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CXCL5 Osmotic Pump HFD Study

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We use this protocol and it's working

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Abstract

This protocol outlines a method for inducing atherosclerosis in CXCL5 heterozygous APOE KO male mice, installing osmotic pumps to administer CXCL5 directly to the bloodstream, and quantifying atherosclerotic lesions in extracted aortic roots. The selection of CXCL5 heterozygous mice simulates the lowest naturally occurring levels of CXCL5 expression in human populations, and this technique simulates the onset of atherosclerosis and the potential therapeutic benefits of direct administration of CXCL5 on atherosclerotic disease burden. Expected results include less atherosclerotic lesion area by percentage of total aortic root area compared to control mice.

Protocol materials

- Gauze **Covidien** **Catalog #**2634
- Dry Ice
- Lab Bench Protector **VWR International (Avantor)** **Catalog #**89126-792
- Major LubriFresh P.M. Sterile Artificial Tears Ointment **Amazon** **Catalog #**B00KA91S0M
- 5mm Goldenrod Lancet **Braintree Scientific** **Catalog #**GR 5MM
- BD Microtainer Capillary Blood Collector (green closure) **Thermo Fisher Scientific** **Catalog #**13-680-62
- BD Microcontainer Clot Activator (gold closure) **Becton Dickinson (BD)** **Catalog #**365967
- 1.7 mL Tubes **Genesee Scientific** **Catalog #**24-282LR
- CO2 Chamber
- 70% Ethanol
- Forceps **Fisher Scientific** **Catalog #**08-953E
- Surgical Scissors **Fine Science Tools** **Catalog #**14002-12
- scalpel blades
- 10% Neutral Buffered Formalin **Fisher Scientific** **Catalog #**STL286001
- Roboz Microscissors **Roboz** **Catalog #**RS-5620
- 18G Needle **Becton Dickinson (BD)** **Catalog #**305195
- Liquid Nitrogen
- 10 mL Plastic Syringe **Fisher Scientific** **Catalog #**14-955-459
- 50 mL Conical Flask **VWR International (Avantor)** **Catalog #**89039-656
- Adjusted Calorie Mouse Food **Envigo (Harlan)** **Catalog #**TD.88137
- Normal Chow Mouse Food
- D-Sucrose (Molecular Biology) Fisher BioReagents **Fisher Scientific** **Catalog #**BP220-1
- Sodium Azide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S8032
- BSA **Thermo Fisher Scientific** **Catalog #**BP9706100
- Distilled Water
- Deionized Water
- Trisodium citrate dihydrate **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S1804
- MgSO4 **Avantor Sciences** **Catalog #**MK607012
- water
- HCl **Fisher Scientific** **Catalog #**A144-212

- Sodium Phosphate Dibasic Anhydrous **Fisher Scientific** **Catalog #**BP332-500
- Potassium Phosphate Dibasic **Thermo Fisher Scientific** **Catalog #**P285-500
- NaH2PO4 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S0751
- CaCl2 **Fisher Scientific** **Catalog #**BP9742
- 1x Phosphate buffered saline pH 7.4
- 45% D-( )-Glucose **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**G8769
- EDTA **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**ED-1KG
- NaHCO3 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S8875
- KCl **Bio Basic Inc.** **Catalog #**PB0440
- Triton X-100 **Fisher Scientific** **Catalog #**BP151-500
- Paraformaldehyde **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**158127
- Propylene Glycol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P4347-500ML
- NaCl **Fisher Scientific** **Catalog #**S640-10
- Oil Red O **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**O0625
- Andwin Scientific Tissue-Tek™ CRYO-OCT Compound **Fisher Scientific** **Catalog #**14-373-65
- 0.22 µM syringe-end filter **Merck Millipore (EMD Millipore)** **Catalog #**SLGV004SL
- 1 mL Syringe **Becton Dickinson (BD)** **Catalog #**309659
- suture
- Mouse Jugular Catheter (Adjustable Length) **Alzet** **Catalog #**0007701
- Osmotic Pumps **Alzet** **Catalog #**7223
- Recombinant Human ENA-78 (CXCL5) **peprotech** **Catalog #**300-22B

Solutions

1 **1X Phosphate Buffered Saline (PBS)** (makes 1 L):

800 mL Deionized Water

8 g NaCl **Fisher Scientific Catalog #**S640-10

0.2 g KCl **Bio Basic Inc. Catalog #**PB0440

1.44 g

Sodium Phosphate Dibasic Anhydrous **Fisher Scientific Catalog #**BP332-500

0.24 g

Potassium Phosphate Dibasic **Thermo Fisher Scientific Catalog #**P285-500

Add HCl **Fisher Scientific Catalog #**A144-212 until pH 7.4

RBC Lysis Buffer (makes 500 mL):

443.35 mL Distilled Water

1.25 g (1.65 mL) BSA **Thermo Fisher Scientific Catalog #**BP9706100

5 mL Triton X-100 **Fisher Scientific Catalog #**BP151-500

50 mL NaCl **Fisher Scientific Catalog #**S640-10

Store at 4 °C

Ringers Solution in 0.1% BSA (makes 250 mL)

1. Add to a 500 mL beaker:

35.994 mg

NaH2PO4 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S0751

30.092 mg MgSO4 **Avantor Sciences Catalog #**MK607012

90.021 mg KCl **Bio Basic Inc. Catalog #**PB0440

2001.57 mg NaCl **Fisher Scientific Catalog #**S640-10

504.05 mg NaHCO3 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S8875

55.492 mg CaCl2 **Fisher Scientific Catalog #**BP9742

450.39 mg

45% D-( )-Glucose **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**G8769

2. Add 200 mL Distilled Water

3. Add 250 mg BSA **Thermo Fisher Scientific Catalog #**BP9706100

4. Adjust to pH 7.4

5. Add Distilled Water until 250 mL total volume

6. Pour into autoclaved 250 mL bottle

Store at 4 °C

30% sucrose in 1x PBS:

30 g

D-Sucrose (Molecular Biology) Fisher BioReagents **Fisher Scientific Catalog #**BP220-1

per 100 mL 1x Phosphate buffered saline pH 7.4

Citrate Anticoagulated Solution:

4 g

Trisodium citrate dihydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S1804

per 100 mL water (autoclaved for sterilization)

0.05% Sodium Azide in 1x PBS:

50 mg

Sodium Azide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S8032

per 100 mL 1x Phosphate buffered saline pH 7.4 (sterile filter)

4% Paraformaldehyde in 1x PBS:

4 g

Paraformaldehyde **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**158127

per 100 mL 1x Phosphate buffered saline pH 7.4 (sterile filter)

Sucrose Formalin:

10 g

D-Sucrose (Molecular Biology) Fisher BioReagents **Fisher Scientific Catalog #**BP220-1

per 100 mL

10% Neutral Buffered Formalin **Fisher Scientific Catalog #**STL286001

4 mM EDTA in 1x PBS:

Prepare 100 mL [M] 0.4 Molarity (M) EDTA by adding 14.61 g EDTA **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**ED-1KG to 70 mL Deionized Water .

Add 50 mL of your [M] 0.5 Molarity (M) EDTA to 950 mL of 1X PBS.

0.5% Oil Red O Solution:

Add 0.5 g of

Oil Red O **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**O0625 to 100 mL

Propylene Glycol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P4347-500ML

85% Propylene Glycol Solution:

Add 15 mL Distilled Water and 85 mL

Propylene Glycol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P4347-500ML

10% Neutral Buffered Formalin:

Mouse Model

2 Mice were CXCL5 heterozygous ApoE Knockout males.

Materials

3 **Mouse Feeding:**

Adjusted Calorie Mouse Food **Envigo (Harlan) Catalog #**TD.88137

Normal Chow Mouse Food

Note

Important Differences Between High-Fat Diet (Adjusted Calorie Mouse Food) and Normal Chow:
High Fat Diet:
- 21.2% fat by weight
- 4.5 kcal/g
- 48.5% carbohydrates

Normal Chow Diet
- 5% fat by weight
- 3.9 kcal/g
- 65% carbohydrates by weight

3.1 **Submandibular Bleeds for Plasma:**

Major LubriFresh P.M. Sterile Artificial Tears Ointment **Amazon Catalog #**B00KA91S0M

5mm Goldenrod Lancet **Braintree Scientific Catalog #**GR 5MM

BD Microtainer Capillary Blood Collector (green closure) **Thermo Fisher Scientific Catalog #**13-680-62

BD Microcontainer Clot Activator (gold closure) **Becton Dickinson (BD) Catalog #**365967

1.7 mL Tubes **Genesee Scientific Catalog #**24-282LR

Gauze **Covidien Catalog #**2634

Dry Ice

Lab Bench Protector **VWR International (Avantor) Catalog #**89126-792

3.2 **Osmotic Pump Implantation**

Osmotic Pumps **Alzet Catalog #**7223

Recombinant Human ENA-78 (CXCL5) **peprotech Catalog #**300-22B

0.22 µM syringe-end filter **Merck Millipore (EMD Millipore) Catalog #**SLGV004SL

1 mL Syringe **Becton Dickinson (BD) Catalog #**309659

scalpel blades

suture

- isoflurane or ketamine (anesthetic)

Mouse Jugular Catheter (Adjustable Length) **Alzet Catalog #**0007701

3.3 **Aortic Root Extraction**

Liquid Nitrogen

Dry Ice

Forceps **Fisher Scientific Catalog #**08-953E

Surgical Scissors **Fine Science Tools Catalog #**14002-12

CO2 Chamber

70% Ethanol

scalpel blades

10% Neutral Buffered Formalin **Fisher Scientific Catalog #**STL286001

Roboz Microscissors **Roboz Catalog #**RS-5620

One per mouse:

18G Needle **Becton Dickinson (BD) Catalog #**305195

10 mL Plastic Syringe **Fisher Scientific Catalog #**14-955-459

50 mL Conical Flask **VWR International (Avantor) Catalog #**89039-656

3.4 **Atherosclerosis Quantification**

Andwin Scientific Tissue-Tek™ CRYO-OCT Compound **Fisher Scientific Catalog #**14-373-65

- Forceps
- Eosin
- Sirius Red

Oil Red O **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**O0625

Propylene Glycol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**P4347-500ML

- Parafilm
- Tissue molds 15×15 mm (Fisher Scientific Cat # 41-741)
- Probe-on-Plus microscope slides (Fisher Scientific Cat # 22-230-900)
- Small weigh boat or petri dish
- Dry Ice
- Styrofoam box
- 6-well plate cell culture lid
- Aluminum foil
- 100% Ethanol
- Thin copper plate
- Gill's or Mayer's hamatoxylin

- glycerin jelly

Equipment

4

Equipment

Micro 17 Microcentrifuge	NAME
Microcentrifuge	TYPE
Sorvall Legend	BRAND
75002431	SKU

Equipment

Compass CX	NAME
Weigh Scale	TYPE
Compass	BRAND
CX221	SKU

- -80 freezer
- BioRad-Plex 200 R&D Luminex
- Alfa Wassermann Vet Axcel Chemistry System
- 4 degree storage

- -20 degree freezer
- Laminar Flow Hood
- Isoflurane Vaporizer
- Dissecting microscope
- Rocker

Timeline

5 **0-10 weeks of age**
- Maintain CXCL5 heterozygous Apoe knockout male mice on Normal Chow Diet.

5.1 **9 weeks of age**
- First RBC Bleeds

5.2 **10 weeks of age**
- First Plasma Bleeds
- Mice are enrolled
- Normal Chow Diet is switched to High Calorie Diet

5.3 **10-16 weeks of age**
- Maintain mice on High Calorie Diet
- Record weights of mice weekly

5.4 **16 weeks of age**
- Osmotic pump implantation

5.5 **16-22 weeks of age**
- Continue maintaining mice on Adjusted Calorie Diet
- Record weights of mice weekly

5.6 **22 weeks of age**
- Final RBC Bleeds
- Final Plasma Bleeds
- Aortic Root Extraction
- Atherosclerosis Quantification

Red Blood Cell (RBC) Bleeds

40m

6 **Preparation**
1. The day before plasma is collected, mouse weight is recorded.
2. After weighing, mice are then fasted overnight. Please be sure not to fast the mice for over 24 hours.
3. Prepare a lab bench with your lab protector.
4. Gather the necessary materials outlined in Section 3.1.

5. Retrieve two 1.7 mL tubes, one green-top BD Microtainer, and one lancet for each mouse.
6. Retrieve LubriFresh (ointment) and gauze.

6.1 Collecting Blood

Safety information

- When working with animals wear proper PPE, such as gloves, a lab coat, and goggles.
- It is important to follow IACUC guidelines regarding the usage of animals.
- If you are bitten by a mouse during the procedure, take off your gloves and check for a break of the skin. Thoroughly wash any bite that breaks the skin and report the injury.

1. Scruff the mouse by using your thumb and forefinger to pinch the loose skin over the shoulders and behind the ear. Hold the tail between your ring and your pinky finger on the same hand you are holding the mouse with.
2. Use your other hand to apply a small amount of ointment to the mouse's cheek. Use the ointment to part the fur up and down to expose a horizontal line of skin in **Figure 1**.
3. Perform a submandibular bleed by inserting the lancet on the line of skin, pointed towards the nose, at the blue spot shown in **Figure 1**. Wiggle the lancet gently after puncturing the skin, still making sure it is never pointed towards the brain, as you could puncture the blood-brain barrier and the mouse will die soon after. If you do not get blood, try again in the same area with a new lancet or switch sides after multiple attempts with a new lancet.
4. Once blood is flowing, collect blood into the green BD Microtainer by tipping the mouse and letting the blood fall freely into the tube, using gravity for collection.
5. Collect ~100uL of blood
6. Once finished, cap the tube and invert it carefully 2-4 times to prevent coagulation. Inverting the tube too fast will damage the sample, by popping the RBCs.
7. Use a gauze square to stop the bleeding. If bleeding persists, apply gentle but firm pressure.
8. Repeat steps 1-7 for each mouse, using a different lancet each time.

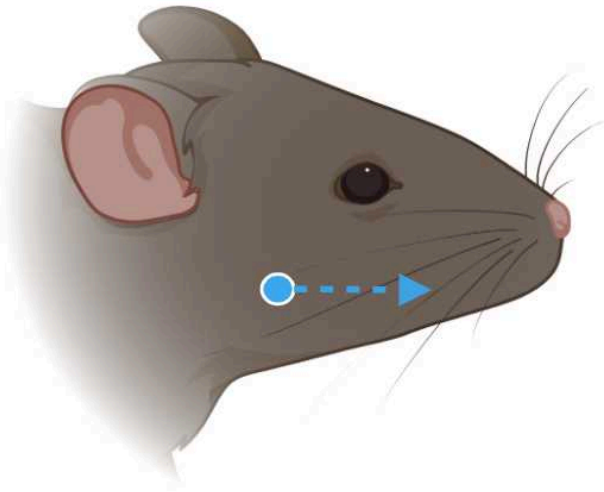

Figure 1. Submandibular Bleed Injection Point. This diagram shows the desired puncture point and lancet direction for performing a submandibular bleed. The puncture point is shown by the blue point encircled in white, and the direction at which the lancet should point upon puncture is shown by the blue dashed arrow pointing to the right of the puncture point.

Note

- A new, sterile lancet must be used for each mouse to avoid potential infection or other complications.
- Failing to point the lancet toward the nose somewhere in the range shown in **Figure 1** will heighten the risk of complications and even possibly terminate the mouse if pointed up toward the brain.

6.2 **Green BD Microtainer Processing** 40m

1. Centrifuge the blood samples at 3 x g for 00:03:00 .
2. Pipette the supernatant plasma into one of the 1.7 mL tubes and store at -80 °C .
3. There will be a jelly-like pellet left in the green BD Microtainer.
4. Add 1 mL of 1x PBS to the green BD Microtainer with the pellet.
5. Centrifuge the pellet and 1x PBS-containing tubes at 1.8 x g for 00:03:00 .
6. Pipette out and discard the remaining supernatant, keeping the pellet in the tube.
7. Repeat steps 4-6.

8. Add RBC Lysis Buffer to the tube. The amount you add should be 10 µL more than the volume of blood you collected for each sample. For example, a sample that had 200 µL of blood should have 210 µL of RBC Lysis Buffer added to it.
9. Let the tubes sit for 00:30:00 at Room temperature .
10. Centrifuge the tubes at 14 x g for 00:04:00 .
11. Pipette the pellet supernatant into the other 1.7 mL tube and store at -80 °C .
12. Store the remaining RBC samples at -80 °C until ready for further analysis.

Plasma Bleeds

5m

7 **Preparation**

1. The day before the bleeds, weigh the mice and record their weights.
2. After weighing, fast the mice overnight. Do not fast for over 24 hours.
3. Prepare a lab bench with a lab protector.
4. Gather the necessary materials outlined in Section 3.1.
5. Retrieve one 1.7 mL tube, one yellow-top BD Microtainer, and one lancet for each mouse.
6. Retrieve lubricant and gauze.

7.1 **Collecting Blood**

See Section 6.1

7.2 **Yellow BD Microtainer Processing**

5m

1. After every four mice centrifuge the four containers at 3 x g for 00:05:00 .
2. Pipette at least 100 µL of the supernatant plasma into the 1.7 mL tube without puncturing the wax.
3. Discard the used yellow BD Microtainers and store the plasma samples at -80 °C until ready for further analysis.

Note

- It is important to avoid puncturing the wax as it will ruin the sample. To do this, pipette the supernatant in two steps, pipetting only an amount you are confident will not be close to the wax the first time, and then the rest the second time. This way, you still have a good sample if you puncture the wax the second time.

Osmotic Pump Implantation

2d 12h

8 **Preparation**

1. Fast mice 24 hours before the procedure?
2. Autoclave gauze to be used as a sterile space to place osmotic pumps.
3. Clear a space in a laminar flow hood to provide an uncluttered and organized workspace. Use 70% ethanol to wipe down the inside of the hood.
4. Place bench protector down on laminar flow hood workspace.
5. Gather the necessary materials outlined in Section 3.2

Safety information

- When working with animals wear proper PPE, such as gloves, a lab coat, and goggles.
- It is important to follow IACUC guidelines regarding the usage of animals.

8.1 **Preparing Solutions**

1. Dissolve Recombinant Human ENA-78 (CXCL5 8-78) in Ringer's Solution in 0.1% BSA. The concentration should be 0.5mg/ml CXCL5 to achieve a mass delivery rate of 90 ng/hour, a rate found by our lab to produce adequate levels of circulating CXCL5 in the bloodstream. For example, if you have 1 mg CXCL5, the method is as follows:
2. Dissolve the 1 mg in 1 mL Ringer's Solution.
3. Mix the solution from step 2 with another 1 mL Ringer's Solution.
4. Make sure your CXCL5 in Ringer's Solution in 0.1% BSA and your Ringer's Solution in 0.1% BSA are both at room temperature prior to the procedure.

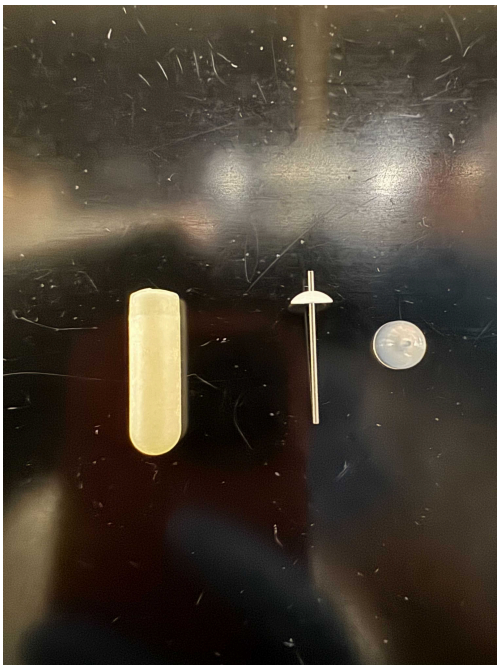

Figure 2. Components of Osmotic Pump. On the left is the storage component of the osmotic pump. In the middle is the flow moderator. On the right is the cap. This is how they will be referred to in future steps.

Note

- It is important to take into account how much solution you are going to need. For the Model 2006 Osmotic Pumps used in this study, you will need 200 µL of solution per pump.
- You will also need approximately 20 µL of solution per catheter.
- The equation used to calculate the concentration needed and to acquire the desired flow rate is as follows: k=Q*C, where k is the mass delivery rate (flow rate, ug/hour), Q is the pumping rate of the pump (0.18 ul/hour for 2006 Alzet pump), and C is the concentration of the drug in the solution.

8.2 Filling the Pumps

Pump filling was done in accordance with the provided guidelines by the pump manufacturer: **Filling & Priming ALZET Pumps - ALZET® Osmotic Pumps**

Note

- Make sure you have on surgical gloves when dealing with osmotic pumps. Skin oils may interfere with the performance of a pump if they accumulate on its surface. If a pump becomes contaminated, its surface may be wiped with an aqueous solution of 70% isopropanol immediately before use.
- Half of the osmotic pumps should receive the CXCL5 In Ringer's Solution in 0.1% BSA and half should receive the Ringer's Solution in 0.1% BSA.

1. Weigh the empty pump together with its flow moderator.
2. Attach a filling tube (supplied with each package of pumps) to your syringe and draw up the solution.
3. With the flow moderator removed, hold the pump in an upright position (exit port pointed vertically).
4. Insert the filling tube through the opening at the top of the pump until it can go no further. This places the tip of the tube near the bottom of the pump reservoir.
5. Slowly push the plunger of the syringe, holding the pump in an upright position. A small amount of backpressure is normal, due to the tight seal at the filling port.

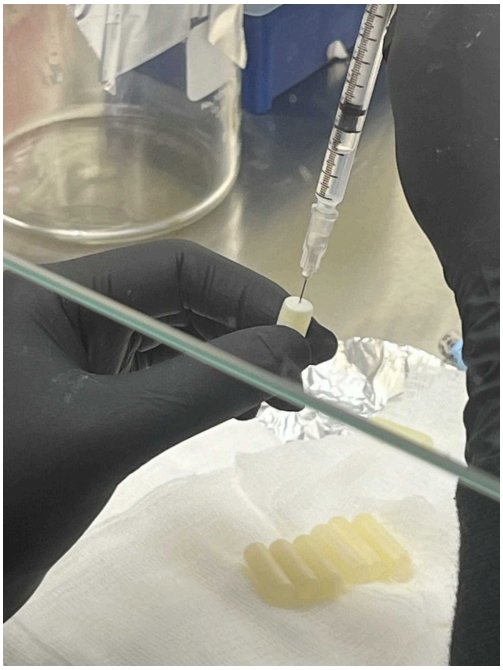

Figure 3. Osmotic Pump Filling.

1. When the solution appears at the outlet, stop filling and carefully remove the tube.
2. Wipe off the excess solution and insert the flow moderator until the cap or flange is flush with the top of the pump. The syringe attached to the distal end of the catheter can now be removed. The insertion of the flow moderator will displace some of the

solution from the filled pump. This overflow should be wiped off. The flow moderator must be fully inserted into the body of the pump.

Note

- If working with an expensive solution, you can conserve it by using your syringe to pipet the excess back up. This is shown in **Figure 4.**

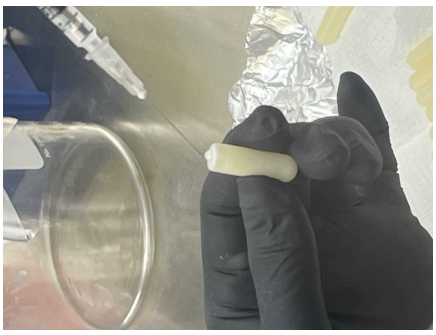

Figure 4. Solution Conservation.

1. Weigh the filled pump with the flow moderator and cap in place. The difference in the weights obtained in this step and the initial weight taken in the first step will give the net weight of the solution loaded. For most dilute aqueous solutions, the weight in milligrams (mg) is approximately the same as the volume in microliters (µl). The fill volume should be more than 90% of the reservoir volume specified on the instruction sheet. If so, the filled pump is ready for use. If not, there may be some air trapped inside the pump. Evacuate the incompletely filled pump and refill (Steps 1-7).

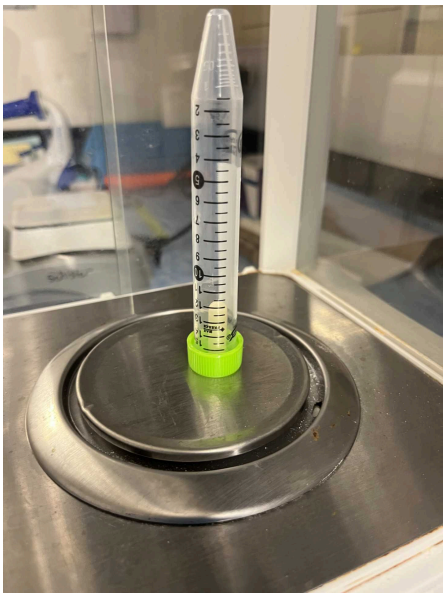

Figure 5. Osmotic Pump Weighing.

Note

- It is essential that each pump be filled completely with drug solution. Air bubbles trapped within the body of the pump, or failure to properly insert the flow moderator into the pump may result in unpredictable pumping rate fluctuations.
- When filling the pump, ensure that the solutions are at room temperature.
- Rapid filling of pumps should be avoided because it can introduce air bubbles into the reservoir.

8.3 **Placing the Catheters**

Catheter placement was done in accordance with the provided guidelines by the pump manufacturer: **Catheter Use - ALZET® Osmotic Pumps**

1. Remove the translucent cap from the end of the flow moderator, revealing a short stainless steel tube protruding from the white flange.
2. Attach the flow moderator to a piece of your catheter tubing. After attachment, the catheter should cover the entire length of the stainless-steel tube above the white flange (or about 3-4 mm).
3. Fill the catheter and attached flow moderator using a syringe. Leave the syringe attached to the distal part of the catheter.

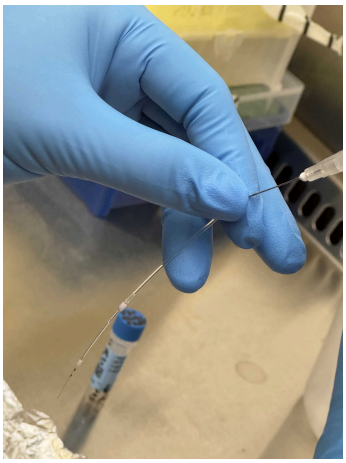

Figure 6. Catheter Filling

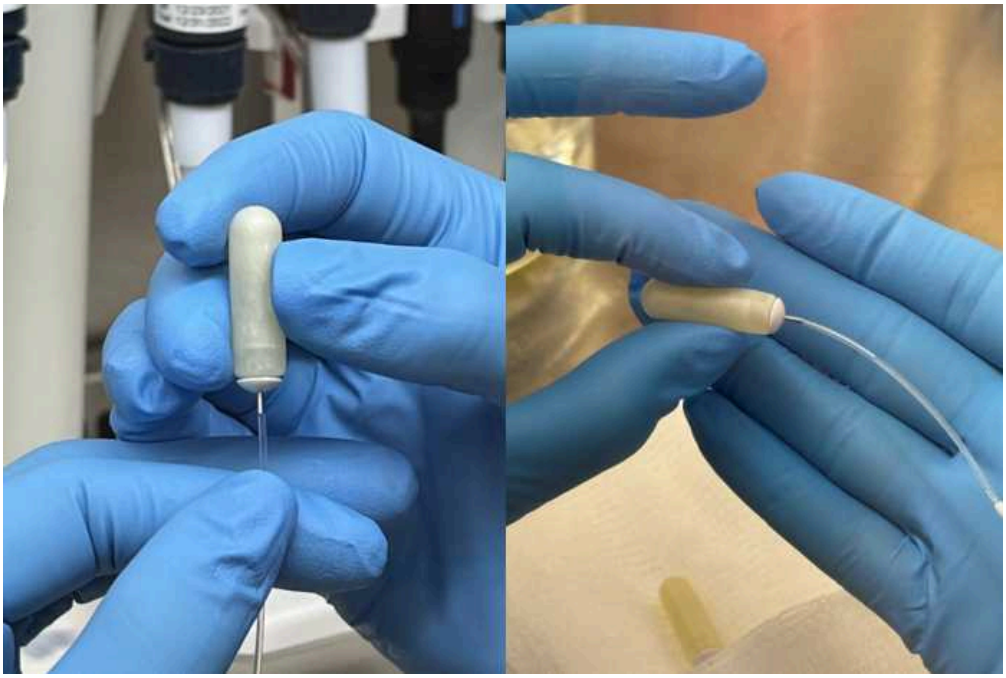

Figure 7. Catheter Attached

8.4 **Priming the Pumps** 2d 12h

Pump priming was done in accordance with the provided guidelines by the pump manufacturer: **Filling & Priming ALZET Pumps - ALZET® Osmotic Pumps**

1. Place the prefilled pumps in 1x PBS solution for 60:00:00 .
2. It is possible to drape the end of the catheter outside the beaker to avoid mixing solutions.

3. Do not be concerned if due to evaporation, fluid is not observed dripping from the end of the catheter, as evaporative loss can occur. Remove the pump from the saline and implant immediately.

Note

- While a small amount of drug solution will be expelled during priming, this will not compromise the administration of your compound for the full delivery period. The pumps are manufactured such that the reservoir holds sufficient solution to deliver beyond the infusion period.

8.5 **External Jugular Vein Pump Placement**

External jugular vein pump placement was generously performed by the local lab vet, Dr. Aung Moe Zaw. Alternatively, sources for performing the operation oneself are provided below:

Implantable Osmotic Release Pump - Conduct Science
THE INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) (ucsf.edu)
Implantation & Explantation - ALZET® Osmotic Pumps

Note

- The location for subcutaneous implantation of osmotic pumps in these mice should be on the dorsum, slightly caudal to the scapulae.

1. Anesthetize the mouse using isoflurane (**Isoflurane Procedures for Safe Use (2023).pdf | Powered by Box**) or ketamine (**UCSF Institutional Animal Care and USe Program-Office of Ethics and Compliance**).
2. Position the animal in dorsal recumbency and elevate the neck to display the ventral neck.
3. Make an incision just lateral to the trachea and dissect down to the external jugular vein
   so that it can be elevated.
4. Ligate the cephalic end of the vein and place two loose ligatures around the cardiac end
   of the vein.
5. Insert the catheter into the jugular vein and control hemorrhage with gentle traction on
   the cephalic suture ends.
6. Tie the cardiac ligatures around the catheter and then tie the cephalic ligature. Trim the

ends of all three ligatures close to the knots.

7. Create a pocket on the dorsum of the animal in the midscapular region. Place the pump
   in this pocket allowing the catheter to reach over the neck to the external jugular vein to
   permit free head and neck movement.
8. Feed the caudal end of the pump through the tunnel into the pocket.
9. Use a two-layer closure with absorbable material in the underlying fascia and an additional layer of closure for the skin using suture.
10. Recover the animal following the UCSF Rodent Anesthesia guidelines and provide analgesia as described in the approved protocol.

For a more detailed walkthrough, reference:
Mouse External Jugular Catheterization/Osmotic Pump Implantation (youtube.com)

Note

- Animals should be monitored for signs of distress or discomfort during and after recovery.
- If needed, administer analgesics per the approved IACUC protocol.
- Animals experiencing post-procedural complications that cannot be alleviated should be euthanized using approved guidelines. (**Guideline - Anesthesia - Rodents.pdf (ucsf.edu)**)

Aortic Root Extraction

9 **Preparation**

1. Gather necessary materials from Section 3.3.
2. Transfer an adequate amount of sucrose formalin to each
   50 mL Conical Flask **VWR International (Avantor) Catalog #**89039-656 and
   store at -20 °C .
3. Prepare syringes with 10 mL of 5 mM EDTA in PBS, 10 mL of 1x PBS, and 10 mL of sucrose formalin (three syringes per mouse) and store at -20 °C .
4. Sucrose formalin fixative and perfusion syringes should be kept on ice during the procedure and made easily accessible.

Safety information

- When working with animals wear proper PPE, such as gloves, a lab coat, and goggles.
- It is important to follow IACUC guidelines regarding the usage of animals.

9.1 **Procedure**

2w 1d 1h 15m

1. Anesthetize the mouse with CO_2.
2. Spray mouse with 70% Ethanol to make cutting easier.
3. Make an incision from the base to the diaphragm and be careful to cut only the peritoneum.

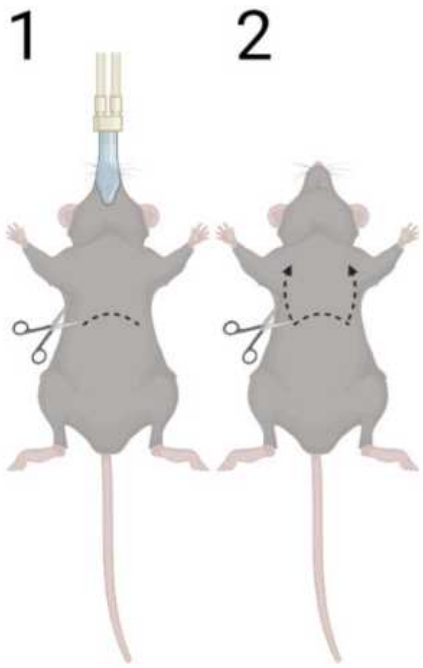

Figure 4. Laparotomy and Thorax Exposing. This figure shows the necessary cuts for exposing the heart. Step 1 shows the performing of a laparotomy. Step 2 represents the cuts of the rib cage to expose the thorax.

1. Rinse the mouse vasculature with 10 mL of cold 5mM EDTA in 1x PBS by gravity perfusion through a puncture of the left ventricle and an incision of the right atrium for drainage. This should be performed with an 18G needle and syringe.
2. Rinse the mouse vasculature with 10 mL of cold 1x PBS by gravity perfusion through a puncture of the left ventricle and an incision of the right atrium for drainage. This should be performed with an 18G needle and syringe.
3. Fix the vasculature by gravity perfusion through the left ventricle with 10mL of sucrose formalin. This should be performed with an 18G needle and syringe.
4. Remove the entire heart with the aorta attached. This will include the
5. Fix the aortic structure with the heart attached in your prepared sucrose formalin at 4 °C for 24:00:00 . The sample should be on a rocker during this time.

6. Drain the fixative and then wash the sample 3x with 1x PBS for 00:10:00 each.
   The PBS should be dumped and replaced between washes.
7. Pour enough 0.5% Triton X-100 in 1x PBS to cover the aortic structure and let it rock for 01:00:00
8. Repeat step 5, but wash for 00:05:00 intervals instead.
9. Transfer the structure to 1x PBS and store at 4 °C .

Note

If storing for a prolonged period of time, transfer the structure to 0.05% sodium azide in 1x PBS and store at 4 °C

1. Surgically extract the aorta with the heart attached from the structure.
2. Take the remaining sample and cryoprotect using 30% sucrose in PBS. This should be done at 4 °C until the heart sinks in the solution.
3. For proper extraction of the aortic root, make a transversal cut on the removed heart and proximal aorta in a manner that joins the lower tips of the right and left atria in a straight line.
4. Cut off the rest of the attached descending aorta at the point where it attaches to the heart. We stored these for possible future analysis, but this is optional.

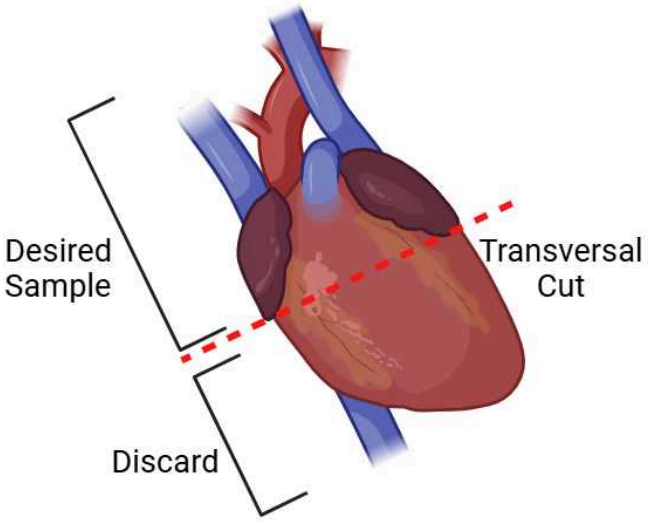

Figure 5. Transversal Cut of Heart and Aorta. This diagram shows the mouse heart and aorta. The dashed red line represents the cut to be made in order to attain the desired sample. The apex of the heart, shown below the dashed red line, is to be removed and discarded. The desired sample is to be fixed in 10% sucrose in NBF.

Note

- The transition from the aortic root extraction step to the atherosclerosis quantification step should be relatively quick, as the sample is only stable in sucrose for approximately one week.

Embedding Samples

2h 45m

10 **Preparation**

1. Gather necessary materials from section 3.4.
2. Gather samples after cryoprotecting in 30% sucrose in 1x PBS and making the transversal cut to isolate the aortic root.
3. Gather a Styrofoam box and fill with dry ice.
4. Fill box substantially with 100% ethanol to create slush.
5. Wrap 6-well plate lid in aluminum foil. Place on top of dry ice slush.
6. Place copper plate on top of aluminum foil-wrapped plate lid.

10.1 **Cryomolds** 35m

1. Make a thin layer of OCT so it coats the bottom of the cryomold.
2. Fill weight boat or petri dish with substantial amount of OCT.
3. Dab the sample to a Kim wipe to remove any leftover sucrose.
4. Place the sample in the weigh boat/petri dish. Use forceps to gently squeeze air bubbles out of the sample. Gently squeeze more OCT on sample if needed.
5. Once air bubbles are squeezed out, swirl around the sample a few times with the forceps.
6. Place the sample in the OCT-filled cryomold. Ideally, align each sample in the same way.
7. Using forceps, lightly push the sample to the bottom of the cryomold.
8. Add additional OCT to cover the whole sample and fill the cryomold.
9. Place the cryomold on the previously prepared copper plate to snap freeze. The block should be frozen and opaque by the end. If dealing with large sample, squeeze more OCT on top as needed.
10. Store at -80 °C for future use.

Note

- To avoid OCT spreading out on the cryomold, wait until the bottom half of the cassette is frozen before adding more.
- OCT embedding can typically be done by histology core facilities.

Slicing and Staining

11 **Slicing and Staining**

All slicing and staining was performed by our local Histology Core. Instructions for desired slicing essentially amounted to the following:

1. Slice each cryomold into 10 µm slices, placing 6 slices per slide on 9 slides, with 0 µm intervals. The slices should be placed on each slide first before moving to the next slot on a given slide. This means the first 9 slices should each occupy the first slot of their own respective slide, the second 9 slices should occupy the second slot, and so on.

11.1 **Oil Red O Staining**

Oil Red O Staining was performed by our local Histology Core. Alternatively, a viable protocol for doing so is available here: **Oil Red O Staining Protocol - IHC WORLD**

11.2 **H&E Staining**

H&E Staining was performed by our local Histology Core. Alternatively, a viable protocol for doing so is available here: **CMSRH-E.pdf**

11.3 **CD68 Staining**

CD68 Staining was performed by our local Histology Core. Alternatively, a viable protocol for doing so is available here: **CP03A-010_(CD68,CD163,PD-L1)_Staining_Protocol_for_BOND_RX-RXm_20220127A.pdf**

Imaging

12 **Imaging**

1. Use Imaging system to scan and image the slides
2. Use application to outline a square section around the aortic root. Can use the left ventricle as a reference. Make sure to include the entirety of the outer membrane of the aortic root.
3. Better to have higher threshold sensitivity than too low.
4. Set calibration points, ideally making over half of the area include the membrane/tissue.
5. Compile into file.

Plaque Analysis and Quantification

13 **Preparation**

Install Fiji imaging software with Bio Formats plugin.

Note

- Dr. Andrew McCall generously helped develop the below macros.

13.1 **Oil Red O**

1) Open the macros files containing the following code in Fiji.

```
run("Hyperstack to Stack");
title=getTitle();
setSlice(1);
run("Duplicate...", "title=1");
selectImage(title);
setSlice(2);
run("Duplicate...", "title=2");
selectImage(title);
setSlice(3);
run("Duplicate...", "title=3");
run("Merge Channels...", "c1=1 c2=2 c3=3 create keep");
selectWindow("Composite");
setTool("freehand");
waitForUser("Draw ROI");
roiManager("Add");
imageCalculator("Subtract create", "1", "2");
selectWindow("Result of 1");
roiManager("select", 0);
waitForUser("Delete Splotches");
//run("Threshold...");
setAutoThreshold("Otsu dark no-reset");
List.setMeasurements("limit");
run("Set Measurements...", "area mean standard modal min
integrated median limit redirect=None decimal=3");
run("Measure");
roiManager("Deselect");
roiManager("Delete");
close(title);
close(1);
close(2);
close(3);
close("Composite");
close("Result of 1");
```

2) Take .vsi file and open it in Fiji
3) Bio Formats window will open, leave all default settings and click ok
4) A new window will open where you will choose which image to analyze. Unselect anything that is selected by default (important)
5) Select the highest resolution version of the sample you want to analyze (normally the first image our of the group of the same sample) and click ok
6) Run the imported macro
7) When prompted by the macro, freehand draw your ROI and click ok
8) Note of any prominent red splotches that are not real signal. When prompted to delete splotches, use the ink tool to cover any of the splotches with black ink and click ok
9) A new window with the results will open and record these values
10) Repeat steps 2-9 for each slide of each sample you want to analyze.

13.2 **CD68**

Macro for CD68:

```
//path=getInfo("image.directory");;
//name=getInfo("image.filename");
title=getTitle();
//select the nuclear channel//
Stack.setChannel(1);
//Get the user to draw the ROI with the freehand tool and save the
ROI to the
ROIManager//
setTool("freehand");
waitForUser("Draw a freehand ROI around the aortic root
perimeter");
roiManager("Add");
//Generate a copy of the CD68 channel//
Stack.setChannel(2);
run("Select All");
run("Copy");
run("Internal Clipboard");
//Obtain the noise/background by selecting everything that's not
the root, and
define noise as avg + 3xSD//
run("Set Measurements...", "area mean standard modal min
integrated median limit redirect=None decimal=3");
roiManager("Select", 0);
run("Make Inverse");
run("Measure");
noise=getResult("Mean") + getResult("StdDev")*3;
run("Select None");
//Subtract the background from copy1 //
run("Subtract...", "value=noise");
//threshold without applying it between 1 and max. 1 is the first
value above
the background average//
//setAutoThreshold("Triangle dark");
setThreshold(1, 65535, "raw");
//apply the previously drawn ROI and measure only in areas that
are not
background and the mean intensity here is the value we want//
roiManager("Select",0);
run("Measure");
//close and reset//
selectImage("Clipboard");
close();
selectImage(title);
close();
```

```
//IJ.deleteRows(0,0)
roiManager("reset");
```

13.3 **H&E**

Macro for H&E:

```
//Adds multi-file
capability, the #@ String is just used so that the #@ File[]
creates a Widget
that lets you have drag and drop capability, instead of a file
dialog box
#@ String (visibility = MESSAGE, value="Add files to be processed,
you can
drag and drop files into the box below.", required=false) msg
#@ File[] files
//Comment out this next command to see the images being processed.
You'll have
to comment out the "close" commands at the end too.
//setBatchMode("hide");
lumen=0;
plaque=0;
print("File,Series,Lumen,Plaque");
for (i = 0; i < files.length; i++) {
      for(s = 14; s < 40; s = s +
5){
run("Bio-Formats Importer",
"open=["+files[i]+"] autoscale color_mode=Default
rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT
series_"
+s);
title=getTitle();
setTool("freehand");
waitForUser("Trace the Lumen");
roiManager("Add");
do {
      finished="No";
      waitForUser("Trace
Plaque");
      roiManager("Add");
      //-- Create and show a blocking
dialog box
      Dialog.createNonBlocking("Click
OK to continue");
      Dialog.addCheckbox("All Plaque
Traced", false);
      Dialog.show();
      //-- When OK is clicked, read out
the status of the dialog
      if
(Dialog.getCheckbox()){finished="Yes";}
```

```
} while (finished=="No");
run("Set Measurements...", "area mean standard integrated
limit redirect=title decimal=3");
roiManager("Select",0);
run("Measure");
lumen=lumen + getResult("Area",0);
n = roiManager('count');
      for (r = 1; r < n; r++) {
        roiManager('select', r);
        run("Measure");
        plaque = plaque +
getResult("Area",r);
      }
print(title + "," + s + "," + lumen +"," +
plaque);
//setResult("Lumen",nResults-1,lumen); // sets the value in
the last line of the results table
//setResult("Plaque",nResults-1,plaque);
      //updateResults(); // updates the
results table to show the new value
close("results");
roiManager("Deselect");
roiManager("Delete");
close(title);
      }
}
path=getDirectory("Choose where to save the results");
selectWindow("Log");
saveAs("Text", path + "results.csv");
close("Log");
setBatchMode("exit and display");
```

Protocol references

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Sectioning of Mouse Proximal Thoracic Aorta.pdf
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Implantable Osmotic Release Pump - Conduct Science
Catheter Use - ALZET® Osmotic Pumps
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