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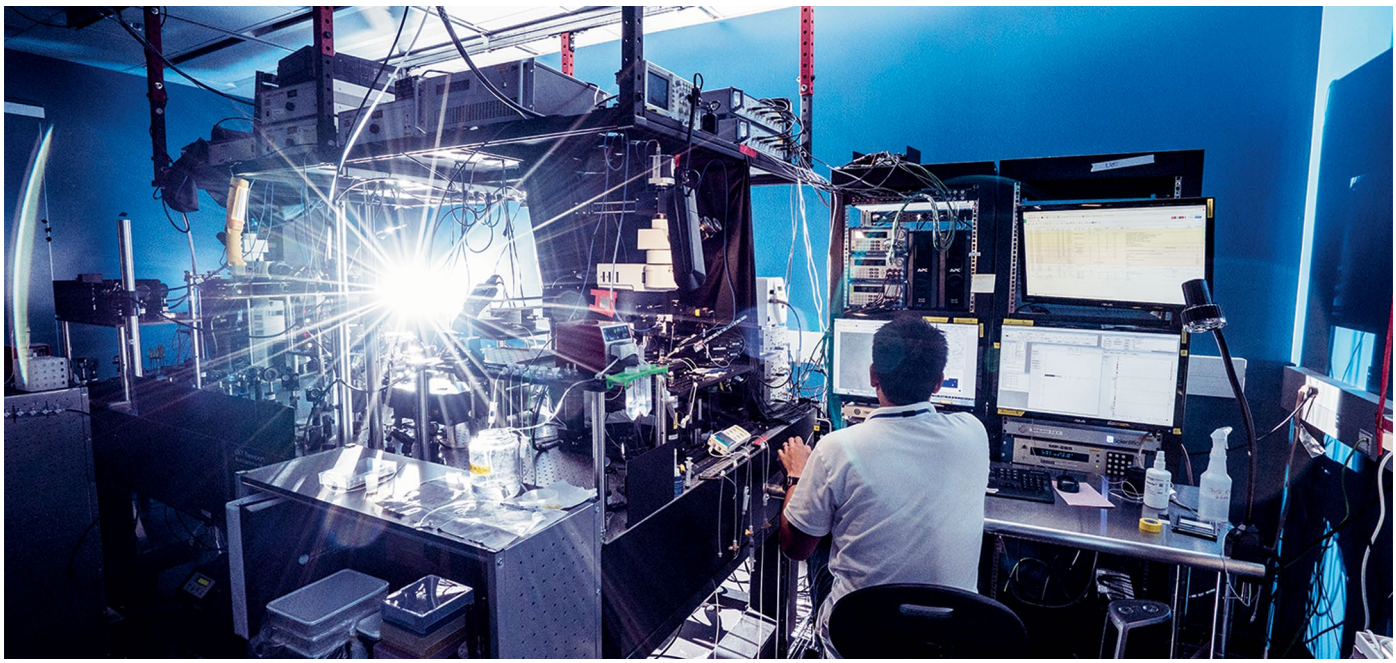

Brian Lee at the Allen Institute for Neuroscience and his colleagues have spent a few years to set up, optimize and scale up a Patch-seq pipeline.

Patch-seq takes neuroscience to a multimodal place

Patch-seq delivers morphology, electrophysiology and transcriptomic data to those with skill, patience and persistence.

By Vivien Marx

Patch-clamp electrophysiology is a neuroscience workhorse for which Bert Sakmann and Erwin Neher received the 1991 Nobel Prize. It takes skill to perform and is one of the "powerful methods in neuroscience," and one that may have fallen a little "out of fashion," says Andreas Tolias, a neuroscientist at Baylor College of Medicine. What's nudging the method back in vogue is its younger cousin Patch-seq. The technique links patch clamping to single-cell transcriptomics approaches that, says Tolias, have been advanced by Karolinska Institutet scientists Sten Linnarsson, Rickard Sandberg and many others.

"The technique is phenomenal," says neuroscientist Mark Cembrowski from the University of British Columbia about Patch-seq. "In one go," he says, from one and the same cell, scientists can acquire an electrophysiological readout with patch-clamp recording, obtain morphological data after infusing dye into the neuron and later using immunohistochemistry, and get a transcriptomic signature from single-cell RNA sequencing (scRNA-seq). Capturing this trio of data types from many cells is part of a multimodal profiling push in neuroscience in both small and large labs, says Tolias. Patch-seq joins together the "different languages of neuroscience," he says: those spoken by anatomists, physiologists, molecular biologists and computational neuroscientists. The technique, says Nathan Gouwens, a computational neuroscientist at the Allen Institute for Brain Science, builds on the "wave of excitement over single-cell transcriptomics" and the expansive view it provides of the "dimensions of diversity" of the brain's cell types. Scientists have been using single-cell transcriptomics alongside other methods to build atlases of cell types in the brains of, for instance, mice, nonhuman primates and people. One needs ever new ways to query how "real" those classifications are, says Gouwens. With Patch-seq, a lab can assess how similar or dissimilar individual cells are to neighbors in terms of their transcriptome, electrical activity and shape. That's a way to validate the "exciting results" from large-scale efforts applying single-cell transcriptomics. Among those efforts is the

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Brain Initiative Cell Census Network, in which teams at multiple institutions, including the Allen Institute, are cataloging the diversity of cell types in the human, monkey and mouse brains. For instance, single-cell transcriptomics has been applied to map the mouse primary motor cortex.

When studying the brain, it can be perilous not to consider using multiple data types. "I think what neuroscience does with single-cell RNA-seq is simply wrong," says Tibor Harkany who has a double appointment at the Medical University of Vienna and the Karolinska Institutet. What often gets lost in scRNA-seq data, given the data's inherent 'noise' or size, are "the really interesting details." As scRNA-seq data are often presented these days, he says, they can lack biological context and enable overinterpretation.

Cembrowski says that studying the brain in ever greater multimodal detail can, for example, reveal that cells "classically" lumped together are actually different cell types[1]. One can query the rules governing those variations. Such insights shed light on the neuronal underpinnings of brain feats such as memory, spatial navigation and learning. To Harkany, "the coolest aspect" of Patch-seq is the way it helps identify useful new cell markers for mouse genetics experiments. The technique connects molecular neuroscience to the long-practiced science and art of neuroanatomy. As a Hungarian scientist, he is proud of his native country's heritage in neuroscience and anatomy. "Thus, we can live a new era of Golgi and Cajal in which we put real colors on the beautiful anatomical drawings in terms of molecular differences even amongst closely mapped neuronal types in any given brain area."

Top: At the University of British Columbia, Mark Cembrowski (center) and his lab use patch clamping and Patch-seq to collect data related to the hippocampus, the subiculum and memory. Lower row (left to right): Brianna Bristow and Kaitlin Sullivan; Adrienne Kinman and Madeline Elder.

How to 1, 2

Patch-seq was developed in two labs independently[2,3]. "There are slight differences in the methods, but it's the same idea," says Tolias. A coincidental lunch between Karolinska Institutet researchers Sandberg and Linnarsson made the groups aware of one another. Tolias and his lab had been working with Sandberg; Harkany and Linnarsson had been collaborating. "Luckily for all involved," says Harkany, the scientists decided to be collegial about the situation. Harkany had sought out Linnarsson for help with "a conceptual conundrum" in his lab about how to use a knockout mouse with a gene deletion for "the best cellular marker known for certain cell types." It's a "pressing challenge" in classic neuroscience, he says, to localize cells in genetics experiments when the "single known cellular marker" is lost. This came up, for example, when they wanted to profile neurons that expressed a specific calcium-binding protein that was the only protein known to be sensitive to psychostimulants. The collaboration led them to alternative neuronal markers to 'find back' cells of interest in knockout mice. In a different experiment, they focused on a 'reference cell type': cholecystokinin-containing GABAergic interneurons, which are thought to be involved in regulating neuronal cortical circuits and may play a role in mood disorders. They used Patch-seq to characterize subpopulations of these interneurons and found new neuron types, too.

Tolias and colleagues used Patch-seq to assess neocortical interneurons in cortical layer 1 (L1) of the mouse brain. They profiled the shape, electrophysiology and gene expression at a single-cell level of two types of inhibitory neurons: elongated neurogliaform cells and single bouquet cells. In this kind of analysis, says Tolias, data can group into small clusters and look like an olive tree, with its many small leaves. But their data clustered into several large groups within which there was transcriptomic diversity. It looks like "a large banana tree," with a few large 'leaves.' Morphology and electrophysiology data from these cells map along these axes, too. As part of the large-scale NIH Brain Research through Advancing Innovative Neurotechnologies (BRAIN) Initiative, scientists at the Allen Institute work with other labs including Tolias's, in areas related to the mouse, nonhuman primate and human brain. For these projects, the researchers have spent a few years tweaking Patch-seq protocols, building and

scaling up a pipeline to increase throughput. One tweak of many: extracting both cytosol and the nucleus into the recording pipette can improve RNA yield and transcriptomic data quality.

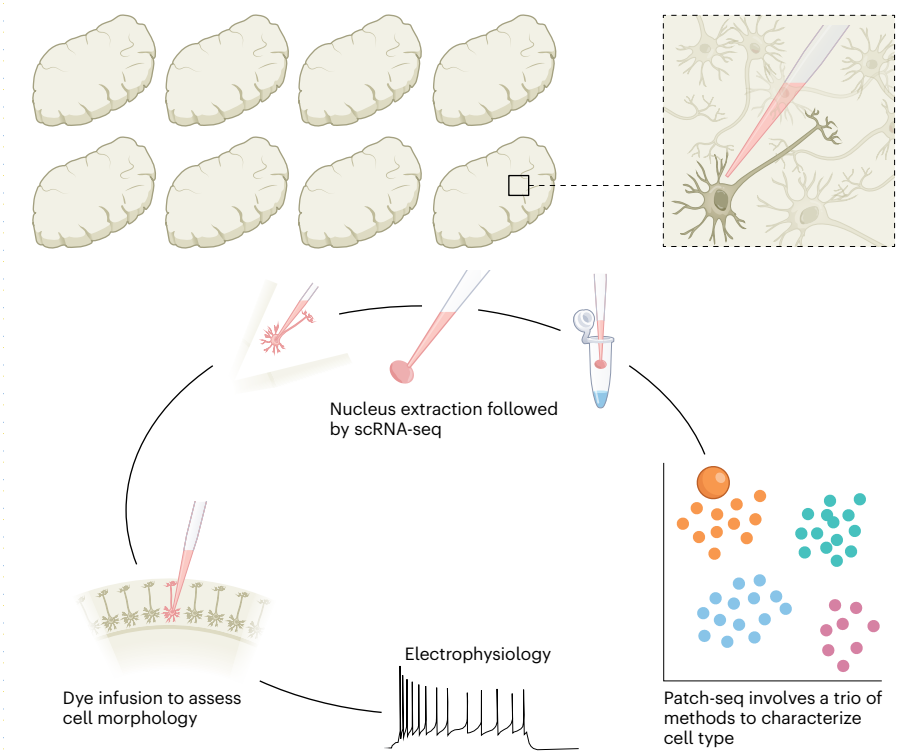
In his view, says Cembrowski, who performs both Patch-seq and patch clamping in his lab, "the Allen is leading the way" in Patch-seq. The approach requires one to combine methods and compromise each one as little as possible. If, after electrophysiology recording, dye infusion and pipette retraction to remove the nucleus for sequencing, the neuron's membrane doesn't seal back up, "all that dye that you worked so hard to put in a cell just leaks out the cell," he says.

Into the pipeline

At the Allen Institute, a team prepares brain slices and hands them off to the next group in the Patch-seq team. Tissue is kept in wells with carbonated medium, where the tissue is "happier" than under the microscope, says Agata Budzillo, a data analyst and trained electrophysiologist who is part of the Allen Institute's Patch-seq team that includes Gouwens, Brian Lee and others. Once the sample is mounted and the microscope is connected to a large screen, the clock starts ticking. Lee says that human and nonhuman primate tissue generally last longer under the microscope than mouse tissue: human tissue has been kept "on the rigs for six, seven hours."

A scientist navigates a pipette to a cell, and adds some suction to gain "electrical access to the neuron," says Lee. Throughout the electrophysiology recording, dye is diffused into the neuron. The dye enables later immunohistochemical analysis to yield shape data about each neuron. Once electrophysiology data are recorded, suction is applied to the pipette to tug the nucleus out of the cell. "It's a very delicate, tedious process where we slowly retract the tip of the pipette," says Lee. "And we hope that the nucleus is on the tip of that pipette." The neuron's membrane seals up, the nucleus is dropped into a buffer and the molecular biology team handles extraction of genetic material, sequencing prep and sequencing.

Among the team's tools is patch-clamp electrophysiology data analysis software that handles data acquisition and does quality control, too. This tool and others, along with protocols, are shared in publications[4–6] and at https://github.com/AllenInstitute/patchseqtools. Patch clamping takes years to master, says Lee. As a graduate student, Budzillo sliced brains from euthanized animals, 'pulled' her own glass pipettes and did patch-clamp recordings and analysis. Variability was unavoidable, she says. It's much lower with multimember teams that handle Patch-seq's individual steps. A rig has to be shielded from any stray electrical interference. In her grad school days, she learned to keep her cell phone away from the rig for this reason, says Budzillo. Being superstitious is also OK. Electrophysiologists are scientists, says Lee, but "some people have a little doll sitting at their rig," because that has led to good recordings.



With Patch-seq, researchers can reap three types of single-cell multimodal data. A researcher navigates a pipette to the cell to be characterized. Electrophysiological recording begins and a dye is infused to allow later assessment of neuron shape with immunohistochemistry. With the same pipette, the nucleus is removed and RNA is sequenced. Among the many challenges: RNase inhibitors can cause cells to swell; electrophysiology data can be variable, as can the amount of extracted RNA; the pipette tip can become contaminated with 'off-target' cells.

Patch-seq protocols differ in the use or non-use of RNase inhibitors. The Allen Institute scientists find these inhibitors helpful for getting high-quality transcriptomic data. But these change the cell's osmolarity, and the cell swells, says Budzillo. The scientists compensate for this by modifying the solution in the pipette used to patch clamp individual cells. Because osmolarity shifts can change patch-clamp efficiency, they also change the size of the recording pipette. Addressing limitations as they pop up, says Lee, means one has to "find some happy medium" to reap cell morphology data, transcriptomic data and e-physiology data from the same cell. Their optimizations can guide other labs, he says, but each experiment and brain region will require its own tweaks. The team is happy to collaborate with other labs, both large and small, and help with Patch-seq experiments. Scientists regularly come to the institute for a week of learning. He and his team would also be happy to run workshops. It's taken a few years and thousands of recordings to get to the point they are at now, says Lee.

Among the many feedback loops between experiment and analysis, mishaps happen. "If the cell ruptures, it's almost a lost cause," says Lee. Collected electrophysiology data can look fine, says Budzillo, but later turn out to be less so. Lee says that at eight

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The Allen Institute Patch-seq team has eight patch-clamping rigs in their Patch-seq pipeline. The team is happy to accommodate visitors to learn the technique, says Brian Lee (right).

patch-clamping rigs, they regularly record from thousands of cells, get morphology data and extract the cells' nuclei for sequencing. The team has "really nailed the parameters of success," he says. When all runs smoothly, electrophysiology recording can be completed in 15–20 minutes, which includes finding and extracting the nucleus. When each person on his team "can get six or seven recordings per day, I would consider that a good day," he says.

Aligning the modalities of Patch-seq data is challenging. The mouse genome has around 30,000 genes, which means a cell such as neuron can be in a 30,000-dimensional space, says Gouwens. That's too big for analysis, he says, but one can "carve up" that data space. "By bringing in those other data types, you can clarify what you're seeing in the transcriptomic data," he says. Meaningful differences between cells are likely to show up in multiple Patch-seq modalities, which helps an experimenter determine "what's actually going on in the system."

Small-lab view

Gouwens says that when Patch-seq came onto the scene, the researchers decided to integrate Patch-seq into their atlas-building pipeline aimed at building a taxonomy of cell types in the brain using mouse lines and single-cell transcriptomic data. But Patch-seq is not just for large labs, he says. Tolias agrees and says that the technique can advance focused scientific questions

Brandeis University neuroscientist Christine Grienberger, who runs a small lab, says she is struck by the brain's ability to learn and store new information. Researchers know that neuron ensembles drive memory-guided behavior. What's yet unknown, for example, is how synaptic plasticity makes some neurons part of these memory ensembles. Patch clamping is, in her experience, the only currently available method to investigate, on a single-cell level, "the relationship between a neuron's action potential output and its intrinsic electrical properties, the synaptic input it receives and any synaptic plasticity that has occurred."

In her work recording from an animal's brain as it performs a task that involves spatial memory, Grienberger was part of a team that discovered a type of synaptic plasticity called behavioral timescale synaptic plasticity (BTSP) that drives the formation of place cells in the hippocampus. The hippocampus is a brain region where episodic memories are formed, and its place cells are viewed, she says, as the "cellular substrate of episodic memories." More recently, together with others, she found that as an animal learns a task, BTSP affects the way a type of neurons called hippocampal CA1 neurons change[7]. Not only is patch clamping low throughput because one records from one cell at a time, it's "a big challenge" to obtain and maintain a high-quality recording while the animal is running, she says. But new methods are making it easier to get high-quality recording for longer times in animals doing tasks. Patch-seq, too, is low throughput, she says, but it's powerful, "and I do have high hopes," she says. Performing Patch-seq in acute brain slices will be useful to tease out the role of transcription in BTSP and to explore whether BTSP induces changes in transcription and whether it is present in different molecularly defined cell types, she says. These questions are currently unanswered, "and these experiments are certainly on my agenda," she says.

With Patch-seq, ideally what labs like his and others seek is in vivo data, says Tolias. Grienberger has tried Patch-seq for in vivo recordings as she sought to leverage RNA-seq data and identify the cell types and understand the cell-type diversity involved in a specific learning task. "Unfortunately," she says, interpreting the RNA-seq results was too challenging. The culprit was likely contamination, "so I decided to put these experiments on hold for the moment" until she and her team find a way to do it well. One option, she says, is to fluorescently label the neuron or neurons of interest, do the in vivo recordings, make acute slices and then proceed with Patch-seq. "This is doable, and that is how I think of performing the experiment," she says. "I just haven't had the time to set it up, since my lab is new."

Patch-seq troubleshooting

Because Patch-seq is both technically challenging and manual, it's hard to prevent contamination by RNases, which degrade sampled mRNA, says Shreejoy Tripathy from the University of Toronto, who has a separate appointment at the independent Centre for Addiction and Mental Health. The technique's many manual steps can be variable, the amount of mRNA captured can vary and it's hard to standardize mRNA extraction efficiency across many cells[8].

With Patch-seq, cells can end up in the pipette that are not the ones you want to study, says Cembrowski. For electrophysiological data, a researcher will want to record from a neuron deep within tissue to avoid the ones on the slice surface that have been cut or likely damaged. From a gene-expression perspective, he says, "as your pipette is pushing in deeply into the slice, you're pushing through this whole micro-environment and contaminating your pipette." It's likely that a pipette is "probably piercing a whole bunch of glia

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on your way down," says Gouwens. It's easier to sort this out in data analysis when the contaminating cells are similar to the one that an experimenter wants to characterize.

To gauge transcriptomic quality, says Budzillo, the Allen Institute team uses a marker-gene-based approach. It was developed in the lab of Paul Pavlidis at the University of British Columbia, and it's for removing cells from the dataset that are likely contaminated. At the time, Tripathy was a postdoctoral fellow in the Pavlidis lab. "Cell-type-specific markers are really helpful, in Patch-seq and in many other contexts, for helping QC and sanity check that the mRNA obtained from single-cell technologies 'makes sense'," says Tripathy.

"Off-target contamination," says Tripathy, is fairly common in Patch-seq datasets. When he and his colleagues pointed this out[9], they were concerned about community reactions. "While it took a bit of time for some of them to come around to our interpretation of off-target contamination and other challenges with Patch-seq, my experience now is that this is now a well-accepted conclusion as it's been widely observed in a lot of Patch-seq datasets since," he says. He and his team advise putting one's experimental Patch-seq data into context by generating or using a transcriptomic cell atlas based on scRNA-seq from dissociated cells. One can distinguish a neuron's action potentials and the neuron-specific marker genes in that cell's transcriptomic profile.

In a recent preprint[10], which has not been peer reviewed, Tripathy and colleagues focused on microglia off-target contamination in Patch-seq datasets. In the three large samples of human and mouse Patch-seq data—published datasets from mouse glutamatergic and GABAergic cells from the motor cortex, GABAergic cells from the visual cortex and superficial pyramidal cells in the medial temporal gyrus from human tissue—they found such contamination to be "widely present" in the sampled neurons' transcriptomes.

Routine evidence of contamination by microglia from a patch-clamped neuron from which the nucleus is aspirated for scRNA-seq is, they note, "an inconvenient truth" about Patch-seq datasets. Such contaminating "off-target mRNA expression" is found only in datasets collected from acute brain slices, not from neurons plated on a dish. Contamination levels differ, for instance, between different neurosurgical donors, which might be due to resection quality.

Overall, says Shreejoy Tripathy, "we treat microglia contamination as kind of a 'signal', instead of a confound."

With the microglia, the scientists found distinct transcriptional and electrophysiological signatures indicative of activated microglia and an inflammation-related cell state. Perhaps, they say, microglia interacting with neurons alter the neuron directly or through signaling molecules and change cellular excitability. Or, microglia may be chemotaxing near neurons that have been changed or damaged, for instance in sample prep.Their study is, as they note, "associational." It's unclear whether microglia are activated and then cause a changed electrophysiology signal, or whether they are attracted to neurons dying due to sample prep. Overall, says Tripathy, "we treat microglia contamination as kind of a 'signal', instead of a confound, and use that as an opportunity to ask how microglia might associate with different types of neurons, and how such associations might impact neuronal function, like electrophysiology."

Says Tolias, commenting on this work, "what is interesting" is the finding of different contamination levels in different cell types. Among many possible causes are neuron–microglia interactions and issues with the way patch clamping was performed. Beyond the indication that cells in different layers need to be measured in different ways, it also informs on the microenvironment. "It's interesting."

Say Harkany, specificity, sensitivity and resolution are "the alpha and omega of each method." Anatomists are wont to say that one can comment on what one sees, not exclude what is unseen. What is seen can be due to the "sensitivity of your tools that limits resolution," he says. Indeed, with Patch-seq, other cells beside the desired ones can end up in the pipette. "We have always filtered our genes to see if we find non-neuronal ones," he says. They have found few, if any, which may be due to the way they amplified the genetic material or to their having had a less contaminated pipette tip to begin with. "But," he says, referring to the Tripathy lab's latest work, "I think the paper you refer to brings up legitimate questions." scRNA-seq datasets, too, face contamination issues, says Harkany. For instance, doublet data, which arise when two cells are mistaken for a single cell, are excluded. Somatic synapses can be missed. Researchers have grown to recognize that mRNA is present in synapses and active translation takes place there. Hence, it's possible that small structures that can deliver 'false signals' escape detection under the microscope. This is why scRNA-seq, and sequencing more generally, he says, "is great to generate hypotheses but not, at least not in solitude, to prove concepts."

"Developing methods for quality control is really essential," says Christine Grienberger.

It's helpful, says Grienberger, that contamination in this analysis stems from a completely different cell type from the one targeted for characterization. The finding highlights, in her view, "that developing methods for quality control is really essential." This could be a role for institutions such as the Allen Institute, with its resources and standing to establish useful quality control metrics. And, she says, reporting standards can emerge that researchers in the field will adopt, and their use can be enforced by funding agencies and journals.

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