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Label-free neuroimaging in mice captures sensory activity in response to tactile stimuli and acute pain

Sensory disorders are clinically common, debilitating conditions. But mouse behavioral models are often insufficient. We demonstrate that label-free, minimally invasive brain imaging in mice could be a promising avenue for sensory research or drug discovery efforts.

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Purpose

We were interested in finding ways to measure various sensations in mice without directly observing their behavior. Specifically, we hypothesized that we could use a microscope to observe brain activity directly in response to vibrating stimuli, capsaicin-induced pain, and histamine-induced itch. To minimize experimental and surgical complexity, we performed *in vivo* flavoprotein autofluorescence imaging, a

label-free and minimally invasive technique. During imaging, we administered tactile stimuli, capsaicin, or histamine to mice to see which brain areas activate (i.e., autofluoresce more) in response. While we found a clear signal in the mouse brain in response to touch and capsaicin-induced pain, our results for histamine-induced itch were inconclusive.

This work may be useful for neuroscientists interested in sensory research. From a technical perspective, we show that imaging the brain response to peripheral capsaicin injections is possible using simple fluorescence microscopy, without labels and relatively non-invasively. This could be an inexpensive and flexible solution to studying pain processing in the brain. Though we're not following up on this work at this time, we welcome others to replicate the activity we observed, test the effect of drugs, and cross-reference our results with other methodologies.

- Imaging **data** from this pub, including raw and processed videos, are available on Zenodo.
- All associated **code** is available in this GitHub repository.

We've put this effort on ice!

#TechnicalGap

Our original goal was to employ this technique to complement traditional mouse behavioral assays for itch. Using widefield autofluorescence microscopy, we observed no consistent brain activity in response to histamine-induced itch. We're therefore icing this concept for now.

Learn more about the Icebox and the different reasons we ice projects.

Background and goals

Our senses serve as our vital link to the surrounding environment. The sensations of touch, pain, and itch are especially evolutionarily important in helping an organism avoid unpleasant or potentially harmful stimuli. At the same time, persistent pain and itch in the absence of a stimulus can become highly uncomfortable or even intolerable **[1][2]**.

Mouse behavioral models are widely used to study the neurological basis of sensation **[3]**. For instance, researchers can administer drugs that induce a particular sensation in a peripheral body part of a mouse and observe its behavior. Unfortunately, behavioral assays are time-consuming and confounded by variables that are difficult to control, such as subtle procedural or environmental differences during animal handling, adaptation, and testing. These subtle but important sources of variability can limit the clinical predictive power of mouse behavioral assays **[4]**. Here, we propose that imaging the mouse brain directly could bypass the need for some behavioral experiments and lead the way to higher-fidelity, more reproducible assays.

Brain imaging in anesthetized mice is a viable strategy to eliminate the variability of behavioral assays by measuring upstream, behavior-independent physiology. Various imaging techniques have been used to study brain activity, identifying specific areas responsible for vision, touch, memory, and higher-order processing.

While chemical dyes or genetically encoded sensors are widely used, intrinsic signal optical imaging (ISOI) is a technique that allows imaging of the unlabeled, genetically unmodified brain **[5]**. ISOI avoids the need for transgenic animals, which may be expensive and have abnormal brain activity themselves **[6]**, and invasive dye or virus injections. A well-known, commonly used subset of ISOI is hemodynamic imaging **[7] [8]**. The principle of hemodynamic imaging is that active brain areas require more oxygenated blood, which leads to an increase in blood flow to those areas. This influx of oxygenated blood to a brain area can then be measured optically. This is also the signal that's measured using functional magnetic resonance imaging (fMRI).

Flavoprotein imaging is a less common subset of ISOI that relies on the autofluorescence of mitochondrial flavoproteins in response to metabolic activity **[9] [10]**. The principle of the technique is as follows: when a brain area becomes more active due to a sensory (or other) stimulus, the metabolic activity of the cells in that area increases, leading to higher autofluorescence of flavoproteins in the

mitochondria. We can measure this activity with a simple fluorescence microscope to determine which brain areas respond to the stimuli.

Pain and itch, as well as sensory perturbations, have been studied before in humans and/or mice using imaging techniques such as fMRI, positron emission tomography (PET) **[11]**, and more recently, multiphoton excitation microscopy **[12][13][14]**. However, those techniques are either very costly (in the case of fMRI and PET) or require labeling the brain with fluorophores (for multiphoton) **[5]**. Due to these limitations, those techniques are likely unsuitable for a reasonably high-throughput, scalable assay.

Therefore, our goal in this study was to see if we could use *in vivo* flavoprotein imaging to study acute pain- and itch-related signaling in the brain.

> **SHOW ME THE DATA**: Access our imaging data containing raw and processed videos on Zenodo (DOI: 10.5281/zenodo.11585535).

The approach

Following procedures from Couto et al. **[15]**, we built an ultra-widefield microscope (UWFM) capable of imaging the entire mouse cortex (the outermost structure of the mammalian brain) with enough resolution to see responses in specific brain regions (Figure 1). We decided to use label-free, through-skull flavoprotein imaging because it was the most economical, least invasive, and most flexible solution that could eventually become a routine assay.

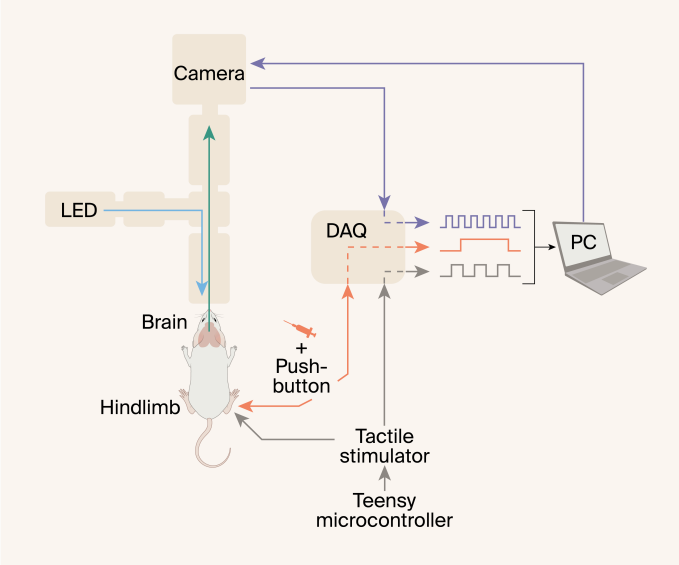


Figure 1

Schematic of the UWFM experimental setup.

The microscope consists of a blue LED for autofluorescence excitation, a camera for capturing brain activity, and additional mirrors, filters, and lenses (not pictured). The Teensy microcontroller drives the stimulator that's used to deliver tactile stimuli. The PC controls the camera, the Teensy microcontroller, and the data acquisition system (DAQ). The microcontroller runs a small program to control the LED and tactile stimulator. The DAQ captures signals from the camera, tactile stimulator, and injection push-button to synchronize the sensory stimulation and imaging data.

Microscope and sensory stimulation hardware

We built the UWFM using the protocol from Couto et al. **[15]** with the following two modifications (Figure 1). First, we used a ZL41 camera (Oxford Instruments Andor)

instead of the pco.Edge 5.5 (Excelitas) used in that study. Second, Couto et al. **[15]** used alternating blue/violet light for imaging, whereas we only needed blue light to capture autofluorescence. We measured the full field of view (FOV) of the microscope to be 13.4 × 11.3 mm (5 µm/px with no binning). For all imaging trials, we cropped the FOV to only include the whole brain and binned the frames 4 × 4. We approximately aligned images to a reference brain atlas **[15][16]** using anatomical landmarks.

For tactile stimulation, we used an inexpensive vibrating motor (#B07Q1ZV4MJ, Amazon) that we adhered to the paw of the mouse using lab tape during the experiment. We controlled the motor with a simple driver circuit powered by two AA batteries. In some trials, we injected the mouse with a pain- or itch-causing substance (see details in "Injection experiments"). For these, we used a simple push-button to mark the approximate injection time.

For subsequent analysis, we had to align the occurrence of the sensory stimulation (tactile pulses as well as injections) to the imaging data to synchronize camera frames to stimulation events. As in Couto et al. **[15]**, we used a microcontroller (PJRC Teensy 4.0) to control the blue light-emitting diode (LED) and the tactile stimulator. A data acquisition board (DAQ; USB-6001, National Instruments), connected to the data acquisition computer, captured the control signals from the microcontroller, along with the injection push-button signal and exposure signal from the camera. We controlled camera acquisition with µManager software (version 2.0) **[17]**.

The entire cost of the imaging system, including the camera, was roughly $30,000, making it relatively affordable compared to single-neuron resolution imaging methods, which could cost hundreds of thousands of dollars.

Tactile stimulation experiments

We performed all animal work in accordance with the Institutional Animal Care and Use Committee of the Charles River Accelerator and Development Lab (CRADL, South San Francisco, CA).

We anesthetized wild-type female mice (N = 5 total used for this study; DBA/2J, The Jackson Laboratory) using 3–4% isoflurane anesthesia. We confirmed that mice were anesthetized through the loss of righting reflex, a breathing rate of approximately one breath per second, and no response to toe pinch. We then head-fixed the mice using

earbars attached to a stereotaxic frame (Kopf Instruments) and used a heating pad (B00075M1T6, Amazon) to maintain the animal's body temperature. We then carefully removed the hair over the scalp using Nair, applied eye ointment (Puralube® Ophthalmic Ointment, Dechra), and injected 1 mL of body-temperature sterile saline subcutaneously to maintain hydration. Subsequently, we injected 0.1 mL of body-temperature 1% lidocaine (Bichsel) subcutaneously under the scalp and removed the scalp using fine-tipped scissors. We cleaned the periosteum of connective tissue and stopped any bleeding with cotton swabs. Finally, we applied a very thin layer of cyanoacrylate glue to the surface of the cleaned skull to maintain optical clarity during imaging.

We then used the UWFM to non-invasively image flavoprotein autofluorescence in the brain. The optical light path of our custom-built microscope (Figure 1) was identical to that of Couto et al. **[18]** Briefly, light from a blue LED (M470L5, Thorlabs) passed through a bandpass filter (ET470/40×, Chroma), reflected towards the brain via a dichroic mirror (T495lpxr, Chroma), and focused using the objective lens (85mm f/1.4 Lens; Rokinon). Autofluorescence light passed through the objective lens and dichroic mirror through an emission filter (86-963, Edmund Optics) and was focused on the camera using an imaging lens (NIKKOR 105mm f/1.4E ED; Nikon).

For each experiment, we started by attaching the tactile stimulator to either the left or right hindlimb of the mouse. The stimulator delivered a sequence of 50 vibrational pulses (2 s on, 4 s off) while we simultaneously imaged with the UWFM at 10 frames per second. Based on literature (e.g., **[19][20]**), we expected to observe brain activity in the S1 region (somatosensory area 1) of the brain. If we didn't detect a cortical response in S1, we performed the following troubleshooting steps and tried again: adjusted the focus depth, adjusted the positioning of the stimulator, reduced the isoflurane level by 0.25% (but never below 1%), or placed the stimulator on a different limb. In most cases, one or more of these steps resulted in a clear signal.

Injection experiments

Once we observed a brain response to a tactile stimulus, we proceeded with chemical stimuli. For capsaicin experiments, we first made a vehicle solution consisting of 80% PBS (phosphate-buffered saline), 10% Tween-80 (278632500, Thermo Scientific), and 10% ethanol (v/v). We dissolved 40 µg of capsaicin (M2028-50MG, Sigma-Aldrich) in 10 µL of the carrier solution for the capsaicin injection. The control for the capsaicin

experiments was the carrier solution alone. In the histamine experiments, we dissolved histamine dihydrochloride (PHR1357-500MG, Sigma-Aldrich) in PBS to create a 27 mM solution (previously determined to be effective at eliciting an itch behavioral response **[21]**). The control for histamine injections was PBS alone. Before starting the imaging trial, we carefully inserted a 1 cc insulin syringe containing the chemical to be injected intradermally into the forelimb, hindlimb, or nape of the mouse. After imaging for five minutes, we slowly injected the solution by depressing the syringe plunger. Simultaneously, we pushed the push-button to mark the injection. We then continued imaging for 10 minutes for control (vehicle) injections and 30 minutes for histamine and capsaicin injections. This additional imaging time was needed to ensure that any longer-term effects of the drug were captured.

Software and analysis

The firmware on the Teensy microcontroller controlling the LED and stimulator is available here. A Python script communicated with the microcontroller via the serial interface. The software NI SignalExpress (National Instruments, 2015) captured signals from the data acquisition system (DAQ) and wrote the output to a CSV file that we used for synchronization in the analysis scripts.

To analyze and interpret the imaging data, we built a Python-based data analysis and visualization pipeline. The pipeline ingested raw image stacks and first averaged them in time by a factor of two frames for tactile stimulation trials and eight frames for injection trials to reduce noise. We then motion-corrected the imaging stack using SIFT-based registration **[22]**. We then automatically masked out portions of each frame that weren't the brain using a simple flood-filling algorithm (`flood` function from scikit-image **[23]**). Because autofluorescence is subject to photobleaching, we performed rudimentary bleach correction by subtracting the bottom 5% of pixels within the brain from each frame. Finally, we subtracted baseline autofluorescence from each frame to make the response more evident.

For tactile stimulation trials, we present results as stimulus-triggered averages, where the response to each stimulus is aligned by its onset time and averaged across all stimulations in a single trial. We selected regions of interest (ROIs) based on the known anatomical locations of the hind and forelimb somatosensory areas. Results are plotted as $\Delta F/F$ (change in autofluorescence over baseline).

For injection trials, we used the entire brain area (defined by the mask above) as the ROI except for the nape capsaicin injection trial, where only the somatosensory area of the brain was responsive. For this experiment, in both vehicle and capsaicin conditions, we chose the ROI to approximately cover just the capsaicin-responsive area. To visualize the results of injection experiments, we adjusted the pixel intensities of the autofluorescence response in the vehicle and drug trials to have the same minimum and maximum values, making them directly comparable. We performed the ΔF/F computation on non-adjusted, baseline-subtracted autofluorescence data.

> All **code** we generated and used for the pub is available in this GitHub repository (DOI: 10.5281/zenodo.12770054), including the code necessary to process the raw imaging data and generate the figures in the pub.

Additional methods

We used ChatGPT to help write and clean up code, and GitHub Copilot to help write and add comments to our code.

The results

> **SHOW ME THE DATA**: Access our imaging data containing raw and processed videos on Zenodo.

Brain activity during tactile stimulation

As a way to validate the assay, we first wanted to see whether we could use the ultra-widefield microscope (UWFM) to observe previously described brain activity patterns in response to tactile stimulation. The mouse primary somatosensory area (S1) is largely somatotopically organized, meaning a sensory stimulus of a particular area (such as a limb) should lead to a reproducible activation of a particular location in the cortex.

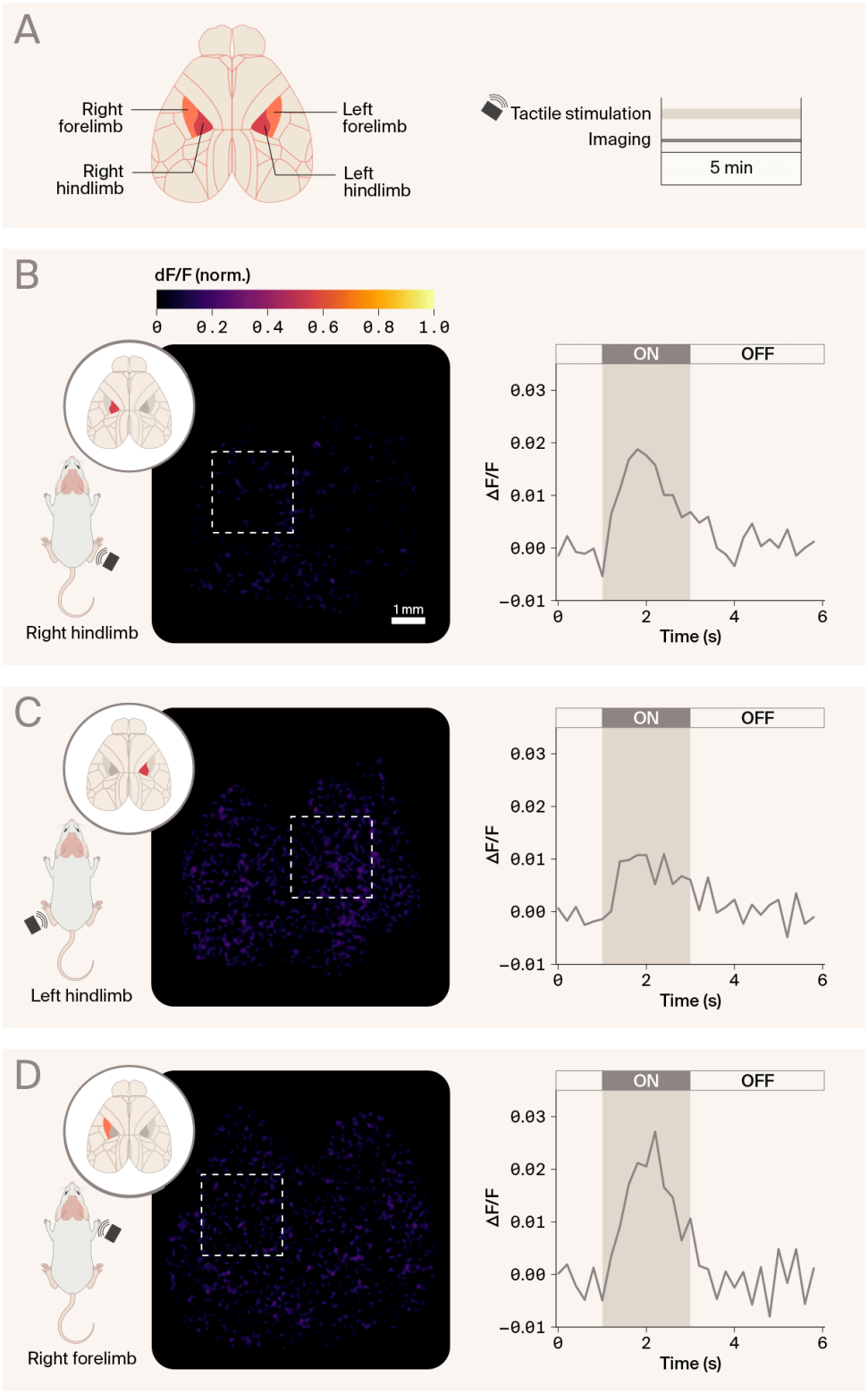


Figure 2

Imaging brain activity in response to tactile stimulation.

(A, left) Brain atlas with the areas responsible for somatosensory representations of the right and left forelimb and hindlimb filled in.

(A, right) Experimental setup. During imaging, a tactile vibrating stimulator adhered to the fore or hindlimb of the mouse delivered the stimulation (2 s on, 4 s off; repeated 50 times).

(B–D, left) Stimulus-triggered average of ΔF/F over 50 trials (1 frame = 100 ms), Inset: Brain atlas with expected activation area.

(B–D, right) Measured fluorescence trace of the outlined region of interest from the left panel. Shaded “ON” area shows the two-second stimulation duration.

(B) Brain activity in response to stimulation of the right hindlimb. N = 1 mouse.

Data ID: 2024-02-29/Zyla_5min_RHLstim_2son4soff_1pt25pctISO_1

(C) Brain activity in response to stimulation of the left hindlimb. N = 1, same mouse as in B.

Data ID: 2024-02-29/Zyla_5min_LHLstim_2son4soff_1pt25pctISO_2

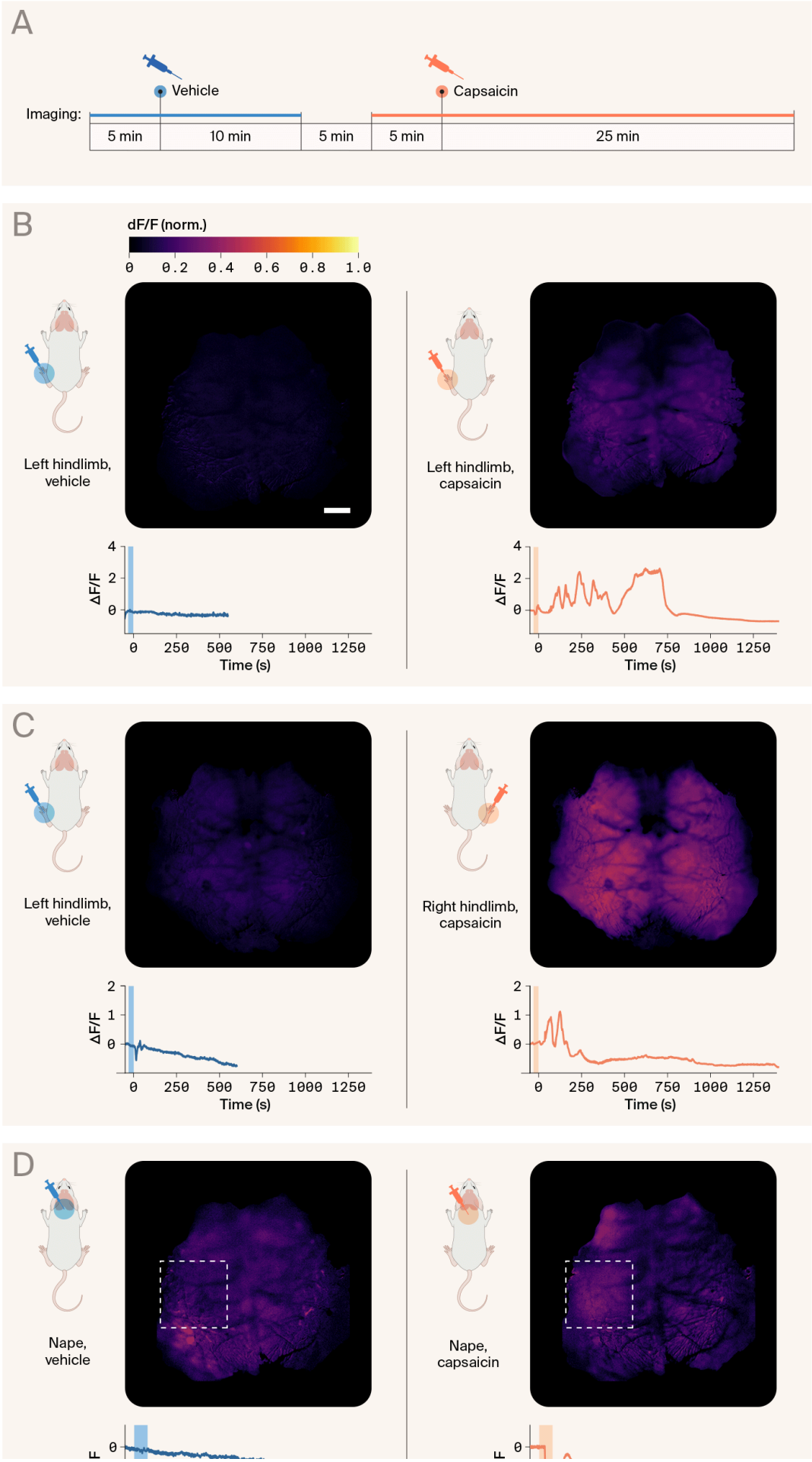
(D) Brain activity in response to stimulation of the right forelimb. N = 1 mouse.

Data ID: 2024-02-21/Zyla_5min_RFLstim_2son4soff_1pt25pctISO_deeper_2

To test this, we imaged the brain during bouts of vibrating tactile stimulation (Figure 2, A). As expected, we observed reliable, localized autofluorescence signals in contralateral brain areas: when stimulating the right hindlimb, we saw activity in the left somatosensory area; when stimulating the left hindlimb, we saw activity in the right area (Figure 2, B–C). When stimulating the right forelimb, we saw activity in the left somatosensory area, slightly lateral to the hindlimb (Figure 2, D). These results are consistent with known anatomical structure **[16]**. The response was a 1–3% increase in autofluorescence above baseline after trial averaging, which is consistent with previous flavoprotein imaging results **[9][10]**.

Brain activity in response to capsaicin-induced pain and histamine-induced itch

We next explored whole-brain activity in response to acute capsaicin-induced stimuli. Mice injected with capsaicin into a hindlimb exhibited widespread, high-intensity cortical activation for ~10 minutes following the injection (Figure 3, A). This activation wasn't present after injecting the vehicle control alone. The activity pattern was complex and oscillatory. Interestingly, the oscillatory activity was more intense for a hindlimb we previously injected with vehicle control (Figure 3, B) than a hindlimb that only experienced the capsaicin injection (Figure 3, C). A mouse injected with capsaicin into the nape of the neck also exhibited oscillatory brain activity, but it was briefer and confined to the somatosensory area of one hemisphere, so we only computed the signal within this area in both the vehicle and capsaicin trials (Figure 3, D; see outlined region of interest). Together, these results suggest that the UWFM can record cortical responses to stimuli that activate acute pain-related pathways.


A
Vehicle
Capsaicin
Imaging:
5 min
10 min
5 min
5 min
25 min
B
dF/F (norm.)
0
0.2
0.4
0.6
0.8
1.0
Left hindlimb, vehicle
Left hindlimb, capsaicin
ΔF/F
Time (s)
C
Left hindlimb, vehicle
Right hindlimb, capsaicin
ΔF/F
Time (s)
D
Nape, vehicle
Nape, capsaicin

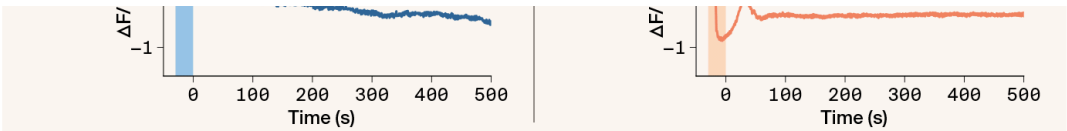


Figure 3

Imaging brain activity in response to capsaicin injection.

(A) Experimental setup. We injected a mouse with vehicle and/or capsaicin into the left hindlimb, right hindlimb, or the nape of the neck 5 minutes after beginning the imaging trial. We imaged the vehicle trials for 15 min and the capsaicin trials for 30 min.

(B–D) Brain activity animations and quantification.

(B–D, top) Animations of brain activity (1 frame = 30 s). Animations on the left and right sides are normalized to use the same color scale to enable a direct comparison between control and capsaicin injections.

(B–D, bottom) Absolute (non-normalized) ΔF/F. Shaded area represents approximate injection time.

(B) Result of vehicle (left) and capsaicin (right) injections into the left hindlimb of the same mouse (N = 1).

Data IDs: 2024-03-18/Zyla_15min_LHL_carrierinj_1pt25pctISO_1 (vehicle), 2024-03-18/Zyla_30min_LHL_capsacirinj_1pt25pctISO_1 (capsaicin)

(C) Results of injections into different limbs. We injected vehicle into the left hindlimb and capsaicin into the right hindlimb. N = 1 mouse.

Data IDs: 2024-03-19/Zyla_15min_LHL_carrierinj_1pt5pctISO_1_1 (vehicle), 2024-03-19/Zyla_30min_RHL_40ugin10uL_1pt5pctISO_1_1 (capsaicin)

(D) Results of injections into the nape of the neck. The ΔF/F trace below the animation is the average of the activity only within the outlined region. Note that the x-axis is different in this panel to highlight the transient response. N = 1, same mouse as in C.

Data IDs: 2024-03-18/Zyla_15min_nape_carrierinj_1pt25pctISO_1 (vehicle), 2024-03-18/Zyla_30min_nape_40ugin10uLcapsacininj_1pt25pctISO_1 (capsaicin)

Finally, we applied the same methodology to observe the brain's response to a stimulus that activates itch pathways (Figure 4, A). Mice injected intradermally with histamine into the left or right hindlimb still exhibited widespread bilateral autofluorescence above baseline. However, the activity patterns were inconsistent between trials, the magnitude of the activation was similar to saline control, and the activation didn't seem to be localized to a particular brain area (Figure 4, B–C). Due to these factors, we believe that a more sensitive imaging methodology or more sophisticated analysis will be necessary to determine whether itch-related brain activity was present.

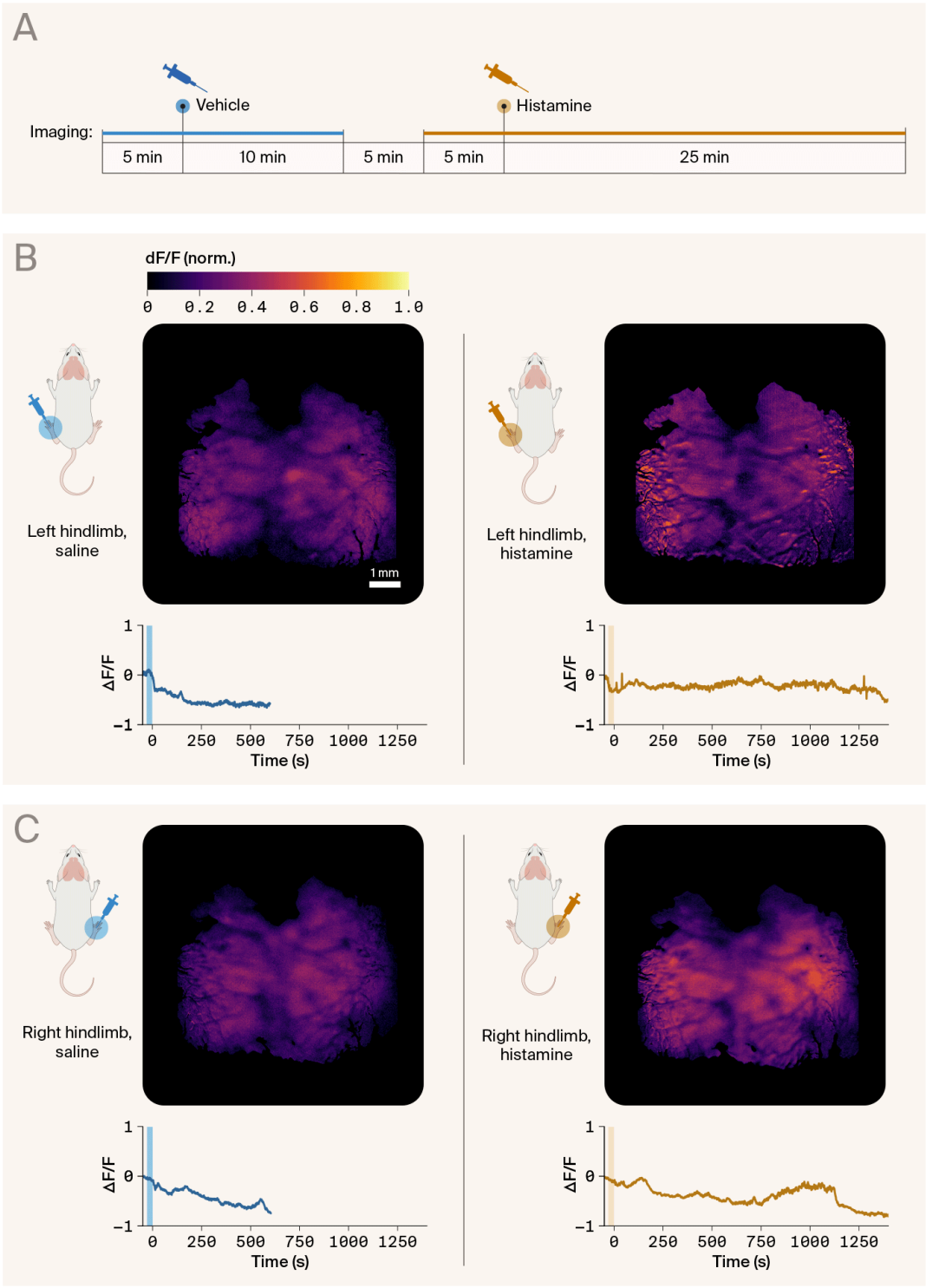


Figure 4

Imaging brain activity in response to histamine injection.

(A) Experimental setup. We injected a mouse with saline and/or histamine into the left or right hindlimb 5 min after beginning the imaging trial. We imaged the saline trials for 15 min and the histamine trials for 30 min.

(B–C) Brain activity animations and quantification.

(B–C, top) Animations of brain activity (1 frame = 30 s). Animations on the left and right side are normalized to use the same color scale to enable a direct comparison between control and histamine injections.

(B–C, bottom) Absolute (non-normalized) ΔF/F. Shaded area represents approximate injection time.

(B) Results of saline (left) and histamine (right) injections into the left hindlimb of a mouse (N = 1).

Data IDs: 2024-02-29/Zyla_15min_LHL_salineinj_withpushbutton_1 (saline), 2024-02-29/Zyla_30min_LHL_50uL27MMHistinj_withpushbutton_1 (histamine)

(C) Results of saline (left) and histamine (right) injections into the right hindlimb of a mouse (N = 1), same mouse as in B.

Data IDs: 2024-02-29/Zyla_15min_RHL_salineinj_withpushbutton_1 (saline), 2024-02-29/Zyla_30min_RHL_50uL27MMHistinj_withpushbutton_1 (histamine)

What does this mean?

Our goal with this study was to determine whether label-free widefield imaging could let us observe brain activity in response to painful and itch-causing stimuli. Overall, we observed a clear, reproducible signal for pain but not itch. From a technical perspective, this work demonstrates that label-free, through-skull flavoprotein imaging can be used to capture a variety of sensory activity in the brain with a less complex imaging setup than what's typically used.

How do these results fit within our understanding of pain and itch biology? Most neurophysiological studies of both pain and itch to date have focused on signaling and cell type identification within the spinal cord **[24][25][26]**. More recently, rodent studies have confirmed that itch sensations and both acute and chronic pain activate neurons within the S1 area of the brain **[11][12][27]**. However, there's evidence that neurons responsive to painful stimuli are more prevalent in the brain than those responsive to itch **[13][28]**, which may explain why we didn't detect reproducible

activity in response to itch using our approach. The widespread bilateral activation of the cortex in response to acute limb pain *was* consistent with other fMRI studies in rodents **[29][30]**. Similar observations of widespread brain activation in response to painful stimuli have also been reported in human fMRI studies **[31]**.

Overall, our findings suggest that flavoprotein imaging in the mouse brain can be used as a new, inexpensive technique to image acute activation of pain pathways in the peripheral nervous system, but more work must be done to see whether we could use it for imaging peripheral itch pathways.

Key takeaways

We used label-free autofluorescence microscopy in anesthetized mice to image the response of the brain to the sensations of touch, pain, and itch. Consistent with previous work by others, we observed localized brain activity in response to tactile stimulation. We found that a capsaicin-induced, painful stimulus evoked strong, consistent oscillatory activity in the brain. Finally, we saw no consistent activity in response to histamine-induced itch. Together, our results suggest that autofluorescence microscopy can be an inexpensive and straightforward technique to measure brain activity in response to various stimuli.

Next steps

We're not pursuing this work further for now because we weren't able to easily observe activity in response to itch. That said, we propose several experiments that can build upon our work:

1. We'd welcome any input on why we saw no reproducible activity in response to itch or how we could improve our methodology to enable this measurement. Because it's known that fewer neurons in the cortex are responsive to itch than pain **[13]**, our current hypothesis is that there isn't enough autofluorescence signal from those few neurons to be detected above the noise. This hypothesis could be tested by repeating the experiments using a highly sensitive genetically encoded calcium indicator like GCaMP **[32]** to increase the signal. Additionally, because

anesthesia is known to suppress cortical activity **[33]**, it would be beneficial to perform the experiment on non-anesthetized animals.

2. It would be informative to repeat the capsaicin experiments to distinguish activity patterns that are truly inherent to the brain from those that are caused by experimental variability. It would be especially interesting to see what factors (such as injection location, dose, and prior injections) contribute to the localization, amplitude, duration, and period of the oscillations we observed.
3. To see whether this neuroimaging technique could be used for drug screening, it would be valuable to test various analgesic drugs to ensure that their application produces expected results in alleviating the pain-related sensory activity in the brain.
4. For clinical applicability, it would be useful to perform this type of imaging on chronic mouse pain and itch models as opposed to the acute models used here to assess whether the imaging can be useful for those conditions.

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