Methods Article

A Minimally Invasive Technique for Serial Intraosseous Perfusion Measurements in the Murine Tibia Using Laser Doppler Flowmetry

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Abstract

In biomedical and preclinical research, the current standard method for measuring blood perfusion inside murine bone, radiolabeled microspheres, is a terminal procedure that cannot be used to monitor longitudinal perfusion changes. Laser Doppler flowmetry (LDF) can quantify perfusion within the proximal tibial metaphysis of mice *in vivo* but requires a surgical procedure to place the measurement probe directly onto the bone surface. Sustained inflammation for over a month following this technique was previously reported, and previous studies have used LDF as an endpoint-only procedure. We developed a modified, minimally invasive LDF procedure to measure intraosseous perfusion in the murine tibia without stimulating local or systemic inflammation or inducing gait abnormalities. This modified technique can be used to measure perfusion weekly for up to at least a month.

- Unlike previous endpoint-only techniques, this modified LDF procedure can be performed weekly to monitor serial changes to intraosseous perfusion in the murine tibia
- The modified LDF technique utilizes a smaller, more localized incision to minimize invasiveness and speed recovery

Keywords

bone blood flow, vascular supply

1 Method Details

2 Background

3 The current standard method for assessing functional blood flow within bone in rodents

4 uses microspheres labeled with fluorescent or radioactive tags that are introduced to the

5 vascular supply, and then the amount of microspheres in tissue samples taken from the

6 animal *ex vivo* are quantified [1,2]. However, this procedure is a terminal one and cannot

7 be used to assess serial changes to intraosseous blood flow. Laser Doppler flowmetry

8 (LDF) has been validated as an accurate technique to quantify *in vivo* perfusion within the

9 murine proximal tibial metaphysis, but signs of local inflammation for over a month after

10 the procedure were reported [3]. Inflammation stimulates angiogenesis, which could alter

11 subsequent perfusion measurements [4]. Similarly, inflammation may affect nearby bone

12 remodeling via cellular signaling [5] or alter limb loading via gait abnormalities [6], both of

13 which could confound study designs focused on bone tissue outcomes. We have modified

14 the LDF technique to minimize invasiveness so that it can be used for serial

15 measurements of bone perfusion at weekly intervals in the same mice without causing

16 inflammation [7].

17

18 Modified LDF Procedure

19 This protocol was approved by the Institutional Animal Care and Use Committee at North

20 Carolina State University. In this study, the LDF procedure was performed on eighteen

21 14-week-old male C57Bl/6J mice (The Jackson Laboratory, Bar Harbor, ME). Mice should

22 be fasted 6-8 hours prior to the procedure. The LDF probe is sensitive to movement,

23 temperature, and light, so care should be taken to ensure consistent conditions across

24   procedures. The LDF monitor should be regularly calibrated with a probe flux standard.

25   Follow the manufacturer's directions to calibrate the probe. Calibrate between studies,

26   not between LDF procedures.

27

28   *LDF Monitor and Software Preparation*

29   1. Connect the LDF monitor to a computer. Turn on the LDF monitor and open the

30   data acquisition software provided by the manufacturer. In the signal processing

31   settings, reduce the bandwidth to one appropriate for the slower blood speeds in

32   rodent bone, about 0.4-1 mm/s [3], by selecting a lower cutoff frequency for the

33   low-pass filter (e.g., 3 kHz) [8], as suggested by the manufacturer. *Note: Perfusion*

34   *data captured with different bandwidth settings should not be compared.*

35   2. Clean the LDF probe with 70% ethanol using a sterile cotton swab and allow to air

36   dry.

37

38   *LDF Measurement Procedure*

39   1. Anesthetize mouse in an induction chamber with isoflurane (3-5%) in pure oxygen.

40   *Note: Different gas mixtures (i.e., compressed air) can be used instead of pure*

41   *oxygen but may alter intraosseous blood supply. Perfusion data should not be*

42   *compared for different anesthesia conditions.*

43   2. Shave the fur over the anterior and medial surfaces of the proximal tibia and knee

44   joint.

45   3. Place the mouse supine on a heated surgical pad, and tape down the hind paws

46   so that the medial surface of the hindlimb is accessible. Maintain anesthesia with

47   isoflurane (1.5-2%). Maintain rectal temperature at 37°C with a feedback-

48   controlled heating system. Apply ophthalmic ointment to the eyes to prevent

49   corneal drying during the procedure.

50   4. Disinfect the incision site three times with povidone-iodine followed by 70%

51   ethanol.

52   5. Gently palpate the anterior surface of the proximal tibia with forceps to find the

53   most proximal surface over the anteromedial surface of the bone that does not

54   have soft tissue beneath the skin. Position the limb (re-tape limb if needed) so that

55   this site is easily accessible (Figure 1A).
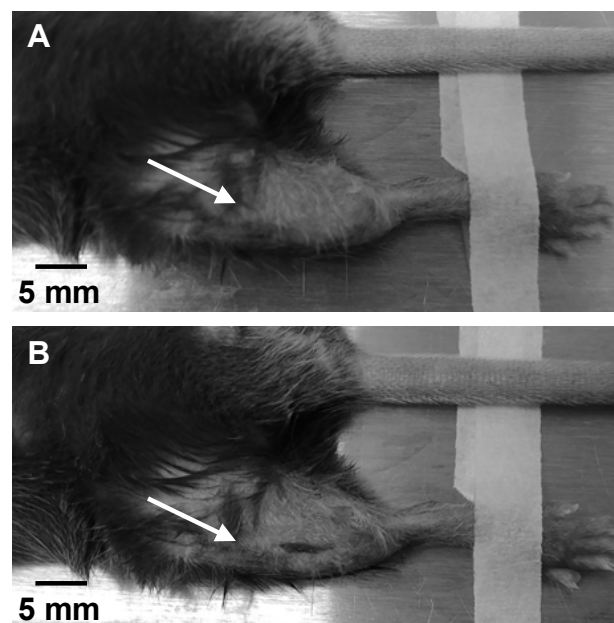
Figure 1: Image showing the measurement site on the right hindlimb A) before and B) after the incision is made. The arrows point to the approximate position of the right knee.

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57   6. Using a #11 or other preferred small scalpel blade, make a 2-5-mm long sagittal

58   skin incision directly over the proximal tibia, starting approximately 3-4 mm below

59   the knee and moving distally (Figure 1B). Use a sterile cotton-tipped applicator to

60   absorb fluid at the incision site. *Note: This incision should not cause excessive*

61   *bleeding. If blood pools in the incision site, making probe placement difficult, firmly*

62   *hold a sterile cotton-tipped applicator or gauze for 15-30 seconds until bleeding*

63   *stops.*

64   7.   Using forceps, gently retract any soft tissue covering the tibia and palpate the tibia

65   to ensure no soft tissue is covering the bone. Using the edge of the scalpel blade,

66   gently scrape a small window in the periosteum that is approximately the same

67   size as the LDF probe tip (0.8 mm diameter). Probe the bone surface gently with

68   the scalpel to confirm the periosteum was removed. Before removal the surface

69   will feel more compliant when compressed, and after it is removed it will feel

70   harder.

71   8.   Using a micromanipulator, position the LDF probe tip in the periosteum-free

72   window, placing it firmly against and perpendicular to the exposed bone surface

73   (Figure 2). Pushing the probe with excessive force into the bone can lower tissue

74   perfusion measurements, so position it just firmly enough to prevent bone

75   movement or probe slipping during recording. *Note: The LDF probe is sensitive to*

76   *motion. Do not touch the LDF probe or fiber optic cable while recording perfusion.*
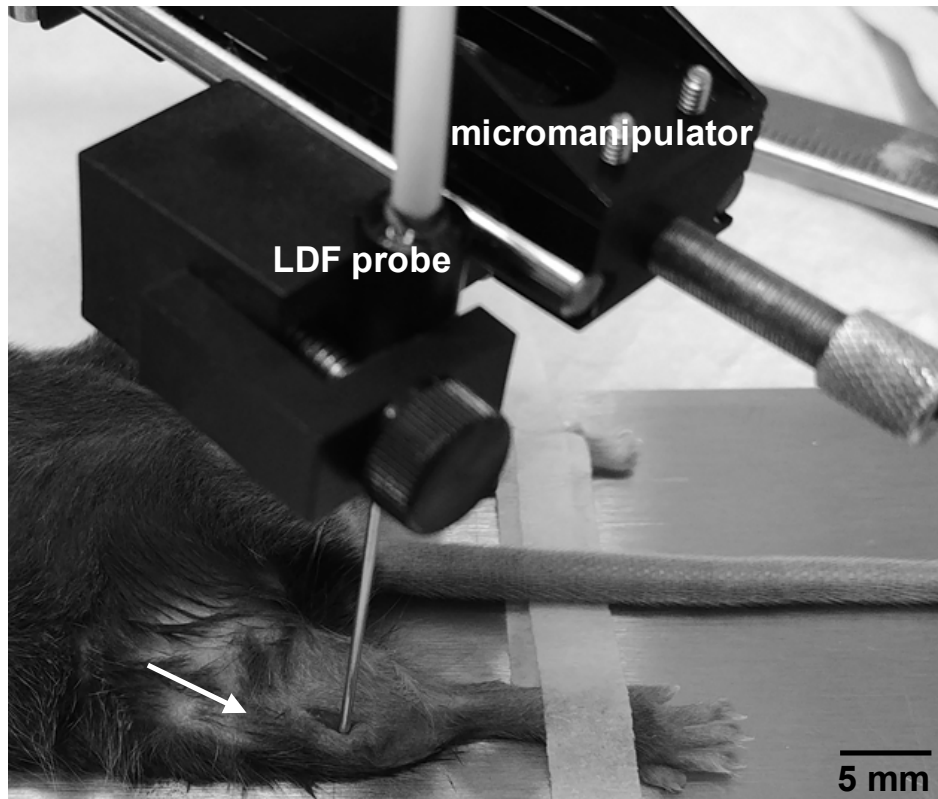
Figure 2: Image showing probe placement during the modified laser Doppler flowmetry technique on the right hindlimb. The arrow points to the approximate position of the right knee.

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78   9. Record perfusion with the LDF monitor's software. We capture 30-second

79   perfusion readings, which are sufficient for a stable average measurement, but

80   longer or shorter readings could be recorded, if desired. *Note: Perfusion readings*

81   *under 5 PU indicate the probe is not placed perpendicular to the medullary cavity,*

82   *and readings over 30 perfusion units (PU) likely indicate the probe slipped off the*

83   *bone and is recording in soft tissue. A noisy signal (greater than 5 PU variation)*

84   *suggests that either the probe is not pressed firmly against the bone, the probe is*

85   *moving, or the probe is not perpendicular to a flat bone surface. In any of these*

86   *cases, reposition and secure the probe until resolved. Large spikes in the signal*

87   *likely indicate limb movement; sufficient anesthesia depth should be confirmed,*

88   *and the hindlimb can be taped down above the knee if additional security is*

89   *needed.*

10. After obtaining a good reading, remove and reposition the LDF probe, and record again. *Note: We recommend repositioning the probe at least one time to ensure consistent placement over the bone. If the two readings are dissimilar (greater than 5 PU difference), then more readings should be taken and averaged.*

11. Remove the probe. Pinch the incision closed with forceps, and apply a small amount of tissue adhesive to the incision. Continue to hold the incision closed for 15 seconds, being careful to avoid contacting the glue with the forceps. *Note: Tissue adhesive should only be applied to the outside of the incision, not within the incision. The glue dries rapidly, and only a small amount is needed to close a small incision.*

12. Apply 2% lidocaine cream and triple antibiotic ointment to the incision site.

13. Remove the tape from the hindlimbs, remove the rectal probe, and turn off the isoflurane. Move the mouse to a heated recovery cage until ambulatory. Return to normal cage and monitor for signs of discomfort twice a day for at least 48 hours and longer if necessary.

Data Processing

1. Select individual readings in the LDF monitor software, avoiding spikes if possible. Export the mean perfusion and length of the reading, which can be either in time units or number of points.

110   2. Tibial perfusion for each mouse is calculated as the weighted mean of each of the

111   readings for each mouse:

$$T_i = \sum_{j}^{1..n} t_{i,j}$$

$$P_i = \frac{p_{i,j} \cdot t_{i,j}}{T_i}$$

114   where T_i is the total length of all n readings for each mouse (i), $t_{i,j}$ is the length of

115   each reading (j) for that mouse, P_i is the weighted mean perfusion over all readings

116   for that mouse, and $p_{i,j}$ is the mean for each perfusion reading for that mouse.

117

118   <u>Validation</u>

119   Our modified LDF technique is based on a procedure that has been proven to accurately

120   measure perfusion in the murine tibia, demonstrated by comparing LDF readings to

121   controlled blood flow rates through *ex vivo* bone samples using a syringe pump [3].

122   Greater cortical thickness will have a lower LDF perfusion measurement, but small

123   variations in cortical thickness do not greatly affect perfusion readings [3]. Therefore, LDF

124   readings should only be compared when cortical thickness is not expected to be

125   significantly different, whether with age, sex, mouse strain, treatment, or other conditions.

126

127   We validated that this modified technique can be used serially in the same mice without

128   inducing inflammation or gait abnormalities [7]. We performed the modified technique in

129   two groups of male C57Bl/6J mice starting at 12 weeks of age. One group received

130   weekly tibial perfusion measurements for four weeks (n=10, *Repeated*), while the other

131   group only had tibial perfusion measured at the fourth week (n=8, *Endpoint*). We found

132   no difference in tibial perfusion between the Repeated and Endpoint groups at the end of

133   the study (t-test p = 0.92, Figure 3), demonstrating that repeated LDF procedures do not

134   affect subsequent measurements, at least up to four weeks. We also showed that the

135   modified technique does not alter hindlimb gait patterns, induce localized inflammation at

136   the incision site, or cause detectible increases in serum concentrations of inflammatory

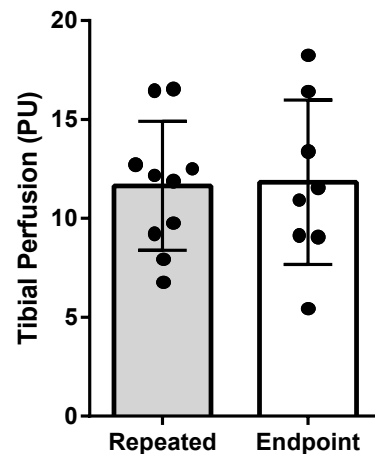137   marker interleukin-6 when performed weekly in the same mice for four weeks.

Figure 3: Perfusion measured in the proximal tibia was similar between mice that received a weekly laser Doppler flowmetry procedure for four weeks (*Repeated*) and mice that received the procedure only once at the final timepoint (*Endpoint*).

138

139   While our modified LDF procedure has only been validated for serial bone perfusion

140   measurements in young adult male C57Bl/6J mice just after skeletal maturity, it may also

141   be useful in other rodent populations. We have applied this technique to serial

142   measurements over four weeks in 24-week-old male and female C57Bl/6J mice and to

143   endpoint measurements in aged male Brown Norway rats at 34 months of age. Other

144   studies have performed endpoint-only LDF procedures in older mice (17 months of age)

145   [3], female mice [3], and rabbits [9]. A full validation should be performed before

146   performing this serial measurement procedure in different populations to ensure the

147   animals do not respond differently to weekly procedures. Because age, sex, and mouse

148   strain affects LDF perfusion measurements [3], studies involving this technique should be

149   designed with caution to avoid comparisons across different populations.

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