MICROKROM

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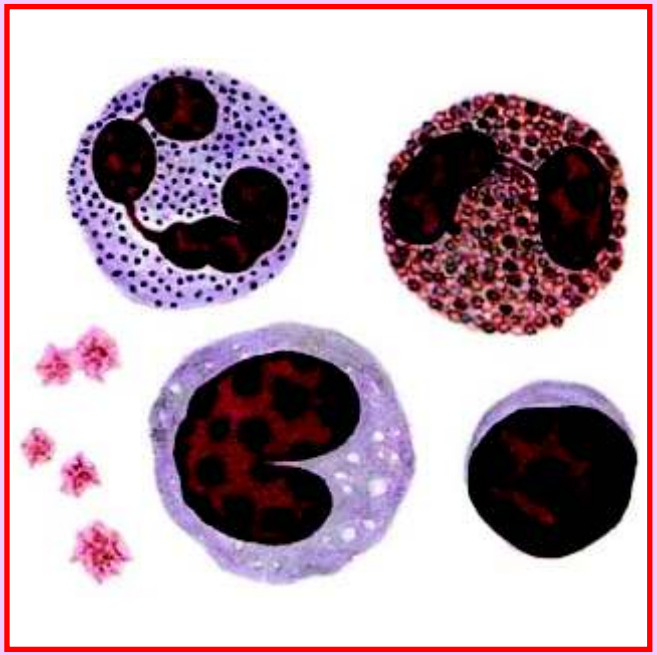
MICROKROM
MICROSCOPY STAINS



Basic Guidelines for Quality Staining with Romanowsky Stains

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Introduction

In microscopic examination of blood smears for blood cell morphology or parasite examination there are a few important parameters that essentially play vital role in blood smear staining.

These parameters being,

- Clean and grease free glass slide
- Good microscope
- Technique for preparing the smears
- Quality of the stains used
- Maintaining appropriate pH by using buffered water to attain a good staining reaction and better differentiation
- Care of glassware and microscope
- Expertise of the person in evaluating the results

All the above mentioned parameters have to be optimized so that the quality of smears, so achieved will lead to better staining and differentiation.

This booklet essentially serves as a refresher for the busy lab professional who is already using microscopy stains. For the students it serves as a useful learning tool to improve microscopy based diagnosis.

We hope that with this scientific booklet we are able to communicate to professionals the importance of basic precautions and guidelines that are essential inorder to achieve quality microscopic staining of thick and thin blood smears.

Cleaning and storing microscopic slides

Microscope slides are usually supplied in boxes. They may be described on the box as “washed” or” pre-cleaned”, but they need to be properly washed, dried and wrapped. It is not possible to make good quality films on dirty microscope slides.

Blood films made on dirty or greasy slides will wash off easily during staining

It is therefore best to discard slides that,

- Have rainbow like colour or appear white or opaque
- Are not properly cleaned
- Are old, with surface scratches or chipped edges

Cleaning slides

In order to clean the slides, the following items are suggested.

- A large plastic basin
- Gauze or cotton wool
- A good quality detergent
- Clean cotton cloth
- Clean water

Cleaning new slides

- All new slides should be washed with detergent and clean water
- After being soaked for 30 minutes to 60 minutes, the slides should be rinsed under running tap water or in several changes of clean water

- Each slide should be individually wiped dry and polished with clean, dry cotton.

Cleaned slides should be handled only by the edges to avoid finger marks or grease being deposited on the slide

Cleaning of used slides

- Used, dirty slides should be soaked for a day or two in water containing detergent (warm water should be used whenever possible)
- After soaking, the slides should be cleaned one by one with a small piece of gauze or cotton wool

All traces of blood film and oil (used during microscopy) should be removed from the slides

➢ *Caution: Do not leave the slides in detergent for too long, soaking should be for a few days only, not weeks. If slides are left in the detergent solution for too long period, water will evaporate, leaving a deposit on exposed slides that is impossible to remove.*

- After cleaning, the slides should be transferred to a fresh solution of detergent and later rinsed under running water or in several changes of water
- Each slide should be individually wiped dry and polished with clean, dry cotton cloth

Wrapping cleaned slides

Items required for wrapping cleaned slides

- Sheets of thin, clean paper
- Empty cardboard slide boxes

- Rubber bands or adhesive tapes

Cleaned slides may be wrapped in packs of 10

Each pack may be secured with adhesive tape or rubber band

Packs may then be placed in the cardboard slide boxes

Storage of clean slides

- Slides should be stored in a dry place
- If stored at room temperature with high humidity, the slides will stick together after a few weeks. As a result they cannot be used unless they are re-washed and dried

Care of the microscope

The microscope is an expensive instrument, which if properly maintained and taken care will be useful for many years.

The following are the important points that need to be considered,

Remove dust and grease

1. When not in use during the day, the microscope should be kept covered with a clean cloth or plastic cover to protect the lenses from dust that settles out of the air.
2. When the microscope is to remain unused for long periods or to be stored overnight, it should be placed inside the box with door tightly closed. To protect the objective lenses, the x4 objective should be rotated to line up with the ocular.
3. Oil and grease from eyelashes and fingers are easily deposited on the lenses and oculars. These parts should be cleaned with very soft cotton cloth.
4. The oil immersion objective should be cleaned after use. If it is not cleaned, the oil will harden and make the objective ineffective. A soft cotton cloth is usually sufficient for cleaning. However the same cloth should never be used to clean the other objectives, the oculars or the mirror, otherwise oil will be transferred to these components. From time to time, dried oil should be removed from the oil immersion objective using xylene. Oil can be washed off the slides with a small amount of xylene, if no xylene is available, the oil smear can be carefully removed by using absorbent paper.

Preventing the growth of fungus

In warm, humid climates it is easy for fungal growths to be established on lenses and prisms. These growths can cause problems and may even become so bad that the microscope can no longer be used.

Fungus cannot grow on glass when the atmosphere is dry, and every effort should be made to store the microscope in a dry atmosphere when it is not being used.

The following are preventive measures that can be adopted,

1. Keep the microscope in a continuously air conditioned room.
2. Place the microscope in a “warm cupboard” (air tight cupboard) in which one or two 25 - watt bulbs are constantly glowing.
3. Keep all the lenses and prism heads in airtight box or dessicator where the air is kept dry by means of silica gel.

Preparation of Blood films or Smears

Examining a stained blood smear or film is a routine part of the technologist to view the elements of blood or components of the blood.

The procedure of studying the blood film involves,

- Preparing a blood film on a microscope slide
- Drying the film (to prevent distortion and washing away of cells)
- Fixing the film
- Staining the film
- Finally the cellular components of blood are observed under microscope

The main objectives of fixation are,

- To kill the cells suddenly and uniformly so that they retain the same appearance which they possessed in living condition
- To preserve the cells by the inhibition of autolytic changes
- To set and hold the intracellular components, by precipitation in the position which they occupied in living condition. Hence facilitating the closest possible study of the cells
- To render the cells and its components resistant to subsequent process such as dehydration, embedding, clearing prior to examination under microscope
- To facilitate differentiation in the refractive indices of certain cell elements which would otherwise be invisible owing to the narrow margin between the refractive index of one type of cell with other type
- To facilitate proper staining

Techniques for estimation of Blood films

Examination of a fixed and stained blood film is an essential part of a haematological investigation. Ideally the blood films must be well spread, well fixed and well stained for microscopic observation.

The films or smears are of two types,

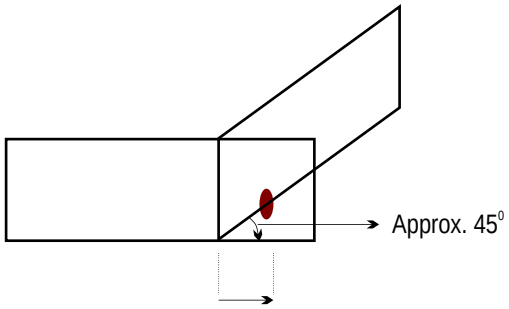
- Thin blood film or smear
- Thick blood film or smear

Preparation of thin blood films

Use clean, grease and moisture free glass slides for accurate diagnosis

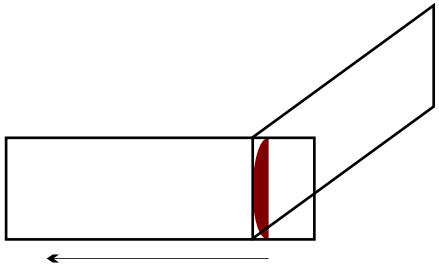
- Place a drop of blood, approximately 3 mm diameter on the slide one-third distance away from the labelled end

- Without delay, place a glass slide or spreader (such as Magispread) at an angle of 45^0 to the slide and move it back to make contact with the drop. The drop should spread out quickly along the line of contact of the spreader with the slide. (Schematic representation on the next page)



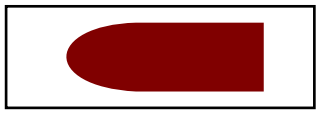
(Move the spreader till it touches the drop of blood)

- The moment this occurs spread the film by a rapid, smooth, forward movement of the spreader.



(Spread the film)

- A thin film appears as follows



- Air-dry the film thoroughly.

Preparation of thick blood films or smears

- Add approximately 5 µl of blood on a clean slide
- With the end of applicator stick or a corner of slide, quickly distribute the blood to make a even thick film about 1cm^2
- Allow the thick film to air dry

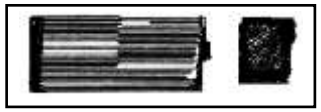
Common faults in making thin and thick blood films

- *Badly positioned blood films*

  Care should be taken that the blood films are correctly sited on the slide for thick or thin film. Improper positioning of the blood drop will lead to difficulty in examination of the film.

- *Too much blood*

  After staining films made with too much blood, the background of the thick film will be excessively blue in colour thereby leading to improper estimation especially in identification of parasites. If the thin film is too thick, red blood cells should be on top of one another and it will be impossible to examine them properly after fixation.



- *Too little blood*

  If too little blood is used to make the films (for thin film preparation the drop of blood should be approximately 3 mm in diameter and for thick film approximately 5 µl of blood) there will not be enough white cells in the thick film leading to improper examination. Also the thin film may be too small for identification.

- *Blood films spread on greasy slides*

  The blood films will spread unevenly on a greasy slide, which makes examination very difficult. Some of the thick film will probably come off the slide during staining process.

- *Edge of spreader slide chipped*

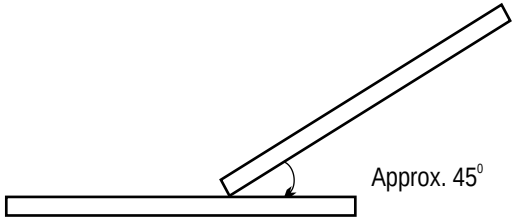
  When the edge of the spreader slide is chipped, the thin film spreads unevenly and has many “tails”. The spreading to the thick film may also be uneven.

Other common faults

- Blood films are made on badly scratched slides
- The thick film is allowed to dry unevenly.
- Auto fixation of thick film occurs with the passage of time through exposure to heat, and then staining becomes difficult or unsatisfactory
- Slides are wrapped together before all the thick films are properly dried, and slides stick to one another.

Thus the necessary precautions that need to be ascertained inorder to attain a good smear are,

- The glass slide must be clean, grease and moisture free
- Usage of appropriate drop size for preparing the respective smears
- Make the smear immediately after putting a drop of blood on the slide (a delay will cause uneven distribution of white cells on the smear)
- The spreader or the spreader slide must be moved steadily, without any jerky movements. Since loss of contact between the spreader slide and blood will lead to poor smears
- The angle of holding the spreader slide essentially determines the thickness of the smear. The approximate angle should be around 45^0. A greater angle will result in too thick a smear, too thin if a smaller angle is employed



- The blood smear should be made preferably immediately after blood collection but certainly not later than four hours of blood collection. Inability to do the smear within four hours will result in distortion of red blood cells and cause hemolysis.
- As far as possible films should be stained as soon as they are air- dried. They certainly should not be left unfixed. If the films are left unfixed at room temperature for a day or more, it may be found that, in addition to distortion of morphology, the background of dried plasma stains a pale blue colour that is impossible to remove thereby spoiling the staining of the blood cells.

Commonly used Romanowsky stains

Stains are primarily chemicals that react with blood components (cellular) at a particular pH and aid in identification of the blood dyscrasias and parasite estimation thereby helping the clinician to diagnose disease or underlying pathology, initiate a therapy and also help in prognosis.

The mechanism by which certain component of a cell structure stain with particular dye and other component fail to do so depends upon,

1. The chemical composition of various cellular organelles.
2. The binding of the dye molecule and chemical components of the cell organelles.

Generally, the acidic groups of the nucleic acid, proteins of the cell nuclei react with the basic component of the dye. Similarly the granules in the cytoplasm are alkaline in nature and react with the acidic component of the dye.

For any chemical reaction to occur one of the most important factor is pH. A pH of 6.8 is usually recommended for general use. When looking for malaria parasites a pH of 7.2 is recommended inorder to see Schuffner's dots.

Inorder that the staining is attained properly buffered water is used which maintains a correct pH to ensure proper staining reaction and blood films can be seen clearly under the microscope with good differentiation.

Romanosky stains are universally employed for staining blood films. Giemsa and Leishman stains are types of Romanosky stain, which is utilized for blood smear staining.

Field stain is used for rapid identification especially for thick film in malarial parasite estimation.

Staining procedure of thin blood films

With Giemsa stain

- Fix the blood smear with methanol for 3-5 min
- Prepare a 10% solution of Giemsa stain with buffered water
- Stain the slides in diluted stain for 30-45 minutes
- Rinse the slide in buffered water
- Blot dry, or air dry in a vertical position

Freshly diluted stains should be used for each day's work. Prepare sufficient amount of one day's use of diluted stain, since the diluted stains are not stable overnight.

With Leishman's stain

- Prepare a thin film and air dry the film
- Flood the slide with Leishman stain (e.g. x ml) on the air dried smear and spread over the film, which acts as a initial fixative
- Add double the volume of buffered (e.g. 2 x ml) and stain the film for approximately 5-7 min. Take care not to allow the stain to dry up during the time
- Then wash it in a stream of buffered water until it has acquired a pinkish tinge (upto 2 min)
- And dry the slide in a vertical position

Washing is done with buffered water because pH must be carefully maintained in Leishman stain inorder to attain a proper staining reaction.

With Field's stain

- Prepare a thin blood film
- Fix blood film in methanol for one minute
- Wash off methanol with tap water
- Cover the blood film with Field's stain B
- Immediately add an equal volume of Field's stain A
- Mix well and stain for one minute
- Rinse the slide with tap water, and place upright to drain and dry

Staining procedure of thick blood films

With Giemsa stain

- Prepare a thick blood film
- Prepare a 10% solution of Giemsa stain in buffered water
- Apply the stain to the unfixed thick smear and stain for 30 minutes
- Remove the slides and wash in buffered water
- Air dry the film

With Field's stain

Requirements

- One staining dish filled with Field's stain A
- One staining dish filled with Field's stain B
- Two dishes filled with neutral Distilled water

Method

1. Dip the unfixed air-dried slide into Field's stain A for 3 seconds
2. Wash gently by dipping (once) into neutral distilled water
3. Dip into Field's stain B for 3 seconds
4. Wash as in step 2
5. Place the slide upright in a draining rack to air dry

Depending upon the moisture/humidity in each laboratory the staining time may be required to be fine tuned further to optimize results.

Artefacts

Blood films may contain many features that can cause confusion and problems in diagnosis. Such features are known as artefacts.

- Fungus will show up artefacts on blood films. The best way to prevent fungal growths on slides is to stain blood films as soon as possible after making and drying them within 48 hours at most
- Dust particles floating in air will settle on blood films while they are drying, either before or after staining. Specks of dirt may be transferred from a patient's finger when the blood sample is taken, or the original slide may not be clean. Hence it is always advisable to take a clean slide and hold the slide by its edges
- Precipitated stain suspensions super-imposed upon red blood cells may cause confusion
- Debris from the patient's skin, also may produce a source of confusion
- Algae and other organisms may grow in staining solutions which have become contaminated and may be introduced in a well produced film in the staining stage

Colour responses of blood cells to Romanowsky staining in thin blood films*

Cellular component	Colour
Nuclei	
Chromatin	Purple
Nucleoli	Light blue
Cytoplasm	
Erythrocytes	Dark blue
Reticulocytes	Pink
Lymphocytes	Blue
Monocytes	Grey blue
Neutrophils	Pink
Basophils	Blue
Granules	
Basophil	Purple black
Eosinophil	Red orange
Neutrophil	Purple
Platelet	Purple

*Table from book Practical Hematology by Sir John V. Dacie / S.M. Lewis, 8th Edition, 1995

Basic precautions to be taken in handling and storage of stain solutions

What you should do

- When the bottle of the stain solution is not being used, keep the screw capped tightly to prevent the evaporation of solvent and oxidation of the stain
- Keep the stain bottle away from sunlight
- Store the solution in cool dry place at all times

What you should not do

- Never add water to the stain stock solution, the smallest amount of water will cause deterioration of the solution
- Do not shake the stain bottle before use. This can lead to resuspending of very small undissolved crystals of stain, which can settle on the blood films during staining and cause problems in microscopic examination
- Never return unused stain to the stock solution bottle, it is better to measure out a small quantity for one or two day's use

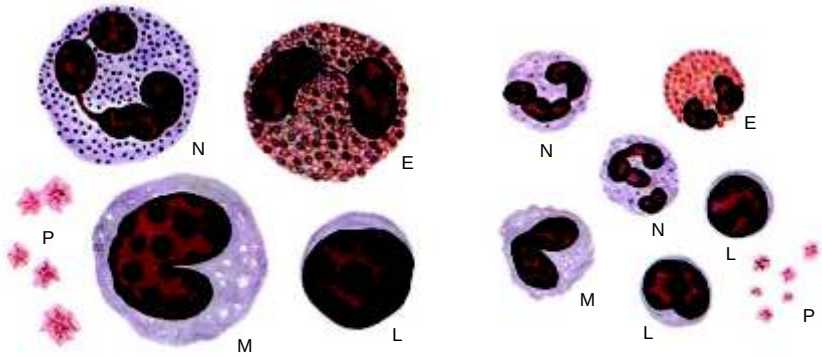
Care of glassware

- Glassware such as measuring cylinders, pipettes and staining troughs must always be clean and dry before use
- Any glassware that has been used for staining purpose should be rinsed in clean water immediately after use to remove as much stain as possible
- The glassware should then be soaked for some time, preferably overnight in a detergent solution

- Then they should be washed with clean water and dried. Washing glassware in detergent gives satisfactory results provided that you rinse it thoroughly with clean water. Deposits of the detergent left on the glassware can upset the pH of buffered water and spoil the staining

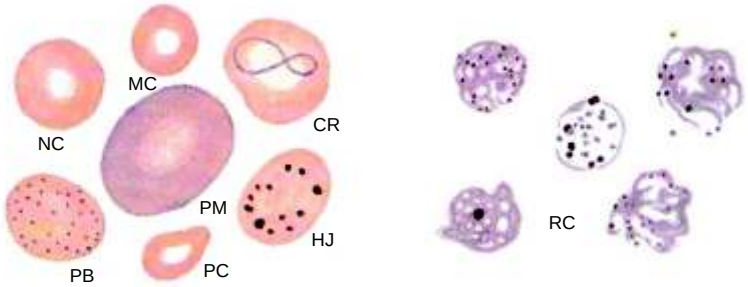
Any stain deposits that are allowed to dry on the glassware will become difficult to remove and may spoil the staining of subsequent films. In such a case soaking the glassware in methanol and then washing with detergent in the normal way can remove them.

Components of Blood



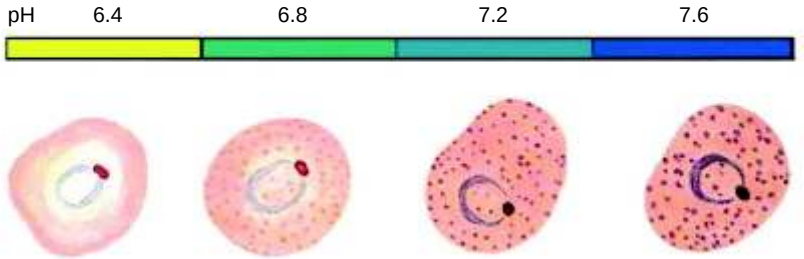
Thin Film LEUKOCYTES Thick Film

N = Neutrophil, E = Eosinophil, M = Monocyte, L = Lymphocyte, P = Platelets



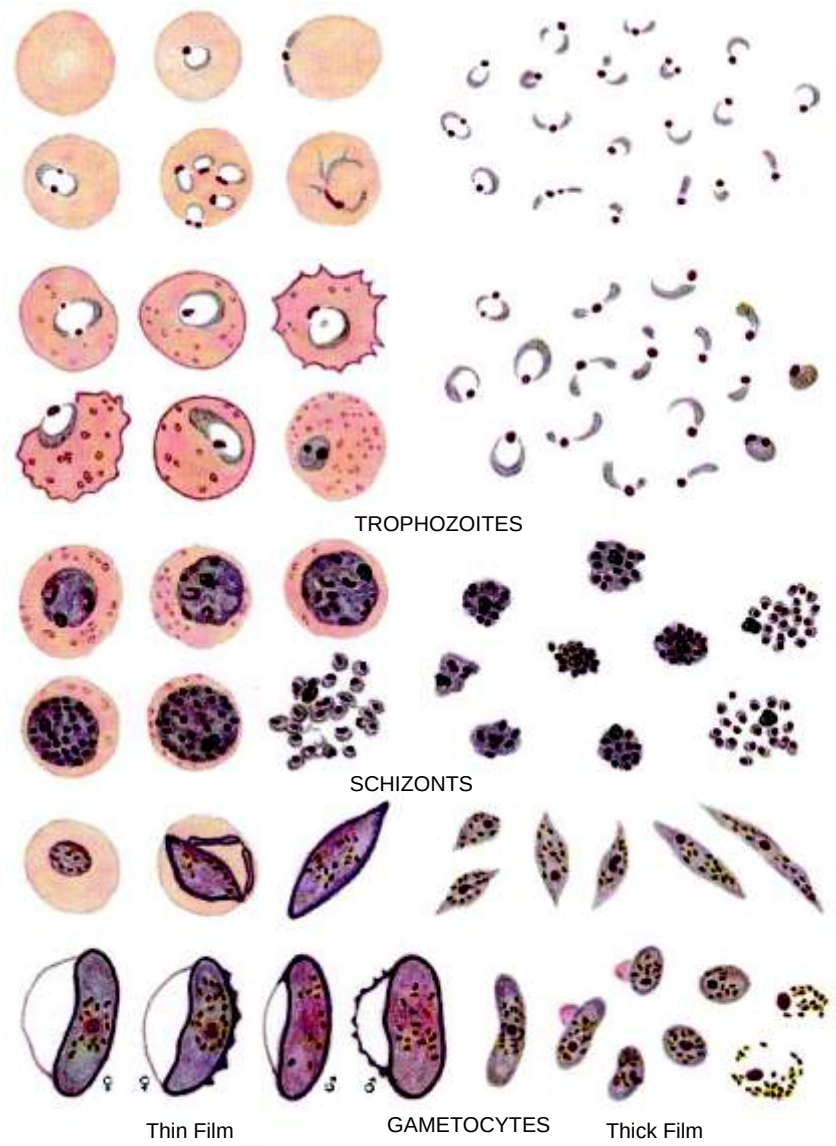
Thin Film ERYTHROCYTES Thick Film

NC = Normocytes, MC = Microcyte, PM = Polychromatic macrocyte, PC = Poikilocyte, PB = Punctate basophilia, CR = Cabot's ring, HJ = Howell-Jolly bodies, RC = Reticular 'clouds' and chromatoid bodies in severe anaemia

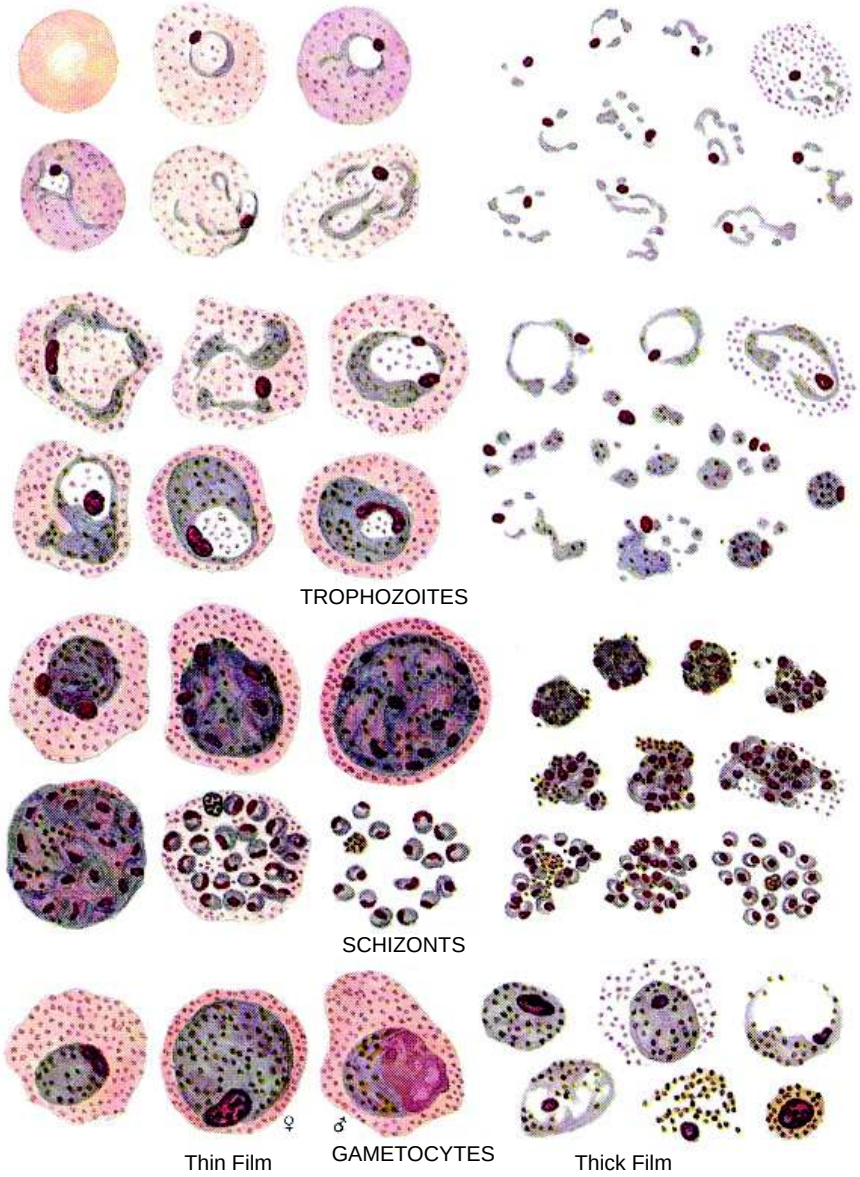


MALARIA STAINING AND pH

Appearance of *Plasmodium falciparum* stages in Giemsa Stained Thin and Thick films

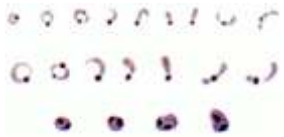


Appearance of *Plasmodium vivax* stages in Giemsa Stained Thin and Thick films



Species identification of malaria parasites in Giemsa stained Thick Blood films

Stage of parasite in peripheral blood

	Plasmodium falciparum Young, growing trophozoites and/or mature gametocytes usually seen	*Plasmodium vivax* All stages seen, Schuffner's stippling in "ghost' of host red cells, especially at film edge
Trophozoite		
	Size: small to medium, *number:* often numerous, *shape:* ring and comma forms common; *chromatin:* often two dots: *cytoplasm:* regular, fine to fleshy; *mature forms:* sometimes present in severe malaria, compact with *pigment:* as few coarse grains or a mass.	*Size:* small to large, *number:* few to moderate, *shape:* broken ring to irregular forms common; *chromatin:* single, occasionally two; *cytoplasm:* irregular or fragmented; *mature forms:* compact dense; *pigment:* scattered, fine.
Schizont		
	Usually associated with many young ring forms. *Size:* small, compact, *number:* few, uncommon, usually in severe malaria; *mature forms:* 12-30 or more merozoites in compact cluster; *pigment:* single dark mass.	*Size:* large, *number:* few to moderate, *mature forms:* 12-30 merozoites, usually 16, in irregular cluster, *pigment:* loose mass.
Gametocyte		
	Immature pointed-end forms uncommon. *Mature forms:* banana-shaped or rounded; *chromatin:* single, well defined; *pigment:* scattered, coarse, rice-grain-like; pink extrusion body sometimes present. Eroded forms with only chromatin and pigment often seen.	Immature forms difficult to distinguish from mature trophozoites. *Mature forms:* round, large; *chromatin:* single, well defined; *pigment:* scattered, fine. Eroded forms with scanty or no cytoplasm and only chromatin and pigment present.

Artefacts that may cause confusion in diagnosis

'Clouds' and chromatoid debris derived from immature erythrocytes in severe anemia

Isolated groups of eosinophilic granules

Blood platelets Lymphocyte for comparison of size

BLOOD ELEMENTS

BACTERIA

SPORES

VEGETABLE CELLS

Hyphae and spores FUNGUS

Dust particles

Giemsa stain crystals

Herring-bone scratches in glass slide

Crystalline 'pits' in devitrified slide

VARIOUS SOURCES

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