Blackbox-Based Gait Analysis Outperforms Observational Scoring In Tracking Murine Post-Fracture Recovery

Xiaohua Gao[1], Sophia Fisher[1], Annemarie Lang[1].
[1]Department of Orthopaedic Surgery - University of Michigan, Ann Arbor, MI
E-Mail: annelang@med.umich.edu

Disclosures: X.G. (N), S.F. (N), A.L. (N)

INTRODUCTION: Reliable assessment of pain in laboratory rodents, particularly mice, remains a significant challenge central to both animal welfare and the translational validity of experimental studies. Precise pain assessment is essential not only to reduce distress, but also to distinguish pain-related from functional deficits throughout the fracture healing process. Current approaches in mouse fracture models combine general clinical parameters with assessments of spontaneous and model-specific behaviors, including altered gait and changes in weight-bearing. Detailed analysis of ambulation and weight-bearing can yield valuable insights into skeletal pain and mechanical limitations, facilitating differentiation between acute pain and gradual functional recovery. While automated gait analysis systems offer the highest precision, their routine use is often limited by equipment requirements and the need for prior animal training. By contrast, observational scoring of limping provides a practical, albeit less granular, method for quantifying locomotor abnormalities in a resource-limited setting. The Blackbox system enables automated behavioral phenotyping and gait analysis, offering the potential for objective and longitudinal assessment of pain and functional recovery after fracture. However, its implementation requires validation and optimization for specific fracture models and experimental setups. In this study, we characterized gait changes using the Blackbox platform in a well-established external fixator (ExFix) femoral fracture model in mice. Our objectives were: (i) to compare automated gait analysis with traditional limping scores; and (ii) to assess gait parameters in both a small observation chamber and an open-field setting, to determine the optimal conditions for future studies of functional recovery.

METHODS: Female C57BL/6J mice (Jackson Laboratory), aged 14-16 weeks (n = 7), were utilized in this preliminary study, with future work planned to include male cohorts. Data was collected in two independent experiments. All procedures were IACUC-approved. An external fixator was placed on the left femur, and the fracture was generated using a 0.44 mm Gigli wire (RISystem). Mice received a single subcutaneous dose of extended-release buprenorphine (Ethiqa XR, 3.25 mg/kg) for post-operative analgesia. Body weight, clinical appearance, and walking behavior were measured at 1, 2, 3, 4, 7, 10, and 14 days post-surgery. A validated metric limping score [1] (0 = normal use; 1 = sporadic limping/complete contact; 2 = persistent limping or hopping; 3 = partial non-use; 4 = complete non-use) was applied during 1-minute observations in a separate cage. For automated assessment, Blackbox gait analysis was performed for 3 minutes per session in two chamber formats: a small box (6 x 6 in) and an open field (12 x 12 in) (Fig. 1a). Only the final two minutes of each session were analyzed (after one minute adaptation). Blackbox data were analyzed using the Palmreader software suite (DeepLabCut and Python package [2, 3]). All mice underwent tunnel handling and three Blackbox training sessions, with two baseline measurements (average day -1) in the week prior to surgery. Statistical analysis was performed using two-way repeated measures ANOVA, with time and box setting as matched factors. Sidak's multiple comparison test was applied for selected group comparisons: (1) between box settings at each time point, and (2) for differences between days 7-14 post-fracture across box settings.

RESULTS: All mice exhibited a slight reduction in body weight over the first 7 days post-surgery, with no recovery by day 14. Clinical scores averaged 1 on day 1 and improved to 0.5 by day 2. Limping scores similarly peaked at day 1 (score of 1), with all subsequent days scoring 0 (data not shown), confirming an acute, transient impairment consistent with previous reports [4]. Automated gait analysis with the Blackbox system provided substantially greater detail than manual limping scores. Distance traveled peaked at 1 day post-fracture (dpf), declined slightly at days 2-4, and returned to baseline by days 7-14; distances were significantly greater in the open field on days 1, 7, and 10 ($p^{*}<0.05$; ANOVA statistics: time $p^{***}<0.001$; box $p^{**}<0.01$; Fig. 1b). Assessment of the left hind paw (fractured limb) via paw luminance (sum pixel intensity) revealed a significant drop at day 1, a peak at day 3, and a decline by day 7, with continuous recovery through day 14 ($p^{*}<0.05$ for 7 vs. 14 dpf; ANOVA statistics: time $p^{*}<0.05$; box $p^{**}<0.01$; Fig. 1c). Differences between chambers were apparent: the small box exhibited sharper within-day fluctuations and higher paw luminance, particularly on day 3 ($p^{*}<0.05$), while the larger box yielded smoother trajectories. The pressure index (pixel intensity/area) mirrored the paw luminance pattern, with a distinct biphasic trend across settings ($p^{*}<0.05$ for 7 vs. 14 dpf large box; ANOVA statistics: time $p^{***}<0.001$; box $p^{**}<0.01$; Fig. 1c). Histological analyses (HE staining) aligned these functional changes with the biological healing cascade: inflammation and hematoma at day 3, resolution by day 7, and cartilage/bone formation by day 14 (Fig. 1d). Functional recovery, as captured by automated gait metrics, commenced in earnest after day 7, aligning with the resolution of inflammation and the onset of tissue regeneration. Notably, extended-release buprenorphine effectively minimized pain-related behaviors during the early post-surgical period, as evidenced by low clinical scores, although physical impairment due to fracture persisted. The pronounced increase in activity (peak distance traveled) observed at 1 dpf may, in part, reflect known side effects of buprenorphine, specifically, drug-induced hyperactivity and restlessness in mice [1].

DISCUSSION: These findings demonstrate that automated gait analysis using the Blackbox platform yields more nuanced, quantitative insights into post-fracture functional impairment and recovery than conventional limping scores. While observational scoring is sufficient for detecting marked pain or severe deficits, automated gait analysis is more informative for monitoring gradual recovery. Our data suggest that the period between days 7 and 14 post-fracture represents a critical window for functional recovery in the ExFix model, corresponding to the transition from inflammation to bone regeneration. This temporal alignment supports integrating functional assessment alongside microCT and histological endpoints for comprehensive preclinical evaluation. Differences in chamber size influenced certain gait parameters, with the open field providing smoother, lower-magnitude patterns and the small box producing sharper peaks, although the overall trends were robust across conditions. Future studies will incorporate male mice and additional treatment groups with established compounds to further validate and refine recovery tracking methodologies.

SIGNIFICANCE/CLINICAL RELEVANCE: Evidence-based, longitudinal, and non-invasive assessment of functional recovery is essential for the design and translation of preclinical fracture repair studies. Optimization of automated gait analysis will advance both scientific rigor and animal welfare, supporting the 3R principle in musculoskeletal research.

REFERENCES: [1] Jirkof, Durst et al. 2019 *Sci. Rep.*; [2] Mathis et al. 2018 *Nat. Neurosci.* [3] Nath, Mathis et al. 2019 *Nat. Prot.* [4] Wolter et al. 2023 *Sci. Rep.*

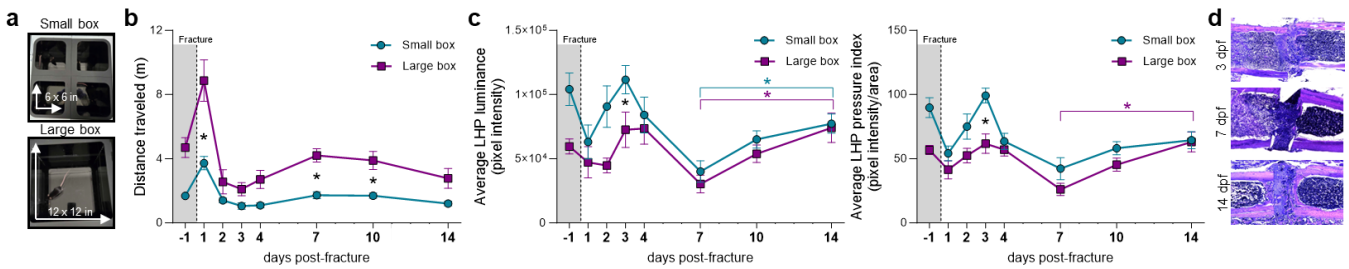

Figure 1: Blackbox gait analysis in a mouse femoral fracture model. (**a**) Images of Blackbox setup and measurement chambers. (**b**) Distance traveled (in meters) during the 2-minute analyzed interval. (**c**) Quantification of paw luminance (sum pixel intensity) and pressure index (pixel intensity/area) for the fractured limb. (**d**) Hematoxylin and eosin (HE) staining of the fracture gap at 3, 7, and 14 days post-fracture (dpf).