SANGER INSTITUTE
STANDARD OPERATING PROCEDURE

SUBJECT: ***Collection and phenotyping of ED18.5 embryos for DMDD***

INTRODUCTION:

The procedure is aimed at phenotyping E18.5 mouse embryos by neurological assessment. This aims to determine if homozygous embryos are surviving to this time point, if they are morphologically normal and if they display any neurological impairment.

The procedure also covers the subsequent processing of the samples to be sent to UCL collaborators as part of DMDD project.

This is a regulated procedure.

ABBREVIATIONS:

DCF = Data Capture Form
DMDD = Deciphering the Mechanisms of Developmental Disorders
PBS = Phosphate buffered solution
PFA = Paraformaldehyde
PPE = Personal Protective Equipment
SOP = Standard operating procedure

QUALITY CONTROL (QC) DURING PROCEDURE:

Refer to the table below for comments or action to be taken during the procedure.

Problem / Issue	Comment on DCF / action to be taken
A welfare issue makes it impossible to perform the procedure or is causing pain/distress to the embryo	Do not perform procedure on embryo. Immediately cull embryo by foetal decapitation. Record on DCF
Procedure is affected by delays due to fire alarms	Do not perform procedure; leave litter submerged in PBS on ice. Cull embryos by foetal decapitation as soon as possible. Record on DCF which embryos were affected.
An equipment failure affecting specific equipment (i.e. Heat Mats)	Do not perform neurological assessment. Harvest litter in ice cold PBS and take to Sulston lab. After minimum 30 min submerged in cold PBS, inspect embryos for gross dysmorphology before foetal decapitation. Record on DCF that the procedure was not performed. Process for shipment as default.
A procedural error affects the welfare of the embryo	If the error is likely to cause any pain to the embryo, do not perform the procedure and cull immediately by foetal decapitation. Record on DCF.
A procedural error affects the procedure	Record error and how it could have affected the procedure on DCF

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.
- New Workers are to be supervised until deemed competent to perform this assay
- Individual Risk Assessment for lone workers, young persons and new or expectant mothers would be performed to define any exclusion for performing this assay.
- Appropriate personal protective equipment (PPE) is to be worn at all times when handling animals. This includes:
    - Overshoes
    - Gown
    - Gloves
- In addition to the above, when sources for LAA (animals or soiled cages) are not contained within Local Exhaust Ventilation Systems (change stations, fume hoods or downflow tables), a respiratory mask, for which you have passed a face fit test, must be worn.
- Check Eye wash stations, fire extinguishers, standard spill kits and solvent/formaldehyde spill kit is located in the local area.
- Lone worker alarms should be used when working alone.
- This procedure can only be performed during Animal house core hours (7:30am-7:30pm). If necessary pregnant dam should be removed from Animal room before 2.30pm due to lock down of the room.
- All electrical equipment is to be inspected for damage before use.

RESPONSIBILITIES:

All staff performing this procedure are responsible for ensuring that this Standard Operating Procedure (SOP) and accompanying Risk Assessment have 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).

For secondary phenotyping, seek confirmation with project manager for deviations from this SOP. Any deviation will be detailed in the Project Authorisation Form (PAF).

RESOURCES:

Equipment:

1. Appropriate PPE
2. Functional downflow tables
3. Large and small heat mats (Livefoods.co.uk)
4. Balance (for embryo weights)
5. Magnifier with LED (Interfocus: 28011-20)
6. Cork boards
7. 50ml tubes (Sanger Stores: No.PLTU0002)
8. 40ml tubes with flat bottom (VWR: No. 11793214)
9. 10 cm plastic petri dishes (Sanger Stores: No. PLDI0012)
10. 6 cm plastic petri dishes (Sanger Stores: No. PLDI0018)
11. Dissection tools (Sanger Stores: 11251-20, 14084-08, 10370-17)
12. Timer

13. Paint Brush (it is important that the hair is synthetic and not animal-derived)
14. Pot accommodating 50mL falcon tube
15. Genotyping plates and plate covers (Sanger Stores: 10363173, PLPL0109)
16. PCR plate cooler blocks
17. Camera
18. Leica microscope (Leica (DFC495 – camera & M205c – Microscope)
19. Computer with Leica software (LAS) and sanger MIG database access
20. Ethanol wipes (Sanger Stores HVPA0062)
21. PBS at 4°C *(LifeTech 10010023)*
22. Polystyrene bucket
23. Leica MZ16A Stereomicroscope with camera
24. Computer with LeicaLAS v4.4 software
25. Light box (Leica CLS 150x)
26. 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.

Staff:

- *Required: Two persons per litter*

NOTE:

- *Embryos are to be kept warm throughout the procedure.*
- *Process one litter at a time and a maximum of two in one day.*
- *Database entry can be done while performing the procedure by the person assisting the phenotyper. Avoid doing so if this is affecting speed and quality of the procedure.*
- *Collection may only occur Monday to Thursday as of collaborator request*

PROCEDURE:

Before performing the procedure, verify that this is the correct procedure at this point in the pipeline by consulting the cage card(s) and confirming that this is the correct harvest date.

SET UP:

1. Set up the downflow table in the experimental room and the bench area. One table will be used to perform the neurological assessment, the other to take genotyping samples decapitate the embryos and computer work

2. Cover cork board with foil and place large heat mat on top. Turn on large heat mat and an additional small heat mat. Place plastic petri dishes on heat mats to warm up and cover with foil

3. Turn on weighing scale and place plastic petri dish on for weighing embryos

4. Collect ice in polystyrene bucket. Fill 40ml flat bottom tubes with cold PBS and place on ice.

5. Fill a 50mL falcon tube with warm PBS and place into a pot of warm water to keep warm.

6. Prepare timer by setting it to 1.00 minute

7. Prepare two genotyping plates on cooler blocks

8. Log onto mouse database

COLLECTION:

9. Locate required pregnant female in the correct animal room and transfer to experimental room.

10. **On the downflow table** in experimental room, sacrifice 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 heart removal

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

Person 1:

11. Remove the whole uterus and count the embryos (and reabsorptions). Record this on the cage card.

12. Place uterus on a dry petri dish. Replace the dish lid and quickly transfer to a heat pad to keep warm. Place a small heat mat on top of the petri dish and cover with foil.

13. Using the paint brush, brush the whole litter with warm PBS to keep it moist

14. As soon as the uterus is removed spontaneous movement of embryos should be seen. If any embryos are not moving, try to stimulate movement by brushing with PBS. Record on cage card any embryos that do not move after stimulation

Person 2:

15. Record litter on the database:
    - Search for the mating
    - Select edit litter and harvest embryos
    - Enter number of embryos collected
    - Ensure embryo harvest procedure is added

PHENOTYPING:

Person 1:

1. Start dissecting from one extremity of the uterus and proceed in a sequential order through to the other end.

2. Dissect one embryo out of uterus and yolk sac, leaving rest of uterus in the petri dish on the heat mat. Hold embryo and place lid and heat mat back on top of petri dish. Ensure the litter is always kept warm and moist

3. While holding the embryo, assess movement and breathing by brushing face and body of the embryo with a warm wet brush for 1 minute.

4. Record:
    - Time of first breath

- Total number of breaths taken in 1 minute
- Any dysmorphology of the embryo

5. Place embryo on petri dish on balance for weight measurement and to pass to person 2

Do not perform the procedure if the welfare of the embryo is affected. (See QC section)

Person 2 – can also perform database tasks during procedure if this does not affect speed and accuracy of phenotyping (see database entry):

6. Record weight of embryo from balance

7. Ensure phenotyping data (breaths and abnormalities) are recorded

8. Examine the embryo using the magnifier to check for dysmorphology

9. Cull embryos by foetal decapitation (Schedule 1)

10. Place head and body on paper towel to absorb the blood. Avoid directly placing the cut surface on paper towel as this can damage the tissue analysed by the collaborator.

11. Take two small pieces of skin from flanks for genotyping and place in correct well of genotyping plate.

12. Place embryos into 40ml flat bottom tube filled with PBS. Mark lid of tube with mouse name and place tube on ice.

DATABASE ENTRY:

Note: Database entry can be performed by person 2 during phenotyping, only if this does not affect the speed and accuracy of the procedure. Otherwise database entry can be done after phenotyping of whole litter is complete.

1. **Print Labels:** Create necropsy task ‘Embryo collection’ and print tube labels

2. **Data Capture:**
    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; record phenotyping data, tick any relevant dysmorphology observed and add any comment if needed. If brushing was not performed for any embryo in the litter ensure that this is recorded.

IMAGING:

13. Images should be taken of all abnormalities (time permitting) using either the camera or microscope.

14. If dysmorphology is not causing discomfort/stress/welfare issues for the embryo, images can be taken with the camera before decapitation. Embryos with severe abnormalities should be culled immediately before imaging.

15. Ensure image taking does not slow down the procedure – ie images should only be taken by person 2 or after all embryos have been processed

16. Images taken on the camera must be uploaded to the database

17. Check that any images taken on the microscope have been uploaded onto the database

Please note:
The below instructions refer to the workflow and processing of E18.5 set up for DMDD project. Processing and shipping of E18.5 samples will no longer be performed as part of primary pipeline work after the end of DMDD project.

PROCESSING and DISPATCH of SAMPLES:

1. Clean room and dispose of waste as appropriate

The residual animal carcass and any biological waste must be disposed of in a cadaver waste bag, sealed, and put in the appropriate freezer.
Dispose of the cage in dirty cage drop off zone

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

2. Transfer samples to Lab

3. Print labels and attach to tubes

4. Remove PBS from tubes and fix embryos in cold 4% PFA pH7.4
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.

5. Use Parafilm to seal tubes' lid (this is a safety measure needed for shipping)

6. Line a polystyrene bucket with a plastic bag, fill with ice and place sealed tubes in the ice

7. Seal polystyrene bucket and affix shipping labels

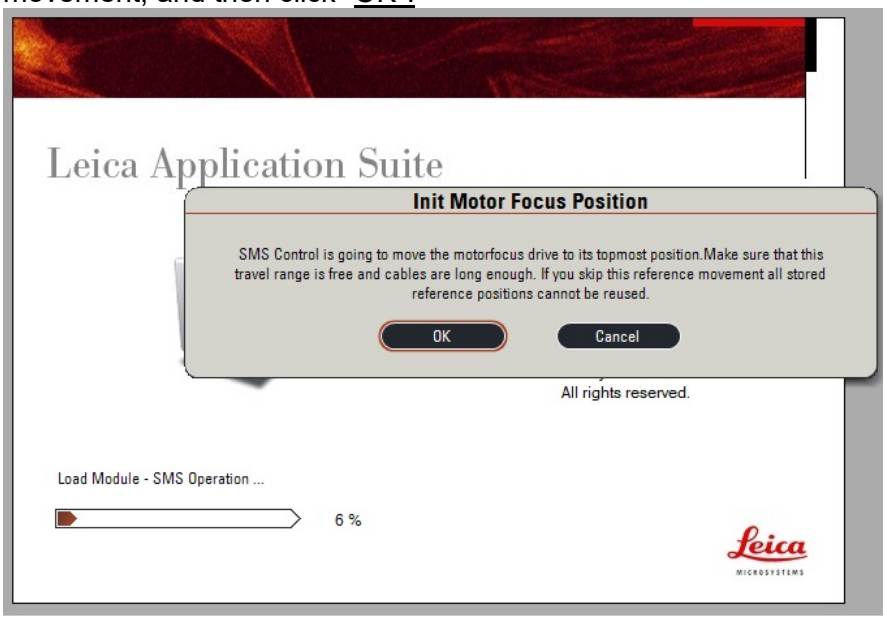
8. Dispatch embryos on the same day as dissection to collaborator

Appendix B: Using the Microscope and Taking images

1. ***Turn on microscope***

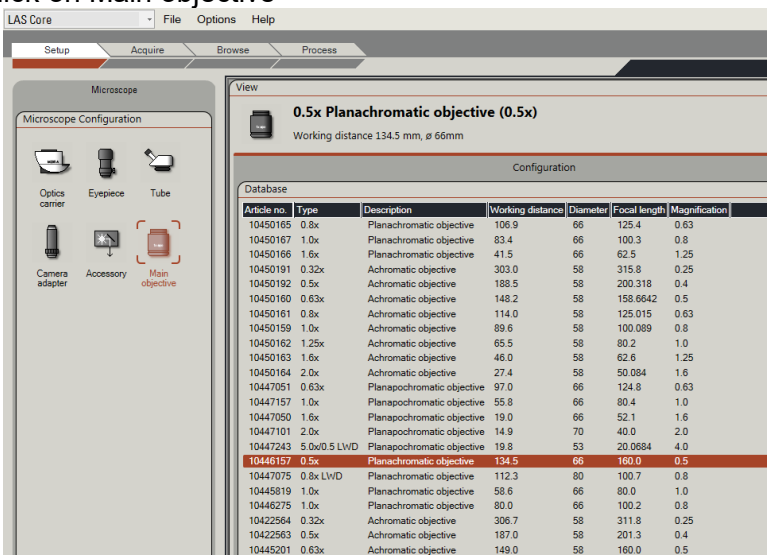
2. ***Start Leica LAS v4.4 software***

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



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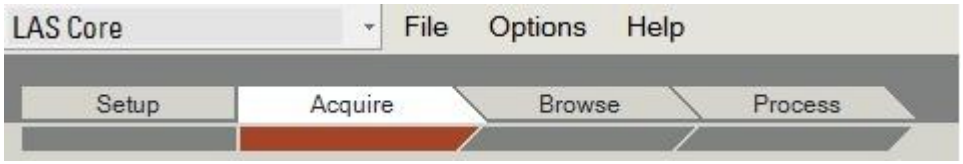
- If not select the correct objective The standard/default objective is 10446157 - 0.5x Planapochromatic objective - working distance 123.5 – Diameter 66 – Focal length 160.0 - Magnification 0.5
- Turn off software and microscope and wait 5 -10 seconds
- Turn on microscope and then the LASV4 software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

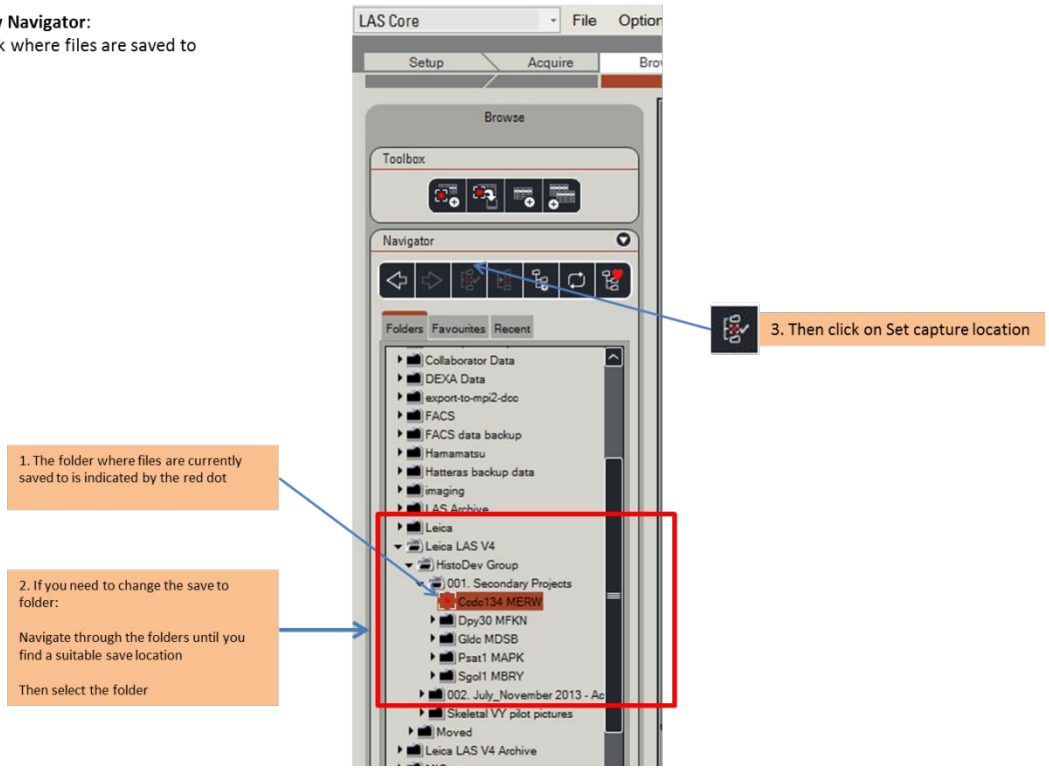
5. Use the ***tabs at the top to navigate*** through the software:



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 drive***
   - Click on the "browse" tab
   - Path to save images to
   - When set to the correct file location, click on the  icon

Show Navigator:
Check where files are saved to

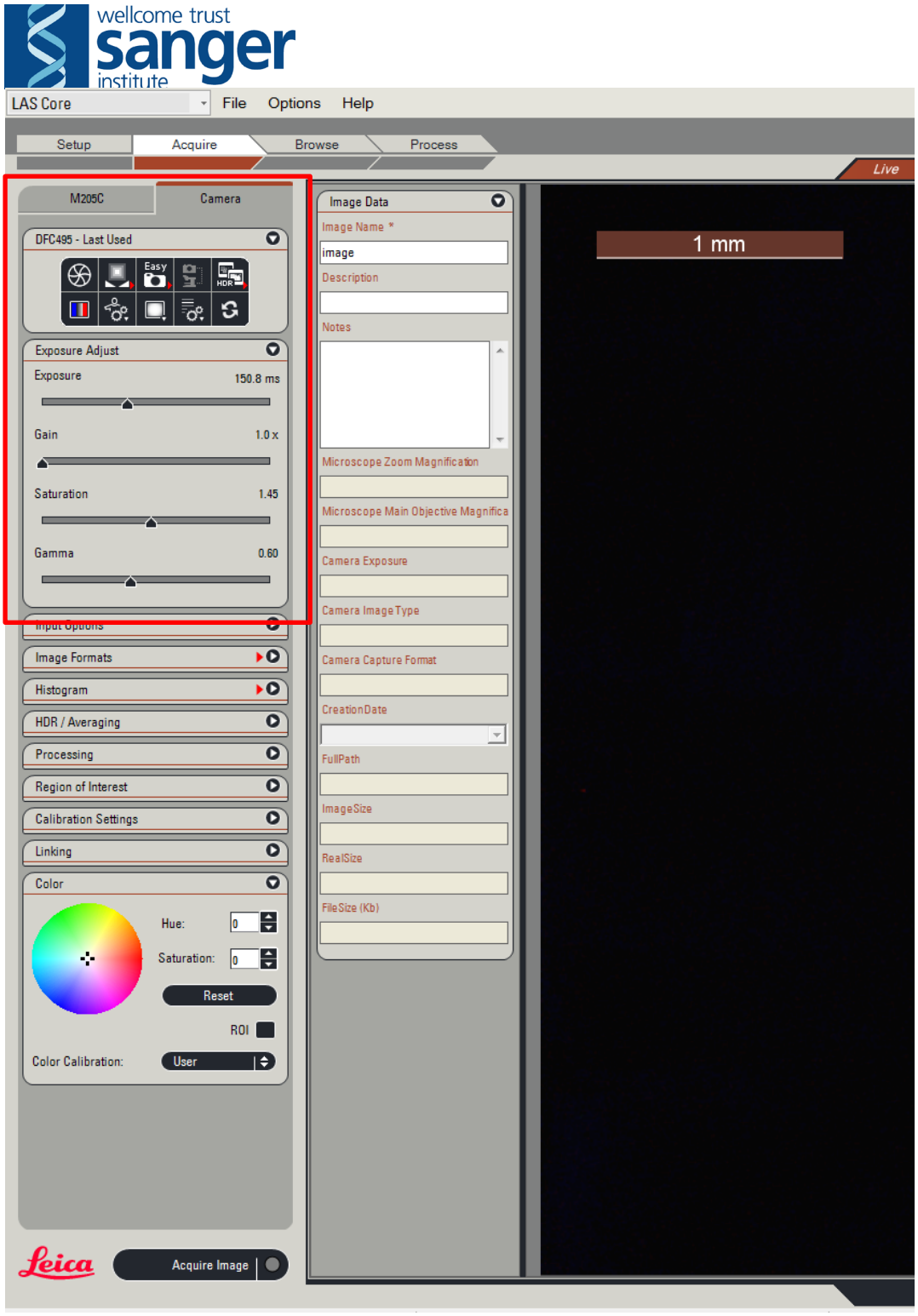


7. ***Capturing an image*** : the "Acquire" Tab

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

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

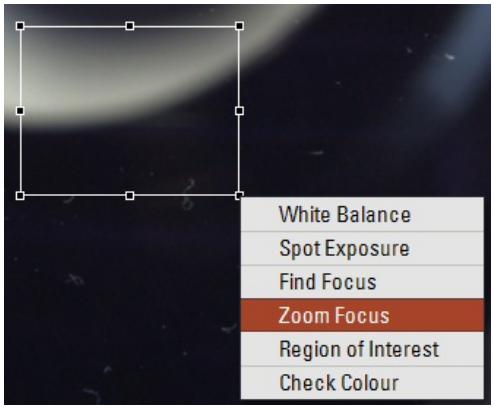
    - Adjust the lighting, microscope diaphragm and exposure time to achieve a well illuminated sample with a uniform black or white background.
        - Using the functions in the camera settings in the acquire tab



- 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. PMCZ31.1a_ (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.