In vivo visualization of single-unit recording sites using MRI-detectable elgiloy deposit marking

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Koyano KW, Machino A, Takeda M, Matsui T, Fujimichi R, Ohashi Y, Miyashita Y. In vivo visualization of single-unit recording sites using MRI-detectable elgiloy deposit marking. J Neurophysiol 105: 1380–1392, 2011. First published December 1, 2010; doi:10.1152/jn.00358.2010.—Precise localization of single-neuron activity has elucidated functional architectures of the primate cerebral cortex, related to vertically stacked layers and horizontally aligned columns. The traditional “gold standard” method for localizing recorded neuron is histological examination of electrolytic lesion marks at recording sites. Although this method can localize recorded neurons with fine neuroanatomy, the necessity for postmortem analysis prohibits its use in long-term chronic experiments. To localize recorded single-neuron positions in vivo, we introduced MRI-detectable elgiloy deposit marks, which can be created by electrolysis of an elgiloy microelectrode tip and visualized on highly contrasted magnetic resonance (MR) images. Histological analysis validated that the deposit mark centers could be localized relative to neuroanatomy in vivo with single-voxel accuracy, at an in-plane resolution of 200 μm. To demonstrate practical applications of the technique, we recorded single-neuron activity from a monkey performing a cognitive task and localized it in vivo using deposit marks (deposition: 2 μA for 3 min; scanning: fast-spin-echo sequence with 0.15 × 0.15 × 0.8 mm³ resolution, 120/4500 ms of echo-time/repetition-time and 8 echo-train-length), as is usually performed with conventional postmortem methods using electrolytic lesion marks. Two localization procedures were demonstrated: 1) deposit marks within a microelectrode track were used to reconstruct a dozen recorded neuron positions along the track directly on MR images; 2) combination with X-ray imaging allowed estimation of hundreds of neuron positions on MR images. This new in vivo method is feasible for chronic experiments with nonhuman primates, enabling analysis of the functional architecture of the cerebral cortex underlying cognitive processes.

OVER THE PAST 50 YEARS, EXTRACELLULAR single-unit recording methods from primates have been used to map the firing patterns of neurons and have provided important insights into how the human brain processes information (Logothetis 1998; Parker and Newsome 1998; Miyashita 2004). Precise localization of recorded neuronal activity has revealed many features of the functional architecture of the cerebral cortex at different levels, including cortical layers (Hubel and Wiesel 1968), columnar organization (Mountcastle 1957; Hubel and Wiesel 1962; Merzenich and Brugge 1973), and interactions between adjacent cortical areas (Zeki 1978; Naya et al. 2001). The currently accepted “gold standard” method for identifying the location of recorded neurophysiological responses is the use of electrolytic microlesions (Hubel and Wiesel 1962), which can be placed at electrode tip positions by passing an electrical current. The lesions can then be detected in postmortem histological sections, allowing reconstruction of recording site positions along a penetration track by interpolating between the lesions. This procedure provides definite locations of recording sites within cortical structures and is sufficient for experiments using acute preparations. However, in chronic recordings, postmortem measurement is often inadequate because the locations remain unknown until all the in vivo experiments are completed. This uncertainty of recording sites is especially crucial for the long-term chronic experiments using behaving monkeys, which often last for several months or years. In addition, the number of detectable recording sites within a local region is severely limited because lesions become undetectable several weeks after the placement, and closely spaced lesions from adjacent tracks are difficult to identify. This limitation in the number of detectable recording sites of the electrolytic lesion method can be improved to some degree by using other marking techniques, such as marking with metal deposition (Hess 1932; Adrian and Moruzzi 1939; Marshall 1940; Green 1958; Brown and Tasaki 1961; Suzuki and Azuma 1987), injecting dyes (Thomas and Wilson 1965; Stretton and Kravitz 1968; Lee et al. 1969) or carbon fiber (Sawaguchi et al. 1986), coating the electrode with fluorescent dyes (Honig and Hume 1989; Snodderly and Gur 1995; DiCarlo et al. 1996; Naselaris et al. 2005), detecting gliosis immunocytochemically (Benevento and McCleary 1992), and the juxtacellular labeling of single cells (Pinault 1996). However, all of these methods still require postmortem analysis and are thus unable to detect recording sites until all the in vivo experiments have been finished.

To overcome the difficulties of postmortem methods in chronic experiments, several alternative techniques have been proposed to localize recording sites in vivo. The easiest and most widely used method is the predictive estimation of the location using stereotaxic coordinates (Horsley and Clarke 1908; Saunders et al. 1990; Asahi et al. 2003). However, the accuracy of this estimation is severely limited, because the method does not consider trajectory variation across each recording penetration, which is especially critical for deep brain areas (for detailed discussion, see Cox et al. 2008). Sonography (Collier et al. 1980; Tokuno et al. 2000; Glimcher et al. 2001) and X-ray imaging (Aggleton and Passingham 1981; Nahm et al. 1994; Cox et al. 2008) are low-cost, convenient, and noninvasive
imaging methods to visualize inserted electrodes in each recording session. However, due to their low tissue contrast for neuroanatomy, these imaging methods need to be complemented with anatomical information from another resource (Nahm et al. 1994; Cox et al. 2008), and thus can only indirectly locate recording sites relative to fine brain structures.

MRI is a promising imaging method for localizing recording sites within the brain, because of its high tissue contrast and high spatial resolution (Fahlbusch and Samii 2007). Previous studies have shown that MRI can visualize inserted microelectrodes within the brain (Jog et al. 2002; Martínez Santiesteban et al. 2006; Tammer et al. 2006; Matsui et al. 2007) and localize them at an accuracy of single-voxel size (50 μm in vitro and 150 μm in vivo; Matsui et al. 2007). These MRI-based approaches perform better than other imaging modalities for directly localizing inserted microelectrodes in relation to fine brain anatomy (Nakahara et al. 2007). However, because MRI requires expensive hardware, it is often difficult to use in a laboratory setting, and everyday use is not a practical choice for many laboratories.

A previous study reported an alternative MRI-based approach that could solve this problem (Fung et al. 1998). Fung et al. found that gradient-echo MRI could detect iron deposits placed within the rat brain by passing small electrolytic currents (5–30 μC) through a stainless steel electrode. Because a large number of deposit marks can be detected simultaneously in a single MRI session, this approach does not require frequent MRI usage, so it is feasible for many laboratories that have limited access to an MRI scanner. However, at the same time, this approach has a potential drawback in practical recording experiments, as pointed out by Fung et al. themselves: stainless steel electrodes are generally thought to deliver noisier electrophysiological recordings than other common electrode materials, such as tungsten, platinum-iridium, or elgiloy, and are generally avoided in vivo recording experiments (for example, see Snodderly and Gur 1995; Geddes and Roeder 2003). Actually, to the best of our knowledge, stainless steel deposits have never been used in vivo to localize recording sites of stainless steel microelectrode directly (but see Pezaris et al. 2000; Pezaris and Reid 2009 for indirect estimation of tungsten/gold-plate tetrode recording sites in separate recording sessions). The recording characteristics of stainless steel microelectrodes should be improved significantly, so as to establish the usefulness of this MRI-based approach, and to promote its widespread adoption in chronic recording experiments.

Cobalt-nickel-iron alloy (elgiloy) is another candidate electrode material for use in MRI-visible metal deposit marking methods. Elgiloy can create iron-containing metal deposits similar to those produced by stainless steel, but possesses superior recording characteristics (Suzuki and Azuma 1976, 1987; Ashford et al. 1985). Indeed, this material has been used in hundreds of primate electrophysiological studies (for example, Suzuki and Azuma 1976; Sugita 1999; Kakei et al. 2001; Ohbayashi et al. 2003; Kamigaki et al. 2009, 2011; Yamagata et al. 2009). In the current study, we extend the approach of Fung et al. by using an elgiloy microelectrode and showed that elgiloy metal deposits can be localized accurately with MRI. Furthermore, we demonstrated two practical applications of this elgiloy deposit mark method to record and localize the activity of single neurons in vivo from a monkey performing a cognitive task: direct reconstruction of recorded neuron positions along the microelectrode track on a magnetic resonance (MR) image, and estimation of hundreds of neuronal positions in combination with X-ray imaging.

METHODS

Animals. We used three macaque monkeys (two Macaca mulatta and one Macaca fuscata, weighing 4.3–9.0 kg) in this experiment. An MRI-compatible head holder and a recording chamber (Crist Instruments, Hagerstown, MD) were attached to the skull under aseptic conditions and general anesthesia with pentobarbital sodium (4 mg·kg⁻¹·h⁻¹ iv) and xylazine (2 mg·kg⁻¹·h⁻¹ iv), supplemented as needed. Monkeys were given postsurgical analgesics (acetaminophen, 20 mg·kg⁻¹·day⁻¹ or pranoprofen, 3 mg·kg⁻¹·day⁻¹, per os) for at least 3 days and postsurgical prophylactic antibiotics (benzylpenicillin, 20,000 U·kg⁻¹·day⁻¹, ampicillin, 100 mg·kg⁻¹·day⁻¹, intramuscular injection or enrofloxacin, 5 mg·kg⁻¹·day⁻¹ sc) for 1 wk. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Review Committee of The University of Tokyo School of Medicine.

Metal deposition and MRI. We used a glass-coated iron-cobalt-nickel alloy (elgiloy) microelectrode (0.3–0.8 MΩ, Suzuki and Azuma 1976; Ashford et al. 1985) for the electrophysiological recordings and metal deposition. Using a hydraulic microdrive manipulator (MO-95; Narishige, Tokyo, Japan), the electrode was inserted into the brain along a stainless steel guide tube, and metal deposits were placed at the electrode tip position by passing an anodic direct current (2–10 mA for 3–30 min) through the microelectrode (Fig. 1A) (Suzuki and Azuma 1987). The current parameters used for each deposition are shown in Table S1 (Supplemental Material for this article is available online at the Journal website). The number of marks placed in each animal and the number recovered are summarized in Table S2.

To detect the metal deposits, we used a 4.7 T MRI scanner (BioSpec 47/40; Bruker BioSpin, Ettlingen, Germany) and actively decoupled a surface receive radiofrequency coil of 50-mm diameter with a volume radiofrequency coil for transmission (Bruker BioSpin). Monkeys were kept under anesthesia during the MRI scan with propofol (5–10 mg·kg⁻¹·h⁻¹ iv), supplemented as needed with xylazine (0.5–2 mg/kg im). Blood pressure, heart rate, and oxygen saturation were continuously monitored, body temperature was kept constant using hot-water bags, and glucose-lactated Ringer solution was given intravenously at a rate of 5–10 ml·kg⁻¹·h⁻¹. A fast low-angle shot gradient-echo (FLASH) and fast spin-echo (FSE) were performed to visualize the metal deposits (see Table S1 for detailed scan parameters). After the MRI, the monkeys were returned to their home cage, and their general state was monitored, by their body temperature kept, until they recovered from anesthesia.

Histology. After each series of experimental sessions, two of the three monkeys were euthanized, and their brains were examined histologically by conventional methods (e.g., Koyano et al. 2005). We could not perform a histological examination on the other monkey, who was kept alive and continues to be used in further experiments. The two monkeys were deeply anesthetized with pentobarbital sodium (60 mg/kg iv) and perfused intracardially with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer. After removal of the skull, brains were postfixed for 48 h in 4% paraformaldehyde at 4°C and were cryoprotected in 30% sucrose in PBS at 4°C until they sank. Brains were cut into 40-μm cryostat sections along the same plane of the previously acquired MR images. Sections were collected for two series and mounted onto slides. One series of sections was stained with cresyl violet for Nissl to show cytoarchitecture. The other series of sections was stained using Prussian blue reaction to detect iron-containing metal deposits as azure spots (Brown and Tasaki 1961; Suzuki and Azuma 1976; Fung et al. 1998). The sections were treated with 2.5% ferricyanide/2.5% ferrocyanide for 10 min, 2.5% ferricya-
The size of a deposit on the MR image was calculated as an average of diameters measured along the major and minor axes of the ellipse-shaped hypointense spot. Because the metal deposit marks were not simply ellipse-shaped in the histological sections, we measured the area instead of the diameter, and calculated the “effective” diameter according to the following formula: \( D_e = 2A^{1/2}/\pi^{1/2} \), where \( D_e \) is the effective diameter and \( A \) is the area of a metal deposit mark. We distinguished blood vessels (which were often visualized as small hypointense spots on MR images) from deposit marks using the following procedures: 1) We acquired a series of multi-slice MR images before the metal deposition to compare with MR images after deposition. 2) In each MR scanning session, we initially acquired low-resolution multi-slice MR images, in which blood vessels were typically visualized as continuous small holes across several MR slices and/or visualized as short winding lines. We examined the coronal and sagittal multi-slice images to determine whether the hypointense spots observed in MR images were blood vessels or not.

We compared the positions of the metal deposits on spin-echo MR images with those on histological sections. The distance between the deposit center and the nearest pial surface was measured, and localization accuracy was calculated by subtracting this distance on the MR image from that on the corresponding histological sections. For the measurement of the distance from the pial surface, we chose the marks that were located within the gray matter of the cerebral cortex to minimize errors arising from global tissue distortion.

**Behavioral task and electrophysiology.** We recorded extracellular discharge of single neurons from one task-trained monkey while performing a visual-visual pair-association task (Sakai and Miyashita 1991; Higuchi and Miyashita 1996; Naya et al. 2001; Takeda et al. 2005). Twenty-four monochrome Fourier descriptors extending \( 5^\circ \times 5^\circ \) were paired arbitrarily into 12 pairs and used as visual stimuli. In each trial, one cue stimulus then two choice stimuli (the paired-associate of the cue stimulus and a distractor) were presented sequentially with a delay of 2.0 s. The monkey was rewarded with fruit juice for touching the correct target (the paired associate of the cue).

Glass-insulated tungsten microelectrodes (0.4–1 MΩ) and elgiloy microelectrodes (0.3–0.8 MΩ) were used for the extracellular recording. The elgiloy electrodes were used for recording tracks where the metal deposits were marked. Of 62 penetrations, 8 penetrations were performed with elgiloy electodes, whereas the other 54 tracks were performed with tungsten electrodes. The microelectrode was inserted vertically into the target region through the intact dura mater along a stainless steel guide tube using a hydraulic microdrive manipulator (MO-95, Narishige). The extracellular action potentials were amplified, band-pass filtered (50–10 kHz), and isolated online with a dual-window discriminator (EN-611F; Nihon Kohden, Tokyo, Japan). Spike waveforms were digitized at 20 kHz by data acquisition board (PCI-6220; National Instruments, Austin, TX) and stored to a hard disk. The spike waveforms were then low-pass filtered offline at 5 kHz, and the quality of the isolations was checked. The signal-to-noise ratio of the action potentials was calculated by dividing the mean maximum amplitude by the standard deviation of the baseline. We recorded from the first well-isolated neuron encountered while searching for the next neuron along each penetration of the microelectrode.

**X-ray imaging.** The location of the electrode track for each recording session was measured on an X-ray image (Aggleton and Passingham 1981; Naya et al. 2001). When the microelectrode reached a certain depth near the target region, we acquired a pair of orthogonal X-ray images at right angles to the sagittal and coronal planes of the monkey’s head using a portable X-ray unit (PX-30N, 2.0-mm focal spot; Acoma X-ray Industry, Tokyo, Japan) and X-ray films (RX-U; Fujifilm, Tokyo, Japan) with intensifying screens (MS-V; Kyokko, Kanagawa, Japan). The height, distance, and orientation of the X-ray source relative to the animal’s head and the film were kept constant across X-ray imaging sessions of different days. This positioning was confirmed each time by measuring distances between the X-ray source...
and the primate chair, and by overlapping backlight-projected positions of a pair of crosshair-shaped fiducial marks on both the closer and further side of the primate chair. The films were exposed with the portable X-ray unit set at 80 kV at 15 mA for 1.6 s. The locations of the microelectrodes were then measured along the orbitomeatal plane on the films and corrected for the magnification factor (coronal image: 102.0%; sagittal image: 104.8%). The anteroposterior position of the inserted microelectrode was then measured on the sagittal image as the distance from the external auditory meatus, and the mediolateral position was measured on the coronal image as the distance from the midline. Since the microelectrode tip was too small to detect from X-ray images, the dorsoventral location of each recording site and metal deposition along the microelectrode penetration direction was estimated from microdrive readings. In addition, at the end of most penetration tracks, the bottom of the cortex was determined from a characteristic “crunching” noise in the local field potential to confirm the dorsoventral location. The localization error of the film-based measurements was evaluated across days from the variance in the measurement of the distance between the external auditory meatus and the posterior tip of the sphenoid bone on the film (Aggleton and Passingham 1981; Yoshiida et al. 2003; 24.50 ± 0.20 mm, means ± SD, n = 62). Thus, the error in the measurement was estimated to be comparable to a standard deviation of 0.2 mm.

MRI-based in vivo track reconstruction. For the recording tracks on which deposit marks were placed, the position of each neuron in MR images was estimated from those of the deposit marks on the same track and the distances driven by the microdrive manipulator in that recording session. The neuronal positions were reconstructed in vivo directly on the MR images by interpolating between the within-track deposit marks, the position of each neuron in MR images and the posterior tip of the sphenoid bone on the film (Aggleton and Passingham 1981; Yoshida et al. 2003; 24.50 ± 0.20 mm, means ± SD, n = 62). Thus, the error in the measurement was estimated to be comparable to a standard deviation of 0.2 mm.

RESULTS

Detection of metal deposits after elgiloy microelectrode electrolysis. We first examined whether the metal deposits of the elgiloy microelectrode were visible on the MR images (Fig. 1). Direct anodic currents of 2–5 μA for 5 min passed through elgiloy microelectrode-yielded hypointense spots on the MR images of 150 × 150 μm² in-plane resolution (Fig. 1, B and D; see Table S1 for detailed scan parameters). A corresponding histochemical section showed iron deposit marks as azure spots by means of Prussian blue reaction (Fig. 1, C and E). The locations of these iron deposit marks were well matched to those of the hypointense spots in the MR images. These results demonstrated that the metal deposit marks created by electrolysis of the elgiloy microelectrode could be detected as hypointense spots on the MR images.

Effects of marking and imaging parameters on metal deposit size. The amount of the metal deposition depends on the total charge used for the electrolysis (Suzuki and Azuma 1987). So we examined the relationships between the passed currents and the appearance of the deposit, by creating metal deposit marks using a range of different charges (Fig. 2). The results showed that passing a current as low as 2 μA for 3 min created metal deposit marks that were visible in both spin-echo (FSE; Fig. 2E) and gradient-echo (FLASH; Fig. 2F) MR images of 176 × 176 μm² resolution (see Table S1 for detailed scan parameters). We found that larger charges tended to produce the larger marks (Fig. 2, B–D, G–J), and the size of the metal deposits on the MR images correlated well with the total charge that was used to create the deposits (for spin-echo sequence, R = 0.927, P < 0.0005, Fig. 2O; for gradient-echo sequence, R = 0.763, P < 0.02, Fig. 2P; n = 10 for each sequence). There was also a weaker correlation between the size of the metal deposits on the histological section and the total charge (R = 0.654, P < 0.05, Fig. 2Q; see also Fig. S1). The electrolytic current for the deposition appeared to cause some damage, exhibited as gliosis around the marks (Fig. S1). The diameter of the gliosis, resulting from the metal deposition procedure performed within 2 wk before death, was also correlated with the total charge (R = 0.634, P < 0.001). In cases where the total charge used for the deposition was smaller than 500 μC, the mean diameter of the gliosis was only 233 ± 65.5 μm. The close relationships between the mark sizes on the MR images and total charges can be useful for creating metal deposit marks of a particular size, depending on the resolution of MR images to be acquired. The size of the metal deposits was larger in the gradient-echo than spin-echo sequence (paired t-test, P < 0.01), and the sizes in the images of both MRI sequences were larger than in the histological section (paired t-test, P < 0.002). These differences in size are consistent with the well-known phenomena whereby ferromagnetic metals appear larger on MR images than their actual size, due to susceptibility artifacts (Luedeke et al. 1985), and gradient-echo sequences are more...
sensitive to susceptibility artifacts than spin-echo sequences (Posse and Aue 1990).

The susceptibility artifact not only depends on scan sequences, but also on several other scan parameters (Luedke et al. 1985; Ericsson et al. 1988; Posse and Aue 1990). Therefore, we next examined the effects of those scan parameters, echo time (TE), repetition time (TR), bandwidth, frequency encoding direction, echo train length (spin-echo only), and flip angle (gradient-echo only), with metal deposit marks created by two different charges (Fig. 3; n = 8 for each condition). Two-way ANOVAs revealed significant main effects of charge in all conditions (P < 0.0001), but no significant interactions between charge and scan parameters in all conditions (P > 0.2). In the spin-echo sequence, bandwidth (F3,56 = 8.47, P < 0.01), frequency encoding direction (F3,28 = 14.05, P < 0.001), and echo train length (F3,42 = 5.49, P < 0.01) showed significant main effects. The mark sizes tended to be smaller at larger bandwidths (post hoc Tukey test, P < 0.01; Fig. 3C) and larger echo train lengths (post hoc Tukey test, P < 0.05; Fig. 3E), and the sizes tended to be smaller and appeared thinner when the frequency encoding direction was set parallel to the penetration track compared with when it was set perpendicular to the track (Fig. 3D). In the gradient-echo sequence, TE showed a significant main effect of scan parameter (F3,56 = 11.14, P < 0.05).

The mark sizes tended to be larger at longer TE (post hoc Tukey test, P < 0.05; Fig. 3F).

Stable detection of the metal deposits over a year. In contrast to short-lived electrolytic lesions, the metal deposits produced by the elgiloy microelectrode remained detectable on histological sections even after a survival period of more than 6 mo (Suzuki and Azuma 1987). We examined the long-term visibility of the metal deposit marks on the MR images and found that the metal deposits remained visible after 18 mo of survival (Fig. 4, see Table S1 for detailed scan parameters). Even the smallest deposits (150 μm in diameter; numbered as 3 in Fig. 4A), which were marked by currents of 2 μA for 5 min, were still visible at 18 mo after the deposition. The long-lasting nature of these metal deposits enables this method to be used in the chronic experiments, which are often performed over several months or even a year.

Accurate localization of metal deposits. The locations of the deposit marks on MR images corresponded well to those on histological sections (Fig. 5, A–C). A deposit mark, located at the layer I/II border near the pial surface on a histological section, was found at almost the same location near the pial surface on the corresponding MR image (Fig. 5, A–C). The location of another deposit mark, farther from the pial surface, also matched well between MRI and histology (Fig. 5, A–C).
We then evaluated the accuracy of the mark positions across 26 data points, whose MR images were acquired at an in-plane resolution of 200 × 200 μm². To quantify the mark positions, we measured and compared the distances from the pial surface between histological sections and the corresponding MR images. This distance on MR images corresponded well with those on the histological sections, and a linear regression line between them fitted well and had a slope of 0.95 (R² = 0.92, P < 0.0001; Fig. 5D). The median difference of this distance along orthogonal to the penetration direction (Fig. S2). The median difference of this distance between the MR images and histological sections was 100.9 ± 7 μm, corresponding to 0.50 voxels (Fig. 5E), and this was significantly smaller than the single voxel size (Wilcoxon signed-rank test, P < 0.0001). We also analyzed relative positioning errors both along and orthogonal to the penetration direction (Fig. S2). The median

Fig. 3. Effects of scan parameters on the metal deposit diameters in the FSE (A–E) and FLASH (F–J) sequences (n = 8 for each point). Metal deposits were marked at 2 μA for 5 min (600 μC, bottom of MR images and blue lines in line plots) or 5 μA for 5 min (1,500 μC, top of MR images and red lines in line plots). ⊥ and ∥ in D and I: perpendicular and parallel frequency encoding direction in relation to the microelectrode penetration direction, respectively. *, **, and †: significant difference of the diameter between scan conditions (P < 0.05 with Tukey post hoc test, P < 0.01 with Tukey post hoc test, and P < 0.001 with 2-way ANOVA, respectively). Scale bar, 1 mm. Error bars, SD.

Fig. 4. Detection of metal deposits (arrows) over a year. A–C: MRI with FSE sequence. D–F: MRI with FLASH sequence. G: postmortem detection with Prussian blue reaction. MRI was performed at 1 (A and D), 7 (B and E), and 18 (C and F) mo after creation of the metal deposits. Numbers in left panel of A correspond to those in right panels. The metal deposits are found even 18 mo after the marking. Current parameters: 2 μA for 7 min (1), 4 μA for 5 min (2), 2 μA for 3 min (3), 2 μA for 10 min (4), 2 μA for 7 min (5), and 2 μA for 15 min (6). Scale bars, 5 mm (left) and 1 mm (right panels of A–F and G).
error along the penetration direction was 50.3 μm and that orthogonal to the penetration direction was 62.4 μm, both of which were significantly smaller than single voxel size (200 μm; Wilcoxon signed-rank test, P < 0.0001).

Practical demonstration of direct track reconstruction on the magnetic resonance image using within-track deposit marks. To demonstrate recording site localization in a practical chronic experimental situation, we penetrated an elgiloy microelectrode into the inferotemporal cortex of a monkey performing a visual-visual pair-association task. During the course of the penetration track, we recorded single-unit neuronal activity and then left three metal deposit marks along the track (Fig. 6A). Subsequent MRI with an in-plane resolution of $150 \times 150 \mu m^2$ detected three corresponding metal deposit marks in a straight line around the rhinal sulcus of the inferotemporal cortex (Fig. 6A). The positions of the recorded neurons were reconstructed on the MR image by aligning the deposit marks on the reconstruction track with those on the MR image (Fig. 6A).

Figure 6B shows an example of recorded spike waveforms of an isolated neuron, which was located lateral to the ventral lip of the rhinal sulcus, the area 36 (Fig. 6A). The amplitude reached ~150 μV at the trough with a signal-to-noise ratio of 9.44, demonstrating a good isolation of the single neuronal unit. Stimulus-selective visual responses were detected in this neuron when the visual stimuli were presented as a cue (1-way ANOVA across 24 stimuli, P < 0.0001). The firing rates increased rapidly when a visual stimulus was presented (t-test against 300-ms period before the cue onset, P < 0.0001; Fig. 6C), whereas another stimulus did not elicit such responses (t-test, P > 0.5; Fig. 6C). The signal-to-noise ratio across 12 units in Fig. 6A was as high as that in the example shown in Fig. 6B, 8.29 ± 2.44. This example demonstrates the usefulness of the elgiloy microelectrode, which can record spike waveforms with a high signal-to-noise ratio, isolate single-unit neuronal activity related to cognitive functions, and localize recorded neurons in vivo directly on highly contrasted brain images by deposit marking.

Alignment of X-ray-based coordinates with MRI using metal deposits as within-brain local positional references. The convenience of this method for a given researcher might depend on their access to an MRI scanner, which varies across laboratories. If it is desirable to avoid frequent use of MRI, other handy in vivo localization methods such as neurosonography (Tokuno et al. 2000; Glimcher et al. 2001) or X-ray imaging (Aggleton and Passingham 1981; Nahm et al. 1994; Cox et al. 2008) can be more practical for everyday use, although the tissue contrast of these methods is far inferior to that of MRI. To complement the anatomical information in these methods, the determined recording site positions can be aligned with MR images using common positional references located outside of the skull (Nahm et al. 1994; Cox et al. 2008). Here we demonstrated that the metal deposit marks in the current paradigm can be used as a within-brain local positional reference to align the recording positions between X-ray and MRI. We performed eight recording penetrations with elgiloy microelectrodes into the anterior inferotemporal cortices of a monkey, while performing a pair-association task, and determined the position of the penetration axes with X-ray imaging (Fig. 7A). A total of 29 metal deposit marks were left by these penetrations, and their positions were determined within highly contrasted brain structures with a subsequent MRI scan of $150 \times 150 \mu m^2$ in-plane resolution (Fig. 7A). An optimal transformation of the metal deposit mark positions in the X-ray-based coordinates was then computed to align with positions in the MRI coordinates, using a least-square estimation of a global rigid-body transformation and translations along each penetration axis (Fig. 7A, see METHODS for detailed transformation procedures). After this alignment, the positions of the metal deposit marks were matched well between X-ray and MRI (Fig. 7B, 16.4 ± 224.8-μm difference in anteroposterior direction; 12.9 ± 200.4 μm in lateromedial direction; 13.8 ± 197.4 μm in dorsoventral direction), showing the feasibility of the coordinate transformation from X-ray to MRI. We then applied this transformation to 62 penetration tracks, whose positions were measured with X-ray imaging, and aligned them to the MR images. A total of 687 neurons from those penetration tracks were reconstructed on the MR images of the inferotemporal cortex (Fig. 7C). Consistent with the anatomy revealed by the MRI, the neurons predominantly located within the inferotemporal cerebral cortices and were not positioned in white matter, sulci, or areas outside the brain.
In this study, we developed a novel MRI-detectable elgiloy deposit marking method for in vivo localization of recording sites. Similar to the currently accepted gold standard method using electrolytic lesion marks, which are detectable in postmortem histology, this MRI-based approach enabled direct localization of recorded neuronal activity in vivo within highly contrasted fine brain structures. Quantitative analysis showed that metal deposits could be localized with single-voxel accuracy at an in-plane resolution of 200 μm². We successfully demonstrated two practical applications of the deposit mark in recording experiments from a behaving monkey: reconstruction of a penetration track directly on MR images using within-track deposit marks, and transformation of X-ray-based neuronal activity positions onto MR images with reference to deposit mark positions. These in vivo applications are feasible for chronic experiments using non-human primates, providing a powerful tool for electrophysiological analysis of the functional architecture of the cerebral cortex underlying cognitive processes.

Advantages of the in vivo localization and its potential drawbacks. In vivo localization offers several advantages over conventional postmortem methods. First, noninvasive imaging provides identification of recording site locations without killing animals, which enables the method to be used repeatedly to localize a large number of recording sites from an individual animal. Second, the immediate feedback during the course of the experimental sessions allows correctional adjustment of the penetration trajectory in subsequent experiments and ensures recordings from target areas at an appropriate density. Third, the use of MRI can replace the laborious histological processing with brief MRI scanning that takes only a few hours. Fourth, localization from living organs is free from the distortion effects of postmortem histological processing, providing positions within accurate 3-D coordinates.

The metal deposition method described here does not require specialized equipment other than an MRI scanner. Standard microdrive manipulators can be used for electrophysiological recordings; elgiloy electrodes are now commercially available from a number of sources; and various types of direct-current power supply can be used for the deposition. Because the deposit marks remain detectable for over 1 year (Fig. 4), there is no need to acquire MR images immediately after deposition. In addition, MRI does not need to be performed frequently because multiple deposit marks can be detected simultaneously in a single MRI session. MR images can be acquired at a convenient day after several penetrations and marking procedures. Therefore, many physiological laboratories that have access to MRI can use the MRI-detectable metal deposits immediately at no additional cost.

The use of elgiloy microelectrodes allowed us to record single-unit neuron activity and substantially extend previous studies using stainless steel microelectrodes to create metal deposit marks (Fung et al. 1998; Pezaris and Dubowitz 1999). In contrast to stainless steel microelectrodes, which are ineffective for neuronal recordings, elgiloy microelectrodes can record single-unit activity as demonstrated in the present study (Fig. 6). The high efficacy of the elgiloy electrodes is further supported by the fact that the elgiloy electrodes have already been used in many single-unit electrophysiological studies (e.g., Suzuki and Azuma 1976; Sugita 1999; Kakei et al. 2001; Yamagata et al. 2009; Hikida et al. 2010) including several studies conducted in our laboratory (Ohbayashi et al. 2003; Fukushima et al. 2004; Kamigaki et al. 2009, 2011). Compared with other standard single-unit electrodes such as tungsten, however, we have found that the efficacy of the elgiloy electrodes can occasionally be slightly worse, although this difference in the efficacy between elgiloy and tungsten electrodes was small and difficult to quantify. This might be a potential downside of the elgiloy electrodes relative to electrodes pro-
Fig. 7. Across-track reconstruction of recording penetrations from a behaving monkey by a combination of MRI and X-ray imaging. A: computation of coordinate transformation from X-ray imaging to MRI by using metal deposits as within-brain local positional reference. The metal deposits were localized within 3-D spaces using MRI (red circles, top left) and X-ray imaging with manipulator readings (blue circles, bottom left), and then the positions of the metal deposit marks in the X-ray coordinates were transformed and aligned to those in the MRI coordinates (right). D, dorsal coordinate from the orbitomeatal plane; A, anterior coordinate form interaural line; L, lateral coordinate from the midline. B: distributions of registration errors, as measured by the difference in the metal deposit coordinates between MRI and transformed X-ray. Means ± SD are 16.4 ± 224.8 μm (AP, anteroposterior direction), 12.9 ± 200.4 μm (LM, lateromedial direction), and 13.8 ± 197.4 μm (DV, dorsoventral direction). C: reconstructed neuronal positions (green circles) on coronal MR images of the inferotemporal cortices. Coordinates of the neurons were determined with X-ray imaging and transformed to MRI. Neurons that were localized within ± 0.5-mm range from the imaging slice are superimposed on each MR image for display purposes. Although some recordings which located further from the center of the MR slice sometimes appeared outside the brain, these were actually located within the brain in other MR image slices that were centered nearer to those recording sites. C, right: schematic drawings of a brain in a lateral view and coronal plane, showing positions of the MR images (red squares). Scale bar, 2 mm (C).

duced from other materials such as tungsten, although the recording efficacy of the elgiloy electrode is high enough for single-unit studies. One of the main advantages of using elgiloy electrodes is the ability to isolate single-unit activity and create deposit marks within the same electrode track. As such, the within-track deposit marks allow the recording track to be reconstructed directly on a MR image in vivo (Fig. 6), just as electrolytic lesion marks do on the histological sections postmortem (Hubel and Wiesel 1962, 1968). This direct identification of recording positions on MR images provides definite locations relative to fine neuroanatomy, and is robust to the potential tissue distortion that occurs in the course of microelectrode advancement (Bourgeois et al. 1999; Tokuno et al. 2000).

Some potential drawbacks of the method described in this study must be considered, particularly when a large number of recording sites are concentrated in a small space. First, closely spaced marks are difficult to distinguish in a single MRI session. One solution for this problem is to perform MRI periodically. Marks made in different recording penetrations can be distinguished by creating an MRI database of the deposit marks at each time point. Second, repeated marking within a restricted region might accumulate damage due to the electrical current, which may affect physiological function. Although the damage around a single mark is likely to be negligible in most cases (~250 μm at <500 μC), this problem is inevitable to some extent. Close relationships between the mark size and total charge used for the deposition (Fig. 2, Fig. S1) may be helpful to minimize the effects of the damage. A fundamental solution for these drawbacks is to combine the technique with other imaging methods, as described in detail below.

Combination of MRI with other noninvasive methods. The combination of the current method with other noninvasive imaging methods for inserted microelectrodes, such as sonography (Collier et al. 1980; Tokuno et al. 2000; Glimcher et al. 2001) or X-ray imaging (Aggleton and Passingham 1981; Nahm et al. 1994; Cox et al. 2008), would be beneficial to compensate for the drawbacks of the deposit marking method. In the current study, we demonstrated the efficacy of combining X-ray imaging and MRI, using the metal deposit marks as a common positional reference (Fig. 7). Such a combination is one of the modes of use we propose for our method, which was able to achieve real-time estimation of the recording site repeatedly without leaving damage in the brain. Neurosonography is another imaging method that could potentially be used in combination with MRI. Although its spatial resolution and tissue contrast are lower than those of MRI, the real-time visualization of electrode penetration with some tissue images can reduce
the risk of vessel damage and prevent severe stroke (Tokuno et al. 2000; Glimcher et al. 2001).

A common problem with these combined approaches is the potential error derived from alignment processes to MR images. This error has not been quantitatively evaluated in previous studies using combined X-ray/MRI approaches for in vivo microelectrode localization (Nahm et al. 1994; Cox et al. 2008). In the current study, we measured the registration error between X-ray imaging and MRI around the target region, and showed that the error was sufficiently small (near-zero mean and standard deviations of ~200 µm) to be acceptable for most applications. One potential reason for this precise registration might be the use of the metal deposits as tissue-based positional references, which can help to map X-ray coordinates onto the tissue. To align the X-ray and MRI coordinates, previous studies used fiducial markers located outside the skull, based on the assumption that the brain and skull can be considered a single rigid body. Although this assumption may be true at a resolution in the order of millimeters, the rigidity of soft brain tissue, which floats inside the skull, should be considered carefully at a submillimeter scale. Indeed, even a simple head direction change can induce a positional change of the human brain within the skull of up to 1.7 mm due to gravitational effects (Schnaudigel et al. 2010). Because the length of the macaque monkey brain is approximately half that of the human brain, a movement of 1.7 mm of the human brain corresponds to 0.85 mm of movement in the macaque brain. Therefore, assuming that soft brain tissue and the skull constitute a single rigid body may be inappropriate when considering the localization accuracy of microelectrode recordings at resolutions in the order of a few hundred micrometers. In the current study, we used metal deposit marks as within-brain local positional references to align X-ray and MRI coordinates, under the assumption that local brain regions can be considered rigid bodies. We found that the X-ray and MRI coordinates were aligned accurately with a small registration error (~0.2 mm, as described above), suggesting that within-brain local positional references are robust against several potential error factors (such as ~0.85-mm tissue movement within the skull, although in some conditions this would be smaller) and feasible for soft and floating brain tissues.

In the present study, we used 29 metal deposits from 8 penetration tracks to calculate the registration. However, in principle, registration could be calculated from fewer metal deposits. Although a single internal fiducial is insufficient due to the degrees of freedom in the rotation angle, more than three linearly independent fiducials enable the positions to be determined in three-dimensional space.

*Application of the reconstruction procedures.* We proposed two modes of reconstruction procedure: the direct reconstruction of a penetration track onto an MR image using within-track marks (Fig. 6), and the transformation of X-ray-based coordinates on MR images using across-track marks as positional references (Fig. 7). These two procedures are complementary to each other. The former procedure has advantages in the direct and definite localization on MR images, which are robust against tissue-based errors, but possesses disadvantages in the reconstruction of a large number of recording sites from a small region of interest. In contrast, the latter procedure has advantages in the reconstruction of a large number of recording sites without causing tissue damage (Nahm et al. 1994; Cox et al. 2008), but possesses disadvantages in the potential for alignment error between X-ray-based and MRI-based coordinates. Usage of our metal deposit marks as a novel tissue-based reference frame reduces the alignment error between the X-ray-based coordinates with MR images.

The most appropriate use of these two procedures depends on the specific experimental situation, including the purpose of the research, the targeted recording areas, number of necessary recording sites, access to an MRI scanner, etc. To clearly illustrate how to use these procedures, several example situations are described below. *Example 1:* in the case of distributed recording from a relatively large area such as the primary visual cortex, a dozen tracks can be reconstructed directly on MR images by leaving one or two marks on each penetration track. By leaving spaces of a few millimeters across the tracks, one can distinguish each track and thus localize all of the dozens of tracks in a single MRI scanning session, which can be performed on a day after recording and marking at the experimenter’s convenience. This mode of use would be feasible for examining fine functional architecture within the cerebral cortex in alert and behaving primates. *Example 2:* in cases where an experimenter cannot access an MRI scanner frequently and/or in case one intends to record a large number of neurons from a small restricted region, it can be useful to combine X-ray imaging and MRI, as reported in several previous studies (Nahm et al. 1994; Cox et al. 2008). Our deposit marks can be used as internal tissue-based fiducials to transfer the X-ray based coordinates onto MR images. Deposit marks can be created during some of the recording sessions and then visualized with MRI at a later date. This approach enables in vivo localization of cortical areas in which neurons were recorded. *Example 3:* if an experimenter wants to record tens of neurons from a small restricted region without causing tissue damage and directly localize the neurons on MR images, it is possible to create marks above and/or below the target region, leaving the region itself intact. By reconstructing actual recording sites from within-track marks and microdrive readings, the location of tens of neurons can be directly mapped onto MR images. In a recently published electrophysiological paper from our laboratory, a small target region (area 35 at the fundus of the rhinal sulcus) was localized in vivo with this procedure (marking onto the amygdala just above the area 35; Fujimichi et al. 2010). The three examples above are not mutually exclusive: e.g., the approach in example 2 could be used first to map recorded neurons in a broader area, followed by the use of the approach in example 3 to examine fine functional structures at important recording areas of interest. This combination would be useful for studying functional architecture within a cluster of neurons related to specific cognitive functions, such as the “face patch,” consisting of face-selective neurons in the inferotemporal cortices (Tsao et al. 2006; Freiwald et al. 2009), and the “hot spot,” consisting of pair-coding neurons in the perirhinal cortex (Naya et al. 2001; Yoshida et al. 2003).

**Metal deposit visualization with MRI.** The deposited metal we detected as azure spots by the Prussian blue reaction is likely to be iron, since the reaction products of other metals produce different colors (Sharpe 1976). Iron and the other ferromagnetic metal materials composing elgiloy, nickel, and cobalt, could potentially cause susceptibility artifacts on MR images (Ho and Shellock 1999; Matsuura et al. 2002). Susceptibility artifacts induce geometrical distortion and signal loss...
due to intravoxel phase dispersion (the so-called T2* effect) at local regions around metal deposits (Luedeke et al. 1985; Posse and Aue 1990), generating hypointense spots as observed on the MR images in this study. The strength of the susceptibility artifacts correlates with the amount of ferromagnetic metal (Allkemper et al. 2004; Hardy et al. 2005), as reflected in the close relationship between the metal deposit appearance and the total charge used for the deposition (Fig. 2). In gradient-echo sequences, the T2* effect is larger than geometrical distortion, and thus strongly depends on TE (Fig. 3E; Ericsson et al. 1988; Posse and Aue 1990). In contrast, spin-echo sequences are less sensitive to susceptibility artifacts, because the T2* effect is reduced by the refocusing pulse (Luedeke et al. 1985; Ericsson et al. 1988; Posse and Aue 1990), resulting in a smaller metal deposit size compared with the gradient-echo sequence (Fig. 2). Because the effect of geometrical distortion is larger than the T2* effect (Posse and Aue 1990), spin-echo sequences depend on parameters related to geometrical encoding, such as bandwidth and frequency encoding direction (Fig. 3, C and D). The number of refocusing pulse repetitions in the FSE, namely the echo train length, also affected the size of the resulting deposit marks (Fig. 3E; Reimer et al. 1996). The use of spin-echo sequences would be appropriate for localizing the deposit mark positions accurately, because they are less sensitive to the gradient magnetic field inhomogeneity and can thus minimize global tissue distortion. Although the gradient-echo sequence is relatively sensitive to the magnetic field inhomogeneity, its larger signal and higher sensitivity to susceptibility artifacts might be useful when searching for metal deposits in initial exploratory scans of each MRI session.

The most appropriate scanning parameters depend on several factors in individual experiments, such as the imaging contrast required, total scanning time available, and/or the specifications of the MRI scanner. Here, we reconstructed recorded neurons on T2-weighted FSE images taken at a spatial resolution of 0.15 × 0.15 × 0.8 mm³ with 120/4,500 ms of TE/TR and 8 echo train lengths (Figs. 6 and 7). In accordance with the high (0.15 × 0.15 mm²) in-plane resolution, we created most of the metal deposits at 2 μA for 180 s, whose diameters ranged between 1 and 3 voxels on the MR images. Using the above scanning and depositing parameters, we successfully visualized metal deposits on highly contrasted fine brain structures.

We used two-dimensional MRI sequences and found that the metal deposits could be localized with single-voxel accuracy at an in-plane resolution of 200 × 200 μm². However, the slice thicknesses were larger than the in-plane resolutions in these two-dimensional imaging sequences. The accuracy along the normal direction to the imaging plane would be expected to be less than that within the plane, since the spatial resolution is worse in the former direction for the two-dimensional imaging sequences. There are two possible methods for achieving higher accuracy along the normal direction to an imaging plane: one method is to acquire MR images with a thinner slice thickness using a higher-gradient magnetic field, at the cost of a lower signal-to-noise ratio of the image; the other method is to also acquire MR images in another direction, at the cost of doubled scanning time.

**Possible future applications.** An increasing number of laboratories have recently been using functional MRI (fMRI) in nonhuman primates as a navigation tool to target microelectrode recordings. Researchers can identify multiple responsive regions at the whole brain level using fMRI, then investigate the electrical activity of neurons with a high spatio-temporal resolution using microelectrode recordings (Sawamura et al. 2006; Tsao et al. 2006; Freiwald et al. 2009). Since the metal deposit marks are directly detectable in the MR images, it is straightforward to use these marks to compare the location of electrophysiological recordings with that of observed fMRI activity.

In this study, we localized metal deposits at an in-plane resolution of 200 μm using a 4.7 T MRI system and a surface receiver radiofrequency coil. MRI technology has continuously advanced in recent decades, as the spatial resolution in recent monkey fMRI studies has been greatly improved by the use of iron oxide (Vanduffel et al. 2001; Ekstrom et al. 2008). Higher spatial resolution and image contrast of anatomical images have been enabled by recent advancements in MRI technologies, such as ultra-high magnetic fields (Logothetis et al. 2002; Pfeuffer et al. 2004; Vaughan et al. 2006), implantable surface coils (Logothetis et al. 2002; Pfeuffer et al. 2004), parallel imaging systems (Ekstrom et al. 2008; Kolster et al. 2009; Wiggins et al. 2009), cryogenic probes (Darrasse and Giniferi 2003; Baltes et al. 2009), and manganese-enhancement imaging (Silva et al. 2008). In the future, these technologies are likely to allow in vivo localization of recorded neurons at a resolution of tens of micrometers (Boretius et al. 2009; Baltes et al. 2009) with highly contrasted cortical layer structures (Fatterpekar et al. 2002; Barbier et al. 2002; Walters et al. 2007; Boretius et al. 2009).

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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