Date: 22 June 2020

To: International Union of Immunological Societies
FAIS Legacy Project
(IUIS- FLP)

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Subject: Report on FAIS fellowship award

It is well recalled that I have been funded by FAIS fellowship award to support my subsistence expenses for the hands-on training on multi-parameter flowcytometry and immunofluorescence microscopy at University of Texas Medical Branch (UTMB), USA.

Once again, I am grateful for the fund. It helped me to learn a lot of laboratory trainings which I couldn't find in Ethiopia. Attached, alongside this document, is the training report the invoice and some pictures at UTMB.

Regards,

cc: Dr. Tamrat Abebe (PhD Advisor)
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Preface

The aim of my trip to UTMB is two-fold. At one hand, I took hands-on training on different techniques including immunofluorescence staining and microscopy, multi-parameter flow cytometer staining, acquiring and analysis of results. On the other hand, I have done optimization of protocols. For instance, in my PhD study, leftover splenic aspirate sample will be used and processed for immunofluorescence microscopy. However, due to the low volume and cell number of the splenic sample, I have tried to check if cell block works and allows us to do immunofluorescence microscopy from sections as many times as necessary. Moreover, immunohistochemistry (IHC) protocols require microwave oven for antigen retrieval. However, there is no microwave in the field where I collect and process samples. In this regard, testing whether boiling in a water bath replaces the microwave antigen retrieval was necessary.

I would like to thank FAIS Legacy Project to give me this opportunity. I believe that we will keep in touch during the progress of my PhD work. I have tried to include the major activities I was involved during my stay at UTMB.

Sincerely,

Muluneh Ademe
Awardee of FAIS Legacy Project fellowship grant

Contents

Section I: Major activities performed during my training at UTMB

Activity 1: Biosafety level 2 (BSL2) practical training

I have attended a 3 day BSL2 practical training organized by the University of Texas Medical Branch (UTMB) international Biosafety training center.

Activity 2: Immunofluorescence Microscopy

Preparing different cell concentrations for Cell block preparation

- A 10ml 4% formaldehyde suspension with 6 x 10^6 cells used
- 7 different cell suspensions were prepared as follows

Volume taken from original concentration	**4% formaldehyde used to make suspension**	**No. of cells in new preparation**
1 ml	-	6 x 10^5
0.5 ml	0.5 ml	3 x 10^5
250 µl	750 µl	1.5 x 10^5
125 µl	875 µl	7.5 x 10^4
62.5 µl	937.5 µl	3.75 x 10^4
31.25 µl	968.75 µl	1.875 x 10^4
15.6 µl	984.4 µl	9.375 x 10^3

1% agar preparation

- 1g agar should be mixed with 100ml deionized water
- We prepared 10ml agar
    - Mix 0.1g agar + 10ml deionized water
    - Melt in microwave until it forms a bubble (~30 seconds)
    - Refrigerate at 4^{o}c until further use

Embedding

- Turn on wax dripper machine and cooling table
- Use forceps to remove cassette from bucket
- Pop off top of cassette and arrange sample in the middle of one of the metal molds
- Flattest surface should be flat on bottom of molds (different size molds can be used)

- Place the bottom portion of the cassette over top of mold and fill with wax
- Place on cooling table until wax pops out easily
- Store until sections
- NB: if sample is not properly oriented after fixation, melt wax and try it again

Sectioning

- Fill a water bath with ultrapure water and heat to 40-45^{o}C. (maintain this temperature in the process)
- Trimming was started at a thickness of 20 µm.
- Sections were cut at a thickness of 5 µm
    - The first few sections will probably be discarded as they are likely to contain holes caused by trimming.
- Using tweezers (FORECEPS), pick up the ribbons of sections and float them on the surface of the water in the water bath so they flatten out. Use the tweezers to separate the sections.
- Use **charged microscope slides** to pick the sections out of the water bath and store upright in a slide rack.
- Place the slide rack into an oven (60-65^{o}C) to melt the paraffin for 1 hr.
- Stain sections

Training on Microtome machine (Microm HM 325) in "Sara's" laboratory

Materials and Reagents

- Water bath
- Container with ice
- Glass microscope slides
- Microtome and blade
- Oven

Sectioning

- Chill paraffin-embedded tissue blocks on ice before sectioning. Cold wax allows thinner sections to be obtained by providing support for harder elements within the tissue

specimen. The small amount of moisture that penetrates the block from the melting ice will also make the tissue easier to cut.

- Fill a water bath with ultrapure water and heat to 40-45^{o}C. (maintain this temperature in the process)
- Place the blade in the holder, ensure it is secure and set the clearance angle. The clearance angle prevents contact between the knife facet and the face of the block. Follow the microtome manufacturer's instructions for guidance on setting the clearance angle. For Leica blades this is normally between 1^{o} and 5^{o}.
- Insert the paraffin block and orientate so the blade will cut straight across the block.
- Carefully, approach the block with the blade and cut a few thin sections to ensure the positioning is correct. Adjust if necessary.
- Trim the block to expose the tissue surface to a level where a representative section can be cut.
- Trimming is normally started at a thickness of 20 µm.
- Cut sections at a thickness of about 4-5 µm (we cut at 5 µm). The first few sections will probably be discarded as they are likely to contain holes caused by trimming.
- Using tweezers (FORECEPS), pick up the ribbons of sections and float them on the surface of the water in the water bath so they flatten out. Use the tweezers to separate the sections.
- Use **charged microscope slides** to pick the sections out of the water bath and store upright in a slide rack.
- Place the slide rack into an oven (60-65^{o}C) for 1hr.
- Stain sections

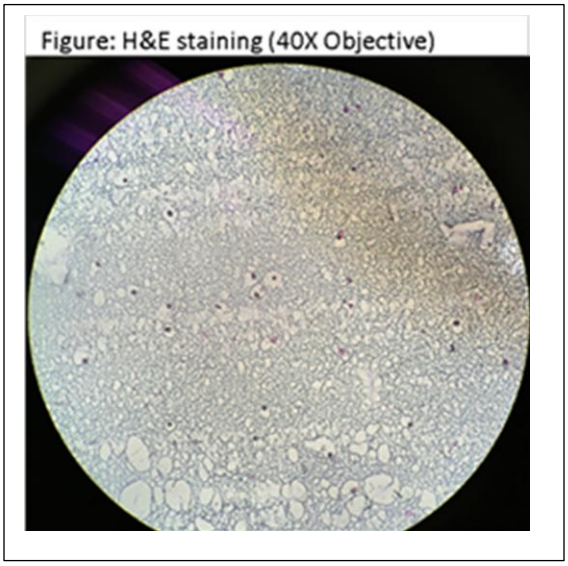
Hematoxylin and eosin staining (H&E) of block sections

- H&E staining comprises of four basic steps
    - Deparaffinization: used to remove paraffin from the section (in this case by clear rite)
    - Rehydration: most staining solutions are aqueous, so to stain the sections, the wax has to be dissolved and replaced with water (in this case a decreasing concentration of flex solution used)
    - Staining by Hematoxylin and eosin (counter stain)

- Dehydration: removes all water from the stained tissue (in this case increasing concentration of flex solution used)

- H&E staining was performed using clear rite and series of flex solutions adopted from "Sara Laboratory" as shown in the following table.

Step	Reagent	Time (min)
1	Clear rite	5
2	Clear rite	5
3	Clear rite	5
4	Flex solution 100%	2
5	Flex solution 100%	2
6	Flex solution 95%	1
7	Flex solution 80%	1
8	Distilled water	2
9	Gills hematoxylin	3
10	Running tap water	4
11	Flex solution 95%	1
12	Eosin	30 sec
13	Flex solution 95%	10 dips
14	Flex solution 100%	1
15	Flex solution 100%	1
16	Clear rite	5
17	Clear rite	5
18	Clear rite	5



Result: As shown in the above figure, the staining formed more debris which may be due to poor de-paraffinization by clear rite, poor rehydration by flex solution or the staining time.

Repeating Hematoxylin and eosin staining (H&E) by increasing the staining time

- Four slides from paraffin section were prepared
- Two slides were processed using "Sara's" lab protocol. The other two slides were processed by increasing the staining time of the same protocol as shown in the following table.

		Old method	New method
Step	**Reagent**	**Time (min)**	**Time (min)**
1	Clear rite	5	5
2	Clear rite	5	5
3	Clear rite	5	10
4	Flex solution 100%	2	5
5	Flex solution 100%	2	5
6	Flex solution 95%	1	1
7	Flex solution 80%	1	2
8	Distilled water	2	2
9	Gills hematoxylin	3	5
10	Running tap water	4	5
11	Flex solution 95%	1	10 dip
12	Eosin	30 sec	30 sec
13	Flex solution 95%	10 dips	5
14	Flex solution 100%	1	3
15	Flex solution 100%	1	3
16	Clear rite	5	5
17	Clear rite	5	5
18	Clear rite	5	5

Result: There was no significant difference in the staining quality of the two methods. This may be due to the less deparaffinising quality of clear rite solution or the use of Flex solution for rehydrating.

Activity 3: Repeat cell block preparation with different cell concentrations

Sample processing for embedding

- Five cell pellets with different cell concentration were prepared
- Cell pellets were mixed with agar with proportionally as shown bellow

Cell concentration	Agar volume
5×10^4	50µl
1×10^5	50µl
2×10^5	50µl
4×10^5	50µl
8×10^5	100µl

- Agar + cell mixture was put in refrigerator (2-8^{o}C) for 20 minutes for agar to solidify
- The sample was placed on plastic cassette labeled with my initials and cell concentrations

- The plastic cassette was put in to a plastic basket and loaded in to a beaker in a machine
- The machine was adjusted to start at 7:10 PM for overnight processing (12hrs.)

  Note: all reagents in Sara's machine was changed with fresh ones

Embedding

- Turn on wax dripper machine and cooling table
- Use forceps to remove cassette from bucket
- Pop off top of cassette and arrange sample in the middle of one of the metal molds
- Flattest surface should be flat on bottom of molds (different size molds can be used)
- Place the bottom portion of the cassette over top of mold and fill with wax
- Place on cooling table until wax pops out easily
- Store until sections
- NB: if sample is not properly oriented after fixation, melt wax and try it again

Sectioning

- Fill a water bath with ultrapure water and heat to 40-45^{o}C. (maintain this temperature in the process)
- Trimming was started at a thickness of 20 µm.
- Sections were cut at a thickness of 5 µm
  - The first few sections will probably be discarded as they are likely to contain holes caused by trimming.
- Using tweezers (FORECEPS), pick up the ribbons of sections and float them on the surface of the water in the water bath so they flatten out. Use the tweezers to separate the sections.
- Use **charged microscope slides** to pick the sections out of the water bath and store upright in a slide rack.
- Place the slide rack into an oven (60-65^{o}C) to melt the paraffin for 1 hr.
- Stain sections

Activity 4: Hoechst staining from cell block

- Paraffin sections prepared from two cell blocks with 1.2 x 106 cells and 2.4 x 106 cells.
- Slides were put in to oven (60-65^{o}C) for 6hrs to melt the paraffin
- Hoechst stain will be done as follows;

- Deparaffinization
  - Xylene 2X for 3 minutes
  - Xylene and 100% ethanol (1:1) for 3 minutes
- Rehydration
  - 100% ethanol 2X for 3 minutes
  - 95% ethanol for 3 minutes
  - 70% ethanol for 3 minutes
  - 50% ethanol for 3 minutes
  - Running water for 5 minutes
- Hydrate with 1x PBS for 10 minutes
- Mark area of sample with hydrophobic pencil
- Add 200 µl Hoechst stain (2µg/ml concentration)
- Incubate in the dark for 10 minutes at RT
- Rinse with 1x PBS
- Mount with antifade mounting medium
- Place coverslip
- Keep the slides in the dark for 24hrs. before microscopy

Activity 5: Hoechst staining from cell pellet

- Pellet cells by centrifugation
- Re-suspend cell pellet in 300 µl 1x PBS for 10 minutes
- Spin at 1500 rpm for 8 minutes
- Discard the supernatant (PBS)
- Re-suspend with 100µl hoechst stain (2µg/ml)
- Incubate for 10 minutes in the dark
- Place one drop on the slide
- Place coverslip
- Evaluate under fluorescent microscope (UV filter) after 24hrs

Activity 6: Fluorescent Microscopy of stained slides

- First, turn on the computer
- Turn on UV light

- Turn on the camera
- Before we begin,
    - The filter selection ring should be set to "1"
    - The 4X objective should be in alignment
    - The fluorescence illumination shutter should be closed
    - The stage should be all the way down.
- Turn on the microscope
- Using 4x objective, bring the image into focus
- Rotate the 10X and then the 40X objectives in to alignment and adjust the fine focus knob for the sharpest image.
- Now we can start fluorescence observation as follows
    - Turn off the illumination
    - Rotate the filter cube selector to "2" to select UV illumination
    - Open the shutter
    - Observe by adjusting the fine focus
    - Note: we can rotate the filter cube selector to "3" to select FITC filter
    - Capture images
    - When we finish work,
        - Turn off the UV light
        - Turn off the camera
        - Turn off the microscope
        - Turn off the computer

Activity 7: Hematoxylin and Eosin staining using xylene and ethanol

- Paraffin sections were put in to oven (60-65oC) for 1 hr. to melt the paraffin
- H&E staining was performed using the following protocol
- The protocol with final dehydration allows to keep the slides permanently. However, the slides without dehydration steps will be used only for short periods.

With final dehydration		
Step	**Reagent**	**Time (min)**
1	Xylene	10
2	Xylene	10
3	Alcohol 100%	5
4	Alcohol 100%	5
5	Alcohol 95%	2
6	Alcohol 70%	2
7	Distilled water	2
8	Gills hematoxylin	4
9	Running tap water	5
10	Alcohol 95%	10 dips
11	Eosin	30 sec
12	Alcohol 95%	5
13	Alcohol 100%	5
14	Alcohol 100%	5
15	Xylene	5
16	Xylene	5

Without dehydration		
Step	**Reagent**	**Time (min)**
1	Xylene	10
2	Xylene	10
3	Alcohol 100%	5
4	Alcohol 100%	5
5	Alcohol 95%	2
6	Alcohol 70%	2
7	Distilled water	2
8	Gills hematoxylin	4
9	Running tap water	5
10	Alcohol 95%	10 dips
11	Eosin	30 sec
12	Running water	2

Result: Slides were prepared from cell blocks with different cell concentrations and their respective cell count is shown below.

label	**Cell block cell concentration**	**Cell count per section**
MAW 0.05	5×10^4	70
MAW 0.1	1×10^5	81
MAW 0.2	2×10^5	150
MAW 0.4	4×10^5	92
MAW 0.8	8×10^5	82

Remark:

1. The background of staining was better than previous H&E stains, however, it has still bubble looking artifacts
2. The cells were small even at 40X

3. Lower cell count for MAW 0.8 will be due to the problem occurred during embedding for this sample. While taking out the agar from Eppendorf tubes, the sample was broken in to pieces.
4. Staining should be repeated from deeper sections

Activity 8: Hoechst staining from different preparations

Hoechst staining for Hematoxylin and eosin stained slides

- H&E stained slides were used
- The following protocol was used for all Hoechst stains
    - Hydrate with 1x PBS for 10 minutes
    - Mark sample area with hydrophobic marker
    - Add 100-300 µl Hoechst stain (4µg/ml)
    - Incubate for 10 minutes in dark area
    - Rinse with 1x PBS to remove excess Hoechst stain
    - Mount with anifade mounting medium
    - Evaluate under fluorescent microscope (UV filter) after 24hrs
    - Note: only H&E stained slides were used as negative control

Hoechst staining for cell pellet (positive control)

- Re-suspend cell pellet in 300 µl 1x PBS for 10 minutes
- Spin at 1500 rpm for 8 minutes
- Discard the supernatant (PBS)
- Re-suspend with 50-100µl hoechst stain (4µg/ml)
- Incubate for 10 minutes in the dark
- Place one drop on the slide
- Place coverslip
- Evaluate under fluorescent microscope (UV filter) after 24hrs

Hoechst staining for Giemsa stained slides

- Prepare Giemsa stained slides as follows
    - Take a cell pellet
    - Make a smear on a slide

    - Air dry
    - Fix with ethanol (100%) for 5 minutes
    - Air dry
    - Cover the sample with undiluted Giemsa stain for 2 minutes
    - Rinse with distilled water
    - Air dry
- Two Giemsa slides were prepared, one for Hoechst stain and the other for negative control.
- Now, the slide is ready for Hoechst stain as described in Activity 1.

Result: the slides will be evaluated the following day together with wright-Giemsa staining slides.

Hoechst staining for wright-Giemsa stained slides

- Prepare cell pellet
- Make a smear on a slide (two slides one for Hoechst stain and the other one for negative control)
- Air dry
- Fix with 100% ethanol for 5 minutes
- Air dry
- Flood the slides with 1 ml wright-Giemsa stain
- Add equal volume (1ml) of buffer immediately
- Mix the stain by blowing for 3-5 minutes
- Wash with water
- Air dry
- Stain one slide with Hoechst stain (as described in Giemsa stained slides) and leave the other one for negative control

Note: the control slides will be mounted with antifade mounting medium.

Result:

	Slides	Hoechst stain	Fluorescence
1	Positive control (cell pellet)	Yes	Yes
2	H & E stained	Yes	No
3	H & E unstained	Yes	Yes
4	Giemsa stained	Yes	Yes
5	Giemsa control	No	No
6	Wright-giemsa stained	Yes	Yes
7	Wright-giemsa control	No	No

Conclusion: Giemsa stained slides and Wright-Giemsa stained slides used for parasitological examination may be used for Immunohistochemistry. However, Hematoxylin and Eosin stained slides do not take immuno-staining.

Activity 9: Cell block preparation from enriched cell populations

CD11b isolation by positive selection

- Since we have frozen cells, it will be thawed with RPMI (10ml)
- Spin at 1500 rpm for 8 minutes and Discard the supernatant
- Re-suspend the cell pellet in 10 ml RPMI
- Prepare a single cell suspension by passing the suspension through 70µm nylon cell strainer
- Spin at 1500 rpm for 8 minutes and Discard the supernatant

- Re-suspend cell pellet in 500µl 1x BD IMag^{TM} buffer
- Count cells in hemocytometer
    - No. of cells were found to be 4.3 x 10^6 ~ 4 x10^6 used for calculations
- Add mouse BD Fc Block at 0.25µg/10^6 cells (in this case 2µl used)
    - Since the number of cells we have is 4 x 10^6 , the mouse BD Fc Block needed will be **1µg**. however, the concentration of **stock** mouse BD Fc Block is 0.5µg/µl. therefore, to get 1µg we need to take **2µl** from the stock mouse BD Fc Block
- Incubate in ice for 15 minutes
- Wash by adding at least equal volume of 1x BD IMag^{TM} buffer
    - In this case, we added 500µl 1x BD IMag^{TM} buffer
    - Spin at 1500 rpm for 8 minutes
- Carefully discard **all** supernatant
- Vortex BD IMag^{TM} anti-mouse CD11b particles
- Add BD IMag^{TM} anti-mouse CD11b particles (50µl particles for every 10^7 cells)
    - Hence, for 4 x 10^6 cells we need **20µl** BD IMag^{TM} anti-mouse CD11b particles
- Mix and refrigerate for 30-40 minutes at 6-12 ^oC.
- Add 1x BD IMag^{TM} buffer at the concentration of 1-8 x 10^7 cell/ml
    - In our case, we need **400µl** 1x BD IMag^{TM} buffer for 4 x 10^6 cells
    - Mix
    - Place in to a magnet for 6-8 minutes at RT
    - Use a glass pipette to take the supernatant with the negative fraction (CD11b –ve cells) and put in to a new test tube
    - Re-suspend the positive fraction (CD11b+ cells attached to the tube) with 500µl 1x BD IMag^{TM} buffer
    - Count both cells in hemocytometer

	Cell count
CD11b+	1.3 x 10^6 cells
CD11b-	3x 10^6 cells

- CD11b+ cells will be used for cell block preparation and Cytospin

Cell block preparation from CD11b+ enriched cell populations

- Cell pellet of 1x 106 cells was mixed with 100µl agar
- Agar allowed to solidify in refrigerator
- Agar + cell pellet was put in to a tissue processing cassette
- The cassette was then put in to a basket
- It was processed in the embedding machine for 12 hrs.
- Embedding will be done tomorrow

Embedding of infiltrated cell

- The infiltrated sample was taken out from the machine
- Embedding was done using paraffin
- Stay at cold to solidify

Testing the CD11b+ cell block with H&E stain and Hoechst stain

Hoechst stain from CD11b+ sample

- Deparaffinized with xylene 2X for 10 minutes each
- Rehydrated by 100% ethanol (2X 5minutes each), 95% ethanol (2min), 70% ethanol(2min)
- Running water for 2 minutes
- 1X PBS for 10 minutes
- Mark sample area with hydrophobic pencil
- Add 200-300µl Hoechst stain (4µg/ml concentration)
- Incubate at RT for 10 min in **dark area**
- Rinse with 1X PBS
- Mount with ANTIFADE mounting medium
- Evaluate

Hematoxylin and eosin staining of CD11b+ sample

- Deparaffinized with xylene 2X for 10 minutes each
- Rehydrated by 100% ethanol (2X 5minutes each), 95% ethanol (2min), 70% ethanol(2min)
- Running water for 2 minutes

- Stain with Gill's hematoxylin for 5 minutes
- Rinse with running water for 5 minutes
- Dip in 95% ethanol 10 times
- Stain with eosin for 30 seconds
- Rinse with running water for 5 minutes
- Evaluate

Result: Both H&E stain and Hoechst stains work well for CD11b cell block

Activity 10: Immunohistochemistry (IHC) using EDTA and Citrate antigen retrieval methods

Buffer preparation for antigen retrieval

a) Citrate Buffer

- Sodium Citrate Buffer (10mM Sodium Citrate, 0.05% Tween 20, pH 6.0):
  - Tri-sodium citrate (dehydrate) --------- 2.94 g
  - Distilled water ---------- 1000 ml
- Mix to dissolve.
- Adjust pH to 6.0 with 1N HCl
- Add 0.5 ml of Tween 20 and mix well.
- Store this solution at room temperature for 3 months or at 4 C for longer storage.

How to prepare 1N HCl from 5N HCL

- Use the formula (N1 V1 = N2 V2)
- We want to prepare 50ml of 1N HCL. So, N1= 1N, V1= 50ml, N2= 5N, V2=???

  1Nx50ml= 5NxV2

  V2= (1Nx50ML)/5N

  V2= 10ml
- Hence, we mix 10ml of 5N HCL with 40ml water to get 50ml of 1N HCL solution

b) EDTA buffer (pH 8)

- EDTA Buffer (1mM EDTA, 0.05% Tween 20, pH 8.0):
  - EDTA (Sigma, Cat# E-5134) ---------- 0.37 g
  - Distilled water ---------- 1000 ml

- Mix to dissolve.
- Adjust pH to 8.0 using 1N NaOH.
- Add 0.5 ml of Tween 20 and mix well.
- Store this solution at room temperature for 3 months or at 4 C for longer storage.

How to prepare 50ml of 1N NaOH

- Molecular weight of NaOH is 40g
- To prepare 1L of 1N NaOH, dissolve 40g in 1000ml distilled water
- In this regard, to prepare 50ml NaOH solution,
    - Dissolve 2g NaOH in 50ml distilled water

Deparaffinization

- Cut paraffin sections in 5µ thickness
- Label 4 slides for EDTA and 4 slides for citrate antigen retrieval as follows: 1^{o} EDTA, 1^{0} Citrate, C1 EDTA, C1 Citrate, C2 EDTA, C2 Citrate, C3 EDTA, C3 Citrate
- Put in to oven (60-65 ^{o}C) for 1 hr. to melt the paraffin
- Deparaffinise with xylene 2X for 10 minutes each
- Rehydrated by 100% ethanol (2X 5minutes each), 95% ethanol (2min), 70% ethanol(2min)
- Running water for 2 minutes

Antigen retrieval through boiling (using EDTA and Citrate buffer)

- Add EDTA buffer and citrate buffer in two beakers (200-300ml)
- Boil until the temperature reaches 95 ^{o}C
- Insert slides (clipping two slides) in to the boiling buffer (4 slides to EDTA buffer and the other 4 slides to citrate buffer)
- Boil for 15 minutes at 95-100 ^{o}C
- Take out slides to cool in running water for 5-10 minutes
- Wash in 1x PBS 2 times for 5 minutes
- Mark the sample area with hydrophobic pencil

Note: the sample should not dry

- Add 3% H_2O_2 solution to cover the sample and incubate for 60 minutes at RT

- Rinse 3 times with 1x PBS
- Add 2 drops of blocking buffer and incubate for 60 minutes
- Remove excess blocking buffer by flicking (Don't wash)
- Add the following and incubate overnight at 2-8 ^{o}C
    - Add 100µl of **1µg/ml** primary antibody (anti-rabbit CD11b in this case) to 1^{o} EDTA and 1^{0} Citrate labeled slides
    - Add 100µlof **1µg/ml** Isotype Rabbit IgG for C1 EDTA and C1 Citrate labelled slides
    - For slides labelled C2 and C3, add blocking buffer for the sample not to dry

Complete IHC from the previous day

- Rinse the sample 3 times with 1x PBS for 10 minutes
- Add 2 drops of poly-HRP conjugate secondary antibody to all except C3 labelled slides
- Incubate for 60 minutes at RT
- Rinse the sample 3 times with 1x PBS for 10 minutes
- Prepare a tyramide working solution according to Table 4. (in our case, we used 6µl 100X Tyramide stock solution, 6µl 100X H2O2 solution, 600µl 1X Reaction Stop Reagent working solution)

  IMPORTANT! Do not use any unused solution after 2 hours of preparation.
- Add 100 µl Tyramide working solution to all except C3 labelled slides
- Incubate for 2-10 minutes in **the dark** at RT (in this case 10 minutes used)
- Remove excess Tyramide working solution by simply flicking
- Add 100 µl Reaction stop reagent for all except C3 labelled slides (~ 1 minute)
- Rinse 3 times with 1x PBS
- Counterstain **all slides** with Hoechst stain
    - Add 100-200 µl Hoechst stain (4µg/ml)
    - Incubate in the dark for 10 minutes
    - Rinse with 1x PBS
    - Mount with antifade mounting medium
    - Apply coverslip

- Evaluate slides (Hoechst stain through UV filter and CD11b+ cells with FITC filter)
- Note: keep slides in dark

How to prepare a tyramide working solution

Table 4. Tyramide working solution

Component	Number of coverslips (18-mm × 18-mm)				
	5	10	20	50	100
100X Tyramide stock solution (Step 1.1)	5 µL	10 µL	20 µL	50 µL	100 µL
100X H_2O_2 solution (Step 1.2)	5 µL	10 µL	20 µL	50 µL	100 µL
1X Reaction buffer (Step 1.3)	500 µL	1 mL	2 mL	5 mL	10 mL

1.1 100X Tyramide stock solution: Dissolve the Alexa Fluor™ tyramide reagent (Component C1) in 150 µL (for 150 slides) or 50 µL (for 50 slides) of DMSO (Component E). Invert the vial several times to dissolve any tyramide that might coat the sides of the vial.

You can store the 100X Tyramide stock solution at 2–8°C for up to 6 months in a sealed vial. Store the vial away from moisture, if possible.

1.2 100X H**2O2 solution:** Add 1 drop (approximately 50 µL) of Hydrogen Peroxide Solution (Component C2) to 1 mL of distilled water.

Note: Prepare the 100X H2O2 solution fresh on the day of use.

1.3 1X Reaction buffer: Add 1 drop (approximately 50 µL) of 20X Reaction buffer to 1 mL of distilled water.

Note: Prepare the 1X Reaction buffer fresh on the day of use. Tris buffer at pH 7.4 can be substituted for Reaction buffer for similar performance. Other HRP enzyme compatible buffers are possible replacements for reaction buffer, but are not tested.

1.4 Reaction **Stop Reagent stock solution:** Add 1.45 mL of 95% ethanol to one vial of Reaction Stop Reagent (Component D). Vortex the vial to dissolve any stop reagent coating the sides of the bottle.

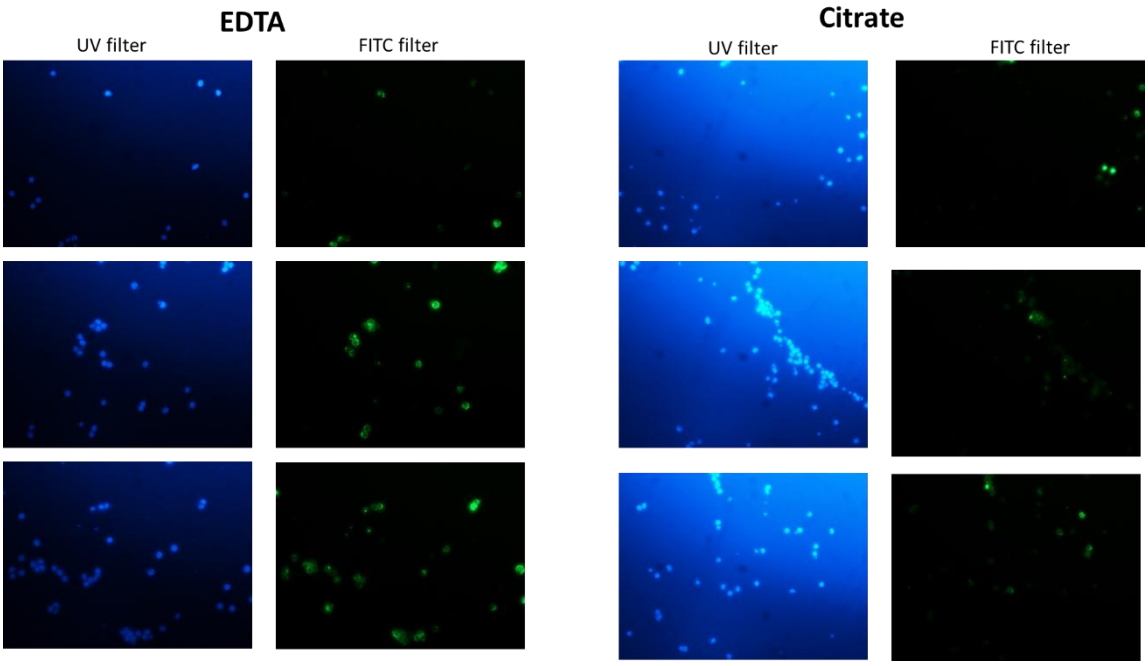
Reaction Stop Reagent stock solution will be diluted 1:11 in PBS before use to prepare a working solution. Unused portion of the stock solution can be stored at –20°C for 6 months.

1.5 Reaction Stop Reagent working solution: Dilute the Reaction Stop Reagent stock solution (prepared in Step 1.4) 1:11 in PBS.

Note: Prepare the Reaction Stop Reagent working solution fresh on the day of use.

Examining IHC stained slides

- All slides evaluated through fluorescent microscopy
- Result:
    - Both EDTA and Citrate Buffer antigen retrieval methods worked well as shown in the following images. However, we noticed a difference in number and intensity of fluorescence in CD11b+ cells.
    - These two methods will be evaluated the following day by counting the number of CD11b+ cells and their fluorescing intensity.



Evaluating IHC slides using EDTA and Citrate antigen retrieval

Cells were counted with both UV filter (all cells) and FITC filter (CD11b+ cells). Moreover, CD11b+ cells were assessed with respect to their brightness as bright and dim. Both EDTA and Citrate methods were evaluated in this regard.

Result:

- ✓ EDTA antigen retrieval method produced more CD11b+ cells as compared to Citrate antigen retrieval method.
- ✓ EDTA antigen retrieval method also produced more bright CD11b+ cells as compared to Citrate antigen retrieval method. See the following tables

		Antigen retrieval method												
		Citrate (PH 6.0)							EDTA (PH 8.0)					
Hoechst		29	22	11	65	105	45	39	61	103	70	25	21	46
CD11b+	Bright	16	5	5	17	7	3	7	9	28	38	15	17	29
	Dim	9	8	3	24	50	13	14	24	64	13	6	3	15
	% CD11b+	86	59	73	63	54	36	54	54	89	73	84	95	96

		Citrate	EDTA
Hoechst		316	326
CD11b+	Bright	60	136
	Dim	121	125
% CD11b+		57%	80%
% bright CD11b+		33%	52%

Conclusion: EDTA antigen retrieval method is better for the retrieval of CD11b+ antigens as compared to Citrate antigen retrieval method.

Activity 11: Comparing Immunohistochemistry from Cytospin and paraffin section

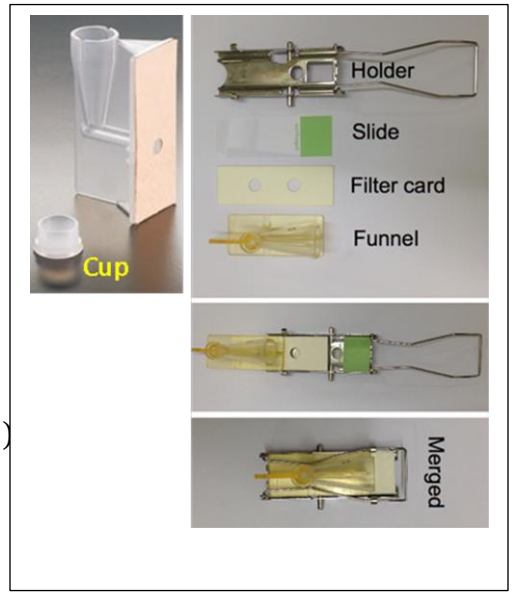
The following slides were compared

- Cytospin (1 slide)
- Paraffin sections from cell block of different cell concentrations (1 slide each)
  - 1 x 10^6 cells (Positive control with CD11b+ cells) --- 1 more slide for isotype included.
  - 4 x 10^5 cells (MAW 0.4)
  - 1 x 10^5 cells (MAW 0.1)
  - 5 x 10^4 cells (MAW 0.05)

Cytospin slides preparation

- Label slides

- Prepare filters card, Holder, Funnel and cytocentrifuge
- Place the slide in to the Holder, the label facing outward
- Place the filter card on top of the slide
- Place the Funnel
- Merge the Holder and the funnel by clipping
- Add sample to the funnel (100 µl ≤ sample ≤ 250µl)
- Add 50 µl (1-2 drops) of medium
- Cup the funnel
- Spin at 800 rpm, medium speed for 5 minutes (**balance**!!)
- Carefully remove slide(s) from cytocentrifuge
- Discard the filter card
- Air dry the sample and process
- In our case, the air dried sample was stored in -20 soaked with ethanol until further processing



Note: be careful, this is not biohazard proof!! Aerosols may be formed.

De-paraffinization (paraffin sections only)

- Paraffin sections were cut in 5µ thickness
- Slides were put in to oven (60-65 ^{o}C) for 1 hr. to melt the paraffin
- Sample area was marked with aluminium pencil
- Deparaffinized with xylene 2X for 10 minutes each
- Rehydrated by 100% ethanol (2X 5minutes each), 95% ethanol (2min), 70% ethanol(2min)
- Washed in running water for 2 minutes

Antigen retrieval through boiling (using EDTA buffer)

- Add EDTA buffer in two beakers (200ml)
- Boil until the temperature reaches 95 ^{o}C
- Insert all slides including cytospin (clipping two slides together) in to the buffer
- Boil for 15 minutes at 95-100 ^{o}C
- Take out slides to cool in running water for 5 minutes

- Wash in 1x PBS 2 times for 5 minutes
- Mark the sample area with hydrophobic pencil
- Add 2 drops of 3% H_2O_2 solution to cover the sample and incubate for 60 minutes at RT
- Rinse 3 times with 1x PBS
- Add 2 drops of blocking buffer and incubate for 60 minutes at RT
- Remove excess blocking buffer by flicking (Don't wash)
- Add the following and incubate overnight at 2-8 ^{o}C (use wet paper towel for the slides not to dry)
    - Primary antibody: add 100µl of **1µg/ml** primary antibody (anti- CD11b rabbit monoclonal antibody) to five slides (except the isotype slide).
        - Dilution of primary antibody: 2.04 µl anti- CD11b rabbit monoclonal antibody + 500 µl blocking buffer
    - Isotype: add 100µlof **1µg/ml** Isotype Rabbit IgG for isotype slide
        - Dilution of Isotype Rabbit IgG: 0.04 µl Isotype Rabbit IgG + 100 µl blocking buffer

Completion of IHC from yesterday

- Rinse the sample 3 times with 1x PBS for 10 minutes
- Add 2 drops of poly-HRP conjugate secondary antibody to all slides and Incubate for 60 minutes at RT
- Rinse the sample 3 times with 1x PBS for 10 minutes
- Prepare fresh tyramide working solution (in our case, we used 6µl 100X Tyramide stock solution, 6µl 100X H2O2 solution, 600µl 1X Reaction Stop Reagent working solution) **IMPORTANT**! Do not use any unused solution after 2 hours of preparation.
- Add 100 µl Tyramide working solution to all slides
- Incubate for 10 minutes in **the dark** at RT
- Remove excess Tyramide working solution by simply flicking
- Add 100 µl Reaction stop reagent for all slides (~ 1 minute)
- Rinse 3 times with 1x PBS
- Counterstain **all slides** with Hoechst stain
    - Add 100-200 µl Hoechst stain (4µg/ml)

- Incubate in the dark for 10 minutes
- Rinse with 1x PBS
- Mount with antifade mounting medium
- Apply coverslip
- Evaluate slides (Hoechst stain through UV filter and CD11b+ cells with FITC filter)
- Note: keep slides in dark before and after evaluation

Activity 12: Multi-parameter Flow cytometer protocol optimization

Flow cytometery will be used to determine the phenotype of myeloid cells (neutrophils, macrophages and myeloid-derived suppressor cells) in the peripheral blood of subjects with active VL (before and after treatment) and age/gender-matched endemic controls. This allows us to see the difference in myeloid cell population before and after initiation of treatment among VL patients. Moreover, the findings will be used to compare the difference in myeloid cell population in the periphery and at the site of visceral infection.

Procedure

Step 1: Label 7 flow cytometer tubes for each study subject and one tube for unstained control as follows

- Panel 1: patient code + MDSC-PDL-1
- Panel 2: Patient code + MDSC-CD66b
- Panel 3: patient code + FMO for MDSC
- Panel 4a: patient code + PD-1 FMO
- Panel 4b: patient code + T-cell-CTLA4
- Panel 5a: patient code + T-cell- PD-1
- Panel 5b: patient code + CTLA4 FMO
- Panel 6: Unstained control (1 control is sufficient per experiment)

Step 2: Surface staining for all tubes

1. Add antibodies as follows

a. For panel 1, panel 2 and panel 3: add **12.5µl of mixed MDSC**
b. For panel 1, add **2.5 µl of PDL-1 PE**
c. For panel 2, add **2.5 µl of CD66b PE**
d. For panel 3, add **2.5 µl of IgG PE**
e. For panel 4a and panel 5a: add **7.5 µl of mixed T-cell**
f. For panel 4a, add **2.5 µl of IgG APC**
g. For panel 5a, add **2.5 µl of PD-1 APC**

2. Add 100µl whole blood to all 6 tubes except panel 4b and 5b
3. Mix using pipette
4. Incubate at RT for 20 minutes in the dark (use aluminum foil)
5. Add 1ml of RBC Lysis buffer
6. Incubate at RT for 10 minutes in dark
7. Add 3 ml FACS buffer (1xPBS)
8. Centrifuge at 1700 rpm for 5 minutes or Angulate rotor (2500rpm for 5 minutes)
9. Decant supernatant
10. Repeat washing
11. For panel 1,2 and 3:
    a. add 1ml of freezing media (DMSO+FBS)
    b. Freeze at -20 with cryovials
12. Panel 4a and 5a
    a. Split residue in to two (one half remain at panel 4a and 5a, transfer the other half at panel 4b and 5b)
    b. For 4a and 5a, add 1ml freezing media and store at-20
    c. For 4b and 5b, undergo cytoplasmic stainig as follows

Step 3: Cytoplasmic staining (only for panel 4b and 5b)

1. Add 400 µl Perm2 buffer to panel 4b and 5b
2. Incubate for 20 minutes at room temperature
3. Add 3ml PBS or FACS buffer to panel 4b and 5b
4. Centrifuge at 2000rpm for 12 minutes or Angulate rotor (3000rpm for 12 minutes)
5. Decant supernatant
6. For 4b, add 2.5µl of CTLA4 PE, for panel 5b, add 2.5µl of IgG1 PE
7. Incubate for 20 minutes
8. Add 3ml FACS buffer or PBS
9. Centrifuge at 2000rpm for 12 minutes or Angulate rotor (3000rpm for 12 minutes)
10. Fix with 0.5ml 2% formaldehyde for 30 minutes
11. Wash by adding 3ml 3ml FACS buffer or PBS
12. Centrifuge at 2000rpm for 12 minutes or Angulate rotor (3000rpm for 12 minutes)
13. Decant supernatant
14. Wash again
15. Decant supernatant
16. Re-suspend in 1ml of freezing media (DMSO+FBS)
17. Freeze at -20 with cryovials

Note: freezing with DMSO was needed because flowcytometry stained samples in the field where sample is collected has to be stored before it is acquired.

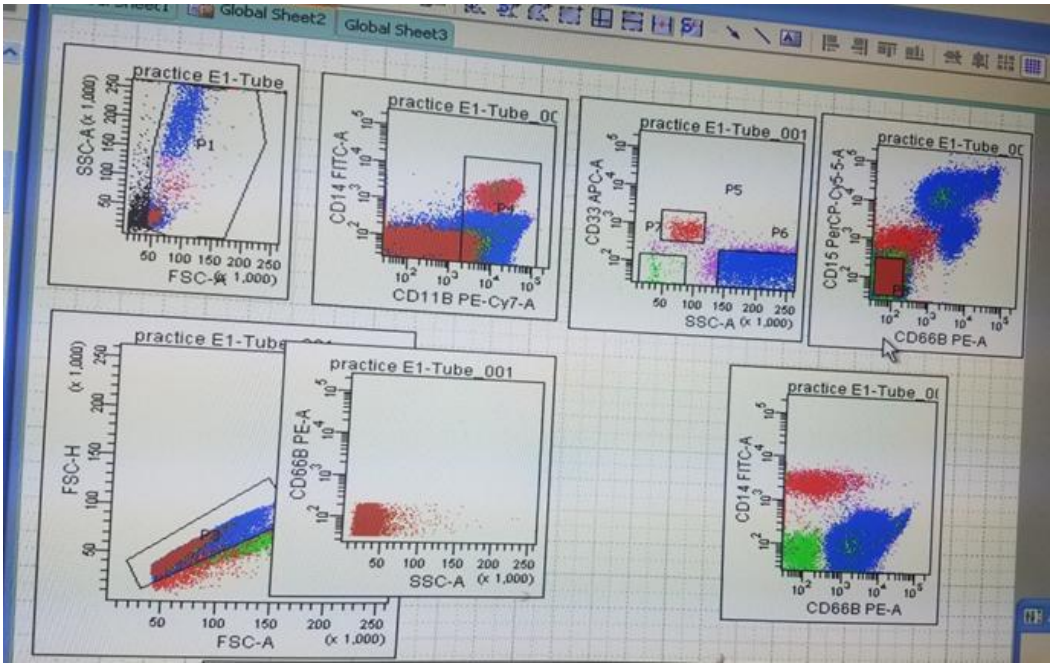


Figure: Mobile picture taken from the BD FACSCanto II Flow Cytometer

Section II: Challenges

1. The peak season for visceral leishmaniasis passed by the time we started phase I sample collection. On the other hand, there was political instability in Ethiopia, which made field works very challenging because road closures were happening here and there. For this reasons, we were forced to postpone first round sample collection which in turn delayed my travel to UTMB for a couple of months than our previous schedule.
2. The local bank here in Ethiopia provided us the grant with local currency, and we incurred additional costs to change back to foreign currency.
3. The COVID-19 situation also changed everything, and we were busy in organizing the Molecular lab for the purpose of testing COVID-19. For this reason, I didn't have sufficient time to sit and organize my reports, and I am sorry for the inconvenience.

Section III: Invoice

INVOICE

Date: 6/22/2020

Invoice issued to:

International Union of Immunological Societies

FAIS Legacy Project

(IUIS- FLP)

Address: Muluneh Ademe Worku

Department of Microbiology, Immunology and Parasitology

Schcool of Medicine, College of Health sciences,

Addis Ababa University

Entoto Road, Churchill Avenue Addis Abab, Ethiopia

e-mail: muluneh.ademe@aau.edu.et

Tel: +251912634868

Award Title: The FAIS Legacy Project fellowship Award

Award Amount: €4,350.00

Description	**Bill Amount**/month	**Duration**	**Total**
Living cost for 3 months (Per diem)	€800.00	3 months	€2,400.00
Home rent	€600.00	3 months	€1800.00
Ground transport (Bus)	€50.00	3 months	€ 150.00
Total Amount Due:			**€4,350.00**

By signing this report, I certify that the report is true, complete, and accurate and the disbursements are for the purposes and objectives set forth in the terms and conditions of the award.

Name: Muluneh Ademe _____ Signature: _____

Annex 1: pictures at UTMB

A picture at UTMB

Visiting NASA, Texas