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Technical note

Thoracotomy reduces intrinsic brain movement caused by heartbeat and respiration: A simple method to prevent motion artifact for *in vivo* experiments

Nobuyoshi Matsumoto[a], Yuji Takahara[a], Norio Matsuki[a], Yuji Ikegaya[a,b,*]

[a] *Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan*
[b] *Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Agency, 5 Sanbancho Chiyoda-ku, Tokyo 102-00075, Japan*

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ABSTRACT

Recent technical advances in electrophysiological recording and functional imaging from the brain of living animals have promoted our understandings of the brain function, but these *in vivo* experiments are still technically demanding and often suffer from spontaneous pulsation, *i.e.*, brain movements caused by respiration and heartbeat. Here we report that thoracotomy suppresses the motion artifact to a practically negligible level. This simple method will be useful in a wide variety of *in vivo* experiments, such as patch-clamp physiology, and optical imaging of neurons, glial cell, and blood vessels.

In vivo recordings from the brain are often perturbed by spontaneous beating of the brain. This brain motion is thought to arise from mechanical instability associated with respiratory and heartbeat pulsation (Dichter, 1973). Consistent with this notion, the intracranial pressure is known to fluctuate in cardiac and respiratory rhythms (Avezaat and van Eijndhoven, 1986).

In order to stabilize *in vivo* recording, researchers have empirically devised a number of strategies. The methods include: making use of mechanical ventilation with small tidal volumes at rapid rates (Kandel et al., 1961; Todd et al., 1981); lifting up the animal's head and suspending the animal's trunk in a hammock (Britt and Starr, 1976; Dichter, 1973); performing bilateral pneumothoraces and ventilating the animals (Britt and Starr, 1976; Dichter, 1973; Kandel et al., 1961); placing liquid agarose on the craniotomized hole to fix the brain into a closed system (Britt and Starr, 1976; Dichter, 1973); draining the cerebrospinal fluid (Britt and Rossi, 1982); acquiring images triggered on cardiac and respiratory oscillation phases (Megens et al., 2010); and developing an online actuator to compensate the position of a recording electrode relative to the brain (Fee, 2000). In addition, respiration noise can be removed by temporarily stopping an artificial respiration apparatus (Taniguchi et al., 1992), although the cardiac noise must be removed offline by subtracting the background obtained from trials without stimuli (Fukunishi and Murai, 1995; Taniguchi et al., 1992) and by using independent component analysis (Inagaki et al., 2003; Maeda et al., 2001), the Lucas–Kanade algorithm (Greenberg and Kerr, 2009), and a hidden Markov model (Chen et al., 2010; Dombeck et al., 2007).

All these methods have been proven valid to some extent, but they may be impractical in terms of their low versatility and procedural complication. In the present study, we demonstrate that the brain pulsation is virtually eliminated by performing thoracotomy in anesthetized mice.

All experiments were performed with the approval of the animal experiment ethics committee at the University of Tokyo (approval number: 19-35) and according to the University of Tokyo guidelines for the care and use of laboratory animals. Male postnatal day 20–27 ICR mice (29.4 ± 5.0 g, Japan SLC, Shizuoka, Japan) or mice expressing membrane-targeted green florescent protein (GFP) (line 21, thy1-GFP transgenic C57B6 mice), a gift from Dr. V. de Paola and Dr. P. Caroni (De Paola et al., 2003), were given free access to food and water and maintained under controlled temperature and light schedules. Mice were anesthetized by intraperitoneal urethane (1.5 g/kg) and given tracheal cannulation. The exposed skull was glued to a metal plate, through which the mice was fixed to a stereotaxic frame. The 1 mm × 1-mm skull was craniotomized

* Corresponding author at: Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Tel.: +81 3 5841 4783; fax: +81 3 5841 4786.
E-mail address: ikegaya@mol.f.u-tokyo.ac.jp (Y. Ikegaya).

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at 3.5 mm posterior, 1.5 mm lateral to the bregma, which corresponded to the primary visual cortex. After the surgical removal of the dura, the exposed cortex was mounted with 1.5% agar at a thickness of 0.5 mm and covered by a 170-μm-thick glass coverslip (Kuga et al., 2011; Takahara et al., 2011). The animals were ventilated using a volume cycled ventilator (120 breaths/min, Shinano, SN-480-type7, Tokyo, Japan). The tidal volume ranged from 12 to 20 ml/kg, but it was initially set to be 16 ml/kg, unless otherwise specified. Rectal temperature was kept at 37 °C by placing the mouse on a heated plate (Thermo plate MATS-505SF; TOKAI HIT, Shizuoka, Japan). Heart rate and blood pressure were monitored through non-invasive recordings using a BP-98AL tail-cuff device (Softron, Tokyo, Japan) (Minamisawa et al., 2011).

Fluorescein isothiocyanate (FITC)-labeled dextran (70 kDa, 0.3%, 600 ml/kg) was intravenously injected into the retro-orbital venous plexus, and the brain blood flow was angiographically imaged at 50 frames per s with a Nipkow-disk confocal unit (CSU-X1; Yokogawa Electric, Tokyo, Japan), a cooled CCD camera (iXon DU887; Andor Technology, Belfast, UK), an upright microscope (Axio Examiner D1, Carl Zeiss, Oberkochen, Germany) with a water-immersion objective (40×, 0.8 numerical aperture, Carl Zeiss), and image acquisition software (SOLIS; Andor Technology). Fluorophores were excited at 488 nm with an argon-krypton laser (10–15 mW, 641-YB-A01; Melles Griot, Carlsbad, CA, USA) and visualized using a 507-nm long-pass emission filter. After obtaining the "baseline" level of pulsation in a few microscopic fields, the mice were thoracotomized, and the same blood vessels were imaged. Region of interests (ROIs) were placed across blood vessel walls, and the fluorescence change $\Delta F/F$ was calculated as $(F_i - F_0)/F_0$, where F_i is the fluorescence intensity at time$_i$ and F_0 is the baseline value averaged across the 20-s period before and after the focused time. Data were analyzed using Image J and Matlab.

Using medical scissors, an incision was made between the costae and the sternum on the right chest from the clavicle to the diaphragm along the body axis 2–3 mm aside from the median line with a caution to avoid injury of the lungs and heart. This surgical procedure did not cause significant hemorrhage, and no specific treatment was required to the incised part.

Data are reported as the means ± standard deviations (SD).

To investigate the effect of thoracotomy on the vital conditions of the animals, we monitored the heartbeat and the blood pressure using a tail-cuff measure device. This apparatus uses an infrared sensor to measure the pulsing of the caudal artery, and the measured blood pressure may not reflect the true values, although the heart rate can be accurately measured. We thus compared the relative values of the mean blood pressure before and after thoracotomy in an arbitrary unit. The mean blood pressures under control and thoracotomized states were 47.3 ± 7.3 and 36.6 ± 2.0, respectively, and the heart rates were 509.4 ± 154.6 min^{-1} and 553.3 ± 138.5 min^{-1}, respectively (mean ± SD = 4 mice). The blood pressure and heart rate tended to be decreased and increased, respectively, after thoracotomy, but these changes were not statistically significant ($P > 0.05$ paired t-test). Thereafter, the animals maintained the blood pressure and heart rates within a 30% change in fluctuations for more than 6 h. Because thoracotomy increases the intrathoracic pressure from the negative value relative to the atmospheric pressure to nearly zero, optimizing the tidal volume may be critical in maintaining the vital conditions of the animals. To examine how the tidal volume influences the heartbeat rate and the blood pressure, we adjusted the tidal volume to 12, 16, and 20 ml/kg. The blood pressures were 38.3 ± 3.8 for 12 ml/kg, 36.5 ± 2.0 for 16 ml/kg, and 33.0 ± 0.9 for 20 ml/kg, whereas the heart rates were 548.2 ± 127.8 min^{-1} for 12 ml/kg, 553.3 ± 138.5 min^{-1} for 16 ml/kg, and 561.0 ± 132.4 min^{-1} for 20 ml/kg. Thus, the tidal volume was unlikely to affect these cardiovascular parameters, and thus, we used 16 ml/kg in the following experiments.

The hydrophilic fluorophore FITC-dextran was intravenously injected into anesthetized mice, and the brain vessel was visualized using a Nipkow-disk confocal microscope and a fast-scanning CCD camera (Fig. 1A). We observed pulse-like movement of vessels in all mice tested. The amplitude of the fluctuations ranged typically from 10 to 30 μm, which corresponded nearly to the diameters of arterioles and capillaries. The degree of the fluctuation varied from animal to animal, but not from vessel to vessel within each animal. This indicates that the movement of vessels arose from pulsation of the cortical surface around the craniotomized region or the entire brain. Consistent with this, we found that neurites and vessels moved spatially in parallel to each other in the brain in a thy1-GFP mouse, which expressed GFP sparsely in a subset of neurons (De Paola et al., 2003) (Supplemental Movie 1). Therefore, the movement of vessels serves as a good index of the pulsation of other brain components, including neurons and glial cells.

To quantify the extent of pulsation, we measured the fluorescence intensity F in ROIs at the borders between vessel walls and brain parenchyma and calculated $\Delta F/F$ as fluorescence changes from the baseline (Fig. 1B). The $\Delta F/F$ values showed periodic oscillations (Fig. 1B inset). The rhythmicity was analyzed with the fast Fourier transform (FFT) and unfolded into the power spectrum. The spectrum exhibited two prominent peaks around 2 Hz and 8 Hz (Fig. 1C). These peaks were thought to correspond to respiration and heartbeat, respectively; note that the ventilator was set at 120 breaths/min (=2 Hz) and the heart rate, as measured above, was about 500 beats/min (≈8 Hz).

We evaluated the strength of the oscillations by calculating the power integral of the peak, *i.e.*, the area under a curve (Fig. 1D). We repeated this analysis for 5 mice and found that the power integral of the heartbeat was 3.2 ± 1.9-fold larger in that of respiration ($T = 0.00$, $P < 0.01$ Wilcoxon signed-ranked test). Thus, the cardiac

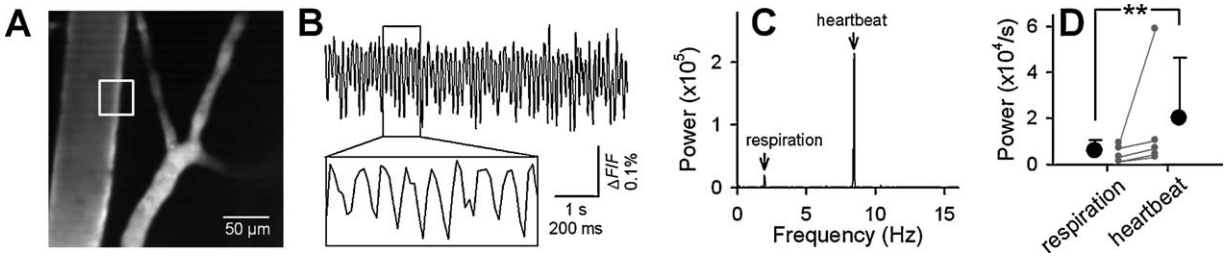

Fig. 1. Quantifying pulsation caused by respiration and heartbeat. (A) Visualization of neocortical blood vessels by intravenous injection of FITC-dextran. (B) Time course of the fluorescence intensity in the ROI in (A). The boxed region is time-expanded in the inset. (C) FFT power spectrum of the fluorescence trace in (B) shows two prominent peaks around 2 and 8 Hz, which correspond to respiration and heartbeat, respectively. (D) Comparison of the power integral (area under the curve) of two peaks. Error bars are standard deviations of 5 mice. Each gray dot indicates a single animal (78 ROIs were selected in 5 mice). $^{**}P < 0.01$, Wilcoxon signed-ranked test.