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Chapter 21

Automated Planar Patch-Clamp Recording of P2X Receptors

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Abstract

P2X receptors are a structurally and functionally distinctive family of ligand-gated ion channels that play important roles in mediating extracellular adenosine 5'-triphosphate (ATP) signalling in diverse physiological and pathophysiological processes. For several decades, the 'manual' patch-clamp technique was regarded as the gold standard assay for investigating ion channel properties. More recently, breakthroughs in the development of automated patch-clamp technologies are enabling the study of ion channels, with much greater throughput capacities. These automated platforms, of which there are many, generate consistent, reliable, high fidelity data. This chapter demonstrate the versatility of one of these technologies for ligand-gated ion channels, with particular emphasis on

protocols that addresses some of the issues of receptor desensitization that are commonly associated with P2X receptor-mediated currents.

Key Words

Automated electrophysiology, Planar patch-clamp, Planar chip, Micro-fluidics, Stacked solution application, Ligand-gated ion channels, P2XR, Voltage-clamp.

1. Introduction

P2X receptors (P2XRs) belong to the ligand-gated ion channel superfamily with distinctive structural and functional properties [1]. In the mammalian system, there are seven different receptor subunits ($P2X_1R$-$P2X_7R$), each of which contains intracellular N- and C-termini and two transmembrane segments linked by a large extracellular domain. P2XR subunits assemble into homo- or hetero-trimeric complexes to form functional channels. The second transmembrane domain from each of the three subunits, in the complex, forms the ion-permeating pathway [2-3]. Extracellular ATP binding specifically activates P2XRs leading to variable permeation of small inorganic cations such as Ca^{2+}, Na^+ and K^+, with the exception of the human $P2X_5R$, which also conducts Cl^- ions [4]. There are three phases associated with gating of P2XRs: the ATP-evoked activation or rising phase that induces a rapid current, the desensitization or decay phase that occurs in the presence of ATP and develops slowly, and a relatively quick deactivation phase elicited by removal of the agonist. The kinetics of these three phases vary considerably among the different P2XR subtypes [5]. In addition, recurrent application of ATP, results in the attenuation of current responses [6]. P2XRs exhibit widespread expression patterns in neuronal and non-neuronal tissue, where they play important roles in mediating a diverse range of physiological functions [2, 7]. While there is evidence for localization in intracellular organelles such as

lysosome, P2XRs are predominantly expressed within the plasma membranes or on the cell surface [8, 9]. A large body of evidence supports the involvement of P2XRs in a number of human pathologies and diseases [2, 7, 10], identifying them as attractive therapeutic targets for precision medicine [11].

The manual patch-clamp technique was developed as the benchmark to measure ionic currents flowing through open channel pores [12-13], thereby enabling the investigation of the biophysical properties of ion channels, testing of therapeutic compounds and examination of mechanisms of action. The manual patch-clamp recording technique has certain limitations, in that extensive technical training is required, and data acquisition is generally low, necessitating a large time investment. Automated technologies have been extensively used for pharmaceutical drug discovery for some time now and as research groups combine resources, automated patch-clamp systems are becoming a more commonplace feature in academic laboratories. Numerous first and second generation platforms have been manufactured over the last two decades, including: QPatch HTX, QPatch II, Qube 384 (Sophion A/S, Copenhagen); PatchXpress® 7000A, IonWorks® Quattro, IonWorks Barracuda™ (Molecular Devices, LLC); NPC-16 Patchliner®, SyncroPatch® 96 SyncroPatch® 384PE (Nanion Technologies GmbH, Munich); CytoPatch™ (Cytocentrics AG, Rostock); Dyna flow® HT (Cellectricon AB, Mölndal); and IonFlux HT (Fluxion Bioscience Inc. USA). Data output from these platforms, is generally highly comparable to the high fidelity data acquired using the manual patch-clamping technique [14]. Here we describe protocols using the first generation platform Patchliner, as an example, to measure agonist-induced P2XR-mediated whole-cell currents from stably transfected or transiently transfected cells. For most automated patch-clamp systems on the market it is challenging to use transiently transfected cells, because of the

blind approach of capturing cells. Interestingly, robust currents from HEK293 cells transiently expressing $P2X_4$Rs were recorded using the Patchliner [15]. Patchliner has built-in robotic, microfluidic, liquid handling capabilities [16], allowing the complete application and washout of agonist in a millisecond time frame [17], making it an ideal platform for measuring rapidly activating, desensitizing, ligand-gated currents. It should, however, be noted that many of the features and notes given in this chapter can be applied to other automated systems, similarly.

2. Materials

2.1 Reagents and Cells

1. Human embryonic kidney (HEK) 293 host cells, HEK293 cells stably expressing the human $P2X_7$ receptor ($hP2X_7R$) and human astrocytoma cells (1321N1) stably expressing $P2X_2R$ and $P2X_3R$ subunits ($P2X_{2/3}R$).
2. HEK293 cell culture media: Dulbecco's Modified Eagles Medium (DMEM), 2 mM L-glutamate and 10% fetal bovine serum (FBS).
3. DMEM/F-12 complete media: DMEM/F-12 liquid, 10% FBS, 2 mM L-glutamate, 10% FBS.
4. 1321N1 cell culture media: DMEM, 10% FBS, 50 U/mL penicillin and 50 μg/mL streptomycin.
5. D-PBS: Dulbecco's phosphate buffer saline Ca^{2+} and Mg^{2+} free.
6. Accutase® cell detachment solution (Invitrogen) or similar.
7. Opti-MEM I reduced serum media (Invitrogen) or similar.
8. Transfection reagent, Lipofectamine 2000 transfection (Invitrogen) or similar.

9. DNA plasmids: empty plasmid vector for mammalian expression of GFP, full-length $P2X_4$-WT-AcGFP (WT), mutant $P2X_4$-G135S (G135S) constructs. DNA contructs were generated in-house.
10. 100 mM Adenosine triphosphate (ATP) and its analogues (e.g. BzATP) in water as stock solutions. Adjust ATP stock solution to pH7.3 with NaOH. Aliquot ATP and BzATP stock solutions in small volumes, and store at -20 ^{o}C. Prepare working solutions with desired concentration by diluting the stock solution in extracellular recording solution.

2.2 Equipment

1. Vented flasks for cell culture (T25). Conical centrifuge tubes (15 mL). Microcentrifuge tubes 1.5 mL.
2. Pipettes and tips 10-, 20-, 200-, and 1000 μL and Easypet pipetting aid and Maxitips 5 mL.
3. High speed bench top centrifuge.
4. Countess^{TM} automated cell counter (ThermFisher Scientifc) or a haemocytometer and trypan blue.
5. NPC®-16 Patchliner Probe Selector/Quattro/Octo (see **Note 1**), PatchControl^{HT} software, single-hole (Fig. 1a), 4-hole ensemble (Fig. 1b) or 8-hole ensemble NPC®-16 chips (see **Note 2**). NPC®-16 electrode set (see **Note 3**) (Nanion Technologies GmbH).
6. Patch-clamp amplifiers (PATCHMASTER, HEKA Instruments) (see **note 4**).
7. Computer with 24-inch thin film transistor monitor (see **Note 5**).
8. Multi-channel stimulation/acquisition software with programmable experiment control and automation (HEKA Instruments) and analysis packages (such as Microsoft

Excel, MatLab R2018a), Igor Pro 6.37 (WaveMetrics Inc.), Adobe Illustrator CS5.1 (Adobe Systems) and GraphPad Prism 7 (Molecular Devices).

9. 50 mL syringe with 0.22-μm-pore diameter filter.
10. Bench top pH metre.
11. Advanced Instruments Osmo1, single sample micro-osmometer.

2.3. Planar Patch-Clamp Solutions

Prepare solutions with deionized water, filter (see **Note 6**) and measure the osmolarity (see **Note 7**) and the pH (see **Note 8**). Solutions can be stored at 4 °C for up to 5 days. Solutions should be warmed to room temperature (20–22 °C) before use. The built-in robotic pipette manages all liquid handling requirements. Solutions are aspirated from the preprogrammed positions on the workstation and dispensed into the appropriate chip chamber. The pipette is capable of aspirating and dispensing a single solution (Fig. 1a, b), double stacked (Fig. 1c) or triple stacked solutions [17], the later allowing the application of a channel modulator prior to agonist.

1. Standard extracellular solution for recording with HEK293 cells stably expressing hP2X$_7$Rs: 147 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 13 mM D-glucose, 10 mM HEPES (pH 7.3 with NaOH; ~298 mOsm).
2. Standard extracellular solution for recording with HEK293 cells transiently expressing P2X$_4$Rs: 145 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 13 mM D-glucose, 10 mM HEPES (pH 7.4 with NaOH; ~298 mOsm).
3. Standard extracellular solution for recording with 1321N1 cells stably expressing P2X$_{2/3}$Rs: 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM D-glucose, 10 mM HEPES (pH 7.4 with NaOH; ~298 mOsm).

4. Standard extracellular solution for enhancing seals: 80 mM NaCl, 3 mM KCl, 10 mM $MgCl_2$, 35 mM $CaCl_2$, 10 mM HEPES (pH 7.4 with HCl) (see **Note 9**).
5. Standard intracellular solution for whole-cell recordings with HEK293 cells stably expressing $hP2X_7Rs$: 85 mM NaCl, 60 mM NaF, 10 mM EGTA, 10 mM HEPES (pH 7.2 with NaOH; ~285 mOsm).
6. Standard intracellular solution for whole-cell recordings with HEK293 cells transiently expressing $P2X_4Rs$: 85 mM NaCl, 60 mM NaF, 10 mM EGTA, 10 mM HEPES (pH 7.2 with NaOH; ~285 mOsm) (see **Note 10**).
7. Standard intracellular solution for whole-cell recordings with 1321N1 cells stably expressing $P2X_{2/3}Rs$: 110 mM KF, 10 mM KCl, 10 mM EGTA, 10 mM HEPES (pH 7.2 with KOH; ~285 mOsm).

3. Methods

3.1 Cell Culture and Transient Transfection

The following procedures should be conducted in a sterile tissue culture fume hood.

1. Culture HEK293 host cells in standard T25 tissue culture flasks in HEK293 cell culture media in humid 37 ^oC, 5% CO_2 tissue culture incubator, until 60% confluent.
2. Culture HEK293 cells, stably expressing $hP2X_7R$, in standard T25 tissue culture flasks in DMEM/F-12 complete media and incubate in a humid 37 ^oC, 5% CO_2 tissue culture incubator, until sub-confluent (70-80%).
3. Culture 1321N1 cells, stably expressing $P2X_{2/3}R$, in standard T25 tissue culture flasks in 1321N1 cell culture media at 37 ^oC in a humidified atmosphere composed of 95% air and 5% CO_2, until sub-confluent (70-80%).
4. Cells should be passaged every 2-3 days using Accutase® cell detachment solution and confluency should be sub-confluent (60-80%) (see **Note 11**).

5. For each transfection, dilute 3 μg of selected cDNA:pAcGFP-N1 empty vector (Mock); $P2X_4$-WT-AcGFP wild-type (WT) or $P2X_4$-G135S (G135S) with Opti-MEM I reduced serum media (total volume 200 μL) in a 1.5 mL tube and in a second tube, add Lipofectamine 2000 Reagent (30 μL) with Opti-MEM I reduced serum media (170 μL), mix contents of each tube thoroughly by gentle pipetting.
6. Incubate both tubes at room temperature for 5 min.
7. Combine contents of the two tubes, mix thoroughly by gentle pipetting and incubate at room temperature for 20 min.
8. Add this transfection media to HEK293 host cells cultured to 60% confluency in a standard T25 flask and return to the incubator.
9. After 24 hours replace the transfection media with normal culture media and return to the incubator for a further 24 hours (see **Note 12**).

3.2 Harvesting Cells for Planar Patch-Clamp

A critical feature determining the success rate of planar patch-clamp recordings is that the healthy cells are maintained at the optimum sub-confluency. The process of capturing cells is completely random, so unlike conventional patch-clamp recording, it is not possible to visually select the healthiest looking cell. In addition, the quality of the seal formed between the cell membrane and the planar chip, which ultimately influences the quality of the recording, is reliant on the health of the cell.

1. Discard the media from the culture flask and, using 5 mL D-PBS, gently wash the cells twice.
2. Discard the D-PBS and dissociate with 0.5 mL pre-warmed Accutase cell detachment solution and gently tilt the flask from side to side to cover all the cells. Incubate for 3 min in a humid 37 ^{o}C, 5% CO_2 tissue culture incubator (see **Note 13**).

3. To neutralize the Accutase, add 5 mL culture media and pipette up and down gently in order to lift and separate the cells.
4. Transfer the cell suspension to a 15 mL conical centrifuge tube and centrifuge at 180 $x\ g$ for 2 min at room temperature and then discard the supernatant by decanting (see **Note 14**).
5. Resuspend the cell pellet by gently pipetting in a mixture of extracellular recording solution and culture media (50:50 ratio, see **Note 15**) at a density of 1 x 10^6 to 5 x 10^7 mL (see **Note 16**). Cells can be counted using a Countess automated cell counter, although a standard hemocytometer is quite adequate.
6. Transfer the cells, in suspension, to the cell hotel (see **Note 17**).

3.3 Automated Planar Patch-clamp using Patchliner

The Patchliner software (PatchControl^{HT}) is coupled to the HEKA amplifier software (Patchmaster), via the optical PCI card in the computer, and when PatchControl^{HT} is opened, Patchmaster opens automatically. This allows the experimental protocols (PatchControl^{HT} Trees), programmed in PatchControl^{HT}, to communicate with the amplifiers. Pre-programmed Trees can be loaded and modified for optimisation with different cell types/characteristics. Selection of appropriate chip cartridges, with the resistance suitable for cells of a particular size, is important (see **Note 18**). The motorized stage on the workstation of the Patchliner ('chip-wagon') has the capacity to hold three chip cartridges and each cartridge allows data acquisition from sixteen cells (eight at any given time), hence allowing for 48 recordings without operator intervention. Chip cartridges are embedded with microfluidic chambers and when a PatchControl^{HT} Tree is activated, the pipetting robot dispenses appropriate recording solutions into the microfluidic chambers of the chip and the cartridge is moved into the measuring head,

which contains the pneumatic and electric contacts and moves up and down to address the chip cartridges. The recording head houses eight headstages, allowing acquisition of data from eight cells simultaneously. Once the intracellular and extracellular solutions have been dispensed, a slight positive pressure is applied to each chip chamber, independently, and the offsets are corrected. Cells from the cell hotel are dispensed into the extracellular chamber and a small suction (-50 mBar) is applied, to attract cells onto each of the eight individual chip apertures, leading to a small increase in the seal resistance. Seal enhancing solution is then added and further suction pulses applied, together with application of negative voltage to aid in the formation of a gigaohm seals. The seal enhancing solution is then replaced with recording solution before additional short suction pulses are applied to achieve the whole-cell access. In some circumstances, it may be necessary to support this process using the zap function to encourage the patch of membrane to rupture (see **Note 19**). The pressure applied during this process is controlled by PatchControl^{HT} parameter settings (e.g. chip resistance, series resistance and slow capacitance), that the user can adjust according to the cell type/characteristics. It is also possible to adjust quality control parameters (e.g. seal resistance, series resistance), so cells that do not meet the specifications are disabled at this stage. Once the whole-cell configuration has been established, the experimental part of the protocol will commence.

1. Load a pre-programmed PatchControl^{HT} ligand Tree (File → load → Tree) and select the edit mode (☒ Edit) to make modifications according to cell/channel/receptor type/characteristics, and experimental paradigm (see **Note 20**).
2. Select chips with the desired resistance and format for cells/receptors and place three chips onto the chip-wagon.
3. Prepare compound solutions directly before each experiment (see **Note 21**).

4. Place recording solutions and compounds in position, according to those defined in the joblist (see **Note 22**).
5. Place the cells into the cell hotel, where they will be aspirated every 30 s throughout the experiment to prevent clumping and sedimentation (see **Note 23**).
6. Select and activate the initialization folder to initialize the robot and wash the pipette. This also generates a new data file within Patchmaster and sets all amplifier and robot parameters to default starting values. This folder only needs to be activated once at the beginning of each day of experiments.
7. The robot will start when the Tree is activated. At the end of each run, it will loop back to the start and continue this process until all chips on the chip-wagon have been used.

Illustrative automated planar patch-clamp recordings from HEK293 cells stably expressing the WT hP2X$_7$R (Fig. 2). Cells were captured on single-hole chips and voltage-clamped at a holding potential of -60 mV while a continuous recording performed data acquisition. BzATP, applied at a rate of 86 μL/s (speed 15), activated inward currents in a concentration-dependent manner. No desensitization was observed in the continued presence of BzATP. These recordings did not utilise a stacked solution application, ligand was applied independently of the external solution.

3.4 Stacked Solution Application

P2XRs exhibit receptor desensitization [5], which is a common characteristic feature of ligand-gated ion channels. The kinetics and level of desensitization of ligand-gated ion channels are determined by ligand concentration and exposure time, or both. For rapidly desensitizing ion channels, it is important that compound application is rapid and short-lived, so that the entire ion channel population is exposed to maximum concentration before entering the desensitized state. Therefore rapid solution exchange combined with

brief drug exposure times can minimise or correct for the deleterious effect caused by receptor desensitisation. This is achieved by using a stacked solution application, whereby two or three zones of solution are aspirated into the pipette before they are dispensed into the chamber, where they have brief and rapid contact with the cell/s (Fig. 1, see **Note 24**).

1. Load a pre-programmed ligand Tree for double stacked solution application. Volumes and speeds of applications can be adjusted (see **Note 25**). In addition, if required, temperature can also be controlled (see **Note 26**).
2. Select medium resistance chips (2-4 MΩ). For HEK293 stably expressing $P2X_4$Rs, single-hole chips were utilized and for 1321N1 cells stably expressing $P2X_{2/3}$Rs 4- or 8-hole ensemble chips were selected. Load three chips onto the chip-wagon.
3. Within the Tree, adjust the holding potential and the required speed of drug/wash delivery. The joblist will contain a continuous recording protocol, for fast ligand activated currents. The duration of the continuous recording can be modified in the Patchmaster pulse generator file.
4. Follow steps 3-6 in section 3.3.

In the example shown in figure 3a, HEK293 cells transiently expressing AcGFP vector, WT $P2X_4$-AcGFP, or $P2X_4$-G135S-AcGFP were voltage-clamped at a holding potential of -50 mV, utilizing single-hole chips. 100 μM ATP applied for 700 ms, rapidly activated robust inward current for WT and mutant h$P2X_4$R, which desensitized slowly in the presence of ATP. No currents were elicited for cells transfected with mock vector control alone, which is reflected in the mean peak current amplitudes (Fig. 3b).

Figure 4 shows the reproducibility of responses to repetitive activation of robust inward currents, from 1321N1 cells stably expressing the $P2X_{2/3}$R, in response to 30 μM ATP applied using a stacked application protocol. The time course demonstrates, very elegantly,

that there is little effect on the peak current amplitude in response to brief repetitive application of agonist.

Representative current responses of an individual cell expressing $P2X_{2/3}$Rs to increasing concentrations of ATP are shown in Figure 5a. The currents exhibit a slow desensitization phase in the continued, but brief, presence of 30 μM ATP. The concentration response curve revealed an EC_{50} for ATP activation of 7.8 ± 1.0 μM (Fig. 5b). $P2X_{2/3}$Rs could be repetitively activated by 30 μM ATP and blocked by suramin (Fig 5c) in a dose-dependent manner. A full concentration response curve to suramin was performed, generating an IC_{50} of 28.0 ± 5.3 μM (Fig. 5d). To achieve short exposure times, solutions were stacked in the robotic pipette. First, wash solution (155 μL) was aspirated, followed by aspiration of the agonist-containing solution (40 μL) and then application to the cell at a speed of 57 μL/s. The cells were pre-incubated with suramin before co-application with 30 μM ATP.

4. Notes

1. NPC®-16 Patchliner Quattro or Octo are being used here, as an example of a validated automated patch-clamp system, but it should be noted that other systems have comparable capabilities.
2. NPC®-16 chips (single use, disposable) are manufactured with different specifications. In general, chips are manufactured with three different megaohm (MΩ) resistance ranges: low (1–2 MΩ), medium (2–4 MΩ) and high (5–6 MΩ). It is also possible to have bespoke chips manufactured according to the specific requirements of the user and/or cell type. Single-hole chips are ideal for use with voltage-gated channels. For ligand-gated channels, ensemble chips are generally preferred, especially if currents are small in amplitude. Ensemble chips are manufactured with either four or eight holes per chip, which is ideal for enhancing current size, because the currents are summated.
3. Electrodes need to be chloridated in bleach filled chambers for 30-60 min and then rinsed with deionized water and air dried before use.
4. Patchliner Quattro and Octo, use either EPC-10 USB Quadro multi-headstage patch-clamp amplifiers or the EPS 16 Probe Selector headstage multiplexer, combined with an EPC 10 Plus amplifier (HEKA Instruments).
5. Any brand of computer can be purchased, but minimum specifications include Windows 10 (Microsoft) with a 64-bit operating system and an optical PCI card for communication with the HEKA amplifiers.
6. Sterile filter all recording solutions using a 50 mL syringe with a 0.22-μm-pore diameter filter attached. This is particularly important for the internal recording solution.

7. Measure the osmolarity of all recording solutions using a freezing-point osmometer. The internal solutions should measure ~285 mOsm/L and the external solutions should measure ~298 mOsm/L. The osmolarity of external solutions should always be higher than the osmolarity of the internal solution.
8. When pH adjusted stock solutions (1 M), of each salt, are used to make the final recording solutions, then the osmolarity should not require further adjustment.
9. A high Ca^{2+}-containing external solution helps in the formation of a strong seal between the cell membrane and the planar chip. This seal enhancing solution is replaced once a gigaohm seal is achieved and before establishing whole-cell access. Calcium in the extracellular solution can be replaced by barium as charge carrier, to avoid calcium-dependant inactivation of voltage-gated calcium channels and increase calcium channel currents, since most voltage-gated calcium channels also conduct barium ions [18-19].
10. Fluoride ions in the intracellular solution improve gigaohm seal formation and stabilise the cell membrane, which in turn results in longer, more stable recordings [20]. The mechanism of this effect is poorly understood.
11. To avoid cells growing in clusters or adhering too tightly to the support substrate, it is vital that they are passaged every 2-3 days. If cells are allowed to grow to a confluency greater than 80%, aggregates commonly form, which in turn lead to lower capture rates, poor seal formation and difficulties breaking into the whole-cell configuration. Furthermore, dissociation buffers encourage cell separation and isolation.
12. A Lipofectamine 2000 Reagent protocol, available at (https://www.ecu.edu/cs-dhs/biochemistry/upload/Transfection-Protocol.pdf), was used to transiently transfect the cells. It should be noted that other transfection kits are also suitable for transient expression of ion channel proteins.

13. Accutase® cell detachment solution gently dissociates mammalian cells from support substrates and from each other. It should be stored at 4^{o}C and used at room temperature in sterile conditions.
14. To prevent shearing of the cell membranes during centrifugation, it is recommended to set the centrifuge acceleration speed to 6 and deceleration speed to 3. The value 0 is equivalent to the lowest acceleration and the value 9 is equivalent to the highest acceleration. The value 9 is equivalent to the shortest possible brake time and the value 0 to longest possible brake time.
15. When cells are resuspended in a mixture of recording solution and culture media (50:50), their bench life is greatly improved, and they remain viable for up to 4 h at room temperature.
16. A standard density of 1 x 10^{6}-5 x 10^{7} cells/mL, for most cell types, works well for use on the Patchliner. When working with primary cells, it is often challenging to harvest such large numbers of cells. It is possible to maintain a good cell capture rate with as few as 1000 cells/mL [21].
17. Cells are housed in the 'cell hotel', where they are kept from clumping and sedimenting by gentle automated pipetting, which improves cell viability. The user can set the pipetting volume and speed.
18. The capacitance of the cell, determines the size of the aperture (chip resistance) required. As an example, medium resistance chip are ideal for use with HEK293 cells and CHO cells [16, 22] and high resistance chips are ideal for use with mouse osteoblasts [23]. For example, if cells are very small, they will pass through a low resistance chip hole when suction is applied, and conversely, large cells will not form good seals on high resistance chips.

19. If it is difficult to establish the whole-cell configuration, following seal formation, then try harvesting the cells one day after plating. In addition, adjust the size of the high voltage pulses to 600–800 mV ('zap'), which can be applied to help rupture the patch of membrane, thus establishing the whole-cell configuration.
20. PatchControl^{HT} comes with a range of pre-programmed protocols ('Trees'), which have numerous experimental paradigms (e.g. Ligand, IV, Pharm), for specific cell characteristics (e.g. cell capacitance, membrane fragility) and channel types (e.g. P2XR, Na^+ or K^+ channels). Features of a Tree can be adjusted, enabling the user to modify amplifier and suction parameters and also allows access to commands that can be inserted into a Tree via a generic drag-and-drop function. In addition, once a Tree has been optimized for a particular cell type, it will not normally require modification for future use with the same cell type. Application notes are available at http://www.nanion.de.
21. Prepare fresh compound solutions, directly prior to use, to avoid precipitation. Compound solutions, where possible should be stored in glassware, because some compounds will adhere to other substrates. An underestimation of concentration may result from adhesion or precipitation of a compound solution.
22. The joblist, within the Tree, defines the position of the compounds, the volume and speed of application and selection of the appropriate pulse generator file in Patchmaster.
23. Regular aspiration of cells retain viability for at least 4 h after they have been prepared in suspension, although some deterioration in success rates has been observed after 3 h.
24. For a double stack with two zones of solution, the wash buffer is aspirated first, directly followed by the ligand resulting in the ligand zone being applied to the cells

first, followed immediately by the wash buffer (Fig. 1c). To examine the effects of a channel modulator, a triple stack with three solution zones should be selected [17]. The wash buffer is aspirated first, followed directly by solution containing both ligand and modulator, followed immediately by modulator.

25. The volumes of the different solution zones can be adjusted, as well as the speed of application, enabling exposure times of as little as 100 ms. Typical volumes for a double stack are 200 µL wash buffer zone, 10-60 µL ligand zone, but these can be optimised for different ligands, accordingly. The maximum volume that the pipette can aspirate at one time is 350 µL. The zone containing the ligand should be applied more rapidly (e.g. 171 µL/s; speed 12) than the other zones (e.g. 19 µL/s; speed 24). In addition it is possible to set the volumes to be dispensed at different rates and these do not need to correspond the volume of the two phases (e.g. 'Vol fast' 120 µL (Speed 12) and 'Vol slow' 140 µL (Speed 24).
26. The recording chamber, chip-wagon and pipette can be heated simultaneously or independently. These modules can be heated to 80 ^{o}C, however, it is not recommended to increase the temperature above 55 ^{o}C. The temperature of the cells and the solutions can also be cooled via an add-on cooling plate. Cooling the cells and compounds improves viability and stability.

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6. Figure Legends

Fig. 1 Schematic of the cross-section of chip chamber micro-domains, illustrating single and double stacked solution application. (**a**) Example of a chip chamber with a cell sealed on the single chip aperture. The robotic pipetting arm dispenses, solutions, cell suspensions and compounds into the chip. With every addition to the external micro-channel, the solution is completely replaced, overflowing into the waste reservoir. The waste reservoir is emptied throughout the experiment. (**b**) Cross-section of a 4-hole ensemble chip, illustrating the arrangement of four cells simultaneously sealed onto four individual apertures in a single chip chamber. (**c**) Illustration of double stacked solution application. The robotic pipette aspirates external solution (Wash) followed by ligand of interest (Drug) before precisely timed application into the external micro-channel. Output from the recording electrode showing the brevity of drug contact with the cell during a continuous recording (Modified from [17])

Fig. 2 BzATP concentration-response curve obtained from WT hP2X$_7$Rs stably expressed in HEK293 cells. Data are fit to the Hill equation with the following EC_{50} value: 69.7 ± 7.5 μM and Hill coefficient: 2.3 ± 0.5 (n = 8 for each data point). Inset shows representative whole-cell currents evoked by BzATP (3–100 μM). Cells were voltage-clamped at a holding potential of -60 mV. (Reproduced from [16])

Fig. 3 P2X$_4$R functional assay using HEK293 cells transiently transfected with AcGFP, WT P2X$_4$-AcGFP, or P2X$_4$-G135S-AcGFP. (**a**) Representative whole-cell current traces from WT P2X$_4$ (dark grey) and G135S mutant P2X$_4$ (light grey) in response to ATP (100 μM) as indicated by the bar above the current traces. No currents were elicited for cells transfected with AcGFP empty vector alone (Mock; black). (**b**) Mean peak current

amplitude for Mock (black; n = 6), WT $P2X_4$ (dark grey; n = 6 cells) and G135S $P2X_4$ (light grey; n = 10 cells) in response to ATP (100 μM). A volume of 60 μL ATP was applied at a rate 86 μL/s; (speed 15), followed immediately by wash (120 μL) at a rate of 19 μL/s (speed 24), using a double stacked protocol. Peak currents were compared and statistical significance marked as $*p<0.01$. (Reproduced from [15] with permission from Wiley)

Fig. 4 Repetitive activation of $P2X_{2/3}R$. (**a**) Current traces induced, in a single 1321N1 cell, by repetitive application of ATP (30 μM) using a double stacked solution application protocol. Black bars above the current traces illustrate ATP (30 μM) contact time. (**b**) Timecourse of the experiment showing 7 reproducible inward currents with consistent current amplitudes of approximately 8 nA

Fig. 5 Effects of ATP and suramin on $P2X_{2/3}Rs$ stably expressed in 1321N1 cells. (**a**) Representative current traces showing activation of $P2X_{2/3}Rs$ by increasing concentrations of ATP (0.1–300 μM). (**b**) Mean dose-response curve for ATP activation. Data are fit to the Hill equation with the following EC_{50} value: 7.8 ± 1.0 μM and Hill coefficient: 1.2 ± 0.065 (n = 10 for each data point). (**c**) Representative traces showing the concentration-dependent block of ATP-induced $P2X_{2/3}Rs$ currents by suramin (1 μM–1 mM). Suramin at increasing concentrations was pre-incubated and then co-applied with 30 μM ATP. (**d**) Concentration-response curve for suramin block. Data are fit to the Hill equation with the following EC_{50} value: 28.0 ± 5.3 and Hill coefficient: 0.82 ± 0.063 (n = 7 for each data point)

7. Figures

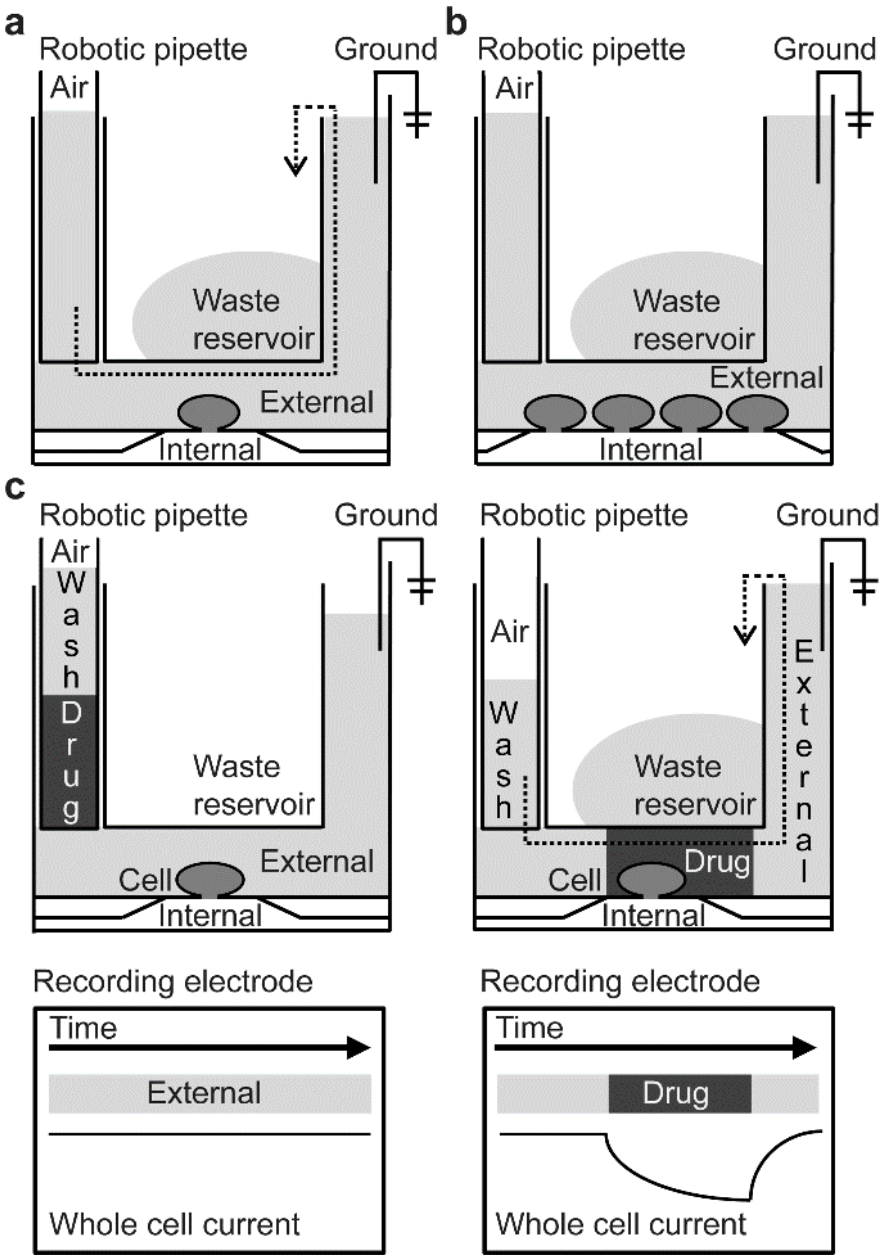

Figure 1

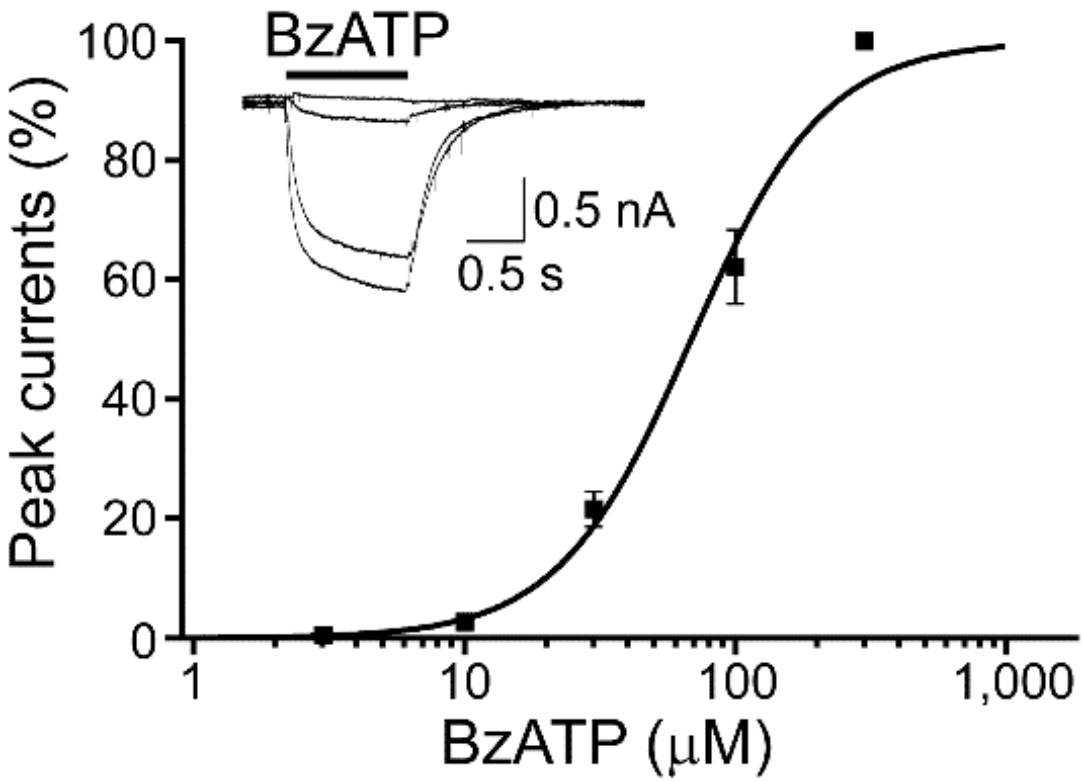

Figure 2

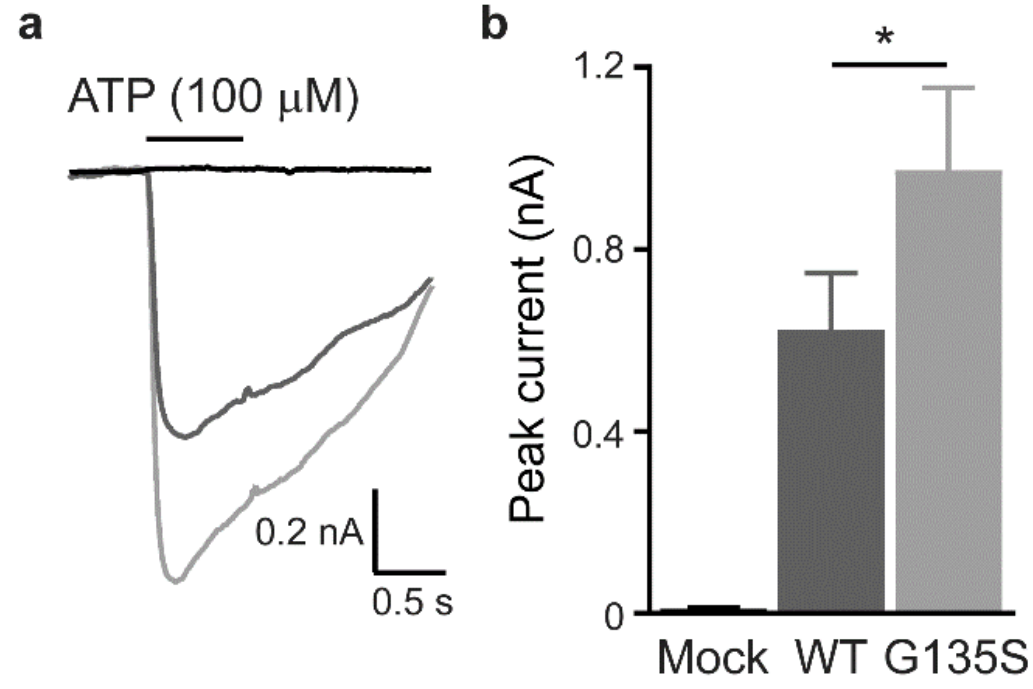

Figure 3

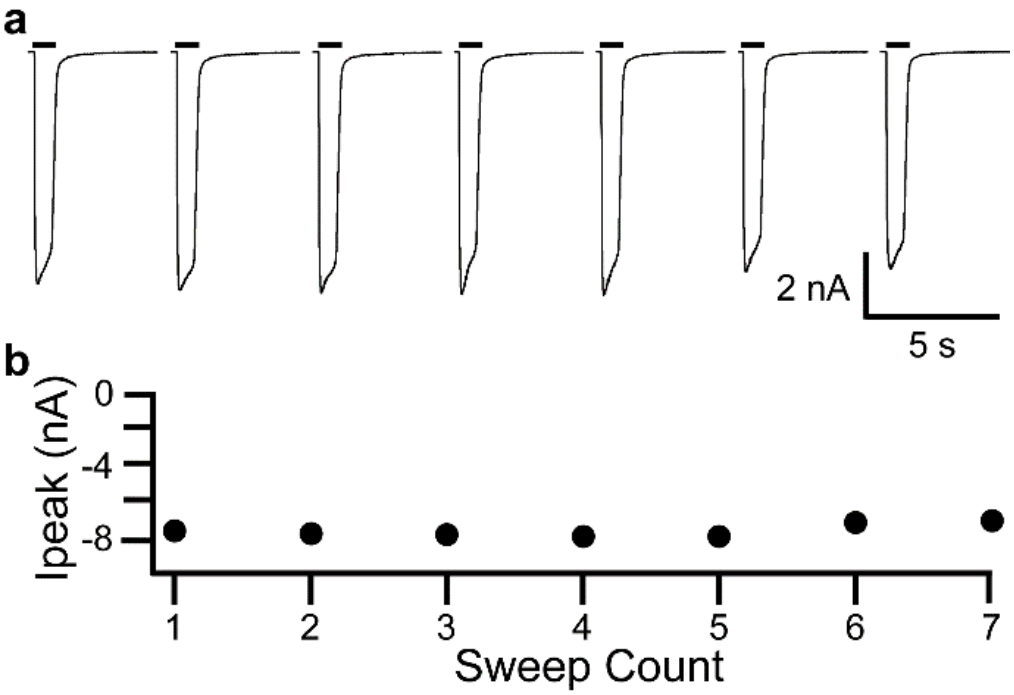

Figure 4

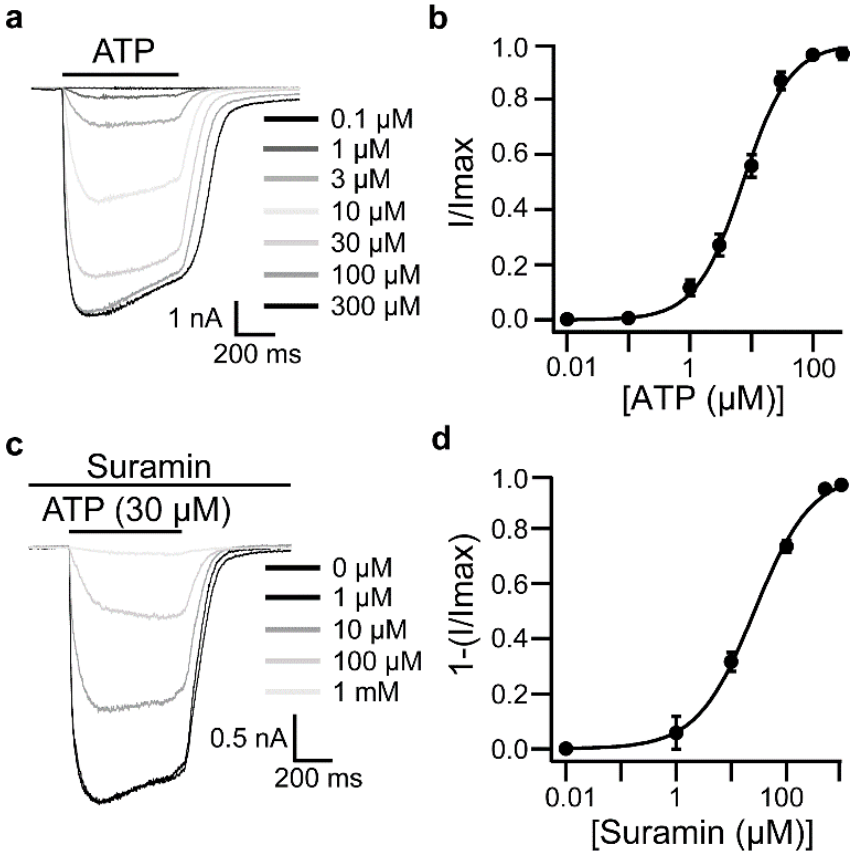

Figure 5