SANGER INSTITUTE
STANDARD OPERATING PROCEDURE

SUBJECT: Recessive Lethality embryo screen

INTRODUCTION:

The main aim of the procedure is to determine whether homozygote mouse embryos are surviving to the embryonic day (E) 14.5. This is achieved by obtaining at least 28 genotyped E14.5 embryos from Het x Het matings. During this process we identify potential embryonic abnormalities which could impair the survival of any homozygous animals, if applicable abnormalities are recorded on data capture forms (DCFs) and images are taken; using a Leica M205c microscope and Leica LAS software.

The embryos are fixed in Bouin's fixative and placentas are fixed in PFA. If applicable, homozygotes and 3 wild types from each knockout line are sent for HREM analysis. All placentas from each hom viable line are sent for histological analysis.

Some litters will have a bleed out procedure applied to them, please see appendix A for this. This procedure is licensed and performed in the animal house.

This is a regulated procedure

ABBREVIATIONS:

DCF – Data Capture Form
Hom – Homozygous
MIG – Mouse informatics group
NaN3 – Sodium Azide
PBS – Phosphate Buffered Saline solution
PFA – Paraformaldehyde
RT – Room temperature
WT– Wild type

HEALTH & SAFETY:

This procedure is covered by the following risk assessment WTSI_1380.

- Appropriate personal protective equipment (PPE) is to be worn at all times when handling the samples (white coat and gloves).
- Access to a functional down-flow table and fume hood is required
- Safety glasses must be worn when handling fixatives
- Eye wash stations, fire extinguishers and spill kits are located in the area.
- New workers are to be supervised until deemed competent to perform this assay.
- Individual risk assessments for lone workers, young persons and new or expectant mothers would be performed to define any exclusion for performing this assay.

RESPONSIBILITIES

All staff performing this procedure are responsible for ensuring that this Standard Operating Procedure (SOP) has been read, understood and where applicable is followed in accordance with the relevant Procedure Project License (PPL).

All staff should be trained and competent to perform the procedure, where applicable they should also be licensed to perform the procedure with a valid Procedure Individual License (PIL).

STAFF: This procedure requires one member of staff per embryo litter to be performed optimally.

RESOURCES

Equipment and reagents

1. Fume hood and down flow bench
2. Polystyrene ice box & ice bucket
3. 50ml falcon tubes (No. PLTU0002.)
4. Ice cold PBS pH 7.4 (Invitrogen: Catalogue No. 70011051.)
5. Dissecting instruments (#5 forceps, scissors, perforated spoon; Fine Science Tools)
6. 10cm plastic petri dishes
7. 6cm plastic petri dishes
8. Bijoux 7ml tubes
9. Yellow and red boxes (Starlab)
10. Genotyping PCR plates (ThermoFisher UK Ltd: 10363173)
11. PCR plate cooler blocks (eppendorf)
12. Leica microscope (Leica (DFC495 – camera & M205c – Microscope)
13. Computer with Leica software (LAS) and sanger MIG database access
14. PBS pH7.4 (Sanger media lab)
15. Incubator (38-39°C)
16. Rocker
17. Large heat mat
18. HBSS no calcium, magnesium or phenol red *(LifeTech 14175095)*
19. EDTA 0.5M *(Life Tech 15575-020)*
20. Bouin's Solution (Sigma: HT10132)
    Bouin's is harmful, carcinogenic, an irritant, a skin sensitizer and explosive in dry form.
    To be used under an effective fume hood and wearing correct PPE.
21. 4% PFA (*EMS 15714-S)* **1 week max shelf life**
    PFA is toxic, harmful, corrosive, an irritant, a skin sensitizer, possibly carcinogenic and can cause damage to organs.
    To be used under a fume hood and wearing correct PPE
22. PBS with 0.01% Sodium Azide (NaN3; Sigma: S8032)
    Sodium Azide is toxic (to aquatic life) and an irritant.
    To be used under an effective fume hood and wearing correct PPE

METHOD - Collection of Embryos in Animal Facility

Before performing the procedure, verify that this is the correct mouse for the procedure.

1. Refer to the daily plan circulated on the mailing list to know about any specific instruction for collection. On a general rule, a maximum of one litter per dissector should be collected at any one time.

2. Locate the female in correct Animal room. Fill in the log book, and transfer the female to the rack in room experimental room. Set up the downflow table for harvesting.

3. Prepare 50 mL falcon tubes of cold PBS pH pH7.4 (one per litter collected) and place on ice. These may be already available and ready to use in the fridge

   Note: Once removed from the mother the uterus should spend **no more than 30 minutes on ice.**

4. **On a down-flow table**, sacrifice the female by cervical dislocation. Check that the female is unresponsive. Damp the fur with 70% Ethanol. Open up the body cavity and confirm death by removal of the heart.

5. Remove the whole uterus. Count the embryos (and resorptions if necessary) and transfer them to the cold PBS. Store on ice. Label the 50ml tube with the litter name to identify the sample.

6. Record the number of embryos on the mating card for database entry later.

   The residual animal carcass must be disposed of in a cadaver waste bag, sealed, and put in the dedicated freezer.

   Clean equipment and surfaces. All dissection instruments should be scrubbed clean in the sink with antibacterial solution.

7. Transfer embryos, on ice, to lab.

METHOD – Embryo Dissection in the Sulston Lab

8. Cut open uterus. Remove the embryo & placenta from uterus by holding onto the uterus with one set of forceps and using the other forceps to guide the embryo & placenta away from the uterus. Be careful not to damage the placenta or embryo.

9. Recount the embryos (and resorptions if necessary). Transfer embryos into a dish of clean PBS (to avoid contamination) and place on ice.

10. Detach embryos from the placenta using fine dissection tools. Remove the yolk sac and the embryonic membrane.

11. Collect two small pieces of yolk sac into two 96 well genotyping plates (on cooler block). One for genotyping and one for back-up. Label plates Yolk 1 and Yolk 2. *Note: Ensure litters of different allele types are placed on different plates*

12. Check the gross morphology of the embryos. If the embryo appears abnormal, take a photo to show the dsymorphology and make notes on the cage card.

13. Transfer embryo, using a perforated spoon, into 7ml Bijoux tubes containing ice cold PBS. Mark the lid of the tube with the colony prefix and embryo name (i.e.MXXX65.1a) for first embryo, and embryo name for the rest of the litter (65.1a).

14. Transfer placenta, using a perforated spoon, into 7ml Bijoux tubes containing ice cold PBS. Mark the lid of the tube with a red pen with the colony prefix and embryo name (i.e.MXXX65.1a) for first placenta, and embryo name for the rest of the litter (65.1a).

15. Repeat until all samples for litter are dissected.

    Note: Clean forceps between embryos to avoid sample cross contamination

    PBS waste containing biological contaminants must be treated with Virkon for 10 minutes, then diluted 5 times with water and disposed of down a laboratory sink.

    Solid biological waste must be frozen in the -20C in a biohazard bag and given to the Sanger Waste Team for disposal.

    Transfer all other waste to a yellow offensive waste bag or clearly labelled waste container.

16. Add litter to mating or mark as 'not pregnant':
    a) Enter any abnormalities observed in the mother (e.g. inflamed uterus).
    b) Cull the female and under Licensing Procedure **ensure the correct procedure and licencing information is added**:

17. Print tube labels for embryo and placenta collections and stick onto tubes.

18. Place Embryo tubes in **yellow "temporary box"** and label box with collection date. Place Placenta tubes in **red "temporary box"** and label box with collection date and litter name

Clean equipment and surfaces. All dissection instruments should be scrubbed clean of blood in the sink soaked in antibacterial solution.

METHOD – Fixation of Tissues

Important Note: Embryos should never be left dry. When changing solutions, always keep embryos in enough residual solution to cover them as they are extremely fragile at this stage and can easily dry out and become damaged.

1. **In a fume hood**, remove the PBS from the embryos using a Pasteur pipette or perforated spoon. Take care not to remove or damage the embryos.

2. Replace the PBS with Bouin's Solution. **Store at RT in a safety cabinet.**

3. **In a fume hood**, remove the PBS from the placentas using a Pasteur pipette. Replace the PBS with 4% PFA Solution and store at 4C.
   Important note: 4% PFA loses its effectiveness with time. Always try and use freshly made 4% PFA solution. If any 4% PFA solution is left from previous days only use it if it's not older than 1 week

4. Mark litters on the white board under the relevant section and leave to fix for 24 hours, or 72 hours over the weekend.

   PBS waste containing biological contaminants must be treated with Virkon for 10 minutes, then diluted 5 times with water and disposed of down a laboratory sink.

METHOD – Database entry

1. **Create cohort:** Enter a description of the cohort (e.g. gene names) and select the MGP Recessive Lethality pipeline. Then add the appropriate litters to the cohort and save it.

2. **Create and register genotyping list:**

   a) If tissues were not collected for any reason, ensure that this is recorded on the genotyping grid.
   b) **Yolk sac genotyping samples:** Print off a copy of the genotyping grid for the samples and spin the plates in the lab centrifuge. Submit both paperwork and genotyping plates to the genotyping team freezer.
   c) **Back-up Yolk sac genotyping samples:** Place the yolk sac genotyping plate in the freezer, in the box labelled Embryo 'x'.

3. **Data Capture:** Add the 'Embryo collection E14.5' DCF to the appropriate litters.
   a) For the first embryo of the litter only (i.e. for "xxxx.1a" only), enter the number of reabsorptions which were observed (enter 0 if there were none).
   b) Fill in the DCF as appropriate with fixatives and any relevant abnormal dysmorphology, add any comment if needed.

4. **Embryo dysmorphology images:** Check that any images taken have been uploaded onto the database.

METHOD – Removal of Fixative

Important Note: Embryos should never be left dry. Embryos are extremely fragile and can easily dry out, causing them to be damaged. When changing solutions, always keep embryos covered in enough residual solution and work through the litter one sample at a time.

1. After 24 hours (or over weekend) fixation, **in a fume hood,** remove Bouin's from the embryos and wash 3 times with PBS pH7.4.

   Bouin's waste must be disposed into the Bouin's waste bucket and given to the Sanger Waste Team for disposal.

2. **In a fume hood** also remove PFA from the placenta and wash 3 times with PBS pH7.4

   PFA waste must be disposed into the PFA waste bucket and given to the Sanger Waste Team for disposal.

3. Remove the final PBS wash from embryos and placentas and fill tubes with PBS containing 0.01% NaN3. Store the embryos at RT in a safety cabinet, and store placentas at 4°C.
   - Consolidate boxes based on colony prefix to facilitate archiving and maximise storage use.
   - Once the safety cabinet is full, transfer embryo boxes for lines that are no more under investigation (finished collection) in the extra storage space at RT
- Once the fridge is full transfer placenta boxes for lines that are no more under investigation (finished collection) to the fridge for collaborator collection

4. Erase the embryo and placenta litters from the white board.

***Appendix A*: Harvest of E14.5 embryos for bleed out procedure & HREM inside the RSF**

The purpose of this procedure is to collect, dissect and bleed out embryo by severing the umbilical cord for improving the quality of HREM imaging.

This is a regulated procedure

Plastic wear and HBSS used during this procedure must be kept in the incubator in so that is pre-warmed. Always stock some plastic wear and at least 1 sealed HBSS in the incubator for future procedures. PBS is to be kept in reagents-fridge at 4°C.

PROCEDURE:

Set Up:

1. Take a polystyrene bucket from RSF store and fill with ice

2. **Go to experimental room and set up for procedure**
   - Turn on the downflow table as it will take a couple of minutes to be fully operative
   - Plug in/turn on microscope, rocker and heat mats.
   - Cover heat mats with foil to prevent heat loss
   - Prepare the warm HBSS + 0.01M EDTA solution: add 5ml of 0.5 EDTA in 500mL HBSS, date the bottle accordingly (dispose of any solution not used within 2 weeks).
   - Fill up a 50mL falcon tube, several 6cm petri dishes, one 6 well plate and one 10cm petri dish with the warm HBSS+EDTA solution. Place back in the incubator to keep them warm.
   - Take several pre-filled 7mL Bijoux tubes from the fridge in R1-58 and place them in the bijoux rack
   - Place 2 genotyping plates on cooler blocks
   - Set up the down-flow table for when you later sacrifice the female

3. **Go to Animal room** and locate all pregnant females, on dispatch rack 8, requiring litters to be bled out. Transfer these females to the holding rack in experimental room

Dissection (1 litter at a time):

4. **On the down-flow table in experimental room** sacrifice female by cervical dislocation. Check the female is unresponsive. Damp the fur with 70% Ethanol. Open up the body cavity and confirm death by heart removal.

5. Remove the uterus and count the embryos and resorptions; record this on the cage card. Transfer litter into 50mL tube filled with HBSS+EDTA solution and immediately return to the incubator. Clean up the down flow table.

The residual animal carcass must be disposed of in a cadaver waste bag, sealed and put in the appropriate freezer in
Clean dissection tools and cork board, scrubbing off any blood residue, in the sink with antibacterial solution

6. Depending on the number of embryos within the uterus, fill up more plastic wear with HBSS + EDTA warm solution if needed.

7. Transfer litter from 50mL tube to 10cm petri dish. Remove the embryos from the uterus under the microscope and transfer each embryo into a separate 6 cm dish filled with HBSS + EDTA solution. Place each dish, with the lid on, back in the incubator until ready for dissection. **Ensure embryos are entirely submerged in warm solution at all times.**

8. Dissect one embryo at a time. Remove the embryos from the yolk sac keeping the yolk sac and placenta intact. Extract embryos from yolk sac and cut the umbilical cord immediately by using 2 forceps to rip it off.

   Do not clamp the umbilical cord (this will aid clotting) and do not cut too close to the belly in order to avoid cutting the intestines (that are still herniated out at this stage).

   Cut as close to the placenta as possible, without causing damage to the tissue. This will allow more space to tear the umbilical cord a second time, closer to the embryo, to assure a complete bleed out of the sample.

   Please record the presence of oedema at this stage as it will be more difficult to spot once the procedure is finished. Any other abnormality will be checked later as it is vital that embryos are transferred immediately on the rocker.

9. Transfer embryo into one of the wells of the six well plate (previously filled with warm HBSS + EDTA solution); note the embryo ID on the plate lid and let them gently shake on the heat mat on the rocker. Cover plate with a tent of foil to trap warmth while the embryos shake.

10. For genotyping, collect two pieces of yolk sac and transfer them to two 96 well genotyping plates. One plate to be designated to the genotyping team (bleed out 1) and the other plate to be used for back-up samples (bleed out 2). Clean forceps with ethanol wipes between different embryos.

11. Transfer the placenta straight into a 7ml tube, pre-filled with cold PBS, mark the lid of the tube with the sample ID and place on ice.

12. Continue the bleed out procedure on the rest of the litter. After every third embryo, go back to the previously dissected embryos to ensure they are still bleeding out. If a clot has formed, or the embryo has stopped bleeding, open up the umbilical cord (being careful not to damage the umbilical hernia) to allow for further blood loss. Return embryos to the heated rocker and continue dissecting.

13. Once embryos are completely bled out, check them for any abnormalities and note them on the cage card. Take any pictures (if needed) that best show the abnormality displayed by the embryos.

14. Transfer the embryo into a 7ml tube, pre-filled with cold PBS, mark the lid of the tube with the sample ID and place on ice

15. Repeat procedure with next female if applicable. If litters can be on the same genotyping plate, record (on cage card) the row order of the genotyping samples. Otherwise, if different alleles, start a new pair of genotyping plates.

After dissection:

16. Turn off instruments, clean up room and wipe incubator with Ethanol wipes to remove any spilt liquid.

17. Seal the genotyping plates and put on ice.

Liquid waste containing biological contaminants must be treated with Virkon for 10 minutes, then diluted 5 times with water and disposed of down R1-59a sink.

Dispose of the cage in the 'dirty cage drop' in room

18. Stock the incubator with new plastic wear (and one sealed HBSS bottle if necessary) for future procedures.

METHOD – Database entry

5. Add litter/s to mating/s:
    a) Enter the number of embryos collected or check the box marked "Not pregnant".
    b) In the main Comments box enter any abnormalities observed in the mother (e.g. inflamed uterus, all embryos in one uterine horn). **Select the appropriate Harvesting Procedure: Regulated or non regulated**.
        1. If Embryos age is <= E13 tick "Unregulated Harvest" box
        2. If your Embryos are protected (>=E13 for our licence), cull the female and under Licensing Procedure **ensure the correct procedure and licencing information is added**: 'Regulated Embryo Harvest', PPL

6. Print tube labels for embryo and placenta.

7. **Create cohort:** Enter a description of the cohort (e.g. gene names) and select the MGP Recessive Lethality pipeline. Then add the appropriate litters to the cohort and save it.
   Please Note: Do not create a new cohort if another cohort already exists for litters dissected that day. In this case, only add your litters to the existing one.

8. ADD "Embryo Exsanguination" REGULATED PROCEDURE TO THE LITTERS.

9. **Create and register genotyping list:**

   a) If tissues were not collected for any reason, ensure that this is recorded on the genotyping grid.
   b) **Yolk sac genotyping samples:** Print off a copy of the genotyping grid for the samples and spin the plates in the lab centrifuge. Submit both paperwork and genotyping plates to the genotyping team freezer.
   c) **Back-up Yolk sac genotyping samples:** Place the yolk sac genotyping plate in the freezer, in the box labelled Embryo 'x'.

10. **Data Capture:** Add the 'Embryo collection E14.5' DCF to the appropriate litters.
    a) For the first embryo of the litter only (i.e. for "xxxx.1a" only), enter the number of reabsorptions which were observed (enter 0 if there were none).
    b) Fill in the DCF as appropriate with fixatives and any relevant abnormal dysmorphology, add any comment if needed.

11. **Embryo dysmorphology images:** Check that any images taken have been uploaded onto the database.

12. Return cage/s to necropsy room and if applicable collect genotyping sheets and labels.

13. Return to Lab carrying embryos, placentas and genotyping plates on ice.

TROUBLESHOOTING:

Unlike with microscope or incubator equipment failure, this procedure can be performed if the rocker is not functional. In this scenario, place the 6 well plate filled with warm HBSS solution in the incubator, with the lid on, and transfer the dissected embryos in as normal.

Every time you are transferring a new sample in, gently stir the warm solution in those wells bearing embryos. Do not touch embryos while doing this step to avoid any damage. The purpose of this operation is to avoid the clot formation that can result from the umbilical cord sticking to the well surface.

Appendix B: Use of LeicaLAS for microscope imaging

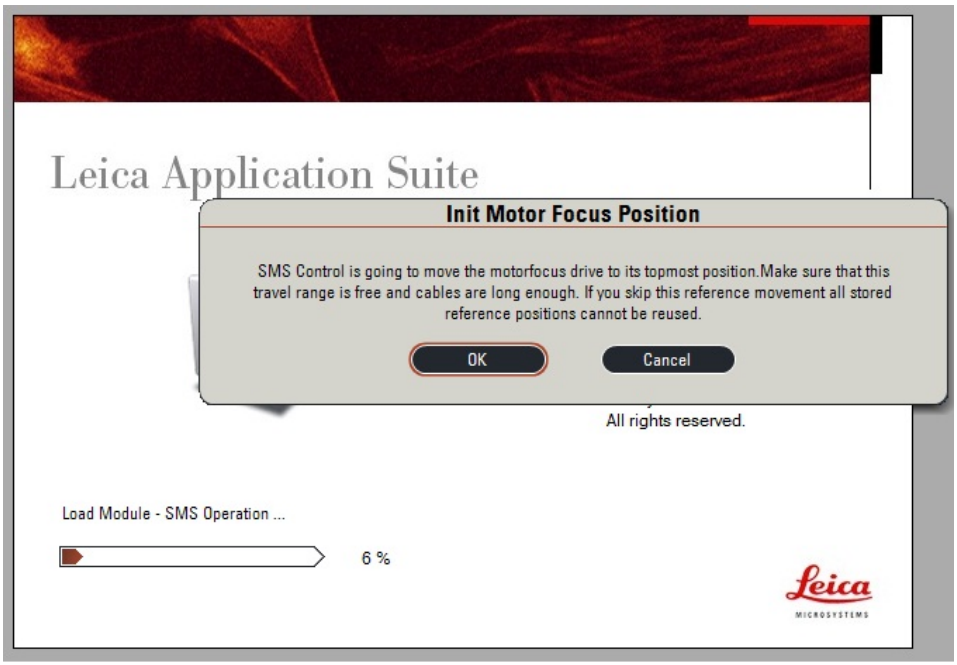
The aim of this procedure is to image tissues and samples under the microscope using Leica LAS v4.4 software.

METHOD

1. ***Turn on microscope***

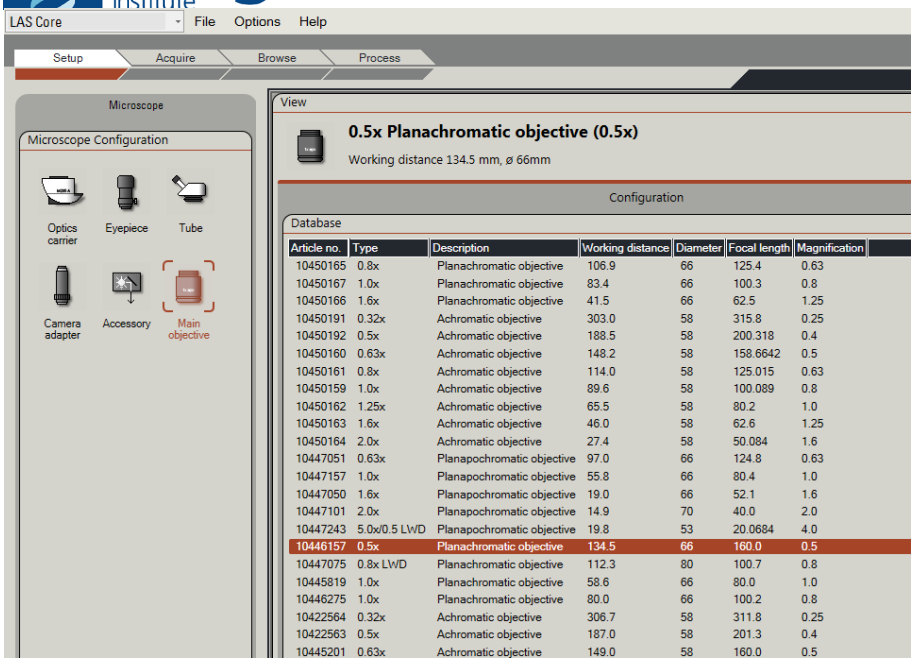
2. ***Start Leica LAS v4.4 software***

3. **Motor Focus Position** - Upon start-up, the software will request to move the microscope so it can calibrate the zoom/focus and scale the images correctly. Ensure the microscope is uncovered and the cables do not restrict its movement, and then click "OK".



4. **Software Settings**

- Check the correct objective is selected
  - Go to the Set up tab
  - Click on Main objective

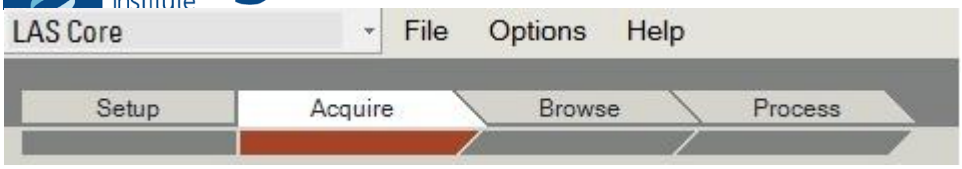


- **The objective displayed** (at the top of the screen in section 'view') **must match the lens on the microscope.**
-
- If not select the correct objective The standard/default objective is <u>10446157</u> - <u>0.5x Planapochromatic objective</u> - working distance 123.5 – Diameter 66 – Focal length 160.0 - Magnification 0.5
- Turn off software and microscope and wait 5 -10 seconds
- Turn on microscope and then the LASV4 software
- Check that the right objective is selected.

- This is the default settings for all the microscopes, as they are all fitted with the same lens. Once you have followed these steps, it should also be the default on your LASV4 software program.
- **<u>If changing the objective</u>** from 0.5x to 1x, or vice versa, make sure you **change *the software settings before changing the lens.*** Avoidance of doing so will result in a wrong scale bar after the change of lens. If you wish to change the objective, <u>our x1 lens is 10447157</u>.
- Change the lens on the main objective tab, then physically change the lens, then turn off the software and the microscope, wait 5-10 seconds and turn back on. **Always check the scale bar using a ruler each time you change the lens** to ensure it has been set up properly. If you change the lens, **always return the 0.5x objective and the software to its default settings at the end of your session**.

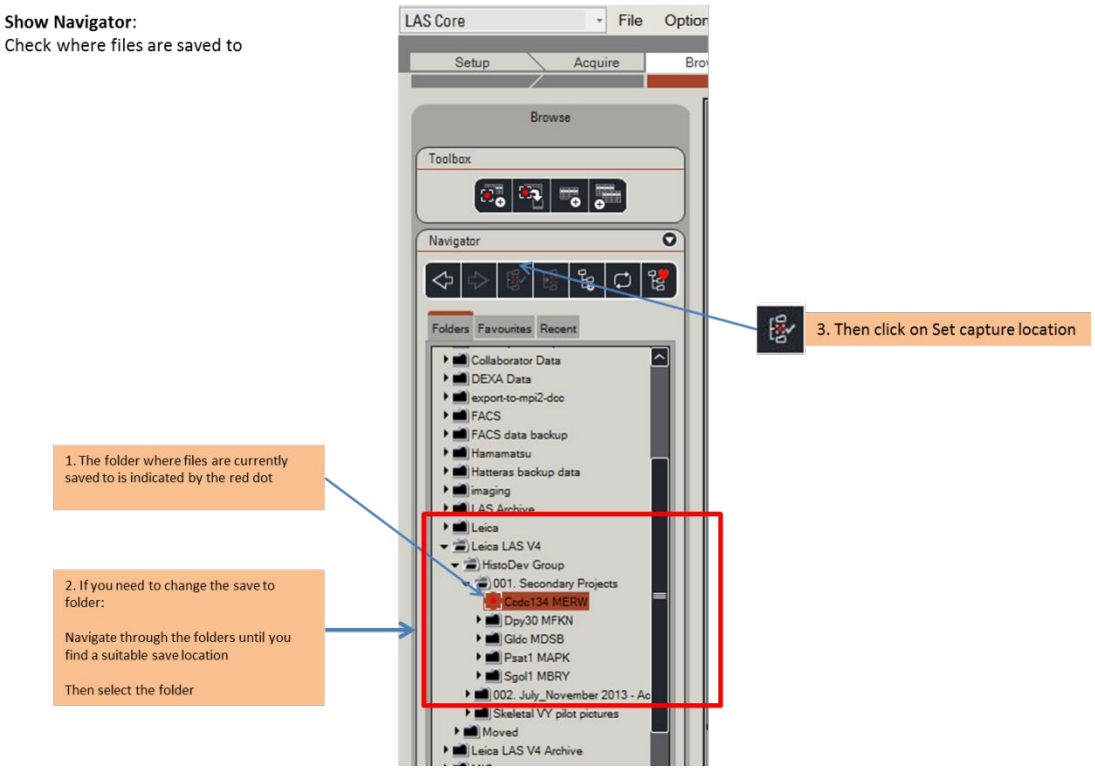
***Important note*:** LASV4 software will save your lens choice, but it does this in a profile specific manner. Therefore <u>you need to check the software settings on your own profile on all 3 microscopes</u>.

5. Use the ***tabs at the top to navigate*** through the software:



Setup: Shows the hardware which is connected – this should be setup already
Acquire: Where images are captured
Browse: Shows where the files are saved to, can be used to view images once they are taken
Process: Used for post-processing images (e.g. cropping, adding arrows etc) – we don't need this

6. ***Saving images to MIG drive*** (see figure below):
   - Click on the "browse" tab
   - When set to the correct file location, click on the  icon

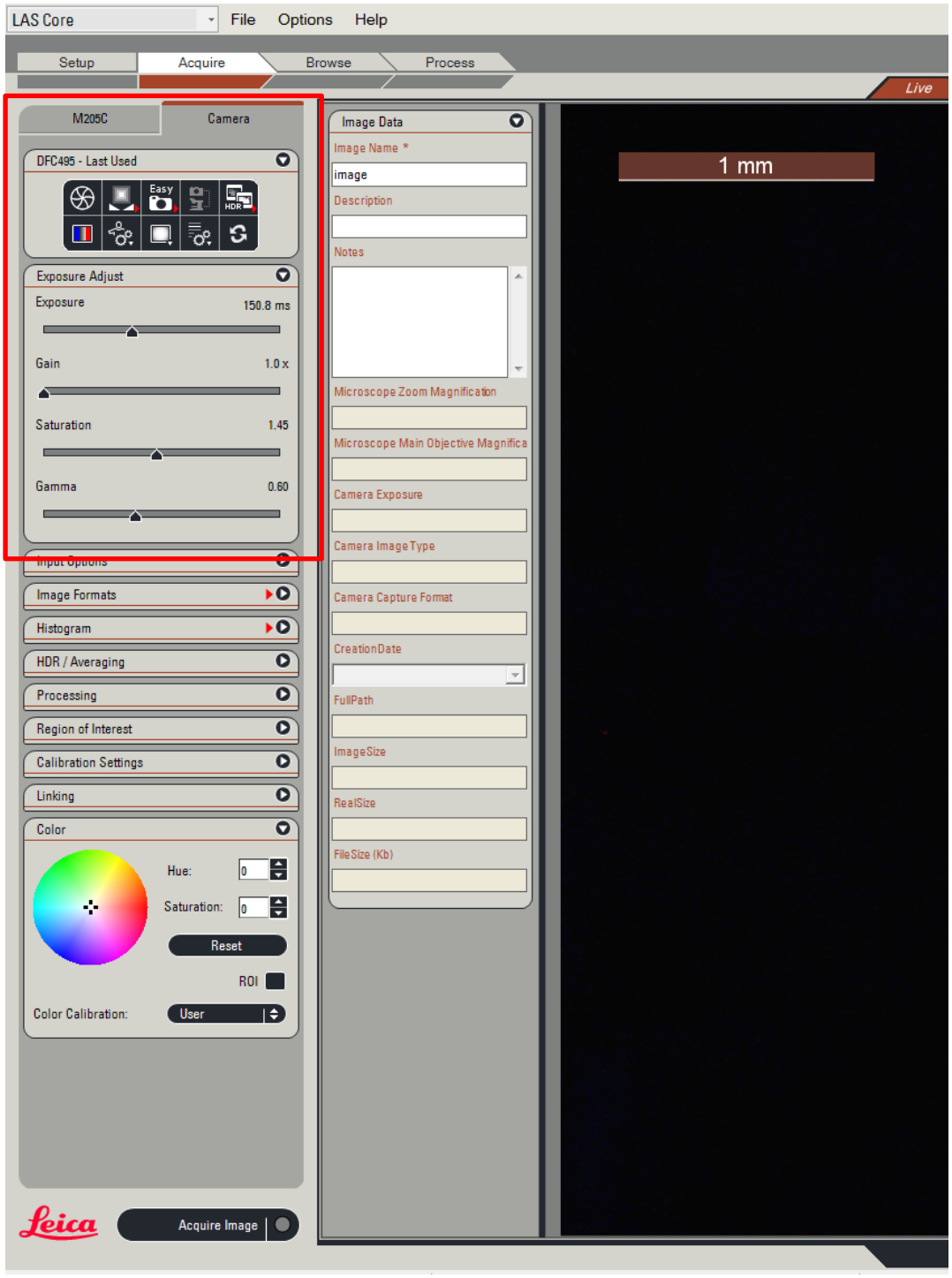


7. ***Capturing an image*** : the "Acquire" Tab

- **Use the guide** 'Leica LAS –Guide to Annotations and Processing' located X:\Team109\Recessive Lethality Pipeline\Equipment and Orders\Microscopes (Leica)

- Setting up the image

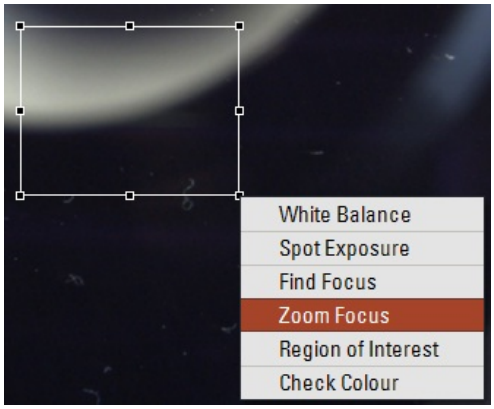
- Adjust the lighting, microscope diaphragm and exposure time to achieve a well illuminated sample with a uniform black or white background.
    - Using the functions in the camera settings in the acquire tab



    - Ensure the image is focussed on the screen (use the zoom focus if necessary)
    - If needed adjust the white balance
        i. Draw a square around the relevant section and click white balance

Draw box on image holding down left mouse button:

- White balance – small box over entirely white/black region
- Zoom focus – large box, with an image edge in the middle. Left click to remove



8. Image Data

- Depending on your experiment, locate the appropriate 2D tags sheet by the microscope ('Embryo Dysmorphology' or 'Wholemount Expression')
- In the form "Image Data" scan in the correct annotations for the image. The annotations will carry over to the next image so be careful to check the annotations are correct before acquiring the image:

    - **Image Name:** Enter the mouse name e.g. PXXX31.1a_ (make sure to place an underscore at the end)
    - **Description:** Scan in the appropriate experiment e.g. Embryo Dysmorphology/Wholemount Expression
    - **Notes:** Scan in the appropriate annotation tags e.g. Time point= E9.5/E14.5, etc.; Tissue = Adult/Embryo/Placenta; Orientation = dorsal/lateral, etc.
    - **Comments:** to add comments manually add '^' at the start and '$' at the end e.g. Comment^'gene name'$ (do not leave gaps)

- **To guarantee all the image annotations are maintained** for the picture you will acquire, press 'Tab' to move the cursor away from the last annotation field you filled in. (i.e. press tab until the cursor moves into the "microscope zoom magnification" box).

- When the image is ready click acquire image button at the bottom left-hand corner of the screen or press 'F3' on the keyboard.

Appendix C: Annotating Embryo Dysmorphology Images

INTRODUCTION

This describes the minimum annotations which are required for embryo dysmorphology images uploaded onto the WTSI Mouse Database.

It details the QC steps required for Embryo Dysmorphology images which are taken for the RL/DMDD phenotyping screen, Recessive Lethality screen, embryos collected for LacZ staining and for other secondary projects.

PROCEDURE

1. Verify images are uploaded on WTSI Mouse Database

2. Check that the number of uploaded images matches the number of images that were taken that day.

3. Verify that the below minimum annotations are present for LacZ stained, RL and DMDD specimens pictures

    a. *Experiment:* Embryo Dysmorphology or Wholemount Expression
    b. *Tissue:* Embryo or Placenta
    c. *Age:* As appropriate (e.g. Embryo E14.5)
    d. *Orientation:* As appropriate (Note: use this tag with discretion; it is supposed to be informative so if the orientation of the tissue is not obvious e.g. due to a strange angle, then do not apply this tag)
    e. *Comment*: use the comment box for extra information, i.e. if photographed with another embryo, state embryo name

4. QC Images:

a. QC Fail images that are:

- Out of focus (cannot see phenotype/other images available)
- Too dark (cannot see phenotype/other better images available)
- Duplicated
- Wrong embryo name (if possible correct embryo name on LAS software so the image can be reloaded with the correct name)

Note: QC of images is performed in batch on a weekly basis. However, everyone taking pictures while dissecting is responsible for their QC failing that same day if any of the above criteria is true.