Two-Photon Targeted, Quad Whole-Cell Patch Clamping Robot

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***Abstract*—We present an automated quad-channel patch-clamp technology platform for *ex vivo* brain slice electrophysiology, capable of both blind and two-photon targeted robotically automated patching. The robot scales up the patch clamp single cell recording technique to four simultaneous channels, with seal success rates for two-photon targeted and blind modes of 54% and 67% respectively. In 50% of targeted trials (where specific cells were required), at least 2 simultaneous recordings were obtained. For blind mode, most trials yielded dual or triple recordings. This robot, a milestone on the path to a true *in vivo* robotic multi-patching technology platform, will allow numerous studies into the function and connectivity patterns of both primary and secondary cell types.**

***Index Terms*—patch-clamp, automation, two-photon imaging, neuroscience, neurotechnology**

I. Introduction

Whole-cell patch-clamp is the gold-standard technique for obtaining high-fidelity electrical recordings of individual neurons. It has enabled the analysis of ion channel biophysics, membrane properties, cell excitability, post and presynaptic responses, neuronal inter-connectivity and high order behavioral states, among many others. Despite providing high-quality data, the patch-clamp technique remains limited by inherently low throughput, steep learning curve and labor intensity.

The combination of patch-clamp automation and two-photon imaging enables the selection of specific cell types, thus presenting the opportunity to resolve single-cell characteristics with brain function by integrating anatomical, pharmacological and physiological data [1], [2]. As a result, hypotheses about the function of circuits involving specific cells or cell types during healthy and pathological states can be tested. This method, known as TPTP (Two-Photon Targeted Patching) [3], [4], directs pipettes filled with a fluorescent dye to patch cells that have been fluorescently labelled with an emission spectrum that allows them to be differentiated. Fluorescence can be induced via intracranial viral injection [4], breeding transgenic animals with cell-specific expression of fluorescent proteins [5], or intravenous injection of blood-brain-barrier-crossing viral vectors [6].

When attempting simultaneous recordings from multiple cells, the benefits of a robotic patch-clamp system may be even more significant [7]–[9]. Pairing patch-clamp recordings allows for precise measures of the connectivity between two neurons as it provides information on linked electrical activity at high temporal resolution, including subthreshold correlations which are not always possible to detect. Other methods, such as genetically encoded calcium indicators (GECIs) like GCaMP or voltage sensors, have not yet consistently shown comparable sensitivity permitting the identification of synaptic connections [12], [13]. In single-cell transcriptomics, paired recordings could also offer much more intricate biological detail [10], [11]. Transcriptomic changes underlying neuronal computation and development, for example, can be effectively investigated. Considering the challenges of carrying out multiple *in vivo* patch-clamp recordings [14]–[16], robotic automation appears to be the main solution for its widespread dissemination.

The use of the patch-clamp technique in live animal preparations has traditionally been limited to 'blind' recordings [17], [18]. In this technique, cells are detected by a change in impedance and the first cells encountered on the electrode path are selected as targets [9], [19]. Blind multi patch-clamp has previously been automated *in vivo* [9]. Although these systems can greatly improve throughput and may be the only option for deep structures in the brain (*in vivo*), they have several disadvantages. Firstly, blind patching leads to a very reduced throughput for specific types of cells, such as interneurons, which are key players in brain function, but only account for approximately 15%-30% of the cortical cell population in rodents [20]. Therefore, in this kind of blind, automated multi-patching which skews towards statistically prevalent cells, the intra and interpopulation connections of secondary cell types cannot be studied. Not only that, but multi-patching without imaging can lead to a perceived false decrease in connectivity as this parameter is highly correlated to inter-somatic distance [16]; patching cells too far away will likely decrease the chances of observing a connection, while patching too close without image guidance can lead to the disruption of axons and dendrites of previously sealed cells with the next pipette, which could destroy the connections. Thus, detailed connectivity studies are not feasible with these systems. Moreover, since blind systems detect cells based on changes in impedance, they tend to patch the part of the

cell membrane first encountered, rather than the center, which has been linked to a decrease in yield [21]. Many issues can thus be solved by the use of two-photon microscopy to target fluorescently labelled neurons. For this reason, we have developed a robot that can do both blind and two-photon targeted multi patch-clamping, extending the system of Annecchino et al. [1], which performed TPTP of single cells *in vivo*. The system has been tested in brain slices with success and will be adapted to *in vivo* multipatching in the near future. We here present the results for both the blind and TPTP *ex vivo* modes.

II. Methods

A. Technological

The platform comprises a Ti:Sapphire laser (Newport/Spectraphysics MaiTai HP), a multi-photon microscope (Scientifica Ltd), and a 3 degrees of freedom (DOF) micromanipulator per channel (Sensapex or Scientifica) fitted with mechanical stability clamps (to improve pipette resistance against vibration, tissue deformation and pressure changes). It incorporates four custom-developed pressure regulators which are a refined and scaled up version from [1], patch-clamp amplifiers and data acquisition (DAQ) hardware. Control is via a custom-developed LabView program which acquires frames directly from the microscope. The graphical user interface has been designed to help the user keep track of the different functionalities by having them in different modules: two-photon imaging, pressure-control, manipulator control, electrophysiology control, etc. To help keep track of the four different channels/pipettes, they are color-coded (Fig. 1).

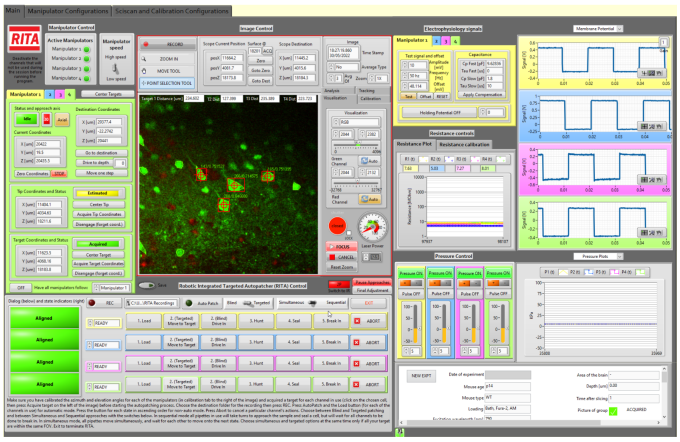

Fig. 1. LabView control software user interface. Functionalities are separated into well defined modules and colour-coded according to channel for ease of use.

In targeted quad *ex vivo* mode (brain slices), a 3D stack of the target cells is acquired, and an image processing algorithm detects their center of mass. The pipettes then simultaneously align in the x/y plane to a position where they have a direct path along the approach angle to their targets. This is followed by a single step movement along said axis, which leaves them in contact. At this point, the sealing protocol is activated. Upon successful seal, the user can choose to break-in or not, whereas break-in will automatically happen if set to auto mode. A two-photon (2P) image of a successfully patched group of four neurons is shown in Fig. 2.

For blind quad *ex vivo* mode, the impedance of the pipettes is closely monitored to detect the presence of a cell near the tip as they move through the brain. When a cell is detected, movement in all other pipettes stops, and the sealing protocol is activated for the tip in question. Once a seal is achieved, the other pipettes resume the hunt, and when all have achieved a seal, break-in is activated in all the channels.

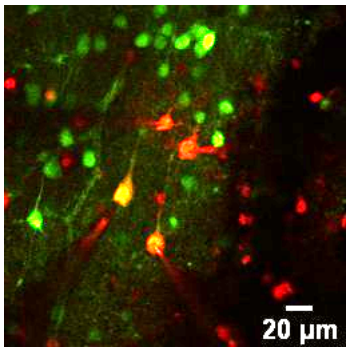

Fig. 2. A two-photon image of four patched neurons. Cells were bath loaded with FURA 2-AM. Alexa 594 Hydrazyde dye (red) from the pipette internal solution diffuses into the cells as direct contact with their intracellular spaces is established, making the green cells yellow.

In TPTP mode, the two-photon images are processed by the software, segmented, and the center of mass, area, contour and contrast of all present cells is extracted in real time owing to their fluorescent print [1]. It is the center of mass that is used as the x/y target coordinates for the pipettes, so as to patch the center of the cell, while the z-coordinate comes from the plane at which the cell is most in focus. This is calculated by maximizing the Contrast Focus Score (CFS) - a parameter that is defined as the difference between the fluorescence of the background surrounding the cell and the fluorescence of the edges of the cell.

After each trial, pipettes are automatically cleaned in an enzymatic solution and then returned to their initial positions, allowing for reuse, following [22]. Clicking one button allows all channels to be offset, their voltage step functions to be activated and the pressure set to the required value, leaving them ready for the next trial. A ‘follow’ function is available to allow the user to move a single pipette and have the rest follow, both in the z and x/y coordinates, to move to a different brain location if needed. Alternatively, pipettes can be automatically sent to a certain depth. Temperature control for the perfusion system is also possible within the software through a Peltier system (Scientifica Ltd). The experimental notes section automatically records the output of each channel in each trial, input resistance, series resistance and membrane potential of successful seals and break ins, as well as an image of the target cells, labelled with the channel that was

used for each cell. This is complemented by the recording module, which automatically documents all other valuable information such as electrophysiology and pressure signals versus time, target coordinates, pipette coordinates and state of the pipette (hunting, sealing, etc) along time. Basic injection and connectivity protocols (Fig. 6) are also available to run within the program, making a complete software package for the basic needs of any experimenter, regardless of skill set.

B. Biological

For targeted experiments, C57BL/6J mice of both sexes of postnatal ages 11-16 were bath loaded with FURA 2-AM according to the protocol by [23]: 2μL of Pluronic F-127 (20% Solution in DMSO) and 13μL of DMSO were added to the 50μg of FURA 2-AM vial and the solution was vortexed and mixed with 2mL of resting artificial Cerebrospinal Fluid (aCSF). This solution was added to the loading chamber, for a slice incubation of 45 minutes at 35ºC. Slicing and resting aCSFs were prepared according to the same protocol. A laser excitation wavelength of 790nm was used in the 2P microscope for optimal visualization.

Imaging (for both targeted and blind) and slicing of blind experiments were done in aCSF with the following mM concentrations: 2 CaCl2 (Calcium chloride), 2.5 KCl (Potassium Chloride), 26 NaHCO3 (Sodium bicarbonate), 1.25 NaH2PO4 (Sodium phosphate monobasic monohydrate), 1 MgCl2 (Magnesium Chloride), 25 Dextrose and 125 NaCl (Sodium Chloride). Osmolarity was adjusted with dextrose to 338mOsm.

Internal solution for the pipettes was prepared in advance and frozen in aliquots with the following mM concentrations: 5 KCl (Potassium Chloride), 115 K-Gluconate (Potassium Gluconate), 10 HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 4 Mg-ATP (Adenosine 5'-triphosphate magnesium salt hydrate), 0.3 Na-GTP (Guanosine 5-triphosphate sodium salt hydrate) and 10 Na- Phosphocreatine (Phosphocreatine disodium salt hydrate) respectively. pH was adjusted with KOH to reach 7.2-7.4 and sucrose was added to adjust the osmolarity to 310mOsm. 10mM Alexa 594 Hydrazyde was added on the day for pipette visualization.

III. Results

A. Quadruple targeted ex vivo patching

As a first step towards an *in vivo* two-photon targeted multi-patching robot, we developed an *ex vivo* mode making use of mouse brain slices. We achieved seal success rates of 54.2% (n=48) for targeted (Fig. 3) and 67.2% (n=64) for blind modes respectively (Fig. 5). This is in line with the success rates for manual patching but with far superior speed and throughput. An average of under 4 minutes was needed for all four channels to attempt seal and break-in in targeted mode, with input resistance averaging at around 65.4 ± 4.75 MΩ and series resistance at 24.89 ± 2.41 MΩ. In 50% of trials (Fig. 3), we obtained two or more simultaneous patches which could potentially be used for connectivity tests.

Example traces of the resistance and pressure signals (along time) of a successful quadruple patch are shown in Fig. 4.

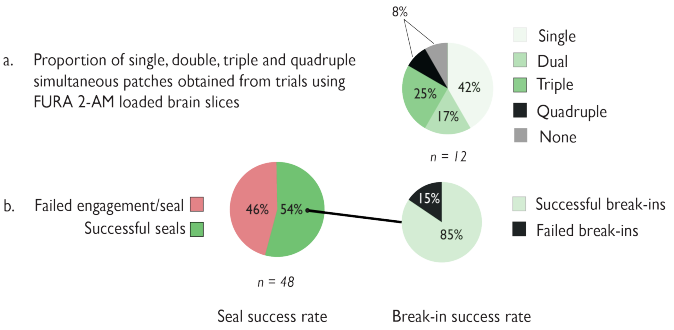

Fig. 3. Success rates for *ex vivo* trials using dye-loaded (FURA 2-AM) brain slices. (a) The different number of simultaneous recordings obtained using four channels. (b) Proportion of successful seal and break-ins.

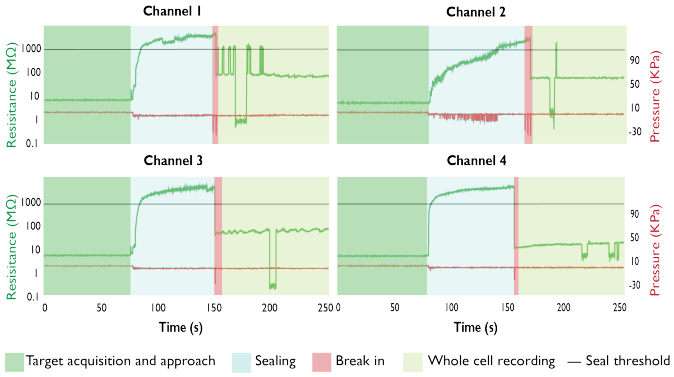

Fig. 4. Stages of automated patching showing pipette resistance and pressure changes. Once the target cell is engaged, sealing automatically begins, releasing positive pressure and applying increasing suction pulses until a gigaseal is formed. Similar but stronger suction pulses are applied for break-in.

B. Quadruple 'blind' ex vivo patching

For blind mode patching in brain slices, we achieved 67.2% (n=64 including 7 quad and 12 triple trials) seal success rate for blind mode (Fig. 5). Daily success rate varied from as low as 50% (n=18, 6 triple trials) to as high as 86.66% (n=15, 5 triple trials), we believe depending largely upon slice quality.

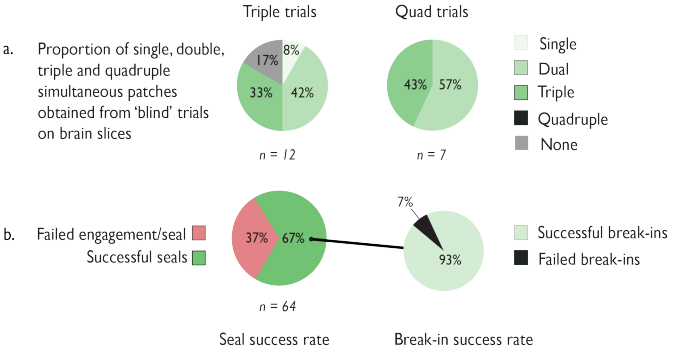

Fig. 5. Blind patch success rate in brain slices. (a) The different number of simultaneous recordings obtained using three and four channels. (b) Proportion of successful seal and break-ins.

Fig. 6 shows typical current injection profiles that the program ran to characterise three patched neurons.

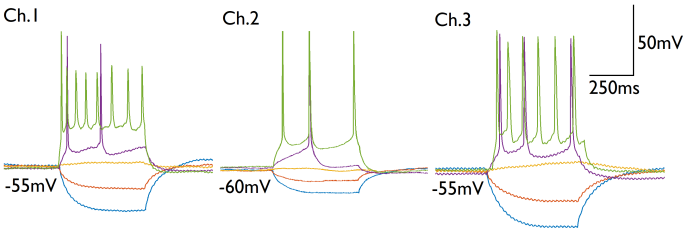

Fig. 6. Example current pulses applied and their responses following successful patching.

Based on these promising statistics, we envisage that our robotic platform will help make multiple patch-clamp electrophysiology accessible to a broader range of laboratories across the world. Additionally, it might be used as an aid by experienced patch-clamp researchers, either by using the pressure module to avoid mouth suction where there may be health safety concerns, or by saving time in numerous ways: automatically recording data, synchronised movement of pipettes, automated cleaning of pipettes for reuse, etc. It will thus make thousands of new neuroscience studies possible and more efficient.

IV. Conclusion

Robotic automation helps mitigate low patch-clamp success rates and offers several benefits, including faster skill assimilation for new operators, standardized recording quality, and improved throughput. It also enables scaling up of the technique to simultaneous patch-clamp recording of multiple cells *ex vivo* and *in vivo*, enabling assays of synaptic coupling and many new research lines in basic and translational neuroscience. With further increase of degree of automation to include other aspects of experimental workflow, such as craniotomies [24], there is the potential for further reduction in human-derived experimental variability.

Our targeted quad patch-clamp system allows scalable and reproducible electrophysiology studies to be conducted across a variety of laboratory settings, offering for the first time, robotically automated recording of subthreshold signals from multiple genetically and optically targeted cells in tandem. As we have made use of only information available *in vivo*, we envisage straightforward extension of the platform from *ex vivo* to *in vivo* application.

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References

[1] L. A. Annecchino, A. R. Morris, C. S. Copeland, O. E. Agabi, P. Chadderton, and S. R. Schultz, "Robotic automation of *in vivo* two-photon targeted whole-cell patch-clamp electrophysiology", Neuron, vol. 95, no. 5, pp. 1048–1055, 2017.

[2] H.-J. Suk, I. van Welie, S. B. Kodandaramaiah, B. Allen, C. R. Forest, and E. S. Boyden, "Closed-loop real-time imaging enables fully automated cell-targeted patch-clamp neural recording *in vivo*", Neuron, vol. 96, no. 1, pp. 244–245, 2017.

[3] T. W. Margrie et al., "Targeted whole-cell recordings in the mammalian brain *in vivo*," Neuron, vol. 39, no. 6, pp. 911–918, 2003.

[4] S. Komai, W. Denk, P. Osten, M. Brecht, and T. W. Margrie, "Two-photon targeted patching (TPTP) *in vivo*", Nat. Protoc., vol. 1, no. 2, pp. 647–652, 2006.

[5] A. H. Meyer, I. Katona, M. Blatow, A. Rozov, and H. Monyer, "*In vivo* labeling of parvalbumin-positive interneurons and analysis of electrical coupling in identified neurons", J. Neurosci., vol. 22, no. 16, pp. 7055–7064, 2002.

[6] D. Goertsen, et al., "AAV capsid variants with brain-wide transgene expression and decreased liver targeting after intravenous delivery in mouse and marmoset", Nat. Neurosci. vol. 25, pp. 106–115, 2022.

[7] H. Markram, J. Lübke, M. Frotscher, A. Roth, and B. Sakmann, "Physiology and anatomy of synaptic connections between thick tufted pyramidal neurones in the developing rat neocortex", J. Physiol., vol. 500, no. 2, pp. 409–440, 1997.

[8] S. Crochet and C. C. H. Petersen, "Correlating whisker behavior with membrane potential in barrel cortex of awake mice", Nat. Neurosci., vol. 9, no. 5, pp. 608–610, 2006.

[9] S. B. Kodandaramaiah et al., "Multi-neuron intracellular recording *in vivo* via interacting autopatching robots", Elife, vol. 7, 2018.

[10] C. R. Cadwell et al., "Electrophysiological, transcriptomic and morphologic profiling of single neurons using Patch-seq", Nat. Biotechnol., vol. 34, no. 2, pp. 199–203, 2016.

[11] J. M. H. Kim et al., "Robust RNA-Seq of aRNA-amplified single cell material collected by patch clamp", Sci. Rep., vol. 10, no. 1, p. 1979, 2020.

[12] M. Z. Lin and M. J. Schnitzer, "Genetically encoded indicators of neuronal activity", Nat. Neurosci., vol. 19, no. 9, pp. 1142–1153, 2016.

[13] H. H. Yang and F. St-Pierre, "Genetically encoded voltage indicators: Opportunities and challenges", J. Neurosci., vol. 36, no. 39, pp. 9977–9989, 2016.

[14] J. F. A. Poulet and C. C. H. Petersen, "Internal brain state regulates membrane potential synchrony in barrel cortex of behaving mice", Nature, vol. 454, no. 7206, pp. 881–885, 2008.

[15] I. van Welie, A. Roth, S. S. N. Ho, S. Komai, and M. Häusser, "Conditional spike transmission mediated by electrical coupling ensures millisecond precision-correlated activity among interneurons *in vivo*", Neuron, vol. 90, no. 4, pp. 810–823, 2016.

[16] J.-S. Jouhanneau and J. F. A. Poulet, "Multiple two-photon targeted whole-cell patch-clamp recordings from monosynaptically connected neurons *in vivo*", Front. Synaptic Neurosci., vol. 11, p. 15, 2019.

[17] X. Pei, T. R. Vidyasagar, M. Volgushev, and O. D. Creutzfeldt, "Receptive field analysis and orientation selectivity of postsynaptic potentials of simple cells in cat visual cortex", J. Neurosci., vol. 14, no. 11, pp. 7130–7140, 1994.

[18] T. W. Margrie, M. Brecht, and B. Sakmann, "*In vivo*, low-resistance, whole-cell recordings from neurons in the anaesthetized and awake mammalian brain", Pflugers Arch., vol. 444, no. 4, pp. 491–498, 2002.

[19] S. B. Kodandaramaiah, G. T. Franzesi, B. Y. Chow, E. S. Boyden, and C. R. Forest, "Automated whole-cell patch-clamp electrophysiology of neurons *in vivo*", Nat. Methods, vol. 9, no. 6, pp. 585–587, 2012.

[20] R. Tremblay, S. Lee, and B. Rudy, "GABAergic interneurons in the neocortex: From cellular properties to circuits", Neuron, vol. 91, no. 2, pp. 260–292, 2016.

[21] G. L. Holst et al., "Autonomous patch-clamp robot for functional characterization of neurons *in vivo*: development and application to mouse visual cortex", J. Neurophysiol., vol. 121, no. 6, pp. 2341–2357, 2019.

[22] C. R. Landry, M. C. Yip, I. Kolb, W. A. Stoy, M. M. Gonzalez, and C. R. Forest, "Method for rapid enzymatic cleaning for reuse of patch clamp pipettes: Increasing throughput by eliminating manual pipette replacement between patch clamp attempts", Bio Protoc., vol. 11, no. 14, p. e4085, 2021.

[23] A. J. Sadovsky and J. N. MacLean, "Mouse visual neocortex supports multiple stereotyped patterns of microcircuit activity", J. Neurosci., vol. 34, no. 23, pp. 7769–7777, 2014.

[24] M. L. Rynes et al., "Assembly and operation of an open-source, computer numerical controlled (CNC) robot for performing cranial microsurgical procedures", Nat. Protoc., vol. 15, no. 6, pp. 1992–2023, 2020.