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July 10th – August 9th 2014
Control of Middle Ear and Jaw Development in *Monodelphis*

PROTOCOL: The use of Laser Capture Microscopy to isolate the proximal Meckel's cartilage from *Monodelphis* pups at the point of detachment of the middle ear from the jaw.
Rational: to isolate RNA from the specific portion of Meckel's cartilage which undergoes break down to allow the detachment of the middle ear form the mandible. RNA can then be used for next generation sequencing to identify genes responsible for transformation.

Step by Step protocol:

Ensure gloves are worn through out and that all surfaces are clean and wiped down with RNAse decontamination solution.

Steps 2-6 should be done within 20 minutes.

1. Collect opossum pups form mother. Minimum biological replicates of 2 for each time point used.
2. Euthanize pups, decapitate and skin heads. Tissue may not sectional correctly if skinning is incomplete.
3. Carefully open cranium along sagittal suture and remove brain without disrupting otic capsule
4. Submerge in freshly opened, pre-chilled Tissue-Tek OCT embedding compound (VWR) in a pre-labeled cryo-mould.
5. Position head for horizontal sections and snap freeze by submerging base of cryo-mould in a slurry of dry ice in 100% ethanol. Be carful not to get alcohol onto OCT compound as this will affect ability to section.
6. Once frozen, wrap in clean foil, labeled and store immediately at -80˚C.
7. Cryostat: external surfaces should be cleaned with RNAse decontamination solution such as RNAase Zap (Life Technologies) or a mild NaOH solution. Internal surfaces should be cleaned with 75% ethanol.
8. Trim block to region of interest. Collect 10µm sections onto prepared PEN membrane slides (Life Technologies). Collect every section proximal Meckel's cartilage over several slides. Once a slide is full, immediately store on dry ice. (Alternatively use immediately for LCM without freezing). Sectioning of each slide should be completed in less than 25 minutes.
9. When ready to carry out LCM, remove one slide from the -80˚C, immediate flick dry slide to that samples do not become wet with condensation as they come to room temperature.
10. Carry out LCM as per specific microscope set up available (e.g. Arcturus Veritas LCM). Pool all slides from one individual into one LCM tissue collection cap. Aim to complete all sectioning and collection form one individual within 45 minutes
of defrosting first slide. Set aside first slide of an individual until all slides from that individual have been captured.
11. Remove the LCM cap form the microscope and if required, assemble the LCM extraction device. If using the Arcturus Picopure LCM RNA extraction kit, add 10µl of extraction buffer to the cap. Pipette a further 10µl of extraction buffer on to a section of the set aside slide. Leave fore 30 seconds, then pipette the extraction buffer off the slide and in to a clean RNAse free 0.5ml centrifuge tube. This will act as your quality control RNA (QC RNA).
12. Close both tubes and incubate for 30 minutes at 42˚C. Samples are now stable and should be stored on dry ice or at -20˚C until all samples are

pipette the extraction buffer off the slide and in to a clean RNAse free 0.5ml centrifuge tube. This will act as your quality control RNA (QC RNA).
12. Close both tubes and incubate for 30 minutes at 42˚C. Samples are now stable and should be stored on dry ice or at -20˚C until all samples are collected.
13. Repeat 9-12 until all desired tissue is collected.
14. Carry out RNA extraction using Arcturus Picopure RNA Isolation kit (Life Technologies) as per protocol. Skip optional DNA digestion step as Arcturus RNA isolation kit does not permit DNA to pass through the column, and gDNA digestion may reduce RNA quality. Elute with 11µl of elution buffer. Immediately store RNA from LCM at <-80˚C. Ensure samples are stored in the back of the -80˚C freezer or in LiN2 to prevent fluctuations in temperature that can result in RN degradation.
15. Use QC RNA to determine RNA quality by use of bioanalyser (e.g. Agilent Technologies 2100 Bioanalyser). RNA quality of QC RNA, as measured by RIN value, will give an indication of the RNA quality of the LCM derived cells, while avoiding loss of the sample RNA. RIN values of >7.5 are suitable for downstream amplification and RNA-Seq. QC-RNA should yield 20-40ng/µl. Sample RNA will yield around 1-3ng/µl for each proximal Meckel’s cartilage / malleus joint region.