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Micro-channel fabrication by femtosecond laser to arrange neuronal cells on multi-electrode arrays

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Abstract We propose and demonstrate micro-channel fabrication by femtosecond-laser processing to arrange neuronal cells on multi-electrode arrays (MEAs). When neuronal cells were seeded in the micro-channels on MEAs, they elongated their neurites along the channel and finally formed the network patterned on MEAs. The spontaneous electrical activity of the neuronal networks on MEAs was evaluated by using electrophysiological systems, indicating that patterned network was functionally activated along the channels. The present method is applicable to analyze network dynamics of living neuronal cells patterned artificially.

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1 Introduction

Neuronal networks are widely studied both experimentally and theoretically to elucidate the basis for brain systems referred to as learning and memory. The functional characteristics in response to spatio-temporal structure of neuronal activity have been investigated by fluorescence imaging and electrophysiological methods [1, 2]. The Multi-Electrode Array (MEA) technology has been intensively used in neuroscience for recording electrical activity in dissociated neuronal cultures and brain slices [3, 4]. This allows us to study plasticity of neuronal networks as electrical communication with all neurons.

We have applied the neuronal network on MEAs to understand developmental connectivity as spatio-temporal dynamics of the electrical activity. The organization of neuronal network on the MEAs and its connectivity has been characterized in previous studies [5, 6]. The promising application of artificial neuronal network is to couple the output signals with bio-robotics systems [6]. In order to realize such application, it is indispensable to control the neuronal network by flexible arrangement of the neuronal cells on the MEAs. As one of them, we have recently demonstrated local modification of neuronal network by direct laser cutting by a focused femtosecond laser [7, 8]. This method can be utilized in local arrangement of the neuronal network of matured neurons at single neurite level. As further improvement, since the methodology to arrange the dissociated neuronal cells on whole area of MEAs has been required, we

considered to make micro-channels to connect specific electrodes on MEAs in advance of the local arrangement. Flexible and rapid prototyping of such micro-channels is realized by masking the MEAs with sticky film, where slits are fabricated. Silicone rubber film is useful as the masking material because of its stickiness and bio-compatibility. In this work, femtosecond-laser processing was applied to make slits in the silicone rubber film for micro-channel fabrication.

We have previously demonstrated femtosecond-laser processing of organic thin film with less thermal damage and high aspect ratio, and clearly indicated the potential with experimental results of time- and space-resolved investigation [9, 10]. These results suggest that the initial modification of the molecular association by single-shot femtosecond-laser irradiation is dominant not by thermal evaporation but by extremely rapid thermoelastic stress increase in a picosecond time range and leads to a mechanical disruption [11, 12]. Silicone rubber should undergo similar ablation upon the femtosecond-laser irradiation. Therefore, we considered femtosecond-laser processing of silicone rubber should be initiated by the mechanical disruption resulting in the thermoelastic stress, which can be used to minimize photo-thermal and chemical damage. It is suitable for realizing the high aspect ratio processing. Furthermore, if the edge of the slits is carbonized by thermal and chemical modifications, the conductivity of the edge may prevent accurate measurement of the electrical activity of neurons. Hence we employed the femtosecond laser to process the silicone rubber.

Here, we demonstrate micro-channel fabrication utilizing a femtosecond-laser processing for arrangement of neurons on MEAs. Neuronal cells were arranged in silicone rubber masking, which was made by the femtosecond-laser processing. When dissociated neurons were cultured in the micro-channels on MEAs, the neuronal network was formed along the channel. Spatio-temporal structures of electrical activity of the neuronal cells on MEAs with micro-channels were compared with those of random arrangement and functional connectivity of the patterned network is discussed.

2 Experimental methods

2.1 Laser processing of silicon rubber

The experimental procedure for the laser processing and arrangement of the transparent silicone rubber film (Momentive) with a thickness of 50 μm is illustrated in Fig. 1. Silicone rubber film was processed by femtosecond-laser pulses generated by a regeneratively amplified Ti:sapphire laser system (Hurricane, Spectra-Physics) with a center wavelength of 800 nm, pulse duration of 120 fs, and repetition rate of 1 kHz. The femtosecond-laser was focused through a convex lens ($f = 150$ mm) on the silicone rubber film placed on a support substrate, as shown in Fig. 1(a), where the sample was positioned slightly above the laser focal point to avoid optical breakdown. The diameter of laser

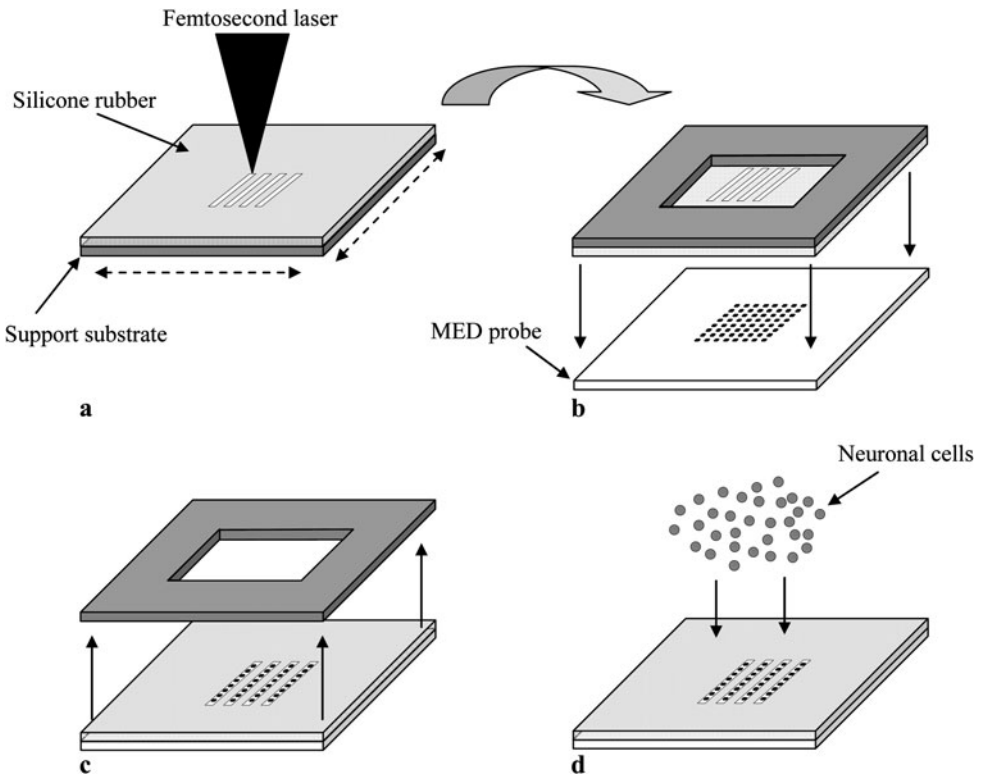

Fig. 1 Schematic diagram to fabricate micro-channels for the cell arrangement on MED probe. (**a**) Silicone rubber film on support substrate is processed by scanning pulse train of the femtosecond laser and slits were made in the film. (**b**) The silicone rubber film was deposited on 8 × 8 microelectrodes in MED, where the slits of the silicone rubber were arranged along column lines of electrodes. (**c**) The support substrate is detached from silicone rubber. (**d**) Neuronal cells are seeded in the micro-channels on MED probe

spot on the film was examined to be 60 μm by a laser pattern formed on a carbon-doped polymer film, which was about twice of the diffraction-limited spot size. The laser beam was scanned by moving the motorized stage with linear velocity of 150 μm/s. After the processing, the silicone rubber film was turned upside-down and set above an intact MEAs dish by home-made positioning systems. To strengthen the adhesion between the silicone rubber film and the surface of the MEAs dish, UV light of Hg lamp irradiated the MEAs for a half day.

2.2 Primary neuron culture and electrophysiological analysis

Hippocampal neurons were isolated from embryos of Wister rat (embryonic day 18) as described previously [5, 6]. The experimental procedures were performed in accordance with the guidelines for the animal care and use of National Institute of Advanced Industrial Science and Technology. Neurons were dissociated with 0.01% trypsin in Ca^{2+}-free and Mg^{2+}-free phosphate-buffered saline minus (PBS^{-}) with 10 mM glucose, dispersed in the PBS buffer, and seeded at a density of 2.5–5.6 × 10^4 cells/mm^2 on a polyethylenimine-coated MEAs dish probe (MED probe, MED-P515A, -P545A, Alpha MED Sciences) [13]. The MED probe has 8 × 8 planar microelectrodes whose size and spacing are 50 × 50 μm and 150 or 450 μm, respectively [14]. We used a glass ring (internal diameter 5 mm, height 10 mm) to form the neuronal network in the central area of MED probe. The ring was removed after 1 day. The neurons were maintained in culture medium containing Dulbecco's modified Eagle's medium and Ham's F12 medium (Invitrogen) with supplements of 5% horse serum, 5% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 μg/ml insulin and incubated at 37°C in a humidified atmosphere with 5% CO_2. For current experiments, the neurons cultured for 9–50 days were used. Phase contrast images of neuronal cells on the MEAs were obtained with an inverted microscope (CKX41, Olympus) equipped with a CCD camera (DP20, Olympus).

For electrophysiological analysis, the extracellular action potentials of neurons were detected through 64 electrodes on MEAs dish and recorded by a 64-channel extracellular recording system (MED64 system, Alpha MED Science) with a sampling rate of 20 kHz for 600 s. The action potentials were analyzed using MEDFAUST [15]. Extracellular spike trains were determined as events when their amplitude exceeded a prespecified threshold for each recording channel.

3 Results and discussion

The silicone rubber film processed with pulse energy of 300 μJ/pulse is shown in Fig. 2. When the silicone rubber at the laser focal point was perfectly removed, a line slit was formed. Since shot number at a position on the scanning line can be estimated to be

$$1000\ [\mathrm{Hz}] \times \frac{60\ [\mu\mathrm{m}]}{150\ [\mu\mathrm{m/s}]} = 400\ [\mathrm{shot}],$$

the silicone rubber is gradually drilled with 400 pulses. Critical photo-thermal damage was not observed at the line edge, because the silicon rubber processing by the femtosecond laser was probably initiated by photomechanical fragmentation due to multi-photon absorption of the femtosecond pulses [9]. Slits of the silicone rubber were set on the electrodes of MED probe and then micro-channels with 8 lines were fabricated on the electrodes as shown in Fig. 2. Since the channel width and depth (film thickness) were ∼85 μm and 50 μm, the aspect ratio (width/depth) could be estimated to be ∼1.7. Hence the precision processing of rubber with narrow aspect ratio (width/depth) was realized under the present experimental condition. Each electrode of 50 × 50 μm size was successfully exposed to the surface, which allows us to attach the neurons on the MED probe.

When the dissociated hippocampal neurons were seeded on the silicone rubber film with channels on the MED probe

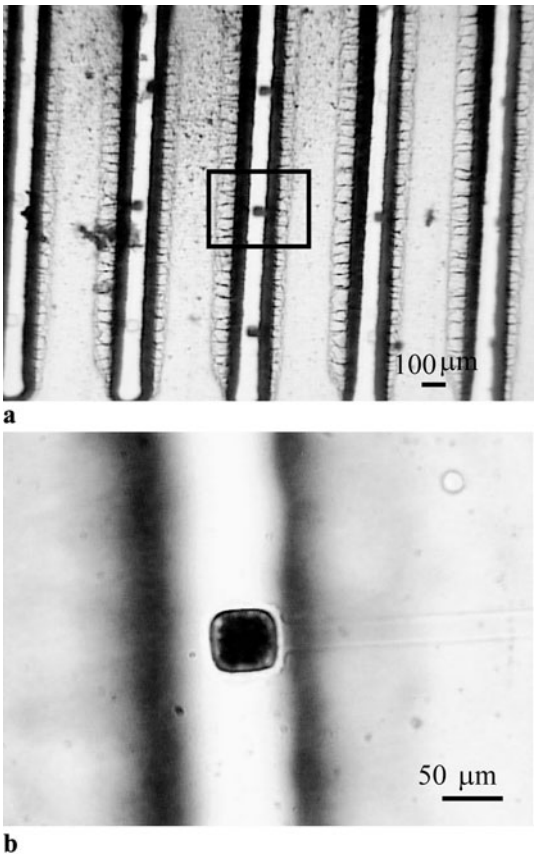

Fig. 2 Microphotograph (**a**) and enlargement one (**b**) of the micro-channel on the MED probe. The *black square* is an electrode with size of 50 × 50 μm

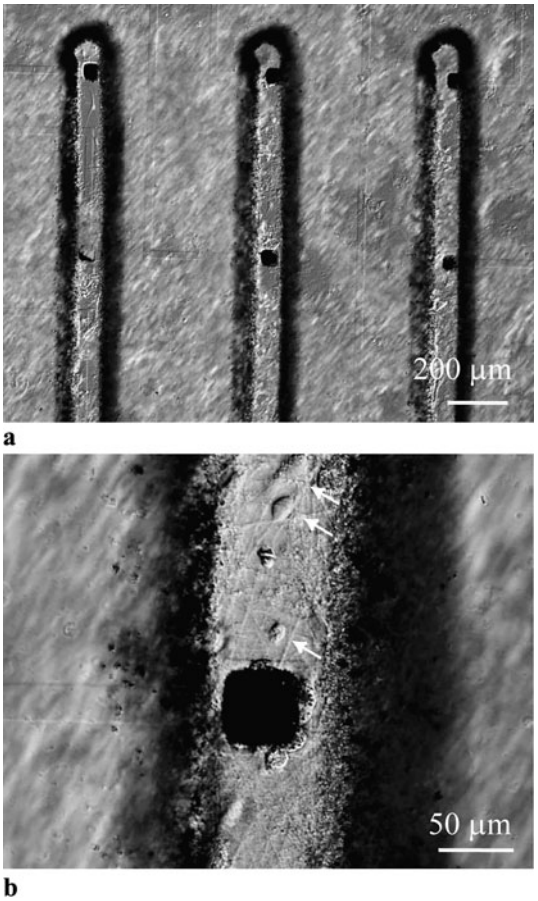

Fig. 3 Microphotographs (**a**) and enlargement one (**b**) of hippocampal neurons at 9 DIV cultured in the micro-channel on polyethylenimine-coated MED probe. The spacing between electrodes is 450 µm. The *white arrow* indicates neurite elongation of neurons

by utilizing the procedure shown in Fig. 1(d), some of neuronal cells were introduced into the channels, while most neurons were attached to the surface of silicon rubber film. This is caused that the probability of introduction into the channels was not too high due to narrow width of the channels, since the neuronal cells can be similarly adherent to both surfaces of the MED probe and the silicone rubber films because of coating of polyethyleneimine in advance. The neurons in the micro-channels elongated their neurites along the channels within a few days in vitro. Figure 3 shows neuronal cells in the channels at 9 days in vitro (DIV). The neuronal cells visualized as phase contrast elongated their neurites in the channel (b). The cells arranging with interval of a few µm demonstrated to be connected by the neurites. Locomotion of the neuronal cell and elongation of their neurites were perfectly blocked by the wall of the silicone rubber. Therefore, it is concluded that a neuronal network is fabricated in the channel.

To evaluate functional connectivity in the neuronal networks, spatio-temporal patterns of electrical activity of neuronal cells in MED probe was measured by recording extracellular field potential near the neurons on an electrode as a voltage, which is called the spontaneous action potentials (SAPs). A representative result of the measurement for 8×8 electrodes arrangement is shown in Fig. 4. Signals of SAPs were observed from approximately 14 DIV, meaning that neuronal cells formed complex and random networks among electrodes. Figure 4(c) showed a snapshot of spatio-temporal patterns of SAPs of neuronal cells on intact (non-patterned) MED probe at 36 DIV and the SAPs at each electrode occurred at high frequency, as we have previously reported [8]. In the case of neuronal cells on MED probe with micro-channels whose connected on column lines (column line 1 to 8) and disconnected on row lines of the electrodes (row line 1 to 8), the spatio-temporal patterns of SAPs at 44 DIV were obtained as shown in Fig. 4(d). For analysis of neuronal network dynamics with and without micro-channels, we investigated the synchronization of SAPs, which occurs in the neuronal network along the column lines.

We have previously analyzed the synchronization among spatio-temporal patterns of SAPs as cross-correlation between them and explained functional synaptic connections on the MEAs [8]. Such synchronization along column or row lines can be evaluated simply as follow: The spike trains of SAPs at each electrode were added in each line (column and row) and divided by the number of electrodes at a line. The mean number of spike trains at 50 µs was counted with time duration of 5 ms step by step. For example, when the signals are synchronized, the mean spike number at a unit time should be increased. Figures 5(a) and 5(b) show the mean spike number corresponding to single lines at C8 and R8 in MED probe, where the electrodes are connected and disconnected by the micro-channels, respectively. The mean spike number at the column line of C8 along micro-channels was significantly larger than that at the row line of R8. In addition, the mean spike number at the row line of R8 without micro-channels gave random and frequent spikes because of the lack of synchronization. This indicates that the spatio-temporal patterns of SAPs are synchronized in the direction of column lines of C8, since a functional network of neuronal cells is formed along the micro-channels. We calculated the time-averaged mean spike numbers of all lines as shown in Figs. 5(c) and 5(d). The number of spikes beyond a particular cut-off value of spike number, which was defined as the average value plus five standard deviations was counted and the time-average within 600 s was obtained. The mean spike number of column lines (C1 to 8) of electrodes depended on the line compared with that of row lines (R1 to 8). In the micro-channel containing few neurons, it

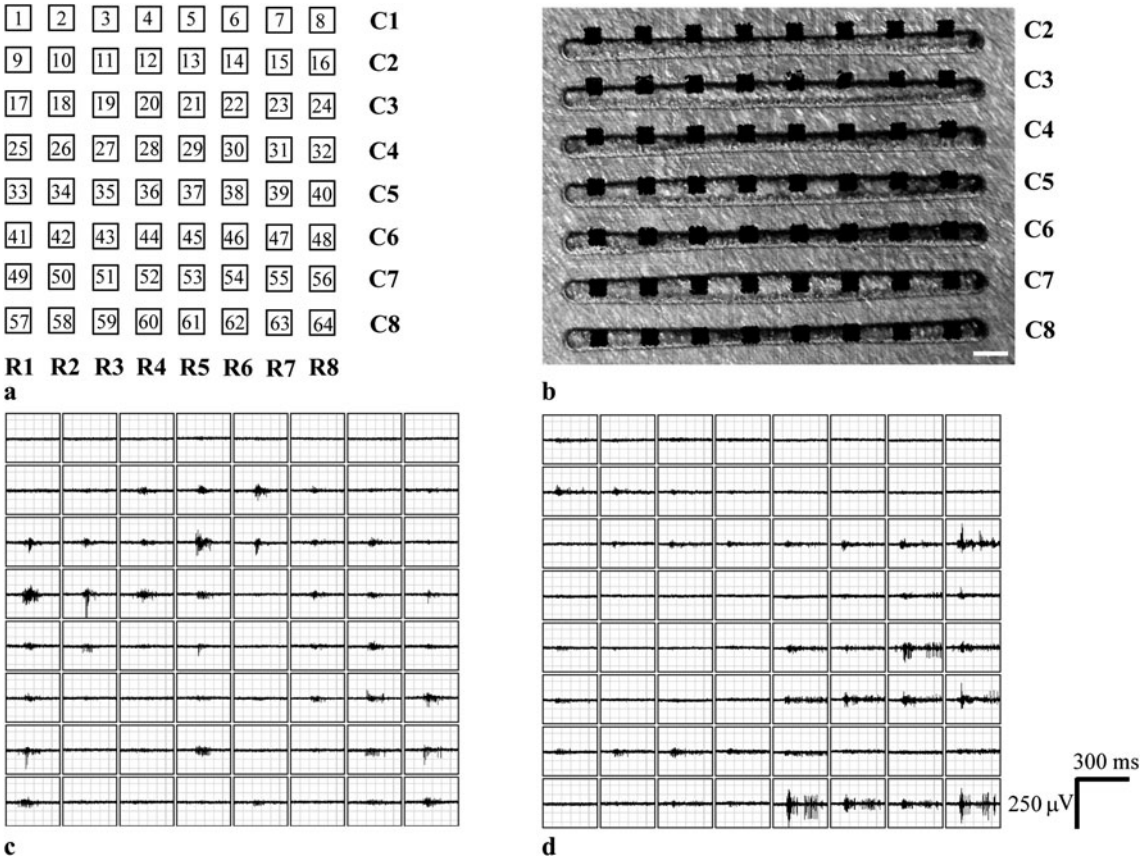

Fig. 4 (**a**) Arrangement of 64 electrodes in a MED probe. Numbers correspond to individual electrodes. C1 to 8 and R1 to 8 show a number of column and row lines of electrodes, respectively. (**b**) Microphotograph of hippocampal neurons at 44 DIV cultured in micro-channels on the MED probe. The *white bar* is 100 µm. The spacing between electrodes is 150 µm. (**c**, **d**) Snapshots of spatio-temporal patterns of spontaneous action potentials (SAPs) of neuronal network cultured on MED probe without micro-channels at 36 DIV (**c**) and that on MED probe with micro-channels at 44 DIV (**d**). The *single box* corresponds to the arrangement of 64 electrodes in (**a**). *Vertical* and *horizontal bars* in single box indicate 250 µV of extracellular voltage and 300 ms of time, respectively

seems that mean spike number was low. The present results indicate that neuronal activity is localized in micro-channels on MED probe, because the temporal behavior of mean spike number reflects on functional connectivity of neurons. Conclusively, the neuronal network can be successfully arranged in micro-channels.

4 Conclusion

We have demonstrated micro-channel fabrication for arranging of neuronal cells on MEAs. The micro-channel was fabricated by processing a silicone rubber film and overlaying it on the MEAs dish. Femtosecond-laser processing achieved the fabrication of the micro-channels with high aspect ratio. When neuronal cells were cultured in the micro-channels on MEAs, they formed the network with elongating their neurites. The morphological change and electrical activity of neuronal cells supported that the formed network was functionally separated along the micro-channels.

The micro-patterning of neuronal cells on a planer plate has been reported by several groups for analysis at cellular assembly and single cell levels by mainly using chemical patterns [16, 17]. In comparison with those methods, rapid prototyping by the femtosecond-laser processing is better for flexible patterning of neuronal network. Additionally, since silicone rubber film can be easily removed after patterning the neuronal network, we can realize further flexible modification of the patterned network, for example, additional elongation of neuritis between the micro-channels and/or additional cell patterning on the network. In the near future, multi-modulation of neuronal network on MEAs will be realized by combining the present and previous patterning method with laser modification techniques for neuronal cells reported us [8].

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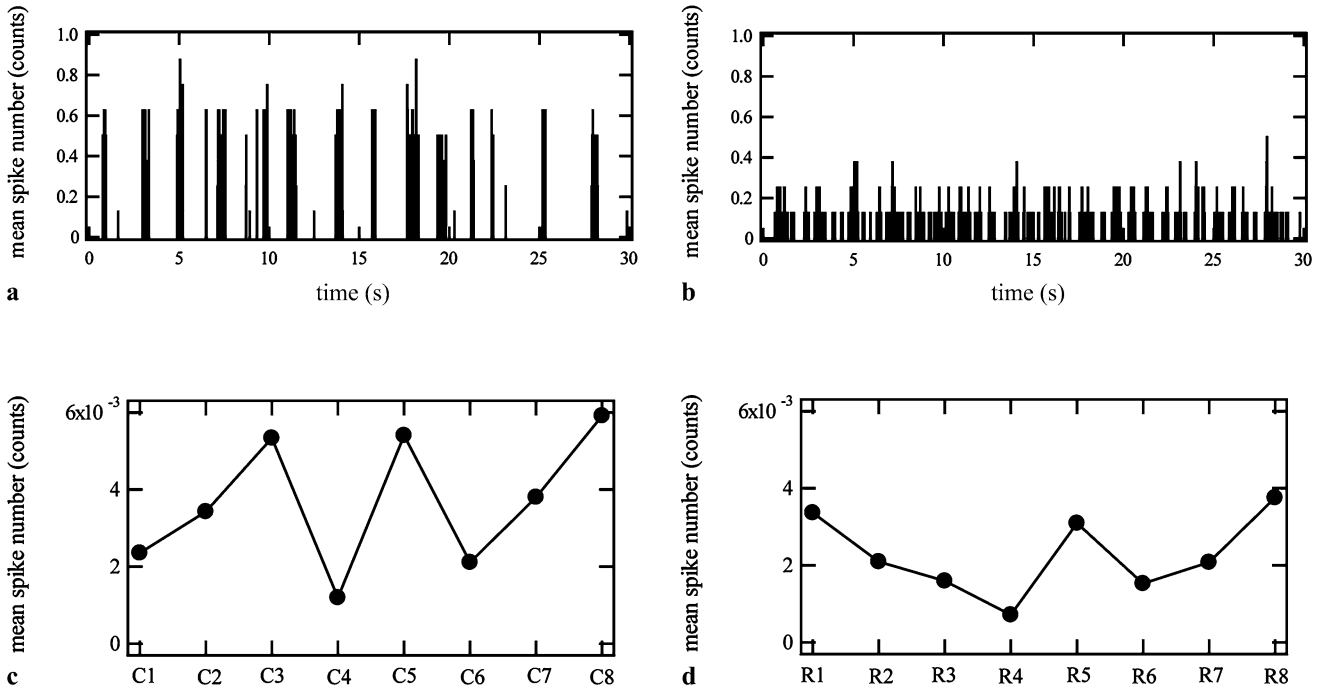

Fig. 5 (**a**, **b**) Representative temporal profiles of mean spike number of 8 electrodes corresponding to single line at C8 (**a**) and R8 (**b**) in MED probe. The bin is 5 ms. (**c**, **d**) The line dependences of mean spike number of electrodes at column line of C (**c**) and row line of R (**d**). The cut-off value of spike number is defined as the normal mean value plus five standard deviations, averaged over 8 electrodes at each line

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