Adhesion Stabilized en Massé Intracellular Electrical Recordings from Multicellular Assemblies

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ABSTRACT: Coordinated collective electrochemical signals in multicellular assemblies, such as ion fluxes, membrane potentials, electrical gradients, and steady electric fields, play an important role in cell and tissue spatial organization during many physiological processes like wound healing, inflammatory responses, and hormone release. This mass of electric actions cumulates in an en masse activity within cell collectives which cannot be deduced from considerations at the individual cell level. However, continuously sampling en masse collective electrochemical actions of the global electrochemical activity of large-scale electrically coupled cellular assemblies with intracellular resolution over long time periods has been impeded by a lack of appropriate recording techniques. Here we present a bioelectrical interface consisting of low impedance vertical gold nanoelectrode interfaces able to penetrate the cellular membrane in the course of cellular adhesion, thereby allowing en masse recordings of intracellular electrochemical potentials that transverse electrically coupled NRK fibroblast, C2C12 myotube assemblies, and SH-SYSY neuronal networks of more than 200,000 cells. We found that the intracellular electrical access of the nanoelectrodes correlates with substrate adhesion dynamics and that penetration, stabilization, and sealing of the electrode–cell interface involves recruitment of surrounding focal adhesion complexes and the anchoring of actin bundles, which form a caulking at the electrode base. Intracellular recordings were stable for several days, and monitoring of both basal activity as well as pharmacologically altered electric signals with high signal-to-noise ratios and excellent electrode coupling was performed.

KEYWORDS: Nanoelectrodes, cell-adhesion, intracellular recordings, en masse signaling

Tight cellular assemblies feature dense networks of intercellular contacts and connections that allow electrochemical coupling between cells, for example, gap junctions, chemical synapses, or tunneling nanotubes. Electrical signal exchange within these networks is involved in several cellular processes such as myogenic contraction, neuronal information processing, vaso- and lymphendothelia contraction as well as collective cell migration during wound healing. Furthermore, the exchange of electrical signals enables cellular synchronization and organization (e.g., simultaneous hormone release in pancreatic beta cells). On a molecular level, cellular electrochemical signaling represents a highly orchestrated process where fluctuations in ion channel permeability and ion concentrations of the cytosol or organelles are triggered by a variety of input signals. Most signals that affect physiological processes act exclusively in a multicellular context. Most importantly, previous studies have shown, that such en masse action and associated feedback mechanisms cannot be deduced from considerations at the single cell level. For example, within interlinked multicellular chimeric state architectures, rhythmic and synchronized actions between single pairs or ensembles of several cells are pivotal for correct physiological signaling. This en masse action spectrum formed in the collective appears to form due to the intercellular signaling (e.g., via gap junctions or chemical synapses). It follows that coherent and noncoherent spatiotemporal patterns emerge, which can only be understood when the activity of the whole assembly is considered. A behavior which is not only true for neuronal networks but probably for the entirety of collective cell activity patterns in multicellular organisms.

Intracellular en masse recordings of electrical actions from cellular collectives harboring several thousand cells have mostly been limited by a lack of suitable electrode interfaces that permit stable and accurate monitoring of en masse potentials in a non-invasive manner over long periods of time. Currently intracellular techniques monitor and manipulate the electrical

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Figure 1. Interfacing adherent cells with gNEIs. (a) Schematic illustration of electrically coupled adherent confluent cell layers interfaced with gNEIs for voltage recordings. (b) Representative image of NRK cells cultured on a gNEI. Membranes (wheat germ agglutinin Alexa555 conjugate), cytosol (CellTracker Green), and nuclei (DAPI) are shown in red, green, and blue, respectively. The scale bar is 20 μm. (c) and (d) Analysis of the viability of NRK cells cultured on glass coverslips (glass), PC-coated glass coverslips (PC), or gNEIs for 24 h after seeding, based on the relative ATP concentrations and propidium iodide (PI) dead cell staining. All values are shown as mean ± s.d. (n = 3 independent experiments). (e) Representative SEM image of a NRK cell cultured on a gNEI; the white arrow points to a typical interfacing electrode. The inset shows the typical tent-shaped apical membrane configuration around the electrodes. The scale bar is 5 μm in the larger image and 1 μm in the inset. (f) SEM image of a fractured cross-section showing basal membrane penetration and the membrane electrode interface. The inset shows basal membrane-associated, filamentous structures anchoring the cell to the electrodes. The scale bar is 1 μm in the larger image and 200 nm in the inset. (g) TEM micrograph of a horizontal section view of the electrode–cell interface where the cutting plane was in some distance from the underlying substrate (upper panel) or in close proximity to the underlying substrate (lower panel). In the upper panel, the direct contact of the electrode tip to the cytosol is obvious (note that there is no membrane surrounding the electrode), whereas the electrode base is clearly enclosed by the cell membrane (lower panel, black arrow). Both scale bars are 200 nm. (h) TEM cross-section of a cell protrusion extending to the electrode’s PC cone and thereby tightly enclosing the penetration site. The basal membrane sticks closely to the PC layer (PC) and ruptures close to where the PC substrate and the gold electrode (Au) join. The scale bar is 200 nm. (i) Vertical TEM sections confirm the close contact between the electrodes and cytoplasm. The inset shows a higher magnification of the electrode–cell interface. The scale bar is 200 nm. (j) Horizontal TEM section of a nanoelectrode penetrating through the nuclear envelope in an area without any ultrastructural reorganization of heterochromatin. The inset shows a higher magnification of the chromatin–electrode interface. The scale bar is 300 nm.

activity of individual cells and have been a valuable tool for in vitro and in vivo physiological studies for more than a century. However, for en masse electrical activity of electrically coupled cells in the majority of tissues, the whole might be greater and the sum of its parts, meaning that information based on single cell resolution is not necessarily indicative for the en masse action of the cell collective.10 Conventional glass pipet-based sharp or patch clamp electrode recordings offer excellent electrode-to-membrane coupling coefficients. Therefore, they have been used to study the full spectrum of electrical activities (e.g., resting membrane potentials, action potentials, excitatory as well as inhibitory postsynaptic potentials) in single cells with high resolution and exquisite signal-to-noise ratio (SNR).11,12 These setups, however, are restricted to observations of only a few cells in parallel and do not allow for continuous recordings over several days. Planar patch clamp arrays have been developed which allow whole cell voltage clamp precision from multiple cells suitable for high-throughput drug screening. However, channel and receptor gating and conductance are not indicative for the intracellular en masse action of cell collectives,13 and cells must be in suspension, limiting their use to recombinant or dissociated cell systems. Extracellular microelectrode arrays have also been employed to monitor electrical signals from larger cell culture systems and tissues in vitro and in vivo. However, such signals only reflect rapid membrane potential changes such as action potentials. Due to their extracellular nature, they do not indicate the membrane potential and cannot access it or manipulate it.13,14 Larger numbers of cells can be monitored by imaging voltage or ion-sensitive fluorescent probes, however, membrane potential cannot be accessed, as its measurement is relative to a difficulty calibration procedure and optogenetic manipulation of membrane potential is limited. We therefore aimed to design a device which does not record with single cell resolution but rather probes the en masse electrical state of a cell collective and provides long-term (days, not minutes) intracellular access to monitor and manipulate membrane potentials en masse.

In recent years, nanosized electrode architectures have been developed for interfacing with numerous electrogenic cells in parallel to acquire intracellular recordings over prolonged time periods via nanoprobe insertion.15 These include field-effect transistor-based vertical electrodes with superior electro-mechanical coupling coefficients, mushroom-shaped gold electrodes for “in cell” recordings, and vertical iridium oxide nanotube electrodes with low impedance.16–19 All of these interfaces have been successfully employed to record signals
from single or a small number of neuronal or myogenic cells cultured on the electrode substrates over a short time period. Means to achieve electrode insertion into the cytoplasm included mechanical and electrical stimulation or biochemical modification of the electrodes. Passive membrane penetration associated with cell adhesion, in contrast, was only rarely observed. Even under penetration-promoting circumstances, stable intracellular access lasted only a few minutes to hours.18,20–24 Yet stable penetration concomitant with adhesion is highly desired, because intracellular access through repeated electroporation or mechanical stimulation affects the cellular physiology. Moreover, previous devices were designed to record electrical activities with single cell resolution but were unable to record en masse activities from several thousands of cells due to the need for independent circuits for each electrode. In contrast, a nanoelectrode surface designed for en masse recordings without separating electrical signals from individual cells is not limited by complex microcircuitry but is instead scalable and in principle is not limited in surface area. Only such an approach has the potential to form a bioelectric interface with an entire cellular monolayer.

We have developed an electrode interface that can be easily implemented and used in combination with other experimental approaches (e.g., microscopy). A long lasting intracellular recording was mediated exclusively by cell adhesion forces which provided mechanically stable seals. The interfaces were designed to provide low impedance paired with superior resolution of electrical signals, whereas the electrode architectures were devised so as to promote membrane sealing at the electrode–cell interface. Our electrode interfaces allow for en masse intracellular recordings of collective multicellular electrochemical activity. To demonstrate the ability of our gold nanoelectrode interfaces (gNEIs) to record intracellular high SNR electrical signals en masse over extended time periods, we selected three distinct electrically interconnected cell types which form monolayers in culture: normal rat kidney (NRK) fibroblasts, which exhibit spontaneous pacemaker activity, C2C12 myoblast assemblies, which form contractile collectives, and SH-SY5Y cells, which are of neuronal origin.

**Results and Discussion.** We recently reported on the fabrication of gNEIs with vertically aligned, monocrystalline, cigar-shaped nanoelectrodes, which were produced by template-based electrochemical deposition.25,26 These arrays feature high conductivity and low impedance (see Figure S1a,b). Moreover, they are easily scalable and thus allow for interfacing and recording of electrical signals from several thousand cells. To electrically monitor mammalian cell culture systems on gNEIs, we designed a bioreactor setup that allows for culturing adhesive cells with continuous medium perfusion over several days (Figure 1a). Our electrode interface design, in which all electrodes are deposited on the supported Au-surface, is especially suited to recorded the en masse activity from all cells in the monolayer simultaneously, as no single electrode information is collected but rather every electrode contributes to the recorded signal. Our gNEIs possess promising electrode architectures and configurations for adhesion-mediated membrane penetration.27,28 To this end, we produced 1.57 cm² gNEIs with an electrode density of 0.94 × 10⁶ cm⁻² (±0.03 × 10⁶, n = 10 single electrode surfaces), electrode diameters of 102 nm (±4 nm, n = 25 single electrodes), and electrode lengths of 2.2 μm (±0.4 μm, n = 16 single electrodes) (see Figure S1c). The electrode density was chosen such as to favor proper cell adhesion and to avoid the so-called “fakir effect”, where a cell’s contact area with the substrate is reduced to the electrode tips. Electrode bases were passivated by an approximately 400 nm-thick bisphenol A-polycarbonate layer. Electrode tips were uncovered by etching, resulting in free-standing, cigar-shaped electrodes with a conic polycarbonate base (see Figure S1d). Mammalian cell lines, in this report mostly fibroblasts, were grown on these poly-L-lysine coated gNEIs. Quiescent, confluent monolayers of density-arrested cells were monitored by bright-field microscopy, and voltage recordings were performed over a period of several days.

Some previous communications have reported dramatic effects of nanowire-structured surfaces on the division, growth rate, migration, viability, and morphology of cultured cells, thereby raising questions about the unbiased nature of results obtained with nanowire arrays.29–33 We found, however, that morphology, viability, and metabolic activity of normal rat kidney (NRK) fibroblasts cultured on gNEIs were comparable to those of cells cultured on glass or electrode-free polycarbonate (PC)-coated surfaces (Figure 1b–d and Figure S2). Obviously apoptotic morphologies and a dramatic reduction in viability were observed, however, when cells were cultured on gNEIs without an isolating PC layer. This is most probably due to a short-circuiting of the membrane potential between penetrating electrodes and the basal gold layer, resulting in a massive disturbance of the cells’ electrophysiology (see Figure S3). Thus, PC-isolated gNEIs appear to have only a little influence on cellular physiology and can be considered to be nontoxic.

When observed by scanning electron microscopy (SEM), NRK cells cultured on gNEIs showed flat morphologies, indicating functional cell adhesion and spreading (Figure 1e). In these images we counted on average, 14 (±1, n = 40 cells) electrodes under each cell. From these images, we could not discriminate between penetrating and nonpenetrating electrodes, however the penetration of nanoscale structures into cells has been reported to depend on the subcellular positioning of the electrodes. In this context, it has been reported that the cell soma is more likely to be penetrated than the cells’ periphery.20,28 We found no preferred location of the cell soma in relation to the electrodes and the apical membrane conformations around electrodes located at the cells’ edges were comparable to those near the cells’ nuclei. Typically, the apical membrane showed a tented structure when covering an electrode (Figure 1e, inset). Similar tented apical membrane structures have previously been associated with passive electrode penetration.34

To further examine the cross-section of the electrode–cell interface, fractured SEM probes were prepared using poly-L-lysine (PLL) coating (Figure 1f). In contrast to focused ion beam cross-sectioning, where ultrastructural information is lost due to melting-induced degradation at the cutting site, fractured sections are well-suited for looking at membranous ultrastructure. We found successful penetration of the basal membrane and prominent membrane-associated filamentous structures used for anchoring the cell to the electrode base (Figure 1f, inset). Ineficiency has previously been a key issue when inserting metal nanoelectrodes, in particular gold nanoelectrodes, passively through cellular adhesion forces.16,35

In addition, even nanoelectrode penetration induced by electroporation commonly culminates in rapid exclusion of the electrode by membrane rescaling. As a result, general
questions about the suitability of gold nanoelectrodes for stable intracellular incorporation have been raised.21,22,36 In contrast to these reports, all gNEI electrodes observed in our fractured SEM probes penetrated the basal membrane (n = 10). This process was also accompanied by anchoring of the cell to the electrode base directly where the PC surface and the gold of the nanoelectrodes join. It can be assumed that these cellular anchoring structures act as a seal around the electrodes, thereby contributing to the long-term stability of electrode penetration into the cell. These findings suggest that the cells actively participate in sustaining the insertion of the electrode into the cytoplasm, rather than a coordinated extrusion of the electrode by membrane reorganization as has been reported in other studies.

To elucidate the electrode–cell interface in greater detail and to further expose potential sealing efforts of the cells, we performed transmission electron microscopy on PLL-coated gNEIs (TEM), which provides high resolution of membranous structures. Accordingly, TEM has recently been used to probe different membrane configurations around nanopillars, but these studies did not find any passive membrane rupture or penetration.23,37 In contrast, in horizontal TEM sections, where the cutting plane was further from the substrate, we found electrodes with direct contact to the cells’ cytoplasm and without any surrounding membranes (Figure 1g, upper panel). In accordance with our SEM observations, in section views where the cutting plane was in close proximity to the substrate, we observed electrode-surrounding membranes, which enclosed the polycarbonate material between the cells and the electrodes (Figure 1g, lower panel). Vertical TEM sections confirmed this layout with the cell’s basal membrane protruding toward an electrode and sticking closely to the electrode base and the same membrane rupturing at the polycarbonate-gold transition sites (Figure 1h). This cellular layout allows for direct intracellular access and simultaneous sealing of the interface. Previous studies have shown that...
cellular components, for example, cytoplasmic proteins and clathrin-associated vesicles accumulate around high aspect ratio structures like nanopillars. However, we found neither structural reorganization of organelles nor ultrastructural variations around membrane penetrating electrodes like accumulated cytoplasmic components or vesicles. This led us to assume that the bioinert gold electrodes did not interfere with processes in the cytoplasm (Figure 1i and Figure S4). Nanopillar architectures have previously been reported to cause perturbations of chromatin structures and nuclear morphologies. However, even if the electrodes were penetrating into the nuclear envelope, we did not observe the gNEI electrodes to induce any alterations of the heterochromatin ultrastructure (Figure 1j). Taken together, these data indicate the first effective and reliable integration of nanoelectrodes into living cells without the utilization of any penetration-promoting electrical or chemical procedures.

In previous studies, membrane penetration by nanoelectrodes was mostly achieved by local electroporation, chemical modification of the electrode’s surface, or mechanical forces (e.g., centrifugation). Effective spontaneous penetration mediated by substrate adhesion, on the other hand, was rarely observed. Detailed quantification studies of unaided nanoprobe penetration into cells showed that only a small fraction of nanotubes gained access to the cytosol via substrate adhesion forces. In fact, mechanic continuum configurations of adherent cells with morbid morphologies. Uncoated PC surfaces prevented all cell adhesion and spreading. Adherent cells with smaller, and both surfaces promoted more spindle-like morphologies. Passivation by PLL-PEG led to a dramatic reduction of cell adhesion as well as the occurrence of semi-adherent cells with morbid morphologies. Uncoated PC surfaces prevented all cell adhesion and spreading.

Interestingly, z-stacks of paxillin-YFP expressing cells showed two distinct FAC arrangements at the electrodes. In 87% of cases ($n = 27$ individual electrodes), fluorescence was exclusively found at the electrode base (Figure 2c), while in 7% of cases, two distinct signals at the electrode base and at its tip, accompanied by prominent anchoring actin fibers, could be observed (Figure 2c). Given that the penetration efficiency in our experiments was very high, we expect the latter configuration to resemble the unpenetrated cellular state. In this state, the cells prevent electrode penetration by using membrane incorporated protein complexes and the recruitment of cytoskeletal elements to the area of highest membrane curvature at the electrode tips to actively promote an intact membrane configuration. Correspondingly, noncontrasted TEM sections occasionally showed stress fibers directed toward electrodes that have not penetrated the cell membrane (see Figure S5b). When paxillin accumulations were exclusively found at the electrode base, we assumed successful penetration, a state where focal adhesions mediate tight sealing of the electrode—membrane interface. Here, the attachment of the cell membrane to the electrode’s polycarbonate cones may be accomplished by integrin-mediated coupling of FACs to the border where the PC and gold meet. Sealing appears to be crucial for preventing electrochemical leakage, which would otherwise lead to massive imbalances in the cells’ electrophysiology (see Figure S3). These findings add to the current perception in which cells are not only able to regulate electrode extrusion and penetration events by orchestrating cytoskeletal elements around the penetration sites, but might also actively participate in sealing the penetrating electrodes after successful insertion.

A correlation between cell adhesion and nanowire-cell coupling was demonstrated in recent publications. Our findings support a direct influence of substrate adhesion mechanisms on nanoprobe penetration. Therefore, we systematically evaluated the impact of alterations in FAC engagement on the intracellular electrical access of nanoelectrodes. To this end, gNEIs were coated either with (1) poly-l-lysine (PLL), which promotes nonspecific electrostatic interactions; (2) fibronectin, which mediates the formation of FACs by RGD-motif-integrin interactions; (3) collagen type I, which mediates specific receptor-based adhesion and signaling via FACs; and (4) PLL-polyethylene glycol (PEG) for passivation and concomitant suppression of FACs formation. These coatings were compared to uncoated controls with a hydrophobic PC isolation layer. On each variant of gNEI coating, cellular adhesion was assessed by measuring the overall cell spreading area in nonconfluent cultures (Figure 2d). As expected, cell spreading was greatest on the PLL-coated surface, on which large uniform lamellipodia were observed. The average surface areas of cells on the collagen and fibronectin coatings were slightly smaller, and both surfaces promoted more spindle-like morphologies. Passivation by PLL-PEG led to a dramatic reduction of cell adhesion as well as the occurrence of semi-adherent cells with morbid morphologies. Uncoated PC surfaces prevented all cell adhesion and spreading.

To quantify the electrical accessibility of our gNEIs to the cytosol, confluent cell layers were cultured on each variant of gNEI coating, and nitro blue tetrazolium chloride (NBT), a colorless dye that turns a deep purple color when reduced, was added to the culture medium. Immediately after NBT addition, a reducing voltage of 150 mV, which is far below the...
voltage needed to electroporate biomembranes (typically on the order of several kV)\textsuperscript{54} and should therefore not cause an artificial voltage-induced insertion of the electrodes, was applied to the gNEIs for 15 min.\textsuperscript{55,56} Intracellular adhesion-inserted electrodes acting as a cathode thereby alter the rate of intracellular NBT reduction, whereby the number of penetrated electrodes is expected to correlate with NBT reduction. By quantifying the amount of reduced NBT for each condition, the number of adhesion-inserted electrodes can be quantified. Figure 2e shows an increased NBT reduction in adhering cells with mature cell spreading.

Integrin ligands like fibronectin, for example, promote the assembly of FACs in a ligand concentration-dependent manner.\textsuperscript{57} In our gNEI chambers, the effect of reducing the fibronectin concentration in the substrate coating is two-fold. A slight decrease in the cell spreading area can be observed, paired with a disproportionally decreased electrical accessibility of the cytosol. Perhaps this can be traced to a reduced recruitment and assembly of adhesive architectures to electrode sites, influenced by FAC ligands that affect their assembly. In turn, changes to the assembly of adhesive architectures would probably affect the regulation of nano-electrode penetration as well as the stabilization of cellular arrangements surrounding the integrated electrode. This may also explain why a collagen substrate coating, which recruits FACs by a different set of integrins ($\alpha_1\beta_1$, $\alpha_2\beta_1$) than fibronectin, results in larger cellular spreading areas, while electrical accessibility to the cytosol is slightly lower than on fibronectin. Variable integrin engagement could alter mechanotransduction signaling and thereby affect electrode penetration. PLL, while promoting a close interaction between the substrate and the cell membrane, impedes the formation of integrin associated FACs. PLL-coated gNEIs gain excessive electrical access to the cytosol, because assembly of FACs that act in a penetration-inhibiting or electrode-extruding manner might fail, while, at the same time, passive mechanical penetration through electrostatic interactions between the cell membrane and the surface is promoted. On the PLL-PEG-coated gNEIs, which inhibit cell adhesion, the electrical accessibility of the cytosol is nearly identical to that of cells on plane gold electrode surfaces. Uncoated controls, in comparison, result in the complete absence of both cell adhesion and

**Figure 3.** En masse gNEI voltage recordings of whole cell layers. (a) Shown is a representative voltage recording from a gNEI plated with NRK cells without medium perfusion. The black arrow under the time scale indicates the time point of cell seeding. After an initial voltage increase and subsequent monolayer formation, a stable membrane potential is recorded. Cell layer dieback after approximately 22 h is due to a lack of perfusion and is accompanied by a voltage decrease toward the initial baseline. Inset 1 shows voltage progression during initial cell-to-nano-electrode coupling. Inset 2 shows voltage oscillations associated with homeostatic calcium signaling after confluent cell layer formation. Inset 3 shows auxiliary voltage oscillations during cell layer dieback. (b) Representative bright-field images of the cells, at indicated time points after seeding, on gNEIs during respective adhesion phases. All scale bars are 100 $\mu$m. (c) Voltage oscillations associated with calcium action potentials after treatment with 1 $\mu$M PF2 (green), 1 $\mu$M BK (orange), and under unstimulated conditions (black). Scale bars are 0.6 mV (vertical) and 3 min (horizontal). (d) gNEI voltage recordings (black) and plane gold surface control recordings (red) of NRK cells stably expressing ChR. The gray time scale is marked with black vertical lines at time points of cyan light illumination. Scale bars are 0.5 mV (vertical) and 60 s (horizontal). (e) gNEI voltage measurement of NRK cells using a recording setup with 10 G$\Omega$ input resistance. The black arrow underneath the time scale indicates the time point of cell seeding. The red inset shows a magnification of the calcium action potential-associated voltage oscillations in the cell monolayer. The violet inset shows an even greater magnification of the superimposed voltage oscillations, and the orange inset illustrates noise levels.
electrical access to the cytosol. These findings further support an adhesion-mediated electrode penetration scenario, where cytosolic access is promoted by substrate adhesion and cells actively participate in the initiation and stabilization of electrode penetration.

For en masse recordings of interlinked intracellular signaling pathways, confluent cell layers of 200,000 NRK cells were seeded on gNEIs, and resulting voltages were recorded with a 1 MΩ input resistance oscilloscope. To monitor initial cell-to-electrode coupling and nanoelectrode integration as well as processes associated with cell layer formation and intercellular signaling within these networks, voltages were recorded over the entire culturing period.

In their study looking at the time window for the cell-to-nanowire coupling processes, Xie et al. observed four distinct phases of cell-to-nanoprobe adhesion.34 During the first 5 min after seeding, cells settled as spheres onto the nanotube substrates (phase I); 5−30 min after addition, they gradually began to adhere to the nanotubes (phase II); in the following 3 h, cells spread onto the arrays (phase III); and only after 9 h following initial substrate contact did the authors finally observe mature cell adhesion (phase IV). In accordance with these results, we recorded a progressive negative voltage increase of approximately −1.5 mV (±0.6 mV, n = 8 independent voltage measurements) briefly after cell seeding (Figure 3a, inset 1, and Figure 3b, phase I). This voltage increase plateaued after approximately 10 min and was followed by another sudden −0.5 mV (±0.8 mV, n = 8 independent voltage measurements) increase, which correlates with phase II of cell-to-substrate adhesion. This suggests that additional electrode penetration takes place at this time (Figure 3a, inset 1, and Figure 3b, phase II). During the subsequent hours, which correspond to phase III of cell adhesion, recordings showed a continuous voltage increment of −3 mV (±1.7 mV, n = 8 independent voltage measurements) (Figure 3a,b, phase III) up to a voltage output of −5 mV (±1.9 mV, n = 8 independent voltage measurements), which remains stable for the following 12 h (Figure 3a,b, phase IV). We next analyzed the proportion of cells of the collective being impaled and recorded intracellularly by our gNEIs. To this end, we analyzed staining intensity of single NRK cells in the monolayer after performing the NBT reduction assay (see Figure 2e) to investigate if all cells are intracellularly electrically contacted and therefore recorded (see Figure S6). When compared to nonreduced cell layers, all gNEI reduced cells showed an increased NBT staining except for cells sitting on top of the monolayer, indicating that intracellular recordings are performed from all cells.

When the reactor was not perfused with fresh culturing medium at 24 h after cell seeding, a regressive voltage change toward the initial baseline was monitored. This is most likely caused by medium depletion and subsequent cell starvation and was accompanied by detachment of cells from the substrate and progressive cell death. Constant perfusion with fresh culture medium led to sustained voltage outputs over several days (see Figure S7), indicating long-term, stable nanoelectrode penetration of the cells. In control experiments with non-adherent lymphocyte cultures, despite an initial voltage response, which we attributed to gravity-induced cell penetration of settling cells, no comparable progression was observed (see Figure S8). This demonstrates the ability of gNEIs to gain intracellular electrical access during cell spreading and cell layer formation, a prerequisite for en masse intracellular recordings.

Fibroblasts represent one of the most abundant cell types of vertebrate organisms. Unfortunately, their electrophysiology remains poorly understood, despite the fact that their malfunctioning is pivotal in a multitude of pathophysiological processes. Because of their complex electrical behavior, NRK cells have frequently been used as model systems to study fibroblast electrogenesis and physiology as well as electrical signaling in connective tissue.58,59 For instance, spontaneous pacemaker activities of single cells within a density-arrested and quiescent NRK monolayer stimulate the synchronized firing of calcium action potentials in “follower cells”, resulting in a wave-like propagation of electrical signals.60 These signals, which are associated with inositol-1,4,5-triphosphate release, require gap junction-based cell connections to transverse a cell layer at a speed of approximately 50 μm/s.61,62 Notably, when performing gNEI voltage measurements on quiescent NRK monolayers, we observed prominent, arrhythmic oscillations, which began during phase III of cell-to-gNEI adhesion and manifested themselves approximately 20 h after cell seeding (Figure 3a, inset 2). To ensure that NRK cells do form gap junctions when cultured on gNEI surfaces, we performed immunofluorescence staining for connexin43 (Figure S9a). We ascribe these oscillations to the wave-like propagation of calcium action potentials associated with electrical signaling. Their emergence during phase IV is probably linked to cell layer tightening and, thus, successive coupling via gap junctions, which is necessary for proper intercellular signal propagation.63 During phase IV of cell-to-nanoelectrode coupling, we monitored oscillations with average amplitudes of 0.3 mV (±0.09, n = 34 single amplitudes) and respective phase lengths of 4.1 min (±0.6 min), which is in agreement with previous reports on signal propagation speed and calcium oscillation frequencies in NRK cell monolayers.51,63 A considerable and rapid amplitude increase to an average of 0.921 mV (±0.14, n = 16 single amplitudes) occurred just before medium depletion, possibly attributable to an altered auxiliary signal exchange. At the same time, the phase lengths of oscillations decreased to 1.51 min (±0.44 min, n = 27 single oscillations) during cell layer dieback (Figure 3a, inset 3).

To confirm that the observed oscillations are indeed associated with excitatory electrical signals generated by the cells’ pacemaker activity and to illustrate the suitability of gNEIs for monitoring pharmacologically altered en masse electrochemical signaling, we examined the dynamics of the voltage oscillations under hormone treatment. The NRK cells’ pacemaker activity is sensitive to a variety of tissue hormones. These include prostaglandin F2α (PF2), an activator of F-type prostanoid receptors and inducer of phosphoinositol turnover during calcium signaling as well as bradykinin (BK), a peptide which is involved in inflammatory signaling and vasorelaxation and whose receptor inhibitor, icatibant, is used for hereditary angioedema treatment.64,65 De Roos et al. and Harks et al. reported a stimulatory effect of PF2 on calcium action potential generation in NRK cells.66,67 BK, on the other hand, has been associated with inhibition and silencing of pacemaker cells.61,66 In line with this, we observed an increase of the average oscillation amplitude under PF2 treatment (0.54 mV, ± 0.08 mV, n = 12 single amplitudes), whereas BK induced an attenuation of the oscillatory activity (0.02 mV, ± 0.01 mV, n = 10 single amplitudes) (Figure 3c). These increased (PF2) or diminished (BK) pacemaking frequencies
are likely to result in altered cell layer excitation states, thus resulting in affected oscillation amplitudes. We therefore conclude that the observed arrhythmic oscillations in fact do represent en masse recordings of long-range calcium signaling-associated excitations between distant cells, provoked by the cell layers pacemaker activity. This not only demonstrates that gNEIs are able to record en masse intracellular electrochemical signals from cellular collectives harboring thousands of cells but also highlights their suitability to reveal diversified intercellular electrochemical signaling during drug treatment.

To confirm that gNEIs record intracellular information about electrical activity, we performed voltage measurements of NRK cells stably expressing a channelrhodopsin (ChR) variant. ChR increases its cation conductance under blue light illumination and thus induces membrane depolarization. When illuminated with LED cyan light (470 nm) for 500 ms, gNEI recordings of ChR+ cells showed prompt depolarization, followed by a sharp re- and hyperpolarization phase after LED shutoff (Figure 3d, black line). This hyperpolarization decayed to the initial resting membrane potential in the following seconds. Repeated illumination lead to comparable biphasic responses. In contrast, control measurements on plane gold surfaces showed inverted and slightly attenuated dynamics (Figure 3d, red line), proving that gNEI recordings truly represent intracellular measurements. Cell-free or ChR− recordings resulted in small monophasic hyperpolarization events during irradiation, followed by brief recoveries after LED shutoff. These events most probably represent photoelectric reactions of the electrodes (see Figure S10). In summary, these data confirm an intracellular positioning of the electrodes and show that en masse gNEI voltage recordings can monitor intercellular activities stemming from multicellular architectures.

Previously developed nano electrode platforms often suffered not only from unstable intracellular electrode insertion but also from poor membrane-to-electrode coupling and insufficient junctional sealing of the cell-electrode interface. These drawbacks resulted in overall unsatisfactory SNR and low coupling coefficients. To examine whether gNEIs provide adequate electrode coupling, we performed voltage measurements with an elaborate recording setup featuring 0.1 μV resolution, 30 ppm (0.003%) accuracy, and >10 GΩ multimeter input resistance. Measurements with this setup showed comparable dynamics to measurements obtained using a 1 MΩ setup (Figure 3e). We recorded stable voltage outputs of up to −40 mV, which approximately correspond to the previous reported membrane potentials of NRK cells, thus suggesting an excellent electrode coupling. Moreover, recordings showed a SNR of >30 dB (as calculated by mean output intensity of 31.2 mV between 12 and 22 h after plating and mean background noise 0.04 mV in the respective time interval) (Figure 3e, orange inset). This suggests a tight electrical sealing of the junctional membrane, possibly consisting of the stabilizing and sealing cytoskeletal components described above. Furthermore, pacemaker-related arrhythmic oscillations recorded with this setup were similar to those recorded with our standard simplified recording setup.

Both recordings showed comparable dynamics, except that the more elaborate high-resolution setup recorded larger oscillation amplitudes of 7.2 mV (±1.1 mV, n = 20 single amplitudes) (Figure 3e, red inset). Remarkably, the high-resolution setup revealed further superimposed oscillatory signals, which were not resolvable with the standard setup (Figure 3e violet inset). Their origin is as of yet unidentified, but local variations in signal transduction or excitatory calcium waves “resonating” within the culturing chamber that contribute to the en masse signal seem a plausible explanation.

To explore the applicability and intracellular accessibility of gNEIs on other electrogenic cell collectives, we performed gNEI measurements on differentiated myotubes and neuronal assemblies. In order to observe collective electric activity in neuronal networks, we cultured 300,000 SH-SY5Y cells on gNEIs and performed voltage recordings over several days to monitor network formation during retinoic acid-induced differentiation. Similar to our NRK voltage recordings, we observed voltage changes correlating with the different phases of cell to substrate adhesion (e.g., a voltage increase of −28.2 mV in phase III) (see Figure S11). After approximately 3 days of culture and differentiation, we found oscillations in gNEI voltage which were absent in nondifferentiated cultures (see Figure S11, insets 1 and 2). These sinusoidal oscillations appeared with striking regular periodicity of 926.7 s (±29.1 s, n = 40 single oscillations) and correlated with the degree of connectivity between the cells (e.g., onset after mature cell–cell contact formation between neurons and progressive decline during cell layer dieback). The high SNR (see Figure S11 inset 3) of these recordings indicates that the sealing of the gNEI intracellular access was excellent also for neuronal cell cultures.

To analyze possible collective actions of myogenic cultures, we cultured 150,000 C2C12 myoblasts on gNEI surfaces and differentiated them to form myotube assemblies as a muscle fiber model system (see Figure S12a). Again, we obtained stable intracellular gNEI voltage measurements of myotubes over several days. After formation of mature myotubes (as assessed by the appearance of elongated multinucleated contractile myotubes), we recorded spontaneous depolarizations (see Figure S12b) and were able to distinguish between single fused and unfused tetanic depolarization events (see Figure S12c,d). Periodic voltage oscillations, as observed in the neuronal and fibrogenic cell cultures, were not observed in the myotube culture system, indicative of a less distinct collective electrochemical activity in these assemblies. The high SNR as well as the spontaneous depolarization events, which we attribute to the contraction events observed with optical microscopy, indicate a tight sealing of the electrode–cell interface and the intracellular nature of the recordings.

Conclusions. Here we report on gNEIs for en masse intracellular recordings of electrical activity in multicellular networks. We demonstrate that gold nanoelectrodes are able to penetrate the basal membrane of cells by way of substrate adhesion forces and without affecting cell viability or morphology. We found that cell adhesion to the gNEI substrate is essential for achieving effectual integration of the nanoelectrodes into the cytoplasm. In contrast to micro-electrode recordings, this gNEI technology is easily adapted to many experimental settings without requiring other skills or equipment than those necessary for standard cell culturing. Moreover, the setup potentially enables sensitive recordings of intercellular en masse electrochemical actions from millions of cells. gNEIs provide a novel powerful tool to examine fundamental en masse 2D intercellular signaling processes of electrogenic cell collectives under in vitro conditions. They provide users with an easy to use technology to study en masse intercellular communication. Furthermore, gNEI technology is not only easily scalable but can also be integrated into parallel
high-throughput analysis pipelines. The simplicity of the circuitry and the scalability of gNEIs gives this technology potential beyond that of current single and multicellular electrode techniques offering individual cell outputs. We see this technology as more suitable for biosensor applications where the required electrical output is dozens of orders of magnitude larger than single cells can produce and where the vast majority of cell types are electrically interconnected into a syncytium which produces electrical signals en masse.

Compared to previous approaches based on nanoelectrode arrays, we achieved a significantly more stable intracellular insertion of the nanoelectrodes. This allowed for continuous and long-term monitoring of the signal exchange within multicellular assemblies over several days. We ascribe the insertion stability to an active participation of the cells toward maintaining an electrically favorable membrane–electrode configuration by recruiting cytoskeletal and mehano-transducing elements to the electrode penetration sites. Given their intricate configuration, an active participation of the cells in stabilizing a favorable electrode–membrane configuration is probable. FACs, major mediators of mechanotransduction, are particularly suited to regulate the local mechanical stress provoked by nanoelectrode tenting and implement. Their recruitment to the membrane–electrode interface could counteract membrane penetration, for example, by stabilizing areas of high membrane curvature or alternatively by stabilizing penetrated electrode configurations. This could be achieved by regulating focalized force exertion through actin assemblies around the electrodes, thereby forming a caulking at the electrode base. The formation of these stabilizing assemblies might, in part, be driven by the nanoelectrode architecture of gNEIs, where hydrophobic polycarbonate at the electrode base merges with the hydrophilic gold electrode surface. The formation of adhesive assemblies is probably responsible for the effective sealing of the cell–electrode interface, resulting in superior electrode coupling and SNRs. However, other critical and cell-type-dependent factors like membrane and cortical stiffness have been associated with successful membrane penetration and might need to be taken into account when fine-tuning adhesion-mediated electrode insertion.68,69 Even though our gNEIs were designed to record en masse signals from cell collectives and nanoelectrode recordings with single cell resolution, as performed by other research teams, were not within the scope of this study,10 the concept of adhesion-mediated electrode insertion might be applied to other vertical nanoelectrode techniques aiming for single cell resolution. For instance, we are currently trying to transfer adhesion-mediated electrode insertion of the nanoelectrodes. This allowed for continuous and stable intracellular insertion of the nanoelectrodes. This allowed for continuous and stable intracellular insertion of the nanoelectrodes.

**Methods.** Cell Culture. Normal rat kidney fibroblasts (NRK) and rat embryonic fibroblasts (REF) as well as REF cells stably expressing YFP-pasillin were routinely cultured in Dulbecco’s modified eagle medium (Thermo Fischer Scientific), supplemented with 1% glutamine (Thermo Fischer Scientific), 1% penicillin/streptomycin (Thermo Fischer Scientific), and 10% fetal bovine serum (Sigma-Aldrich) at 37 °C and in 5% CO₂ atmosphere. NRK cells expressing channelrhodopsin were supplemented with 300 μg/mL Geneticin sulfate (Sigma-Aldrich) for routine culturing and for gNEI measurements. SH-SY5Y neuroblasts (ATCC CRL-2266) were cultured in a 1:1 mixture of Dulbecco’s minimal essential medium and F12 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin/streptomycin (Thermo Fischer Scientific) at 37 °C and 5% CO₂ atm. For differentiation of SH-SY5Y neuroblasts on gNEI surfaces, 10 μM all-trans retinoic acid was added to the culture medium with reduced fetal bovine serum concentration (1%). C2C12 myoblasts were cultured in Dulbecco’s modified eagle medium supplemented with 10% fetal bovine serum (Sigma-Aldrich), 1% penicillin/streptomycin (Thermo Fischer Scientific), 1% L-glutamine (Thermo Fischer Scientific), and 1 mM sodium pyruvate (Thermo Fischer Scientific) at 37 °C and 5% CO₂ atm. For differentiation and myotube formation on gNEIs, C2C12 cells were allowed to attach on a gNEI surface in fully supplemented culture medium. After 12 h, the culture medium was exchanged with Dulbecco’s modified eagle medium supplemented with 2% horse serum (Thermo Fischer Scientific) and 1 μM insulin (Sigma-Aldrich). This differentiation medium was exchanged every 24 h for 6 days to allow the formation of mature myotubes.

For morphological observations, cellular membranes were stained with wheat germ agglutinin (WGA) AlexaFluo555-conjugates (Life Technologies), Hoechst 33342 (Sigma-Aldrich), and CellTracker Green CMFDA (Life Technologies) following the manufacturers recommendations. For staining of actin architectures, cells were rinsed twice with PBS and then fixed with 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, the fixed cells were incubated with 33 nM Phalloidin-TRITC (Sigma-Aldrich) overnight at room temperature, subsequently rinsed twice with PBS, and mounted in glass coverslips and imaged.

**Fluorescence and Confocal Laser Scanning Microscopy.** Fluorescence microscopy was performed with a Leica DM6000B upright bright-field microscope equipped with a 40×/0.75 NA water immersion objective, a HBO 100 W mercury arc lamp, and suitable fluorescent excitation/emission dichromatic filters controlled by LAS X software. Confocal laser scanning microscopy was performed with a Leica TCS SP2 laser scanning spectral confocal microscope (Leica Microsystems) equipped with a 63×/1.32 NA objective, at 8-bit resolution, zyx scan mode, 1.0 airy pinhole, and 400 kHz scan speed. Z-projections of confocal stacks, contrast enhancement of electron microscopy images, and local minimum filtering in a 3 × 3 neighborhood of fluorescence microscopy data were performed by using ImageJ software.

**gNEI Fabrication.** Detailed descriptions of the gNEI manufacturing process have been published previously.25,26 Briefly, vertically aligned nanoelectrodes were grown inside track etch membrane nanopores (ip4it, Belgium) with diameters of 100 nm by electrochemical deposition on gold sputtered glass surfaces. Porous membranes where subsequently dissolved by dichlormethane (Merck Millipore) incubation. The electrode arrays were then isolated with bisphenol A-polycarbonate (Sigma-Aldrich) via spin coating.
and electrode tips were etched by 4 N NaOH treatment for 10 min at room temperature.

**gNEI Voltage Measurements.** A custom-tailored double-chamber bioreactor setup with a total culturing volume of 8 mL was used for long-time gNEI voltage monitoring. Voltage measurements were performed either with an easy to use Mephisto UM202 oscilloscope (Meilhau) or a Keithley 2000 data acquisition system (Tektronix) controlled by a LabView (National Instruments) software. For this, cells were seeded on the PLL-coated gNEI chamber, and voltages were measured against a plane gold surface with a total surface area of 1.57 cm² inside a cell culture incubator (37 °C, 5% CO₂ atmosphere). For long-time measurements, the reactor was perfused with 300 μL/h fresh culture medium. For pharmacological studies, prostaglandin F2α (Sigma-Aldrich) and Bradykinin (Sigma-Aldrich) were dissolved in cell culture medium and perfused into the culture chamber at 200 μL/min to give a final concentration of 1 μM. Simultaneous monitoring by bright-field microscopy was performed with a J uli Br live cell analyzer (NanoEnTek).

**ATP Assay.** Cellular ATP concentrations were measured with the CellTiter-Glo Luminescent Cell Viability Assay (Promega), following the manufacturer’s protocol. Luminescence was quantified with a GloMax detection system (Promega) equipped with a luminescence detection module.

**PI Assay.** Cell viability on gNEIs was measured by incubating the cells with a 50 μg/mL propidium iodide (PI)/300 nM Hoechst 33342 solution (Sigma-Aldrich) for 10 min at 37 °C. After rinsing with PBS, PI positive cells were observed and counted by fluorescence microscopy.

**Electron Microscopy.** For SEM, cells plated on gNEIs were fixed in 2.5% glutaraldehyde dissolved in a 0.1 M Na₃PO₄ solution for 30 min at room temperature. Samples were dehydrated by successively replacing the fixation medium with 50%, 60%, 70%, 80%, 90%, and 100% ethanol and dried by CO₂ critical point drying with a CPD030 (Leica). For imaging purposes, samples were additionally covered with a 5 nm-thick Au layer by metal sputtering. For cross-sectional examinations of the electrode cell interface, SEM probes were fractured by hand and imaged at a 90° angle.

For TEM analysis, gNEIs with cultured cells were fixed in 0.4% uranyl acetate overnight after glutaraldehyde fixation, as described above. Fixed cells were subsequently dehydrated with ethanol and embedded in resin overnight at 60 °C. 85 nm ultrathin sections were prepared and contrasted with lead acetate or osmium tetroxide.

**gNEI Coating.** For adhesion studies, gNEIs were coated with respective substances directly in the mounted bioreactor. For poly-l-lysine (PLL, Sigma-Aldrich) and PLL-polyethylene glycol (SuSoS Surface Technology) coatings, arrays were incubated with 1 mL of a 100 μg/mL PLL solution for 10 min at room temperature and subsequently rinsed twice with 1 mL PBS. For fibronectin (from bovine plasma) coating, arrays were incubated with 1 mL of a 1 μg/mL or a 0.1 μg/mL solution (Sigma-Aldrich) overnight at 4 °C, subsequently rinsed twice with PBS, and then blocked with 1% BSA (Carl Roth) solution for 1 h. Bovine collagen coating was performed by incubating gNEI arrays with 0.01% (w/v) collagen solution (Thermo Fischer Scientific) for 3 h at room temperature, followed by rinsing with PBS and blocking with a 1% BSA (Carl Roth) solution for 1 h.

Total cell area was assessed by staining cellular membranes of nonconfluent cultures with WGA-AlexaFluor555 (Thermo Fischer Scientific) after fixation with 4% paraformaldehyde. Stained cells were imaged by bright-field fluorescence microscopy, and the cell area was calculated based on global threshold segmentation of respective images in ImageJ.

**Nitro Blue Tetrazolium (NBT) Reduction Assay.** NBT (Sigma-Aldrich) was dissolved to a final concentration of 200 mg/mL in fresh culture medium and added to the gNEI bioreactor. Subsequently a reducing current of 150 mV was applied on the gNEIs for 15 min. Immediately after reduction, the bioreactor was rinsed twice with PBS, and cells were lysed in 2 mL 2% SDS, 1 M NaOH solution, or alternatively imaged with a Zeiss Axiovert A1 equipped with plan-apochromat 10X/0.45 objective. NBT crystals were dissolved by adding 20 μL DMSO (Sigma-Aldrich) into the culturing chamber. Respective absorptions were measured at 595 nm with an UltraSpec 3000 (Pharmacia Biotech) photometer in glass cuvettes and normalized to the total protein content (determined by Bradford assay).

**Data Availability.** The relevant data that support the findings of this study are available from the corresponding author upon reasonable request.

**Immunocytochemistry.** Immunocytochemical analysis for gap-junctional connectivity was performed by pre-extracting the cells for 1 min with 0.5% NP40, 1 mM MgCl₂, 5 mM EGTA solution buffered with 80 mM PIPES/KOH (pH 6.8) and subsequent fixing with 4% paraformaldehyde/4% sucrose for 30 min. Cells were rinsed twice with PBS and incubated for 10 min with a 50 mM NH₄Cl quenching solution, followed by 1 h incubation with 1% BSA solution. Anti-connexin43 antibodies (C6219, Sigma-Aldrich) were dissolved according to the manufacturer’s recommendations in blocking solution for 90 min with the cells. After 2X rinsing with blocking solution, cells were incubated with Alexa Fluor 488 coupled secondary antibody and 100 ng/mL DAPI.

**Morphological Analysis.** NRK cell areas were quantified by culturing nonconfluent NRK cells on glass, PC, and gNEI substrates for 24 h, followed by fixation and staining with 1 μM WGA-Alexa647 for 20 min. Automated global threshold segmentation of respective confocal microscopy pictures and automated particles analysis (total particle area and particle circularity) was performed with build in ImageJ PlugIns.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.9b00784.

- Figures S1–S12 (PDF)
- Video S1: Actin fibers anchor at penetrated electrode (AVI)

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**Author Contributions**

O.S. performed the experimental work, and S.W. performed the impedance spectrometry and cyclic voltammetry characterization. C.P.B. and H.B. helped with the channelrhodopsin experiments and interpretation of the calcium signaling data. O.S., A.R., and J.P.S. designed the research. H.B. provided the...
research equipment and facilities. A.R. and J.P.S. led the study and supervised the research. O.S. and J.P.S. wrote the manuscript with the help from all authors.

**Notes**

The authors declare no competing financial interest.

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