

# Automated Assessment of Post-Fracture Pain in Mice using Novel Imaging and Machine Learning Tools

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**INTRODUCTION:** Fractures are one of the most common injuries worldwide with *The Lancet* Global Burden of Diseases reporting 178 million new fractures and 445 million prevalent fractures in 2019. Currently, opioids are the standard of care for addressing post-fracture pain, due in part to evidence that NSAIDs delay bone repair. Opioid-based analgesics introduce a potential for medication tolerance, dependence, addiction, and abuse. To address and reduce post-fracture opioid prescriptions, there is a critical technology gap for tools that can reliably measure fracture pain in order to understand factors that exacerbate pain and ultimately find novel ways to address pain. Currently, there is a paucity of studies that attempt to study post-fracture pain and those that are published use the von Frey up-down method and/or gait analysis. However, in our testing, these assays were not sufficiently reliable and sensitive for evaluating pain in a fractured limb. Our objective for this abstract is to develop and validate a sensitive and unbiased methodology to measure pain following fracture in rodent models. We hypothesize that by recording freely moving mice in a setting designed to promote natural behavior coupled with DeepLabCut™ machine learning to quantify behavior in an unbiased manner, we can achieve more reliable post-fracture pain measurements. Here we present our first data using a novel technology, the BlackBox System, coupled with DeepLabCut™ machine learning to monitor how quantifiable behavioral endpoints of pain shift after bone fracture. This technology allows for the collection of continuous data streams and automatically analyzes them to assess musculoskeletal pain behaviors rapidly and objectively in a natural setting.

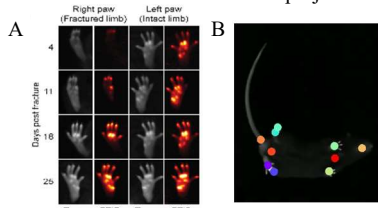
**METHODS:** In adult mice, we performed unstabilized tibia fractures and longitudinally assessed pain-related behaviors and gait patterns at 4-, 11-, 18-, and 25-days post fracture in accordance with our approved IACUC protocol. We used the BlackBox One system to perform high spatial and temporal recording of pain behaviors in freely moving mice, which captures both animal pose (body position) and paw weightbearing (paw pressure). The grayscale images produced are the transmitted light illumination of the hindpaw that captures animal pose and the heatmap images indicate paw pressure of frustrated internal reflectance (FTIR) with red indicating less pressure and yellow/white indicating greater pressure (**Figure 1A**). We then performed automated extraction of hindpaw position and trained these algorithms to track changes in paw pressure and kinematic phenotypes using machine learning algorithms in DeepLabCut (**Figure 1B**). There were 5-8 mice in each the fractured (experimental) and unfractured (control) group at each time point. Data was analyzed for statistical significance using a two-way repeated measures ANOVA with correction for multiple comparisons.

**RESULTS:** First, we observe a significant decrease in weight bearing on the fractured limb as compared to the contralateral, non-fractured hindlimb in the fractured mice (red line) compared to the unfractured mice (black line) at day 4 that resolves around day 18 (**Figure 2A: p = 0.0003 d4, p = 0.0016 d11**). Decreased weight bearing on an injured limb is a well-established measure of pain that has been used in many studies of chronic pain in rodents (i.e., spared nerve injury, complex regional pain syndrome). Secondly, we observe a significant increase in guarding (raised, folded paw) of the hindpaw of the fractured limb with peaks at days 4 & 11 in our fractured mice, a similarly well-characterized pain behavior in rodents (**Figure 2B: p=0.0001 d4, p=0.0001 d11, p=0.0304 d18, p=0.0366 d25**). The concurrence of these two behaviors demonstrates that fracture-related pain drives mice to shift their posture to avoid weight bearing on the fractured hindlimb. Next, we probed how pain-related changes in posture translate to changes in gait patterns during walking. To compensate for the loss of weight bearing on the injured hindlimb, we observed that fractured mice will often hop with their uninjured hindlimb. During this hopping behavior, mice will increase the stride length and maximum swing speed of the uninjured paw while bracing with their forepaws. Fractured mice had a significant increase in this hopping behavior at day 4 compared to the non-fractured mice that resolved by day 11 (**Figure 2C: p<0.0001 d4**). Lastly, the combined effect of these fracture-induced changes culminates in a loss of gait synchrony, exhibited by frequent switching between synchronous hopping and alternating hindpaw movements during walking. The fractured mice in our study showed a significant increase in loss of gait synchrony compared to the unfractured mice that resolved over time (**Figure 2D: p<0.0001 d4, p=0.0056 d11**). Importantly, weight bearing, paw guarding, hopping, and gait asynchrony peak initially and resolve over time and the recovery of these measures tracks with critical milestones in the bone fracture healing process. We also looked at total distance travelled, walking speed, stride length, and stepping rate but saw no significant differences between the fractured and unfractured mice.

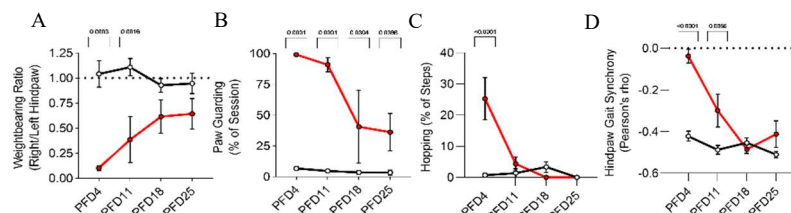
**DISCUSSION:** Our data demonstrate how coupling the BlackBox system with powerful, high-throughput machine-learning tools (DeepLabCut) greatly enhances our ability to detect chronic pain behaviors in mice after fracture. This will allow for a more comprehensive analysis with greater sensitivity in quantifying induced and spontaneous pain-related behaviors than currently available methods (i.e., von Frey fibers, CatWalk/DigiGait, etc). By using the BlackBox, we will be able to assess the efficacy of non-opioid alternatives and therapeutics for post-fracture pain.

**SIGNIFICANCE/CLINICAL RELEVANCE:** Given the high prevalence of fractures, and that opioids are the standard-of-care to address pain following fracture, having a reliable tool to measure preclinical pain response to fracture is the critical first step to subsequently studying the mechanistic drivers of pain and testing effective non-opioid alternatives to abate chronic and induced pain.

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**Figure 1. Tracking pain after tibia fracture in mice.** (A) Longitudinal assessment of weightbearing for the fractured (right hindpaw) and intact limbs (left hindpaw) with transmitted light illumination of the hindpaw (grayscale) and heatmap images indicating paw pressure (FTIR). (B) Automated tracking of mouse behavior using DeepLabCut.



**Figure 2. Assessing locomotion and pain behaviors after tibia fracture in mice.** (A) Weightbearing ratio is the ratio of summed intensity from FTIR images from each hindpaw. (B) Paw guarding determined by the absence of stereotypical toe spreading during weightbearing. (C) Hopping as a percentage of total steps. (D) Synchrony of hindpaw movements during walking. Values near zero (dotted line) indicate loss of synchronous gait, negative values indicate alternating hindpaw movements during walking. (A-D) Data are shown for Naïve (black lines) and Fractured (red lines) mice. N = 3 mice/group. Data displayed as mean ± SEM, p-values shown.