A Cell-Based Bar Code Reader for High-Throughput Screening of Ion Channel–Ligand Interactions

Jon Sinclair, Johan Pihl, Jessica Olofsson, Mattias Karlsson, Kent Jardemark, Daniel T. Chiu, and Owe Orwar*

Department of Physical Chemistry, and Microtechnology Centre, Chalmers University of Technology, SE-412 96 Göteborg, Sweden, Department of Chemistry, University of Washington, Seattle, Washington 98195-1700, and Department of Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden

This paper presents a microfluidics-patch clamp platform for performing high-throughput screening and rapid characterization of weak-affinity ion channel–ligand interactions. This platform integrates a microfluidic chip consisting of multiple channels entering an open volume with standard patch clamp equipment. The microfluidic chip is placed on a motorized scanning stage and the method relies on the ability to scan rapidly, on the order of milliseconds, a patch-clamped cell across discrete zones of different solutions created in the open volume. Under ideal conditions, this method has the capacity to obtain kinetically resolved patch clamp measurements and dose–response curves of up to $10^3$ ligand solutions in a single day.

The human genome codes for at least 406 different types of ion channels and recent crystallographic studies at 2.8Å resolution have gained new insights into the structure and mechanics of certain K⁺ channels. Since ion channels are important drug targets, it is of fundamental interest to develop methods that identify lead compounds from drug libraries with sufficient speed and accuracy for use in primary and secondary screening against ion channels. Traditional high-throughput screening (HTS) methods based on fluorescence measurements or raw binding suffer from many shortcomings because they are either indirect (such as fluorescence) or are carried out in a nonfunctional context (e.g., raw binding) and often provide inaccurate information on potential drug–target interactions. Binding of a compound (i.e., agonist or modulator) not only evokes conformational changes in the protein that lead to flux of ions across the cell membrane but also causes the ion channel to desensitize, that is, to reside in a long-lasting ligand-bound yet shut and nonconducting state. Both activation and desensitization of many types of ion channels occur within a few milliseconds. Thus, to efficiently study such rapid receptor interactions with high throughput, methods are required that can change a large number of solutions containing different ligands around cells in the millisecond time range.

Patch clamp is a superior detection technique for registration of ion channel activity and has the sensitivity to measure ionic currents passing through a single channel in the femtoampere (10⁻¹⁵ A) range with submillisecond time resolution. This technique also is ideally suited for monitoring the potential efficacy and affinity of drugs, because it provides a direct measurement on the activity of ion channels. The main drawback of patch clamp measurements, however, is the tedious nature by which measurements are made. This fact underlies current efforts in developing parallel patch clamp devices that aim to automate and multiplex the number of patch-clamped cells and thereby increase throughput in screening applications.

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We have taken a different approach toward developing a patch clamp-based HTS platform that also addresses the demand for rapid solution exchange around patch-clamped cells. Rather than using parallel patch clamp devices, we integrate a conventional single-cell-based patch clamp technique with microfluidic systems designed to provide rapid sequential delivery of ion channel agonists or antagonists onto the patch-clamped cell. Our system is based on high-speed scanning of a single patch-clamped cell across a laminar stream of different solution environments, which alternately contain ion channel ligands and pure buffer solution for washing the cell between each ligand exposure. To generate these conditions, we have fabricated a microfluidic chip with tightly packed microchannels exiting into an open bath volume. When working in the low-Reynolds number regime, the fluid streams will immediately viscously couple to each other upon exiting from the channels. In contrast to previous studies and applications of laminar flows in closed microchannels, we rely on the formation of laminar flows in open volumes, which has different requirements and results in characteristics different from closed-volume laminar flows. Since the flow in the open volume is confined by wall boundaries in only two directions, the flow will not only propagate forward but also sideways and upward. However, for our system design, the flow from the outer channels will act to support the flow from the inner channels, resulting in a flow propagating mainly forward and upward. Close to the channel outlets, a cross section of the stream can be considered to contain equally sized, discrete zones of different solution environments.

As a patch-clamped cell is scanned across different ligand zones, exposure times for the cell to each agonist can be as short as a few milliseconds, in which case the detection speed may be limited only by the inherent rapid channel gating kinetics. In essence, the system operates as a sequential bar code reader where the detection element is a single patch-clamped cell and the bars are represented by interdigitated columns of ligands and ligand-free buffer solutions.

In addition, this method enables very long recording times from a single cell, up to hours, as a result of increased stability of patch seals under the flow conditions we used. The microfluidic chip is scalable and can be made with 96 or 384 channels and containers to fit industry-standard microwell plates for drug storage and drug dispensing. We have been able to obtain more than 300 responses from individual ligand channels in ~30 min using a 32-channel chip, and conservative estimates are that compound libraries of 109–1010 weak-affinity ion channel ligands can be screened for in a single day. This represents an improvement of 1–2 orders of magnitude over current technologies. Furthermore, we demonstrate that dose–response curves can be obtained rapidly from which agonist/antagonist affinity and efficacy can be obtained.

100 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 11 mM EGTA, and 10 mM HEPES; pH was adjusted to 7.2 with KOH. All experiments were performed at room temperature (18–22 °C). Signals were recorded with an Axopatch 200 A (Axon Inc.) patch clamp amplifier, at a holding potential of −70 mV, and were digitized and stored on the computer hard drive (sample frequency 10 kHz, filter frequency 5 kHz using a four-pole Bessel filter) and analyzed using a PC and Clampfit 8.1 software (Axon Inc.). The experimental chamber containing the microchannel structure was mounted on an inverted microscope stage equipped with 40× and 10× objectives (Leica, DM IRB, Wetzlar, Germany). This equipment together with micromanipulators (Narishigi, Tokyo, Japan) was placed on a vibration-isolated table inside a Faraday cage. The patch clamp amplifier, the Digidata board, and the PC were kept outside the cage to minimize interference from line frequency.

The channel structure was loaded with HBS and agonist dissolved in HBS interdigitated in every second channel. The structure was placed on the microscope on a motorized scanning stage (Proscan, Prior Scientific Cambridge, U.K.) and interfaced to a syringe pump (CMA/100, microinjection pump, Carnegie Medicine, Cambridge, U.K.). The flow was driven by pressurized air corresponding to a flow velocity of 3 mm/s. PC-12 cells were patch-clamped in whole-cell configuration and placed in the HBS flow at ~20 µm from the channel outlets.

**Cell Culture.** Adherent PC-12 cells were cultivated on circular cover slips in Petri dishes for 4–8 days in (DM EM / F 12) medium supplemented with antibiotics and antimycotin (0.29%), fetal calf serum (10%), and ₯-glutamine. Before the patch clamp experiments, cells were washed and detached in a HEPES–saline buffer (HBS) containing (in mM) 10 HEPES, 140 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, and 10 g glucose (pH 7.4) and placed in the open-volume buffer reservoir at the outlet of the microchannel structure.

**Materials.** Four-inch, low-reflective blank soda lime masks coated with 1000 Å of chrome were obtained from Nanofilm (Westlake Village, CA). 3-in. CZ, [100], two sides polished, N-Ph 1–10 Ω-cm silicon wafers were from Topsil Semiconductor Materials A/S (Frederikssund, Denmark). Three-inch, 500µm-thick Corning No. 7740 wafers were purchased from Präzisions Glas & Optik GmbH (Iserlohn, Germany). Electron beam resists, photoresists, and developers were from Shipley (Shipley Co., L.L.C., Marlborough, MA). Other chemicals used in the chip fabrication were all in VLSI grade from Merck unless otherwise stated. All chemicals used in the patch clamp and fluidics experiments were purchased from Sigma-Aldrich Ltd. or Merck.

**Characterization of the Microfluidic System.** The chip was loaded with 100 mM fluorescein in every other channel and was placed directly on the stage of an inverted microscope (Leica DM IRB). Pressurized air corresponding to a flow velocity of 3 mm/s was applied. The bath chamber was filled with acidic buffer solution (pH 4) to quench the background fluorescence. The 488-nm line of an Ar⁺ laser (2025-05, Spectra-Physics Lasers Inc., Mountain View, CA) was used for epifluorescence illumination. To break the coherence and scatter the laser light, a spinning disk was placed in the beam path. The light was reflected off a polychromatic beam splitter and sent through a 5× objective (Leica PL Fluotar) to excite the fluorophores. The same objective collected and directed the fluorescence onto a CCD camera (C2400-41H, Hamamatsu Photonics K.K.) controlled by an Argus-20 image processor (Hamamatsu Photonics Norden AB, Solna, Sweden). Recordings were made using a Super VHS (Panasonic SVHS AG-5700, Stockholm, Sweden) Digital image editing was performed using the Argus-20 system, Adobe Premiere, Adobe Photoshop graphic software.

**Safety Considerations.** Microfabrication of the silicon chips developed in this study involves chemical and plasma etching as well as electron beam and photolithography. Such processing should be performed by trained personnel, and necessary safety precautions should be followed.

**RESULTS AND DISCUSSION**

**Design of the Microfluidic Chip.** Figure 1A shows a schematic drawing of a microfluidic chip used in combination with patch clamp recordings together with a programmable scanning microscope stage for device translation. Panels B and C of Figure 1 show a photograph of a prototype hybrid microfluidic chip fabricated in silicon/glass/polydimethylsiloxane and a SEM image of the microchannels (50 µm wide × 100 µm high) entering the open-volume recording chamber, respectively. To perform patch clamp measurements using this microfluidic system, the chip was first mounted on a motorized scanning stage to allow controlled and rapid movement of the channel outlets relative to the patch-clamped cell. Fluid was then driven through the channels using pressurized air applied through a retractable pump head. By using a microfluidic device operating in the low-Reynolds number regime and by tightly packing the microchannels together, the fluid streams immediately coupled with each other upon exiting from the channel outlets into the open volume. This coupling created one laminar liquid stream containing different solution environments in the interfacial region between the channel outlets and the open volume. This behavior is illustrated for a 32-channel device in Figure 1D, which shows a fluorescence image of the multiple streams entering the open volume and consists of alternating bands of fluorescein and fluorescein-free solutions. The collimation distance of the fluid streams was proportional to the applied pressure and inversely proportional to the square root of the diffusion coefficients of the substances contained within the stream. When a cell sensor is scanned across microchannel outlets in a direction perpendicular to the channels, it moves in to and out of discrete zones of different solution environments following approximately a step function. Thus, the system operates as a sequential bar code reader where the detection element is a single patch-clamped cell and the bars are represented by interdigitated columns of ligands and ligand-free buffer solutions.

**Effect of the Flow on Patch-Clamped Cells.** The fluid streams leaving the outlets of the channels as well as the translation of the patched cell perpendicular to the direction of fluid flow generates a drag force on the patch-clamped cell. This force can be readily calculated from the equation for Stokes flow past a sphere, F = 6πrνγ, where F is the force, r the radius of the spherical cell, γ the viscosity of the fluid, and ν the velocity of the fluid relative to the cell. When a cell is scanned across the fluid

stream while the pipet is held with its longitudinal axis in the direction of the fluid flow, this drag force can be divided into two orthogonal directions (Figure 1A). The force along the first direction is created by the scanning movement and acts along the direction of the scan. Experiment shows that this force does not deflect the cell even for scan rates as high as 4 mm/s. The second force is generated by the flow from the channel outlets and acts in the direction of the fluid flow. A flow rate of 2 mm/s past a 5-μm-radius spherical cell in an aqueous medium with a viscosity of 10⁻³ N s/m² will result in a force of approximately 200 pN. In comparison, the sedimentation force acting on the same cell is 0.4 pN. Remarkably, it was found that this force, pushing the cell toward the seating surface of the pipet, effectively stabilizes the cell–pipet seal allowing patch clamp measurements to be performed for prolonged periods. We could routinely keep cells in the whole-cell configuration in excess of 40 min and sometimes as long as 110 min. This is a marked improvement when compared to stability times in traditional patch clamp of a free-hanging cell under no applied external forces, which typically is in the range of 10–20 min in our hands. This increased mechanical seal stability also often results in increased electrical seal resistances.

**Patch Clamp Measurements on the Chip.** Initial patch clamp screening experiments were performed using a microfluidic device with seven microchannels to investigate the behavior of the patched cell under different constant-velocity scanning conditions. As detectors, we employed patch-clamped PC-12 cells expressing rapidly desensitizing nicotinic acetylcholine receptors (nAChR). To date, 11 members of the neuronal nAChR family have been identified and cloned from vertebrate genomes.¹⁶ The physiological and pharmacological properties of nAChR responses, including activation and desensitization, depend on both the α (α₁–α₉) and the β (β₂–β₄) subunits and on the degree of phosphorylation.²⁰ The time constant for the fast desensitization of the nAChR with saturating concentration of agonists is ~50 ms,²⁰ and the time constant for recovery from desensitization is ~800 ms.²¹ A patch-clamped PC-12 cell held in the whole-cell configuration was scanned at constant velocity at five different

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outlets with cell was scanned nonlinearly, i.e., rapidly across agonist channel second channel interdigitated by HBS buffer to wash the cell. The microfluidic device loaded with 1 mM acetylcholine (ACh) in every peak responses from a single cell in channels 1, 3, 5, and 7 contained HBS buffer. (F) A recording of 165 was used where channels 2, 4, and 6 contained ACh (1 mM) and constant scanning velocity. A microfluidic chip with seven channels about micromanipulator at a distance of channel outlets (Figure 2A), and even at the highest scanning rate (Figure 2G). Interestingly, this slow rate of response attenuation between receptor activations, indicating that the wash times were too short. Simultaneously, higher scan rates with shorter exposure times resulted in a lesser degree of receptor desensitization and a correspondingly smaller variance in peak amplitudes (Figure 2B). For the nAChR system, therefore, we found it is optimal to use a nonlinear scanning program by specifically lowering \( t_{\text{exp}} \) at ligand channel outlets and increasing \( t_{\text{exp}} \) at buffer channel outlets. A 32-channel microfluidic device was used with \( t_{\text{exp}} \) ~600 ms at ligand channel outlets and \( t_{\text{exp}} \) ~1.5 s at buffer channel outlets. Out of the 32 channels, 15 were loaded with 1 mM ACh. Every other channel (17 in total) contained HBS solution to intermittently resensitize the receptors. A whole-cell patch-clamped PC-12 cell was scanned across the channel outlets. Figure 2F shows recordings from 11 forward and backward scans resulting in 165 current responses from one cell in ~9 min. M over, the amplitude of the current responses decreased only by 24% at a rate of ~2.7 pA/min between the first and the eleventh scan (Figure 2G). Interestingly, this slow rate of response attenuation represents roughly the loss of only one nAChR/min. We have been able to extract >300 responses from individual ligand channels using a single patch-clamped cell in ~30 min. The device was also used for screening of three different nAChR agonists, nicotine (1mM), charbachylophiline (1mM), and ACh (1mM). Figure 2H shows current responses from forward and backward scans, where more than 70 responses were obtained in a single recording.

**Dose—Response Curves and \( EC_{50} \) Values Generated from a Simple Search Protocol.** The microfluidic patch clamp ap-

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Figure 2. (A–E) Patch clamp inward currents recorded at a constant scanning velocity. A microfluidic chip with seven channels was used where channels 2, 4, and 6 contained ACh (1 mM) and channels 1, 3, 5, and 7 contained HBS buffer. (F) A recording of 165 peak responses from a single cell in ~9 min using a 32-channel microfluidic device loaded with 1 mM acetylcholine (ACh) in every second channel interdigitated by HBS buffer to wash the cell. The cell was scanned nonlinearly, i.e., rapidly across agonist channel outlets with \( t_{\text{exp}} = 633.3 \pm 72.18 \) ms and slowly across HBS-containing microchannel outlets \( t_{\text{exp}} = 1.5 \pm 0.27 \) s. The scan was performed back and forth 15 times. The time between each scan was 9.9 \pm 2.9 s. The channel flow rate was 3 mm/s. (G) A plot of the mean peak current and standard deviation versus time of each scan in the 165-response trace in (F). Amplitude decreases 24% over the duration of the experiment at a rate of ~2.66 pA/min obtained from linear curve fit. (H) Screening with different agonists. The device was loaded with four channels containing 1 mM nicotine (Nic), four channels containing 1mM charbachylophiline (CCh), and one channel containing 1 mM ACh interdigitated by channels containing HBS. The cell was scanned eight times back and forth at increasing scan rates. The last trace was obtained at \( v_{\text{scan}} = 3.6 \) mm/s. All patch clamp data were recorded using PC-12 cells in the whole-cell configuration. The holding potential was ~70 mV, and the traces were digitized at 10 kHz and filtered at 0.2 kHz.

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proach also facilitates rapid dose–response measurements in determining agonist efficacies and EC\textsubscript{50} values, as well as in the characterization of antagonist actions and IC\textsubscript{50} values. By having the ability to screen over a large concentration range, greater than 10 orders of magnitude, a complete characterization of the concentration sensitivity of a receptor can be obtained rapidly. Using a 32-channel device, we obtained concentration-dependent whole-cell patch clamp current responses to ACh in the concentration range of 10\textsuperscript{-3}–10\textsuperscript{-10} M in steps of one decade (Figure 3 A). The cell started to respond to 10 \mu M ACh, and a fully saturated response was obtained at 1 mM. From this screen over eight decades, the search interval was thus narrowed down to four decades for finding the EC\textsubscript{50} value for the receptor and a concentration interval from 1 \mu M to 1 mM in steps of 9–500 \mu M was used (Figure 3B). A dose–response curve was generated from these data (Figure 3C), yielding an EC\textsubscript{50} value of 58 ± 2 \mu M, which is in accordance with previous data from characterization studies of nAChRs expressed in PC-12 cells.\textsuperscript{23} The search sequence is generic for finding EC\textsubscript{50} and IC\textsubscript{50} values, and a consecutive patch clamp experiment (over two different concentration ranges) for finding the EC\textsubscript{50} value for ACh on PC-12 cells took less than 0.5 h to perform.

CONCLUSIONS

This paper demonstrated a microfluidic method for rapidly and sequentially changing the local aqueous solution environment around patch-clamped single-cell biosensors. This method is suitable for high-throughput screening of weak-affinity receptor agonists, receptor antagonists, receptor modulators, or other bioactive molecules, such as drugs and pharmaceutically active substances. The programmable sequence of presentation of different agents and buffer solutions to the biosensors yields high screening rates that are applicable to competitive assays, and to dose–response characterizations from which agonist/antagonist affinity and efficacy as well as receptor blockage mechanisms can be obtained. This microfluidic platform is also suitable for kinetically resolved measurements of ion channel activation and desensitization.

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