

Supporting Information

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SI Materials and Methods

In Vivo Imaging of Neuronal Activity. The mouse strain was BAC-EGR-1-EGFP [Tg(Egr1-EGFP)GO90Gsat/Mmucd, from Genesat project, distributed from Jackson Laboratories]. Animal care was in accordance with the Institutional Guidelines of Tsinghua University. Three- to 5-mo-old mice received cranial window implantation as previously described (1), and recording began 1 mo later. Only the mice with a clear window were used for imaging; we achieved 70% success. To implant the cranial window, the mouse was immobilized in custom-built stage-mounted ear bars and a nosepiece, similar to a stereotaxic apparatus. A 1.5-cm incision was made between the ears, and the scalp was reflected to expose the skull. One circular craniotomy (6~7 mm in diameter) was made using a high-speed drill and a dissecting microscope for gross visualization. A glass coverslip was attached to the skull with glue. For surgeries and observations, mice were anesthetized with 1.5% (vol/vol) isoflurane. The preparation for imaging was restricted to 20 min after anesthetization for each test. The mice were anesthetized 40 min after training and subjected to optical imaging. Six cortical volumes were recorded during each imaging session within 60 min. The same sets of volumes were measured after different training trials to track neuronal EGFP signals in the same neurons at different training sessions. During imaging, a wax ring was placed on the edges of the coverslip of the cortical window and filled with distilled water to create a well for water immersion lens. EGFP fluorescent intensity (FI) was imaged with an Olympus Fluoview 1000MPE with prechirp optics and a fast AOM mounted on an Olympus BX61WI upright microscope, coupled with a 2-mm working distance, 25× water immersion lens (numerical aperture, 1.05). A mode-locked titanium/sapphire laser (Tsunami; Spectra-Physics) generated two-photon excitation at 920 nm, and three photomultiplier tubes (Hamamatsu) collected emitted light in the range of 380–480, 500–540, and 560–650 nm. The output power of the laser was maintained at 1.56 W, and power reaching the mouse brain ranged from 7.8 to 12.5 mW. For each cortical volume, the laser power was consistent during time-lapse images under multitasks. To facilitate location of cortical volumes from section to section, dextran Texas red (Dex Red; 70,000 molecular weight; Invitrogen) was injected into a lateral tail vein to create a fluorescent angiogram, as previously described (1).

Purification of Cortical EGFP-Positive Neurons from the EGR1-EGFP Mice. Three-month-old transgenic mice were anesthetized with halothane, and the brains were removed and sectioned in the coronal plane at 500 μm on a Vibratome (Leica) in perfusion buffer (choline-chloride, 115 mM; KCl, 2.5 mM; NaH₂PO₄, 1.25 mM; NaHCO₃, 26 mM; D-(+)-glucose, 10 mM; MgSO₄, 8 mM; L-ascorbate-Na, 1 mM; Na-pyruvate, 3 mM), pH 7.2–7.4, osmolarity 290–310, and bubbled with 5% CO₂/95% O₂. Cortical tissues were collected at 0.5-mm sections and subjected to enzyme digestion dissociated for 45 min at 37 °C with Papain enzyme (Papain Dissociation System; Worthington Biochem) in Earls balanced salt solution (EBSS) with DNase according to the manufacture's protocol. EBSS was as follows: NaCl, 116 mM; KCl, 5.4 mM; NaHCO₃, 26 mM; NaH₂PO₄, 1 mM; CaCl₂, 1.5 mM; MgSO₄, 1 mM; EDTA, 0.5 mM; glucose, 25 mM; cysteine, 1 mM). The slices were rinsed with EBSS for three times.

Brain slices were triturated with three glass pipettes of decreasing tip diameter. To remove excess debris, cell pellets were resuspended in EBSS, DNase, and albumin ovomucoid inhibitor (AOI) at 4 °C. Cell pellets were resuspended in the EBSS buffer

media and filtered through a 100-μm mesh. Cells were treated with propidium iodide (PI) (20 μg/mL) and DAPI to label dead cells and normal cells. Neurons were sorted on a FACSAriaII cell sorter (Becton Dickson) for FITC signals (detecting EGFP), DAPI signals (detecting DAPI), and PE signals (detecting PI). Cells were kept on ice before and after sorting.

For imaging of the FACS-sorted cells, presort and postsort cells were resuspended in the EBSS and fixed with 4% paraformaldehyde (PFA) immediately after sorting, followed by rinsing in 0.1 M PBS. Cells were imaged under a fluorescent microscope (Olympus).

RT-PCR. Sorted GFP-positive and -negative cells were lysed with the TRIzol reagent (Invitrogen), following the standard protocol. cDNA was obtained through standard protocol, using SuperScript II reverse transcriptase (Invitrogen) with oligo dT. The cDNA samples were subjected to semiquantitative PCR for quantification.

Primer sequences used for PCR were as follows: EGR1, 5'-CTGCACCCCGCATGTAAC-3' and 5'-TGTGGTCAGGTG-CTCATAGG-3'; GFP, 5'-TACGGCGTGCAGTGCTTCAG-3' and 5'-GGCGGATCTGAAGTTCACC-3'; and GAPDH, 5'-GGTGAAGGTCGGTGTGAACG3' and 5'-CTCGCTCCTGGA-AGATGGTG-3'.

Neuronal Culture and Stimulation. Embryonic cortices (E17) of EGR1-GFP BAC transgenic mice (Genesat Project) were isolated using standard procedures and triturated with trypsin/DNase digestion. Cortical neurons were plated at a density of 10,000 cells per well in black/clear bottom plates coated with the poly-D-lysine (Costar) in neurobasal medium (1.6% B27, 2% glutamax, 1% pen/strep, and 5% heat-inactivated FCS) and in the neurobasal medium without serum 24 h later. Under these culture conditions, the percentage of glia was estimated to be in the range of 5–25. The chemical stimulation was performed on days in vitro (DIV) 6 by changing the culture medium containing 100 μM dihydroxyphenylglycine (DHPG) or 1 μM TTX or 20 μM CNQX and 15 μM MK-801 for 10 min. After stimulation, the original culture medium was returned to the culture dish after washing with warmed MEM for three times. EGFP signals in each cell were imaged at different time points after stimulation and quantified by Image J. The data were normalized to the control (medium addition).

The optogenetic method was used to perform the light-induced stimulation. pLenti-CaMKIIa-hChR2-mCherry-WPRE was a gift from Karl Deisseroth, Stanford University Stanford, CA (2, 3). ChR2-mCherry vector was transfected in the cultures. An array of LED lamps (~480 nm, 3.0–3.2 V, 1,000–1,200 mcd/lamp, 25 mW) were placed 10 cm above the culture dish. LED lamps were controlled by the SCM control electric circuit (single chip mi-cyoco, specifically, AT89S52 and AT89C52). Light pulses were given at 5 ms/pulse. The cultured neurons were transfected at DIV4 and stimulated at DIV7. After the transfection, neurons were kept in a dark box. Cells were fixed in 4% PFA/4% sucrose in PBS. Fixative was washed away with the PBS (three wash cycles) and processed for EGR1-GFP imaging as described previously in detail (4). Images were quantified using Image J.

Behavior Tasks. Context-dependent fear conditioning in context A. Training consists of a 3-min exposure of mice to the conditioning box (context, TSE system) followed by a foot shock (2 s, 0.8 mA, constant current). The memory test was performed 24 h later by reexposing the mice for 3 min into the conditioning context. Freezing, defined as a lack of movement except for heart beat and

respiration associated with a crouching posture, was recorded every 10 s by two trained observers (one was unaware of the experimental conditions) during 3 min (a total of 18 sampling intervals). The number of observations indicating freezing obtained as a mean from both observers was expressed as a percentage of the total number of observations. Different context was achieved by changing the shape and the color of the chamber wall and the smell in the chamber. For the retrieval trial, the mouse was placed in the chamber for 3 min. For the contextual fear training in context B, the shape of the arena was switched from a square to round shape. The inner color and texture were changed to white paper board. The floor was covered with the plastic grid. The smell of the chamber was switched from ethanol to acetic acid. **Passive avoidance (context C experiment).** The mice were placed in the dark-light chambers for 20 min. The chamber receiving the electrical shock cycled between the light and dark part, 2 s after a brief tone cue (1 kHz). The mice learned the sound and the sequence of shock chambers to avoid the electric shock. Twenty trials were performed each day. Mice were anesthetized 40 min after the last trial.

Environment exploring task in a big chamber (context E experiment). During each trial, the mice were placed in the enriched cages equipped with various toys for 24 h. The enriched cages were more than fourfold larger in space than the home cage. The toys include a running wheel, a ladder, a running ball, and colorful blocks.

Tunnel environment task (context D experiment). Mice were placed in a white box with a Y-shaped tunnel. A food pellet was placed in one branch of the tunnel. The placement of the food branch was not changed during the training and retrieval. For the training trial, after food deprivation for 24 h, the mice were placed in the tunnel until they retrieved the food. Five training trials were performed during a training section.

Algorithm of Cell Recognition Used in Cell Detection. We designed a modified neuronal network algorithm for the pattern recognition (Dataset S1). Each labeled sample for learning was placed in the center of the receptive field (30×30 pixels; Fig. S4), according to the signal values of each pixel (S_i). S_i in the receptive field was standardized to a representation value, (A_i), in a unique chain of numbers under a Gaussian distribution (Fig. S4). In detail, we defined a ranking function (mapping) for each of the signals (S_i) in the picture. The function was defined as $\sigma: \{1, \dots, n\} \rightarrow \{1, \dots, n\}$, so that for signals in the picture (S_i), $S = \{S_i | 1 \leq i \leq n\}$, there was an order $S_{\sigma(1)} \leq S_{\sigma(2)} \leq \dots \leq S_{\sigma(n)}$ and $rank(S_i) = \sigma^{-1}(i)$. Then, the gray signals (S_i) were represented by a new set of values (A_i) under the standard normal distribution: $A_i = \Phi^{-1}\left(\frac{1}{n+1}rank(S_i)\right) = \Phi^{-1}\left(\frac{1}{n+1}\sigma^{-1}(i)\right)$, where

$\Phi(x)$ is the cumulative distribution function (CDF) of the standard normal distribution, and $\Phi^{-1}(p)$ is its inverse, known as the probit function. The increase of synaptic weights between the output neuron j , which recognize the signal pattern of a neuron, and the receptive field neurons were generated as $dW_i = A_i$. After learning the accumulated synaptic weights of each output neuron j was normalized from 20 labeled samples for each category (the neuron, the neuritis, the background, and the vessel); the accumulated synaptic weights of each output neuron j were normalized to the same level, $W'_i = W_i/\Sigma W_i$.

Therefore, weight for four categories was formed according to the training samples.

Next, for the recognition, all of the input signals from the images were standardized to the representation A_i . The output signals of a specific output neuron j were calculated as

$$output_j = \sum_{i=1}^m w_{ij} \cdot A_i.$$

The difference in the output signals was determined by the spatial similarity between the input signal and the learned synaptic pattern as a winner-take-all method (WTA). The classification of the target signal was determined by the identity of the output neuron j , which showed the highest activity comparing to all of the other output neurons.

After detection of the cells in all of the images, the cell center was determined in the image stacks by aligning the centers of each identified neuron in the z-stacks. The center positions in the middle of adjacent points were picked up for the position of the neuron. The identified positions of each neuron were plotted on the original image stacks and subjected to manual modification.

Quantification of Population Correlation. The correlation of spatial activation pattern is defined as linear correlation between two vectors. The correlation between each two training trials is calculated based on the EGFP signal in all neurons within the same layer (signals of neurons in each trials treated as sequences of values, x_s, x_t), and the correlation is calculated as follows:

$$C_{st} = \frac{(x_s - \bar{x}_s)(x_t - \bar{x}_t)'}{\sqrt{(x_s - \bar{x}_s)(x_s - \bar{x}_s)' \sqrt{(x_t - \bar{x}_t)(x_t - \bar{x}_t)'}}$$

where

$$\bar{x}_s = \frac{1}{n} \sum_j x_{sj} \quad \text{and} \quad \bar{x}_t = \frac{1}{n} \sum_j x_{tj}.$$

1. Xie H, et al. (2013) Rapid cell death is preceded by amyloid plaque-mediated oxidative stress. *Proc Natl Acad Sci USA* 110(19):7904–7909.
2. Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K (2005) Millisecond-timescale, genetically targeted optical control of neural activity. *Nat Neurosci* 8(8):1263–1268.

3. Zhang F, Wang LP, Boyden ES, Deisseroth K (2006) Channelrhodopsin-2 and optical control of excitable cells. *Nat Methods* 3(10):785–792.
4. Guan JS, et al. (2009) HDAC2 negatively regulates memory formation and synaptic plasticity. *Nature* 459(7243):55–60.

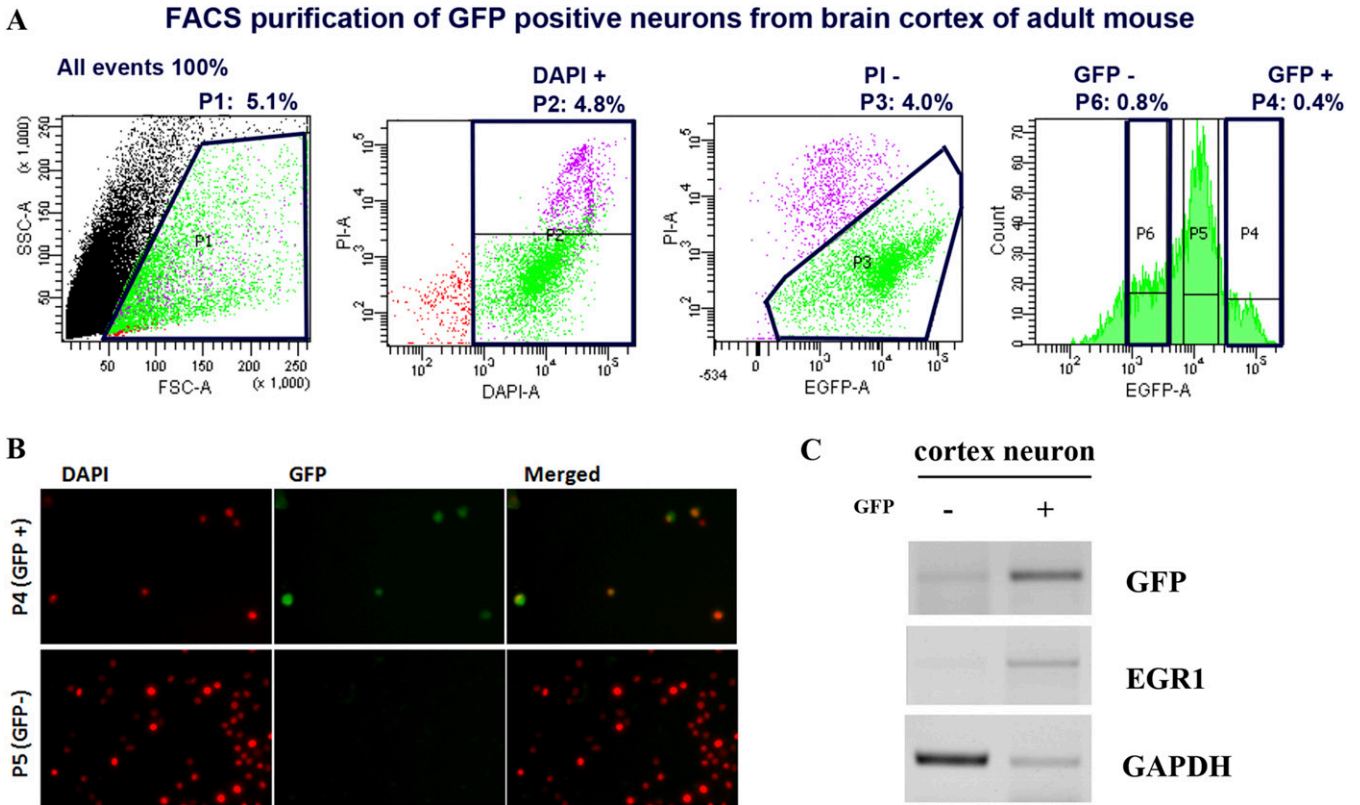


Fig. S1. The EGFP signal within the cells derived from EGR1-EGFP BAC transgenic mice were subjected to the EGR1 transcription. **(A)** Cortical neurons were dissected from the adult mice. The EGFP-positive neurons and EGFP-negative neurons were separated by a FACS system (BD FACSArial) in the P4 and P6 fraction. Neurons were stained with DAPI and PI dyes. Cell debris without DAPI staining were discarded. The isolated intact neurons were in the population with high DAPI signal and low PI signal. The neurons with different EGFP signal intensities were collected, according to the EGFP intensity in each neuron via a FACS sorter. The EGFP-positive cells were in the P4 (0.2% of all events). The EGFP-negative cells were in the P5 (0.7% of all events). **(B)** The example of the isolated EGFP⁺ and EGFP⁻ neurons. All neurons were stained with DAPI. Only the P4 fraction showed the EGFP-positive signal. **(C)** The RT-PCR experiments to examine the mRNA expressions within the collected cell populations with low GFP signals and high GFP signals in the cortical and the hippocampal tissues.

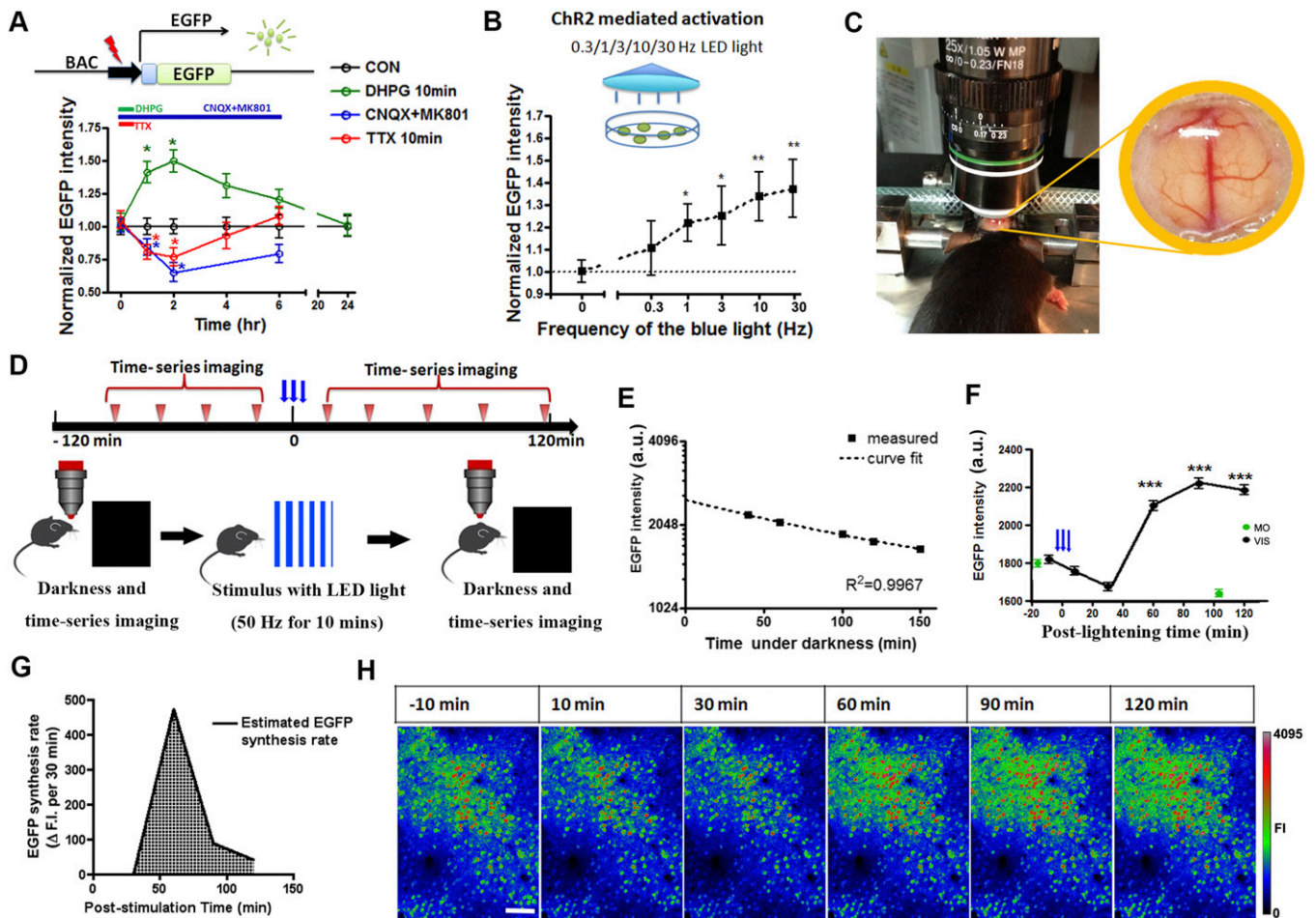


Fig. S2. Activity-induced EGR1-EGFP signal in cultured neurons and living mice. (A) Time course of EGR1-EGFP fluorescence intensity in response to neuronal activation or inhibition in the cortical neuron cultures. Cultured EGR1-EGFP-expressing neurons at DIV 10 were activated by a 100 μ M DHPG pulse for 10 min or inhibited by 1 μ M TTX or 20 μ M CNQX plus 15 μ M MK-801 for indicated periods, and the EGFP signals were recorded ($n \geq 82$ neurons from three cultures). (B) ChR2-mCherry-expressing neurons were stimulated with LED (\sim 480 nm) for 10 min at the indicated frequency (5-ms duration for each pulse) and imaged at 1.5 h after stimuli ($n \geq 24$ neurons from three cultures). (C) For in vivo imaging, a craniotomy was implanted on the skull. (D) Procedure to measure light induced response in the visual cortex. Mice were anesthetized in a dark room for 2 h and then stimulated by 50-Hz blue light for 10 min. (E) The decay rate of EGR1-EGFP in each neuron in visual cortex under the dark condition ($n = 450$ neurons from three mice). Dotted line shows the curve fit with single exponential decay. (F) Changes in the EGFP signal after the light stimuli ($n = 450$ neurons from three mice). Blue arrows indicate light stimuli. MO, motor cortex; VIS, visual cortex. (G) The estimated EGFP production rate after the visual stimuli. Production rate was showed as actual signal increase during 30 min. (H) Time series images showed the change of the EGFP signal in the visual cortex. Visual stimuli was applied at 0 min. (Scale bar, 70 μ m).

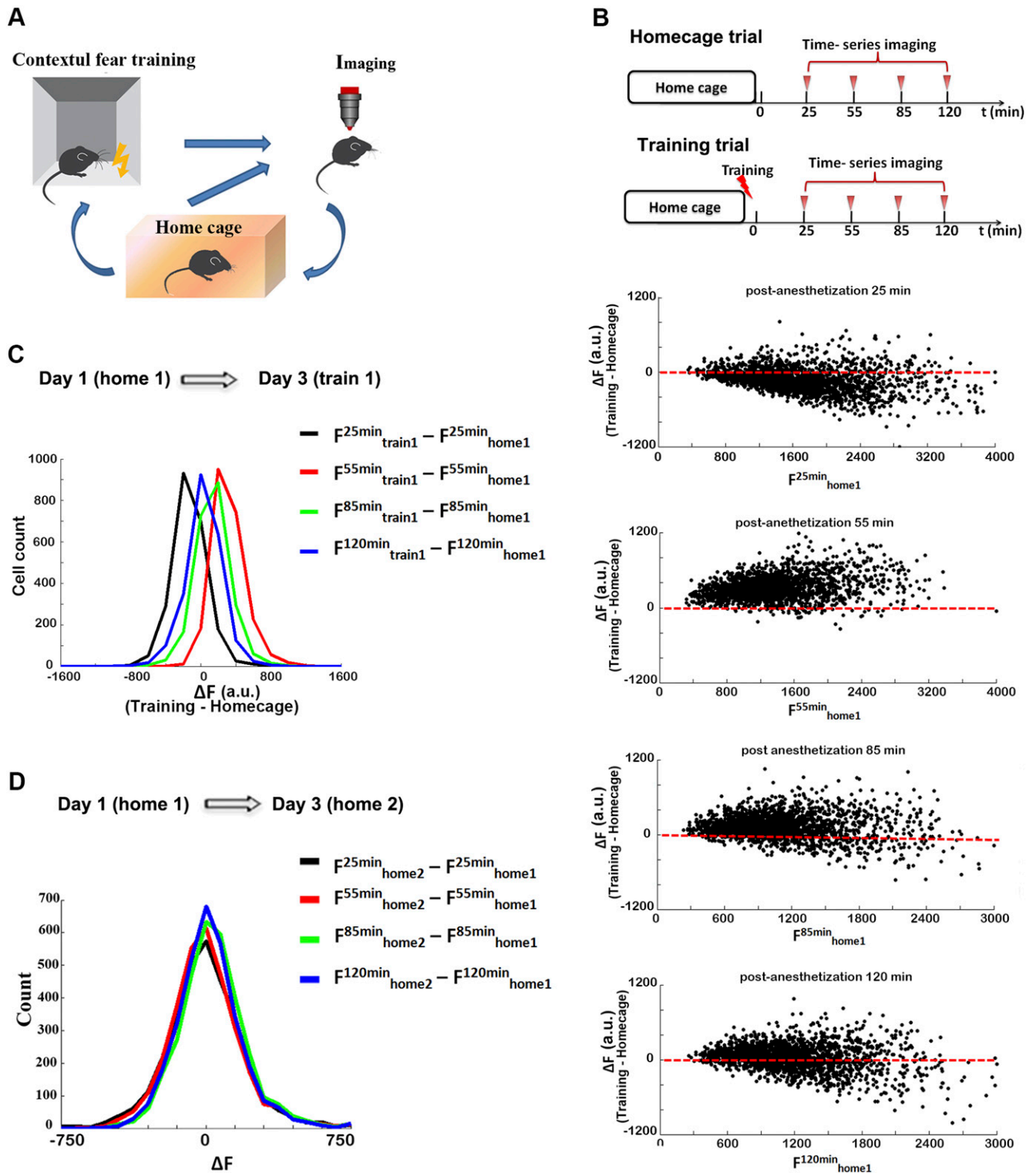


Fig. S3. Task-induced cellular EGFP signal changes in a visual volume of freely moving mice. (A) The experimental procedure. (B) The same cortical volume was recorded in the homeage trial on day 1 and day 3. The mouse received contextual fear conditioning training on day 3 and was anesthetized immediately after the training. Images were taken at 25, 55, 85, and 120 min after the anesthetization. Each dot indicated the signal changes in one cell. (C) The histogram of signal intensity changes for all neurons in the same volume. Different color showed the histogram for different time points after the training ($n = 3,323$ neurons). (D) The mouse was anesthetized directly from the homeage for each trial in day 1 and day 3. Images were taken at 25, 55, 85, and 120 min after the anesthetization. The changes of the EGFP signals in each neuron in the visual cortex were quantified in the histogram ($n = 3,633$ neurons). No significant shifting of signals was observed in all four time points.

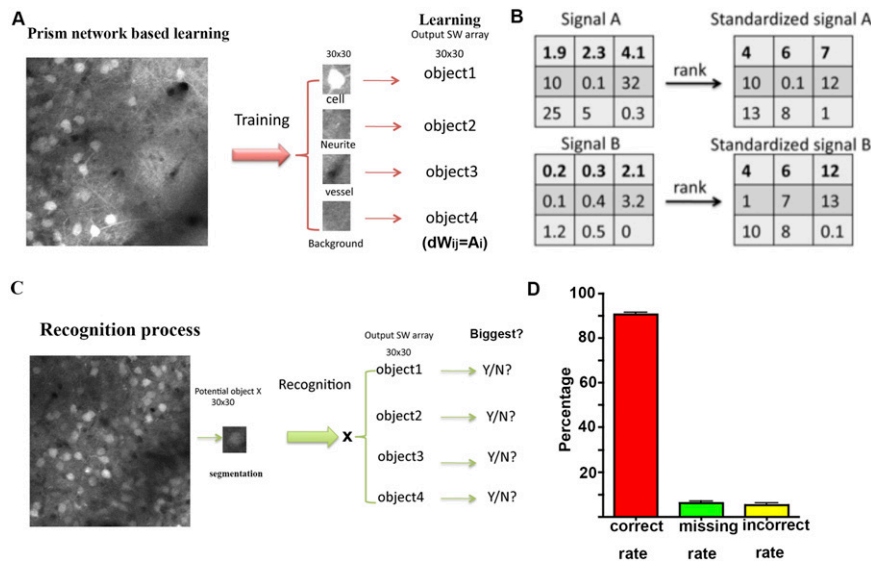


Fig. 54. The computer-based recognition process. (A) Training sample. Pictures were divided into small patches (30×30 ; the receptive field), where the object was placed in the center of the small patch. Synaptic weights were accumulated as $\Delta w_{ij} = A_i$ using the labeled samples. Each output neuron was trained with 20–30 labeled samples in the same category. After the training, the synaptic maps were normalized in each neuron and were stored in a 30×30 array. (B) Signals in the receptive field were standardized, according to the rank of each signal in this receptive field. A unique series of values was used for the entire task in the representation layer, so that the spectrum of the representation layer was kept the same without any changes on various inputs. The input signals (S_i) from the bit map of the images were transformed into the unique distribution of the same series of values according to their rank. Thus, the input only induced the redistribution of the same serial values in the 30×30 map. This process is called the standardization. The standardized signals were used for the synaptic weight adjustment and the signal recognition. (C) Image processing and recognition. The original images (512×512) were segregated into 30×30 small patches. The small images were subjected to recognition. Input signals were standardized and magnified by the synaptic weight maps in each output neuron. The identity of a cell was determined if the output neuron for the cell showed the highest value in all of the output neurons. (D) The recognition rate and missing cells in 20 images. Images were manually recognized after the computer processing. The missing rate was defined as the neurons missed in the automatic system but could be manually recognized. The incorrect neuron indicated the noise signals, which were recognized by the system but rejected by the manual adjustment. The correct rate was defined as the number of correctly recognized neurons (automatically generated and passing the manual adjustment) in all of the recognized cells by this system.

EGR1-EGFP signal population correlation between trials

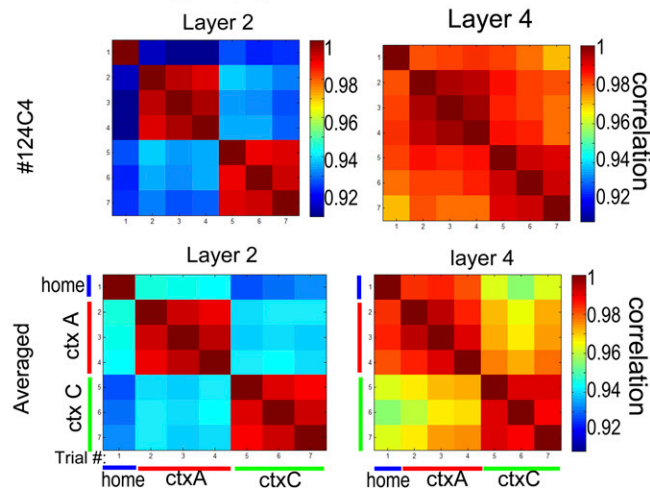


Fig. 55. The population correlations of L4 and L2 neurons in different training trials. Invariant representation of behavior tasks in the cortical volume is shown as the population coding. The example of one volume and the averaged correlations in the visual cortex between each trial were shown [$n = 5$ volumes from four mice; each volume, EGFP signals (>400 neurons) in $500 \times 500 \times 50\text{-}\mu\text{m}$ volume were quantified for each layer]. The color matrix showed the EGFP pattern correlation in deep layer (layer 4) neurons and superficial layer (layer 2) neurons for each trial in the same cortical volume. Hot color indicated higher correlation. Two tasks in different context were performed. Each task was repeated for three trials in different days. Task A trials induced neuronal activation showed higher correlation between A trials. Task C trials induced high correlation of activation between C trials. All of the activation patterns in A trials showed low correlation with patterns in C trials. Ctx A, context A trial; ctx C, context C trial.

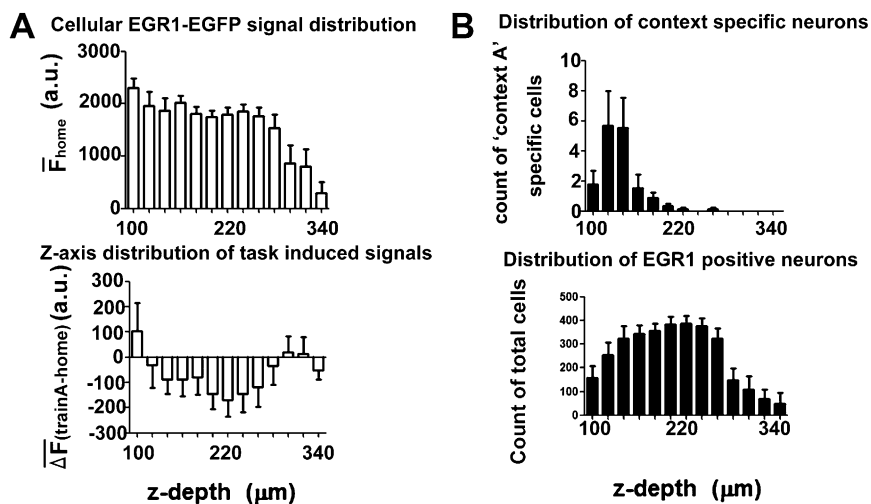


Fig. S8. The location of the identified memory trace cells in a visual volume. (A) Histogram showed the dorsal-ventral distribution of the averaged cellular EGFP signal (Upper; $n = 9$, for each volume neuron number, $n > 3,000$ in $500 \times 500 \times 350\text{-}\mu\text{m}$ volume) and averaged cellular EGFP changes (ΔF ; Lower; $n = 9$). (B) Histogram showed the dorsal-ventral distribution of memory trace cells (Upper, $n = 9$) and the number of total cells (Lower, $n = 9$).

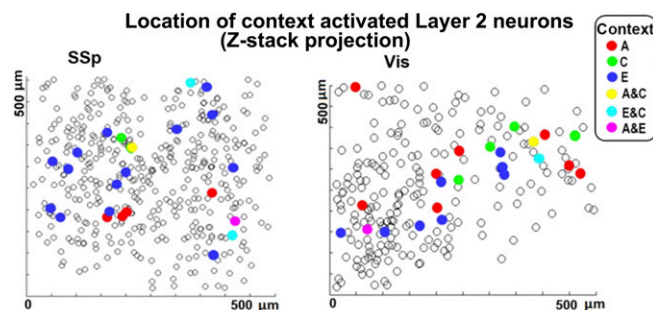


Fig. S9. Distribution of the memory traces for different contexts in layer II neurons. All neurons (within 120 μm below pia mater) were marked with black circle. Identified memory traces for different contexts were marked with different colors. Few neurons showed response to two contexts. Memory traces were determined as the commonly activated neurons (>2.8 -fold SD) in two trials for the same context. For context A, EGFP signals in the trials on day 5 and day 12 in context A were used. For context C, EGFP signals in the trials on day 40 and day 43 in context C were used. For context E, EGFP signals in the trials on day 45 and day 47 were used.

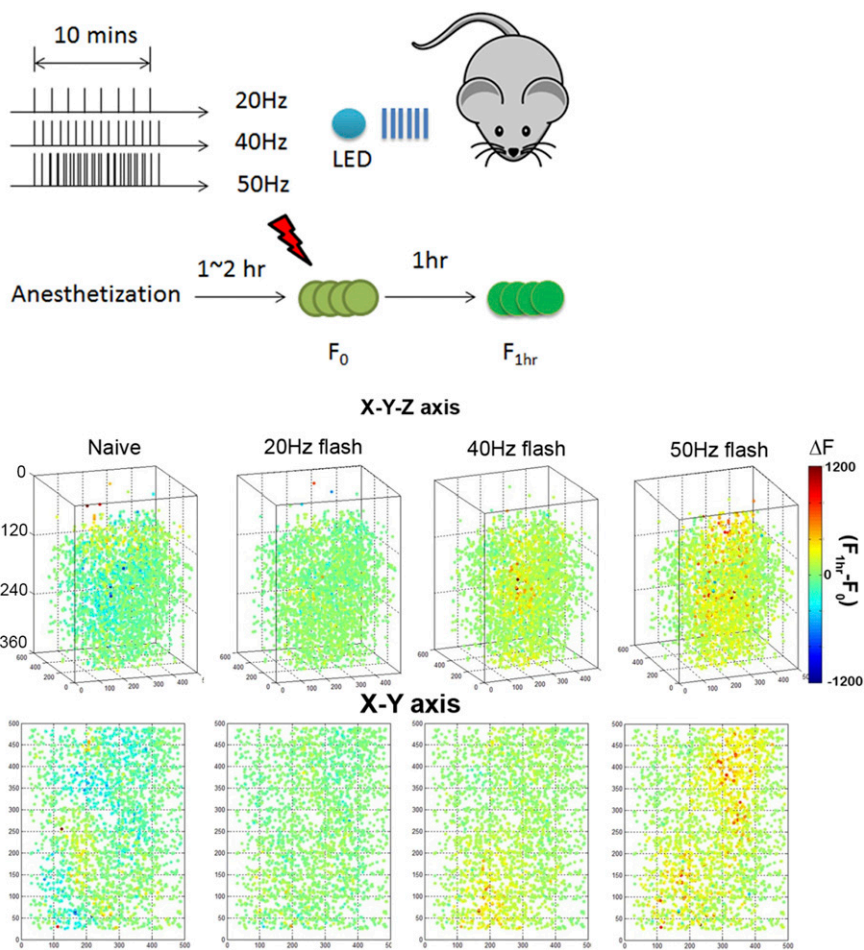


Fig. S10. Simple stimulus-evoked EGR1-EGFP signal changes in the cortical volume. The same mouse was anesthetized using isoflurane and received single visual stimulus for 10 min. Flash light with different frequencies induced a signal increase in both layer 4 and layer 2/3 neurons. Stimuli with different frequencies induced a different activation pattern.

Other Supporting Information Files

[Dataset S1 \(DOCX\)](#)