Preparation and shipping of mouse tibiae for multiphoton microscopy V.1

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VERSION 1
NOV 06, 2023

Protocol Citation: Chad A Lerner, Sofiya Galkina, Gen Matsumae, Simon Melov, Tamara Alliston, Akos A Gerencser 2023. Preparation and shipping of mouse tibiae for multiphoton microscopy. **protocols.io** https://protocols.io/view/preparation-and-shipping-of-mouse-tibiae-for-multi-c4ibyuan

Protocol status: In development
We are still developing and optimizing this protocol

Created: Nov 03, 2023

ABSTRACT

Isolation and packaging of mouse tibiae for overnight shipment for bioenergetic analysis of live in situ osteocytes.

MATERIALS

Consumables

1. 2ml tubes (1 per bone)
2. 15ml and 50ml tubes (2)
3. 10ml syringes (2)
4. 22G needle (1): such as CareTouch hypodermic needle, 22G, 1" #CTHN221
5. Syringe filter (0.2um) (1)
6. 60ml Petri/culture dish (1)
7. Lab tape, marker pen
8. Cryopak phase 22 (8)
9. Transport plate: 96-well, deep, round-well (~2ml) microplate (1): # Thermo Scientific™ Nunc™ 278743
10. Universal (or matching) plate lid (1): Thermo Scientific™ Nunc™ Microplate Lids #250002
11. Breathable film (2): Excel Scientific BS-25, AeraSeal Sealing Film (Genesee Sci. #12-631)

Reagents:

1. PBS
2. Custom BGJb nutrient medium (HEPES-buffered, normal glucose)
3. BSA (fatty-acid free)
4. ITS (100x for cell culturing)
5. penicillin/streptomycin/amphotericin B (100x for cell culturing)

Last Modified: Nov 06, 2023

PROTOCOL integer ID: 90403

Consumables and Tools

1 **Animal dissection tools:**

1. Serrated/toothed forceps
2. Scissors

Bone cleaning tools:

1. Straight tweezers
2. Curved tweezers
3. Small scissors

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Common lab tools: pipettes, scale, dissection microscope, tube racks

Reagents: PBS, Custom BGJb nutrient medium, BSA, ITS, penicillin/streptomycin/amphotericin B

2 Dissection tools and surfaces should be clean, **free of detergents, alcohol or disinfectants**.

Tool cleaning:

1. Detergent wash
2. Alcohol wash (ethanol or isopropanol)
3. Water
4. PBS

3

1. Label one 2ml tube for each bone and fill with 1.8ml of nutrient medium (**Keep the bottle of nutrient medium sterile. Open it in the hood only**).
   - Leave the tubes uncapped, but covered with a small square of self-adhesive membrane (cut one breathable membrane up with scissors).
   - Keep the tubes at RT.
1. Fill two 50ml tubes with nutrient medium. This can be used on the bench to fill the 60ml dishes and the syringe when cleaning the bones.

4 In a 15ml conical supplement nutrient medium to make transport medium:

1. Measure 40mg BSA (0.4 % w/v final)

2. add 10ml nutrient medium
3. add 100µl ITS
4. add 100µl penicillin/streptomycin/amphotericin B
5. Sterile filter using a syringe and syringe filter

Dissection of mice - vivarium

5 Mouse is euthanized in CO_2 chamber according to the IACUC approved protocol.

> **Note**
>
> The section below should be completed where the animals handled, and a samples can wait and to be transferred to lab bench for further cleaning.

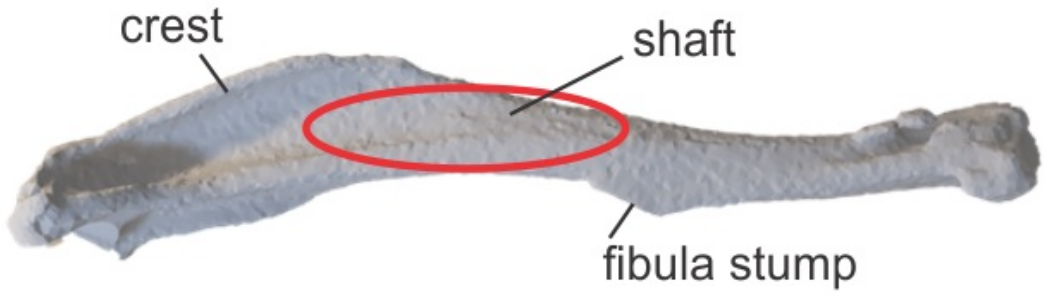
6 Mouse hindlimbs are rinsed with PBS, fixed to a dissection board with pins. **Do not use alcohol or disinfectants.**

7 Cut through the entire hind limb and expose the muscles underneath with the help of scissors.

8 Carefully remove the skin as well as any hair or fur.

9 Coarsely remove muscles without damaging bones by holding and cutting corresponding tendons.

10 Expose tibia and fibula and cut them through knee and ankle joints. Keep tibia and fibula together for now. **Take extra care not to hold the tibia on the surface that is going to be analyzed**, indicated in the picture by the ellipse (flat part of the shaft between crest and fibula stump:

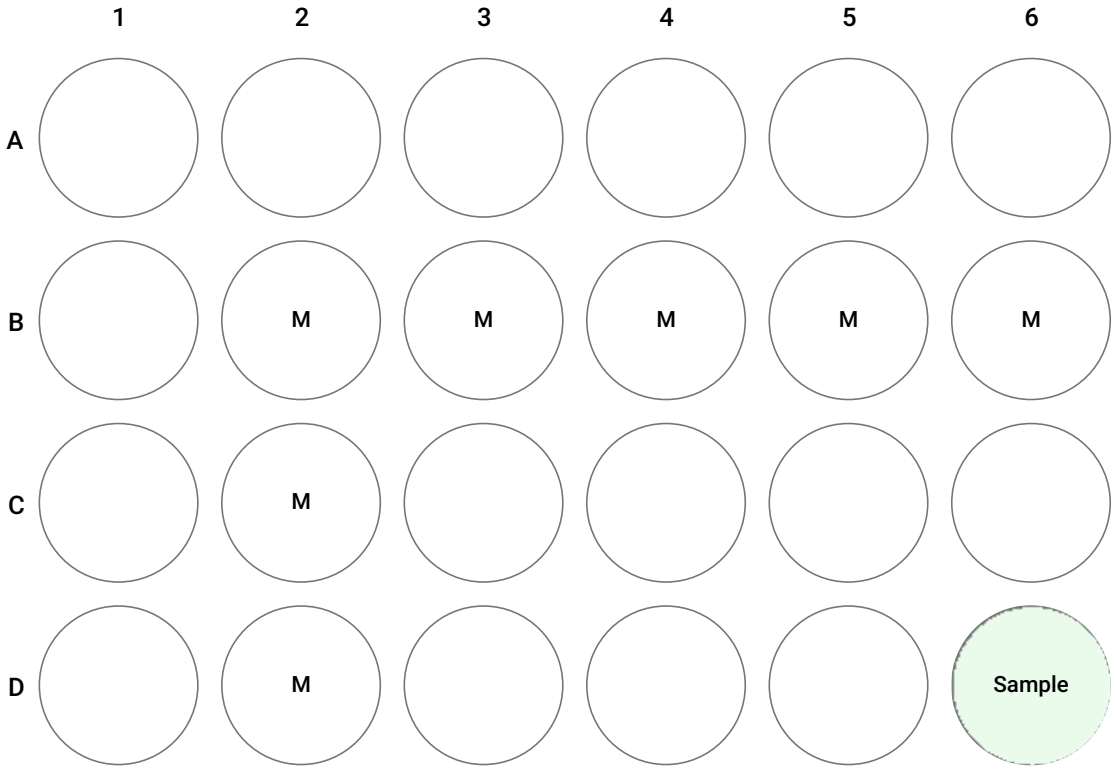


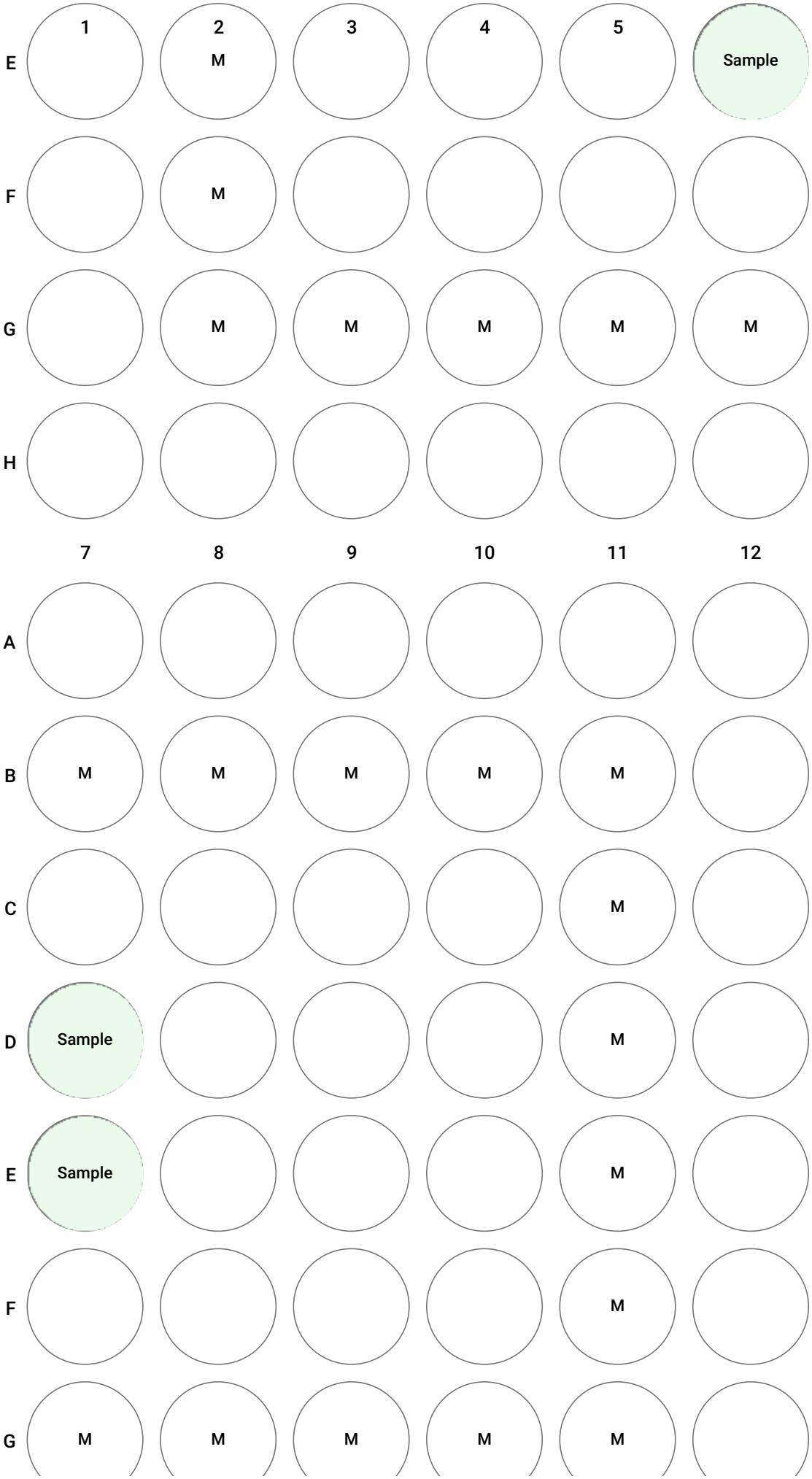
uCT of mouse tibia. Note that at this step fibula and some soft tissues are still attached.

11 Transfer bone specimens into the tube with transport medium. **Do not cap** the tubes, just replace the membrane. Keep tubes at room temperature (**do not use ice**). Samples should wait here for completion of all dissection.

Cleaning of bones - lab bench

12 Fill one well per tibia in the center of the transport plate (deep round well 96-well plate) with transport medium. Fill the edge or second from edge wells with nutrient medium + antibiotics. Cover the plate with lid (but not yet with film). Handle the transport plate with minimizing bacterial contamination.

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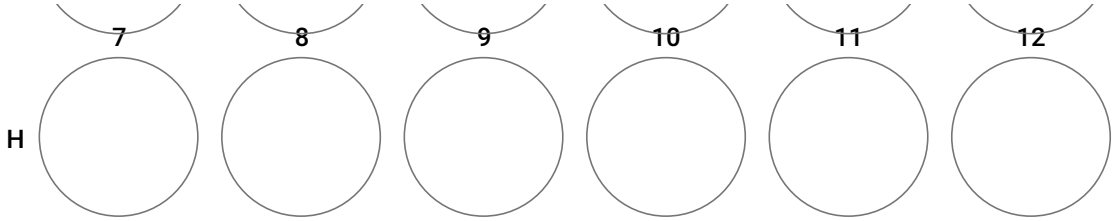


13 Place a 60mm dish on a dissection microscope and half-fill it with room temperature nutrient medium and transfer one bone with tweezer into it.

> **Note**
>
> The whole cleaning procedure is using the nutrient medium and clean tools and is carried out at room temperature. Handle the tissue under the medium. Do not use PBS. Only steel tools should touch the samples, no Kimwipes or gloves touching. Tools may be wiped with gauze. This section is repeated for each bone, with discarding the medium and tissues remaining in the plate.

14 With a small scissors cut off fibula proximally:

1. Grab on the fibula that will be discarded (hold vertically knee pointing upwards; in terms of microscope view field)
2. Insert open scissors vertically between tibia and fibula, cutting upwards
3. Cut off fibula by cutting through knee joint and remaining muscles in the way

15 With a small scissors cut off the fibula distally:

1. Insert open scissors vertically between tibia and fibula, cutting downwards
2. Cut off fibula leaving only a small ~1mm stomp

16 Cut off the knee joint

1. Hold tibia at the distal end, below the fibula stump with tweezers
2. Hold tibia perpendicular to the scissors, in an angle that you will cut in the plane of the crest, so cutting it rather than crushing it
3. Cut as close as possible to the knee joint
4. The cut should expose marrow.

17 Cut distal end ~2mm below the fibula stump

1. Hold tibia at the level of the fibula stump
2. Cut below, carefully not to crack.
3. The cut should expose big enough hole so the marrow can be blown out

18 Clean off remaining muscle / soft tissues.

1. Hold tibia at the distal end, below the fibula stump with tweezers
2. Pull away or cut off remaining muscle or tendons

3. **Do not peel up the periosteum**

19 Blow out marrow

1. Fill a 10ml syringe with nutrient medium and attach a 22G needle.
2. Hold tibia at the level of the fibula stump
3. Insert a needle into the marrow from the proximal end of the tibia and blow out marrow
4. Some peripheral marrow may remain. Do not crack the bone by pushing the needle too deep.

20 Rinse

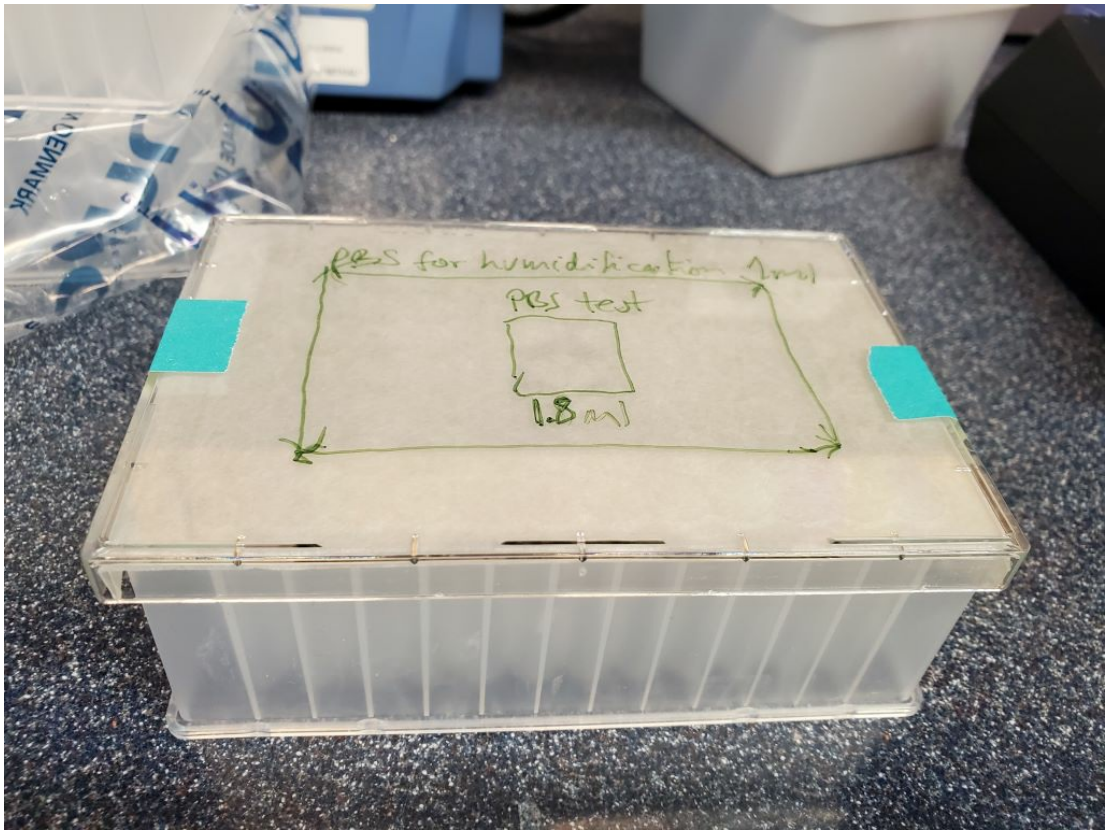
1. Lift up the tibia, rinse it with spraying medium from needle onto it.
2. Transfer the tibia to one "Sample" well of the transport plate.
3. Mark the lid with the code of the sample (please use blinded codes)

21 Discard the contents of the 60mm dish, refill with clean nutrient medium and repeat this section for the next bone.

Packaging for shipping

22 Seal the transport plate

1. Make sure that the top of the transport plate is dry.
2. Peal backing of breathable film
3. Holding at the edges attach the film on the plate. Massage the film that it attaches evenly in between wells
4. Secure the lid on two sides with lab tape, not obstructing air gaps.



23 In a Styrofoam box surround the lidded, sealed transport plate with 4 liquid and 4 solid Cryopak Phase 22 packages.