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An automaton for preclinical pain testing

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In this issue of *Cell Reports Methods*, Dedek et al. present RAMalgo—an AI-powered, automated platform for quantifying nociceptive behaviors in mice. With integrated video tracking and mechanical, thermal, and optogenetic stimulation, RAMalgo has the potential to increase standardization and throughput of pain behavior measurement in rodents.

Pain is a global health burden, with 1 in 5 people experiencing some form of chronic pain disorder during their lifetime. The development of effective pain therapies requires a mechanistic understanding of pain etiology and effective preclinical screening of therapies, both of which rely on accurate measurements of pain in rodents. Classically, measuring pain in rodents has relied on evoked measures, where nociceptive stimuli are applied to the animal, and the threshold of that stimulus required to elicit a withdrawal or nocifensive response is quantified. For example, the difference in withdrawal thresholds between baseline and analgesic treatment is then inferred as a proxy of analgesic or anti-nociceptive efficacy. A major limitation of these assays is that they are typically low-throughput and highly sensitive to experimenter factors, such as subjectivity and sex or experience level of the experimenter.[1,2] These investigator-related confounds increase experimental variability, reducing signal to noise ratio of the assay. Limited throughput and potentially low sample sizes can further compromise reproducibility within and across laboratories.

To address these challenges Dedek et al.[3] present an approach called RAMalgo that automates each stage of nociceptive testing—detection of the affected paw, application of the stimulus, and quantification of withdrawal latency—thereby offering the potential to improve standardization and objectivity of nociceptive testing. RAMalgo utilizes a motorized stage positioned below the test platform and is equipped to deliver mechanical, thermal (via infrared [IR] beam), or optogenetic (via blue light-emitting diode [LED]) stimulation at precise stimulus intensities across subjects and trials. Integration of a red LED assists in aiming RAMalgo, while a photodetector capturing red light reflectance precisely detects paw withdrawal. Experimenters can manually aim RAMalgo, using substage video tracking and the red LED for visualization for accuracy. However, RAMalgo has also leveraged pose estimation to identify paw position from the substage video in real time. Motorized actuators iteratively position RAMalgo until the paw center falls in the photostimulation zone, removing the need for experimenters to manually aim the device. Head-to-head comparison determined that automated aiming resulted in more consistently delivered stimulus intensities than manual aiming.

A primary benefit of RAMalgo is the ability to precisely titrate and apply radiant heat or mechanical stimuli, which is critical since noxious thermal and mechanical stimuli are signaled from the periphery via distinct populations of nociceptors. Further, the ability to deliver optogenetic stimulation allows for selective activation of genetically defined subpopulations of nociceptors expressing optogenetic effectors. The intensity of these stimuli can be standardized by ensuring consistent distance from the stimulated paw and can be precisely graded by modulating stimulus pulse shape and intensity. When trained experimenters applied laser stimulation by aiming RAMalgo at a paw-shaped target with a joystick, intensities of effective laser power (detected by a photodiode on the target) were significantly more uniform than when those same experimenters applied the stimuli manually—as is currently standard practice. This inter-experimenter validation underscores that a significant reduction in experimental variability is achieved by automating stimulus application.

Finally, RAMalgo precisely detects paw withdrawal latency by integrating the red LED used for aiming with a photodetector to measure changes in red-light reflectance. The authors simultaneously recorded high-speed video from the side of the animal and the intensity of reflectance from the bottom of the paw (both recorded at 1 kHz). Withdrawal latency was defined either by the trajectory of paw height in video or as a sharp drop in reflectance relative to baseline. These two metrics were highly correlated ($R = 0.966$), bolstering confidence in the ability of red-light reflectance to capture withdrawal latency with equivalent temporal precision afforded by high-speed videography.

Together, the capabilities allow for closed-loop control of stimulus application and withdrawal threshold detection for fully unbiased nociceptive testing. As experimenter intervention is not required for positioning the stimulus underneath the paw, applying the stimulus, or detecting the withdrawal response, RAMalgo significantly reduces or eliminates variability due to experimenter factors. Automation also promises to improve experimental throughput, allowing for larger sample sizes with equivalent experimenter effort.

Determining nociceptive thresholds remains a standard in analgesic testing and for understanding the neurobiology of nociception. However, the clinical

validity of these evoked pain responses for understanding chronic pain disorders has been questioned, given that the predominant cause of suffering is ongoing, spontaneous pain.[4] To this end, Dedek et al.[3] establish the proof of principle that RAMalgo's substage video can be leveraged not just for paw detection but also for quantifying spontaneous pain behaviors. Using pose estimation and subsequent unsupervised behavioral classification using machine learning, the authors extracted and quantified behavioral motifs associated with spontaneous non-evoked pain from substage video. Consistent with prior studies, withdrawal latencies were very weakly correlated with the expression of spontaneous pain behaviors, pointing to distinct neural mechanisms mediating evoked and ongoing pain.[5–7] Deep- and machine-learning approaches are being increasingly adopted to objectively quantify pain behaviors.[6–8] As this field matures, RAMalgo offers a straightforward integration of spontaneous and evoked pain behaviors.

Any novel method is subject to careful consideration before adoption. For example, specialized adaptors are required for RAMalgo's integration of Von Frey filaments, and it is not immediately clear how other stimulus modalities (i.e., pinprick, brush, chemical, or cold stimulus) may be integrated. This is critical, as certain neuropathic conditions are defined principally by cold sensitivity or allodynia.[9] Since red light is used for aiming, RAMalgo may not be appropriate to use with red-shifted optogenetic effectors, limiting flexibility with respect to intersectional genetic targeting of nociceptor populations. Finally, certain models of chronic pain, such as the widely used spared nerve injury, induce hypersensitivity only in subterritories of the paw,[10] and may thus require exclusive stimulation of these subterritories. Whether automated aiming can accurately be applied to paw subterritories remains to be determined. However, even with these limitations considered, RAMalgo represents a promising advance in improving the objectivity and standardization of measuring pain in rodents. Testing standardization promises to improve reproducibility within and across laboratories, which will ultimately be critical for identifying novel targets for pain treatment and for assessing the efficacy of novel therapeutics.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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