Chapter title: Histological analysis of trophoblast cells in the mouse placental labyrinth zone

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Abstract

The mouse is a common animal species used for translational studies. In reproductive studies, this animal is normally preferred over other models as the rodent placenta shows similarities to the human, but has a relative short gestational period. In mice, the transport of oxygen and nutrients between mother and fetus occurs in a restricted area of the placenta, called the labyrinth zone. Here, we provide a detailed protocol to study labyrinth zone trophoblast proliferation and syncytial trophoblast identification using paraffin-embedded histological sections of the mouse placenta and immunohistochemistry. By describing step by step how to collect the mouse placenta, process and analyse the labyrinth zone, we hope to help other scientists to understand the contribution of changes in placental transport function in their experimental model, and therefore advance our understanding of mechanisms underlying pregnancy complications.

Introduction

The placenta is a vital, transient, external fetal organ that regulates the exchange of nutrients and gases between the mother and fetus. The mouse is a common animal species used for translational studies, including those related to pregnancy. The mouse placenta is haemochorial and therefore similar to the human. However, in the mouse, the exchange of gases, nutrients and wastes takes place in the placental labyrinth zone (Lz), a vascular region composed of fetal vessels and maternal blood spaces separated by two layers of syncytial trophoblast (SynT-I and SynT-II) and a superficial layer of cytotrophoblast cells. Previous work performed by Coan et al., ***[1]*** has established that the gross structure of the definitive mouse placenta is achieved by gestational day (GD) 12.5, which includes the identification of different labyrinth trophoblast lineages. Placental weight reaches its maximum on GD16.5, although the Lz keeps growing at least until GD18.5 (term is ~GD20) ***[1]***. Multiple mouse models have shown that defects in placental formation (including thickened syncytial trophoblast separating the maternal and fetal circulations), and reduced substrate delivery to the fetus affects fetal growth and, in the worst case scenario, results in the death of the fetus ***[2, 3]***. Therefore, understanding the phenotype of the placenta, which includes Lz cellular composition, would be highly useful for developing medical treatments to prevent obstetric problems, such as placental insufficiency, fetal growth restriction and stillbirth.

We and others have published different methods using histological sections to analyse the structure of the mouse placental Lz (e.g. identification of fetal capillaries or maternal blood spaces) ***[1, 4, 5]***. However, a detailed protocol assessing placental cell populations is missing. In this chapter, we provide details on how to collect and process mouse placental samples, and analyse proliferation (assessed with Proliferating Cell Nuclear Antigen, PCNA, marker for cells synthesizing DNA) and the abundance of

SynT-I and SynT-II populations (layer-specific markers: *Slc16a1* - Monocarboxylate transporter 1 (MCT1) and *Slc16a3* - Monocarboxylate transporter (MCT4)) using immunofluorescence on paraffin embedded-sections.

1. Materials

1.1. Equipment

1.1.1. Metzenbaum scissors

1.1.2. Polystyrene weighing boat

1.1.3. Razor blade

1.1.4. Analytical balance

1.1.5. Laboratory shaker

1.1.6. Tissue processor

1.1.7. Histosette – tissue processing/embedding cassettes

1.1.8. Paraffin wax

1.1.9. Bijou container (7ml container)

1.1.10. Petri dish 140 mm size

1.1.11. Disposable 12-well cell culture plate

1.1.12. Steel or plastic mould (StatLab, 21A00J072 or PELCO, 27186)

1.1.13. Tissue embedding centre

1.1.14. Cold plate for modular tissue embedding system

1.1.15. Microtome

1.1.16. Kimtech science precision wipes (Kimtech, 7551)

1.1.17. Paraffin section flotation bath

1.1.18. Microtome blades

1.1.19. Instramed bonney toothed forceps

1.1.20. Histopathology laboratory fume hood

1.1.21. Plastic staining jars

1.1.22. Vertical slide staining rack

1.1.23. Superfrost plus microscope slides

1.1.24. Microscope cover glasses

1.1.25. Fluorescent microscope or scanner

1.1.26. Slide storage box

1.1.27. Measuring cylinder

1.1.28. Pasteur pipettes

1.1.29. Pipettes (200-1,000µl)

1.1.30. Hydrophobic barrier PAP pen (Vector, H-4000)

1.1.31. Incubator set at 37ºC

1.2. Specific solutions and reagents

1.2.1. 4% paraformaldehyde (PFA) pH 7.4

1.2.2. Distilled water (dH2O)

1.2.3. Xylene

1.2.4. Histoclear

1.2.5. Ethanol (100% - 90% - 70% - 50%: diluted in dH2O)

1.2.6. Pepsin (0.04% in preheated 0.01M HCl)

1.2.7. MCT1 antibody (Merck; AB1286-I)

1.2.8. MCT4 antibody (Merck; AB-3314P)

1.2.9. PCNA antibody (Proteintech; 24036-1-AP)

1.2.10. Secondary antibody Alexa 568 (Fisher Scientific, A-11011)

1.2.11. Secondary antibody Alexa 488 (Fisher Scientific, A-11039)

1.2.12. DAPI (Fisher Scientific, D1306)

1.2.13. SlowFade gold anti-fade mountant (Fisher Scientific, S36936)

1.2.14. Clear nail varnish

1.2.15. Phosphate-buffered saline (PBS)

1.2.16. Tween20

1.2.17. Goat serum

1.2.18. Blocking buffer containing:

- 2% bovine serum albumin
- 1% skimmed dry milk
- 0.1% Tween20

1. Methods

1.1. Tissue collection

1.1.1. Set time-mating experiments, ideally 1:1 female-male per cage.

1.1.2. Perform plug checks in the morning to ensure accuracy of the gestational age (ideally between 08.00 and 10.00h).

1.1.3. The presence of a copulatory plug in the dam's genital tract indicates GD1.

1.1.4. Euthanize the pregnant mouse according to institutionally approved method and desired day for tissue collection.

1.1.5. Open the ventral abdominal wall using Metzenbaum scissors and forceps.

1.1.6. Remove the gravid uterus, and place it gently into a Petri dish with PBS.

1.1.7. Place each conceptus in a labelled polystyrene weighing boat.

1.1.8. Dissect each placenta from the fetus and clear any fetal membranes.

1.1.9. Weigh the placenta.

1.1.10. Cut the placenta along the short axis into two equal halves with a razor blade.

1.1.11. One half can be snap frozen in liquid nitrogen for gene/protein expression analysis if required.

1.1.12. Place the other half of the placenta in either a Bijou container containing >3ml of 4% PFA (see note 1) or in the well of 12-well cell culture plate containing 4% PFA (if the researcher is planning to keep all the placentas from the litter) (Figure 1).

1.1.13. Ensure that the placental halves are fully submerged in the PFA and that the tubes/culture plate are appropriately labelled (with mother and fetal IDs)

1.1.14. Leave the tubes/culture plate overnight at 4ºC with gentle movement (if a laboratory shaker is available) to make sure adequate fixation of the tissue.

1.1.15. The day after, remove the PFA and wash each fixed placental half with PBS 3 times (5 minutes/wash).

1.1.16. Label tissue processing/embedding cassettes using a pencil and carefully put each placental half in its own cassette (see note 2).

1.1.17. Place the embedding cassettes in 70% ethanol at 4ºC (use a cold-room or fridge) until processing.

1.2. <u>Processing for paraffin sectioning</u>

1.2.1. Tissue processing requires multiple steps that can be performed manually or using automated tissue processors.

1.2.2. Regardless, it is important to make sure that all solutions used are clean or newly prepared before the researcher starts the protocol.

1.2.3. Embedding cassettes will be transferred into the following solutions (if possible provide gentle movement in all steps):

- 90% ethanol (1 hour).
- 100% ethanol (1 hour).
- 100% ethanol (1 hour).
- Histoclear (1 hour).
- Histoclear (1 hour).
- Wax 60ºC (2 hours).
- Wax 60ºC (2 hours).

1.2.4. Take the cassettes to the tissue embedding center (see note 3).

1.2.5. Open the cassettes to reveal the sample.

1.2.6. Using heated forceps, gently transfer the placental half into steel or plastic embedding moulds (Figure 2A) that are part filled with liquid paraffin wax.

1.2.7. Position the sample with the cut edge facing down which will enable you to identify placental layers (Figure 2B).

1.2.8. Move the mould from the hot plate to a cold plate to allow the wax to set whilst ensuring that the orientation of the tissue is not lost during the movement.

1.2.9. Place the empty cassette bottom on top of the mould and gently add more molten wax.

1.2.10. Move the mould to the cold plate until the paraffin is totally hardened.

1.2.11. If the orientation of the placental half is lost at any time, move the mould back to the hot plate, re-liquify the paraffin wax and repeat previous steps.

1.3. Sectioning paraffin blocks

1.3.1. With a razor blade, remove the excess paraffin wax from the margins of the block to make sure that the block can correctly be placed in the block holder of the microtome (Figure 2C).

1.3.2. Write the ID of the sample and the thickness of the section on the slide.

1.3.3. Section the block at 3-5 µm thickness (thinner sections work better for Lz analysis).

1.3.4. To facilitate the cutting of the block, gently touch the paraffin block with a Kimtech wipes wet with dH2O before the tissue is cut (Figure 2D).

1.3.5. Drop the section in a container with 70% ethanol before then transferring the section into a pre-warmed water flotation bath (set temperature between 40-56ºC). (see note 4)

1.3.6. Collect 2-3 sections per sample onto each superfrost plus slide (see note 5) (Figure 2E).

1.3.7. Set the tissue sections on the slides by incubating them minimum overnight in an oven at 37ºC.

1.3.8. Samples can then be stored in a slide storage box.

1.4. Fluorescent immunohistochemistry

1.4.1. *Dewaxing:* Make sure that staining jars are properly cleaned and dried before adding the solutions.

1.4.2. All jars should be properly labelled ahead of the dewaxing process.

1.4.3. Note that the dewaxing steps should be performed in the histological fume hood to avoid breathing toxic products.

1.4.4. Select the slides and put them in a slide staining rack.

1.4.5. Immerse the rack into the staining jars and proceed with the following steps (longer times are not a problem; however do not reduce the timings as this will affect the staining).

- Xylene I (5 minutes).
- Xylene II (5 minutes).
- 100% ethanol (2 minutes).
- 100% ethanol (2 minutes).
- 90% ethanol (2 minutes).
- 70% ethanol (2 minutes).
- 50% ethanol (2 minutes).
- dH20 (5 minutes).
- PBS (5 minutes) – Samples can be kept in PBS for up to 2 hours.

1.4.6. *Antigen retrieval*: Each antibody is different; therefore, the antigen retrieval conditions should be determined by the researcher in a previous trial to obtain optimal results from the immunohistochemistry (IHC).

1.4.7. The methods that are most commonly used in IHC are described in Table 1.

1.4.8. For PCNA, MCT-1 and MCT-4 staining, antigen retrieval with pepsin in a humidified chamber works well (Sigma, 0.04% in pre-heated 0.01M HCl, 10min, 37ºC) (see note 6).

1.4.9. *Permeabilization of the tissue*: After antigen retrieval has been performed, permeabilize the sample sections by washing the slides 3 times in PBS-Tween 20 (0.1%) (5 minutes each wash).

1.4.10. *Blocking non-specific binding sites*: With the use of a hydrophobic barrier PAP pen, draw a circle around each tissue section (see note 7).

1.4.11. Perform non-specific blocking by incubating tissue sections with a buffer containing 5-10% animal serum (e.g. goat or donkey serum – selection should be based on species that secondary antibody is raised in) in PBS or blocking buffer with dH2O containing 2% bovine serum albumin, 1% skimmed dry milk and 0.1% Tween20 (see note 8).

1.4.12. The duration of the blocking is variable, but usually ranges from 20 to 60 minutes.

1.4.13. *Antibody staining*: The concentration of the antibody required for each IHC should be determined by the researcher based on previous trials and/or according to the recommended dilution supplied by the antibody manufacturer.

1.4.14. For PCNA staining, an antibody dilution of 1:50 in PBS (use 80-100µl volume per sample) applied to the tissue sections and incubated overnight at 4ºC in humidified chamber works well.

1.4.15. For MCT-1 and MCT-4 staining, a combined stock with each antibody diluted at 1:100 in PBS, applied to the tissue sections and incubated for 2h in oven at 37ºC works well.

1.4.16. Following primary antibody incubation, sections should be washed 3 times (5 min each wash with PBS).

1.4.17. For the secondary antibody, applying Alexa 568-488 diluted to 1:400 in PBS on tissue sections and incubating for 1.30h at room temperature (avoid light exposure) works well.

1.4.18. Following secondary antibody incubation, sections should be washed 3 times in PBS.

1.4.19. Add DAPI solution (1µl/ml) to the sections and incubate for 5 minutes at room temperature, avoid light exposure.

1.4.20. Finally, rinse the sections in PBS, apply anti-fade mounting media to avoid rapid photobleaching, apply a coverslip, and fix the coverslip with nail varnish.

1.4.21. Imaging of the IHC: It is recommended to wait 12-24hours after mounting to ensure that the nail varnish is hardened.

1.4.22. Samples can be kept in the fridge protected from the light inside a dark box or covered in aluminium foil.

1.4.23. Approximately 30 minutes before imagining, acclimatize the slides to room temperature.

1.4.24. Images can be then taken with a fluorescent microscope.

1.4.25. Images captured with an EVOS cell imagining system works well (Figure 3).

1.5. Image analysis

There are different ways and software to analyse IHC images (e.g. stereological analysis or manually/automated cell counting). The most commonly used software are NIH Image, ImageJ, CellProfiler and Fiji ***[6–8]***. We believe that 10 to 20 pictures, obtained randomly and from across the entire Lz (at x20 magnification) are ideal for analysis. Regardless, any type of image analysis should always be conducted with the investigator blinded to experimental groups.

Two different methods to analyse the images are provided below.

1.5.1. *Cell counting* (e.g. for PCNA analysis see Figure 4)

- In ImageJ, open the image that needs to be counted.
- Convert the image into 16-bits.
- Set the threshold (toolbar: image / adjust / threshold).
- Subtract the background (process / substract background).
- Remove outliers (process / noise / remove outliers) (see note 9).
- Analyze particles (analyse / analyse particles), whilst keeping the size and circularity constant from image to image.
- For proliferation analysis (PCNA), calculate the total number of cells (DAPI) and total positive cells (Txred) and then express in percentage the proliferation rate as a ratio between positive/total cells.

1.5.2. *Stereological analysis* (e.g. for MCT1/MCT4 analysis see Figure 5)

- Open the image that needs to be counted in ImageJ.
- Open Plugins / Analyze / Grid.
- Select grid type/colour preferred by the research (e.g. points or crosses).
- Set the grid to give you 63 points per picture (Area per point: 20000).
- In excel or a lab book, record the number of points that are positive for MCT1 or MCT4, as well as the number of points that did not fall on any of these two areas
- Express values with respect to the total number of points counted (i.e. 63). For example:
- Total number of points positive for MCT1: 21
- Total number of points positive for MCT4: 24
- Total number of points in image analysed (that is not positive for MCT1 or MCT4): 18

MCT1 (21/63=33.33%) / MCT4 (24/64=38%) / Space (18/63=28.57%)

These volume densities can be corrected by the volume of the Lz if the researcher has previously analysed this structure ***[4]***.

Notes

1. Ensure that the operator does not inhale the PFA, always work with gloves and lab coat. If possible, use protective glasses and work in a fume hood to not inhale PFA. After preparation of PFA, it can aliquoted in Falcon tubes and stored in the -20ºC. If the solution is going to be used in the next weeks it can be kept at 4ºC.
2. Do not use pen as the ink will be washed off with the ethanol.
3. Paraffin wax at the embedding center should be at ≈60ºC.
4. The ethanol step will help to remove wrinkles and flatten the paraffin section.
5. Adding this number of sections per slide will be beneficial for IHC, as the researcher will have the ability to have a positive and negative control on the slide in similar conditions.

6. A humidified chamber can be made by using a slide storage box containing tissue paper which is made damp using dH2O.
7. It is important to not let the sections dry at any stage. In case of emergency, put some drops of PBS on top of the section or return the slides to a jar containing PBS.
8. The blocking step can be performed in different ways. The researcher should perform a trial and evaluate the staining based on the commercial antibody employed.
9. Ensure that the ratios and threshold are kept constant across the different images.

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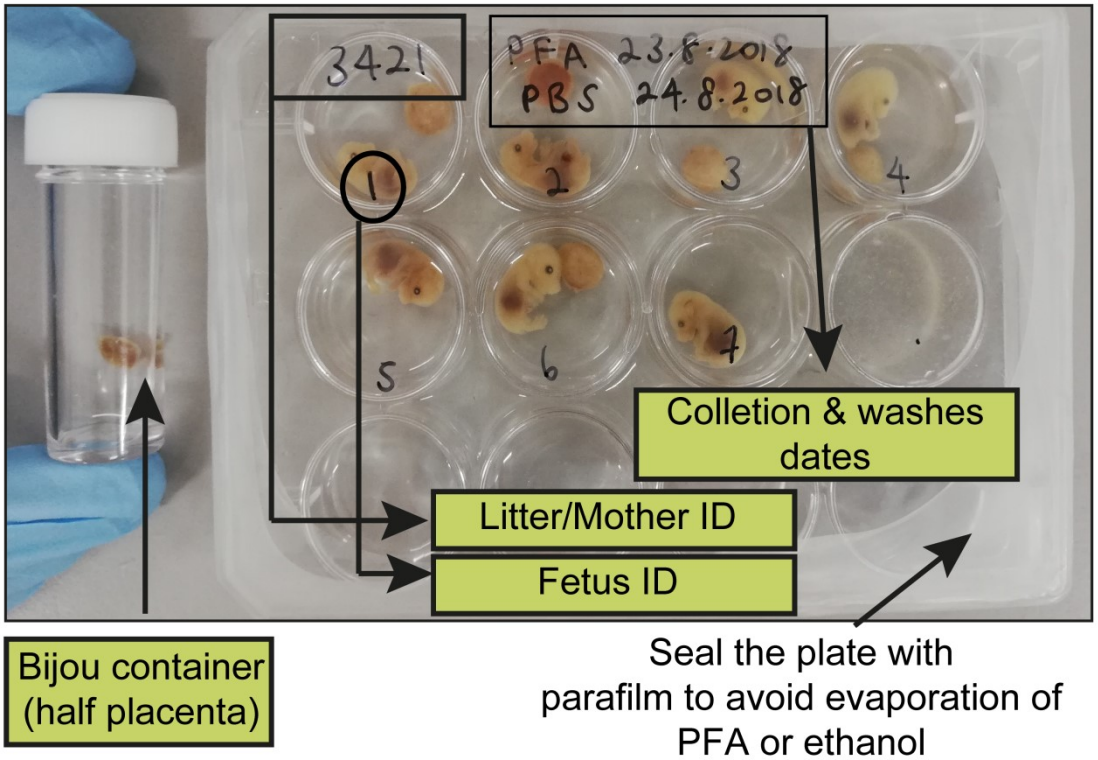
Table 1. Methods for antigen retrieval

Method	Reagent	Conditions	Conditions
Heat-induced	-Sodium citrate -Tris/EDTA	Microwave Water bath (90-100ºC) Pressure cooker	5-30minutes* *assessed by trial. Make sure slides cool down to room temperature before proceeding with the IHC protocol
Enzymatic reaction	-Pepsin -Trypsin -Pronase	Oven or water bath (37ºC)	10-15 minutes* *assessed by trial

346 **Figure legends**

347 **Figure 1. Different options to storage samples before wax-processing.**



348

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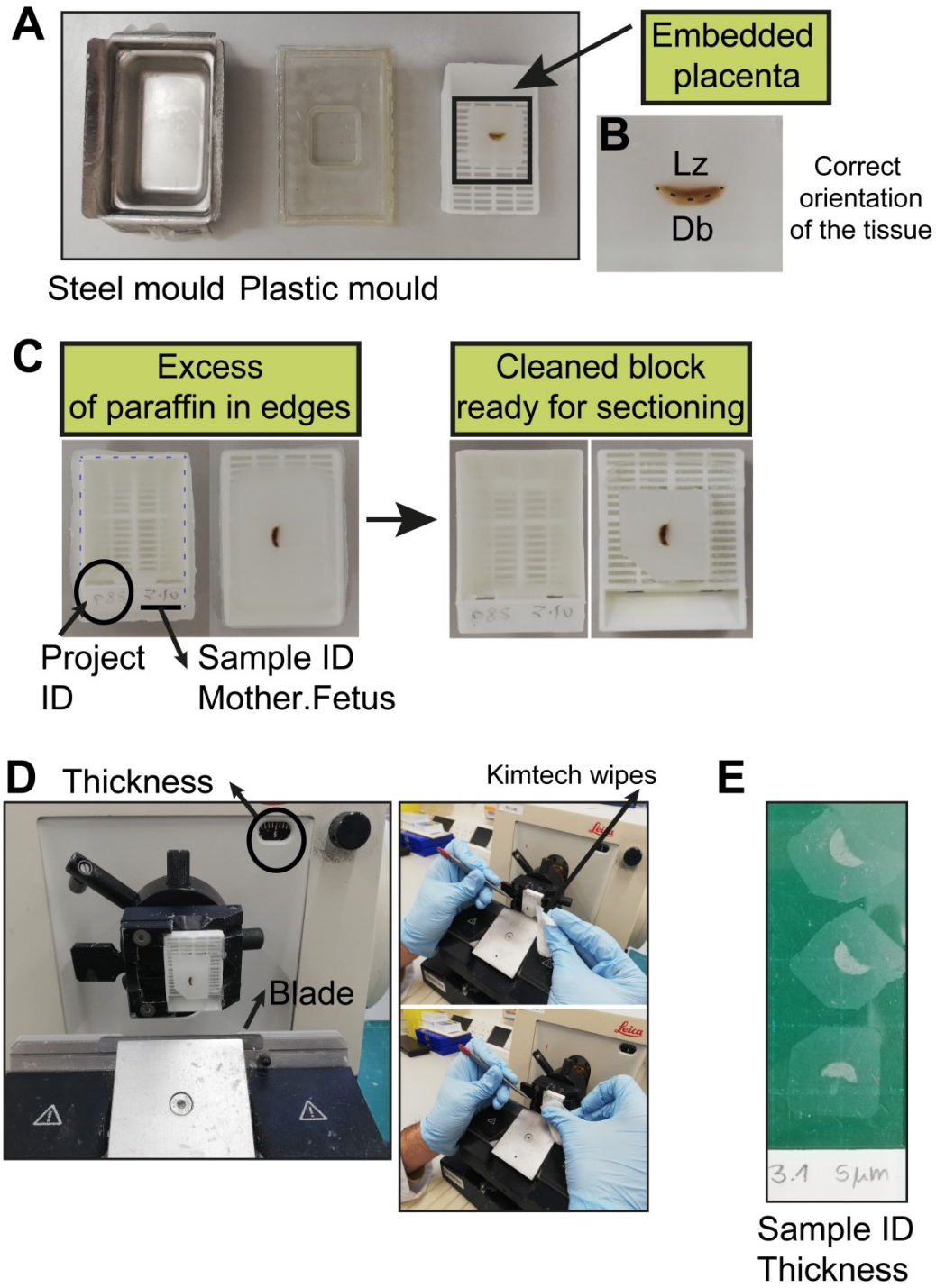
Figure 2. Critical steps in the processing and cutting of the sections.



353 **Figure 3. Staining for PCNA (proliferation marker), and MCT1 and MCT4 (SynT-I**

354 **and SynT-II populations)**

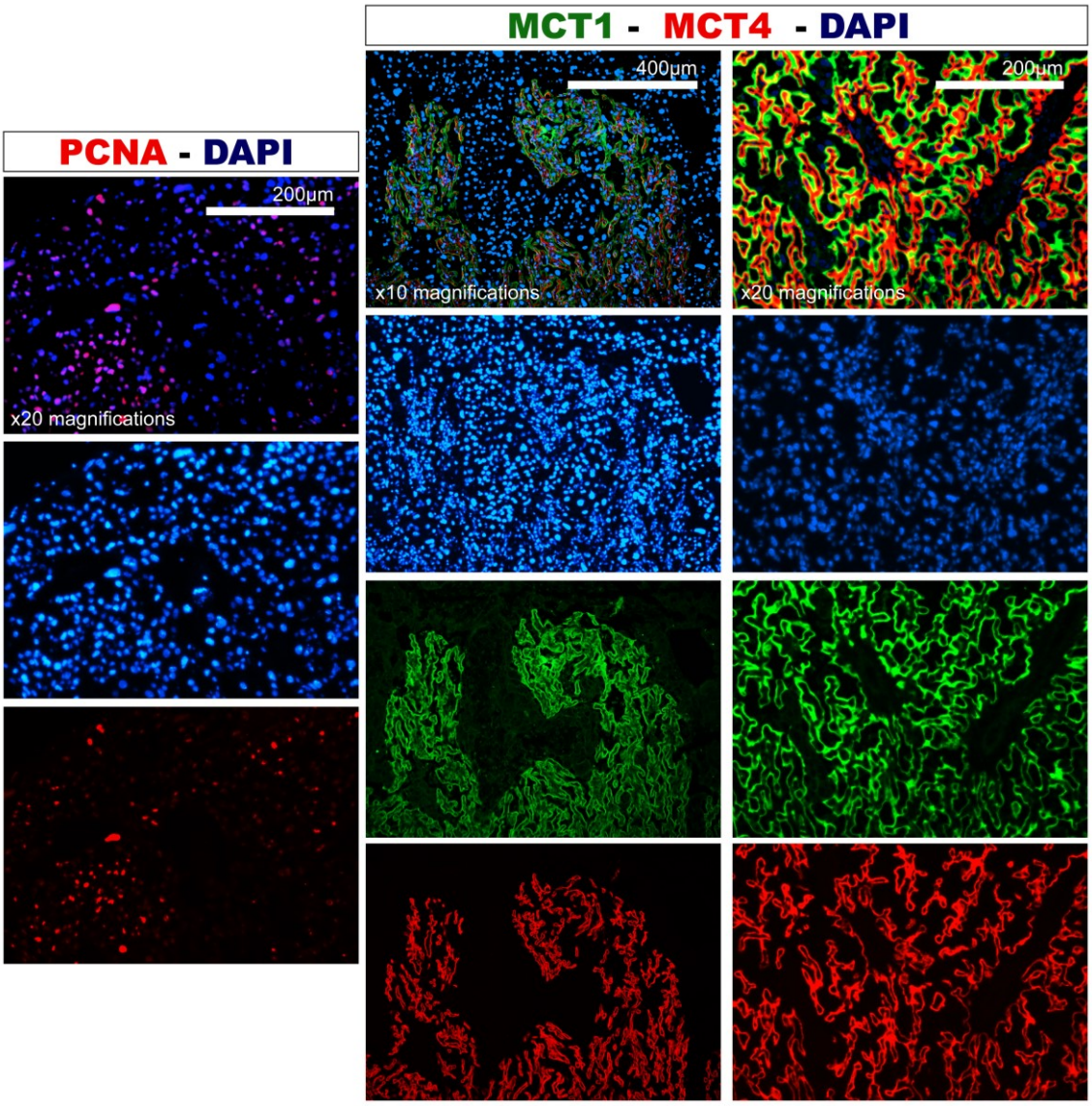


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Figure 4. Cell-counting analysis using ImageJ

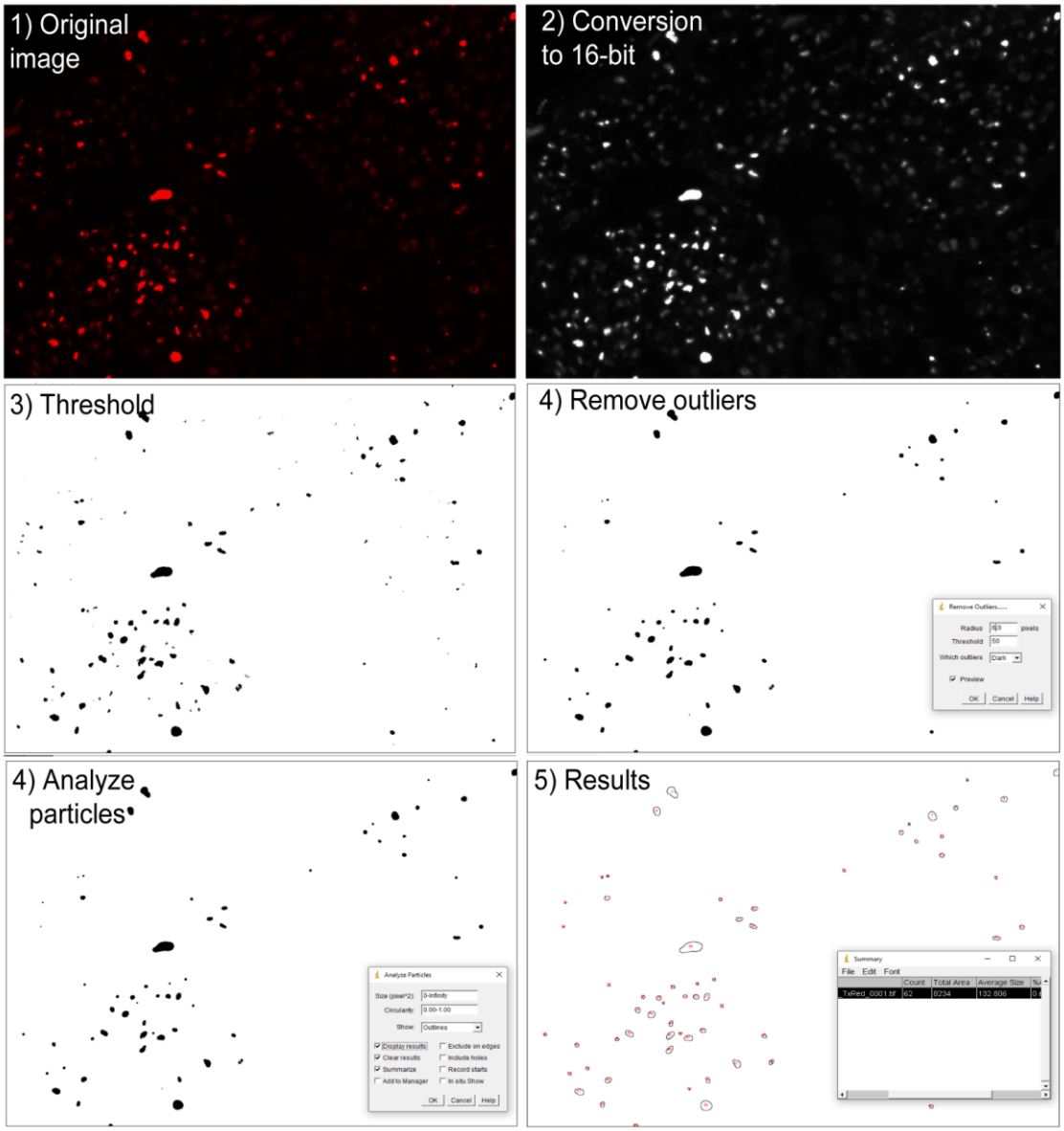

Figure 5. Stereological analysis of MCT1 and MCT4 for SynT-I and SynT-II populations