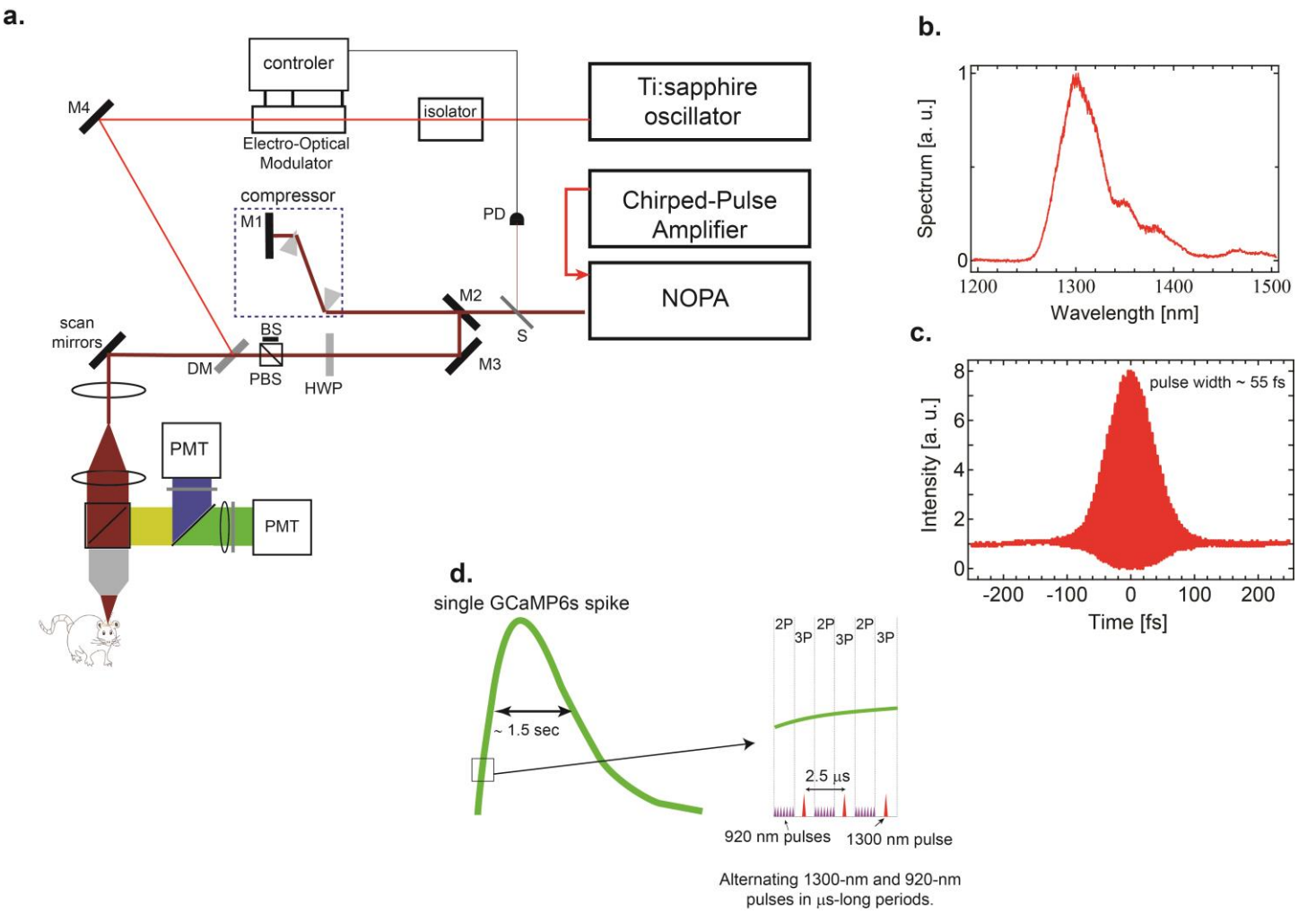


Supplementary Figure 1

Experimental setup

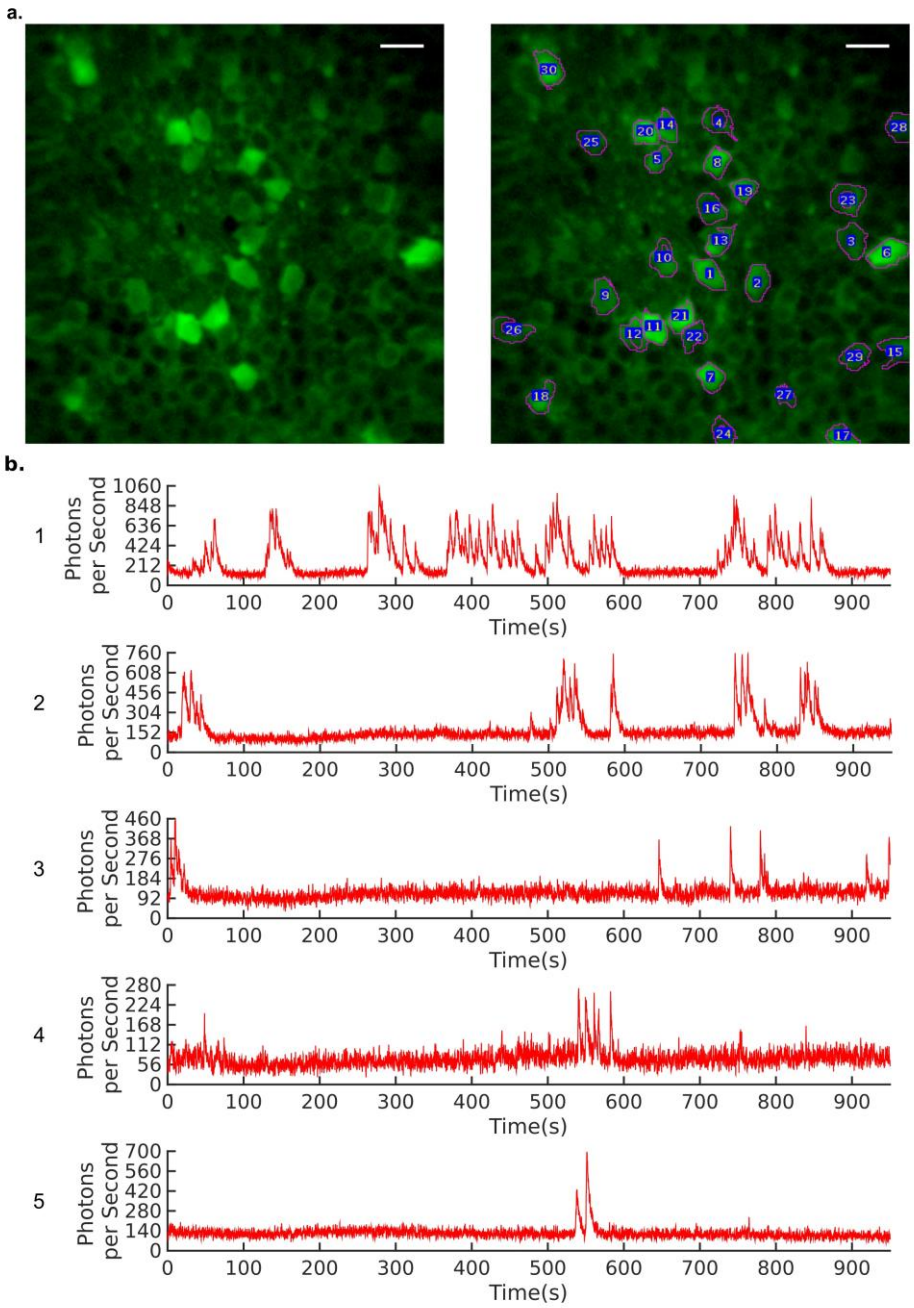
a, Schematic of the excitation sources and the basic optical path of the imaging setup.

b, Measured NOPA output spectrum.

c, Measured interferometric second-order autocorrelation trace of the pulse at the objective focus, with dispersion pre-compensation.

d, Time division multiplexing (TDM) for simultaneous 2PM and 3PM.

M – mirror, HWP – half-wave plate, PBS – polarizing beamsplitter cube, BS – beam stop, DM – dichroic mirror, PMT – photomultiplier tube, S – sampler, NOPA – non-collinear optical amplifier, PD – photo-diode

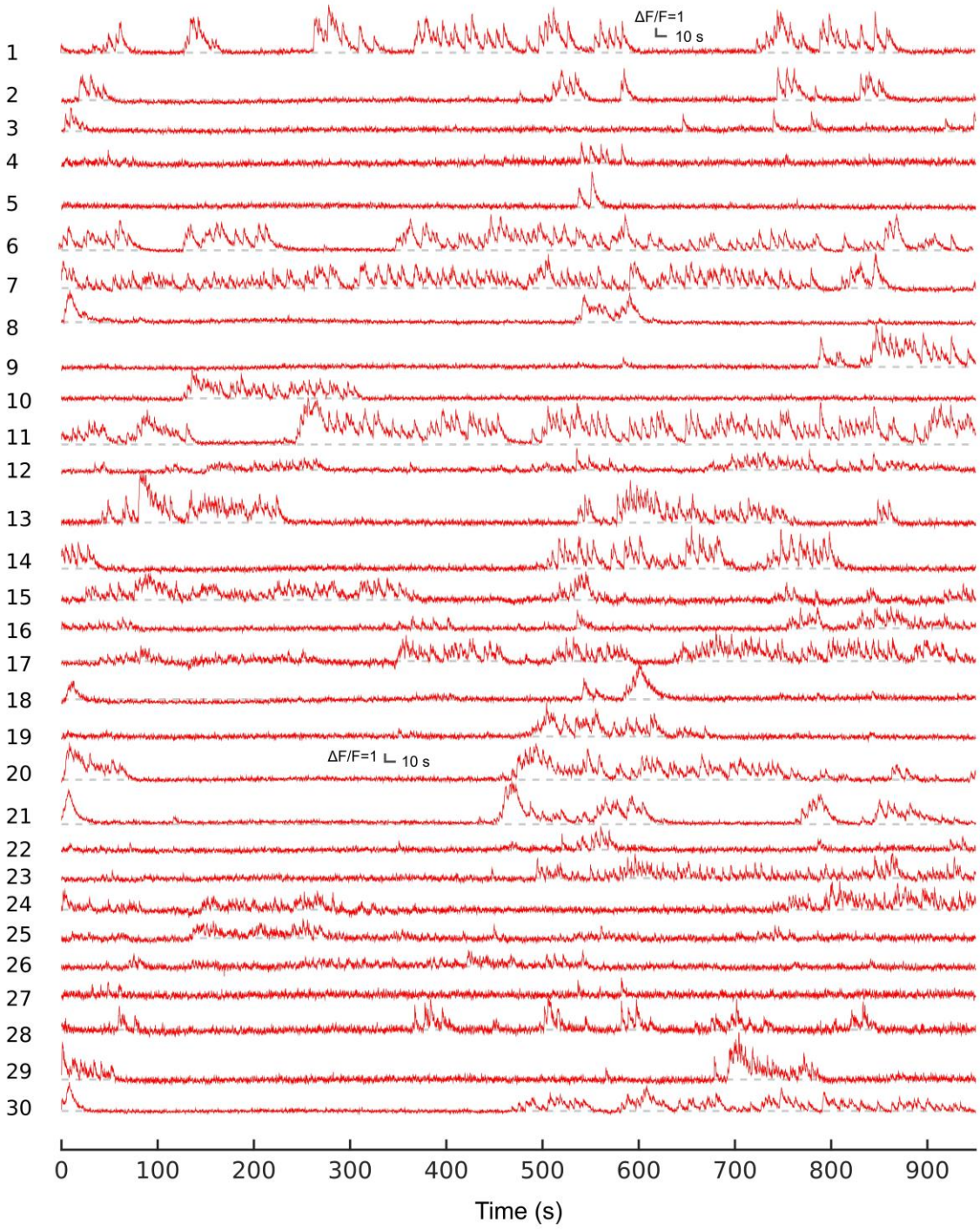


Supplementary Figure 2

3PM imaging of spontaneous activity in GCaMP6s-labeled neurons in the SP layer of the CA1 region of the mouse hippocampus.

a, 3PM image of neuronal population in the CA1 region of the hippocampus located at 984 μm beneath the dura (left). Neurons are indexed (right) for reference to traces in (b), Supplementary Figure 3 and 4. Average power of 50 mW at 800 kHz repetition rate was used for imaging. The field-of-view (FOV) was 200x200 μm. Scale bar, 20 μm.

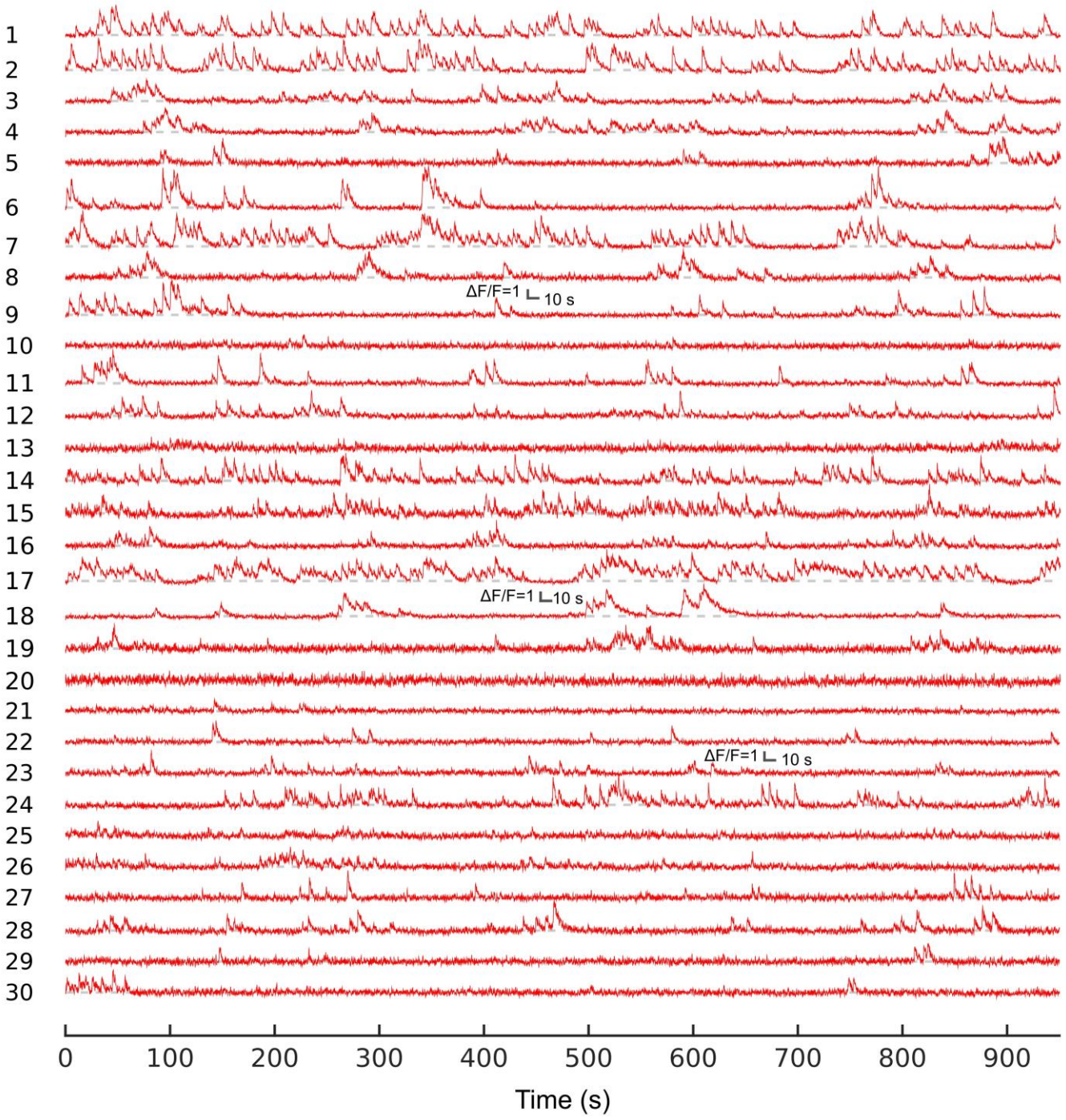
b, Spontaneous activity traces recorded from neuron 1-5 in (a) during approximately the first 16 minutes of a 48-minute recording session, at a frame rate of 8.49 Hz. The five neurons represent a range of activity level and brightness. All traces were low-pass filtered with a hamming window with a 0.59 s time constant, and fluorescence intensity was converted to photon counts per neuron per second.



Supplementary Figure 3

Spontaneous activity traces of the hippocampal neurons shown in Supplementary Figure 2

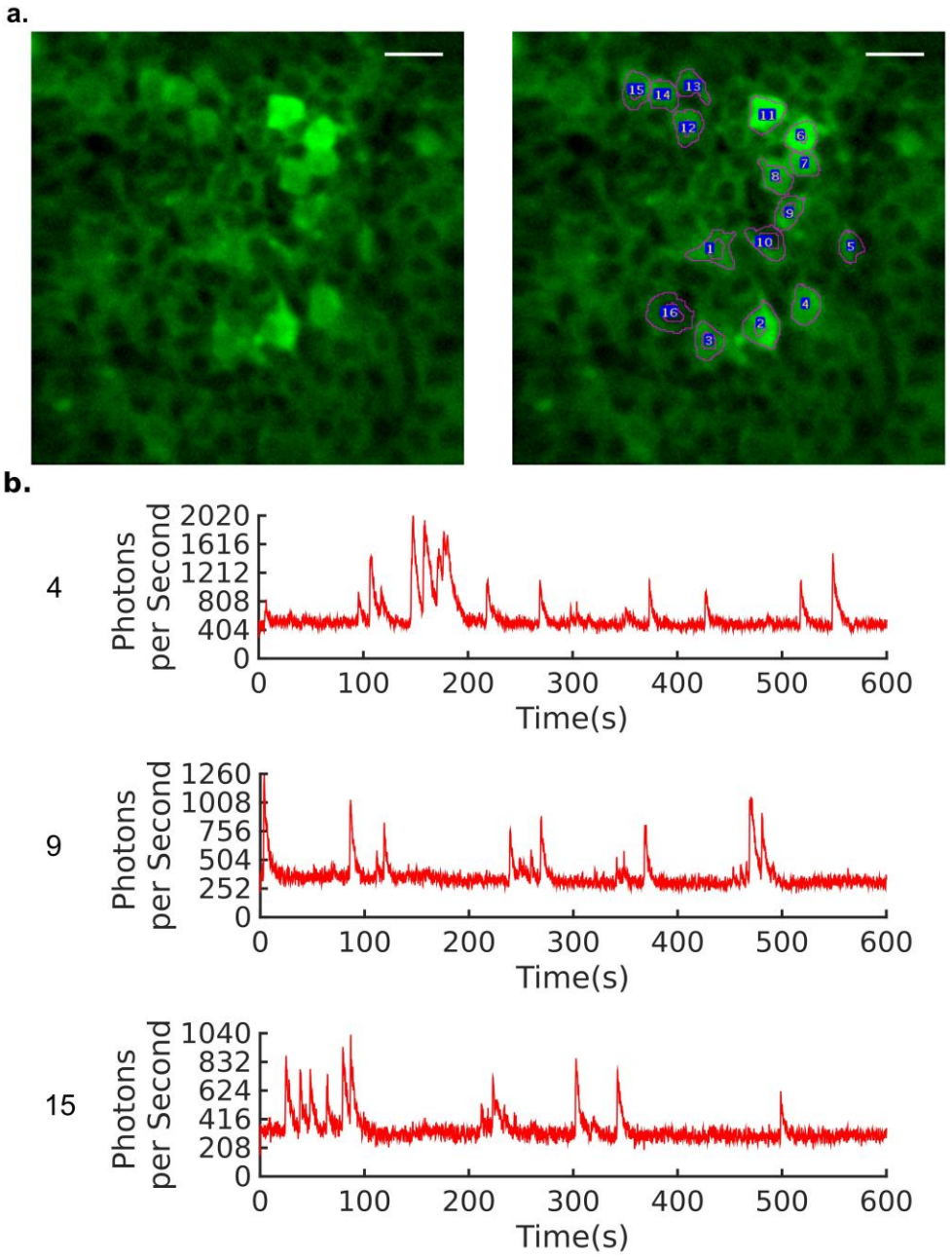
Spontaneous activity traces recorded from all indexed neurons in Supplementary Figure 2 during approximately the first 16 minutes of a 48-minute recording session, at a frame rate of 8.49 Hz. To the left of each trace is the index of the neuron. All traces were low-pass filtered with a hamming window with a 0.59 s time constant, and then normalized to each individual baseline.



Supplementary Figure 4

Continued recording of spontaneous activity traces of the hippocampal neurons after Supplementary Figure 3

Spontaneous activity traces recorded from the same neurons in Supplementary Figure 3 during approximately 16 minutes after 32 minutes of continuous recording (i.e., approximately the last 16 minutes of the 48-minute recording session starting in Supplementary Figure 3). All traces were processed in the same way as in (c).

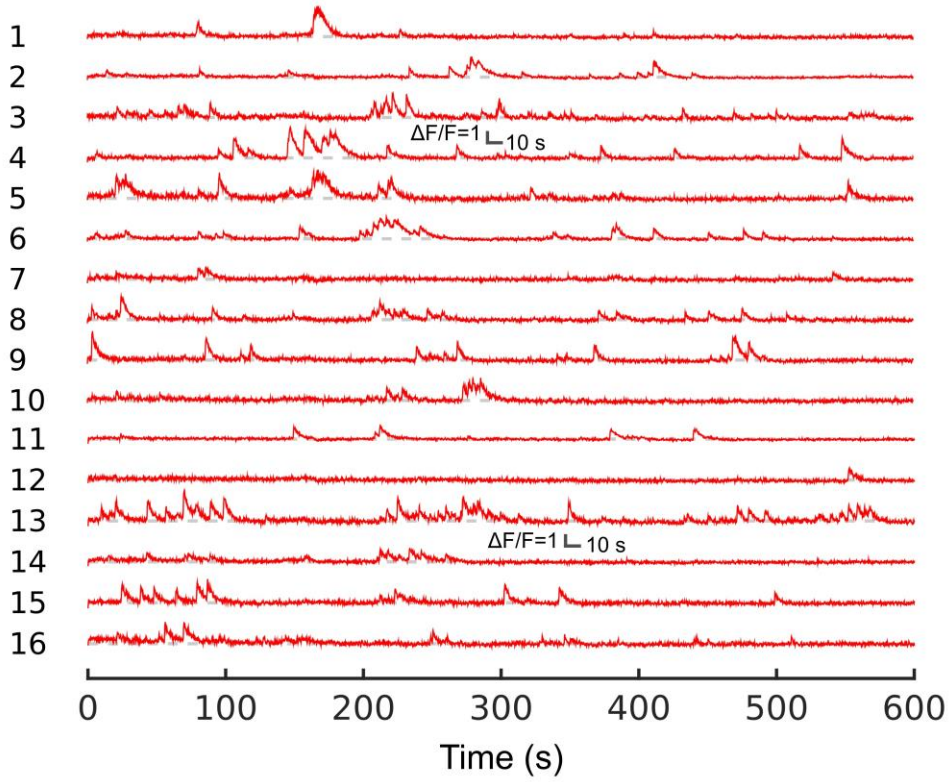


Supplementary Figure 5

Revisit of the same population of neurons in the SP layer of the CA1 region of the hippocampus after one week.

a, Activity recording site in the SP layer of the hippocampus located at 984 μm beneath the dura (left). The neuronal population was the same as that shown in Figure 2(c) and Supplementary Figure 2, but imaged one week later. Neurons are indexed (right) for reference to their traces in (b) and Supplementary Figure 6. Average power of 58 mW at 800 kHz was used for imaging. The FOV was 150x150 μm. Scale bar 20 μm.

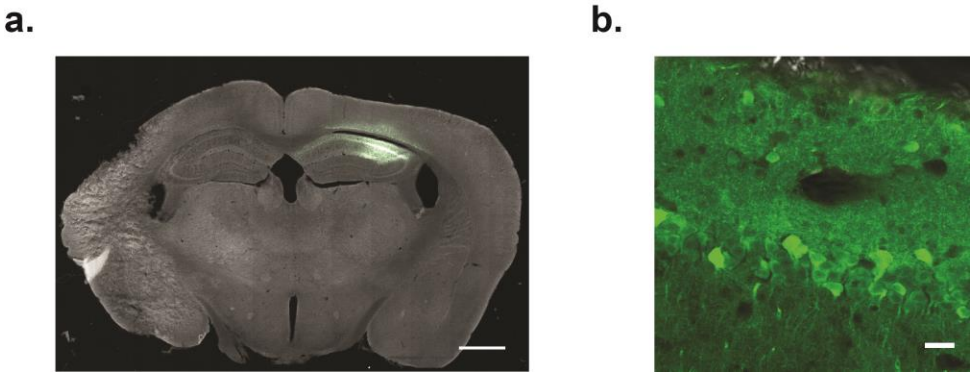
b, Spontaneous activity traces recorded from three indexed neurons in (a) for 10 minutes, at a frame rate of 8.49 Hz. The three neurons represent a range of activity level and brightness. To the left of each trace is the index of the neuron. All traces were low-pass filtered with a hamming window with a 0.59 s time constant, and fluorescent intensity was converted to photon counts per neuron per second.



Supplementary Figure 6

Spontaneous activity traces of the hippocampal neurons shown in Supplementary Figure 5

Spontaneous activity traces recorded from all indexed neurons in Supplementary Figure 5 for 10 minutes, at a frame rate of 8.49 Hz. All traces were low-pass filtered with a hamming window with a 0.59 s time constant, and then normalized to each individual baseline.



Supplementary Figure 7

Histological images of coronal mouse brain sections of the GCaMP6s-labeled site imaged by 3PM in this study

a, Confocal microscopy image of post mortem fixed brain tissue. Scale bar, 1 mm.

b, High resolution ex vivo confocal image from the CA1 region of the hippocampus. Scale bar, 20 μm.