Supplemental Information and legends

Spatiotemporally quantitative *in vivo* imaging of mitochondrial fatty acid β-oxidation at cellular-level resolution in mice

Ayumi Matsumoto, Isao Matsui, Shohei Uchinomiya, Yusuke Katsuma, Seiichi Yasuda, Hiroki Okushima, Atsuhiro Imai, Takeshi Yamamoto, Akio Ojida, Kazunori Inoue, and Yoshitaka Isaka.

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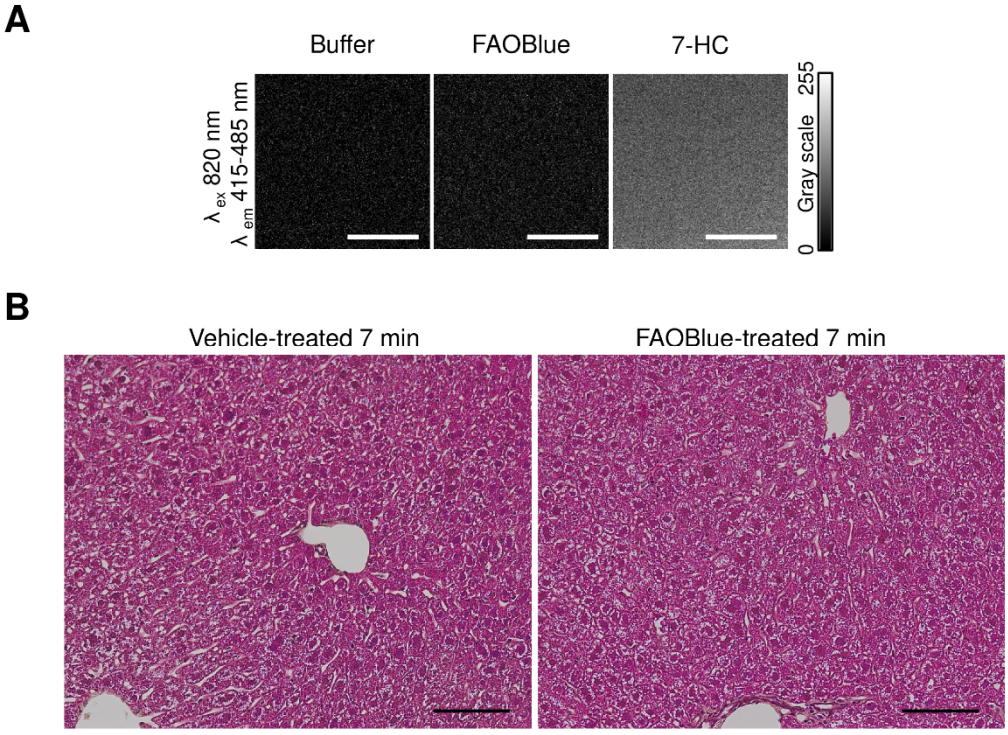
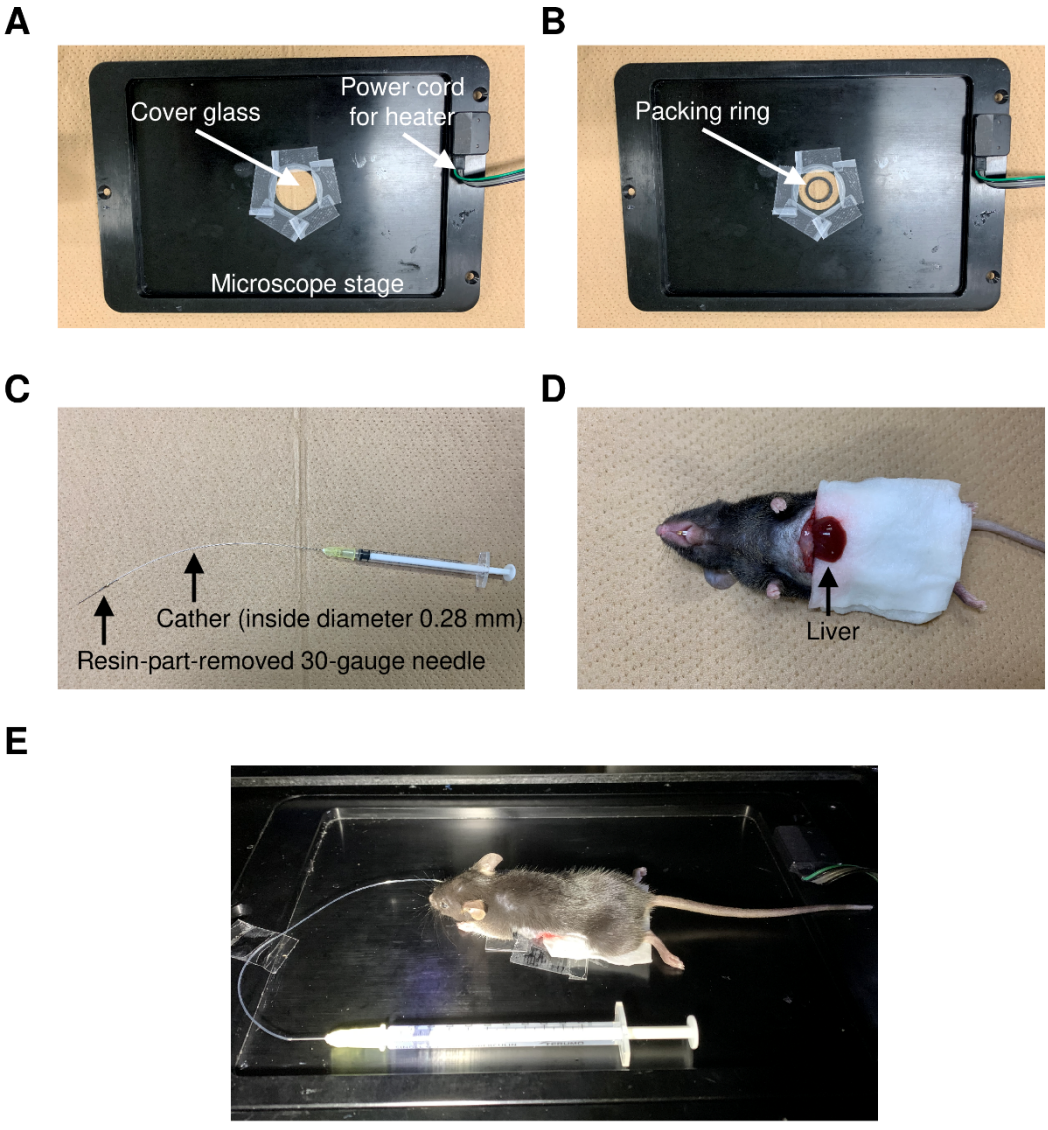
Supplemental Figure S1



Supplemental Figure S1. Additional experiments related to FAOBlue

(A) Multiphoton microscope image of dilution buffer, 62.5 µM FAOBlue, and 0.3125 µM 7-Hydroxycoumarin-3-(N-(2-hydroxyethyl))-carboxamide (7-HC) with two-photon excitation (λ_{ex}) at 820 nm and a bandpass emission filter (λ_{em}) of 415–485 nm. Shown images are 300-pixel square (Scale bars:50 µm). (B) HE-stained liver sections from vehicle or FAOBlue (62.5 µM, 100 µl/animal) injected mice at 7 min. Representative micrographs from three independent experiments are shown (Scale bar: 100 µm).

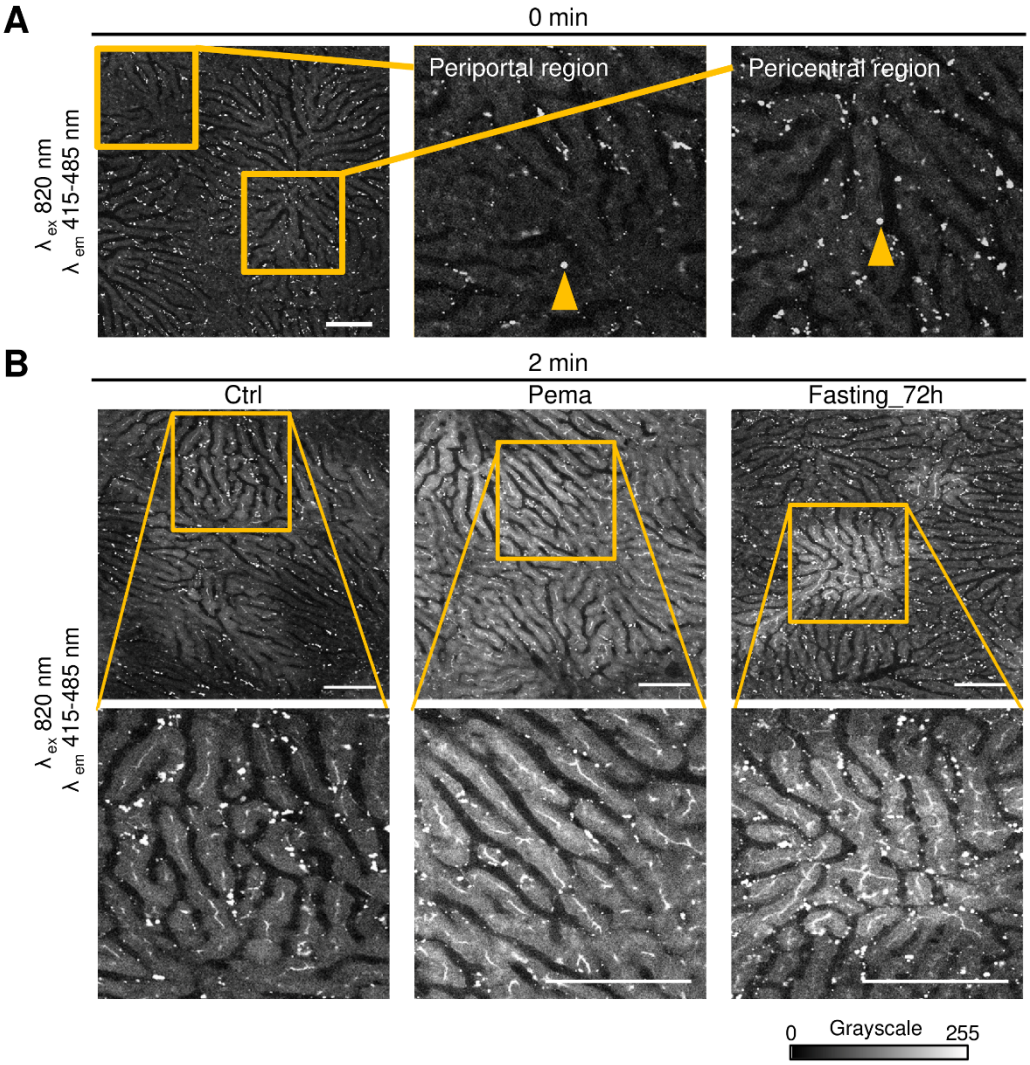
Supplemental Figure S2

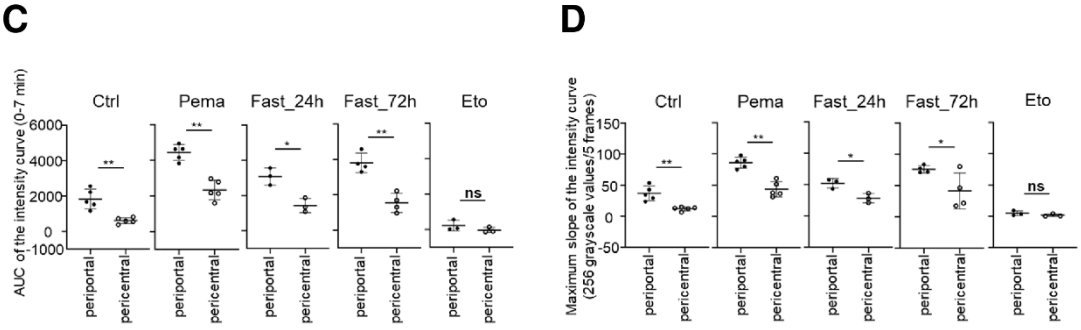


Supplemental Figure S2. Set up for FAOBlue-based analyses

Photographs related to the mouse settings. (A) Circular cover glass taped onto the microscope stage. (B) Water supply packing ring glued onto the cover glass. (C) A catheter used for reagent injection. (D) Example of the exposed liver tissue. (E) Example of mouse setting.

Supplemental Figure S3

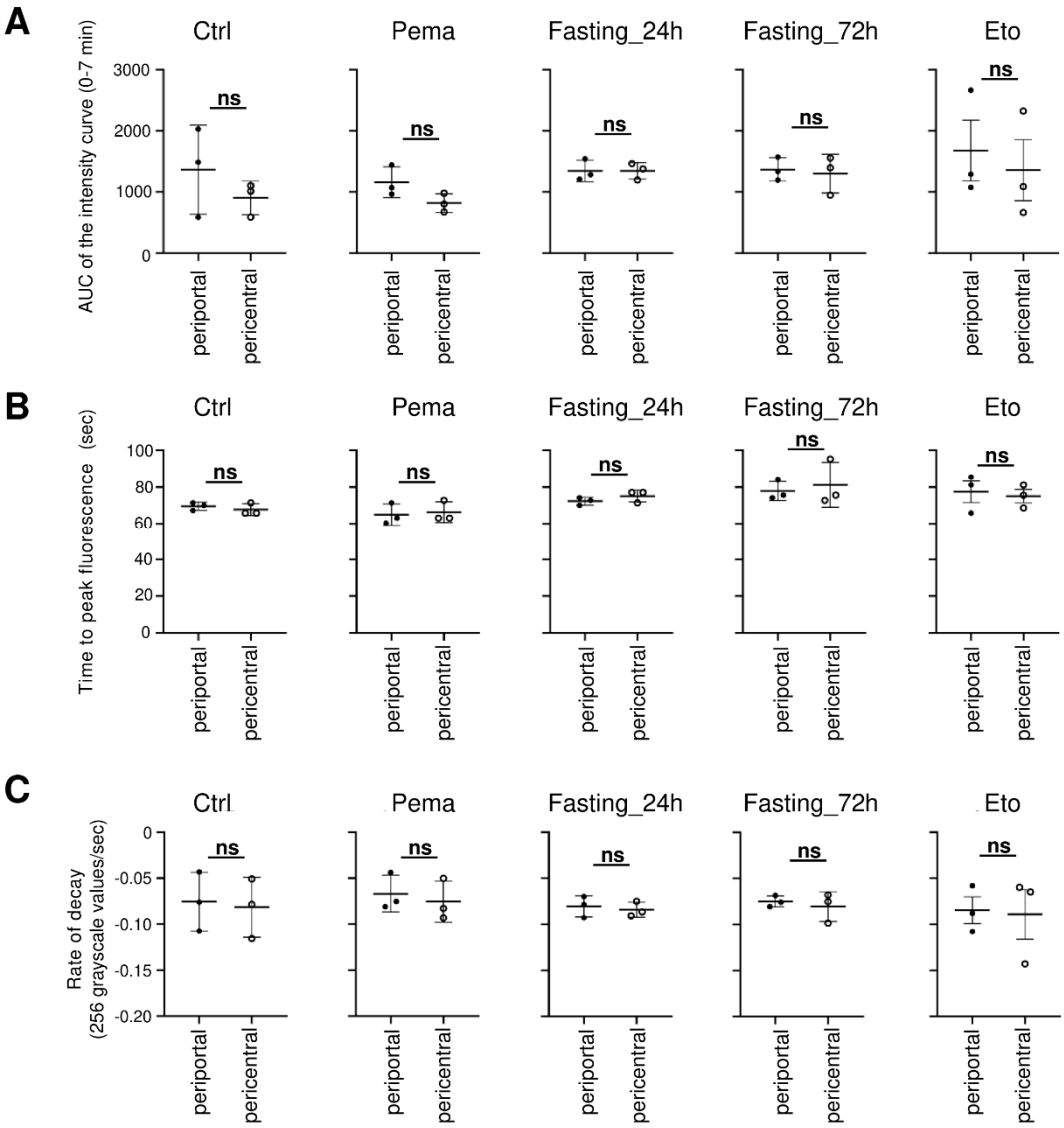




Supplemental Figure S3. Additional analyses of FAOBlue-injected mouse livers

(A) Examples of ROI for quantifying fluorescence intensity. Orange arrowheads indicate circular ROIs with a radius of 4 pixels. (B) Figures 2B, E, and K are shown enlarged. (C) AUCs and (D) maximum slopes shown in Figures 3B and 3C were compared between the periportal and pericentral regions (N = 3–5 biological replicates in each group: ns* $P < 0.05$, ** $P < 0.01$, t-test). ROI: region of interests, AUC: area under the curve.

Supplemental Figure S4



Supplemental Figure S4. Additional analyses of 7-HC-injected mouse livers

(A) AUCs, (B) time to peak fluorescence, and (C) rate of decay shown in Figures 5B-D were compared between the periportal and pericentral regions (N = 3 biological replicates in each group: ns, not significant: *t*-test).

Supplemental Table S1. Primer Nucleotide Sequences

gene	Forward	Reverse
Cpt1a	5'- GACTCCGCTCGCTCATTC -3'	5'- TCTGCCATCTTGAGTGGTGA -3'
Cpt2	5'- CAATGAGGAAACCCTGAGGA -3'	5'- GATCCTTCATCGGGAAGTCA -3'
Ppara	5'- TGTATGAAGCCATCTTCACG -3'	5'- GGCATTGAACTTCATAGCGA -3'
Ppargc1a	5'- TGAAAGGGCCAAACAGAGAG -3'	5'- GTAAATCACACGGCGCTCTT -3'
Cd36	5'- GATGACGTGGCAAAGAACAG -3'	5'- TCCTCGGGGTCCTGAGTTAT -3'
Rps18	5'- TTCTGGCCAACGGTCTAGACAAC -3'	5'- CCAGTGGTCTTGGTGTGCTGA -3'

All primers were designed according to the sequence of each gene deposited in the GenBank database. Gene names are shown in italics. Abbreviations: *Cpt1a*, carnitine palmitoyltransferase 1a; *Cpt2*, carnitine palmitoyltransferase 2; *Ppara*, peroxisome proliferator-activated receptor alpha; *Ppargc1a*, peroxisome proliferative activated receptor gamma coactivator 1 alpha; *Cd36*, cluster of differentiation 36; *Rps18*, ribosomal protein S18.

Supplemental Video S1. Visualization of liver blood flow

The liver of the normal mice intravenously injected with Texas Red™ dextran (70 kDa) was visualized by multiphoton microscopy under the $\lambda_{ex}/\lambda_{em}$ = 1080/570-616 nm condition. The static image in the left half (reference) is the same region observed under the $\lambda_{ex}/\lambda_{em}$ = 820/415-485 nm condition. The video is shown at a 21× rate (Scale bars:100 μm).

Supplemental Video S2. Visualization of FAO activity in the liver

FAO activity in the liver of the Ctrl, Pema, Fasting_24h, Fasting_72h, and Eto groups was visualized by multiphoton microscopy under the $\lambda_{ex}/\lambda_{em}$ = 820/415-485 nm condition. The video is at 42× rate (Scale bars:100 μm). FAO: fatty acid β-oxidation, Ctrl: Control, Pema: Pemafibrate, Fasting_24h: fasting for 24 hours, Fasting_72h: fasting for 72 hours, Eto: Etomoxir.

Supplemental Video S3. Visualization of intravenously injected 7-HC in the liver

Mice were pretreated as in the FAOBlue administration experiments and then injected with 7-HC through the right jugular vein. Their livers were visualized using a

multiphoton microscope under the $\lambda_{ex}/\lambda_{em}$ = 820/415-485 nm condition. The video is at 42× rate (Scale bars:100 μm).

Step-by-step instructions and troubleshooting tips

Step-by-step instructions and troubleshooting tips are summarized.

Multiphoton microscope set up

1. A Circular cover glass (Matsunami, Osaka, Japan) was taped to the observation window of the microscope stage (Supplemental Figure S2A).
2. The water supply packing ring (outer diameter, 17 mm; inner diameter, 13 mm; thickness, 2 mm; Sanei, Osaka, Japan) was sliced in half (1 mm thick) to the cover glass with instant adhesive (Supplemental Figure S2B). This ring reduces the pressure on the liver caused by body weight.

Preparation of 30-gauge catheter

1. The resin at the base of a 30-gauge intraocular needle (Nipro, Osaka, Japan) was heated with a kitchen lighter for a few seconds to melt it. Subsequently, the metal needle was obtained. The resin remaining on the needle was scraped off as much as possible.
2. The needle base was inserted about 5 mm into a catheter (outer diameter 0.61 mm, inner diameter 0.28 mm, Natsume, Tokyo, Japan) and cut to a length of 12 cm (Supplemental Figure S2C). Owing to the possibility of FAOBlue being adsorbed

onto the catheter, the experiment was performed with a constant catheter length.

3. The cut end of an intraocular needle (30-gauge), not heat-processed as described above, was inserted into the lumen on the opposite side of the catheter and connected to a syringe to fill the catheter with dilution buffer (Supplemental Figure S2C).

Surgical procedures

1. The mice were anesthetized by intraperitoneal injection of midazolam (Maruishi, Osaka, Japan), butorphanol tartrate (Meiji Animal Health, Tokyo, Japan), and medetomidine hydrochloride (Kyoritsu, Tokyo, Japan). The anesthetic was prepared by diluting 40 µg midazolam, 5 µg butorphanol tartrate, and 3 µg medetomidine hydrochloride in saline to a total volume of 100 µl. A diluted anesthetic of 100 µl per 10 g of BW was injected intraperitoneally.
2. The abdomen and right side of the neck was shaved.
3. The mice were placed in the lateral position and the skin on the right side of the neck was incised to expose the right jugular vein.
4. A 30-gauge catheter was inserted into the right jugular vein, and the needle was secured using a surgical instant adhesive (Aron Alpha A, Daiichi-Sankyo, Tokyo,

Japan).

5. The mice were placed in the supine position and a 3 cm transverse incision was made approximately 5 mm caudal to the xiphoid process (Supplemental Figure S2D).
6. Kimwipes moistened with saline solution were placed on the caudal side of the skin incision and the abdomen was gently compressed to expose the liver on the kimwipes (Supplemental Figure S2D). This was done under extreme caution to avoid touching or drying the liver surface. Fine positioning was performed on the underside of the liver using a moistened cotton swab.
7. Vetbond (3M, MN, USA) was applied using a cotton swab approximately 3 mm wide to the inside of the ring attached to the cover glass, the part that would be on the head side when the mouse was placed on it. This inhibits liver movement during imaging.
8. Each mouse was placed on a microscope stage such that the exposed liver was inside the ring (Supplemental Figure S2E).

Observations

1. The microscope stage was heated to maintain body temperatures at 36–38°C. It is

recommended that another heater be used to maintain the temperature inside the microscope cover at 36–38°C. Temperature maintenance is important for assessing metabolism.

2. Positioning and focusing was performed.
3. The syringe and needle were removed and replaced with a 30-gauge U-100 insulin syringe filled with FAOBlue solution. FAOBlue was diluted immediately before filling the U-100 syringe.
4. Following baseline observations, FAOBlue was injected and FAO activity was visualized. To reduce phototoxicity, a multiphoton laser should be used at the lowest possible power.