Optical validation of *in vitro* extra-cellular neuronal recordings

Nitzan Herzog\(^1,2\), Mark Shein-Idelson\(^2\) and Yael Hanein\(^2\)

\(^1\) Department of Biomedical Engineering, Tel-Aviv University, Tel Aviv 69978, Israel
\(^2\) School of Electrical Engineering, Tel-Aviv University, Tel-Aviv 69978, Israel

E-mail: nitzan.herzog@gmail.com

Received 5 April 2011
Accepted for publication 20 July 2011
Published 12 August 2011
Online at stacks.iop.org/JNE/8/056008

Abstract
Simultaneous calcium imaging and extra-cellular recordings from cultured cortical rat neurons were performed to directly map the efficacy of extra-cellular recordings with microelectrodes. For the first time, we can associate extra-cellular recordings with neuronal activity of specific neurons in the vicinity of the electrode. We demonstrate that recorded cells can be identified by correlating the electrical signals and the calcium response. Our data demonstrate that in sparse cultures, microelectrodes record exclusively from cells which reside at very close proximity to the recording electrode. Moreover, we show that recording appears to be limited to only a partial subset of the cells residing in this range. We further show that even in cases of strong neuron–electrode coupling, extra-cellular signals recorded from single, well-identified neurons vary in shape over time rendering spike sorting and network activity rate analysis incongruous. As multi-electrode array technology is becoming increasingly widespread, the visualization technique we report here will help users better understand the limits of this versatile and useful method.

Online supplementary data available from stacks.iop.org/JNE/8/056008/mmedia

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Extra-cellular (EC) microelectrode arrays (MEAs) are widely used for multi-site neuronal recordings both *in vitro* and *in vivo*. Their small dimensions and ease of fabrication make them well suited for studying the activity of specific cells within large cell populations (Buzsáki 2004, Morin \textit{et al} 2005). With the addition of micro-fluidic channels, micro-fabricated devices offer exciting prospects in the realm of neuronal investigations. In particular, *in vitro* MEA investigations offer an attractive model system for drug testing and screening (Johnstone \textit{et al} 2010, Stett \textit{et al} 2003).

Since MEA devices consist of a finite and limited number of electrodes, only a small fraction of cells out of the entire cell population are recorded. However, each electrode may record from several cells simultaneously. To distinguish between different cells recorded by the same electrode, data analysis methods are often employed (Lewicki 1998, Hulata \textit{et al} 2002). These methods rely on a fundamental paradigm which associates a unique EC signal shape with each neuron. The shape of the EC signal is attributed to the manner in which the cell is coupled to a recording electrode (i.e. sealing), to the electro-chemical properties of the electrodes and to whether a cell is firing isolated action potentials or whether it is firing very rapidly and in synchrony with other cells (Vassanelli and Fromherz 1998, Fromherz 1999, Harris \textit{et al} 2000, Cohen \textit{et al} 2006). The conductivity of the medium and the surrounding tissue can also dramatically affect the recorded signal. All the factors mentioned above affect the ability to properly identify the number of active cells, their activity rate and the manner in which different neuronal sub-groups function within a larger network. Understanding the link between the recorded EC signals and the original intracellular signals is crucial for interpreting MEA recorded data. Owing to the growing use of MEA technology for *in vitro* neuronal investigations, in particular for applications involving vertebrate networks,
we aim to address the following critical and long standing questions: How many of the neurons in the vicinity of an electrode are active? The spikes of how many of these are detected? With what reliability specific recorded spikes can be assigned to a particular neuron?

A possible approach to address these questions is to utilize optical imaging of neuronal activity in conjunction with EC recordings. Optical investigations of neuronal dynamics offer important benefits that have been so far rarely used in the realm of thin-film multi-electrode array investigations (Cossart et al 2005, Ikegaya and Bon-Jego 2005, Takahashi et al 2007). However, since in vitro MEA recordings are compatible with optical microscopy, the two techniques can be readily combined and used to elucidate the efficacy of EC MEA recording.

In particular, high-affinity aceto-methylized calcium-sensitive dyes have efficient loading performances and high fluorescence signals and therefore can be readily and effectively used to record single action potentials in dissociated cell cultures. These qualities have allowed extensive use of these dyes in spike train reconstruction (Smetters et al 1999, Sasaki et al 2008, Vogelstein et al 2009, Ranganathan and Koester 2010, Rothschild et al 2010).

In this work, we employed a special setup combining upright calcium imaging microscopy and a conventional in vitro MEA recording apparatus. Combining these two techniques, in conjunction with signal analysis, we were able to visually identify individual firing cells and directly associate EC with Ca data. We used this technique to show that not only is the recording restricted to a close proximity of the recording electrode, but also that recording is achieved from only a partial subset of the cells residing in the electrode range. Finally, we used the combined recording method to explore the soundness of activity rate and spike sorting analysis in MEA recordings.

2. Methods

2.1. Preparation and growth of cultured networks

Dissociated cortical cultures were prepared as follows: the entire cortices of (E18) Sprague Dawley rat embryos were finely removed. The cortical tissue was digested with 0.065% trypsin (Biological Industries, Beit Haemek, 03-046-1) in phosphate buffered saline (Beith Haemek, 02-023-1) for 15 min, followed by mechanical dissociation by trituration. Cells were re-suspended in a modified essential medium with Ear’s salts (Beit Haemek 04-004-1), 5 mg ml⁻¹ gentamycin (Beith Haemek 03-035-1), 500 μM glutamine (Beith Haemek 03-020-1) and 0.02 mM glucose (BDH101174Y), and plated on poly-d-lysine (PDL, Sigma, catalog no p-7889) covered multi-electrode arrays (500/30R-Ti or HD 30/10R-ITO, by Multi Channel Systems) with a cell density of 3000–4000 cells mm⁻² (~1.5 × 10⁶ cells per dish). Cultures were maintained at 37 °C with 95% CO₂. The growth medium was partially replaced every three to four days.

2.2. Ca imaging

Recordings were performed in an open air environment, necessitating culture medium replacement to buffered-ACSF medium (containing, in mM, 10 HEPES, 4 KCl, 2 CaCl₂, 1 MgCl₂, 139 NaCl, 10 d-glucose, adjusted with sucrose to an osmolarity of 325 mOsm and with NaOH to a pH of 7.4). Cultures were washed three times to remove traces of incubation medium and were then incubated in ACSF supplemented with 3 μM Fluo-4 AM (Invitrogen F14201, one vial with 7.6 μl anhydrous-DMSO for stock of 6 mM) or Oregon-Green BAPTA-1 AM (Invitrogen 06807, same stock preparation) and same volume of pluronic acid F-127 (Biotium 59000, stock 10% w/v after mixing 1 g vial in 10 ml DDW) for 1 h. Following incubation, cultures were washed again and kept in ACSF. During recordings cultures were kept at 37 °C. Time lapse data were taken with an Olympus upright microscope (BX51WI) fitted with an EMCCD camera (Andor Ixon-885) and a ×40 water immersion objective (Olympus, LUMPLFL NA 0.8). This setup allows the visualization of cells residing on top of non-transparent electrodes. Fluorescent excitation was provided via a 120 W mercury lamp (EXFO x-cite 120PC) coupled to a dichroic mirror with a filter to match the dye spectrum (Chroma T495LP). Camera control utilized Andor propriety SOLIS software. Time lapse recordings were performed at either 2 × 2 binning mode for resolution of 500 × 502 and 35–40 fps or 4 × 4 binning mode obtaining 250 × 251 resolution and 100–103 fps. Time lapse sequences were collected via a dedicated 12-bit Andor data acquisition card installed on a personal computer, spooled to a high capacity hard drive (typically >1 TB) and stored as uncompressed multi-page tiff file libraries.

2.3. Electrophysiological recording and spike detection

EC recordings were conducted utilizing a low noise pre-amplifier board (MEA-1060-BC, amplifier, gain ×1100 with a band-pass filter of 200 Hz to 5 kHz, by Multi Channel Systems). Signals collected from the microelectrodes were sampled at a 10 kHz sampling rate and stored on a personal computer equipped with a 128-channel, 12-bit data acquisition board (MC_Card, by Multi Channel Systems) and MC_Rack data acquisition software (Multi Channel Systems, version 3.8.0). Preliminary spike detection was performed in real time using MC_Rack whereby only 100 ms windows around threshold crossings were stored. Thresholds were first manually selected according to a channel’s signal-to-noise ratio (SNR). A second filtering step utilized the wavelet packet decomposition method introduced in Hulata et al (2002) to exclude noise. Detected spike trains were presented in the form of raster plots where, for display purposes, spikes are collected according to the time of occurrence into bins sized 30–100 ms. Bins are then displayed on a time axis with a color coding which indicates the number of spikes registered in each bin. The bin size was selected manually to allow optimal visualization of all recorded events.
2.4. Ca data analysis

Ca imaging data were stored in the form of an uncompressed tiff library, where pixel values represent fluorescence intensity. Somata boundaries were manually delineated on the time-averaged Ca image. Raw Ca traces were extracted for every cell by averaging over all pixels within the cell boundary. Raw cell traces typically exhibit a decaying baseline characteristic of bleaching effects. This artifact was corrected by employing a centered running minima analysis, computing each time point to be \( (F - F_0) / F_0 \), where \( F \) is the raw fluorescence intensity and \( F_0 \) is the minimum of the raw fluorescence in the surrounding 20 s time-window. Since analyzed traces are computed as relative to a local minimum, they are strictly non-negative.

2.5. Ca data reconstruction from spike timings

Ca events time boundaries were identified (see S1 in the supplementary material available at stacks.iop.org/JNE/8/056008/mmedia) and events in which only a single EC spike occurred were collected, averaged and fitted to a three-parameter model kernel function:

\[
K(M, t_0, \tau, t) = \begin{cases} 
  M_l & t \leq t_0 \\
  M_l e^{-\frac{t - t_0}{\tau}} & t > t_0
\end{cases}
\]

This expression serves as a kernel for the convolution calculation (figure 2(A), inset). EC spike trains were converted to a digital input vector by accumulating spikes over the entire recording into bins sized according to the corresponding Ca imaging exposure times. This spike count vector was convoluted with the fitted kernel in Matlab to produce the reconstructed Ca trace (see also S1 in the supplementary material).

2.6. EC–Ca correlation maps

Correlation between EC and Ca signals cannot be performed directly using the original signals, and transforming both into comparable and continuous versions is required. Ca traces of all cells for each field of view were de-convoluted into rate plots. This procedure required the use of a generic kernel as an accurate kernel is not available for all cells (unlike identified cells). The generic kernel used is a single decaying exponent with an amplitude of 10% and a time constant evaluated separately for each cell, by averaging the decay time of the peak in all detected events (see S1 in the supplementary material). The time duration of the kernel is five time constants, allowing it to reach the baseline. The decay (clearance) time constant seemed to vary significantly even for events in the same cell, with smaller events exhibiting typical values of 1 s and bigger events reaching time constants as high as 3 s. This dichotomy was not addressed in the analysis performed in this work. De-convolution was performed in Matlab by dividing the fast Fourier transform of each Ca trace by that of the kernel and inverse transforming the result. The EC spike timings binned into a spike count vector (section 2.5) and then further filtered with a symmetric Gaussian smoothing kernel (std = 200 ms, width = 700 ms). The Pearson correlation coefficient between the Ca-derived rate plot and the smoothened spike count vector was calculated for each cell and displayed on a cell outline map (see S2 in the supplementary material available at stacks.iop.org/JNE/8/056008/mmedia).

2.7. Neuron–glia classification

Distinction between neuronal and glia cells was achieved by analyzing their Ca traces. A front difference trace was computed (differences between consecutive samples) and the obtained values were collected into a 3000-bin histogram. Histograms from glia traces are nearly perfect Gaussians reflecting slow changes. Neuronal histograms, on the other hand, contained a small yet noticeable subset of high values (superimposed on the positive tail of the Gaussian, see figures S6(C) and (D) in the supplementary material). These outliers correspond to the fast transients in the fluorescence occurring during the rising phase of neuronal events. These features allowed automatic separation between neuronal and glial traces (see S2 in the supplementary material). This classification was validated using immunostaining (see section 2.10, and also S2 in the supplementary material).

2.8. Ca–EC data temporal synchronization

To achieve precise synchronization between calcium imaging and EC recordings, the TTL output from the CCD was fed into a MEA acquisition board. A synchronized imaging session was conducted as follows: the MEA was slotted in the electrical recording unit and placed under the microscope. Activity was shortly inspected at electrodes displaying observable spike waveforms. The microscope field of view was centered and focused on the chosen electrode and the MCS acquisition was started just prior to the camera recording thus allowing accurate collection of camera exposure timings.

2.9. Pharmacology

Suppression of synapse efficacy was attained using one of the three options: (1) adding \( \sim 1 \mu M \) 6-cyano-7-nitroquinolinic acid-2,3-dione (CNQX, AMPA antagonist) and \( \sim 3 \mu M \) (2R)-4-amino-5-phosphonovaleric acid (APV, NMDA antagonist) to the recording medium. This approach was shown to reduce functional connectivity in random neuronal cultures (Breskin et al 2006). (2) Using low calcium ACSF medium formulation where CaCl\(_2\) salt is present in a lowered concentration (0.1–0.2 mM) with extra HEPES used to compensate for the osmotic difference. Lowered calcium contents were previously shown to reduce synchronicity in random neuronal cultures (Canepari et al 1997). (3) Addition of 20–100 \( \mu M \) carbachol, a competitive acetylcholin synaptic agonist (Tateno et al 2005). In some cases, elevation of intrinsic neuronal excitability was induced via the addition of \( \sim 2 \) mM KCl to culture media. This concentration was found to produce a substantial increase in activity, whereas higher concentrations resulted in decreased activity or silencing (results not shown).
2.10. Immunostaining

Samples were washed in a phosphate buffered solution (PBS) and then fixed with 4% paraformaldehyde and 4% sucrose solution for 20 min. Next they were permeabilized and blocked with 0.25% Triton (Sigma), 10% normal goat serum (NGS, Biological Industries Ltd, Israel) solution in the PBS for 20 min and further blocked by incubation in 10% NGS in the PBS. They were then washed with 1% NGS in the PBS and incubated in primary antibodies overnight at 4 °C. To detect glial cells, mouse anti-glial fibrillary acidic protein monoclonal antibody was used (GFAP, 1:400, Chemicon, MAB3402). Neurons were detected using chicken rat anti-MAP2 polyclonal antibody (1:10 000, Zotal, AB5392). After overnight incubation, cultures were washed three times with the PBS and then incubated for 1 h in the dark with the appropriate secondary antibodies: Alexa fluor 488 goat anti-mouse IgG (1:800, Molecular Probes, A-11029) for the detection of GFAP, and Cy-3 donkey anti-chicken IgG (1:500, Chemicon, AP194) for MAP2. For nuclei visualization, cultures were incubated with DAPI (0.01 μg ml⁻¹, Sigma, D9542) for 5 min. Imaging was performed using the upright fluorescent microscope described in section 2.2.

2.11. Spike waveform analysis using PCA and spike identification

To enable computation of waveform PCA, all EC data from a recording session (10–30 min) were subjected to the spike detection analysis described by Hulata et al (2002) based on thresholding over wavelet packet decomposition. All spikes were trimmed and aligned to 5 ms around a point of maximal voltage absolute value. These data were subjected to PCA using Matlab’s statistics toolbox. To collect spikes originating from an identified neuron (identified spikes), its Ca events were compared to their reconstruction counterpart. Spikes occurring during events with amplitude discrepancy of no more than 25% between Ca data and reconstruction were classified as identified and were marked as red dots in figures 4(C1) and (C2). An example for this procedure is shown in figure S1 in the supplementary material.

3. Results

Simultaneous electrical and optical recordings from rat neurons were performed between 10 and 21 days in vitro (DIV). Typical data are presented in figure 1. Figure 1(A) shows an overlay of fluorescent and bright field images of one particular recorded field of view. The Ca traces of five specific cells (marked in figure 1(A)) are shown in figure 1(B) together with simultaneously recorded EC data (C)–(E). A temporal correspondence between the Ca and the EC data is readily apparent. Moreover, the amplitude of the Ca events is correlated with the number of spikes recorded in the corresponding EC events. Large Ca events (up to 120% increase in fluorescence) were observed in concurrence with as many as 250 EC spikes.

It is also apparent from the data in figure 1 that the activity in our cultures is characterized by short time windows of intense neuronal firing separated by longer intervals of sporadic firing. These network bursts (NBs) are typical for such cultures (Maeda et al 1995, Eytan and Marom 2006, Shein et al 2008) and were even shown to intensify in cultures immersed in ACSF media such as the one used here for recording (Latham et al 2000). Due to the synchronized nature of the neuronal activity (figure 1(B)), and the limited Ca imaging time resolution, a NB is represented in the Ca recordings by a large Ca transient.

The synchronized activity introduces a major challenge: if an electrode records from more than one neuron, it is difficult to associate specific EC spikes with the specific cells it is recording from. To overcome this challenge, we used pharmacological treatment to tamper with the synaptic function. In particular, we used an antagonist to desynchronize the neurons in the network. It has been previously shown (Tatemura et al 2005) that the addition of 20–100 μM of carbachol, a competitive agonist of acetylcholine, induces decoupling of neurons in culture. This method proved to be most effective at inducing unsynchronized activity in our cultures (effect of carbachol is illustrated in supplementary movies 1 and 2 available online at stacks.iop.org/JNE/8/056008/mmedia). By introducing de-synchronization it becomes possible to use isolated firing events to relate each EC spike to a Ca response of a specific cell. Figure 2(A) shows two Ca traces of two specific cells after the addition of carbachol. Unlike the synchronized patterns of the undisturbed spontaneous activity (figure 1), in which all the cells fire only during NBs, in figure 2 it is possible to clearly identify events in which a specific cell (blue) fires in between NBs. Moreover, EC data (figure 2(C)) appear to closely correspond with those inter-NB firings. Asterisks indicate events which can clearly help associate the EC with the Ca data. In the data presented here, owing to the use of high-affinity dyes, even single EC spikes (single action potentials) had corresponding, small Ca events (5–20% relative increase in fluorescence). We refer to these events as Ca spikes. The ability to record these events is instrumental to perform the analysis discussed below.

The identification of isolated Ca events which exhibit close correspondence with the EC data is the first important step toward direct association between the optical and the electrical data. However, one should take into account the possibility that poorly stained cells (therefore hidden) or remote cells may, in fact, contribute to the EC spikes thus skewing the interpretation of the data. Therefore, to validate our identification procedure, a more direct method is needed. To achieve such validation we aimed to fully reconstruct the Ca response from the electrically recorded raster plot.

To reconstruct the Ca data from the EC recordings we utilized a spike detection algorithm (Hulata et al 2002) to identify the timings of single spikes in the EC data. The time series was convoluted with a kernel corresponding to a typical Ca-spike. The kernel was extracted from the calcium data by averaging over all Ca spikes (see section 2.5). A reliable identification of this kernel is mandatory to achieve EC–Ca correspondence. This is the ‘forward’ convolution equivalent to the classical routine used to infer electrical
Figure 1. Simultaneous Ca and EC recordings. (A) A fluorescence image overlaid on a bright field image of neurons cultured on a MEA substrate. The image shows a 30 μm diameter electrode (circumscribed) with several nearby cell somata. Five specific cell bodies are marked (circumscribed with solid lines) for later reference. Scale bar is 20 μm. (B) Calcium data recorded from the five neurons marked in A. Data were shifted for clarity. (C) EC electrical recordings (thresholded) obtained from the electrode depicted in (A) for the same time window as in (B). (D) and (E) Zoomed data from (C). Culture was 15 DIV.

Figure 2. Simultaneous Ca and EC recordings with 20 μM carbachol. (A) Ca data of two specific neurons. Asterisks mark specific features in the lower trace which are conspicuously missing in the upper trace. A kernel defined by averaging over single Ca spikes from the blue trace (inset). (B) Convoluted data derived from a binned raster plot. (C) Raster plot of recorded EC data (bin size = 1 s). (D1, D2) Correlation maps showing several neurons in the vicinity of two different recording electrodes (electrode diameters are 30 and 10 μm for D1 and D2, respectively). D2 shows multiple electrodes from a high density MEA grid. The analyzed electrode is circumscribed. Open circles mark cells without any detected events. Culture was 15 DIV. Black scale bars in D1–D2 are 20 μm. Two cells are marked by arrows and are discussed in figure 3.

rate activity from recorded Ca imaging traces (Yaksi and Friedrich 2006). Figure 2(B) depicts the result of such a reconstruction. The convoluted trace matches closely the Ca trace of only one particular neuron throughout the recorded data, in particular for isolated events. The reconstruction also holds for larger events comprising several spikes. During NBs, the correspondence between the convoluted trace and the real Ca data is limited. This effect will be referred to, in more detail, later in the text.

To optimize the procedure described above we computed Ca–EC correlation maps, which use a generic mono-exponential kernel to correlate the EC spikes time
Figures 2(D1) and (D2) show two such maps. In several cases of carbachol (figure 2(D1)), cells with high correlation were found to have a high Ca–EC correlation despite the use of carbachol (figure 2(D2)). In most cases, several cells were identified as non-recorded neurons. It has been previously shown that neuronal somatic Ca traces can be distinguished from glia originating ones based solely on features of the activity (Rothschild et al. 2010). To check whether such a method could be applied to our data, we examined recorded Ca traces of cells which were positively classified by immunostaining with neuronal and glia specific antibodies (see figure S5 in the supplementary material available at stacks.iop.org/JNE/8/056008/mmedia). Inspecting such immunostaining data, we observed that with standard ACSF medium, glia Ca traces did not substantially differ from neuronal traces, but appeared as low amplitude manifestation of the neural activity (Göbel and Helmchen 2007) rather than actual intrinsic glia signals. However, upon application of de-synchronization, which caused both a decrease in neuropil signals as well as the activation of the glia cells, a marked difference between glia and neuronal signals emerged. Specifically, glia events were conspicuously distinct from neuronal ones in shape and were characterized by slower dynamics, particularly in the rising phase (section 2.7).

After carefully screening our data, a classification table can be constructed. Overall, 35 electrodes (from nine different MEAs) with high EC-SNR (>5) spiking activity were recorded and analyzed, out of which only 55% contained data with neurons unambiguously identified as recorded or not recorded (table 1).

The data in table 1 are presented in figure 3(A). Distances are measured between the cell’s soma center and the closest point on the electrode perimeter. Positive distances relate to cells positioned outside the electrode’s perimeter, whereas negative ones relate to cells positioned inside the perimeter, i.e. directly placed on the electrode.

<table>
<thead>
<tr>
<th>Culture name</th>
<th>Electrode number</th>
<th>Most remote recorded cell in μm (°)</th>
<th>Closest non-recorded cell in μm (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>500-30-2</td>
<td>37</td>
<td>−4 (1)</td>
<td>12 (1)</td>
</tr>
<tr>
<td>500-30-3</td>
<td>42</td>
<td>−</td>
<td>22 (6)</td>
</tr>
<tr>
<td>500-30-Sync-4</td>
<td>62</td>
<td>−</td>
<td>70 (1)</td>
</tr>
<tr>
<td>500-30-Stim-2</td>
<td>42</td>
<td>12 (1)</td>
<td>48 (3)</td>
</tr>
<tr>
<td>500-30-Sim-2</td>
<td>54</td>
<td>−11 (1)</td>
<td>14 (1)</td>
</tr>
<tr>
<td>500-30-Sync-5</td>
<td>66</td>
<td>13 (1)</td>
<td>107 (1)</td>
</tr>
<tr>
<td>30-10-1</td>
<td>13</td>
<td>12 (1)</td>
<td>−2 (7)</td>
</tr>
<tr>
<td>30-10-1</td>
<td>22</td>
<td>−</td>
<td>13 (7)</td>
</tr>
<tr>
<td>30-10-2</td>
<td>35</td>
<td>16 (2)</td>
<td>24 (3)</td>
</tr>
<tr>
<td>30-10-2</td>
<td>14</td>
<td>5 (1)</td>
<td>46 (1)</td>
</tr>
<tr>
<td>30-10-2</td>
<td>32</td>
<td>−</td>
<td>3 (7)</td>
</tr>
<tr>
<td>30-10-2</td>
<td>16</td>
<td>−</td>
<td>21 (5)</td>
</tr>
<tr>
<td>500-30-7</td>
<td>51</td>
<td>−3 (1)</td>
<td>33 (14)</td>
</tr>
<tr>
<td>500-30-7</td>
<td>61</td>
<td>0 (1)</td>
<td>5 (1)</td>
</tr>
<tr>
<td>500-30-8</td>
<td>38</td>
<td>−</td>
<td>−6 (10)</td>
</tr>
<tr>
<td>500-30-8</td>
<td>61</td>
<td>−</td>
<td>50 (1)</td>
</tr>
</tbody>
</table>

The data of figure 3(A) demonstrate that EC electrodes can record from cells located as far as 16 μm from the electrode boundary. Therefore, a direct or very intimate contact between the cell soma and the electrode appears to be a prerequisite for recording.

An additional notable observation in our data is that active cells which reside very close or even on the electrodes are not always recorded. Figure 3(B) shows the Ca data of two cells placed next to an electrode as well as the signal reconstructed from the EC data (the same procedure as in figure 2). Events in the Ca data of the upper trace (a cell placed directly on top of the electrode) are clearly not reflected in the EC raster plot. Overall, the number of neurons identified as non-recorded was significantly greater than that of the recorded ones. This effect is associated with the fact that only a small proportion of the neurons is physically in contact with the electrode. Therefore, although all neurons may be characterized by the same propensity to elicit isolated events, only a small proportion of them are recorded. This fact severely skews the identification rate of recorded neurons. Thus, only nine electrodes provided data with identified recorded neurons. Out of these, only one electrode showed data with two different recorded neurons (table 1).

Overall, Ca data reconstruction can be typically categorized into two distinct groups. In the first group both the height of the Ca data waveform and the time ascent are reconstructed to a good extent from the EC data (figures 2(A) and (B), events marked with asterisks). This means that all EC recorded signals are accounted for and therefore these recordings are attributed to a single, well-identified cell. In the second group, consisting of NBs, we observed a marked discrepancy both in the decay phase and
in amplitude between the recorded Ca and the reconstructed data with the second being significantly greater. Two factors may be accountable for this apparent discrepancy: the first is multi-cell recording during NB and the second is a frequency-dependent association between the EC and the Ca data which is not accounted for in our reconstruction scheme. The second factor includes phenomena such as nonlinearities in cellular calcium dynamics and saturation in Ca$^{2+}$ ion binding to the fluorescent dye. To test the degree to which saturation plays a role in Ca dynamics during NBs, we used electrical stimulation to artificially produce events of increased magnitudes (see figure S3 in the supplementary material available at stacks.iop.org/JNE/8/056008/mmedia). The elicited events saturated at amplitudes of 120% ($\Delta F/F_0$), similar to the saturation in spontaneous NB amplitudes, implying that indeed saturation is a major factor determining the shape of Ca-NBs. The apparent saturation is a clear result of the high affinity of the dyes used. While using low-affinity dyes may facilitate better imaging of NBs, such recordings will be of little use in our current analysis which heavily relies on the Ca event detection achieved by the high-affinity dye. As we further explain in section 4, low-affinity dyes cannot resolve the challenge of EC–Ca validation during bursting events.

Owing to the limitations described above it is impossible to associate spikes with particular cells within NBs, but some insight can still be gained as to the properties of the EC data within and outside NBs. Typical raw EC data recorded during an NB are shown in figure 4(A). We next extracted from the EC data all the recorded spikes (figure 4(B)). Finally, we performed PCA over waveforms of the entire EC data (figure 4(C)). Marked as red dots in figure 4(C) are spikes which were able to associate with a specific neuron with the help of the Ca data (see section 2.11). These spikes form a well-identified sub-group in the PCA space. We repeated the analysis for other electrodes (see more examples in figure S4 in the supplementary material available at stacks.iop.org/JNE/8/056008/mmedia). In figures 4(A2)–(C2), we present data from which we were able to identify isolated spikes of two particular cells (red and green dots). The fact that this is a multi-cell recording is further corroborated by the detection of spikes at temporal proximity closer than 2 ms (figure 4(C2)), which is incompatible with single cell activity due to refractory period constraints. We again note that low-affinity dyes could facilitate a more reliable imaging of NBs, but owing to the large number of firing cells in the vicinity of each electrode, it will not allow one to readily deduce which neurons participate in the NBs if more than one cell is contributing to the EC data.

It is readily apparent that the inter-NB spikes (red dots in figures 4(C1) and (C2)) occupy a small sub-domain of the PCA space, whereas the variability of the entire spike data is significantly greater. Indeed, during NBs, there is a marked decline in the EC amplitude over time (increasing spike shape variability) while inter-NB spikes have a relatively well-identified EC signature. Finally, we note that visual inspection of the PCA space in the multi-neuronal recording case (figure 4(C2)) does not separate the data points into two clear groups. Overall, inter-NB spikes in all analyzed recordings formed a subgroup within the entire spike data (see also figure S4 in the supplementary material). This example demonstrates that it is questionable if EC spike shape can be used to distinguish between neurons.

4. Discussion

Using combined Ca imaging and EC recordings we were able to formulate several powerful assertions about EC recordings. First, we observed cases of cell somata placed directly on an electrode and displaying Ca spikes without any manifestation in the EC signal. This effect occurs even when high SNR recordings are achieved. Overall, recording was limited to a range of $\sim 16$ $\mu$m (direct contact with the electrode), but even within this range, recording was not assured, and took place only for $\sim 50\%$ of the cells showing isolated activity. These results might be explained by the sensitivity of EC recordings...
Figure 4. Waveform PCA results of EC data recorded from a single cell (A1–C1) and from several cells (A2–C2). (A1 and A2) EC data excerpt recorded by the analyzed electrode, showing typical spike train obtained during NBs and demonstrating an extensive waveform shape change. (B1 and B2) Overlay of all spikes extracted from the EC data. In B2 a single EC trace is accentuated showing occurrence of two spikes less than 3 ms apart. Due to refractory period violation, they are unlikely to originate from the same cell. (C1 and C2) Waveform PCA domain plot of EC spikes from B1 and B2 showing the top two principal components (comprising 59% and 82% of spike shape variance, respectively). The red dots indicate spikes which were unambiguously attributed to a specific neuron with the help of Ca data. C2 also shows a green dot which was associated with an additional distinct neuron, complying with the inference that this is a multi-cell recording. Number of spikes in analysis: \( n_1 = 1466, n_2 = 1628 \) for C1 and C2, respectively.

to the exact manner in which the neurons are coupled to the electrode. Indeed, past experimental and modeling work showed that the EC signal amplitude and shape depend on the seal resistance, distribution of ion channels in the membrane (Vassanelli and Fromherz 1998, Fromherz 1999) and geometry of axonal extensions (Cohen et al 2006). Variability in these parameters might give rise to the results described above.

The limited range at which microelectrodes can pick up signals is yet another important result of this study. These results are seemingly inconsistent with the general notions that EC recordings can be obtained from very distant cells. Previous studies with neural slices where the identification of recorded neurons can be corroborated via patch clamp recordings have reported the ability to consistently record EC signals from neurons located markedly away from the electrode with the signal amplitude inversely proportional to the electrode–neuron distance (Harris et al 2000). The discrepancy between these results and ours can be readily attributed to the difference in the electrode–neuron distance (Harris et al 2000). The combined optical and electrical recording technique was then used to look at the validity of spike sorting, i.e. how well spike sorting approaches are able to associate spikes with different source neurons. To this end, EC spikes recorded during small isolated events, thus allowing their assignment to a specific neuron, were inspected in a PCA space along with all recorded spikes (figure 4). This analysis showed that the notion that every neuron possesses a characteristic spike waveform indeed holds for small firing events. However, intensive bursting involves with a reduction in spike amplitude and shape alteration, effectively eliminating the ability to differentiate between neurons. In some cases, the amplitude may diminish down to sub-SNR levels causing an altogether failure to perform spike detection. Although it is very well known that considerable waveform alteration during NBs takes place (Harris et al 2000, Buzsáki 2004), our data demonstrate this effect directly for MEA recordings, for the first time. This instability of the EC signal may be attributed to the dependability of spike shape on volatile factors such as ion channel distribution in the junctional membrane, which is likely to change during periods of intensive firing or over long
recording time spans. This fact should be taken into account when employing signal analysis in multi-unit EC recordings. The limited ability to derive the exact number of recorded cells for each electrode also renders spike rate analysis cumbersome.

It is important to note that owing to limited optical SNR levels our analysis is restricted to activity recorded from cell soma, while ignoring the extensions. We note that significant background of cellular extensions exists in our cultures which can generate some undesired signal interference. However, our success to reliably associate EC and the Ca data implies that these effects can be cautiously disregarded.

While the combined Ca–EC technique described here was used to explore the efficacy of EC data, the employment of this technique might prove useful in researching neural systems, allowing the collection of more elaborate information than is possible using MEA recording or Ca imaging alone. Moreover, the technique may be utilized to better explore the details of the Ca imaging technique. To relate Ca to EC data we utilized linear convolution which is a standard practice in the field (Yaksi and Friedrich 2006, Holekamp et al 2008, Mukamel et al 2009, Grewe et al 2010). However, it is conspicuously inaccurate for NB-Ca data. This inaccuracy is largely the result of dye binding saturation, but probably also involves altered Ca clearance kinetics during exceptionally large NBs which typically constitute the activity patterns of neuronal cultures. Further understanding of these nonlinearities is also needed to fully relate the Ca and EC data. This knowledge may also serve to improve tools of spike train prediction from Ca recordings (Sasaki et al 2008, Vogelstein et al 2009). We plan to use our combined Ca–EC system to explore these issues. Using low-affinity dye in future experiments, it may be possible to address the details of the NBs, in particular variability in the activation of different cells, and to derive the number of recorded cells.

It should be noted that the investigation presented here relied heavily on high-affinity Ca dyes as they allowed us to achieve single action potential recording. We therefore used sporadic, inter-burst activity, to associate the Ca and the EC data. This scheme is instrumental for our investigation and could not be achieved using low-affinity dyes or voltagesensitive dyes (VSDs). Indeed, low-affinity dyes would allow us to better record neuronal activity during bursts; however, it would not allow us to simply associate the Ca and the EC data due the following reasoning: neurons’ synchronization during bursting activity implies that many neurons are active nearly simultaneously. To identify the cells contributing to the EC data an exact reconstruction must be achieved from the Ca to the EC data. To achieve this reconstruction an accurate kernel of the Ca events is needed. This kernel can be extracted only from the high-affinity dye isolated-event recordings. Even if an approximated kernel was used, it is likely that nonlinearity in Ca dynamics, occurring during intensive bursting, would render such analysis unreliable.

A technique combining low- and high-affinity dyes has the potential to benefit future investigations. VSDs are an additional alternative to the high-affinity dyes used in this investigation. They are directly sensitive to electrical signals, whereas Ca-sensitive dyes reflect Ca fluctuation associated with neuronal spiking. Indeed, preliminary single trial spike detection has already been demonstrated with a two-photon setup (Fisher et al 2008). Nevertheless, VSDs suffer from inferior staining capabilities and low fluorescence dynamic range, requiring long exposure times (resulting in slow frame rates) or averaging over consecutive stimulations to achieve satisfactory SNR (Fisher et al 2011). Such approaches are not applicable to our current work since the recorded activity is spontaneous and not stimulated and fast acquisition is required to faithfully capture the fast neuronal signals. Further chemical improvement of VSDs (Obaid et al 2004) may allow their use for extracting spontaneous spike data.

To conclude, using high-affinity Ca imaging in conjunction with MEA recordings we were able, for the first time, to directly assess the efficacy of EC recordings in culture. We have demonstrated a novel approach to achieve, despite a high degree of synchrony, an association between Ca and EC data. This was based on the examination of isolated Ca events and identifying their EC counterparts.

Acknowledgments

The authors thank Inna Brainis for technical assistance with culture preparations and Professor Ari Barzilai and Professor Eshel Ben-Jacob for fruitful discussions.

References

Buzsáki G 2004 Large-scale recording of neuronal ensembles Nat. Neurosci. 7 446–51
Cossart R et al 2005 Calcium imaging of cortical networks dynamics Cell Calcium 37 451–7
Eytan D and Marom S 2006 Dynamics and effective topology underlying synchronization in networks of cortical neurons J. Neurosci. 26 8465–8
Göbel W and Helmchen F 2007 In vivo calcium imaging of neural network function Physiology 22 358
Grewe BF et al 2010 High-speed in vivo calcium imaging reveals neuronal network activity with near-millisecond precision Nat. Methods 7 399–405
Harris K et al 2000 Accuracy of tetrode spike separation as determined by simultaneous intracellular and extracellular measurements J. Neurophysiol. 84 401
Holekamp T et al 2008 Fast three-dimensional fluorescence imaging of activity in neural populations by objective-coupled planar illumination microscopy Neuron 57 661–72
Johnstone A F M et al 2010 Microelectrode arrays: a physiologically based neurotoxicity testing platform for the 21st century Neurotoxicology 31 331–50
Maeda E et al 1995 The mechanisms of generation and propagation of synchronized bursting in developing networks of cortical neurons J. Neurosci. 15 6834
Mukamel E A et al 2009 Automated analysis of cellular signals from large-scale calcium imaging data Neuron 63 747–60
Ranganathan G N and Koester H J 2010 Optical recording of neuronal spiking activity from unbiased populations of neurons with high spike detection efficiency and high temporal precision J. Neurophysiol. 104 1812
Sasaki T et al 2008 Fast and accurate detection of action potentials from somatic calcium fluctuations J. Neurophysiol. 100 1668
Shein M et al 2008 Management of synchronized network activity by highly active neurons Phys. Biol. 5 036008
Smetters D et al 1999 Detecting action potentials in neuronal populations with calcium imaging Methods 18 215–21
Stett A et al 2003 Biological application of microelectrode arrays in drug discovery and basic research Anal. Bioanal. Chem. 377 486–95
Yaksi E and Friedrich R 2006 Reconstruction of firing rate changes across neuronal populations by temporally deconvolved Ca\(^{2+}\) imaging Nat. Methods 3 377–83