Efficient and accurate extraction of *in vivo* calcium signals from microendoscopic video data

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Abstract In vivo calcium imaging through microendoscopic lenses enables imaging of previously
 inaccessible neuronal populations deep within the brains of freely moving animals. However, it is
 computationally challenging to extract single-neuronal activity from microendoscopic data, because
 of the very large background fluctuations and high spatial overlaps intrinsic to this recording

- modality. Here, we describe a new constrained matrix factorization approach to accurately
 separate the background and then demix and denoise the neuronal signals of interest. We
- separate the background and then demix and denoise the neuronal signals of interest. We compared the proposed method against previous independent components analysis and
- constrained nonnegative matrix factorization approaches. On both simulated and experimental
- ³⁴ data recorded from mice, our method substantially improved the quality of extracted cellular
- ³⁵ signals and detected more well-isolated neural signals, especially in noisy data regimes. These
- ³⁶ advances can in turn significantly enhance the statistical power of downstream analyses, and
- ³⁷ ultimately improve scientific conclusions derived from microendoscopic data.
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39 Introduction

Monitoring the activity of large-scale neuronal ensembles during complex behavioral states is 40 fundamental to neuroscience research. Continued advances in optical imaging technology are 41 greatly expanding the size and depth of neuronal populations that can be visualized. Specifically, 42 in vivo calcium imaging through microendoscopic lenses and the development of miniaturized 43 microscopes have enabled deep brain imaging of previously inaccessible neuronal populations of 11 freely moving mice (Flusberg et al., 2008; Ghosh et al., 2011; Ziv and Ghosh, 2015). This technique 45 has been widely used to study the neural circuits in cortical, subcortical, and deep brain areas, such 46 as hippocampus (Cai et al., 2016: Rubin et al., 2015: Ziv et al., 2013), entorhinal cortex (Kitamura 47 et al., 2015: Sun et al., 2015), hypothalamus (lennings et al., 2015), prefrontal cortex (PFC) (Pinto 48 and Dan. 2015), premotor cortex (Markowitz et al., 2015), dorsal pons (Cox et al., 2016), basal 49 forebrain (Harrison et al., 2016), striatum (Barbera et al., 2016: Carvalho Povraz et al., 2016: Klaus 50 et al., 2017), amygdala (Yu et al., 2017), and other brain regions. 51 Although microendoscopy has potential applications across numerous neuroscience fields (Ziv 52 and Ghosh, 2015), methods for extracting cellular signals from this data are currently limited and 53 suboptimal. Most existing methods are specialized for 2-photon or light-sheet microscopy. However, 54 these methods are not suitable for analyzing single-photon microendoscopic data because of its 55 distinct features: specifically, this data typically displays large, blurry background fluctuations due 56 to fluorescence contributions from neurons outside the focal plane. In Figure 1 we use a typical 57 microendoscopic dataset to illustrate these effects (see S1 Video for raw video). Figure 1A shows an 58 example frame of the selected data, which contains large signals additional to the neurons visible 59 in the focal plane. These extra fluorescence signals contribute as background that contaminates 60 the single-neuronal signals of interest. In turn, standard methods based on local correlations for 61 visualizing cell outlines (Smith and Häusser, 2010) are not effective here, because the correlations 62 in the fluorescence of nearby pixels are dominated by background signals (Figure 1B). For some 63 neurons with strong visible signals, we can manually draw regions-of-interest (ROI) (Figure 1C). 64 Following (Barberg et al., 2016: Pinto and Dan, 2015), we used the mean fluorescence trace of the 65 surrounding pixels (blue, Figure 1D) to roughly estimate this background fluctuation; subtracting 66 it from the raw trace in the neuron ROI vields a relatively good estimation of neuron signal (red. 67 Figure 1D). Figure 1D shows that the background (blue) has much larger variance than the relatively 68 sparse neural signal (red); moreover, the background signal fluctuates on similar timescales as the 69 single-neuronal signal, so we can not simply temporally filter the background away after extraction 70 of the mean signal within the ROI. This large background signal is likely due to a combination of 71 local fluctuations resulting from out-of-focus fluorescence or neuropil activity, hemodynamics of 72 blood vessels, and global fluctuations shared more broadly across the field of view (photo-bleaching 73 effects, drifts in z of the focal plane, etc.), as illustrated schematically in Figure 1E. 74 The existing methods for extracting individual neural activity from microendoscopic data can 75 be divided into two classes: semi-manual ROI analysis (Barbera et al., 2016: Klaus et al., 2017: 76 Pinto and Dan, 2015) and PCA/ICA analysis (Mukamel et al., 2009). Unfortunately, both approaches 77 have well-known flaws (*Resendez et al.*, 2016). For example, ROI analysis does not effectively 78 demix signals of spatially overlapping neurons, and drawing ROIs is laborious for large population 79 recordings. More importantly, in many cases the background contaminations are not adequately 80 corrected, and thus the extracted signals are not sufficiently clean enough for downstream analyses. 81 As for PCA/ICA analysis, it is a linear demixing method and therefore typically fails when the neural 82 components exhibit strong spatial overlaps (*Pnevmatikakis et al., 2016*), as is the case in the 83 microendoscopic setting 84 Recently, constrained nonnegative matrix factorization (CNMF) approaches were proposed 85 to simultaneously denoise deconvolve and demix calcium imaging data (Pneymatikakis et al. 86 **2016**). However, current implementations of the CNMF approach were optimized for 2-photon 27

and light-sheet microscopy, where the background has a simpler spatiotemporal structure. When



Figure 1. Microendoscopic data contain large background signals with rapid fluctuations due to multiple sources. (**A**) An example frame of microendoscopic data recorded in dorsal striatum (see Methods and Materials section for experimental details). (**B**) The local "correlation image" (*Smith and Häusser, 2010*) computed from the raw video data. Note that it is difficult to discern neuronal shapes in this image due to the high background spatial correlation level. (**C**) The mean-subtracted data within the cropped area (green) in (**A**). Two ROIs were selected and coded with different colors. (**D**) The mean fluorescence traces of pixels within the two selected ROIs (magenta and blue) shown in (**C**) and the difference between the two traces. (**E**) Cartoon illustration of various sources of fluorescence signals in microendoscopic data. "BG" abbreviates "background."

⁸⁹ applied to microendoscopic data, CNMF often has poor performance because the background is

⁹⁰ not modeled sufficiently accurately (*Barbera et al., 2016*).

In this paper, we significantly extend the CNMF framework to obtain a robust approach for

- 92 extracting single-neuronal signals from microendoscopic data. Specifically, our extended CNMF
- ⁹³ for microendoscopic data (CNMF-E) approach utilizes a more accurate and flexible spatiotemporal

⁹⁴ background model that is able to handle the properties of the strong background signal illustrated

in Fig. 1, along with new specialized algorithms to initialize and fit the model components. After a

⁹⁶ brief description of the model and algorithms, we first use simulated data to illustrate the power

of the new approach. Next, we compare CNMF-E with PCA/ICA analysis comprehensively on both

⁹⁸ simulated data and four experimental datasets recorded in different brain areas. The results show

⁹⁹ that CNMF-E outperforms PCA/ICA in terms of detecting more well-isolated neural signals, extracting

¹⁰⁰ higher signal-to-noise ratio (SNR) cellular signals, and obtaining more robust results in low SNR

regimes. Finally, we show that downstream analyses of calcium imaging data can substantially

¹⁰² benefit from these improvements.

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Name	Description	Domain
d	number of pixels	\mathbb{N}_+
Т	number of frames	\mathbb{N}_+
Κ	number of neurons	\mathbb{N}
Y	motion corrected video data	$\mathbb{R}^{d \times T}_+$
Α	spatial footprints of all neurons	$\mathbb{R}^{d \times K}_+$
С	temporal activities of all neurons	$\mathbb{R}^{K \times T}_+$
В	background activity	$\mathbb{R}^{d \times T}_+$
E	observation noise	$\mathbb{R}^{d \times T}$
W	weight matrix to reconstruct <i>B</i> using neighboring pixels	$\mathbb{R}^{d \times d}$
\boldsymbol{b}_0	constraint baseline for all pixels	\mathbb{R}^{d}_{+}
\boldsymbol{x}_i	spatial location of the <i>i</i> th pixel	\mathbb{N}^2
σ_{i}	standard deviation of the noise at pixel x_i	\mathbb{R}_+

Table 1. Variables used in the CNMF-E model and algorithm. \mathbb{R} : real numbers; \mathbb{R}_+ : positive real numbers; \mathbb{N} : natural numbers; \mathbb{N}_+ : positive integers.

103 Model and model fitting

104 CNMF for microendoscope data (CNMF-E)

The recorded video data can be represented by a matrix $Y \in \mathbb{R}^{d \times T}_{\perp}$, where d is the number of 105 pixels in the field of view and T is the number of frames observed. In our model each neuron i is 106 characterized by its spatial "footprint" vector $a_i \in \mathbb{R}^d$ characterizing the cell's shape and location, 107 and "calcium activity" timeseries $c_i \in \mathbb{R}^T_i$, modeling (up to a multiplicative and additive constant) cell 108 *i*'s mean fluorescence signal at each frame. Here, both a_i and c_j are constrained to be nonnegative 109 because of their physical interpretations. The background fluctuation is represented by a matrix 110 $B \in \mathbb{R}^{d \times T}$. If the field of view contains a total number of K neurons, then the observed movie data 111 is modeled as a superposition of all neurons' spatiotemporal activity, plus time-varying background 112 and additive noise: 113

$$Y = \sum_{i=1}^{K} a_{i} \cdot c_{i}^{T} + B + E = AC + B + E,$$
(1)

where $A = [a_1, ..., a_K]$ and $C = [c_1, ..., c_K]^T$. The noise term $E \in \mathbb{R}^{d \times T}$ is modeled as Gaussian, $E(t) \sim \mathcal{N}(\mathbf{0}, \Sigma)$. Σ is a diagonal matrix, indicating that the noise is spatially and temporally uncorrelated.

Estimating the model parameters A, C in model (1) gives us all neurons' spatial footprints and their denoised temporal activity. This can be achieved by minimizing the residual sum of squares (RSS), aka the Frobenius norm of the matrix Y - (AC + B),

$$\|Y - (AC + B)\|_F^2,$$
 (2)

while requiring the model variables *A*, *C* and *B* to follow the desired constraints, discussed below.

¹²⁰ Constraints on neuronal spatial footprints *A* and neural temporal traces *C*

121 Each spatial footprint *a*, should be spatially localized and sparse, since a given neuron will cover

only a small fraction of the field of view, and therefore most elements of a_i will be zero. Thus we

need to incorporate spatial locality and sparsity constraints on *A* (*Pnevmatikakis et al., 2016*). We
 discuss details further below.

Similarly, the temporal components c_i are highly structured, as they represent the cells' fluorescence responses to sparse, nonnegative trains of action potentials. Following (*Vogelstein et al.,* **2010**; *Pnevmatikakis et al., 2016*), we model the calcium dynamics of each neuron c_i with a stable

¹²⁸ autoregressive (AR) process of order *p*,

$$c_i(t) = \sum_{j=1}^p \gamma_j^{(i)} c_i(t-j) + s_i(t),$$
(3)

- where $s_i(t) \ge 0$ is the number of spikes that neuron fired at the *t*-th frame. (Note that there is no
- further noise input into $c_i(t)$ beyond the spike signal $s_i(t)$.) The AR coefficients $\{\gamma_j^{(i)}\}$ are different
- for each neuron and they are estimated from the data. In practice, we usually pick p = 2, thus

incorporating both a nonzero rise and decay time of calcium transients in response to a spike; then

Eq. (3) can be expressed in matrix form as

$$G_{i} \cdot c_{i} = s_{i}, \text{ with } G_{i} = \begin{bmatrix} 1 & 0 & 0 & \cdots & 0 \\ -\gamma_{1}^{(i)} & 1 & 0 & \cdots & 0 \\ -\gamma_{2}^{(i)} & -\gamma_{1}^{(i)} & 1 & \cdots & 0 \\ \vdots & \ddots & \ddots & \ddots & \vdots \\ 0 & \cdots & -\gamma_{2}^{(i)} & -\gamma_{1}^{(i)} & 1 \end{bmatrix}.$$
(4)

The neural activity s_i is nonnegative and typically sparse; to enforce sparsity we can penalize the ℓ_0 (*Jewell and Witten, 2017*) or ℓ_1 (*Pnevmatikakis et al., 2016*; *Vogelstein et al., 2010*) norm of s_i , or limit the minimum size of nonzero spike counts (*Friedrich et al., 2017b*). When the rise time constant is small compared to the timebin width (low imaging frame rate), we typically use a simpler AR(1) model (with an instantaneous rise following a spike) (*Pnevmatikakis et al., 2016*).

139 Constraints on background activity *B*

In the above we have largely followed previously-described CNMF approaches (*Pneymatikakis* 140 et al., 2016) for modeling calcium imaging signals. However, to accurately model the background 141 effects in microendoscopic data we need to depart significantly from these previous approaches. 142 Constraints on the background term B in Eq. (1) are essential to the success of CNMF-E, since 143 clearly, if B is completely unconstrained we could just absorb the observed data Y entirely into 144 B, which would lead to recovery of no neural activity. At the same time, we need to prevent 145 the residual of the background term (i.e., $B - \hat{B}$, where \hat{B} denotes the estimated spatiotemporal 146 background) from corrupting the estimated neural signals AC in model (1), since subsequently. 147 the extracted neuronal activity would be mixed with background fluctuations, leading to artificially 148 high correlations between nearby cells. This problem is even worse in the microendoscopic context 149 because the background fluctuation usually has significantly larger variance than the isolated 150 cellular signals of interest (Figure 1D), and therefore any small errors in the estimation of B can 151 severely corrupt the estimated neural signal AC. 152

In (*Pnevmatikakis et al., 2016*), B is modeled as a rank-1 nonnegative matrix $B = b \cdot f^T$. where 153 $b \in \mathbb{R}^d$ and $f \in \mathbb{R}^T$. This model mainly captures the global fluctuations within the field of view 154 (FOV). In applications to 2-photon or light-sheet data, this rank-1 model has been shown to be 155 sufficient for relatively small spatial regions; the simple low-rank model does not hold for larger 156 fields of view, and so we can simply divide large FOVs into smaller patches for largely-parallel 157 processing (Pnevmatikakis et al., 2016; Giovannucci et al., 2017b), (See (Pachitariu et al., 2016) 158 for an alternative approach.) However, as we will see below, the local rank-1 model fails in many 159 microendoscopic datasets, where multiple large overlapping background sources exist even within 160 modestly-sized FOVs. 161

Thus we propose a new model to constrain the background term *B*. We first decompose the background into two terms:

$$B = B^f + B^c, (5)$$

where B^{f} represents fluctuating activity and $B^{c} = b_{0} \cdot \mathbf{1}^{T}$ models constant baselines ($\mathbf{1} \in \mathbb{R}^{T}$ denotes a vector of T ones). To model B^{f} , we exploit the fact that background sources (largely due to blurred out-of-focus fluorescence) are empirically much coarser spatially than the average neuron soma size l. Thus we model B^{f} at one pixel as a linear combination of the background fluorescence in pixels which are chosen to be nearby but not nearest neighbors:

$$B_{it}^f = \sum_{j \in \Omega_i} w_{ij} \cdot B_{jt}^f, \ \forall t = 1 \dots T,$$
(6)

where $\Omega_i = \{j \mid \text{dist}(\mathbf{x}_i, \mathbf{x}_i) \in [l_n, l_n + 1)\}$, with $\text{dist}(\mathbf{x}_i, \mathbf{x}_i)$ the Euclidean distance between pixel *i* and *j*. 169 Thus Ω_i only selects the neighboring pixels with a distance of I_{i} from the *i*-th pixel (the green dot 170 and black pixels in Figure 2B illustrate i and Ω_i , respectively); here I_i is a parameter that we choose 171 to be greater than *I* (the size of the typical soma in the FOV), e.g., $l_n = 2I$. This choice of l_n ensures 172 that pixels *i* and *j* in (6) share similar background fluctuations, but do not belong to the same soma. 173 We can rewrite Eq. (6) in matrix form: 174

$$B^f = W B^f, \tag{7}$$

where $W_{ii} = 0$ if dist $(\mathbf{x}_i, \mathbf{x}_i) \notin [l_n, l_n + 1)$. In practice, this hard constraint is difficult to enforce 175

computationally, and is overly stringent given the noisy observed data. We relax the model by 176 177

replacing the right-hand side B^{f} with the more convenient closed-form expression

$$B^{f} = W \cdot (Y - AC - \boldsymbol{b}_{0} \cdot \boldsymbol{1}^{T}).$$
(8)

According to Eq. (1) and (5), this change ignores the noise term E; since elements in E are spatially 178

uncorrelated, $W \cdot E$ contributes as a very small disturbance to \hat{B}^f in the left-hand side. We found 179

this substitution for \hat{B}^{f} led to significantly faster and more robust model fitting. 180

Fitting the CNMF-E model 181

Now we can formulate the estimation of all model variables as a single optimization meta-problem:

minimize _{A,C,S,B^f,W,b₀}	$\ Y - AC - \boldsymbol{b}_0 \cdot \boldsymbol{1}^T - \boldsymbol{B}^f\ _F^2$	(P-All)
subject to	$A \ge 0, A$ is sparse and spatially localized	
	$c_i \ge 0, \ s_i \ge 0, \ G^{(i)}c_i = s_i, \ s_i$ is sparse $\forall i = 1 \dots K$	
	$B^f \cdot 1 = 0$	
	$B^f = W \cdot (Y - AC - \boldsymbol{b}_0 \cdot \boldsymbol{1}^T)$	
	$W_{ij} = 0$ if dist $(\mathbf{x}_i, \mathbf{x}_j) \notin [l_n, l_n + 1)$.	

We call this a "meta-problem" because we have not yet explicitly defined the sparsity and spatial 182 locality constraints on A and $S = [s_1, \dots, s_K]^T$; these can be customized by users under different 183 assumptions (see details in Methods and Materials). Also note that s, is completely determined 184 by c_i and $G^{(i)}$, and B^f is not optimized explicitly but (as discussed above) can be estimated as 185 $W \cdot (Y - AC - b_0 \cdot \mathbf{1}^T)$, so we optimize with respect to W instead. 186

The problem (P-All) optimizes all variables together and is non-convex, but can be divided into 187 three simpler subproblems that we solve iteratively: 188

Estimating A, b_0 given \hat{C}, \hat{B}^f

 $\|Y - A \cdot \hat{C} - \boldsymbol{b}_0 \cdot \boldsymbol{1}^T - \hat{B}^f\|_F^2$ minimize (P-S)

 $A \ge 0$, A is sparse and spatially localized subject to

Estimating C, \boldsymbol{b}_0 given \hat{A}, \hat{B}^f

 C, S, b_0

minimize W, B^f, b_0 subject to

$$\begin{array}{ll} \underset{C, S, b_0}{\text{minimize}} & \|Y - \hat{A} \cdot C - b_0 \cdot \mathbf{1}^T - \hat{B}^f\|_F^2 \\ \text{subject to} & c_i \ge 0, \ s_i \ge 0 \end{array}$$
 (P-T)

Estimating W, b_0 given \hat{A}, \hat{C}

$$\|Y - \hat{A} \cdot \hat{C} - \boldsymbol{b}_0 \cdot \boldsymbol{1}^T - \boldsymbol{B}^f\|_F^2 \tag{P-B}$$

$$B^{i} \cdot \mathbf{1} = \mathbf{0}$$

$$B^{f} = W \cdot (Y - \hat{A} \cdot \hat{C} - \mathbf{b}_{0} \cdot \mathbf{1}^{T}).$$

$$W_{ii} = 0 \text{ if } \operatorname{dist}(\mathbf{x}_{i}, \mathbf{x}_{j}) \notin [l_{n}, l_{n} + 1)$$

 $G^{(i)}c_i = s_i, \ s_i \text{ is sparse } \forall i = 1 \dots K$

For each of these subproblems, we are able to use well-established algorithms (e.g., solutions 189 for (P-S) and (P-T) are discussed in (Friedrich et al., 2017a: Pneymatikakis et al., 2016)) or slight 190 modifications thereof. By iteratively solving these three subproblems, we obtain tractable updates 191 for all model variables in problem (P-All). Furthermore, this strategy gives us the flexibility of 192 further potential interventions (either automatic or semi-manual) in the optimization procedure 193 e.g., incorporating further prior information on neurons' morphology, or merging/splitting/deleting 194 spatial components and detecting missed neurons from the residuals. These steps can significantly 195 improve the guality of the model fitting: this is an advantage compared with PCA/ICA, which offers 196 no easy option for incorporation of stronger prior information or manually-guided improvements 197 on the estimates. 198

Full details on the algorithms for initializing and then solving these three subproblems are provided in the Methods and Materials section.

201 **Results**

202 CNMF-E can reliably estimate large high-rank background fluctuations

We first use simulated data to illustrate the background model in CNMF-E and compare its performance against the low-rank NMF model used in the basic CNMF approach (*Pnevmatikakis et al.,* 2016). We generated the observed fluorescence *Y* by summing up simulated fluorescent signals of multiple sources as shown in Figure 1E plus additive Gaussian white noise (Figure 2A).

An example pixel (green dot, Figure 2A,B) was selected to illustrate the background model in 207 CNMF-E (Eq. (6)), which assumes that each pixel's background activity can be reconstructed using its 208 neighboring pixels' activities. The selected neighbors form a ring and their distances to the center 209 pixel are larger than a typical neuron size (Figure 2B). Figure 2C shows that the fluorescence traces 210 of the center pixel and its neighbors are highly correlated due to the shared large background 211 fluctuations. Here for illustrative purposes we fit the background by solving problem (P-B) directly 212 while assuming $\hat{A}\hat{C} = 0$. This mistaken assumption should make the background estimation more 213 challenging (due to true neural components getting absorbed into the background), but nonetheless 214 in Figure 2 we see that the background fluctuation was well recovered (Figure 2D). Subtracting this 215 estimated background from the observed fluorescence in the center yields a good visualization 216 of the cellular signal (Figure 2D). Thus this example shows that we can reconstruct a complicated 217 background trace while leaving the neural signal uncontaminated. 218

For the example frame in Figure 2A, the true cellular signals are sparse and weak (Figure 2E). 219 When we subtract the estimated background using CNMF-E from the raw data, we obtain a good 220 recovery of the true signal (Figure 2D.F). For comparison, we also estimate the background activity 221 by applying a rank-1 NMF model as used in basic CNMF: the resulting background-subtracted 222 image is still severely contaminated by the background (Figure 2G). This is easy to understand: the 223 spatiotemporal background signal in microendoscopic data typically has a rank higher than one. 224 due to the various signal sources indicated in Figure 1F), and therefore a rank-1 NME background 225 model is insufficient 226

A naive approach would be to simply increase the rank of the NMF background model. Figure 2H demonstrates that this approach is ineffective: higher-rank NMF does yield generally better reconstruction performance, but with high variability and low reliability (due to randomness in the initial conditions of NMF). Eventually as the NMF rank increases many single-neuronal signals of interest are swallowed up in the estimated background signal (data not shown). In contrast, CNMF-E recovers the background signal more accurately than any of the high-rank NMF models.

In real data analysis settings, the rank of NMF is an unknown and the selection of its value is a nontrivial problem. We simulated data sets with different numbers of local background sources and use a single parameter setting to run CNMF-E for reconstructing the background over multiple such simulations. Figure 2I shows that the performance of CNMF-E does not degrade quickly as we have more background sources, in contrast to rank-1 NMF. Therefore CNMF-E can recover the



Figure 2. CNMF-E can accurately separate and recover the background fluctuations in simulated data. (A) An example frame of simulated microendoscopic data formed by summing up the fluorescent signals from the multiple sources illustrated in Figure 1E. (B) A zoomed-in version of the circle in (A). The green dot indicates the pixel of interest. The surrounding black pixels are its neighbors with a distance of 15 pixels. The red area approximates the size of a typical neuron in the simulation. (C) Raw fluorescence traces of the selected pixel and some of its neighbors on the black ring. Note the high correlation. (D) Fluorescence traces (raw data; true and estimated background; true and initial estimate of neural signal) from the center pixel as selected in (B). Note that the background dominates the raw data in this pixel, but nonetheless we can accurately estimate the background and subtract it away here. Scalebars: 10 seconds. Panels (E-G) show the cellular signals in the same frame as (A). (E) Ground truth neural activity. (F) The residual of the raw frame after subtracting the background estimated with CNMF-E; note the close correspondence with E. (G) Same as (F), but the background is estimated with rank-1 NMF. A video showing (E-G) for all frames can be found at S2 Video. (H) The mean correlation coefficient (over all pixels) between the true background fluctuations and the estimated background fluctuations. The rank of NMF varies and we run randomly-initialized NMF for 10 times for each rank. The red line is the performance of CNMF-E, which requires no selection of the NMF rank. (I) The performance of CNMF-E and rank-1 NMF in recovering the background fluctuations from the data superimposed with an increasing number of background sources.

²³⁸ background accurately across a diverse range of background sources, as desired.

²³⁹ CNMF-E accurately initializes single-neuronal spatial and temporal components

Next we used simulated data to validate our proposed initialization procedure (Figure 3A). In this 240 example we simulated 200 neurons with strong spatial overlaps (Figure 3B). One of the first steps in 241 our initialization procedure is to apply a Gaussian spatial filter to the images to reduce the (spatially 242 coarser) background and boost the power of neuron-sized objects in the images. In Figure 3C, we 243 see that the local correlation image (Smith and Häusser, 2010) computed on the spatially filtered 244 data provides a good initial visualization of neuron locations; compare to Figure 1B, where the 245 correlation image computed on the raw data was highly corrupted by background signals. 246 We choose two example ROIs to illustrate how CNMF-E removes the background contamination 247 and demixes nearby neural signals for accurate initialization of neurons' shapes and activity. In the 248

- ²⁴⁹ first example, we choose a well-isolated neuron (green box, Figure 3A+B). We select three pixels
- located in the center, the periphery, and the outside of the neuron and show the corresponding



Figure 3. CNMF-E accurately initializes individual neurons' spatial and temporal components in simulated data. (**A**) An example frame of the simulated data. Green and red squares will correspond to panels (**D**) and (**E**) below, respectively. (**B**) The temporal mean of the cellular activity in the simulation. (**C**) The correlation image computed using the spatially filtered data. (**D**) An example of initializing an isolated neuron. Three selected pixels correspond to the center, the periphery, and the outside of a neuron. The raw traces and the filtered traces are shown as well. The yellow dashed line is the true neural signal of the selected neuron. Triangle markers highlight the spike times from the neuron. (**E**) Same as (**D**), but two neurons are spatially overlapping in this example. Note that in both cases neural activity is clearly visible in the filtered traces, and the initial estimates of the spatial footprints are already quite accurate (dashed lines are ground truth). (**F**) The contours of all initialized neurons on top of the correlation image as shown in (**D**). Contour colors represent the rank of neurons' SNR (SNR decreases from red to yellow). The blue dots are centers of the true neurons. (**G**) The spatial and the temporal cosine similarities between each simulated neuron and its counterpart in the initialized neuron. (blue) and other areas (green). The red lines are the thresholding boundaries for screening seed pixels in our initialization step. A video showing the whole initialization step can be found at S3 Video.

²⁵¹ fluorescence traces in both the raw data and the spatially filtered data (Figure 3D). The raw traces

are noisy and highly correlated, but the filtered traces show relatively clean neural signals. This is

²⁵³ because spatial filtering reduces the shared background activity and the remaining neural signals

²⁵⁴ dominate the filtered data. Similarly, Figure 3E is an example showing how CNMF-E demixes two

- overlapping neurons. The filtered traces in the centers of the two neurons still preserve their own
- 256 temporal activity.

After initializing the neurons' traces using the spatially filtered data, we initialize our estimate of their spatial footprints. Note that simply initializing these spatial footprints with the spatially-filtered

data does not work well (data not shown), since the resulting shapes are distorted by the spatial 259 filtering process. We found that it was more effective to initialize each spatial footprint by regressing 260 the initial neuron traces onto the raw movie data (See Methods and Materials for details). The 261 initial values already match the simulated ground truth with fairly high fidelity (Figure 3D+F). In 262 this simulated data. CNME-E successfully identified all 200 neurons and initialized their spatial and 263 temporal components (Figure 3F). We then evaluate the quality of initialization using all neurons' 264 spatial and temporal similarities with their counterparts in the ground truth data. Figure 3G shows 265 that all initialized neurons have high similarities with the truth, indicating a good recovery and 266 demixing of all neuron sources. 267

Thresholds on the minimum local correlation and the minimum peak-to-noise ratio (PNR) for 268 detecting seed pixels are necessary for defining the initial spatial components. To quantify the 269 sensitivity of choosing these two thresholds, we plot the local correlations and the PNRs of all pixels 270 chosen as the local maxima within an area of $\frac{l}{l} \times \frac{l}{l}$, where l is the diameter of a typical neuron, in 271 the correlation image or the PNR image (Figure 3H). Pixels are classified into two classes according 272 to their locations relative to the closest neurons: neurons' central areas and outside areas (see 273 Methods and Materials for full details). It is clear that the two classes are linearly well separated 274 and the thresholds can be chosen within a broad range of values (Figure 3H), indicating that the 275 algorithm is robust with respect to these threshold parameters here. In lower-SNR settings these 276 boundaries may be less clear, and an incremental approach (in which we choose the highest-SNR 277 neurons first, then estimate the background and examine the residual to select the lowest-SNR 278 cells) may be preferred; this incremental approach is discussed in more depth in the Methods and 279 Materials section. 280

CNMF-E recovers the true neural activity and is robust to noise contamination and neuronal correlations in simulated data

Using the same simulated dataset as in the previous section, we further refine the neuron shapes (*A*) and the temporal traces (*C*) by iteratively fitting the CNMF-E model. We compare the final results with PCA/ICA analysis (*Mukamel et al., 2009*) and the original CNMF method (*Pnevmatikakis et al., 2016*).

After choosing the thresholds for seed pixels (Figure 3H), we run CNMF-E in full automatic mode. 287 without any manual interventions. Two open-source MATLAB packages, CellSort 1 (Mukamel, 2016) 288 and ca source extraction ² (*Pnevmatikakis, 2016*), were used to perform PCA/ICA (*Mukamel et al.*, 289 2009) and basic CNMF (Pneymatikakis et al., 2016), respectively. Since the initialization algorithm 290 in CNMF fails due to the large contaminations from the background fluctuations in this setting 291 (recall Figure 2), we use the ground truth as its initialization. As for the rank of the background 292 model in CNMF, we tried all integer values between 1 and 16 and set it as 7 because it has the best 293 performance in matching the ground truth. We emphasize that including the CNMF approach in this 294 comparison is not fair for the other two approaches, because it uses the ground truth heavily, while 295 PCA/ICA and CNMF-E are blind to the ground truth. The purpose here is to show the limitations of 296 basic CNMF in modeling the background activity in microendoscopic data. 297

We first pick three closeby neurons from the ground truth (Figure 4A, top) and see how well 298 these neurons' activities are recovered. PCA/ICA fails to identify one neuron, and for the other 299 two identified neurons, it recovers temporal traces that are sufficiently noisy that small calcium 300 transients are submerged in the noise. As for CNME the neuron shapes remain more or less at the 301 initial condition (i.e., the ground truth spatial footprints), but clear contaminations in the temporal 302 traces are visible. This is because the pure NMF model in CNMF does not model the true background 303 well and the residuals in the background are mistakenly captured by neural components. In contrast, 30/ on this example. CNMF-F recovers the true neural shapes and neural activity with high accuracy. 305

¹https://github.com/mukamel-lab/CellSort

²https://github.com/epnev/ca_source_extraction



Figure 4. CNMF-E outperforms PCA/ICA analysis in extracting individual neurons' activity from simulated data and is robust to low SNR. (**A**) The results of PCA/ICA, CNMF, and CNMF-E in recovering the spatial footprints and temporal traces of three example neurons. The trace colors match the neuron colors shown in the left. (**B**) The intermediate residual sum of squares (RSS) values (normalized by the final RSS value), during the CNMF-E model fitting. The "refine initialization" step refers to the modification of the initialization results in the case of high temporal correlation (details in Methods and Materials). (**C**) The spatial and the temporal cosine similarities between the ground truth and the neurons detected using different methods. (**D**) The pairwise correlations between the calcium activity traces extracted using different methods. (**E**-**G**) The performances of PCA/ICA and CNMF-E under different noise levels: the number of missed neurons (**E**), and the spatial (**F**) and temporal (**G**) cosine similarities between the extracted components and the ground truth. (**H**) The calcium traces of one example neuron: the ground truth (black), the PCA/ICA trace (blue), the CNMF-E trace (red) and the CNMF-E trace without being denoised (cyan). The similarity values shown in the figure are computed as the cosine similarity between each trace and the ground truth (black). Two videos showing the demixing results of the simulated data can be found in S4 Video (SNR reduction factor=1) and S5 Video (SNR reduction factor=6).

We also compare the number of detected neurons: PCA/ICA detected 195 out of 200 neurons, while CNMF-E detected all 200 neurons. We also quantitatively evaluated the performance of source extraction by showing the spatial and temporal cosine similarities between detected neurons and ground truth (Figure 4C); we find that the neurons detected using PCA/ICA have much lower similarities with the ground truth (Figure 4C). We also note that the CNMF results are much worse



Figure 5. CNMF-E is able to demix neurons with high temporal correlations. (**A**) An example simulation from the experiments summarized in panel (**B**), where $corr(c_1, c_2)$ is 0.9: green and red traces correspond to the corresponding neuronal shapes in the left panels. The blue trace is the mean background fluorescence fluctuation over the whole FOV. (**B**) The extraction accuracy of the spatial (a_1 and a_2) and the temporal (c_1 and c_2) components of two close-by neurons, computed via the cosine similarity between the ground truth and the extraction results.

than those of CNMF-E here, despite the fact that CNMF is initialized at the ground truth parameter
values. This result clarifies an important point: the improvements from CNMF-E are not simply
due to improvements in the initialization step. Furthermore, running the full iterative pipeline of
CNMF-E leads to improvements in both spatial and temporal similarities, compared with the results
in the initialization step.
In many downstream analyses of calcium imaging data, pairwise correlations provide an important metric to study coordinated network activity (*Warp et al., 2012; Barbera et al., 2016; Dombeck*

et al., 2009; Klaus et al., 2017). Since PCA/ICA seeks statistically independent components, which forces the temporal traces to have near-zero correlation, the correlation structure is badly corrupted in the raw PCA/ICA outputs (Figure 4D). We observed that a large proportion of the independence

₃₂₁ comes from the noisy baselines in the extracted traces (data not shown), so we postprocessed

the PCA/ICA output by thresholding at the 3 standard deviation level. This recovers some nonzero correlations, but the true correlation structure is not recovered accurately (Figure 4D). By contrast, the CNMF-E results matched the ground truth very well due to accurate extraction of individual neurons' temporal activity (Figure 4D). As for CNMF, the estimated correlations are slightly elevated relative to the true correlations. This is due to the shared (highly correlated) background fluctuations that corrupt the recovered activity of nearby neurons.

Next we compared the performance of the different methods under different SNR regimes 328 Because of the above inferior results we skip comparisons to the basic CNMF here. Based on 329 the same simulation parameters as above, we vary the noise level Σ by multiplying it with a SNR 330 reduction factor. Figure 4E shows that CNMF-E detects all neurons over a wide SNR range, while 331 PCA/ICA fails to detect the majority of neurons when the SNR drops to sufficiently low levels. 332 Moreover, the detected neurons in CNMF-E preserve high spatial and temporal similarities with 333 the ground truth (Figure 4F-G). This high accuracy of extracting neurons' temporal activity benefits 334 from the modeling of the calcium dynamics, which leads to significantly denoised neural activity 335 If we skip the temporal denoising step in the algorithm, CNMF-F is less robust to noise, but still 336 outperforms PCA/ICA significantly (Figure 4G). When SNR is low, the improvements yielded by 337 CNMF-F can be crucial for detecting weak neuron events, as shown in Figure 4H. 338

Finally, we examine the ability of CNMF-E to demix correlated and overlapping neurons. Using 339 the two example neurons in Figure 3E, we ran multiple simulations at varying correlation levels 340 and extracted neural components using the CNMF-E pipeline and PCA/ICA analysis. The spatial 341 footprints in these simulations were fixed, but the temporal components were varied to have 342 different correlation levels (γ) between calcium traces by tuning their shared component with the 343 common background fluctuations. For high correlation levels ($\gamma > 0.7$), the initialization procedure 344 tends to first initialize a component that explains the common activity between two neurons and 345 then initialize another component to account for the residual of one neuron. After iteratively refining 346 the model variables. CNMF-F successfully extracted the two neurons' spatiotemporal activity even 347 at very high correlation levels ($\gamma = 0.95$; Figure 5A,B). PCA/ICA was also often able to separate two 348 neurons for large correlation levels ($\gamma = 0.9$. Figure 5B), but the extracted traces have problematic 349 negative spikes that serve to reduce their statistical dependences (Figure 4A). 350

351 Application to dorsal striatum data

We now turn to the analysis of large-scale microendoscopic datasets recorded from freely behaving 352 mice. We run both CNME-E and PCA/ICA for all datasets and compare their performances in detail. 353 We begin by analyzing in vivo calcium imaging data of neurons expressing GCaMP6f in the 354 mouse dorsal striatum. (Full experimental details and algorithm parameter settings for this and the 355 following datasets appear in the Methods and Materials section.) CNMF-E extracted 692 putative 356 neural components from this dataset; PCA/ICA extracted 547 components (starting from 700 initial 357 components, and then automatically removing false positives using the same criterion as applied in 358 CNME-F). Figure 6A shows how CNME-F decomposes an example frame into four components: the 350 constant baselines that are invariant over time, the fluctuating background, the denoised neural 360 signals, and the residuals. We highlight an example neuron by drawing its ROI to demonstrate the 361 power of CNMF-E in isolating fluorescence signals of neurons from the background fluctuations 362 For the selected neuron, we plot the mean fluorescence trace of the raw data and the estimated 363 background (Figure 6B). These two traces are very similar, indicating that the background fluctuation 364 dominates the raw data. By subtracting this estimated background component from the raw data. 365 we acquire a clean trace that represents the neural signal. 366

To quantify the background effects further, we compute the contribution of each signal component in explaining the variance in the raw data. For each pixel, we compute the variance of the raw data first and then compute the variance of the background-subtracted data. Then the reduced variance is divided by the variance of the raw data, giving the proportion of variance explained by the background. Figure 6C (blue) shows the distribution of the background-explained variance



Figure 6. Neurons expressing GCaMP6f recorded *in vivo* in mouse dorsal striatum area. (**A**) An example frame of the raw data and its four components decomposed by CNMF-E. (**B**) The mean fluorescence traces of the raw data (black), the estimated background activity (blue), and the background-subtracted data (red) within the segmented area (red) in (**A**). The variance of the black trace is about 2x the variance of the blue trace and 4x the variance of the red trace. (**C**) The distributions of the variance explained by different components over all pixels; note that estimated background signals dominate the total variance of the signal. (**D**) The contour plot of all neurons detected by CNMF-E and PCA/ICA superimposed on the correlation image. Green areas represent the components that are only detected by CNMF-E. The components of 14 example neurons that are only detected by CNMF-E. The components of 14 example neurons that are only detected by CNMF-E. The spatial and temporal components of 14 example neurons that are only detected by CNMF-E. The spatial and temporal components of 14 example neurons that are only detected by CNMF-E. The satisf is increased in (**C**), which shows the spatial and temporal components of 14 example neurons that are only detected by CNMF-E. These neurons all correspond to green areas in (**D**). (**F**) The signal-to-noise ratios (SNRs) of all neurons detected by both methods. Colors match the example traces shown in (**G**), which shows the spatial and temporal components of 10 example neurons detected by both methods. Scalebar: 10 seconds. See S6 Video for the demixing results.

over all pixels. The background accounts for around 90% of the variance on average. We further

³⁷³ remove the denoised neural signals and compute the variance reduction; Figure 6C shows that

³⁷⁴ neural signals account for less than 10% of the raw signal variance. This analysis is consistent with

our observations that background dominates the fluorescence signal and extracting high-quality

³⁷⁶ neural signals requires careful background signal removal.

The contours of the spatial footprints inferred by the two approaches (PCA/ICA and CNMF-E) are depicted in Figure 6D, superimposed on the correlation image of the filtered raw data. The indicated area was cropped from Figure 6A (left). In this case, most neurons inferred by PCA/ICA

were inferred by CNMF-E as well, with the exception of a few components that seemed to be false 380 positives (judging by their spatial shapes and temporal traces and visual inspection of the raw 38 data movie: detailed data not shown). However, many realistic components were only detected by 382 CNMF-F (shown as the green areas in Figure 6D). In these plots, we rank the inferred components 383 according to their SNRs: the color indicates the relative rank (decaying from red to yellow). We see 384 that the components missed by PCA/ICA have low SNRs (green shaded areas with vellow contours). 385 Figure 6E shows the spatial and temporal components of 14 example neurons detected only by 386 CNMF-E. Here (and in the following figures), for illustrative purposes, we show the calcium traces 387 before the temporal denoising step. For neurons that are inferred by both methods, CNMF-E shows 388 significant improvements in the SNR of the extracted cellular signals (Figure 6F), even before the 389 temporal denoising step is applied. In panel G we randomly select 10 examples and examine their 390 spatial and temporal components. Compared with the CNMF-E results. PCA/ICA components have 391 much smaller size, often with negative dips surrounding the neuron (remember that ICA avoids 392 spatial overlaps in order to reduce nearby neurons' statistical dependences, leading to some loss of 393 signal strength: see (*Pneymatikakis et al.*, 2016) for further discussion). The activity traces extracted 394 by CNMF-E are visually cleaner than the PCA/ICA traces: this is important for reliable event detection. 395 particularly in low SNR examples. See (Klaus et al., 2017) for additional examples of CNMF-E applied 396 to striatal data. 397

³⁹⁸ Application to data in prefrontal cortex

We repeat a similar analysis on GCaMP6s data recorded from prefrontal cortex (PFC, Figure 7). 390 to quantify the performance of the algorithm in a different brain area with a different calcium 400 indicator. Again we find that CNMF-E successfully extracts neural signals from a strong fluctuating 401 background (Figure 7A), which contributes a large proportion of the variance in the raw data (Figure 402 7B). Similarly as with the striatum data. PCA/ICA analysis missed many components that have 403 very weak signals (33 missed components here). For the matched neurons, CNMF-F shows strong 404 improvements in the SNRs of the extracted traces (Figure 7D). Consistent with our observation in 405 striatum (Figure 6G), the spatial footprints of PCA/ICA components are shrunk to promote statistical 406 independence between neurons, while the neurons inferred by CNMF-E have visually reasonable 407 morphologies (Figure 6F). As for calcium traces with high SNRs (Figure 7F, cell 1 - 6). CNMF-F traces 408 have smaller noise values, which is important for detecting small calcium transients (Figure 7F, cell 409 4). For traces with low SNRs (Figure 7, cell 7 – 10), it is challenging to detect any calcium events 410 from the PCA/ICA traces due to the large poise variance: CNME-E is able to visually recover many of 411 these weaker signals. For those cells missed by PCA/ICA, their traces extracted by CNME-E have 412 reasonable morphologies and visible calcium events (Figure 7F). 413

The demixing performance of PCA/ICA analysis can be relatively weak because it is inherently a 414 linear demixing method (*Pnevmatikakis et al., 2016*). Since CNMF-E uses a more suitable nonlinear 415 matrix factorization method, it has a better capability of demixing spatially overlapping neurons. 416 As an example. Figure 7G shows three closeby neurons identified by both CNMF-E and PCA/ICA 417 analysis. PCA/ICA forces its obtained filters to be spatially separated to reduce their dependence 418 (thus reducing the effective signal strength), while CNME-E allows inferred spatial components 419 to have large overlaps (Figure 7G, left), retaining the full signal power. In the traces extracted 420 by PCA/ICA, the component labeled in green contains many negative "spikes," which are highly 421 correlated with the spiking activity of the blue neuron (Figure 7G, yellow). In addition, the green 422 PCA/ICA neuron has significant crosstalk with the red neuron due to the failure of signal demixing 423 (Figure 7G, cvan); the CNMF-E traces shows no comparable negative "spikes" or crosstalk. See also 474 S8 Video for further details. 425

426 Application to ventral hippocampus neurons

⁴²⁷ In the previous two examples, we analyzed data with densely packed neurons, in which the neuron ⁴²⁸ sizes are all similar. In the next example, we apply CNMF-E to a dataset with much sparser and



Figure 7. Neurons expressing GCaMP6s recorded *in vivo* in mouse prefrontal cortex. (**A**-**F**) follow similar conventions as in the corresponding panels of Figure 6. (**G**) Three example neurons that are close to each other and detected by both methods. Yellow shaded areas highlight the negative 'spikes' correlated with nearby activity, and the cyan shaded area highlights one crosstalk between nearby neurons. Scalebar: 20 seconds. See S7 Video for the demixing results and S8 Video for the comparision of CNMF-E and PCA/ICA in the zoomed-in area of (**G**).

more heterogeneous neural signals. The data used here were recorded from amygdala-projecting
neurons expressing GCaMP6f in ventral hippocampus. In this dataset, some neurons that are
slightly above or below the focal plane were visible with prominent signals, though their spatial
shapes are larger than neurons in the focal plane.

This example is somewhat more challenging due to the large diversity of neuron sizes. It is 433 possible to set multiple parameters to detect neurons of different sizes (or to e.g. differentially 434 detect somas versus smaller segments of axons or dendrites passing through the focal plane), 435 but for illustrative purposes here we use a single neural size parameter to initialize all of the 436 components. This in turn splits some large neurons into multiple components. Following this 437 crude initialization step, we updated the background component and then picked the missing 438 neurons from the residual using a second greedy component initialization step. Next we ran CNMF-439 E for three iterations of updating the model variables A, C, and B. The first two iterations were 440 performed automatically; we included manual interventions (e.g., merging/deleting components) 441 before the last iteration, leading to improved source extraction results (see S10 Video for details 442



Figure 8. Neurons expressing GCaMP6f recorded *in vivo* in mouse ventral hippocampus. (**A**) Contours of all neurons detected by CNMF-E (red) and PCA/ICA method (green). The grayscale image is the local correlation image of the background-subtracted video data, with background estimated using CNMF-E. (**B**) Spatial components of all neurons detected by CNMF-E. The neurons in the first three rows are also detected by PCA/ICA, while the neurons in the last row are only detected by CNMF-E. (**C**) Spatial components of all neurons detected by PCA/ICA; similar to (**B**), the neurons in the first three rows are also detected by CNMF-E and the neurons in the last row are only detected by PCA/ICA method. (**D**) Temporal traces of all detected components in (**B**). 'Match' indicates neurons in top three rows in panel (**B**); 'Other' indicates neurons in the fourth row. (**E**) Temporal traces of all components in (**C**). Scalebars: 20 seconds. See S9 Video for demixing results.

on the manual merge and delete interventions performed here). In this example, we detected 24 443 CNMF-E components and 24 PCA/ICA components. The contours of these inferred neurons are 444 shown in Figure 8A. In total we have 20 components detected by both methods (shown in the first 445 three rows of Figure 8B+C); each method detected extra components that are not detected by the 446 other (the last rows of Figure 8B+C). Once again, the PCA/ICA filters contain many negative pixels in 447 an effort to reduce spatial overlaps; see components 3 and 5 in Figure 8A-C, for example. All traces 448 of the inferred neurons are shown in Figure 8D+E. We can see that the CNMF-E traces have much 449 lower noise level and cleaner neural signals in both high and low SNR settings. Conversely, the 450 calcium traces of the 3 extra neurons identified by PCA/ICA show noisy signals that are unlikely to 451 be neural responses. 452

453 Application to footshock responses in the bed nucleus of the stria terminalis (BNST)

Identifying neurons and extracting their temporal activity is typically just the first step in the analysis 454 of calcium imaging data; downstream analyses rely heavily on the quality of this initial source 455 extraction. We showed above that, compared to PCA/ICA, CNMF-E is better at extracting activity 456 dynamics, especially in regimes where neuronal activities are correlated (c.f. Figure 4D). Using 457 in vivo electrophysiological recordings, we previously showed that neurons in the bed nucleus of 458 the stria terminalis (BNST) show strong responses to unpredictable footshock stimuli (Jennings 459 et al., 2013). We therefore measured calcium dynamics in CaMKII-expressing neurons that were 460 transfected with the calcium indicator GCaMP6s in the BNST and analyzed the synchronous activity 461 of multiple neurons in response to unpredictable footshock stimuli. We chose 12 example neurons 462 that were detected by both CNMF-E and PCA/ICA methods and show their spatial and temporal 463



Figure 9. Neurons extracted by CNMF-E show more reproducible responses to footshock stimuli, with larger signal sizes relative to the across-trial variability, compared to PCA/ICA. (**A-C**) Spatial components (**A**), spatial locations (**B**) and temporal components (**C**) of 12 example neurons detected by both CNMF-E and PCA/ICA. (**D**) Calcium responses of all example neurons to footshock stimuli. Colormaps show trial-by-trial responses of each neuron, extracted by CNMF-E (top, red) and PCA/ICA (bottom, green), aligned to the footshock time. The solid lines are medians of neural responses over 11 trials and the shaded areas correpond to median ± 1 median absolute deviation (MAD). Dashed lines indicate the shock timings. (**E**) Scatter plot of peak-to-MAD ratios for all response curves in (**D**). For each neuron, Peak is corrected by subtracting the mean activity within 4 seconds prior to stimulus onset and MAD is computed as the mean MAD values over all timebins shown in (**D**). The red line shows y = x. Scalebars: 10 seconds. See S11 Video for demixing results.

⁴⁶⁴ components in Figure 9A-C. The activity around the onset of the repeated stimuli are aligned and

shown as pseudo-colored images in panel D. The median responses of CNMF-E neurons display

⁴⁶⁶ prominent responses to the footshock stimuli compared with the resting state before stimuli onset.

⁴⁶⁷ In comparison, the activity dynamics extracted by PCA/ICA have relatively low SNR, making it more

challenging to reliably extract footshock responses. Panel E summarizes the results of panel D;

we see that CNMF-E outputs significantly more easily detectable responses than does PCA/ICA. This is an example in which downstream analyses of calcium imaging data can significantly benefit

This is an example in which downstream analyses of calcium imaging data can significantly benefit from the improvements in the accuracy of source extraction offered by CNMF-F. (*Sheintuch et al.*)

(2017) recently presented another such example, showing that more neurons can be tracked across

⁴⁷³ multiple days using CNMF-E outputs, compared to PCA/ICA.)

474 Conclusion

Microendoscopic calcium imaging offers unique advantages and has quickly become a critical 475 method for recording large neural populations during unrestrained behavior. However, previous 476 methods fail to adequately remove background contaminations when demixing single neuron 477 activity from the raw data. Since strong background signals are largely inescapable in the context of 478 one-photon imaging, insufficient removal of the background could yield problematic conclusions 479 in downstream analysis. This has presented a severe and well-known bottleneck in the field. We 480 have delivered a solution for this critical problem, building on the constrained nonnegative matrix 481 factorization framework introduced in **Pneymatikakis et al.** (2016) but significantly extending it in 482 order to more accurately and robustly remove these contaminating background components. 483

The proposed CNMF-F algorithm can be used in either automatic or semi-automatic mode, and 484 leads to significant improvements in the accuracy of source extraction compared with previous 485 methods. In addition, CNMF-E requires very few parameters to be specified, and these parameters 48F are easily interpretable and can be selected within a broad range. We demonstrated the power 487 of CNMF-E using data from a wide diversity of brain areas (subcortical, cortical, and deep brain 488 areas). SNR regimes, calcium indicators, neuron sizes and densities, and hardware setups. Among 489 all these examples (and many others not shown here). CNME-E performs well and improves 490 significantly on the standard PCA/ICA approach. Considering that source extraction is typically 491 just the first step in calcium imaging data analysis pipelines (Mohammed et al., 2016), these 492 improvements should in turn lead to more stable and interpretable results from downstream 493 analyses. Further applications of the CNMF-E approach appear in (*Cameron et al., 2016: Donghue* 494 and Kreitzer, 2017. limenez et al., 2016, 2017. Klaus et al., 2017. Lin et al., 2017. Murugan et al., 495 2016, 2017; Rodriguez-Romaguera et al., 2017; Tombaz et al., 2016; Ung et al., 2017; Yu et al., 2017; 496 Mackevicius et al., 2017: Madangopal et al., 2017: Roberts et al., 2017: Rvan et al., 2017: Roberts 497 et al., 2017; Sheintuch et al., 2017). 498

We have released our MATLAB implementation of CNMF-E as open-source software (https: //github.com/zhoupc/CNMF_E (*Zhou*, *2017a*)). A Python implementation has also been incorporated into the CalmAn toolbox (*Giovannucci et al.*, *2017b*). We welcome additions or suggestions for modifications of the code, and hope that the large and growing microendoscopic imaging community finds CNMF-E to be a helpful tool in furthering neuroscience research.

504 Methods and Materials

⁵⁰⁵ Algorithm for solving problem (P-S)

In problem (P-S), \mathbf{b}_0 is unconstrained and can be updated in closed form: $\hat{\mathbf{b}}_0 = \frac{1}{T} (\tilde{Y} - A \cdot \hat{C} - \hat{B}^f) \cdot \mathbf{1}$. By plugging this update into problem (P-S), we get a reduced problem

$$\begin{array}{ll} \underset{A}{\text{minimize}} & \|\tilde{Y} - A \cdot \tilde{C}\|_F^2 & (\text{P-S'}) \\ \text{subject to} & A \geq 0, \text{ A is local and sparse,} \end{array}$$

where $\tilde{Y} = Y - \hat{B}^f - \frac{1}{T}Y\mathbf{1}\mathbf{1}^T$ and $\tilde{C} = \hat{C} - \frac{1}{T}\hat{C}\mathbf{1}\mathbf{1}^T$. We approach this problem using a version of "hierarchical alternating least squares" (HALS; *Cichocki et al.* (2007)), a standard algorithm for nonnegative matrix factorization. *Friedrich et al.* (2017b) modified the fastHALS algorithm (*Cichocki and Phan, 2009*) to estimate the nonnegative spatial components *A*, *b* and the nonnegative temporal

activity *C*, *f* in the CNMF model $Y = A \cdot C + bf^T + E$ by including sparsity and localization constraints. We solve a problem similar to the subproblem solved in *Friedrich et al.* (2017b):

$$\begin{array}{ll} \underset{A}{\text{minimize}} & \|\tilde{Y} - A \cdot \tilde{C}\|_{F}^{2} & (\text{P-S''}) \\ \text{subject to} & A \ge 0 \\ & A(i,k) = 0 \; \forall \; \mathbf{x}_{i} \notin P_{k} \end{array}$$

where P_k denotes the the spatial patch constraining the nonzero pixels of the *k*-th neuron and restricts the candidate spatial support of neuron *k*. This regularization reduces the number of free parameters in *A*, leading to speed and accuracy improvements. The spatial patches can be determined using a mildly dilated version of the support of the previous estimate of *A* (*Pnevmatikakis et al., 2016; Friedrich et al., 2017a*).

Algorithms for solving problem (P-T)

In problem (P-T), the model variable $C \in \mathbb{R}^{K \times T}$ could be very large, making the direct solution of (P-T) 512 computationally expensive. Unlike problem (P-S), the problem (P-T) cannot be readily parallelized 513 because the constraints $G^{(i)}c_i > 0$ couple the entries within each row of C, and the residual term 514 couples entries across columns. Here, we follow the block coordinate-descent approach used in 515 (*Pnevmatikakis et al., 2016*) and propose an algorithm that sequentially updates each c_i and b_{0} . 516 For each neuron, we start with a simple unconstrained estimate of c_i , denoted as \hat{y}_i , that minimizes 517 the residual of the spatiotemporal data matrix while fixing other neurons' spatiotemporal activity 518 and the baseline term b_0 , 519

$$\hat{\boldsymbol{y}}_{i} = \underset{c_{i} \in \mathbb{R}^{T}}{\operatorname{argmin}} \|\boldsymbol{Y} - \hat{\boldsymbol{A}}_{\backslash i} \cdot \hat{\boldsymbol{C}}_{\backslash i} - \hat{\boldsymbol{a}}_{i} \boldsymbol{c}_{i} - \hat{\boldsymbol{b}}_{0} \cdot \boldsymbol{1}^{T} - \hat{\boldsymbol{B}}^{f} \|_{F}^{2} = \hat{\boldsymbol{c}}_{i} + \frac{\hat{\boldsymbol{a}}_{i}^{T} \cdot \boldsymbol{Y}_{\mathsf{res}}}{\hat{\boldsymbol{a}}_{i}^{T} \hat{\boldsymbol{a}}_{i}}, \tag{9}$$

where $Y_{\text{res}} = Y - \hat{A}\hat{C} - \hat{b}_0 \mathbf{1}^T - B^f$ represents the residual given the current estimate of the model 520 variables. Due to its unconstrained nature, \hat{y}_i is a noisy estimate of c_i , plus a constant baseline 521 resulting from inaccurate estimation of b_0 . Given \hat{y}_i , various deconvolution algorithms can be applied 522 to obtain the denoised trace \hat{c}_i and deconvolved signal \hat{s}_i (Vogelstein et al., 2009; Pnevmatikakis 523 et al., 2013; Deneux et al., 2016; Friedrich et al., 2017b; Jewell and Witten, 2017); in CNMF-E, we use 524 the OASIS algorithm from (*Friedrich et al., 2017b*). (Note that the estimation of c_i is not dependent 525 on accurate estimation of b_0 , because the algorithm for estimating c_i will also automatically estimate 526 the baseline term in \hat{y}_{i} .) After the c_i 's are updated, we update b_0 using the closed-form expression 527 $\hat{\boldsymbol{b}}_0 = \frac{1}{T} (\tilde{Y} - \hat{A} \cdot \hat{C} - \hat{B}^f) \cdot \boldsymbol{1}.$ 528

529 Estimating background by solving problem (P-B)

Next we discuss our algorithm for estimating the spatiotemporal background signal by solving problem (P-B) as a linear regression problem given \hat{A} and \hat{C} . Since $B^f \cdot \mathbf{1} = \mathbf{0}$, we can easily estimate

532 the constant baselines for each pixel as

$$\hat{\boldsymbol{b}}_0 = \frac{1}{T} (Y - \hat{A} \cdot \hat{C}) \cdot \boldsymbol{1}. \tag{10}$$

Next we replace the b_0 in (P-B) with this estimate and rewrite (P-B) as

$$\begin{array}{ll} \underset{W}{\text{minimize}} & \|X - W \cdot X\|_{F}^{2}, \\ \text{subject to} & W_{ii} = 0 \text{ if } \text{dist}(\boldsymbol{x}_{i}, \boldsymbol{x}_{i}) \notin [l_{n}, l_{n} + 1), \end{array}$$

where $X = Y - \hat{A} \cdot \hat{C} - \hat{b}_0 \mathbf{1}^T$. Given the optimized \hat{W} , our estimation of the fluctuating background is

 $\hat{B}^f = \hat{W} X$. The new optimization problem (P-W) can be readily parallelized into d linear regression

problems for each pixel separately. By estimating all row columns of W_{i} , we are able to obtain the

⁵³⁶ whole background signal as

$$\hat{\boldsymbol{B}} = \hat{\boldsymbol{W}}\boldsymbol{X} + \hat{\boldsymbol{b}}_0 \boldsymbol{1}^T. \tag{11}$$

In some cases, *X* might include large residuals from the inaccurate estimation of the neurons' spatiotemporal activity *AC*, e.g., missing neurons in the estimation. These residuals act as outliers and distort the estimation of \hat{B}^f and b_0 . To overcome this problem, we use robust least squares regression (RLSR) via hard thresholding to avoid contaminations from the outliers (*Bhatia et al.*, **2015**). Before solving the problem (P-W), we compute $B^- = \hat{W}(Y - \hat{A} \cdot \hat{C} - \hat{b}_0 \mathbf{1}^T)$ (the current estimate of the fluctuating background) and then apply a simple clipping preprocessing step to *X*:

$$X_{it}^{clipped} = \begin{cases} B_{it}^{-} & \text{if } X_{it} \ge B_{it}^{-} + \zeta \cdot \sigma_i \\ X_{it} & \text{otherwise} \end{cases}$$
(12)

Then we update the regression estimate using $X^{clipped}$ instead of X, and iterate. Here σ_i is the 543 standard deviation of the noise at x_{i} and its value can be estimated using the power spectral density 54/ (PSD) method (Pnevmatikakis et al., 2016). As for the first iteration of the model fitting, we set 549 each $B_{it}^- = \frac{1}{|\Omega_i|} \sum_{j \in \Omega_i} \tilde{X}_{jt}$ as the mean of the \tilde{X}_{jt} for all $j \in \Omega_i$. The thresholding coefficient ζ can be 546 specified by users, though we have found a fixed default works well across the datasets used here. 547 This preprocessing removes most calcium transients by replacing those frames with the previously 548 estimated background only. As a result, it increases the robustness to inaccurate estimation of AC. 549 and in turn leads to a better extraction of AC in the following iterations. 550

551 Initialization of model variables

Since problem (P-All) is not convex in all of its variables, a good initialization of model variables is 552 crucial for fast convergence and accurate extraction of all neurons' spatiotemporal activity. Previous 553 methods assume the background component is relatively weak, allowing us to initialize \hat{A} and \hat{C} 554 while ignoring the background or simply initializing it with a constant baseline over time. However, 555 the noisy background in microendoscopic data fluctuates more strongly than the neural signals (c.f. 556 Figure 6C and Figure 7B), which makes previous methods less valid for the initialization of CNME-F. 557 Here we design a new algorithm to initialize \hat{A} and \hat{C} without estimating \hat{B} . The whole procedure 558 is illustrated in Figure 10 and described in Algorithm 1. The key aim of our algorithm is to exploit 559 the relative spatial smoothness in the background compared to the single neuronal signals visible 560 in the focal plane. Thus we can use spatial filtering to reduce the background in order to estimate 561 single neurons' temporal activity, and then initialize each neuron's spatial footprint given these 562 temporal traces. Once we have initialized \hat{A} and \hat{C} , it is straightforward to initialize the constant 563 baseline b_0 and the fluctuating background B^f by solving problem (P-B). 56/

565 Spatially filtering the data

⁵⁶⁶ We first filter the raw video data with a customized image kernel (Figure 10A). The kernel is generated

567 from a Gaussian filter

$$h(\mathbf{x}) = \exp\left(-\frac{\|\mathbf{x}\|^2}{2(l/4)^2}\right).$$
 (13)

Here we use h(x) to approximate a cell body: the factor of 1/4 in the Gaussian width is chosen to 568 match a Gaussian shape to a cell of width *l*. Instead of using h(x) as the filtering kernel directly. 569 we subtract its spatial mean (computed over a region of width equal to /) and filter the raw data 570 with $\tilde{h}(\mathbf{x}) = h(\mathbf{x}) - \bar{h}(\mathbf{x})$. The filtered data is denoted as $Z \in \mathbb{R}^{d \times T}$ (Figure 10B). This spatial filtering 571 step helps accomplish two goals: (1) reducing the background B, so that Z is dominated by neural 572 signals (albeit somewhat spatially distorted) in the focal plane (see Figure 10B as an example); (2) 573 performing a template matching to detect cell bodies similar to the Gaussian kernel. Consequently, 574 Z has large values near the center of each cell body. (However, note that we can not simply e.g. 575 apply CNMF to Z, because the spatial components in a factorization of the matrix Z will typically 576 no longer be nonnegative, and therefore NMF-based approaches can not be applied directly.) More importantly, the calcium traces near the neuron center in the filtered data preserve the calcium 578 activity of the corresponding neurons because the filtering step results in a weighted average of 579 cellular signals surrounding each pixel (Figure 10B). Thus the fluorescence traces in pixels close to 580



Figure 10. Illustration of the initialization procedure. (**A**) Raw video data and the kernel for filtering the video data. (**B**) The spatially high-pass filtered data. (**C**) The local correlation image and the peak-to-noise ratio (PNR) image calculated from the filtered data in (**B**). (**D**) The temporal correlation coefficients between the filtered traces (**B**) of the selected seed pixel (the red cross) and all other pixels in the cropped area as shown in (**A-C**). The red and green contour correspond to correlation coefficients equal to 0.7 and 0.3 respectively. (**E**) The estimated background fluctuation $y_{BG}(t)$ (green) and the initialized temporal trace $\hat{c}_i(t)$ of the neuron (red). $y_{BG}(t)$ is computed as the median of the raw fluorescence traces of all pixels (green area) outside of the green contour. (**F**) The decomposition of the raw video data within the cropped area. Each component is a rank-1 matrix and the related temporal traces are estimated in (**E**). The spatial components are estimated by regressing the raw video data against these three traces. See S3 Video for an illustration of the initialization procedure.

neuron centers in Z can be used for initializing the neurons' temporal activity directly. These pixels
 are defined as seed pixels. We next propose a quantitative method to rank all potential seed pixels.

583 Ranking seed pixels

A seed pixel x should have two main features: first, Z(x), which is the filtered trace at pixel x, should have high peak-to-noise ratio (PNR) because it encodes the calcium concentration c_i of one neuron; second, a seed pixel should have high temporal correlations with its neighboring pixels (e.g., 4 nearest neighbors) because they share the same c_i . We computed two metrics for each of these two features:

$$P(\mathbf{x}) = \frac{\max_t(Z(\mathbf{x},t))}{\sigma(\mathbf{x})}, \ L(\mathbf{x}) = \frac{1}{4} \sum_{\text{dist}(\mathbf{x},\mathbf{x}')=1} \text{corr}\Big(Z(\mathbf{x}), Z(\mathbf{x}')\Big).$$
(14)

Recall that $\sigma(\mathbf{x})$ is the standard deviation of the noise at pixel \mathbf{x} ; the function **corr**() refers to Pearson correlation here. In our implementation, we usually threshold $Z(\mathbf{x})$ by $3\sigma(\mathbf{x})$ before computing $L(\mathbf{x})$ to reduce the influence of the background residuals, noise, and spikes from nearby neurons.

Most pixels can be ignored when selecting seed pixels because their local correlations or PNR 592 values are too small. To avoid unnecessary searches of the pixels, we set thresholds for both 593 $P(\mathbf{x})$ and $L(\mathbf{x})$, and only pick pixels larger than the thresholds P_{\min} and L_{\min} . It is empirically useful 594 to combine both metrics for screening seed pixels. For example, high PNR values could result 595 from large noise, but these pixels usually have small L(x) because the noise is not shared with 596 neighboring pixels. On the other hand, insufficient removal of background during the spatial 597 filtering leads to high L(x), but the corresponding P(x) are usually small because most background 598 fluctuations have been removed. So we create another matrix $R(x) = P(x) \cdot L(x)$ that computes the 599 pixelwise product of P(x) and L(x). We rank all R(x) in a descending order and choose the pixel x^* 600 with the largest R(x) for initialization. 601

Algorithm 1 Initialize model variables A and C given the raw data

Require: data $Y \in \mathbb{R}^{d \times T}$, neuron size <i>l</i> , the minimum local	l correlation L_{min} and the minimum PNR P_{min} for
selecting seed pixels.	
1: $h \leftarrow$ a truncated 2D Gaussian kernel of width $\sigma_x = \sigma_y = \frac{1}{4}$; $h \in \mathbb{R}^{l \times l}$ ▷ 2D Gaussian kernel
2: $\tilde{h} \leftarrow h - \bar{h}; \ \tilde{h} \in \mathbb{R}^{l \times l}$	mean-centered kernel for spatial filtering
3: $Z \leftarrow \text{conv}(Y, h); Z \in \mathbb{R}^{d \times T}$	spatially filter the raw data
4: $L \leftarrow$ local cross-correlation image of the filtered data Z ;	$L \in \mathbb{R}^d$
5: $P \leftarrow PNR$ image of the filtered data $Z; P \in \mathbb{R}^d$	
6: $k \leftarrow 0$	⊳ neuron number
7: while True do	
8: if $L(\mathbf{x}) \le L_{min}$ or $P(\mathbf{x}) \le P_{min}$ for all pixel \mathbf{x} then	
9: break;	
10: else	
11: $k \leftarrow k+1$	
12: $\hat{a}_k \leftarrow 0; \mathbf{a} \in \mathbb{R}^d$	
13: $\mathbf{x}^* \leftarrow \operatorname{argmax}_{\mathbf{x}}(L(\mathbf{x}) \cdot P(\mathbf{x}))$	select a seed pixel
14: $\Omega_k \leftarrow \{x x \text{ is in the square box of length } (2l+1) \text{ su}$	urrounding pixel x^* > crop a small box near x^*
15: $\mathbf{r}(\mathbf{x}) \leftarrow \operatorname{corr}(Z(\mathbf{x}, :), Z(\mathbf{x}^*, :))$ for all $\mathbf{x} \in \Omega_k$; $\mathbf{r} \in \mathbb{R}^{ \Omega }$	k
16: $\mathbf{y}_{BG} \leftarrow \frac{\sum_{\{\mathbf{x} \mid \mathbf{r}(\mathbf{x}) \le 0.3\}} Y(\mathbf{x},:)}{\sum_{\{\mathbf{x} \mid \mathbf{r}(\mathbf{x}) \le 0.3\}} 1}; \ \mathbf{y}_{BG} \in \mathbf{R}^T$	▷ estimate the background signal
17: $\hat{c}_k \leftarrow \frac{\sum_{\{\mathbf{x}\mid \mathbf{x}(\mathbf{x}) \ge 0.7\}} Z(\mathbf{x},:)}{\sum_{\{\mathbf{x}\mid \mathbf{r}(\mathbf{x}) \ge 0.7\}} 1}; \ \hat{c}_k \in \mathbf{R}^T$	⊳ estimate neural signal
18: $\hat{a}_k(\Omega_k), \hat{b}^{(f)}, \hat{b}^{(0)} \leftarrow \operatorname{argmin}_{a, b^{(f)}, b^{(0)}} Y_{\Omega_k} - (a \cdot \hat{c}_k^T + b^{(f)}) Y_{\Omega_k} $	$(f) \cdot \boldsymbol{y}_{BG}^T + \boldsymbol{b}^{(0)} \cdot \boldsymbol{1}^T) \ _F^2$
19: $\hat{a}_k \leftarrow \max(0, \hat{a}_k)$	\triangleright the spatial component of the k-th neuron
20: $Y \leftarrow Y - \hat{a}_k \cdot \hat{c}_k^T$	> peel away the neuron's spatiotemporal activity
21: update $L(\mathbf{x})$ and $P(\mathbf{x})$ locally given the new Y	
22: $A \leftarrow [\hat{a}_1, \hat{a}_2,, \hat{a}_k]$	
23: $C \leftarrow [\hat{c}_1, \hat{c}_2, \dots, \hat{c}_k]^T$	
24: return <i>A</i> , <i>C</i>	

602 Greedy initialization

Our initialization method greedily initializes neurons one by one. Every time we initialize a neuron, 603 we will remove its initialized spatiotemporal activity from the raw video data and initialize the next 604 neuron from the residual. For the same neuron, there are several seed pixels that could be used 605 to initialize it. But once the neuron has been initialized from any of these seed pixels (and the 606 spatiotemporal residual matrix has been updated by peeling away the corresponding activity), the 607 remaining seed pixels related to this neuron have lowered PNR and local correlation. This helps 608 avoid the duplicate initialization of the same neuron. Also, P(x) and L(x) have to be updated after 609 each neuron is initialized, but since only a small area near the initialized neuron is affected, we can 610 update these quantities locally to reduce the computational cost. This procedure is repeated until 611 the specified number of neurons have been initialized or no more candidate seed pixels exist. 612 This initialization algorithm can greedily initialize the required number of neurons, but the 613 subproblem of estimating \hat{a}_i given \hat{c}_i still has to deal with the large background activity in the 614 residual matrix. We developed a simple method to remove this background and accurately initialize 615

neuron shapes, described next. We first crop a $(2l + 1) \times (2l + 1)$ square centered at x^* in the field 616 of view (Figure 10A-E). Then we compute the temporal correlation between the filtered traces of 617 pixel x^* and all other pixels in the patch (Figure 10D). We choose those pixels with small temporal 618 correlations (e.g., 0.3) as the neighboring pixels that are outside of the neuron (the green contour in 619 Figure 10D). Next, we estimate the background fluctuations as the median values of these pixels 620 for each frame in the raw data (Figure 10E). We also select pixels that are within the neuron by 621 selecting correlation coefficients larger than 0.7, then \hat{c}_i is refined by computing the mean filtered 622 traces of these pixels (Figure 10E). Finally, we regress the raw fluorescence signal in each pixel onto 623 three sources: the neuron signal (Figure 10E), the local background fluctuation (Figure 10F), and a 624 constant baseline. Our initial estimate of \hat{a}_i is given by the regression weights onto \hat{c}_i in Figure 10F. 625

626 Modifications for high temporal or spatial correlation

⁶²⁷ The above procedure works well in most experimental datasets as long as neurons are not highly

spatially overlapped and temporally correlated. However, in a few extreme cases, this initialization

may lead to bad local minima. We found that two practical modifications can lead to improved
 results.

High temporal correlation, low spatial overlaps: The greedy initialization procedure assumes
 that closeby neurons are not highly correlated. If this assumption fails. CNMF-E will first merge

nearby neurons into one component for explaining the shared fluctuations, and then the following

initialized components will only capture the residual signals of each neuron. Our solution to this

issue relies on our accurate background removal procedure, after which we simply re-estimate each

neural trace c_i as a weighted fluorescence trace of the background-subtracted video $(Y - \hat{B}^f - \hat{b}_0 \mathbf{1}^T)$,

$$\hat{c}_i = \frac{\tilde{a}^T \cdot (Y - \hat{B}^f - \hat{b}_0 \mathbf{1}^T)}{\tilde{a}^T \cdot \tilde{a}},$$
(15)

⁶³⁷ where \tilde{a}_i only selects pixels with large weights by thresholding the estimated \hat{a}_i with $\max(\hat{a}_i)/2$ (this

reduces the contributions from smaller neighboring neurons). This strategy improves the extraction of individual neurons' traces in the high correlation scenarios and the spatial footprints can be

 $_{640}$ corrected in the following step of updating \hat{A} . Figure 4B and Figure 5 illustrate this procedure.

High spatial overlaps, low temporal correlation: CNMF-E may initialize components with
 shared temporal traces because they have highly overlapping areas. We solve this problem by
 de-correlating their traces (following a similar approach in (*Pnevmatikakis et al., 2016*)). We start

by assuming that neurons with high spatial overlap do not fire spikes within the same frame. If so

only the inferred spiking trace with the largest value is kept and the rest will be set to 0. Then we

initialize each c_i given these thresholded spiking traces and the corresponding AR coefficients.

647 Interventions

⁶⁴⁸ We use iterative matrix updates to estimate model variables in CNMF-E. This strategy gives us the

⁶⁴⁹ flexibility of integrating prior information on neuron morphology and temporal activity during the

model fitting. The resulting interventions (which can in principle be performed either automatically

or under manual control) can in turn lead to faster convergence and more accurate source extraction.

⁶⁵² We integrate 5 interventions in our CNMF-E implementation. Following these interventions, we

⁶⁵³ usually run one more iteration of matrix updates.

654 Merge existing components

⁶⁵⁵ When a single neuron is split mistakenly into multiple components, a merge step is necessary to

rejoin these components. If we can find all split components, we can superimpose all their spa-

tiotemporal activities and run rank-1 NMF to obtain the spatial and temporal activity of the merged

- neuron. We automatically merge components for which the spatial and temporal components are
- 659 correlated above certain thresholds. Our code also provides methods to manually specify neurons
- to be merged based on human judgment.

661 Split extracted components

⁶⁶² When highly correlated neurons are mistakenly merged into one component, we need to use spatial

information to split into multiple components according to neurons' morphology. Our current

implementation of component splitting requires users to manually draw ROIs for splitting the

spatial footprint of the extracted component. Automatic methods for ROI segmentation (Apthorpe

et al., 2016; Pachitariu et al., 2013) could be added as an alternative in future implementations.

⁶⁶⁷ Remove false positives

⁶⁶⁸ Some extracted components have spatial shapes that do not correspond to real neurons or tempo-

ral traces that do not correspond to neural activity. These components might explain some neural

⁶⁷⁰ signals or background activity mistakenly. Our source extraction can benefit from the removal of

- these false positives. This can be done by manually examining all extracted components, or in prin-
- ₆₇₂ ciple automatically by training a classifier for detecting real neurons. The current implementation
- relies on visual inspection to exclude false positives. We also rank neurons based on their SNRs and
- set a cutoff to discard all extracted components that fail to meet this cutoff. As with the splitting
- step, removing false positives could also potentially use automated ROI detection algorithms in the
- ⁶⁷⁶ future. See S10 Video for an example involving manual merge and delete operations.
- ⁶⁷⁷ Pick undetected neurons from the residual
- ⁶⁷⁸ If all neural signals and background are accurately estimated, the residual of the CNMF-E model
- ⁶⁷⁹ $Y_{\text{res}} = Y \hat{A}\hat{C} \hat{B}$ should be relatively spatially and temporally uncorrelated. However, the initializa-
- tion might miss some neurons due to large background fluctuations and/or high neuron density.
- After we estimate the background \hat{B} and extract a majority of the neurons, those missed neurons
- have prominent fluorescent signals left in the residual. To select these undetected neurons from
- the residual Y_{res} , we use the same algorithm as for initializing neurons from the raw video data, but
- typically now the task is easier because the background has been removed.
- ⁶⁸⁵ Post-process the spatial footprints
- Each single neuron has localized spatial shapes and including this prior into the model fitting of 686 CNMF-E, as suggested in (Pnevmatikakis et al., 2016), leads to better extraction of spatial footprints. 687 In the model fitting step, we constrain A to be sparse and spatially localized. These constraints 688 do give us compact neuron shapes in most cases, but in some cases there are still some visually 689 abnormal components detected. We include a heuristic automated post-processing step after 690 each iteration of updating spatial shapes (P-S). For each extracted neuron A(:, k), we first convert 691 it to a 2D image and perform morphological opening to remove isolated pixels resulting from 692 noise (Haralick et al., 1987). Next we label all connected components in the image and create 693 a mask to select the largest component. All pixels outside of the mask in A(:,i) are set to be 0. 694 This post-processing induces compact neuron shapes by removing extra pixels and helps avoid 695 mistakenly explaining the fluorescence signals of the other neurons. 696

697 Further algorithmic details

- ⁶⁹⁸ The simplest pipeline for running CNMF-E includes the following steps:
- ⁶⁹⁹ 1. Initialize \hat{A}, \hat{C} using the proposed initialization procedure.
- 2. Solve problem (P-B) for updates of \hat{b}_0 and \hat{B}^f .
- 3. Iteratively solve problem (P-S) and (P-T) to update \hat{A} , \hat{C} and \boldsymbol{b}_0 .
- 4. If desired, apply interventions to intermediate results.
- ⁷⁰³ 5. Repeat steps 2, 3, and 4 until the inferred components are stable.

⁷⁰⁴ In practice, the estimation of the background *B* (step 2) often does not vary greatly from iteration to ⁷⁰⁵ iteration and so this step usually can be run with fewer iterations to save time. In practice, we also ⁷⁰⁶ use spatial and temporal decimation for improved speed, following (*Friedrich et al., 2017a*). We ⁷⁰⁷ first run the pipeline on decimated data to get good initializations, then we up-sample the results ⁷⁰⁸ \hat{A}, \hat{C} to the original resolution and run one iteration of steps (2-3) on the raw data. This strategy ⁷⁰⁹ improves on processing the raw data directly because downsampling increases the signal to noise ⁷¹⁰ ratio and eliminates many false positives.

Step 4 provides a fast method for correcting abnormal components without redoing the whole analysis. (This is an important improvement over the PCA/ICA pipeline, where if users encounter poor estimated components it is necessary to repeat the whole analysis with new parameter values, which may not necessarily yield improved cell segmentations.) The interventions described here themselves can be independent tasks in calcium imaging analysis; with further work we expect many of these steps can be automated. In our interface for performing manual interventions, the most frequently used function is to remove false positives. Again, components can be rejected following

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Name	Description	Default Values	Used in
l	size of a typical neuron soma in the FOV	30µm	Algorithm 1
l_n	the distance between each pixel and its neighbors	60µm	Problem (P-B)
P_{\min}	the minimum peak-to-noise ratio of seed pixels	10	Algorithm 1
L_{\min}	the minimum local correlation of seed pixels	0.8	Algorithm 1
ζ	the ratio between the outlier threshold and the noise	10	Problem (P-B)

Table 2. Optional user-specified parameters.

visual inspection in PCA/ICA analysis, but the performance of CNMF-E can be improved with further
 iterations after removing false positives, while this is not currently an option for PCA/ICA.

We have also found a two-step initialization procedure useful for detecting neurons: we first 720 start from relatively high thresholds of P_{\min} and L_{\min} to initialize neurons with large activity from the 721 raw video data; then we estimate the background components by solving problem (P-B); finally we 722 can pick undetected neurons from the residual using smaller thresholds. We can terminate the 723 model iterations when the residual sum of squares (RSS) stabilizes (see Figure 4B), but this is seldom 724 used in practice because computing the RSS is time-consuming. Instead we usually automatically 725 stop the iterations after the number of detected neurons stabilizes. If manual interventions are 726 performed, we typically run one last iteration of updating B, A and C sequentially to further refine 727 the results. 728

729 Parameter selection

Table 2 shows 5 key parameters used in CNME-F. All of these parameters have interpretable meaning 730 and can be easily picked within a broad range. The parameter *l* controls the size of the spatial filter 731 in the initialization step and is chosen as the diameter of a typical neuron in the FOV. As long as l 732 is much smaller than local background sources, the filtered data can be used for detecting seed 733 pixels and then initializing neural traces. The distance between each seed pixel and its selected 734 neighbors l_{i} has to be larger than the neuron size l and smaller than the spatial range of local 735 background sources; in practice, this range is fairly broad. We usually set I_n as 21. To determine the 736 thresholds P_{\min} and L_{\min} , we first compute the correlation image and PNR image and then visually 737 select very weak neurons from these two images. P_{\min} and L_{\min} are determined to ensure that 738 CNMF-E is able to choose seed pixels from these weak neurons. Small P_{\min} and L_{\min} yield more false 739 positive neurons, but they can be removed in the intervention step. Finally, in practice, our results 740 are not sensitive to the selection of the outlier parameter ζ , thus we frequently set it as 10. 741

742 Complexity analysis

In step 1, the time cost is mainly determined by spatial filtering, resulting in O(dT) time. As for the 743 initialization of a single neuron given a seed pixel, it is only (O(T)). Considering the fact that the 744 number of neurons is typically much smaller than the number of pixels in this data, the complexity 745 for step 1 remains O(dT). In step 2, the complexity of estimating \hat{b}_0 is O(dT) and estimating \hat{B}^f scales 746 linearly with the number of pixels d. For each pixel, the computational complexity for estimating 747 W_i . is O(T). Thus the computational complexity in updating the background component is O(dT). 748 In step 3, the computational complexities of solving problems (P-S) and (P-T) have been discussed 749 in previous literature (*Pnevmatikakis et al.*, 2016) and they scale linearly with pixel number d and 750 time T, i.e., O(dT). For the interventions, the one with the largest computational cost is picking 751 undetected neurons from the residual, which is the same as the initialization step. Therefore, the 752 computational cost for step 4 is O(dT). To summarize the complexity for running CNMF-F is O(dT). 753 i.e. the method scales linearly with both the number of pixels and the total recording time. 754

Dataset	Striatum	PFC	Hippocampus	BNST
Size $(x \times y \times t)$	$256 \times 256 \times 6000$	$175 \times 184 \times 9000$	$175 \times 184 \times 9000$	$175 \times 184 \times 9000$
(# PCs, # ICs)	(2000, 700)	(275, 250)	(100, 50)	(200, 150)
PFC/ICA	986	181	174	53
CNMF-E	726	221	335	435

Table 3. Running time (sec) for processing the 4 experimental datasets.

Implementations 755

Our MATLAB implementation supports running CNMF-E in three different modes that are optimized 756 for different datasets: single-mode, patch-mode and multi-batch-mode. 757

Single-mode is a naive implementation that loads data into memory and fits the model. It is fast 758 for processing small datasets (< 1GB). 759

For larger datasets, many computers have insufficient RAM for loading all data into memory 760 and storing intermediate results. Patch-mode CNMF-E divides the whole FOV into multiple small 761 patches and maps data to the hard drive (Giovannucci et al., 2017b). The data within each patch are 762 loaded only when we process that patch. This significantly reduces the memory consumption. More 763 importantly, this mode allows running CNMF-F in parallel on multi-core CPUs, vielding a speed-up 764 roughly proportional to the number of available cores. 765

Multi-batch mode builds on patch-mode, and is optimized for even larger datasets. especially 766 data collected over multiple sessions/days. This mode segments data into multiple batches tem-767 porally and assumes that the neuron footprints A are shared across all batches. We process each 768 batch using patch mode and perform partial weighted updates on A given the traces C obtained in 769

each batch. 770

All modes also include a logging system for keeping track of manual interventions and interme-771 diate operations. 772

The Python implementation is similar; see (Giovannucci et al., 2017b) for full details. 773

Running time 774

785

To provide a sense of the running time of the different steps of the algorithm, we timed the code on 779 the simulation data shown in Figure 4. This dataset is 253×316 pixels $\times 2000$ frames. The analyses 776 were performed on a desktop with Intel Xeon CPU E5-2650 v4 @2.20GHz and 128GB RAM running 777

Ubuntu 16.04. We used a parallel implementation for performing the CNMF-E analysis, with patch 778

size 64×64 pixels, using up to 12 cores. PCA/ICA took ~ 211 seconds to converge, using 250 PCs and 779

220 ICs. CNMF-E spent 55 seconds for initialization, 1 second for merging and deleting components. 780

110 seconds for the first round of the background estimation and 40 seconds in the following 781 updates, 8 seconds for picking neurons from the residual, and 10 seconds per iteration for updating

782 spatial (A) and temporal (C) components, resulting in a total of 258 seconds. 783

Finally, Table 3 shows the running time of processing the four experimental datasets. 784

Simulation experiments

Details of the simulated experiment of Figure 2 786

The field of view was 256×256 , with 1000 frames. We simulated 50 neurons whose shapes were 787

simulated as spherical 2-D Gaussian. The neuron centers were drawn uniformly from the whole FOV 788

and the Gaussian widths σ_x and σ_y for each neuron was also randomly drawn from $\mathcal{N}(\frac{1}{2},(\frac{1}{\log}\frac{1}{2})^2)$, 789

where l = 12 pixels. Spikes were simulated from a Bernoulli process with probability of spiking per 790

timebin 0.01 and then convolved with a temporal kernel $g(t) = \exp(-t/\tau_{t}) - \exp(-t/\tau_{t})$, with fall time 791

 $\tau_{d} = 6$ timebin and rise time $\tau_{r} = 1$ timebin. We simulated the spatial footprints of local backgrounds 792

as 2-D Gaussian as well, but the mean Gaussian width is 5 times larger than the neurons' widths. As 793

for the spatial footprint of the blood vessel in Figure 2A, we simulated a cubic function and then 79/

⁷⁹⁵ convolved it with a 2-D Gaussian (Gaussian width=3 pixel). We use a random walk model to simulate

⁷⁹⁶ the temporal fluctuations of local background and blood vessel. For the data used in Figure 2A-H,

⁷⁹⁷ there were 23 local background sources; for Figure 2I, we varied the number of background sources.

⁷⁹⁸ We used the raw data to estimate the background in CNMF-E without subtracting the neural

⁷⁹⁹ signals $\hat{A}\hat{C}$ in problem (P-B). We set $I_n = 15$ pixels and left the remaining parameters at their default ⁸⁰⁰ values. The plain NMF was performed using the built-in MATLAB function nnmf, which utilizes

⁸⁰¹ random initialization.

⁸⁰² Details of the simulated experiment of Figure 3, Figure 4 and Figure 5

⁸⁰³ We used the same simulation settings for both Figure 3 and Figure 4. The field of view was 253×316 ⁸⁰⁴ and the number of frames was 2000. We simulated 200 neurons using the same method as the ⁸⁰⁵ simulation in Figure 2, but for the background we used the spatiotemporal activity of the background ⁸⁰⁶ extracted using CNMF-E from real experimental data (data not shown). The noise level Σ was also ⁸⁰⁷ estimated from the data. When we varied the SNR in Figure4D-G, we multiplied Σ with an SNR

⁸⁰⁸ reduction factor.

We set l = 12 pixels to create the spatial filtering kernel. As for the thresholds used for determining seed pixels, we varied them for different SNR settings by visually checking the corresponding local correlation images and PNR images. The selected values were $L_{min} = [0.9, 0.8, 0.8, 0.6, 0.6]$ and $P_{min} = [15, 10, 10, 8, 6, 6]$ for different SNR reduction factors [1, 2, 3, 4, 5, 6]. For PCA/ICA analysis, we set the number of PCs and ICs as 600 and 300 respectively.

The simulation in Figure 5 only includes 2 neurons (as seen in Figure 3E) using the same simulation parameters. We replaced their temporal traces c_1 and c_2 with $(1 - \rho)c_1 + \rho c_3$ and $(1 - \rho)c_2 + \rho c_3$, where ρ is tuned to generate different correlation levels (γ), and c_3 is simulated in the same way as c_1 and c_2 . We also added a new background source whose temporal profile is c_3 to increase the neuron-background correlation as ρ increases. CNMF-E was run as in Figure 4. We used 20 PCs and ICs for PCA/ICA.

820 In vivo microendoscopic imaging and data analysis

For all experimental data used in this work we ran both CNME-F and PCA/ICA For CNME-F we 821 chose parameters so that we initialized about 10-20% extra components, which were then merged 822 or deleted (some automatically, some under manual supervision) to obtain the final estimates. 823 Exact parameter settings are given for each dataset below. For PCA/ICA, the number of ICs were 824 selected to be slightly larger than our extracted components in CNMF-E (as we found this led to 825 the best results for this algorithm), and the number of PCs was selected to capture over 90% of the 826 signal variance. The weight of temporal information in spatiotemporal ICA was set as 0.1. After 827 obtaining PCA/ICA filters, we again manually removed components that were clearly not neurons 828 based on neuron morphology. 829

We computed the SNR of extracted cellular traces to quantitatively compare the performances of two approaches. For each cellular trace y, we first computed its denoised trace c using the selected deconvolution algorithm (here, it is thresholded OASIS); then the SNR of y is

$$SNR = \frac{\|\boldsymbol{c}\|_{2}^{2}}{\|\boldsymbol{y} - \boldsymbol{c}\|_{2}^{2}}.$$
 (16)

For PCA/ICA results, the calcium signal y of each IC is the output of its corresponding spatial filter, while for CNMF-E results, it is the trace before applying temporal deconvolution, i.e., \hat{y}_i in Eq. (9). All the data can be freely accessed online **Zhou et al.** (2017).

836 Dorsal striatum data

837 Expression of the genetically encoded calcium indicator GCaMP6f in neurons was achieved using a

recombinant adeno-associated virus (AAV) encoding the GCaMP6f protein under transcriptional

control of the synapsin promoter (AAV-Syn-GCaMP6f). This viral vector was packaged (Serotype 1)

and stored in undiluted alignots at a working concentration of > 1012 genomic copies per ml at 840 -80°C until intracranial injection, 500//l of AAV1-Syn-GCaMP6f was injected unilaterally into dorsal 841 striatum (0.6 mm anterior to Bregma, 2.2mm lateral to Bregma, 2.5mm ventral to the surface of the 842 brain). 1 week post injection, a 1mm gradient index of refraction (GRIN) lens was implanted into 843 dorsal striatum $\sim 300 \mu$ m above the center of the viral injection. 3 weeks after the implantation, the GRIN lens was reversibly coupled to a miniature 1-photon microscope with an integrated 475nm 845 LED (Inscopix) Using nVistaHD Acquisition software images were acquired at 30 frames per second 846 with the LED transmitting 0.1 to 0.2 mW of light while the mouse was freely moving in an open 847 field arena. Images were down sampled to 10Hz and processed into TIFFs using Mosaic software. 848 All experimental manipulations were performed in accordance with protocols approved by the 849 Harvard Standing Committee on Animal Care following guidelines described in the US NIH Guide 850 for the Care and Use of Laboratory Animals. 851

The parameters used in running CNMF-E were: l = 13 pixels, $l_n = 18$ pixels, $L_{min} = 0.7$, and $P_{min} = 7.728$ components were initialized from the raw data in the first pass before subtracting the background, and then additional components were initialized in a second pass. Highly-correlated nearby components were merged and false positives were removed using the automated approach described above. In the end, we obtained 692 components.

857 Prefrontal cortex data

Cortical neurons were targeted by administering 2 microiniections of 300 ul of AAV-DI-Camklla-858 GCaMP6s (titer: 5.3 x 1012, 1:6 dilution, UNC vector core) into the prefrontal cortex (PFC) (coordi-859 nates relative to bregma; injection 1: +1.5 mm AP. 0.6 mm ML. -2.4 ml DV; injection 2: +2.15 AP. 0.43 860 mm ML, -2.4 mm DV) of an adult male wild type (WT) mice. Immediately following the virus injection 861 procedure, a 1 mm diameter GRIN lens implanted 300 um above the injection site (coordinates 867 relative to bregma: +1.87 mm AP. 0.5 mm ML, -2.1 ml DV). After sufficient time had been allowed for 863 the virus to express and the tissue to clear underneath the lens (3 weeks), a baseplate was secured 864 to the skull to interface the implanted GRIN lens with a miniature, integrated microscope (nVista, 865 473 nm excitation LED. Inscopix) and subsequently permit the visualization of Ca2+ signals from 866 the PEC of a freely behaving mouse. The activity of PEC neurons were recorded at 15 Hz over a 867 10 min period (nVista HD Acquisition Software, Inscopix) while the test subject freely explored an 868 empty novel chamber. Acquired data was spatially down sampled by a factor of 2, motion corrected. 869 and temporally down sampled to 15 Hz (Mosaic Analysis Software, Inscopix). All procedures were 870 approved by the University of North Carolina Institutional Animal Care and Use Committee (UNC 871 IACUC). 872

The parameters used in running CNMF-E were: l = 13 pixels, $l_n = 18$ pixels, $L_{\min} = 0.9$, and $P_{\min} = 15$. There were 169 components initialized in the first pass and we obtained 225 components after running the whole CNMF-E pipeline.

876 Ventral hippocampus data

The calcium indicator GCaMP6f was expressed in ventral hippocampal-amygdala projecting neurons 877 by injecting a retrograde canine adeno type 2-Cre virus (CAV2-Cre: from Larry Zweifel, University 878 of Washington) into the basal amydala (coordinates relative to bregma: -1.70 AP. 3.00mm ML. 879 and -4.25mm DV from brain tissue at site), and a Cre-dependent GCaMP6f adeno associated virus 880 (AAV1-flex-Synapsin-GCaMP6f, UPenn vector core) into ventral CA1 of the hippocampus (coordinates 881 relative to bregma: -3.16mm AP, 3.50mm ML, and -3.50mm DV from brain tissue at site). A 0.5mm 882 diameter GRIN lens was then implanted over the vCA1 subregion and imaging began 3 weeks after 883 surgery to allow for sufficient viral expression. Mice were then imaged with Inscopix miniaturized 884 microscopes and nVistaHD Acquisition software as described above; images were acquired at 15 885 frames per second while mice explored an anxiogenic Elevated Plus Maze arena. Videos were motion 886 corrected and spatially downsampled using Mosaic software. All procedures were performed in 887 accordance with protocols approved by the New York State Psychiatric Institutional Animal Care 888

and Use Committee following guidelines described in the US NIH Guide for the Care and Use of
 Laboratory Animals.

The parameters used in running CNMF-E were: l = 15 pixels, $l_n = 30$ pixels, $\zeta = 10$, $L_{min} = 0.9$, and $P_{min} = 15$. We first temporally downsampled the data by 2. Then we applied CNMF-E to the downsampled data. There were 53 components initialized. After updating the background component, the algorithm detected 6 more neurons from the residual. We merged most of these components and deleted false positives. In the end, there were 24 components left. The intermediate results before and after each manual intervention are shown in S10 Video.

897 BNST data with footshock

Calcium indicator GCaMP6s was expressed within CaMKII-expressing neurons in the BNST by 898 injecting the recombinant adeno-associated virus AAVdi-CaMKII-GCaMP6s (packaged at UNC Vector 899 Core) into the anterior dorsal portion of BNST (coordinates relative to bregma: 0.10mm AP, -0.95mm 900 ML. -4.30mm DV). A 0.6 mm diameter GRIN lens was implanted above the injection site within the 901 BNST. As described above, images were acquired using a detachable miniature 1-photon microscope 902 and nVistaHD Acquisition Software (Inscopix). Images were acquired at 20 frames per second while 903 the animal was freely moving inside a sound-attenuated chamber equipped with a house light and 904 a white noise generator (Med Associates). Unpredictable foot shocks were delivered through metal 905 bars in the floor as an aversive stimulus during a 10-min session. Each unpredictable foot shock 906 was 0.75 mA in intensity and 500 ms in duration on a variable interval (VI-60). As described above. 907 images were motion corrected, downsampled and processed into TIFFs using Mosaic Software. 908 These procedures were conducted in adult C57BL/6I mice (lackson Laboratories) and in accordance 900 with the Guide for the Care and Use of Laboratory Animals, as adopted by the NIH, and with 910 approval from the Institutional Animal Care and Use Committee of the University of North Carolina 911 at Chapel Hill (UNC). 912 The parameters used in running CNMF-E were: l = 15 pixels, $l_n = 23$ pixels, $\zeta = 10$, $L_{min} = 0.9$, and 913

 $P_{\text{min}} = 15$. There were 149 components initialized and we detected 29 more components from the residual after estimating the background. there were 127 components left after running the whole pipeline.

917 Code availability

⁹¹⁸ All analyses was performed with custom-written MATLAB code. MATLAB implementations of ⁹¹⁹ the CNMF-E algorithm can be freely downloaded from https://github.com/zhoupc/CNMF E (*Zhou*,

2017a). We also implemented CNMF-E as part of the Python package CalmAn (Giovannucci et al.,

2017b), a computational analysis toolbox for large scale calcium imaging and behavioral data
 (https://github.com/simonsfoundation/CalmAn (*Giovannucci et al., 2017a*)).

The scripts for generating all figures and the experimental data in this paper can be accessed from https://github.com/zhoupc/eLife submission(*Zhou*, *2017b*).

925 Supporting information

⁹²⁶ S1 Video. An example of typical microendoscopic data. The video was recorded in dorsal

- striatum; experimental details can be found above.
- 928 **MP4**

929 S2 Video. Comparison of CNMF-E with rank-1 NMF in estimating background fluctuation in

simulated data. Top left: the simulated fluorescence data in Figure 2. Bottom left: the ground

- ⁹³¹ truth of neuron signals in the simulation. Top middle: the estimated background from the raw ⁹³² video data (top left) using CNMF-E. Bottom middle: the residual of the raw video after subtracting
- ⁹³² video data (top left) using CNMF-E. Bottom middle: the residual of the raw video after subtracting the background estimated with CNMF-F. Top right and top bottom: same as top middle and bottom.
- ⁹³⁴ middle, but the background is estimated with rank-1 NMF.
- 935 MP4

S3 Video. Initialization procedure for the simulated data in Figure 3. Top left: correlation image of the filtered data. Red dots are centers of initialized neurons. Top middle: candidate seed pixels (small red dots) for initializing neurons on top of PNR image. The large red dot indicates the current seed pixel. Top right: the correlation image surrounding the selected seed pixel or the spatial footprint of the initialized neuron. Bottom: the filtered fluorescence trace at the seed pixel

- or the initialized temporal activity (both raw and denoised).
- 942 MP4
- 943 S4 Video. The results of CNMF-E in demixing simulated data in Figure 4 (SNR reduction fac-
- ⁹⁴⁴ tor=1). Top left: the simulated fluorescence data. Bottom left: the estimated background. Top
- ⁹⁴⁵ middle: the residual of the raw video (top left) after subtracting the estimated background (bottom
- ⁹⁴⁶ left). Bottom middle: the denoised neural signals. Top right: the residual of the raw video data (top
- ₉₄₇ right) after subtracting the estimated background (bottom left) and denoised neural signal (bottom
- ⁹⁴⁸ middle). Bottom right: the ground truth of neural signals in simulation.
- 949 MP4
- S5 Video. The results of CNMF-E in demixing the simulated data in Figure 4 (SNR reduction
 factor=6). Conventions as in previous video.
- 952 MP4

S6 Video. The results of CNMF-E in demixing dorsal striatum data. Top left: the recorded fluorescence data. Bottom left: the estimated background. Top middle: the residual of the raw video (top left) after subtracting the estimated background (bottom left). Bottom middle: the denoised neural signals. Top right: the residual of the raw video data (top right) after subtracting the estimated background (bottom middle). Bottom right: the denoised neural signals while all neurons' activity are coded with pseudocolors.

959 MP4

⁹⁶⁰ **S7 Video. The results of CNMF-E in demixing PFC data.** Conventions as in previous video.

961 **MP4**

⁹⁶² S8 Video. Comparison of CNMF-E with PCA/ICA in demixing overlapped neurons in Figure 7G.

- Top left: the recorded fluorescence data. Bottom left: the residual of the raw video (top left) after
- ⁹⁶⁴ subtracting the estimated background using CNMF-E. Top middle and top right: the spatiotemporal ⁹⁶⁵ activity and temporal traces of three neurons extracted using CNMF-E. Bottom middle and bottom
- right: the spatiotemporal activity and temporal traces of three neurons extracted using PCA/ICA.
- 967 MP4

S9 Video. The results of CNMF-E in demixing ventral hippocampus data. Conventions as in S6
 Video.

970 MP4

971 S10 Video. Extracted spatial and temporal components of CNMF-E at different stages (ven-

tral hippocampal dataset). After initializing components, we ran matrix updates and interventions

- ⁹⁷³ in automatic mode, resulting in 32 components in total. In the next iteration, we manually deleted ⁹⁷⁴ 6 components and automatically merged neurons as well. In the last iterations, 4 neurons were
- merged into 2 neurons with manual verifications. The correlation image in the top left panel is
- computed from the background-subtracted data in the final step.
- 977 MP4

978 S11 Video. The results of CNMF-E in demixing BNST data. Conventions as in S6 Video.

979 MP4

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