Title: Protocol for smearing, staining and counting malaria slide

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Objectives: To describe the methodology of smearing, staining, counting and staging malaria slide.

General description:

Blood: Should be:

a) Fresh blood from finger prick or the left over blood in the syringe but the blood still not clots.

b) Blood collected in EDTA or ACD (Acid Citrate Dextrose) tube. Try not to use blood from heparin or fluoride tube because the stain will be red and can not see the parasites clearly especially thick film.

Smear: Put one tiny drop of blood (≈ 6 ul) on one end of the slide near to the frosted end side. Use toothpick to smear the blood around into a circle about 1.2 cm in diameter and not very thick (can see through the smear around the border). Put another tiny drop of blood (≈ 4 ul) near to the thick smear (≈ 1 cm away). Use another glass slide or spreader to smear. Hold the spreader about 45° angle and smear. Do not press the spreader too hard otherwise the red cells will be distorted. Try to spread the smear into a long thin end and dry the smear quickly with hair dryer. Leave the slide about 5 minutes for thick film to dry, if urgent, blow with cold air then with hot air to make it dry properly before fixing the thin film otherwise the wet blood from thick film will drop down to thin film. Label the smear with patient's code, date and time of blood taken and time of study (e.g. adm, 4 H, 24H etc).
Fix: Fix thin film in absolute Methanol (100%) for ≈ 5 seconds.

Do not dip the slide too close to thick smear otherwise the thick smear will be fixed, too.

Leave the slide to dry on the rack or blow dry with hair dryer.

Stain:

1. Giemsa Stain

Preparing the Working Stain
- Prepare solution freshly before use.
- Filter stock Giemsa.
- Prepare 10% Giemsa stain in Buffer pH 7.2

Procedure

1. Staining:
   - Arrange the slides on a staining rack with the slides face up.
   - Cover the slides with 10% Giemsa stain.
   - Leave slides to be stained for 20 minutes.

2. Washing:
   - Pour clean water (drinking water) gently on the slides from the thin blood film side, in order to remove most of the Giemsa solution.
   - Individually wash each slide to prevent cross contamination.

3. Drying:
   - Put slides upright to dry (thin film up)
   - Or blow dry with Hair Dryer.

NB % Giemsa stain can be prepared and modified for convenience of each lab but should be prepared freshly everyday.
2. Modified Field’s Stain

Field’s “A” 5.0 gm + 500ml water. Field’s “B” 4.8 gm + water (dist H₂O, water for battery or drinking water is OK)

Filter both stains with filter paper (Whatman no.1) everyday to prevent bacteria growing

Before staining; make sure that the slide is dried properly especially thick film otherwise the smear will come off while staining. For thick smear, if urgent, leave smear to dry completely first and use hot air (hair dryer) to blow for ≈ 30 seconds before staining.

Procedure

1. Dip the slide on thin smear part into Field's stain "B" for 5 seconds.
2. Dip in tap water jar and wash for 5 seconds.
3. Dip the thin smear part into Field's stain "A" for 30 seconds then put the whole slide including thick smear into the stain for 20 – 30 seconds.
4. Wash for 5 seconds.
5. Dip the thick smear part into Field's stain "B" for 5 seconds.
6. Wash.
7. Dry on the rack or blow dry with hot air.
**Count:**

**Thin film**

For high parasitaemia (> 20 parasites / 10 fields, in thick film)

Count parasitized red cells / 1,000 red cells.

Count only 1 parasitized red cell even though there are more than 1 parasite in the red cell.

**Procedure:** Start from the bottom end of the smear and move ----> along the slide. Look for the field that red cells line nicely and easy to count. Count parasitised red cells along with normal red cells until 1,000 normal red cells.

**Report stage of parasite if possible**

Parasitaemia / µl = no. of parasitised red cells /1,000 rbc × Hct x 125.6

or

= no. of parasitised red cells /1,000 rbc × absolute rbc ÷ 1,000

**NOTE:** If you find only 1 / 1,000 rbc, continue counting to 2,000 rbc, if you find 2/2,000 rbc, report 1/ 1,000 rbc but, if you still find 1/ 2,000 rbc go to count in thick film.

The reason is: if you stop counting at 1,000 rbc you may find the parasite 1/1,000 rbc by chance (it may be 1/5,000 or /10,000 or 1 parasite in the whole thin film). But when you find 2/2,000 rbc, it is more accurate that you find 1/1,000 rbc.

**Thick film**

For low parasitaemia (find 1 parasite /2,000 rbc, in thin film)

Count parasites / 200 or / 500 white cells (because red cells are all lysed).

Count every parasite.

**Procedure:** Start from the bottom end of the smear and move along ----> try to count around the border where the smear is not too thick. Count parasites along with white cells until 200 white cells if only 5 or lower (0) parasites found continue to 500 white cells. If 0/ 500 wbc, report “Negative” (16 parasites/ul).

**For gametocyte:** Count gametocytes/200 or /500 wbc

Parasitaemia / µl = no. of parasites /200 wbc × 40

or

= no. of parasites / 500 wbc × 16

or

= no. of parasites / 200 wbc × absolute wbc ÷ 200

or

= no. of parasites / 500 wbc × absolute wbc ÷ 500
Counting Procedure:

- **Admission:**
  a. Start with thick film first to confirm that slide is positive, if there is > 20 parasites/10 fields, go to thin film and count parasites/1,000 rbc. If there are 1 infected red cell / 2,000 rbc go back to thick film and count parasites/200 wbc.
  b. If there are <5 parasites (4 or lower) /200 wbc continue counting to 500 wbc.
  c. If there is 0 parasite/ 500 wbc report to the doctor that parasites are negative (0/500 wbc).
  d. Look at both thick film and thin film to see the number of parasites and species. Mixed infection is easy to find in thick film and species is easy to identify in thin film.

- **Follow up:**
  a. Count parasites in thin film until there is 1 infected red cell / 2,000 rbc, go to thick film to count.
  b. If it is 0/500 wbc, report to the doctor that the slide is first time negative. The smear has to be negative 2 times before the doctor stops taking slide.

Do not forget to do Hematocrit every time you do the smear.

**Parasite morphology in blood smears by light microscopy:**

1. **Assessment of parasite stage development in thin blood smears:**

   This process is used to assess the stage development of malaria parasites that are still intact inside the red blood cells and the morphology of the parasites can be identified clearly. It is normally used for high parasitaemias (> 0.1% or 1/1,000 rbc).

   Thin smears that contain *P. falciparum* parasites will be assessed for stage development using criteria from Silamut et al (1999) which divides the developmental cycle of the parasite into 8 stages, based on morphology of cytoplasm, the appearance of malarial pigment and number of nuclei. For Schizont stages, the number of nuclei will be counted and recorder on the staging sheet. The number of gametocytes found will be included in the total number of parasites staged.
Criteria for stage development of *P. falciparum* in thin blood smear, as assessed by light microscopy (Silamut et al, 1999).

<table>
<thead>
<tr>
<th>Stage development</th>
<th>Cytoplasm</th>
<th>Nucleus</th>
<th>Pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tiny ring;</td>
<td>Ring form, width &lt; 1/2 of diameter of the nucleus</td>
<td>1 – 2, round at one side of cytoplasm</td>
<td>No</td>
</tr>
<tr>
<td>Small ring</td>
<td>Ring form, width ≥ 1/2 of diameter of the nucleus</td>
<td>1 – 2, round at one side of cytoplasm</td>
<td>No</td>
</tr>
<tr>
<td>Large ring</td>
<td>Ring form, width ≥ diameter of the nucleus</td>
<td>1 – 2, round or elongated</td>
<td>No</td>
</tr>
<tr>
<td>Early trophozoite</td>
<td>Spherical</td>
<td>1 – 2, inside cytoplasm</td>
<td>Faint, pale brown</td>
</tr>
<tr>
<td>Middle trophozoite</td>
<td>Spherical, enlarged, stained dark</td>
<td>1 – 2, pale inside cytoplasm</td>
<td>Brown, clumps</td>
</tr>
<tr>
<td>Late trophozoite</td>
<td>Spherical, ≅ 1/2 of host RBC, stained dark</td>
<td>1 – 2, pale inside cytoplasm</td>
<td>Dark brown clumps</td>
</tr>
<tr>
<td>Early schizont</td>
<td>Spherical, &gt;1/2 of the host RBC, stained dark</td>
<td>3 – 5, irregular, inside cytoplasm</td>
<td>Dark brown clumps</td>
</tr>
<tr>
<td>Late schizont</td>
<td>Spherical, nearly fills the host RBC, stained dark</td>
<td>&gt; 5, round or oval, inside cytoplasm</td>
<td>Dark brown clumps</td>
</tr>
</tbody>
</table>
**P. falciparum at different stages in thin blood smear (Kamolrat Silamut, 1999)**

How to stage parasite:

One hundred (100) parasites will be staged using the 8-channel electronic differential counter. The abnormality of parasite morphology will be marked. The results will be put in the working sheet with parasite count (/1000 rbc or /200 wbc) for analysis.

**To assess malaria parasites in thin blood smear**

1. Assess parasites in the middle of the field that parasites are seen clearly.
2. Assess only parasites that infected red cells are normal (not shrink or distort) and 1 parasite / infected red cell.
3. Try to strict to the same area in every field that we assess until 100 parasites. (see explanation no. 6)
4. Moderate - high parasitaemia (50 - 200 /1,000 rbc); assess the parasites in the middle of the field (eg. area A or B). Do not assess >20 parasites in each field.
5. Very high parasitaemia (>200 /1,000 rbc); Strictly assess the parasites in a straight line along the middle part of the field (line C only) (not >20 parasites) and move to another field until staging up to 100 parasites. (see explanation no. 6)
6. If there are other stages of parasites seen out of the assessing areas eg Schizont is in area A, but not in areas B and C. Do not count but make a note after finish, e.g. Tiny ring 87, Small ring 13 (1 Late schizont found after 100 parasites). This is to mark that there is also schizont stage found in patient’s blood but less than 1%
2. **Assessment of parasite stage development in thick blood smears:**

This process is used to stage the parasites that are loose outside red cells because the red cells are haemolysed. Normally, the parasites appear compact and it is not easy to identify their stages. This method will be used for low parasitaemias (<0.1% or 1/1,000 rbc) or slides that have only thick smears.

Thick blood smears containing *P. falciparum* parasites will be assessed for stage developmental stage using modified criteria from Silamut et al (1999) which divides the parasites into 3 asexual developmental stages and 1 sexual stage (gametocytes), based on the morphology of the cytoplasm, the appearance of malarial pigment and the number of nuclei.

**NOTE:** Number of neutrophils with malaria pigment will be counted/100 neutrophils
Criteria for identifying developmental stages of *P. falciparum* in thick blood smears as assessed by light microscopy (modified from Silamut et al, 1999)

<table>
<thead>
<tr>
<th>Stage development</th>
<th>Cytoplasm</th>
<th>Nucleus</th>
<th>Pigment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ring</td>
<td>Blue, ring shape</td>
<td>Red dot; can be elongated, 1-2 dots.</td>
<td>No</td>
</tr>
<tr>
<td>Trophozoite</td>
<td>Blue, round shape, stained darker than ring (on the same slide).</td>
<td>1-2 red, big dots or irregular shape inside cytoplasm. Sometimes cannot see.</td>
<td>From pale brown to clumped and dark brown, inside cytoplasm.</td>
</tr>
<tr>
<td>Schizont</td>
<td>Blue, round shape, size up to that of red cell.</td>
<td>At least 3 nuclei, purple-blue colour</td>
<td>Dark brown, clumps in the middle of the parasite</td>
</tr>
<tr>
<td>Gametocyte</td>
<td>Crescent shape like a banana</td>
<td>Cannot see</td>
<td>Elongated, like a chopstick, in the middle</td>
</tr>
</tbody>
</table>
Stage development of *P. falciparum* in thick smears

Malaria pigment in neutrophils in thick smears
How to stage parasites in thick films

- One hundred (100) parasites will be staged as: ring, trophozoite, schizont or gametocyte, using the 8-channel electronic differential counter.
- The number of neutrophils with pigment will be counted/100 neutrophils.
- The results will be recorded on the working sheet with parasite count (/1000 rbc or /200 wbc)

To assess malaria parasites in thick smear

1. Assess parasites in the middle of the field in which parasites are seen clearly.
2. Keep strictly to the same area in every field that is assessed. (see explanation no. 6)
3. For moderate to high parasitaemia (4+ or 11-100 parasites/field); assess the parasites in the middle of the field (eg. area A or B). Do not assess >20 parasites in each field, move to another field until a maximum of 100 parasites are staged.
4. For very high parasitaemia (> 4+ or >100 parasites/field); strictly assess the parasites in a straight line along the middle part of the field (line C only) (not >20 parasites), move to another field until a maximum of 100 parasites are staged. (see explanation no. 6)
5. For low parasitaemia (not more than 20 parasites / field) assess all parasites.
6. If there are other stages of parasites seen outside of the assessed areas (eg Schizont in area A, but not in areas B or C), do not count them but make a note in the ‘Note’ column that they are present, e.g. ring 87, troph 13 (1 schizont found after 100 parasites- This is to mark that there are also schizonts found in the patient’s blood, but less than 1%).

Number of parasites staged

1. For slides that have high → very high numbers of parasites (WHO plus system = 4+ → > 4+ or 11-100 to >100 parasites/field), stage 100 parasites.
2. For mid range numbers of parasites (3+ or approximately 1 – 10 parasites / field), stage 100 parasites.
3. For low numbers of parasites (2+ or approximately 11 - 100 parasites/100 fields), stage 50 parasites.
4. For very low numbers of parasites (1+ or approximately 1-11 parasites/100 fields), stage 10 -25 parasites.

NOTE: After staging, report the total number of parasites staged and the number of parasites in each stage as % of this total.
Area to stage parasites in thick blood smear

To clean slides after finish counting and staging:
Put slide face down onto a piece of paper (A4 or news paper) and dab a few times on the paper to absorb the oil - DO NOT WIPE. Clean oil off with Histoclear or Xylene then put the slide on tissue paper to dry. DO NOT WIPE!!!

Reference

Form to record malaria parasite count and parasite stage development

<table>
<thead>
<tr>
<th>Parasite Staging version 1.0 (18 June 2012)</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Site</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Subject No.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Planned time point</th>
<th>Date on slide</th>
<th>Time (24 hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D___ H___</td>
<td>[___</td>
<td>___</td>
</tr>
<tr>
<td></td>
<td>(eg. 01-Jan-2011)</td>
<td>(eg. 14:00)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parasite Count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barcode ID</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Species (more than one allowed)</th>
<th>HCT (%)</th>
<th>If no HCT, please provide Hb g/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ Negative</td>
<td>□ PF</td>
<td>□ PV □ PO □ PM</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pf. parasite count</th>
<th>1,000 RBC</th>
<th>200 WBC</th>
<th>500 WBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ NC</td>
<td>__________</td>
<td>/1,000RBC</td>
<td>/200WBC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pf. gametocytes</th>
<th>1,000 RBC</th>
<th>200 WBC</th>
<th>500 WBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>□ NC</td>
<td>__________</td>
<td>/1,000RBC</td>
<td>/200WBC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Slide quality</th>
<th>□ Good</th>
<th>□ Bad smear</th>
<th>□ Bad staining</th>
<th>□ No smear</th>
<th>□ Many WBC</th>
<th>□ Broken slide</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Parasite Staging</th>
</tr>
</thead>
<tbody>
<tr>
<td>T Ring: __________</td>
</tr>
<tr>
<td>S Ring: __________</td>
</tr>
<tr>
<td>L Ring: __________</td>
</tr>
<tr>
<td>E Troph: __________</td>
</tr>
<tr>
<td>M Troph: __________</td>
</tr>
<tr>
<td>L Troph: __________</td>
</tr>
<tr>
<td>Gam: __________</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sick cyto pyknotic</th>
<th>Sick cyto irregular</th>
<th>Dead no cyto pyknotic</th>
<th>Dead no cyto irregular</th>
<th>Normal morpho</th>
<th>Unidentified</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature: ____________________________</td>
</tr>
</tbody>
</table>
