ICoRD: Iterative Correlation-Based ROI Detection Method for the Extraction of Neural Signals in Calcium Imaging

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Abstract

In vivo calcium imaging is a standard neuroimaging technique that allows the simultaneous observation of neuronal population activity. In calcium imaging, the activation signals of neurons are key information for the investigation of neural circuits. For efficient extraction of the calcium signals of neurons, selective detection of the region of interest (ROI) pixels corresponding to the active subcellular region of the target neuron is essential. However, current ROI detection methods for calcium imaging data exhibit relatively low extraction performance from neurons with a low signal-to-noise power ratio (SNR). This is problematic because a low SNR is unavoidable in many biological experimental settings. Therefore, we propose an iterative correlation-based ROI detection (ICoRD) method that robustly extracts the calcium signal of the target neuron from a calcium imaging series with severe noise. ICoRD extracts calcium signals closer to the ground truth than the conventional method from simulated calcium imaging data in all low SNR ranges. Additionally, this study confirmed that ICoRD robustly extracts activation signals against noise, even within in vivo environments. ICoRD showed reliable detection from neurons with low SNR and sparse activation, which were not detected by the conventional methods. ICoRD will facilitate our understanding of neural circuit activity by providing significantly improved ROI detection from noisy images.

Introduction

Intracellular calcium concentration remains low in the resting state and increases abruptly upon neuronal activity (Helmchen, Imoto, & Sakmann, 1996; Maravall, Mainen, Sabatini, & Svoboda, 2000). Therefore, calcium imaging is a standard neuroimaging method for measuring multi-neuronal activities. The compatibility of calcium imaging with behavioral tasks allows the assessment of neural activity during specific behavioral periods.

For precise extraction of information on activity, a selective grouping of pixels corresponding to the intracellular calcium change of a target structure, such as the cell body or dendrite, is required. The group of pixels has been defined as the region of interest (ROI). In general, the averaged signal of the pixels detected in a given ROI is used as the calcium activation signal of the target neuron. Detecting the pixels of neuronal cell bodies with a large calcium deflection is straightforward; however, depending on the experimental

conditions and size of the target structure, the signal-to-noise power ratio (SNR) of the calcium signals is often suboptimal.

A reduction in the SNR of a neural signal in calcium imaging caused by the wide field of view (FOV) of the microscope, low expression performance of the indicator, short dwell time of each neuron, small target structure, or a combination of these causes (Harris, Quiroga, Freeman, & Smith, 2016; Stringer & Pachitariu, 2019). A large FOV lowers the SNR by reducing the number of photons reached per pixel from the individual neurons. The current calcium imaging technique allows researchers to observe a wider FOV but records a narrower FOV because of the limitation of the signal extraction performance of conventional methods for low-SNR data. In general, the number of neurons used in neural signal analysis is nearly ten times less than the number of neurons that can technically be scanned using a microscope (Deneux et al., 2016). Heterogeneity in calcium indicator expression causes SNR reduction in neurons with low expression. The performance of the calcium indicator of neurons with low indicator expression reduces the activation signal itself, resulting in a lower SNR. The shorter dwell time of the microscope causes the SNR to decrease for each neuron. In addition, experimental factors such as inflammatory reactions and blurring due to the regeneration of the dura lower the SNR. Neurons with low SNRs often appear as blurry outlines in mean images of calcium imaging movies; however, the signal extraction performance of these neurons has not been extensively discussed in previous studies.

Calcium imaging technology was developed to monitor the activities of a greater number of neurons with high temporal resolution (Cardin, Crair, & Higley, 2020; Langer et al., 2013; Lillis, Eng, White, & Mertz, 2008; Sofroniew, Flickinger, King, & Svoboda, 2016). In fact, most calcium image data sets with resonance scanners are acquired at 50 Hz, whereas biological analysis is performed with reduced temporal resolution (~5 Hz) to increase the SNR (for example, a moving average filter) (Dana et al., 2016; Terada, Kobayashi, Ohkura, Nakai, & Matsuzaki, 2018). Therefore, a new ROI detection and denoising method for low-SNR images will increase the ability to monitor a greater number of neurons with temporal resolution. If the activation signal of neurons can be extracted reliably even at a low SNR, a larger number of neurons can be analyzed from calcium imaging data with a wider FOV. The ability to simultaneously analyze a larger number of neurons will help to examine more complex neural circuits.

Efforts to extract the signals of target neurons from calcium imaging data began with the principal component analysis (PCA) and independent component analysis (ICA)-based ROI detection methods (Mukamel, Nimmerjahn, & Schnitzer, 2009). The PCA/ICA technique was developed for the effective separation of signals from the overlapped area of the neurons; however, the denoising performance was not sufficient for low-SNR data. Since the development of the PCA/ICA technique, many ROI detection methods have been proposed to improve denoising capability. However, once the constrained non-negative matrix factorization (CNMF) method based on calcium dynamics modeling was proposed, this has been mainly used for calcium imaging analyses (Maruyama et al., 2014; Pnevmatikakis et al., 2016). CNMF is effective in detecting the shape of many neurons and simultaneously extracting signals from those neurons. Despite these benefits, the signal extraction performance remains insufficient for neurons with low SNRs. These methods detect morphologically clustered pixels, such as neurons, which results in the inefficiency of signal extraction from low-SNR images.

Signals from the pixels that cover intracellular organelles, where there is no calcium indicator, will reduce the SNR despite the pixels being clustered morphologically. In contrast, signals from proximal dendrites or axons may reflect the same calcium deflection as somata; thus, incorporating these pixels would enhance the SNR. Indeed, several pixels were found to cluster in the shape of a neuron containing severe noise, such as pepper salt noise. Detecting these noisy pixels as an ROI lowered the SNR of the detected pixels' average signal. Conversely, detecting pixels outside the shape of a neuron as an ROI can reduce noise when calculating the average of the ROI pixels. Detecting clustered and non-clustered pixels as a criterion for increasing the SNR can consequently improve the signal extraction performance.

In this paper, an iterative correlation-based ROI detection (ICoRD) method is proposed, which aims to maximize the SNR of target neurons by iteratively estimating the ground truth signal (true activation signal without noise). ICoRD detects correlated pixels to maximize the SNR based on the estimated activation signal. The performance of ICoRD was verified on a simulated dataset with the ground truth, and ICoRD was also applied to in vivo mouse calcium images to validate the signal extraction performance. The results of ICoRD were compared with those of CNMF. Compared to CNMF, ICoRD showed a substantially higher signal extraction performance, which was evaluated by calculating the correlation coefficient with the ground truth signal, especially when the SNR was lower than -15 dB. The lower the SNR of the raw data, the greater the difference in performance between the two methods. It is expected that neuroscientists will detect neurons with low SNR using ICoRD, resulting in research results from a larger number of neurons. The currently proposed ICoRD method will increase the number of analyzable neurons in a set of experiments and effectively enhance the temporal resolution by reducing the minimum SNR for an accurate measurement of neuronal activity.

Materials and Methods

Overview of the iterative correlation-based ROI detection method for extracting neural signals in calcium imaging

A novel iterative correlation-based ROI detection method that iteratively detects ROIs to obtain signals closer to the ground truth calcium signal is proposed (Fig. 1A). First, a reference signal was defined as the signal from a user-defined pixel from a neuron of interest. Within the observation range (red dotted box in Fig. 1A), which is a group of pixels in which the cell body of the neuron can be best visualized with space around it, pixels were selected to maximize the correlation between the average signal of the selected pixel and the reference signal. When the correlation threshold was too low (high number of pixels in the ROI in Fig. 1B), pixels with no signal were averaged together, which reduced the mean correlation. Similarly, when the number of pixels in the ROI was too low, white noise was pronounced, also resulting in a low correlation (Fig. 1B). A set of pixels over the threshold was chosen as an ROI at a given iteration to achieve the highest correlation. Then, new reference signal was set as either the average ROI signal or the constrained deconvoluted average ROI signal (Fig. 1A). This process was repeated until the reference signal was close to the ground truth signal.

Initial parameter setup

To select the first reference signal, the ICoRD algorithm began by choosing the centroid pixel of the target neuron. The average signal of nine pixels adjacent to the selected pixel was used as the initial reference signal. The observation range that best visualizes the neuron was selected by checking the movie and average pixel values over time (the mean image in Fig. 2D) and the correlation coefficient values with four adjacent pixels (correlation image in Fig. 2D). When a certain number of pixels had to be excluded for various reasons, such as overlapping with neighboring neurons, the area was selected for rejection, and the algorithm was executed without the rejected range (Fig. S1).

Iterative correlation-based ROI detection

In ICoRD, the correlation coefficients are calculated for the temporal calcium signals corresponding to the target neuron's initial reference signal and target range pixels (that is, the observation range) determined by the user. To detect the ROI that maximizes the correlation coefficient with the ground truth calcium signal, a correlation-based ROI detection method is proposed. The correlation-based ROI detection method only detects pixels with a correlation coefficient higher than the optimal threshold as an ROI. A detailed explanation of the correlation-based ROI detection method is provided in the next paragraph. The optimal threshold that maximizes the correlation coefficient between the average signal of the ROI and the estimated ground truth calcium signal was determined. To estimate the ground truth calcium signal of the target neuron in in vivo data, a constrained deconvolution (CD) method was applied to the average signal of the detected ROI using a correlation-based ROI detection method (Pnevmatikakis et al., 2016). Because this CD method has a denoising effect, its application helped in estimating the ground truth. ICoRD repeated this series of ROI detection until the reference effectively estimated the ground truth and iteratively updated the average signal or CD signal to the reference signal such that the reference approximated the ground truth signal. The criterion for selecting the reference signal was the information difference between the average calcium signal of the previous iteration and this iteration. The information difference was 1 minus the correlation coefficient between the average temporal signal of the ROI detected in the current iteration ($Rawc$) and the previous iteration ($Rawp$) (Equation 1).

$$Information\ difference\ (Rawp, Rawc) = 1 - \frac{\sum_i^n (Rawp_i - \overline{Rawp})\,(Rawc_i - \overline{Rawc})}{\sqrt{\sum_i^n (Rawp_i - \overline{Rawp})^2}\sqrt{\sum_i^n (Rawc_i - \overline{Rawc})^2}}. \quad (1)$$

A detailed algorithm flowchart of ICoRD is presented in Fig. S2. ICoRD eventually provided the CD result reconstructed from the average signal of the ROI with the maximum correlation using the estimated ground-truth calcium signal from the last iteration.

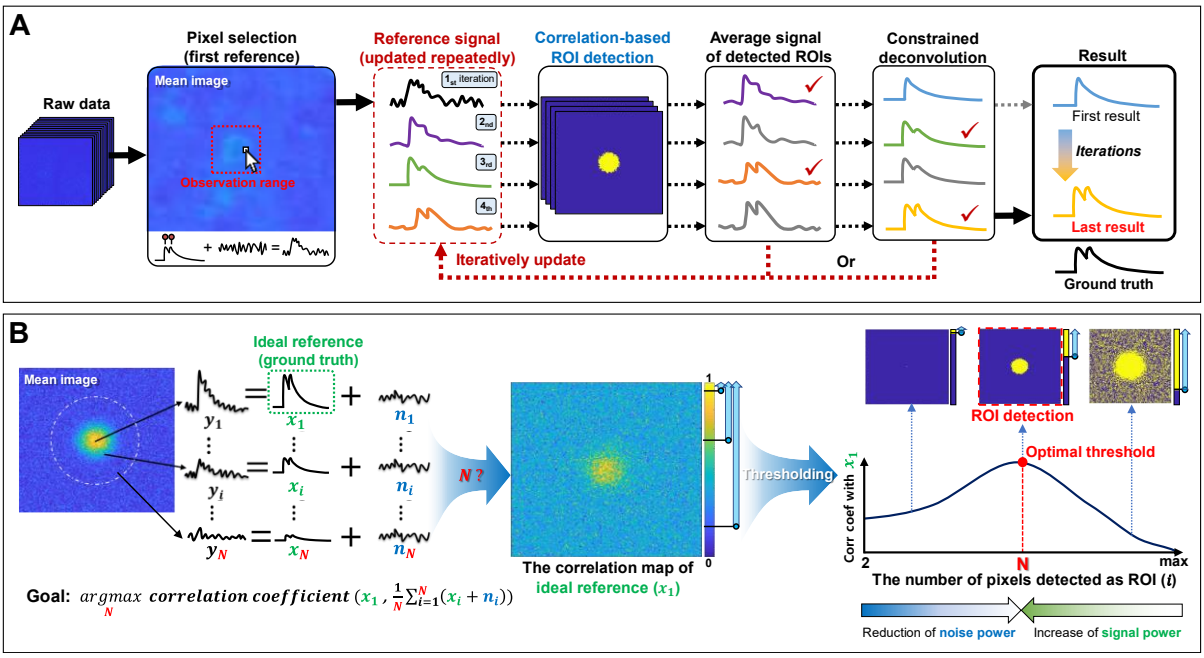

Figure 1. Schematic representation of the proposed ICoRD algorithm for neural signal extraction

A. Framework of the ICoRD algorithm for estimating accurate activation signals from the target neuron. A representative pixel and observation range were manually selected (left). ICoRD used the temporal calcium signal of the chosen representative pixel as the first reference signal for ROI detection. The reference signal was iteratively updated to approximate the ground-truth calcium signal. For ROI detection, a correlation-based ROI detection method was applied, which detected the ROI to have an average signal most similar to the reference signal. The average signal of the pixel corresponding to the ROI and the constrained deconvolution (CD) signal were computed in each iteration. The reference signal of the next iteration was selected from the average signal or CD of the previous iteration. When the iteration was completed, the CD signal of the final iteration was output as the inferred signal of the target neuron (right). **B**. Correlation-based ROI detection to find the set of pixels that maximizes the correlation coefficient between the average of the pixels and the reference signal (see text for detail). The correlation coefficient of each pixel with the reference signal was color-coded as an example image (middle). The ideal threshold was determined to maximize the correlation coefficient between the reference signal and the averaged pixels over the threshold (right).

Correlation-based ROI detection

Fig. 1B shows the correlation-based ROI detection method theorized in Fig. 1A. An ROI detection method is proposed, which effectively extracts the calcium signal of a target neuron even in extremely noisy environments. The ROI detection technique proposed in this study aims to detect the neuron signal closer to the ground-truth calcium signal. ICoRD was assumed to acquires a reference signal close to the ground truth upon iteration, and an ROI detection method that maximizes the correlation coefficient with the reference signal was first

derived.

In calcium imaging, the denoising techniques for individual neurons are evaluated based on the correlation coefficient of the denoised result and the ground truth calcium activation signal. However, most existing calcium imaging analysis methods extract neural signals through post-processing after selecting an ROI based on the neuron shape. The proposed correlation-based ROI detection method detects the ROI in which the correlation coefficient between the reference signal and the average signal of the ROI is the maximum. In a noise-free environment, the ROI detection method that maximizes the correlation coefficient is used to detect a single pixel as an ROI that maximizes the correlation coefficient between the ground-truth signal and the average signal of the detected ROI. However, in an environment where noise exists, the ROI detection method that maximizes the correlation with the ground-truth signal should consider the noise reduction effect by averaging the signals of the pixels detected as the ROI. Therefore, only pixels with a correlation coefficient above a certain threshold, at which the correlation coefficient between the average signal of the ROI and the ground-truth signal was maximized, were detected. The correlation coefficient trend between the ground-truth signal and the average signal of the detected ROI according to the thresholds had a concave shape, as shown in the right figure of Fig. 1B. The concave shape represents the trade-off relationship between the reduction in noise power due to the averaging effect and the increase in signal power owing to the detection of a highly correlated signal. Fig. 1B shows the optimal number of pixels (N), which determines the ROI that has the maximum correlation coefficient with the ideal reference signal (x_1) of the target neuron. Here, the temporal signal (y) of each pixel (i) is composed of a signal (x) and noise (n). The pixel order (i) is sorted in descending order based on the Pearson correlation coefficient of each pixel's signal (y_i) and ground-truth signal (x_1). The correlation-based ROI detection method maximizes the correlation coefficient between the average signal of the ROI and the ground-truth calcium signal by detecting pixels that have a correlation coefficient above the optimal threshold.

Results

Performance of the iterative ROI detection algorithm in simulated calcium imaging datasets

Having established a new ROI detection algorithm, its performance at various noise levels was investigated. For a fair measurement of the event detection accuracy of ICoRD, a ground-truth signal and various noise levels were required. Therefore, a realistic model that simulated calcium imaging data with a designated noise level was built. The simulated calcium imaging data were generated by modeling and multiplying the spatial and temporal components of the calcium dynamics. The spatial component was modeled as a Gaussian shape of light propagation owing to the calcium indicator at the central location (75,75) in 150 horizontal and 150 vertical spaces (Fig. 2A). The temporal component for single activation was modeled as a 10-Hz sampling rate based on the rising tau ($\tau_r = 550 \pm 52$ ms) and decaying tau ($\tau_d = 179 \pm 23$ ms) values of pGP-CMV-GCaMP6s (GCaMP6s) (Fig. 2B, Equation 2) (Chen et al., 2013).

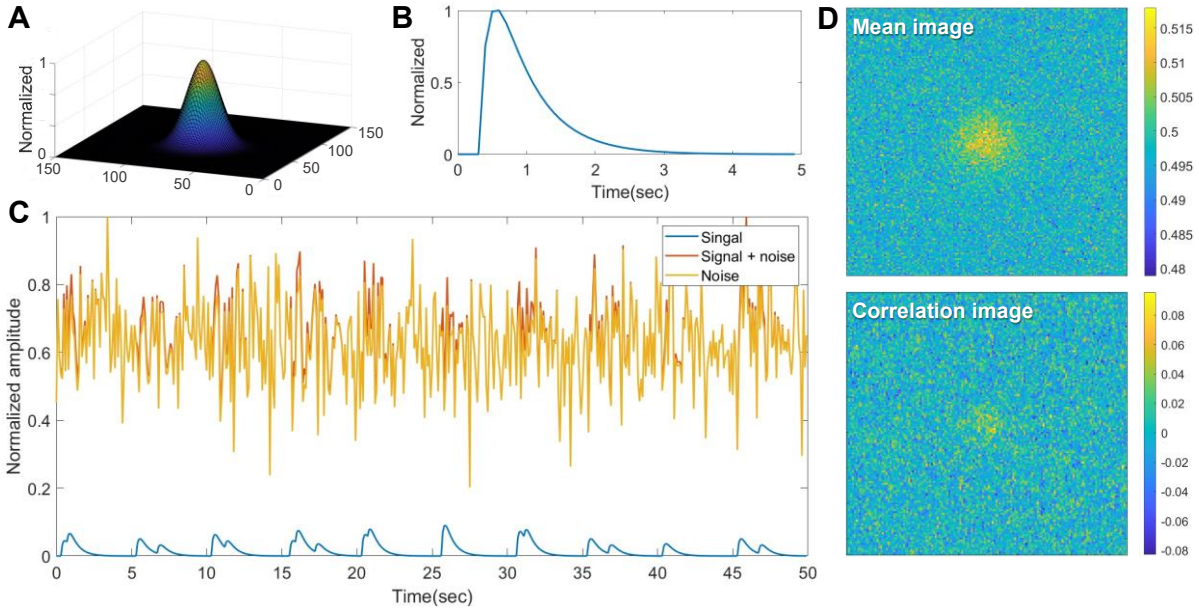

Figure 2. Simulated calcium dynamics at a severe noise level

A. The spatial component of the calcium dynamics model. **B.** The temporal component of the calcium dynamics model derived from the kinetics of GCaMP6s. **C.** An example of the simulated calcium trace of the center pixel with an SNR of -30 dB (signal, raw, noise). **D.** An example of the mean and correlation images of the simulation data with an SNR of -30 dB.

$$h(t) = \begin{cases} e^{-t/\tau_d} - e^{-t/\tau_r}, & t > 0 \\ 0, & t \leq 0 \end{cases}. \tag{2}$$

The resulting signal and noise of the central pixel's simulated calcium trace from the neuron with an SNR of -30 dB is illustrated in Fig. 2C. The ratio of neuron signal power and white Gaussian noise power was calculated as the SNR to generate a simulated calcium movie with various SNRs (Equation 3).

$$SNR_{dB} = 10\log_{10}\left(\frac{P_S}{P_N}\right), \tag{3}$$

Where P_S denotes the signal power, and P_N is noise power. The modeled temporal dynamics were generated as simulation data for 50 s based on a 10-Hz sampling rate by convoluting the calcium events at various intervals. If selective ROI detection using ICoRD increases the sensitivity of the event detection, the events could be resolved in severely noisy images, and closely interleaved events could be resolved better than the conventional CNMF method. To build a testing platform, two or more events occurring within 5 s with various intervals and noise levels between -30 and 0 dB SNRs were modeled. Fig. 2D shows the mean and correlation images of the simulated calcium movie with an SNR of -30 dB. The mean image for each pixel represents the average value of the time-axis signal. The correlation image for each pixel was computed by averaging the correlation coefficient of each pixel with its four immediate pixels. In general, the mean image provides an estimate of the location and shape of the neuron because the average pixel value of the neuron's signal tends

to be higher than the background signal. In the correlation image, the higher the activation frequency of neurons or the stronger the signal intensity versus noise, the clearer the appearance of the neuron.

The performance of the proposed ICoRD algorithm was examined in comparison with CNMF using the above-mentioned simulated calcium imaging data. A comparison was conducted between the extracted traces of the ROIs set by the two methods from the simulated calcium images with an SNR of -30 to 0 dB (Fig. 3). Using the calcium images with severe noise (-30 dB), CNMF automatically determined and rejected all the pixels in the image with an SNR of -30 dB as noise. Conversely, ICoRD detected the Gaussian signal closely, although a substantial number of background pixels were included within the ROIs (Fig. 3A). Furthermore, when the selected pixels were restored using CNMF, the extracted signal from the ICoRD-selected ROI correlated significantly better with the ground truth than the restored ROI from CNMF (Fig. 3B, correlation coefficient with the ground truth from CNMF and ICoRD was 0.8267 and 0.9834, respectively).

Subsequently, performance of ICoRD at various noise levels was tested. At all noise levels, signals extracted from ROIs detected by ICoRD were closer to the ground truth than those detected by CNMF (Fig. 3C-D), demonstrating that ICoRD-based ROI detection is more robust and suitable for noisy signals, as in many in vivo calcium images. As the neuron's SNR decreased, ICoRD lowered the noise level by detecting not only the pixel corresponding to the neuron but also the surrounding pixels to maximize the correlation coefficient with the reference signal. In the case of CNMF, all ROIs in the data with SNRs of -30 dB and -25 dB were automatically determined as noise and rejected; therefore, the rejected ROIs were restored once again. CNMF detected a smaller number of pixels as the SNR decreased.

Fig. 3D shows the correlation coefficient of the ground-truth signal and the temporal results detected by the two methods from 20 datasets (140 total) with white Gaussian noise added to each SNR (-30 to 0 dB). At all noise levels, the temporal result of ICoRD had a higher correlation coefficient with the ground-truth signal than the temporal result of CNMF. In Fig. 3D, the difference in performance between the two methods is particularly large in the SNR range of -30 dB to -20 dB. Because the proposed method is a correlation-based ROI detection method that aims at true calcium signal extraction, this shows excellent performance at severe noise levels (SNR of -30 to -20 dB).

Parameters that change during iteration in ICoRD

Fig. 4 shows the trend of the estimated correlation coefficient and ground truth correlation coefficient, and the information difference during the iteration of the proposed method in simulated calcium imaging data with an SNR of -30 dB. The estimated correlation coefficient indicates the correlation coefficient between the CD result of the last iteration and the CD results of all iterations. The ground-truth correlation coefficient is the correlation coefficient between the ground-truth signal and the CD results of all iterations. During an iteration, the CD-based update of the reference has a pink background, and the average (Avg)-based update has a white background. A detailed description of the update algorithm process is presented in Fig. S2.

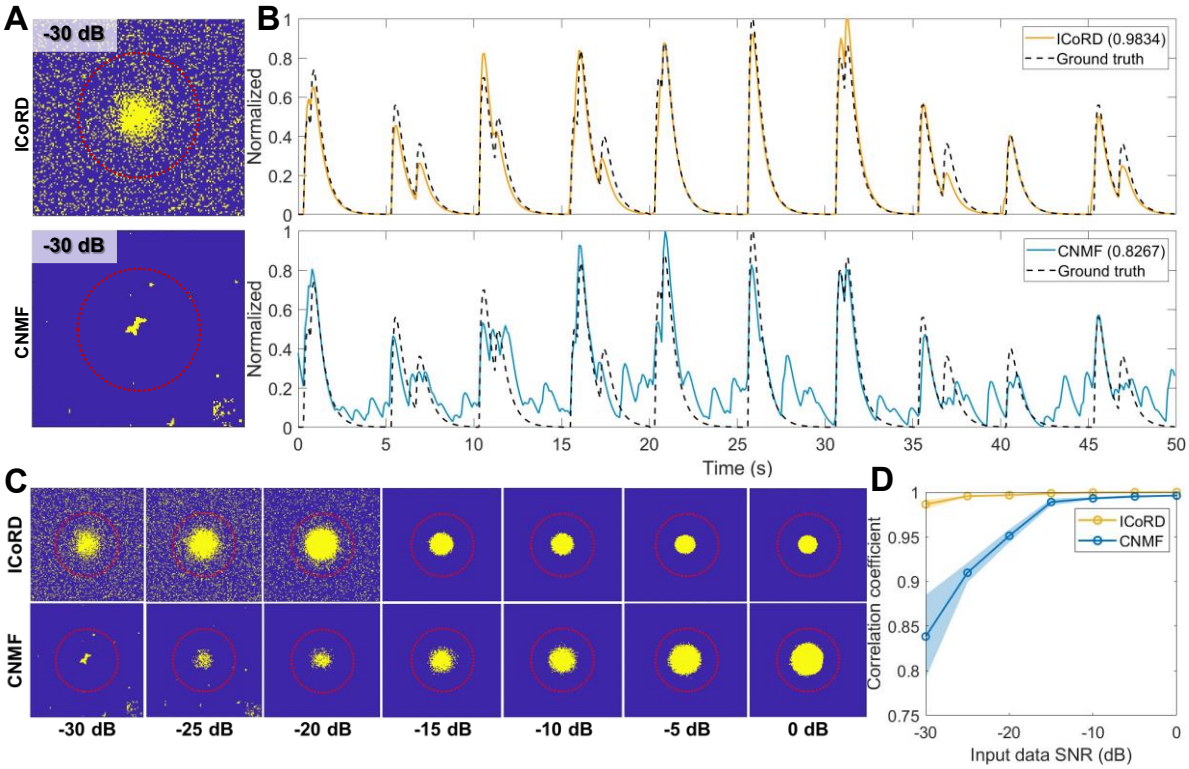

Figure 3. Results of the proposed ICoRD method and CNMF in simulated calcium imaging datasets

A. The pixels included in the ROIs from the simulated calcium images (yellow) with an SNR of -30 dB using ICoRD (upper) and CNMF (lower). The red dotted line indicates the range in which the neuron's signal exists in a Gaussian shape. **B**. The extracted calcium traces from the selected pixels of the simulated calcium images with an SNR of -30 dB using ICoRD (upper) and CNMF (lower). **C**. Detected ROIs using ICoRD (upper) and CNMF (lower) from the simulated calcium imaging data with SNRs of -30 to 0 dB. The red dotted lines are as in A. **D**. Mean (solid line) and standard deviation (shading) of the correlation coefficient between the ground-truth and extracted signals using ICoRD (yellow) and CNMF (blue).

Fig. 4A shows the trend of the estimated correlation coefficient and the ground-truth correlation coefficient. The estimated correlation coefficient generally increased with iteration, similar to the ground-truth correlation coefficient. The correlation coefficient between the ground truth and each iteration result approached 1 as the iteration increased and then saturated at approximately 1. This trend of the ground truth correlation coefficient reveals that the iterative algorithm has been effectively designed to approximate the ground-truth signal of the target neuron. The ground truth correlation coefficient had a maximum correlation coefficient (0.9889) at seventeen iterations. ICoRD calculates the moving average, which is the average of the estimated correlation coefficient at each iteration as an optional condition to effectively stop before the maximum number of iterations. Because the window length for calculating the moving average was set to five, the algorithm stopped

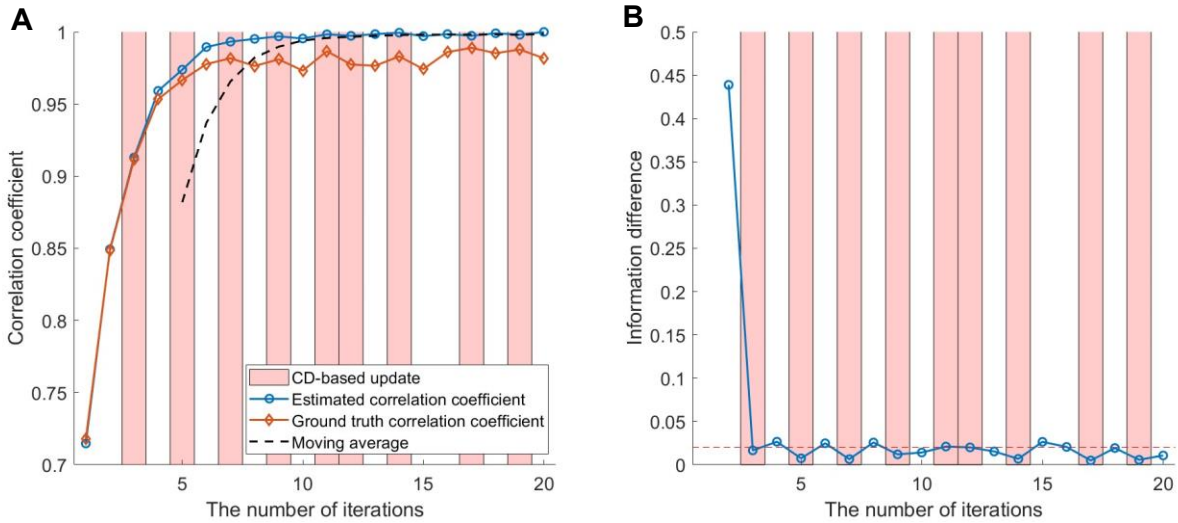

Figure 4. Trend of estimated correlation coefficient, ground-truth correlation coefficient, and information difference during iterations in the simulation data with an SNR of -30 dB.

A. Comparison of estimated correlation coefficient and ground-truth correlation coefficient according to iteration in the simulation data. **B.** The information difference according to iteration in the simulation data.

at the 19th iteration, and the algorithm selected the 18th result with the maximum estimated correlation coefficient as the final result. The 18th result had a sufficiently high ground truth correlation coefficient (0.9852).

Fig. 4B shows the general trend of the information difference, which is the criterion for selecting CD or raw as the reference signal (Equation 1). If the information difference is less than a certain threshold (for example, 0.02), the algorithm determines that there is no more information to be obtained from the previous reference source and updates it with another data source (Raw→CD, CD→Raw). The algorithm updates the reference with a different data type to avoid converging to the local optimum if the information difference is higher than in the previous iteration. This conversion of the appropriate data source allows ICoRD to estimate the ground-truth signal with a high correlation coefficient.

Application of the ICoRD algorithm to in vivo two-photon calcium imaging data

It has been verified that the proposed method has a powerful advantage in terms of SNR in the simulation data. In this section, the practicality of the proposed method is examined using two-photon calcium imaging data measured in a mouse brain.

All procedures involving mice were approved by the Korea Brain Research Institute, Institutional Animal Care and Use Committee (approval number: IACUC-19-00041). Adult (older than P60) transgenic mice expressing GCaMP6f (C57BL/6J-Tg [Thy1-GCaMP6f] GP5.5Dkim/J, The Jackson Laboratory) underwent surgeries to implant a cranial window and a headplate. A 3-mm craniotomy was performed over the posterior parietal cortex (PPC)

area centered at 2 mm posterior and 1.7 mm lateral to the bregma. The window covering the craniotomy area was constructed by bonding 3-mm and 5-mm diameter No. 0 coverslips (Warner Instruments). The titanium headplate was attached to the skull over the window using opaque dental cement (Super-Bond, Sun Medical) and covered all exposed tissue.

Images were acquired with a Nikon 16X (0.8 NA, 3.0 mm WD) objective lens attached to a microscope (HyperScope, Scientifica) running ScanImage (2019a). The Ti-Sapphire LASER (Chameleon Vision II, Coherent) operated at 910 nm was used as the light source for the imaging microscope. The head-fixed mouse was awake during the imaging procedure of the PPC, and it was kept warm with a custom-made body restrainer. The imaging area was approximately 200 μm deep and centered at the PPC; it was adjusted to avoid thick blood vessels appearing in the ROI. The resolution of the images was 512 × 512 pixels, and the FOV was 700 μm square. The frame rate of the microscope was set to 30 Hz. The proposed ICoRD algorithm was applied to raw in vivo calcium imaging data in the same algorithmic process as the simulation-based validation.

The spatial and temporal results of the selected neurons using ICoRD and CNMF in in vivo calcium imaging data were compared (Fig. 5). To compare ICoRD with CNMF, a neuron with frequent calcium activity (neuron 1) and a neuron with relatively sparse temporal activity (neuron 2) were selected among neurons clearly detected by CNMF (Fig. 5A). A neuron with a low SNR and sparse activation (neuron 3) was detected by ICoRD but not CNMF. Fig. 5B shows the zoomed images (mean, correlation) around three target neurons in the in vivo calcium imaging movie and the ROIs detected by ICoRD and CNMF. The location of the target neuron has been marked in the mean and correlation images with a red dotted line. The SNR of neuron 1 was sufficiently high to identify it in both the mean image and the correlation image. The ROI of neuron 1 detected by ICoRD and CNMF were similar. The results of the two methods in high-SNR neurons in vivo were the same as the detection trends observed in the simulation environment. Neuron 2 only exhibited the blurry shape of the target neuron in the mean image. ICoRD detected the clustered pixels and several of the surrounding pixels outside the target neuron, whereas CNMF only detected the clustered pixels of neuron 2. Neuron 3 only showed a blurry neuron morphology in the mean image, and its ROI was only detected by ICoRD. ICoRD extracted signals from neuron 3, despite this neuron being sparsely activated at high noise levels. ICoRD detected the ROI of neuron 3 with clusters and surrounding pixels, similar to the ROI of neuron 2. These ROI detection results with the surrounding pixels of neurons 2 and 3 were similar to the ROI detection results in the simulation verification with low SNRs. Because ICoRD detects the ROI that maximizes the correlation coefficient with the estimated reference by detecting the pixels that have a high correlation with the estimated reference, it detects surrounding pixels in the case of very low SNR neurons and takes advantage of the noise reduction effect.

Fig. 5C shows the raw signal of the selected pixel as a 'Raw' (grey line) of the three neurons in the ICoRD algorithm and each CD result of the ROI detected by ICoRD (orange line) and CNMF (blue line). We present the first reference and result of the last iteration of ICoRD to explain how the estimated reference changes with iteration. Each signal was standardized by subtracting the mean and dividing it by the standard deviation. A CD-based SNR estimation method is proposed to evaluate neurons in an in vivo environment (Fig. S3).

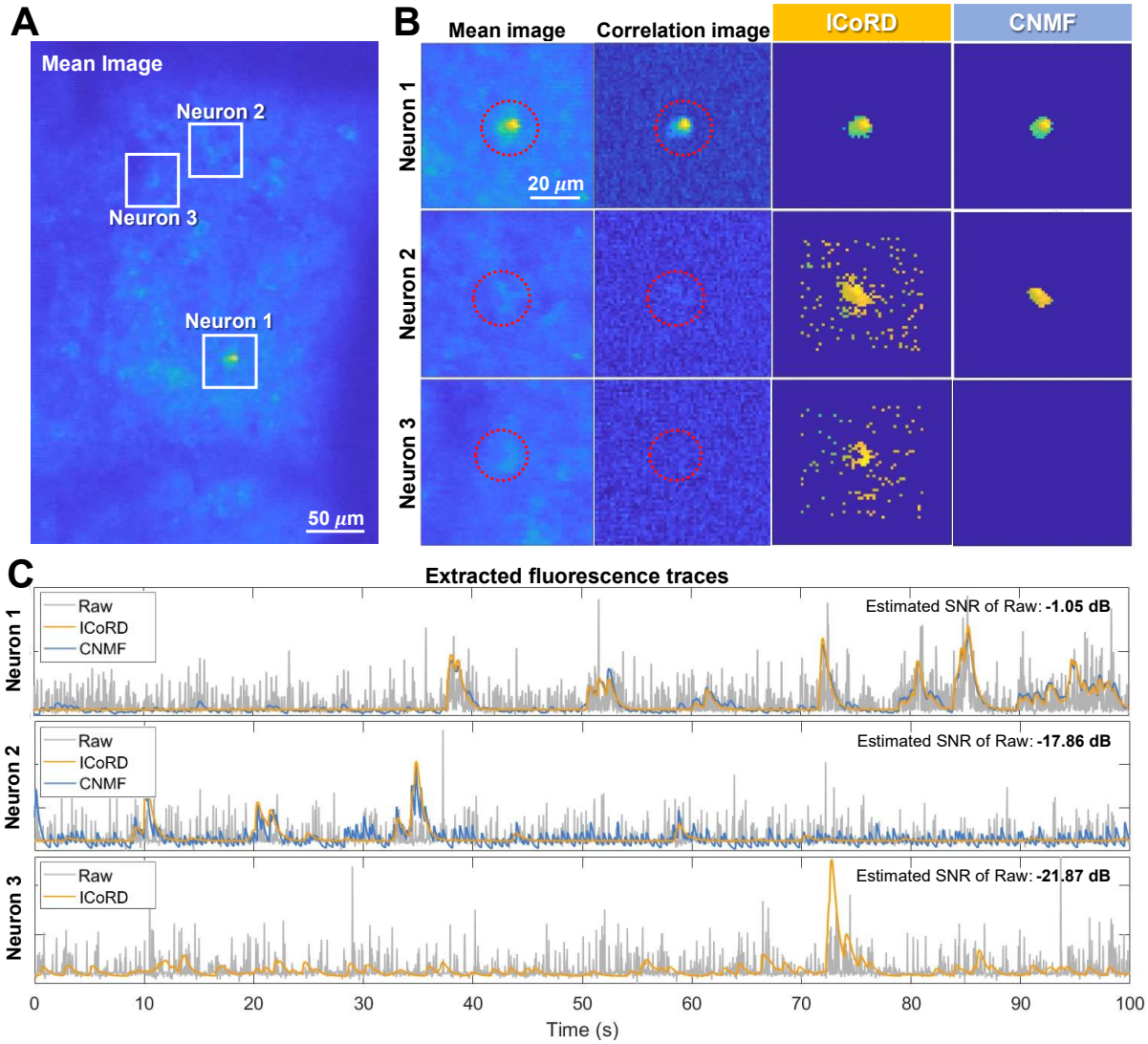

Figure 5. Application to in vivo two-photon calcium imaging data

A. Mean image of the in vivo calcium imaging movie and three target neurons. **B.** The zoomed mean and correlation images around the target neurons (1,2,3) and the ROIs detected by ICoRD and CNMF. **C.** The first reference (raw signal of the selected pixel) of the target neurons (1,2,3) and extracted temporal result using ICoRD and CNMF. The estimated SNR of neuron 1, neuron 2, and neuron 3 were -1.05 dB, -17.86 dB, and -21.87 dB, respectively.

The estimated SNR of individual neurons in the Raw signal was -1.05 dB in neuron 1, -17.86 dB in neuron 2, and -21.87 dB in neuron 3. The signals extracted by ICoRD and CNMF in neuron 1 with a high SNR and frequent calcium activity exhibited similar trends (correlation coefficient: 0.9608). However, for neuron 2 with a relatively infrequent and low amplitude calcium activation signal, the signal trends detected by the two methods were relatively different (correlation coefficient: 0.7241). Furthermore, only ICoRD, not CNMF, detected neuron

3 as an ROI. This result is similar to those of CNMF, indicating low signal extraction performance for an SNR below -20 dB in a simulation environment. Although the calcium signal of neuron 3 was highly sparse at high noise levels, ICoRD effectively extracted the calcium trace after iteration. Although ICoRD and CNMF apply the same CD method, the signal obtained from ICoRD appears to have a higher SNR. This is because ICoRD provides a better input signal for the CD method by detecting the ROI that maximizes the correlation coefficient with the predicted ground-truth signal of the target neuron. ICoRD was confirmed to effectively denoise signals from neurons with a low SNR in in vivo calcium imaging data.

Discussion

In the field of neuroscience, the calcium imaging technique is actively used because it can show the simultaneous activity of numerous neurons. When attempting to observe a larger number of neurons at once, neurons with low SNRs are inevitably observed; however, analyzing these low-SNR neurons is currently a challenging task. Existing ROI detection methods for calcium imaging data are aimed at the automatic detection of many neurons; hence, the extraction of signals from neurons with low SNRs often fails. If the calcium signal of neurons with sparse and weak activity can be extracted, the results of various calcium imaging experiments can be analyzed more reliably using the signals of more neurons. Therefore, we developed an algorithm to reliably estimate ground-truth calcium activity even in neurons with a low SNR.

An iterative correlation-based ROI detection method that effectively extracts calcium signals, even in calcium imaging data with a low SNR, was investigated. ICoRD performed reliable signal extraction (correlation coefficient with ground truth > 0.98) of the target neuron from simulated calcium imaging data with an SNR of -30 dB to 0 dB, which was difficult to analyze using the conventional method. Because ICoRD successfully estimated the ground truth according to the iteration, it was possible to detect the ROI that improves the SNR based on the estimated reference. ICoRD was confirmed to robustly extract activation signals, even from neurons with sparse activity, in an in vivo environment with severe noise levels. ICoRD extracted signals from neurons with a low SNR, which CNMF was not able to detect.

Because the proposed algorithm aims to extract temporal calcium activity, the focus of this study was not on detecting the shape of the spatial component. The proposed method clearly estimated the signal of a neuron with a low SNR, which was difficult using the conventional method. ICoRD detected neurons with very sparse activity, which were blurry in the mean image and not visible in the correlated image. The robust extraction performance of ICoRD may have been possible because the proposed algorithm does not require the ROI to be clustered like a neuron. The ROI detection method that maximizes the correlation coefficient with the reference signal regardless of the shape of the ROI is very effective when extracting the calcium signal from a single neuron.

ICoRD provides calcium signals with a high SNR from the target neuron; however, the user must determine the location of the target neuron for the first reference signal. If the target neuron greatly overlaps with other neurons, the user must choose a range of pixels to reject. These initial parameters of ICoRD can be laborious

if thousands of neurons need to be detected. Therefore, ICoRD is recommended when users need to clearly extract signals from hundreds or fewer individual neurons with low SNRs. If the user identifies the target neuron in two-photon calcium imaging data, but the conventional method fails to detect neurons with low SNRs, ICoRD will provide a calcium trace with high reliability.

ICoRD is designed intuitively so that users can easily apply it to their own calcium imaging data. It is hoped that using ICoRD, neuroscientists can analyze more neurons, including those with low SNRs and derive abundant research results with higher reliability.

References

Brondi, M., Moroni, M., Vecchia, D., Molano-Mazon, M., Panzeri, S., & Fellin, T. (2020). High-Accuracy Detection of Neuronal Ensemble Activity in Two-Photon Functional Microscopy Using Smart Line Scanning. *Cell Rep, 30*(8), 2567-2580 e2566. doi:10.1016/j.celrep.2020.01.105

Cardin, J. A., Crair, M. C., & Higley, M. J. (2020). Mesoscopic Imaging: Shining a Wide Light on Large-Scale Neural Dynamics. *Neuron, 108*(1), 33-43. doi:10.1016/j.neuron.2020.09.031

Chen, T. W., Wardill, T. J., Sun, Y., Pulver, S. R., Renninger, S. L., Baohan, A., . . . Kim, D. S. (2013). Ultrasensitive fluorescent proteins for imaging neuronal activity. *Nature, 499*(7458), 295-300. doi:10.1038/nature12354

Dana, H., Mohar, B., Sun, Y., Narayan, S., Gordus, A., Hasseman, J. P., . . . Kim, D. S. (2016). Sensitive red protein calcium indicators for imaging neural activity. *Elife, 5*. doi:10.7554/eLife.12727

Deneux, T., Kaszas, A., Szalay, G., Katona, G., Lakner, T., Grinvald, A., . . . Vanzetta, I. (2016). Accurate spike estimation from noisy calcium signals for ultrafast three-dimensional imaging of large neuronal populations in vivo. *Nat Commun, 7*, 12190. doi:10.1038/ncomms12190

Harris, K. D., Quiroga, R. Q., Freeman, J., & Smith, S. L. (2016). Improving data quality in neuronal population recordings. *Nat Neurosci, 19*(9), 1165-1174. doi:10.1038/nn.4365

Helmchen, F., Imoto, K., & Sakmann, B. (1996). Ca2+ buffering and action potential-evoked Ca2+ signaling in dendrites of pyramidal neurons. *Biophys J, 70*(2), 1069-1081. doi:10.1016/S0006-3495(96)79653-4

Langer, D., van 't Hoff, M., Keller, A. J., Nagaraja, C., Pfaffli, O. A., Goldi, M., . . . Helmchen, F. (2013). HelioScan: a software framework for controlling in vivo microscopy setups with high hardware flexibility, functional diversity and extendibility. *J Neurosci Methods, 215*(1), 38-52. doi:10.1016/j.jneumeth.2013.02.006

Lillis, K. P., Eng, A., White, J. A., & Mertz, J. (2008). Two-photon imaging of spatially extended neuronal network dynamics with high temporal resolution. *J Neurosci Methods, 172*(2), 178-184. doi:10.1016/j.jneumeth.2008.04.024

Maravall, M., Mainen, Z. F., Sabatini, B. L., & Svoboda, K. (2000). Estimating intracellular calcium concentrations and buffering without wavelength ratioing. *Biophys J, 78*(5), 2655-2667. doi:10.1016/S0006-3495(00)76809-3

Maruyama, R., Maeda, K., Moroda, H., Kato, I., Inoue, M., Miyakawa, H., & Aonishi, T. J. N. N. (2014). Detecting cells using non-negative matrix factorization on calcium imaging data. *Neural Networks, 55*, 11-19.

Mukamel, E. A., Nimmerjahn, A., & Schnitzer, M. J. (2009). Automated analysis of cellular signals from large-scale calcium imaging data. *Neuron, 63*(6), 747-760. doi:10.1016/j.neuron.2009.08.009

Pnevmatikakis, E. A., Soudry, D., Gao, Y., Machado, T. A., Merel, J., Pfau, D., . . . Paninski, L. (2016). Simultaneous Denoising, Deconvolution, and Demixing of Calcium Imaging Data. *Neuron, 89*(2), 285-299. doi:10.1016/j.neuron.2015.11.037

Sofroniew, N. J., Flickinger, D., King, J., & Svoboda, K. (2016). A large field of view two-photon mesoscope with subcellular resolution for in vivo imaging. *Elife, 5*. doi:10.7554/eLife.14472

Stringer, C., & Pachitariu, M. (2019). Computational processing of neural recordings from calcium imaging data. *Curr Opin Neurobiol, 55*, 22-31. doi:10.1016/j.conb.2018.11.005

Terada, S. I., Kobayashi, K., Ohkura, M., Nakai, J., & Matsuzaki, M. (2018). Super-wide-field two-photon imaging with a micro-optical device moving in post-objective space. *Nat Commun, 9*(1), 3550. doi:10.1038/s41467-018-06058-8

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Author contribution

Seongtak Kang, Conceptualization, Data curation, Software, Formal analysis, Validation, Investigation, Visualization, Methodology, Writing-review and editing; Jiho Park, Conceptualization, Data curation, Software, Formal analysis, Validation; Kyungsoo Kim, Conceptualization, Software, Writing-review and editing; Sung-Ho Lim, Methodology, Writing-review and editing; Joon Ho Choi, Resources, Writing-review and editing; Jong-Cheol Rah, Supervision, Project administration, Writing-review and editing; Ji-Woong Choi, Supervision, Validation, Methodology, Writing-review and editing.

Supplemental Information

Demixing of overlapped neurons using iterative correlation-based ROI detection (ICoRD)

When the target neuron overlaps with other neurons, ICoRD runs the algorithm after excluding the overlapping pixels. If the user can identify two overlapping neurons, ICoRD can distinguish the signals of the overlapping neurons. Fig. S1A shows that the rejection range is determined when overlapping neurons (neuron A: left neuron, neuron B: right neuron) are found in the compressed image (mean, max). Fig. S1B shows the result of selecting the ROI of neuron A among the pixels, excluding the rejection range. Fig. S1C shows the 100-s ground-truth signals of the two simulated neurons. Fig. S1D shows the result of detecting neuron A through ICoRD and the ground truth. ICoRD was able to extract the signal of the desired neuron among the two overlapping neurons in a noisy environment (-10 dB) with high accuracy (correlation coefficient with ground truth: 0.9884).

Details of the ICoRD algorithm

For each iteration, the reference signal is updated with the average signal (Avg) or CD result in the previous iteration (Fig S2). For the initial iteration, the Avg signal of the ROI is updated with the reference signal because the estimation accuracy of CD in the first iteration tends to be low. However, repetitive updates to only the Avg signal can easily reach a local optimum because there is no function to estimate the signal. Iterative reference updates based only on the CD result can repeatedly find the ROI biased to the initially estimated calcium dynamics model. Thus, starting from the second iteration, the Avg signal or CD result is selectively updated as a reference signal according to the information difference between each temporal Avg result of the current and previous iterations.

When the information difference is less than a certain threshold (for example 0.02), the algorithm determines that there is no more information available from the Avg-based iteration and updates the CD result in the next iteration. The CD-based update also changes to an Avg-based update on the next iteration when the information difference falls below a threshold. ICoRD maintains the reference source only when the information difference is larger than the threshold and is smaller than the previous information difference. The estimated correlation coefficient is used to stop the iterations of the ICoRD algorithm for time efficiency. If the average value of the estimated correlation coefficient among the last five iterations is lower than that of the previous iteration, the iterations cease and the result of the maximum estimated correlation coefficient is outputted. Users can change the length of the sliding window to calculate the moving average. If the user does not use the moving average function, the algorithm stops at a predetermined maximum iteration. At this point, the CD result is outputted owing to the algorithm.

Signal-to-noise power ratio (SNR) estimation of calcium imaging data

To estimate the SNR of calcium activity (Raw), the result of constrained deconvolution (CD) was defined as a signal, and the data of the lower 25% of temporal raw data subtracted by CD was defined as noise (Brondi et al., 2020). Equation S1 represents the proposed SNR estimation equation

$$Estimated\ SNR\ (dB) = 10\log\frac{(CD)^2}{\left(bottom\ 25\%\ of(Raw - CD)\right)^2}. \tag{S1}$$

The SNR estimation performance was verified by generating simulation data every 0.2 dB from -30 to 10 dB. All simulation data generation conditions were the same as those in Fig. 2. In Fig S3, defining the bottom 25% of Raw-CD as noise revealed better SNR estimation performance than defining Raw-CD as noise. The total estimation error of the proposed method at -30 dB to 10 dB was 1.3493 ± 2.2625 dB. Because CD cannot perfectly estimate the signal, the signal may remain after subtracting CD. Therefore, the noise power was greater because a part of the signal was determined as noise, and the larger noise power resulted in a lower estimation of the SNR. Through SNR estimation method, we compared the relative SNRs of calcium signals of target neurons within in vivo calcium imaging data.

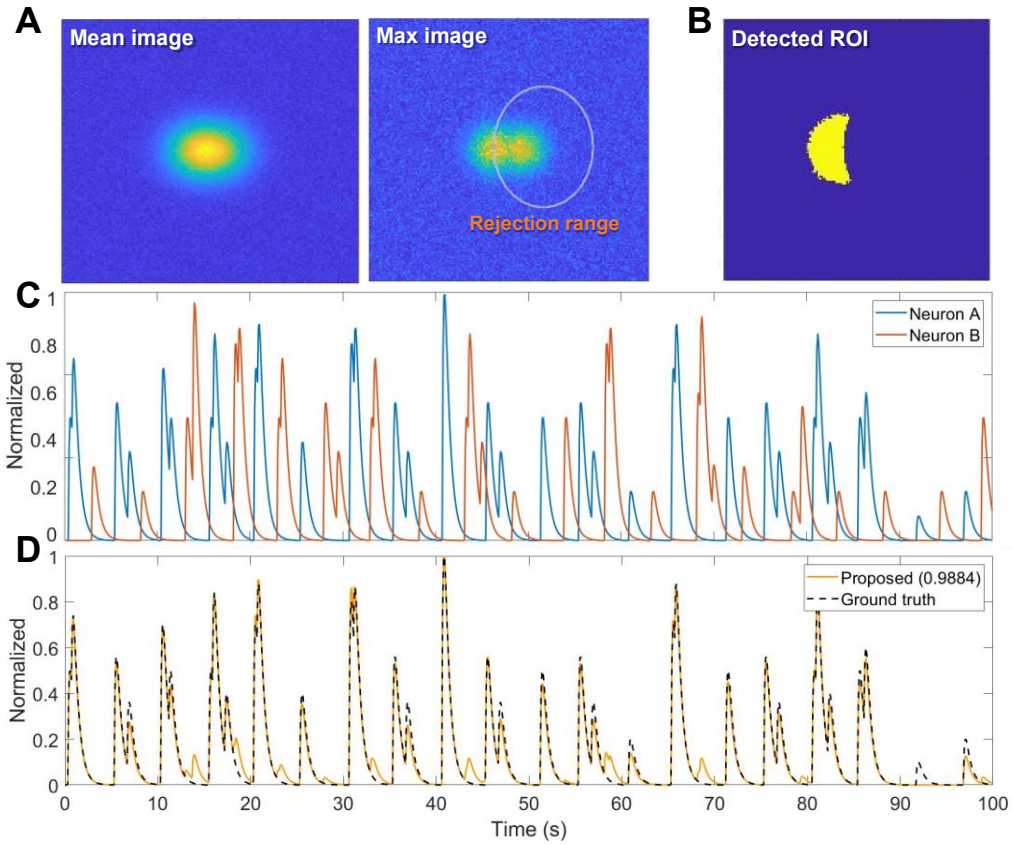

Figure S1. Demixing of two overlapped neurons (Neuron A, Neuron B) in simulation data using ICoRD

A. Mean and max images of two overlapped neurons with a SNR of –10 dB**. B.** Detected ROI of neuron A using the ICoRD method. **C.** Simulated temporal calcium dynamics of the two neurons (Neuron A, Neuron B). **D.** Temporal result of the detected ROI of neuron A using the ICoRD algorithm.

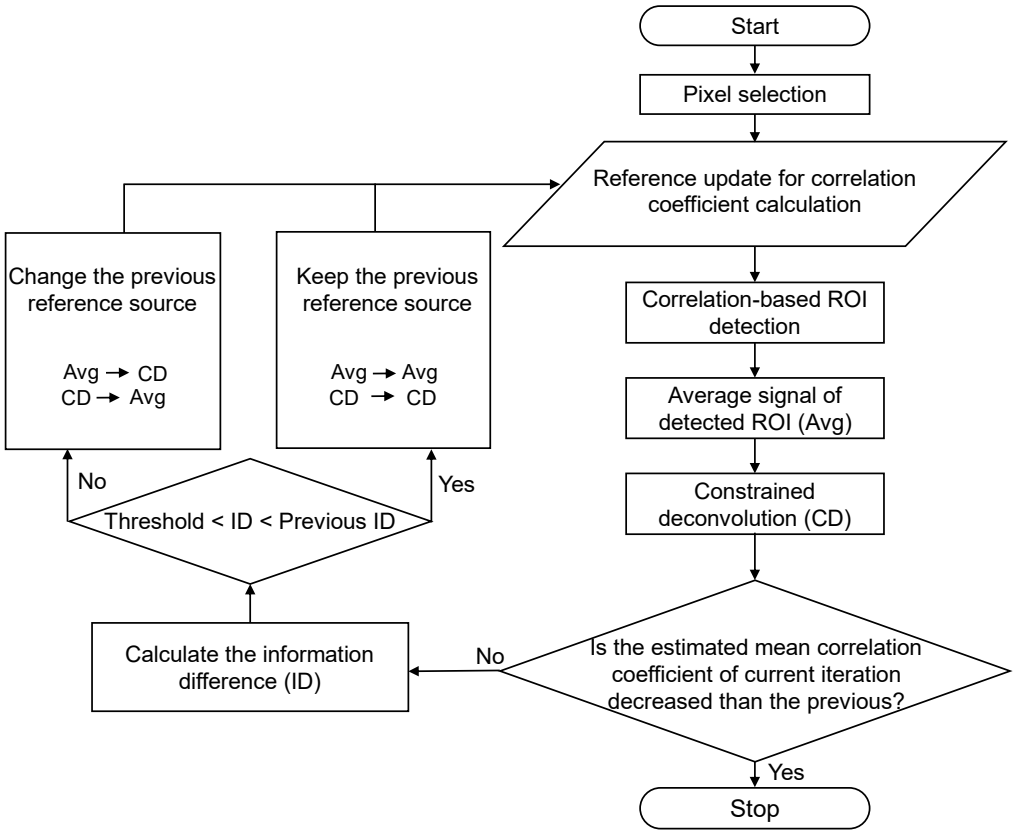

Figure S2. Detailed flowchart of the proposed ICoRD algorithm

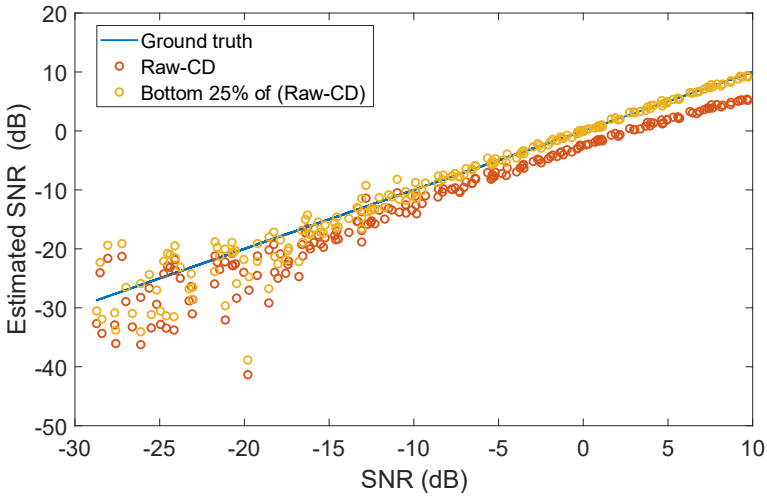

Figure S3. Proposed SNR estimation method according to ground-truth SNR

The blue line indicates the ground-truth SNR of the simulated calcium traces. The red circles represent the SNR estimation results when Raw-CD is defined as noise for each simulated calcium trace. The yellow circles show the SNR estimation results when the lower 25% of Raw-CD is defined as noise for each simulated calcium trace.