash with water

-Drop lugol (wait 2 min)

-Wash with water

-Decolorate the preparation with alcohol (30 sec)

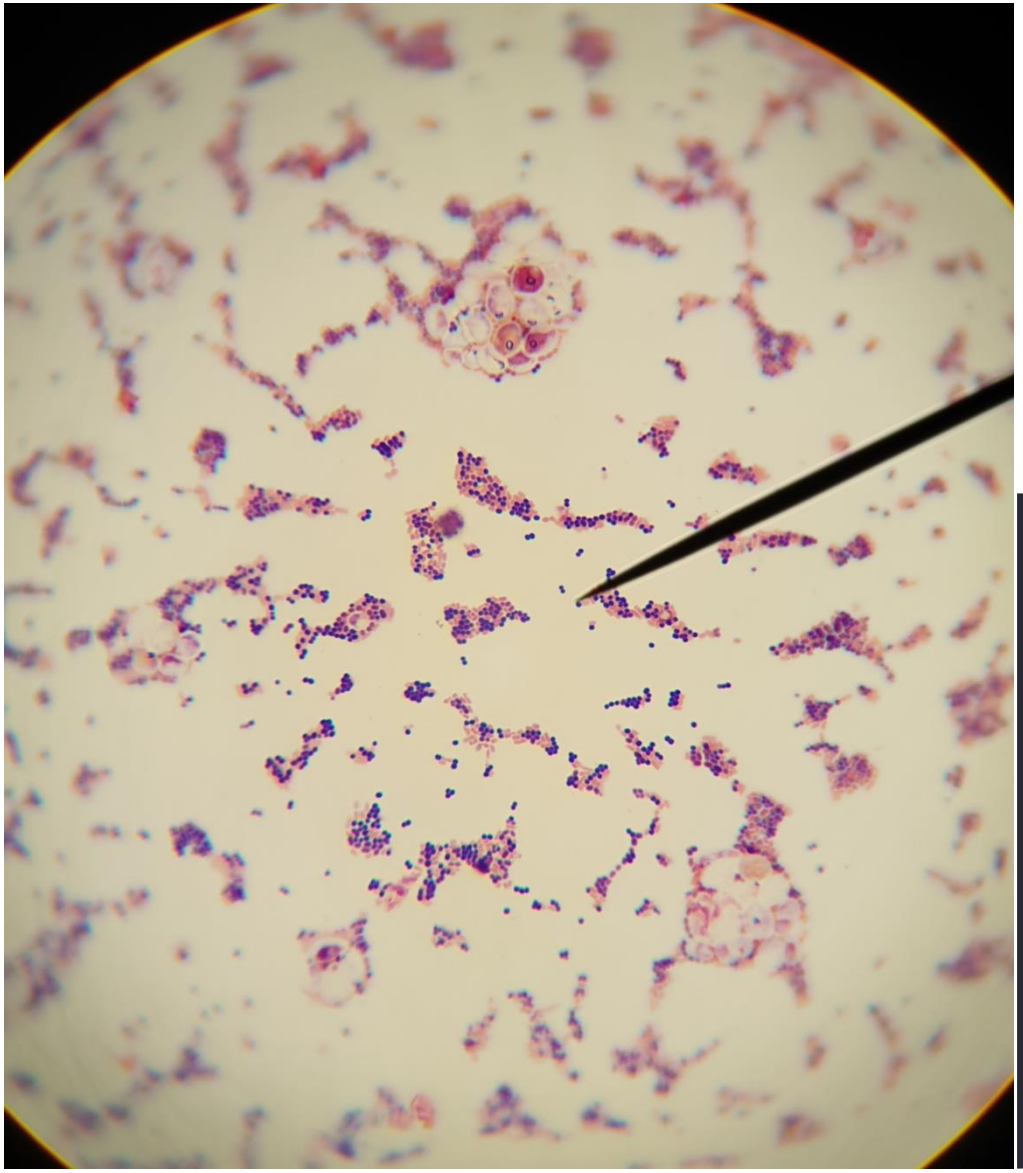
-Wash with water

-Drop safranine (1 min)

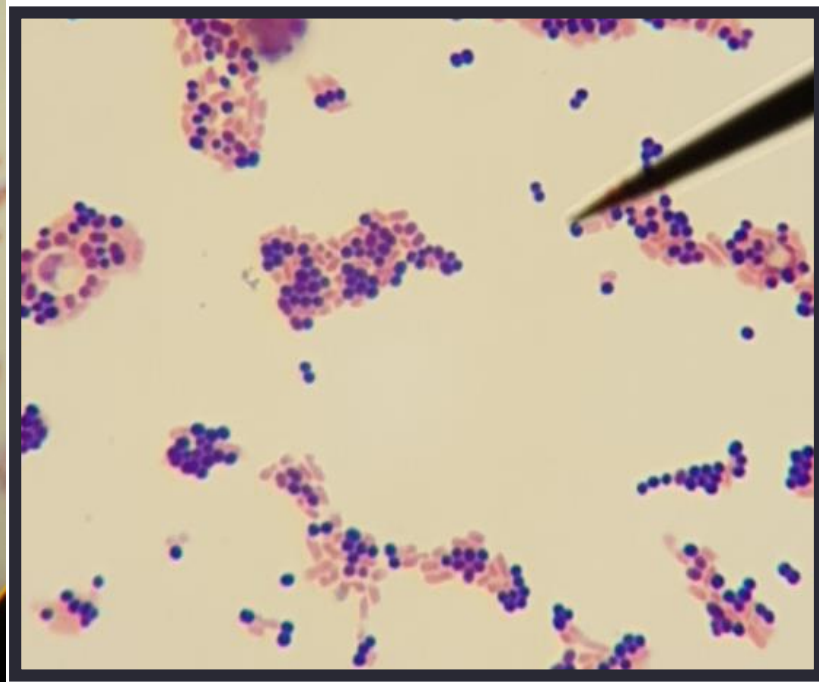
-Wash with water, dry,

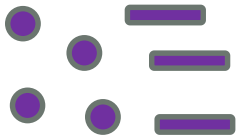
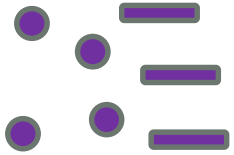
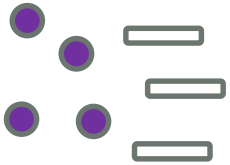
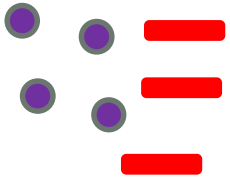
-Drop the immersion oil examine in 100X objective.

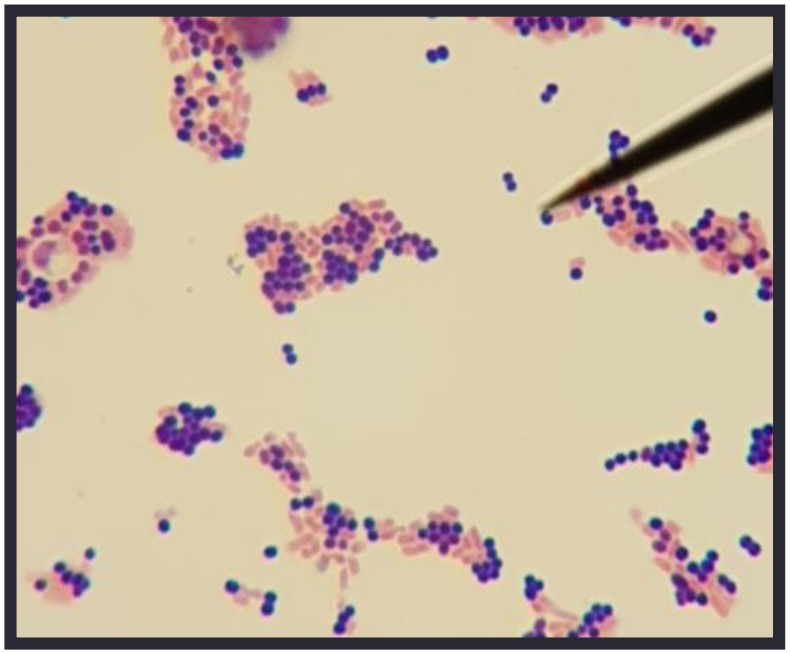
# Gram staining Microscope Image



**Gram Staining**



| | Gram staining steps | Bacteria cells |
|---|---|---|
|  | **1. Crystal violet** (primary stain) | Cells stain purple |
|  | **2. Lugol** (Mordant) | Cells remain purple |
|  | **3. Alcohol** (Decolorizer) | Gram(+) cells remain purple; Gram (-) cells become colorless |
|  | **4. Safranine** (counterstain) | Gram(+) cells remain purple; Gram (-) cells appear red |



# REFERENCES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- Tıbbi Mikrobiyoloji Laboratuvar Kılavuzu, Doç.Dr. Ömer Poyraz, TC Cumhuriyet Üni.Yayınları, 1998.