1 Localized detection of ions and biomolecules with a 2 force-controlled scanning nanopore microscope

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ABSTRACT:

Proteins, nucleic acids, and ions secreted from single cells are the key signaling factors to determine the interaction of cells with their environment and the neighboring cells. It is possible to study individual ion-channels by pipette clamping, but it is difficult to dynamically monitor the activity of ion-channels and transporters across the cellular membrane. Here, we show that a solid-state nanopore integrated in an atomic force microscope (AFM) can be used for stochastic sensing of secreted molecules and the activity of ion-channels in arbitrary locations both inside and outside of a cell. The translocation of biomolecules and ions through the nanopore is observed in real-time in live cells. The versatile nature of this approach allows detection of specific biomolecules under controlled mechanical confinement, and monitoring ion-channel activities of single cells. Moreover, the nanopore microscope is used to image the surface of the nuclear membrane via high-resolution scanning ion conductance measurements.

KEYWORDS:

Nanopore AFM; single-cell mapping; molecular confinement; nucleus mapping; scanning nanopore; ion-channels monitoring

Localized detection of ions and biomolecules is of paramount importance in understanding the physics of life at cellular and molecular scales. For example, a large number of biological processes in living cells are regulated and mediated by localized membrane transport.¹ Trafficking of macromolecules (such as proteins, RNA and DNA) and ions routed through the cell membrane determine the interaction of cells with each other, their environment and immunological response.² Any dysfunction of these trafficking routes causes severe problems leading to a variety of diseases, such as cancer and autoimmunity.³ To understand the underlying biological mechanism of cell signaling, it is essential to identify and characterize transmembrane pathways at single-cell level.⁴ Protein secretion and/or ion-channel activities are often irregular processes and the neighboring cells within a tissue may secrete significantly different amount/types of proteins/ions in a given time.⁵ Currently, there is no method for localized detection of ionic and biomolecular pathways through the cell membrane.

Patch-clamp - the gold-standard technique in studying ion-channels - uses pulled glass pipettes for recording the ionic current between an electrode in the pipette and one in the bath solution. The technique enables single-channel recording, however, it lacks efficiency and sensitivity for protein identification.⁶ Traditional methods for protein identification and characterization include mass spectroscopy, nuclear magnetic resonance (NMR), X-ray spectroscopy, flow cytometry and immunoassays, which are impractical for time-resolved measurements of secreted proteins from single cells.⁵ These obstacles significantly limit the understanding of dynamics of the cell membrane's permeability, which is a particularly important aspect of the immunological response. The grand challenge in this field is to dynamically identify the proteins/ions that are secreted from a single-cell into the extracellular environment. Recent methods developed for single-cell proteomics include: optical interferometric detection of scattered light from secreted

proteins,⁵ optical imaging through molecular binding on the sensor surface,^{7,8} and ionic current recording using a nanopore embedded in a microfluidic incubator.⁹ Despite their success and the innovative approaches, none of these techniques can be used for localized detection of biomolecules and they lack sensitivity for monitoring ion-channel activities.

In this pilot study, we introduce a force-controlled scanning nanopore microscope which enables localized detection of the activity of ion-channels and membrane transporters by locally detecting ions and macromolecules such as DNA and proteins. This article focuses on the fabrication and the different areas of application of this force-controlled scanning nanopore microscope in molecular biology, such as: (1) localized confinement and detection of biomolecules (*e.g.* DNA and protein sensing), (2) extracellular recording from single cells to monitor the transmembrane activities (*e.g.* permeation of proteins/ions), (3) controlled localization of the nanopore probe into the cytoplasm and nucleus of the cell for intracellular sensing, injection and mapping of the nuclear membrane.

Detection of molecules under tunable mechanical confinement

The platform builds on a Fluid Force Microscope (FluidFM, **Supplementary Fig. 1**).¹⁰ The AFM cantilevers used in this study, are made of silicon nitride and contain an embedded microfluidic channel inside.¹¹ The nanopores were fabricated via “ion-beam sculpting”¹² using a Helium-Ion-Microscope (HIM).^{13,14} Briefly, the apex of the tip was flattened by ion sputtering, producing a thin film of silicon nitride (with $\sim 150 \times 150 \text{ nm}^2$ lateral dimensions and $\sim 20 \pm 10 \text{ nm}$ thickness). A small hole was then fabricated in the middle of the thin film using focused He^+ ions. It is possible to shrink the pores to a desired diameter by raster scanning of He^+ ions across

the pore.¹¹ Secondary electrons produced during the fabrication process provide a simultaneous visual feedback with a high resolution (< 1 nm) which allows precise control over the pore diameter while shrinking. Pores as small as 5 ± 1 nm in diameter were produced using this method (**Figure 1**). The fabricated nanopores are similar to the conventional on-chip solid-state pores (**Supplementary Fig. 2**), yet they are located at the apex of AFM cantilevers. The introduced system also resembles the glass pipette pores in various aspects, such as adaptability for scanning ion conductance microscopy (SICM),¹⁵ which can be used for imaging surfaces, detection and on-demand delivery of biomolecules.^{16–20} However, the AFM cantilevers are two orders of magnitude softer than glass pipettes, allowing flexibility and mechanical engagement with the samples. Moreover, the wide opening angle of the fabricated pores provides higher ionic sensitivity compared to the conical glass pores.^{21,22}

The nanopore AFM enables nano-confinement of biomolecules between the apex and the underlying surface. Pre-confinement of biomolecules results in slower translocation speeds through the nanopore, which in general provides longer times for the sequence-based read-out.^{23–26} Molecular confinement prior to the translocation also reduces the conformational variations in passing molecules by setting a barrier for the folded DNA/protein configurations.²⁷ **Figure 2a** schematically shows a situation where the nanopore AFM generates a nano-confined region near the surface of a glass substrate and sets a barrier for the molecules before their entrance to the nanopore. A representative *I-V* curve for a pore near the surface and the voltage dependence of protein translocation through such a constrained geometry are shown in **Figure 2b** (see **Supplementary Fig. 3** for noise analysis and **Supplementary Fig. 4** for DNA confinement). The produced geometrical constraint at contact with the surface limits the ionic current and therefore increases the resistance of the system. In this experiment Fibronectin protein (Fn) was

introduced to the bath solution (*cis*) and a positive bias was applied to the *trans* side (electrode placed in the probe reservoir), triggering (di)electrophoretic translocation of the charged proteins towards the pore. At lower voltages the protein translocation was suppressed, suggesting reduced penetration depth of the electric field into the bulk solution and consequently a reduced capture rate of diffusing molecules. In a blank buffer solution, the number of recorded events was significantly suppressed, confirming that the observed jumps in the current traces are due to protein translocation through the nanopore (**Supplementary Fig. 5**). The translocation time τ in confinement is long compared to a non-confined event, particularly evident at higher applied voltages. For example, the mean translocation time of Fn under mechanical confinement of the AFM tip is $\sim 2.2 \pm 0.6$ ms at 400 mV in 1x PBS (note $\tau \sim 0.95 \pm 0.92$ ms at same conditions far from the surface). On the other hand, the frequency of translocation events is lower in the confined geometry (< 5 1/s), implying that protein molecules encounter an energy barrier under the mechanical confinement (see **SI** for discussion on capture rates). The dynamics of the confined translocations are distinct from those with no confinement. The event duration (τ) exhibits an exponential decay with the applied voltage (V) (**Figure 2b**), unlike the linear dependency observed in non-confined experiments in this study and also in other solid-state nanopore experiments²⁵ (but similar to the translocations observed with protein nanopores²⁸). This difference can be attributed to the higher energy barrier for the translocating molecules induced by the mechanical confinement between the substrate surface and the nanopore chip.

According to Kramer's theory ($\tau \sim e^{\frac{(\Delta G - qV)}{k_B T}}$), an energy barrier (ΔG) can hinder translocation of a polymer (with an effective charge q), therefore the frictional forces between the confining surfaces and the passing molecules can alter the translocation dynamics, consistent with the

friction-dominated translocation through protein nanopores.^{28–30} The interaction of proteins with the probe walls can be a source of friction.³¹

By increasing the set-point (SP) forces on the AFM cantilever, the distance between the nanopore and the substrate surface decreases, which is corroborated by reduction of the background current. Correspondingly, the protein/DNA translocation time increases, suggesting the strong interaction between protein and the surfaces of the confined region. The confinement prior to translocation not only reduces the speed of biomolecules, but also more accurately determines the translocation pathways for the molecules. As a result, the standard deviation of the current transients reduces significantly (**Figure 2c**). Therefore, it is concluded that the mechanical confinement of the molecules enables longer read-times for the detection which will be exploited to monitor translocation of proteins released from cells.

Extracellular mapping and detection of secreted products

Imaging of live cells using scanning probe microscopy provides a wealth of understanding about the nanoscale organization of biomolecules.^{32–34} The scanning nanopore microscope enables nanoscale monitoring of live cells using simultaneous force and ion current feedback, allowing generation of the topographical images and the concomitant ionic current maps (**Figure 3a,b**). Molecular-level cellular activities, such as protein secretion, can be monitored by the nanopore microscope through distinctive blockades/enhancement in the current traces (**Figure 3c**).

Protein secretion from individual cells is particularly interesting and has significant importance in cellular functionality, but monitoring these proteins with nanopores requires several technical considerations. Importantly, proteins and other biomolecules tend to adsorb on the surfaces of materials within a relatively short amount of time.^{35,36} Protein adsorption on the surface of the nanopore chip was observed in our live cell experiments, which led to the permanent blockade of the used pores within the course of a few minutes after exposure to the extracellular environment. To avoid clogging issues, the surfaces of the nanopore chips were coated with an antifouling layer using a polymer PAcrAm-g-(PMOXA, NH₂, Si)^{37,38} with known protein-resistant properties. The PMOXA side chains of this molecule are 9 nm in size and are covalently bonded to the surface via the silane groups of the PAcrAm backbone. The nanopores coated with this polymer layer could be used for live cell studies for several weeks without any permanent clogging. **Figure 3c** shows an example of current recorded near the surface of a cell membrane, in which several current spikes are visible during the minutes-long measurements. To make sure that the recorded current transients correspond to the cellular activity of a single cell, low density of cells were cultured and prior to the measurement the culture medium was washed

and replaced with a buffer solution without proteins (*e.g.* PBS). In this buffer, negligible current transients were observed at distances far from the cells (*e.g.* at 10 μm distance) in a culture dish.

The events gathered near the outer membrane of a live cell can be attributed to translocation of variety of biomolecules, such as proteins or fragmented RNA/DNA, exosomes or even temporal ionic fluxes through the ion channels. These events may be distinguished by the shape of the translocation peak.³⁹ Proteins possess range of charges and molecular weights which can characteristically influence the duration and magnitude of a translocation event.³⁹ The nanopores exhibit a bias towards specific proteins based on their charge and molecular weights (**Supplementary Fig. 6**). For instance, Bovine Serum Albumin (BSA, a negatively charged protein at pH 7.4) can be only detected at positive applied voltages, while Lysozyme (a positively charged protein at pH 7.4) detection requires a negative applied voltage. There are other proteins, such as Fibronectin (Fn), which possess large amount of negative and positive charges on their surface and can be detected at both positive or negative voltages. The magnitude of the current change can be used for specific detection of the biomolecules as well. For example, magnitude of the current change is much higher for serum Fn dimers (~440 kDa) compared to smaller proteins such as BSA (~65 kDa) and Lysozyme (~15 kDa).

Classification of the peaks by their shape can also be used as another method to distinguish the source of an event. For example, a correlation clustering algorithm (Louvain method⁴⁰) was used to analyze the current transients obtained from multiple single cells (**Figure 3d**). Two significantly distinct types of events were observed in all the measurements: (I) One type of event exhibits a single-step peak in the current transient, which has a shape correlation with the blockade events acquired from pure protein solutions. (II) The second class of the current

transient consists of a current reduction which is followed by an immediate enhancement before reaching the background level (not observed in pure protein solutions).

In order to distinguish between various types of proteins in a crowded environment of secreted products, we examined Fn secretion of mouse embryonic fibroblasts knockout for fibronectin (MEFs Fn^{-/-}) versus MEFs Fn^{+/+} (**Figure 4**). Fn is one of the major components of the extracellular matrix (ECM) of fibroblasts which is expressed and assembled into fibrillar structure in early stage of ECM formation.⁴¹ Since in MEFs Fn^{-/-} cells are unable to secrete fibronectin, it can be used as a control to see if Fn can be specifically detected in MEFs Fn^{+/+}.

The secreted products of MEFs Fn^{-/-} vs. MEFs Fn^{+/+} are compared by Kernel density estimate plots (KDE) of the events distribution (**Figure 4** and **Supplementary Fig. 7**). The difference in the density plots of the events is particularly evident at the region with higher current change where one would expect to detect larger biomolecules, such as Fn. Since the major difference of MEFs Fn^{-/-} to MEFs Fn^{+/+} is the lack of fibronectin secretion, the increased density of the events at a particular region of the KDE plot in MEFs Fn^{+/+} is most likely due to Fn secretion of the cells.

To further confirm that the increased cell secretome in MEFs Fn^{+/+} is related to Fn release, translocation of purified Fn solution was tested with the same nanopore (**Supplementary Fig. 7**). The results indicated accumulation of events in the density plots in a similar region where the difference between MEFs Fn^{-/-} to MEFs Fn^{+/+} is the most significant. There are minor differences in density plots of the pure Fn solution and the cells secretome, which most likely originate from variations in conformation of the proteins in native and purified states,⁴² or other physical differences in the experimental setup, such as interfaces of the confined regions.

Localized detection of ion channels

The working principle of nanopore sensors is based on temporal changes of ionic current/potential within (or nearby) the pore. Therefore, ion channel activities in the cell membrane are expected to produce a signal in a nanopore system. As a proof-of-principle application of nanopores in monitoring the ion-channel activities of single cells, the action potentials of individual hippocampal neuron cells was recorded (**Supplementary Fig. 8**). Interestingly, the recorded events during neuron ‘firing’ exhibit similarities to the shape of the class II current transient observed in **Figure 3d**. Class II events were not observed in pure protein solutions. However, during the extracellular recording from live cells, these class of events can be observed even at 0 mV applied bias, in which little contribution from biomolecular translocation is expected. Therefore, the class II events can be attributed to the temporal changes of current/potential in the vicinity of the cell membrane, most likely due to ion channel activities.

To further demonstrate the capability of nanopore in localized detection of ion channels, we acquired average-current maps for the cells with controlled number of ion channels (**Figure 5a**). HEK293 with overexpressing and knockout Piezo1 ion-channels were used. Overexpressing cells were generated by transient transfection and the knockout cells were obtained using CRISPR/Cas9. The number of Piezo1 ion-channels in the overexpressing cells is five times higher than the normal cells, and by far more than the knockout cells. The nanopore was positioned over an arbitrary location on the cell and it was scanned over a small area ($3 \times 3 \mu\text{m}^2$, with 2 nN set-point force). It is evident from the recorded current maps that the cells with the excess of ion-channels exhibit abundance of “hot” regions (with currents higher than the background). Particularly, as is shown in **Figure 5b**, the ratio of class II/class I events is generally higher for the cells with excess of ion channels compared to the knockout cells. These

data suggest that the ion-channel activity of single cells can be monitored with the nanopore microscope in a ‘loose’ patch configuration (*i.e.* with the probe positioned in close contact to the cell surface without gigaseal formation). However, at present we cannot decide whether or not it is possible to record single ion-channels using this technique. The nanopore is very sensitive to minor changes in the ionic flux, meaning the activities of the neighboring channels may influence the recording. Given that the dimension of the nanopore tips ($150 \times 150 \text{ nm}^2$) is much bigger than the size of ion-channels ($< 10 \text{ nm}$) and also considering ion channel clustering,⁴³ the likelihood of measuring multiple neighboring ion channels under the nanopore probe is plausible. It is anticipated that the strongest signals are correlated with the activities of the nearest channels to the probe, but contribution from neighboring channels and from cell membrane movements cannot be excluded.

Intracellular recording, mapping and injection

Finally, we assessed the feasibility of intracellular recording using both the ionic current and the force deflection feedback (**Figure 6**). By monitoring the force feedback in single-cell studies, the nanopore microscope enables intracellular recordings at particular parts of the target cells. **Figure 6a** shows typical force and current curves acquired during probe insertion into the cell and the subsequent withdrawal. The force feedback is related to the interaction between the nanopore tip and the cell. Four distinct instants are indicated in this curve which can be associated with the following steps: (i) the penetration of the tip through the outer cell membrane (and the nuclear membrane), (ii) maintaining the set-point and resting at the contact point, (iii) withdrawal from the contact point and then (iv) the cell. Optical images suggest that the tip insertion inside of the cell did not result in any observable change in the cellular activity

(**Supplementary Fig. 9** and Guillaume-Gentil et al.⁴⁴). After several measurements, it was found that penetration through the HEK cell membrane occurs at > 100 nN force, while penetration through the nuclear membrane requires forces > 300 nN. By taking an intermediate value for the set-point (*e.g.* 150 – 250 nN), the tip can penetrate the cell membrane and stay in contact with the nucleus without further penetration. Precise insertion of the nanopore on top of the nucleus led us to directly obtain the current maps of the nucleus membrane as shown in **Supplementary Fig. 10**. The current leakage through the cell membrane enables the circuit functionality for ion-flux recording inside of the cytoplasm. The intracellular sensing can be done similar to the extracellular recording. Once the nanopore is placed near the surface of the basal or apical nuclear membrane, a large number of current spikes are observed which are most probably due to the translocation of intracellular elements through the nanopore (**Figure 6b**). Although the precise characterization of the interior contents of the cells requires additional considerations, the technique can provide statistical information on many stochastic events in different cells, and therefore enable single cell analysis in heterogeneous cell populations. As shown here and in other studies,⁴⁵ voltage control can be used for better statistical classification of the events due to the sensitivity of the biomolecules to the applied bias. Although we are not able to determine specific types of the intracellular molecules at this stage, it is envisioned that selectivity can be achieved by functionalizing the nanopores,⁴⁶ molecular tagging,⁴⁷ antibody labeling,⁴⁸ or complementary methods such as mass-spectrometry,⁴⁹ post qPCR amplification²³ and implementation of learning algorithms in data analysis.⁵⁰

Conclusions:

The demonstrated concept for the realization of an AFM-controlled scanning nanopore microscope features several advantages. The use of an AFM cantilever to control the location of the nanopore makes the approach directly applicable for localized sensing of biomolecules. A nano-confinement can be readily produced to mechanically control the translocation of the molecules prior to their entrance to the nanopore. With this approach, the speed of molecular translocations reduces significantly, as well as the standard deviation in the current transients. Also, the extracellular signaling of single cells can be monitored by recording the activity of their ion channels and counting the amount of proteins secreted by the cell, based on stochastic translocation analysis. The spatial organization of ion channels and membrane transporters can be visualized by scanning the nanopore tip over the cell membrane while recording transient current events. Voltage control can further improve the quantitative assessment of such activities by enabling better classification of the events. The laser deflection from the cantilever provides a sensitive feedback for precise positioning of the nanopore inside of the cell, enabling intracellular sensing and mapping at different locations, such as outer membrane, in the cytoplasm, on the nuclear membrane or inside the nucleus. As an application, the ion conductance image indicative of nanoscale features in the nuclear membrane of a living cell was obtained.

References:

1. Alberts, Johnson, Lewis, Raff, Roberts, Walter Lefers, M. F.-F. T. '*Molecular Biology of the Cell.*' (Garland Science, 2009).
2. Kollmannsberger, P., Bidan, C. M., Dunlop, J. W. C., Fratzl, P. & Vogel, V. Tensile forces drive a reversible fibroblast-to-myofibroblast transition during tissue growth in engineered clefts. *Sci. Adv.* **4**, eaao4881 (2018).
3. Catterall, W. A. From ionic currents to molecular mechanisms: The structure and function of voltage-gated sodium channels. *Neuron* **26**, 13–25 (2000).
4. Robert, C., Hue, I., McGraw, S., Gagné, D. & Sirard, M.-A. Quantification of cyclin B1 and p34(cdc2) in bovine cumulus-oocyte complexes and expression mapping of genes involved in the cell cycle by complementary DNA macroarrays. *Biol. Reprod.* **67**, 1456–64 (2002).
5. McDonald, M. P. *et al.* Visualizing Single-Cell Secretion Dynamics with Single-Protein Sensitivity. *Nano Lett.* **18**, 513–519 (2018).
6. Actis, P. *et al.* Compartmental genomics in living cells revealed by single-cell nanobiopsy. *ACS Nano* **8**, 546–553 (2014).
7. Gatterdam, V. *et al.* Focal molography is a new method for the in situ analysis of molecular interactions in biological samples. *Nat. Nanotechnol.* **12**, 1089–1095 (2017).
8. Li, X. *et al.* Label-Free Optofluidic Nanobiosensor Enables Real-Time Analysis of Single-Cell Cytokine Secretion. *Small* **14**, 1800698 (2018).
9. Kennedy, E. *et al.* Method for Dynamically Detecting Secretions from Single Cells Using a Nanopore. *Nano Lett.* **18**, 4263–4272 (2018).
10. Meister, A. *et al.* FluidFM: Combining atomic force microscopy and nanofluidics in a universal liquid delivery system for single cell applications and beyond. *Nano Lett.* **9**, 2501–2507 (2009).
11. Dorwling-Carter, L., Aramesh, M., Han, H., Zambelli, T. & Momotenko, D. Combined Ion Conductance and Atomic Force Microscope for Fast Simultaneous Topographical and Surface Charge Imaging. *Anal. Chem.* **90**, 11453–11460 (2018).
12. Li, J. *et al.* Ion-beam sculpting at nanometre length scales. *Nature* **412**, 166–169 (2001).
13. Aramesh, M. Ion-Beam Sculpting of Nanowires. *Phys. Status Solidi - Rapid Res. Lett.* **12**, 1700333 (2018).
14. Aramesh, M., Mayamei, Y., Wolff, A. & Ostrikov, K. Superplastic nanoscale pore shaping by ion irradiation. *Nat. Commun.* **9**, (2018).

- 323 15. Ossola, D. *et al.* Simultaneous Scanning Ion Conductance Microscopy and Atomic Force
324 Microscopy with Microchanneled Cantilevers. *Phys. Rev. Lett.* **115**, 238103 (2015).
- 325 16. Lan, W. J., Kubeil, C., Xiong, J. W., Bund, A. & White, H. S. Effect of surface charge on
326 the resistive pulse wavelshape during particle translocation through glass nanopores. *J.*
327 *Phys. Chem. C* **118**, 2726–2734 (2014).
- 328 17. Ivanov, A. P. *et al.* On-demand delivery of single DNA molecules using nanopipets. *ACS*
329 *Nano* **9**, 3587–3594 (2015).
- 330 18. Klausen, L. H., Fuhs, T. & Dong, M. Mapping surface charge density of lipid bilayers by
331 quantitative surface conductivity microscopy. *Nat. Commun.* **7**, 1–10 (2016).
- 332 19. Perry, D., Al Botros, R., Momotenko, D., Kinnear, S. L. & Unwin, P. R. Simultaneous
333 Nanoscale Surface Charge and Topographical Mapping. *ACS Nano* **9**, 7266–7276 (2015).
- 334 20. Freedman, K. J. *et al.* Nanopore sensing at ultra-low concentrations using single-molecule
335 dielectrophoretic trapping. *Nat. Commun.* **7**, 1–9 (2016).
- 336 21. Steinbock, L. J., Steinbock, J. F. & Radenovic, A. Controllable shrinking and shaping of
337 glass nanocapillaries under electron irradiation. *Nano Lett.* **13**, 1717–1723 (2013).
- 338 22. Dorwling-Carter, L. *et al.* Simultaneous Scanning Ion Conductance and Atomic Force
339 Microscopy With a Nanopore: Effect of the Aperture Edge on the Ion Current Images. *J.*
340 *Appl. Phys.* **124**, 174902 (2018).
- 341 23. Nadappuram, B. P. *et al.* Nanoscale tweezers for single-cell biopsies. *Nat. Nanotechnol.*
342 **14**, 80 (2018).
- 343 24. Pud, S. *et al.* Mechanical Trapping of DNA in a Double-Nanopore System. *Nano Lett.* **16**,
344 8021–8028 (2016).
- 345 25. Wanunu, M., Morrison, W., Rabin, Y., Grosberg, A. Y. & Meller, A. Electrostatic
346 focusing of unlabelled DNA into nanoscale pores using a salt gradient. *Nat. Nanotechnol.*
347 **5**, 160–165 (2010).
- 348 26. Feng, J. *et al.* Identification of single nucleotides in MoS₂ nanopores. *Nat. Nanotechnol.*
349 **10**, 1070–1076 (2015).
- 350 27. Briggs, K. *et al.* DNA Translocations through Nanopores under Nanoscale
351 Preconfinement. *Nano Lett.* **18**, 660–668 (2018).
- 352 28. Maglia, G., Restrepo, M. R., Mikhailova, E. & Bayley, H. Enhanced translocation of
353 single DNA molecules through α -hemolysin nanopores by manipulation of internal charge.
354 *Proc. Natl. Acad. Sci.* **105**, 19720–19725 (2008).
- 355 29. Kasianowicz, J. J., Brandin, E., Branton, D. & Deamer, D. W. Characterization of
356 individual polynucleotide molecules using a membrane channel. *Proc. Natl. Acad. Sci.* **93**,

- 13770–13773 (1996).
30. Meller, A., Nivon, L. & Branton, D. Voltage-driven DNA translocations through a nanopore. *Phys. Rev. Lett.* **86**, 3435–3438 (2001).
 31. Balme, S. *et al.* Influence of Adsorption on Proteins and Amyloid Detection by Silicon Nitride Nanopore. *Langmuir* **32**, 8916–8925 (2016).
 32. Toyoda, Y. *et al.* Genome-scale single-cell mechanical phenotyping reveals disease-related genes involved in mitotic rounding. *Nat. Commun.* **8**, 1266 (2017).
 33. Klotzsch, E., Stiegler, J., Ben-Ishay, E. & Gaus, K. Do mechanical forces contribute to nanoscale membrane organisation in T cells? *Biochim. Biophys. Acta - Mol. Cell Res.* **1853**, 822–829 (2015).
 34. Karner, A. *et al.* Tuning membrane protein mobility by confinement into nanodomains. *Nat. Nanotechnol.* **12**, 260–266 (2017).
 35. Aramesh, M., Shimoni, O., Ostrikov, K., Prawer, S. & Cervenka, J. Surface charge effects in protein adsorption on nanodiamonds. *Nanoscale* **7**, 5726–36 (2015).
 36. Aramesh, M., Tran, P. A., Ostrikov, K. (Ken) & Prawer, S. Conformal nanocarbon coating of alumina nanocrystals for biosensing and bioimaging. *Carbon* **122**, 422–427 (2017).
 37. Konradi, R., Pidhatika, B., Mühlebach, A. & Textor, M. Poly-2-methyl-2-oxazoline: A peptide-like polymer for protein-repellent surfaces. *Langmuir* **24**, 613–616 (2008).
 38. Weydert, S. *et al.* Easy to Apply Polyoxazoline-Based Coating for Precise and Long-Term Control of Neural Patterns. *Langmuir* **33**, 8594–8605 (2017).
 39. Yusko, E. C. *et al.* Real-time shape approximation and fingerprinting of single proteins using a nanopore. *Nat. Nanotechnol.* **12**, 360–367 (2017).
 40. Blondel, Vincent D., Guillaume, Jean-Loup, Lambiotte, Renaud & Lefebvre, Etienne. Fast unfolding of communities in large networks. *J. Stat. Mech. Theory Exp.* **2008**, P10008+ (2008).
 41. Sakai, T. *et al.* Plasma fibronectin supports neuronal survival and reduces brain injury following transient focal cerebral ischemia but is not essential for skin-wound healing and hemostasis. *Nat. Med.* **7**, 324–330 (2001).
 42. Ruoslahti, E. Fibronectin and its Receptors. *Annu. Rev. Biochem.* **57**, 375–413 (1988).
 43. Gottlieb, P. A. in *Current Topics in Membranes* (ed. Gottlieb, P. A. B. T.-C. T. in M.) **79**, 1–36 (Academic Press, 2017).
 44. Guillaume-Gentil, O. *et al.* Force-controlled fluidic injection into single cell nuclei. *Small* **9**, 1904–1907 (2013).

- 390 45. Larkin, J., Henley, R. Y., Muthukumar, M., Rosenstein, J. K. & Wanunu, M. High-
391 Bandwidth Protein Analysis Using Solid-State Nanopores. *Biophysj* **106**, 696–704 (2014).
- 392 46. Wei, R., Gatterdam, V., Wieneke, R., Tampé, R. & Rant, U. Stochastic sensing of proteins
393 with receptor-modified solid-state nanopores. *Nat. Nanotechnol.* **7**, 257–263 (2012).
- 394 47. Bell, N. A. W. & Keyser, U. F. Digitally encoded DNA nanostructures for multiplexed,
395 single-molecule protein sensing with nanopores. *Nat. Nanotechnol.* **11**, 645–651 (2016).
- 396 48. Ren, R. *et al.* Nanopore extended field-effect transistor for selective single-molecule
397 biosensing. *Nat. Commun.* **8**, 586 (2017).
- 398 49. Guillaume-Gentil, O. *et al.* Tunable Single-Cell Extraction for Molecular Analyses. *Cell*
399 **166**, 506–517 (2016).
- 400 50. Misiunas, K., Ermann, N. & Keyser, U. F. QuipuNet: Convolutional Neural Network for
401 Single-Molecule Nanopore Sensing. *Nano Lett.* **18**, 4040–4045 (2018).

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Author Contributions

M.A. and J.V. designed the experiments. M.A., C.F., L.D-C., I.L., T.S., S.J.I., I.S. and V. H. performed the experiments. M.A., C.F. and S.J.I. performed the statistical analysis and coding with support from J.V. All the authors discussed the results and commented on the manuscript. M.A. wrote the manuscript with support from J.V.

Competing interests

The authors declare no competing financial interests.

Data availability

All data needed to evaluate the conclusions in the paper are present in the paper. The additional data and other findings of this study are available from the corresponding authors upon reasonable request.

Additional Information:

Supplementary information is available in the online version of the paper. Reprints and permission information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to M. Aramesh and J. Vörös.

447 **Supplementary Information**

448 A listing of the contents of each file supplied as Supporting Information:

449 S1- Experimental Section

450 S2- Supplementary figures and notes

451 2-1- Noise characterization

452 2-2- DNA confinement near-the-surface

453 2-3- Voltage dependence of protein (Fn) translocation

454 2-4- Protein discrimination based on charge and molecular weight

455 2-5- Fibronectin secretion from Fibroblast cells

456 2-6- Recording action potentials of neurons

457 2-7- Cell survival upon tip insertion

458 2-8- Current map of the nuclear membrane

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Figures:

Figure 1- Nanopore fabrication on the AFM cantilevers. (a) Shows a SEM image of the cantilever in an angle perpendicular to the long axis of the cantilever. (b) shows the cut-view of a pyramidal apex with the embedded microchannel. (c) shows flattening of the pyramid apex with Ga^+ irradiation, and the subsequent pore drilling with a focused Helium ion beam. (d) shows an example of the “ion-beam sculpting” process in which the pre-fabricated pore could be shrunk in diameter by raster scanning of He^+ ions across the pore. The scale bars are: 10 μm in (a), 1 μm in (b), 50 nm in (c) and 20 nm in (d).

Figure 2. Localized detection of proteins near the surface under mechanical confinement with a nanopore AFM. (a) shows the schematics of the experimental setup and the concept of protein confinement using mechanical forces. The approach of the tip to the surface creates a nano-confined area which produces an energy barrier for biomolecular translocation prior to entrance to the nanopore. (b) shows from left to right the I-V curve obtained when the tip is far from the surface ($R = 45 \text{ M}\Omega$, blue curve) and near the surface ($R \approx 78 \text{ M}\Omega$, red curve), the current change (ΔI_b) due to local blockage of the pore during Fibronectin (Fn) translocation, the frequency (f) and duration (τ) of the blockage events. Voltage-dependence of Fn translocation near-the-surface (set-point of 2 nN) exhibits an exponential decay ($\tau \sim e^{-0.003V}$) which is indicative of surface interactions with the biomolecules. The error bars indicate the standard deviations for each experiment. (c) The Kernel density estimate (KDE) plots of the events distribution with/without confinement. Tip-surface distance (controlled by the set-point force, SP in nN) influences the dynamics of the translocation, particularly evident from the translocation time (τ) and the standard deviation. n is number of the detected events at $+400 \text{ mV}$. The pore diameter (d_{pore}) was $\sim 20 \text{ nm}$.

Extracellular recording (protein secretion)

Figure 3. Extracellular recording from single cells. (a) shows a schematic of a polymer-coated nanopore AFM on top of a cell membrane (The schematic is not to scale and depicts the possibility of recording multiple channels). (b) A nanopore AFM is located on top of a fibroblast cell to simultaneously record topographical (AFM feedback) and ionic current maps (the scale bar is 1 μm , the scale height is 1 μm in the AFM image and 100 pA in the current image). (c) Localized detection of biomolecules and ionic fluxes on an arbitrary location on top of a fibroblast cell ($V = +100$ mV, $d_{\text{pore}} \sim 20$ nm). (d) The scattered plot obtained from sporadic events indicates occurrence of two significantly different types of events. Class I has a single peak profile while class II has double peaks.

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500 Figure 4. Extracellular recording from Mouse embryonic fibroblasts (MEFs) with/without Fibronectin (Fn)
501 expression. (a) Confocal images of fixed cells that express Fn (MEFs $Fn^{+/+}$) (top image) and fixed cells
502 that are knocked-out for Fn expression (MEFs $Fn^{-/-}$) (bottom image). The cell nuclei are stained in blue
503 with DAPI, and the Fn component of the ECM is stained in red with antibody immunostaining. The scale
504 bar is 10 μ m. (b) Exemplary density plots of the event distribution, comparing the secretome of the cells
505 with/without Fn expression. The current traces were recorded at +200 mV ($d_{pore} \sim 20$ nm). The increased
506 density of the events distribution in the KDE plot of $Fn^{+/+}$ is shown by a red circle, which is mostly due to
507 Fn expression, and it is comparable with the signals obtained in pure Fn solutions. (N.b. The small events
508 are excluded here.)

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Figure 5. (a) Confocal images of HEK cells with overexpressing Piezo1 ion channels (top) and HEK cells with knocked-out Piezo1 ion channels (bottom). The scale bar is 20 μm . The green color is the fluorescence of the GFP molecules that are specifically bound to the corresponding ion-channels, and the red color is the cell membrane tagged with Vybrant DiI Cell-labeling solution. The corresponding ion-current maps (integrated over 10 msec) from HEK cells with/without excess of the ion-channels shown on top and bottom, respectively (The left and right images were taken from different samples. The scale bar is 1 μm). The probability of observing “hot” regions (increased current changes) is higher for the cells with excess of ion-channels ($d_{\text{pore}} \sim 20 \text{ nm}$). (b) shows comparison at different applied voltages of the sporadic current events near the cell membrane for the HEK cells (with excess and lack of the ion-channels). The number of class II events (shown by percentage) is higher for the cells with more ion channels.

Intracellular recording (injecting, mapping and sensing)

(a) Probe insertion (simultaneous force and current feedback)

(b) Intracellular sensing

Figure 6. Intracellular recording with nanopore AFM. (a) Laser deflection from the AFM cantilever reveals mechanical engagement of the tip with different compartments of the cell. In a typical force-spectroscopy experiment, five distinct areas can be observed at sufficiently high set-point forces (typically > 300 nN). These areas are interpreted as: (I) the penetration of the tip through the cell and nuclear membranes, respectively, (II-III) the resting time at the set-point value and (IV) the retraction of the tip from the nucleus and cell membranes, respectively. The corresponding simultaneous current recorded during the tip insertion inside of the cell is shown. The reduction of the current between (I) and (II) is due the approach of the tip from the bulk to the surface of the cell membrane (i.e. increased resistance). Similarly, the current increases back to the initial value during the withdrawal stage (III) to (IV). (b) Demonstration of the intracellular monitoring of biomolecules inside a single cell (HEK). The current transients due to the nano-confined molecular translocations through the nanopore near the surface of the nuclear membrane are shown at two different voltages ($d_{\text{pore}} \sim 20$ nm, SP= 500 nN and $V = \pm 800$ mV, the red line is the detection threshold which can be decided arbitrarily). The density plots of the events are shown for the threshold value of 75 pA.