SANGER INSTITUTE
STANDARD OPERATING PROCEDURE

Preparation of E9.5 Embryos in to RNA later for Transcriptomics or Bouins for HREM & E9.5 Placentas into PFA for Histology

INTRODUCTION

The aim of the procedure is to dissect embryonic mouse tissue and to preserve its RNA material for later extraction and transcriptomic analysis or to fix the embryonic tissue for HREM analysis.

Embryos are dissected and then stored in either RNA Later solution or fixed in Bouin's solution for 24 hours (or 72h if over the weekend). The embryos in RNAlater will be initially stored at 4°C and then moved after 24 hours (or 72h if over the weekend) into lab freezer, awaiting collection for analysis.

Placentas are dissected and fixed for 24 hours (or 72h if over the weekend) in 4% Paraformaldehyde in preparation for histological analysis.

ABBREVIATIONS:

DCF – Data Capture Form
DMDD – Deciphering Mechanisms of Developmental Disorder
NaN_3 - Sodium Azide
PBS – Phosphate Buffered Saline solution
PFA – Paraformaldehyde
PPE – Personal Protective Equipment
RT – Room temperature

HEALTH & SAFETY:

This procedure is covered by the following risk assessment **WTSI_1380**

- Entry procedure to the animal house should be followed including the wearing of scrubs.
- Appropriate personal protective equipment (PPE) is to be worn at all times when handling the samples (white coats 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.
- 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 litter to be performed optimally.

RESOURCES

Equipment and reagents

1. Fume hood and down flow table
2. Polystyrene ice box & ice bucket
3. 50ml falcon tubes (Sanger Stores: No. PLTU0002.)
4. Ice cold PBS pH 7.4 (Sanger Media Team)
5. Polystyrene bucket and ice bucket
6. Dissecting instruments (#55 forceps: Fine Science Tools; Sanger Stores: 11295-10, 14084-08, 10370-17)
7. Sterile 10cm plastic petri dishes (Sanger Stores: No. PLDI0012)
8. Sterile 6cm plastic petri dishes (Sanger Stores: No. PLDI0018)
9. Sterile Plastic Pasteur pipettes (Sanger Stores)
10. Sterile 2ml Eppendorf tubes
11. 2ml Eppendorf tubes
12. Genotyping PCR plates (ThermoFisher UK Ltd: MPP-114-101R)
13. PCR plate cooler blocks
14. RNA Zap (*Life technologies (Ambion) AM9780*)
15. RNA Later (*Life technologies (Ambion) AM7021*)
16. Tween20 (Sanger Stores)
17. p200 pipette & filter tips (Sanger Stores)
18. Computer with MIG access and Microscope and camera and software LASV4
19. 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.
20. 4% PFA (*EMS 15714-S)* **1 Week max shelf life**
    Paraformaldehyde 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.
21. PBS with 0.01% Sodium Azide (NaN3; Sigma: S8032)
    Sodium Azide is toxic and causes damage to organs.
    To be used under an effective fume hood and wearing correct PPE
22. Virkon Powder
    Virkon powder is harmful, corrosive, an irritant and causes damage to organs
    To be dissolved under an effective fume hood and wearing correct PPE

METHOD - Collection of Embryos in Animal house

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

1. Collect polystyrene bucket from the store and fill with ice

2. In experimental room, turn on the downflow table and prepare the work station (i.e. dissection board and equipment, cadaver waste bag and 70% ethanol spray bottle)

3. In experimental room, prepare 50 mL falcon tubes of cold PBS pH pH7.4 (one per litter to be collected) and place on ice

4. Go to animal room and locate all pregnant females requiring litters to be harvested.

   Note: *E9.5 embryos should be harvested between 9:00-10:30 am and, once removed from the mother, the uterus should spend* ***no more than 30 minutes on ice.***
   A maximum of one litter per dissector should be collected at a time.

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

6. Remove the uterus. Count the embryos and transfer them to the cold PBS. Store on ice.

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

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

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

   Note: *if any of the females are not pregnant communicate this to lab. Depending on other pregnancies due that day, you might be instructed to collect additional litters.*

8. Transfer the tubes containing the litters, on ice, to lab in a polystyrene bucket.

METHOD – Set Up working area in the Lab:

9. Wipe all working areas with ethanol wipes. **Wearing PPE**, spray RNA ZAP on all surfaces you will come in contact with during dissection (microscope, tools, marker, keyboard…) Wear gloves at all times from this point onwards to avoid RNA degradation.

10. Supply every working station needed with sterile plastic wear

11. If embryos are going into RNAlater **Wearing PPE,** fill sterile, autoclaved 2ml Eppendorf tubes with RNA Later solution:
    - RNA later is stored in 15ml aliquots at RT.
    - With a 200µl filter-tip fill up the tube with 200µl RNA later solution.

    If you think you may have contaminated an aliquot of RNA later or 2ml tubes, DO NOT RETURN THEM TO THE DRAWER.
    Dispose of them in an offensive waste bag

12. If embryos are going into Bouins:
    - Fill in a box with some empty 1.5 mL tubes (these will contain embryos)

13. Fill in a box with some empty 2.0mL tubes (these will contain placentas)

METHOD – Embryo Dissection in the Sulston Lab

12. Place the litter in a clean 10cm petri dish filled with ice cold PBS. Cut open uterus and recount the embryos (and resorptions if applicable). Transfer dissected out embryos into a 6cm dish filled with clean PBS (to avoid contamination) and place on ice.

13. Extract embryos from the placenta using extra fine dissection tools. Remove the yolk sac and the embryonic membrane. Use ethanol wipes to clean forceps between samples.

14. Collect two small pieces of yolk sac into two separate 96 well genotyping plate (on cooler block). One for genotyping and one for back-up. Label plates Yolk 1 and Yolk 2. **Use ethanol wipes to clean forceps between samples**.

Note: *if 2 persons are dissecting the same litter, use a marker to highlight on the genotyping plate which wells have already a yolk sac sample in them to avoid any genotyping contamination.*

15. Check the gross morphology of the embryos. If the embryo appears abnormal, take a photo to best represent the dsymorphology (section of taking images) and note the abnormality on the cage card.

16. Stage the embryo by counting the number of somites (Appendix B). Record the somite number on the cage card.

17. Transfer embryo, using a sterile Pasteur pipette, into the sterile tubes set up in above section either one of the options below:

    - 2ml Tube filled with RNA Later solution. **Transfer as little PBS as possible in the tube**. Mark the lid of the tube with embryo's ID. Place tube on ice.
    - Empty 1.5 ml tubes, containing ice cold PBS. Mark the lid of the tube with embryo's ID. Place on ice.

18. Transfer placenta, using a different Pasteur pipette, into 2ml tubes containing ice cold PBS. Mark the lid of the tube with embryo's ID. Place tube on ice.

19. Repeat through the whole litter.

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 -20ºC in a biohazard bag and given to the Sanger Waste Team for disposal.

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

After dissection:

20. Turn off instruments, and wipe area with Ethanol wipes to remove any spilt liquid.

21. Add litter to mating:
    Cull the female by entering Harvested by box and date. This will then bring up the age of the embryo. This work is at E9.5 so register as 'unregulated harvest'.

22. Print tube labels for embryo and placenta collections and stick onto tubes. Place Embryo tubes in a relevant box and label box with date of death. Place placentas in red box.

23. Transfer the samples in the fridge (4ºC)

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.*

24. **In a fume hood wearing PPE and safety goggles**, remove the PBS from the placentas and embryos if going into Bouin's using a Pasteur pipette.

25. Replace the PBS with Bouin's Solution (if embryos are to in fixed in Bouin's). **Store at RT in a safety cabinet. In yellow box, labelled with DOD and litter name, and fixative**

26. Replace the PBS with 4% PFA solution (placentas) and store at 4C.
    Important note: 4% PFA loses its effectiveness with time. Always try and use freshly made 4% PFA solution.

27. Note litter on the white board. Mark the litter on the tick sheet as well, adding the date of collection.

28. Leave samples in fixative for 24 hours (or 72h if 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

29. **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.

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

    - If tissues were not collected for any reason, ensure that this is recorded on the genotyping grid.
    - **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.
    - **Back-up Yolk sac genotyping samples:** Print off a copy of the genotyping grid for the samples which will be stapled with the days mating cards. These are then stored in the archive boxes. Place the yolk sac genotyping plate in the archive freezer.

31. **Data Capture:** Add the 'Embryo collection Pre_E10.5' DCF to the appropriate litters.
    - 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).
    - Fill in the DCF as appropriate with fixatives and any relevant abnormal dysmorphology, add any comment if needed.

32. **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. 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*

33. After 24hours (or 72h if over the weekend) at 4ºC, transfer the embryos in RNAlater to the freezer.

34. After 24/72 hours fixation, **in a fume hood wearing PPE and safety goggles,** remove the Bouin's solution from the embryos, using a Pasteur pipette, and wash 3 times with PBS pH7.4 Add the litters to the long term storage chemical cabinet, consolidate boxes to maximise space.

    Bouins waste must be disposed of into the Bouins waste container and given to the Sanger Waste Team for disposal.

35. **In a fume hood wearing PPE and safety goggles** remove PFA from the placentas, using a Pasteur pipette, and wash 3 times with PBS pH7.4

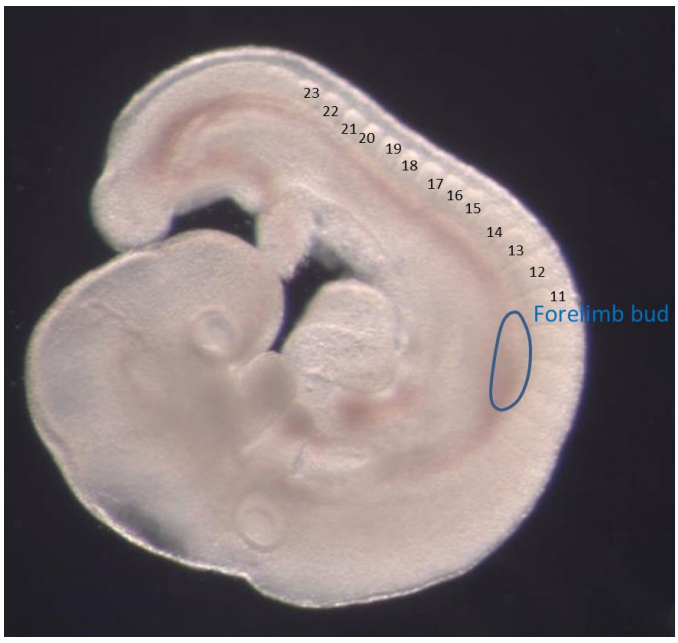
PFA waste must be disposed of into the PFA waste bucket and given to the Sanger Waste Team for disposal.

36. **In a fume hood** fill Bouin's fixed embryo and PFA fixed placenta tubes with PBS containing 0.01% NaN3 and store at 4C.

    - Add placentas to their colony box, adding the litter number to the side labelling. If starting a new line please label the box on the top with colony prefix and pipeline and on the slide the colony prefix and litter numbers.
    - Add placentas to their colony box, adding the litter number to the side labelling. If starting a new line please label the box on the top with colony prefix and pipeline and on the slide the colony prefix and litter numbers.
    - Once the fridge is full or the line has finished samples can be put into long term storage in the extra storage space at RT.

Appendix A: Somite counting

- For training of counting somites, see the trainers and assessors within the team.
- Perform somite counting for DCFs only once you are signed off to do so.
- Somites can be counted around the whole embryo, starting at somite 11 by the caudal side of the fore limb bud.



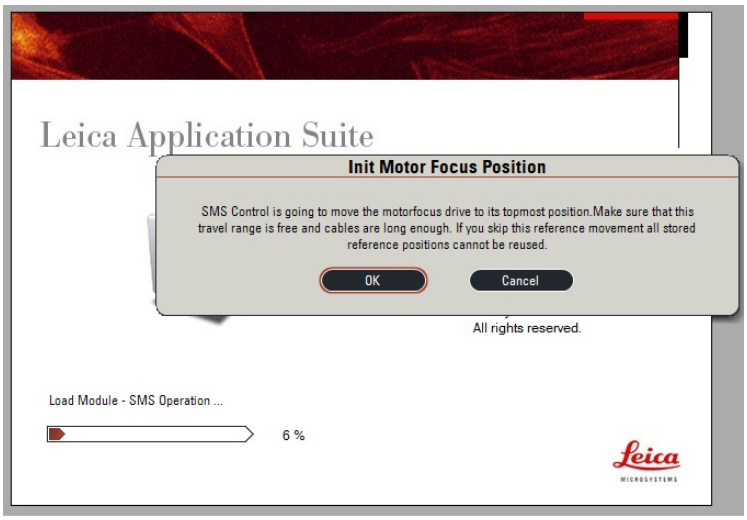
- Only count completely developed somites (i.e. those for which you can clearly see defined edge on both sides)
- Always double check the somite number by counting them twice
- If in doubt, double check somite counts with a colleague

Appendix B: Using the Microscope and Taking images

1. ***Turn on microscope***

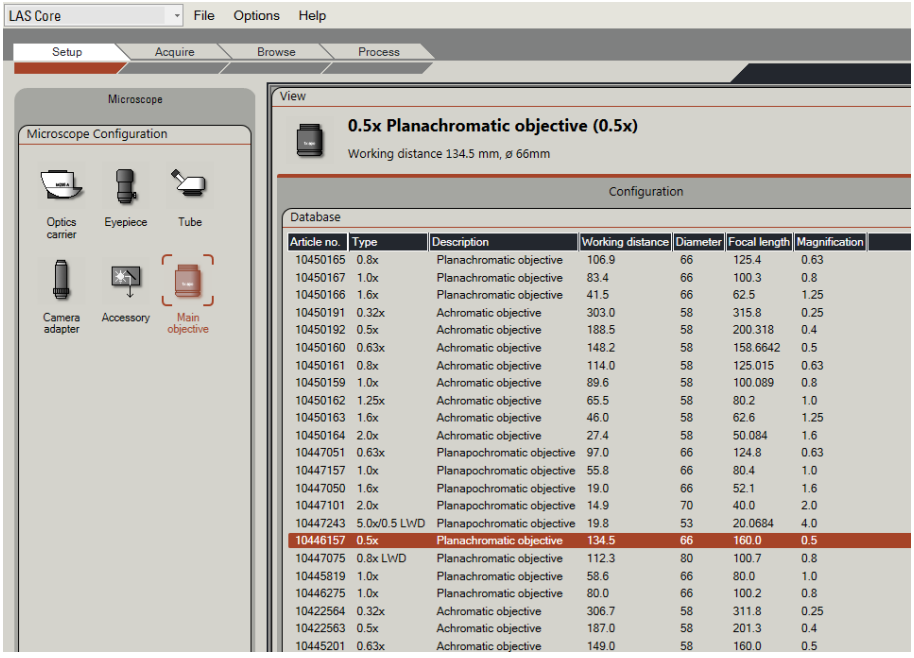
2. ***Start Leica LAS v4.4 software***

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



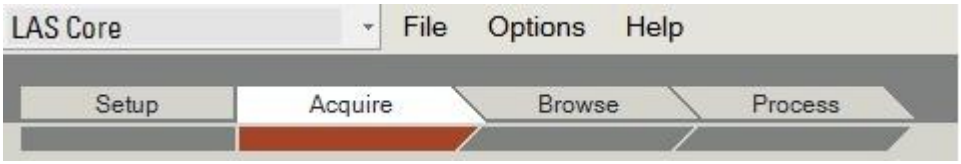
Article no.	Type	Description	Working distance	Diameter	Focal length	Magnification
10450165	0.8x	Planachromatic objective	106.9	66	125.4	0.63
10450167	1.0x	Planachromatic objective	83.4	66	100.3	0.8
10450166	1.6x	Planachromatic objective	41.5	66	62.5	1.25
10450191	0.32x	Achromatic objective	303.0	58	315.8	0.25
10450192	0.5x	Achromatic objective	188.5	58	200.318	0.4
10450160	0.63x	Achromatic objective	148.2	58	158.6642	0.5
10450161	0.8x	Achromatic objective	114.0	58	125.015	0.63
10450159	1.0x	Achromatic objective	89.6	58	100.089	0.8
10450162	1.25x	Achromatic objective	65.5	58	80.2	1.0
10450163	1.6x	Achromatic objective	46.0	58	62.6	1.25
10450164	2.0x	Achromatic objective	27.4	58	50.084	1.6
10447051	0.63x	Planapochromatic objective	97.0	66	124.8	0.63
10447157	1.0x	Planapochromatic objective	55.8	66	80.4	1.0
10447050	1.6x	Planapochromatic objective	19.0	66	52.1	1.6
10447101	2.0x	Planapochromatic objective	14.9	70	40.0	2.0
10447243	5.0x/0.5 LWD	Planapochromatic objective	19.8	53	20.0684	4.0
10446157	0.5x	Planachromatic objective	134.5	66	160.0	0.5
10447075	0.8x LWD	Planachromatic objective	112.3	80	100.7	0.8
10445819	1.0x	Planachromatic objective	58.6	66	80.0	1.0
10446275	1.0x	Planachromatic objective	80.0	66	100.2	0.8
10422564	0.32x	Achromatic objective	306.7	58	311.8	0.25
10422563	0.5x	Achromatic objective	187.0	58	201.3	0.4
10445201	0.63x	Achromatic objective	149.0	58	160.0	0.5

- **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 10446157 - 0.5x Planapochromatic objective - 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.
- **If changing the objective** 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, our x1 lens is 10447157.
- 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 you need to check the software settings on your own profile on all 3 microscopes.

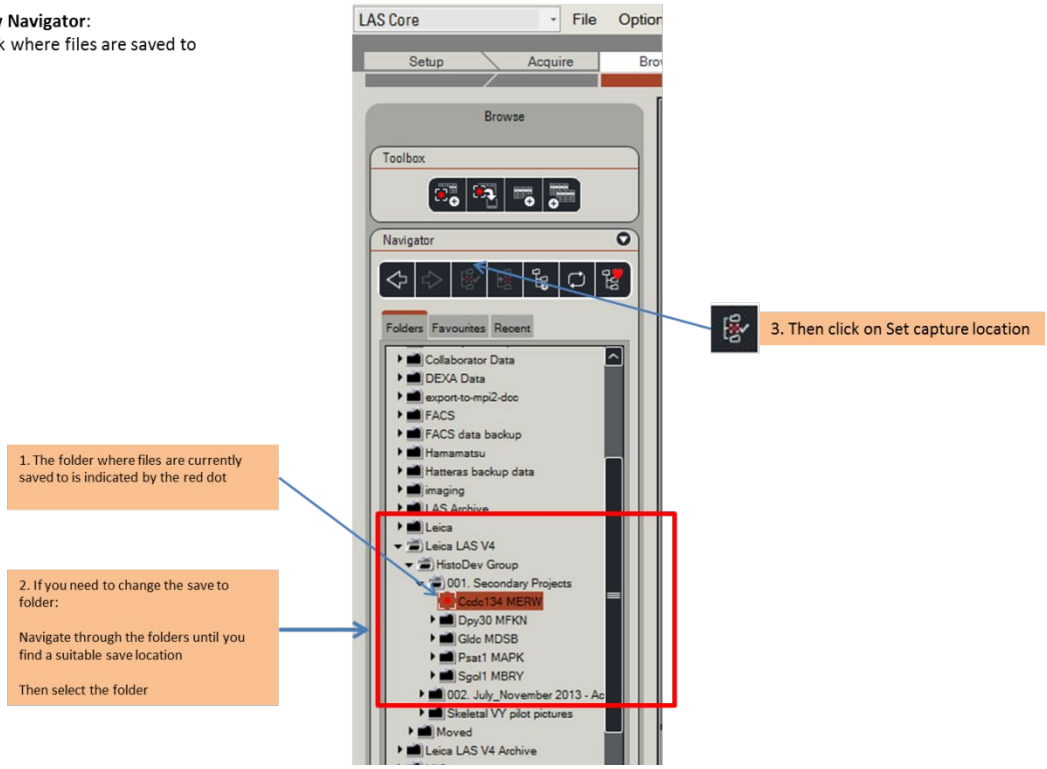
5. Use the ***tabs at the top to navigate*** through the software:



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

Show Navigator:
Check where files are saved to

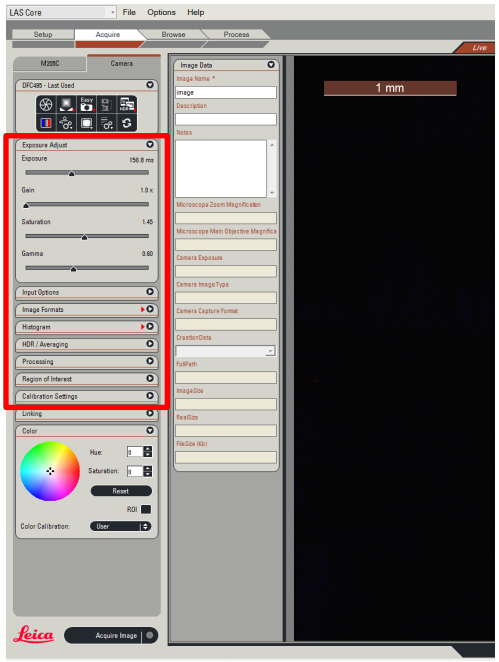


7. ***Capturing an image*** : the "Acquire" Tab

- **<u>Use the guide</u>** 'Leica LAS –Guide to Annotations and Processing'

- <u>Setting up the image</u>

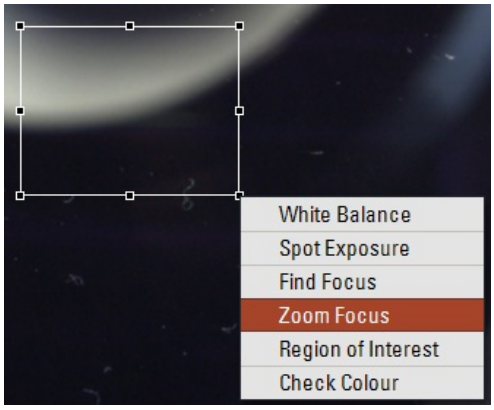
    - Adjust the lighting, microscope diaphragm and exposure time to achieve a well illuminated sample with a uniform black or white background.
        - Using the functions in the camera settings in the acquire tab



- 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 '$' at the end e.g. Comment^ccdc134$ (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

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

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.