Mapping Cerebellar Modules at Single-Cell Resolution with Barcoded Neuroanatomy

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1 Introduction: Anatomy of the Cerebellum

The cerebellum is an important site for information processing in the brain. Once thought to contribute only to motor control, the cerebellum is now thought to play a role in many areas of cognitive function [12]. Information from across the nervous system, including the spinal cord and subcortical and cortical structures of the cerebrum is projected to the cerebellum through the pons. The pontine nuclei give rise to mossy fiber efferents which expand massively to the granule cells in the cerebellar cortex. Granule cells give rise to parallel fibers, which are read out by Purkinje cells, the sole outputs of the cerebellar cortex. Purkinje cells are also innervated by climbing fibers arising from the inferior olive. Climbing fibers initiate complex spikes in Purkinje cells, which are thought to instruct plasticity at the parallel fiber-Purkinje cell synapses. Finally, Purkinje cells project to the Deep Cerebellar Nuclei (DCN), which are the sole outputs of the cerebellum.

In the rostro-caudal direction, the cerebellum is divided into four "transverse zones," which are distinguished by their developmental origin and gene expression patterns [1]. The transverse zones are further divided into ten "lobules" (Anterior Zone: lobules I-V in Mouse; Central Zone: lobules VI, VII; Posterior Zone: lobules VIII to dorsal IX; Nodular Zone: ventral lobule IX and lobule X). Despite their visible separation, the lobules within a transverse zone have no obvious physiological differences, and it has been suggested that the cerebellum's lobulation is merely the solution to the problem of fitting a large cerebellar cortex into a confined space [1]. Laterally, each half of the cerebellar cortex can be divided into four longitudinal zones (A, B, C, and D) on the basis of their projections to the cerebellar nuclei (Fig. 1.B) [9]. Each of these zones also receives climbing fiber inputs from distinct subdivisions of the inferior olive, which also receive inhibitory connections from the Purkinje cells corresponding cerebellar nucleus target zone (Fig. 1C) [10]. Within each zone, multiple "microzones" approximately 200 micrometers in width, were distinguished by their inputs. Electrophysiological recordings showed that each microzone receives climbing fiber inputs from distinct regions of the inferior olive, and themselves project to finer subdivisions of the cerebellar nuclei [9]. More modern tracing experiments have confirmed that neurons in a spatially restricted portion of the inferior olive project to stripes of Purkinje cells in the cerebellar cortex which are restricted to a narrow sagittal band of approximately 200 micrometers in width, but extending longitudinally across many lobules, and even across transverse zones (Fig. 1F) [14].

More recently, the striped organization within and between the transverse zones of the cerebellum have been revealed with molecular staining methods. Most prominently, when the cerebellar cortex is stained for Aldolase C (aka Zebrin II), vertical stripes alternating between AldoC positive and AldoC negative appear [3], suggesting a second subdivision of the cerebellar cortex along the mediolateral axis. However, it was later shown that the projection zones of the inferior olive align with the Aldolase C stripe pattern [16]. In addition, a comprehensive map of the projections from fine-grained regions of the inferior olive to the cerebellar cortex has been assembled, finding that these projection zones align with the Aldolase C stripes in the cerebellar cortex [13]. The apparent congruence between the anatomically and molecularly defined stripes of the cerebellum have led to a "one-map hypothesis" [1], suggesting that a single anatomical map underlies the vertical stripes of the cerebellum, whether revealed by molecular stains or anatomical connectivity.

These studies paint a vivid picture of the cerebellum as a highly repetitive structure consisting of thousands of narrow "microzones," each consisting of a stripe of Purkinje cells in the cerebellar cortex, as well as it's connecting portion of the inferior olive and target portion of the the deep cerebellar nucleus. However, they leave many questions open about the structure *within* these microzones, and their relationship to each other. Two studies (to my knowledge) have begun to elucidate the finer-grained organization within the cerebellar microzones. First, Sugihara et al.'s 2001 study visualized the whole trajectories of 34 individual olivocerebellar axons using biotinylated dextran amine anterograde tracer injections. They found that the axons of inferior olive neurons typically split into multiple climbing fibers (ranging from 2 to 17, with an average of about 7 per axon) with each innervating a different Purkinje cell target. While these target Purkinje cells were always restricted to a narrow sagittal section of the cerebellar cortex, climbing fibers from the same inferior olive axon often split into multiple lobules, and 12 of the 34 traced axons split across multiple transverse zones. A second, more recent study mapped the connectivity of individual neurons in the deep cerebellar nucleus to the different lobules of the cerebellar cortex by coupling optogenetic stimulation of the lobules with voltage-clamp recordings from the cerebellar nuclei [8]. Specifically, they removed a thin sagittal slice from the mouse vermis and stimulated the lobules individually using optogenetic techniques, and measured the resulting inhibitory postsynaptic currents (IPSCs) in individual neurons in the fastigial nucleus. Reminiscent of Sugihara et al.'s findings in the inferior olive, Gruver et al. found that about half of the recorded cerebellar nucleus neurons received inputs from a single cerebellar lobule. The other half received inputs from multiple lobules, and neurons receiving projections from all four transverse zones (anterior, central, posterior, and nodular) were over-represented relative to chance level. Their results suggest that the cerebellar nuclei are a site of integration across modalities.

While these works suggest that connectivity within cerebellar microzones are structured (i.e. non-random) and functionally important, they have significant limitations, including low-throughput yields and confinement to a single sagittal slice. The fine-grained structure of the cerebellar microzones, as well as any potential connections between them therefore remain unclear.

2 Barcoded Anatomical Tracing Techniques

In conventional anterograde molecular tracing experiments (such as ref. [14]), a tracer is injected into a spatially restricted group of neurons, and is transported along the axons of those neurons to the targets of their projections. The brain is then cut into sections and the pattern of projections can be visualized under a microscope. While this allows one to determine the targets of projections which arise from a spatially restricted region, there is no way to distinguish between the projections of different neurons within the injection site.

Barcoded anatomical tracing methods present a clever solution to this problem – injecting tracers which consist of viruses containing diverse, random RNA "barcodes" that uniquely label each of the neurons they infect [6]. For anterograde tracers such as those based on the sindbis virus, infected cells replicate the uniquely barcoded virus in the cell body, and they are transported along the axons. Single-cell RNA sequencing at the injection site reveals which barcodes infected which neurons. Bulk RNA-seq is then performed on small tissue punches from downstream structures and the recovered barcodes matched to their somatic partners, revealing the projection patterns of neurons at the injection site at single-cell resolution. My first proposed experiment uses a variant of this procedure known as "BarSeq2" [15], in which the injection site is analyzed while intact using in-situ RNA sequencing. This preserves the spatial relationship between the projection neurons.

Recently, barcoded connectivity techniques based on the rabies virus have made it possible to

trace retrograde synaptic connectivity at single-cell resolution as well. Unlike sindbis virus, which remains confined to a single neuron, rabies moves across the synapse in the retrograde direction, infecting pre-synaptic partners. Currently available methods use a version of the rabies virus deficient of a glycoprotein which is required for transport across the synapse. Over-expressing the glycoprotein in *trans* in the injected cells allows the virus to jump to the pre-synaptic partners of injected cells, but no further. My second proposed experiment will rely on a recent iteration of this technique which combines barcoded tracing of connectivity with single-cell RNA sequencing that can determine the gene expression profiles of the cells at the injection site and their labeled pre-synaptic partners [11].

3 Proposed Experiments

The cerebellar vermis, caudal medial accessory olive (cMAO), and fastigial nucleus constitute a highly interconnected zone of the cerebellum originally termed the "A" module [7, 9]. Recent genetic and tracing studies have revealed that the Fastigial Nucleus of the deep cerebellar nuclei contains five distinct spatially localized populations of neurons, which can be distinguished by the expression of a handful of molecular markers (Fig. 3a,b). Furthermore, each of these subpopulations connects to a unique subset of the stripes in the cerebellar cortex, and a unique subset of the cells in the inferior olive (Fig. 3c,d,e). The corresponding regions of the cerebellar cortex can be classified on the basis of the alternating Aldolase C stripes (Fig. 3c). The corresponding regions of the inferior olive corresponded to four subdivisions of the caudal medial accessory olive (cMAO-a, b, c, d) as well as the beta nucleus. The spatial and molecular characterization of the modular organization within the Fastigial nucleus, and their connections to the inferior olive and cerebellar cortex provide a useful springboard from which to investigate the structure of cerebellar olivo-cortico-nuclear loop at single-cell resolution.

3.1 Experiment 1: Olivo-Cortical Projections With BarSeq2

While the tracing experiments of Sugihara et al. provided important insights into the projection patterns of single climbing fibers, the method used is low-throughput, the axons of only 34 Olivocerebellar axons, and provided no genetic information about the participating inferior olive neurons. Projection patterns of Inferior Olive neurons have been mapped with high throughput, but at the cost of losing single-cell resolution [13]. Anterograde Tracing with Barcoded Neuroanatomy provides an avenue for high-throughput mapping of Olivocerebellar axons without the loss of single-cell resolution.

My first proposed experiment applies the BarSeq2 protocol [15] to the projections from the inferior olive to the cerebellar cortex. Specifically, the barcoded anterograde tracers are injected into the cMAO and beta nucleus of the inferior olive. The tracers will be transported along the inferior olive axons and into the climbing fibers of the molecular layer of the cerebellar cortex. At the injection site in the inferior olive, spatial gene expression patterns are determined using spatial transcriptomics to simultaneously map RNA barcodes and endogenous gene expression. This will allow for RNA barcodes to be associated with a single subdivison of the cMAO or beta nucleus. Furthermore, this may reveal a useful genetic distinction between the subdivisions of the inferior olive that could underlie their differential connectivity to their synaptic partners in the cerebellar cortex and nuclei. To analyze connectivity, the cerebellar cortex can then be flattened and sectioned longitudinally along the borders between the Aldolase C positive and negative stripes, and in the transverse direction at the borders between the ten lobules. Each lobule/stripe pair will then be separately processed by RNA sequencing to recover random RNA barcodes.

This experiment will provide a comprehensive mapping of the connectivity of individual cells in the medial inferior olive to the different stripes and lobules of the cerebellar cortex, along with detailed spatial and genetic information about the sources of the projections. The high-throughput data may reveal rare connectivity patterns that were missed in previous experiments. For example, do any inferior olive neurons project to multiple cortical stripes? The recovered connectivity will also allow for an extensive characterization of the statistics of inferior olive axonal connections. For example, the data could be used to test whether the set of lobules targeted by climbing fibers stemming from a single axon is random, or whether certain combinations of lobules are over-represented. Furthermore, one could recover how these statistics vary by the source of the climbing fiber within the cMAO or beta nucleus. Finally, the simultaneous measurement of gene expression patterns of the inferior olive could reveal the genetic basis of climbing fiber target localization to thin sagittal bands, as well as their targeting to different lobules within a band. One possibility is that the lobules targeted by the climbing fibers originating from a single inferior olive axon are selected by a random branching process, but the detailed genetic and anatomical information recovered in this experiment could potentially find differential gene expression in inferior olive neurons whose climbing fibers target different patterns of lobules. This would suggest genetically encoded wiring specificity *within* cerebellar microzones.

3.2 Experiment 2: Corticonuclear Connectivity

While Gruver et al. managed to map the connectivity of single neurons in the fastigial nucleus to the lobules of the cerebellar cortex, doing so required the removal of a thin sagittal slice of the cerebellum. This limited the number of fastigial nucleus neurons they were able to record from and severed any axons that were not fully contained within the removed slice. Furthermore, they did not resolve the genetic identity (see Fig. 3A,B) of the fastigial nucleus neurons they were recording from. Barcoded retrograde tracing with rabies-based tracer can overcome these limitations.

Specifically, I propose to inject barcoded Rabies Virus-based tracers widely into the Fastigial Nucleus. These will infect and reproduce in a large number of neurons in the fastigial nucleus before retrograde trans-synaptic transport into the Purkinje cells in the cerebellar cortex. Single-cell RNA sequencing is then performed on the fastigial nucleus. While breaking down to single cells will destroy the spatial information within the Fastigial nucleus, each sequenced cell can be assigned to one of the five relevant subdivisions of the fastigial nucleus using expression levels of a handful of marker genes (SSPI, SANC, and CALB2) in different combinations (see Fig. 3b) [5]. Because rabies virus is transported across the synapse, this method can resolve connectivity at single-cell resolution for Purkinje cells as well. Sectioning the cerebellar cortex along the Aldolase C stripes and lobular divisions as in experiment 1 and performing single-cell RNA sequencing, the connectivity between neurons of the fastigial nucleus and all of their pre-synaptic partners in each stripe/lobule of the cerebellar cortex can be mapped at single-cell resolution.

The resulting data would answer questions beyond the reach of previous experiments. For example, Fig. 3d shows that retrograde tracer injected into the ventrolateral fastigial nucleus (vlFN) is transported to both the 1+ and 2+ Aldolase C stripes in the cerebellar cortex [5]. This could be due to Purkinje cells from both stripes converging on single neurons in the fastigial nucleus, but it is also possible that these stripes converge on spatially intermixed, but distinct, populations of fastigial nucleus neurons. The combination of single-cell connectivity and gene expression profiling could detect this possibility, and may explain the genetic basis of the fine-grained wiring specificity within the vlFN, of any other subpopulation of the fastigial nucleus with similar properties.

This experiment would also allow for a more comprehensive mapping of integration across transverse zones. Gruver et al.'s results suggest that about half of fastigial nucleus neurons integrate

inputs from more than one transverse zone, and that four-zone convergence is over-represented relative to chance level. However, their results may be specific to the section of the fastigial nucleus that was included in their section. Combining Purkinje cell convergence data with genetic profiling of fastigial nucleus neurons could reveal important difference in the statistics of Purkinje cell convergence onto different populations of fastigial nucleus neurons, which are known to participate in a diverse set of motor and cognitive behaviors [5].

4 Conclusion and Future Directions

My proposed experiments aim to further our understanding of both the olivo-cortical and cortico-nuclear projections of a cerebellar subcircuit. However, they do not address the alignment between these two sets of projections. An ideal experiment would simultaneously map connectivity in the full loop between the inferior olive, cerebellar cortex, and cerebellar nuclei at single-cell resolution. This could, for example, elucidate whether Purkinje cells which share a common climbing fiber input more likely to converge on a common cerebellar nucleus output. However, simultaneous injections into the inferior olive and cerebellar cortex are unlikely to label multiple common neurons due to the requirement of sparse labeling to ensure unique association between barcodes and individual neurons. Furthermore, multi-step barcoded tracers are not yet available. Another technical hurdle for future experiments will be to distinguish between connections made directly between the deep cerebellar nuclei and inferior olive, and connections through a Purkinje cell intermediary. However, when these tools are available, it will be possible to map the olivo-cortico-nuclear loop with even greater precision and detail.

Already, though, the availability of barcoded axonal projection tracers and retrograde monosynaptic tracers can reveal details of cerebellar organization left ambiguous by previous experiments. Indeed, this has already begun with a barcoded connectivity measurement to the mouse ponto-cerebellar circuit [4]. The experiments proposed here would provide a new level of detail to our understanding of cerebellar cortex, its inputs from the inferior olive, and outputs to the cerebellar nuclei. Specifically, the combination of barcoded connectivity measurements and single-cell expression profiling could reveal new details of cerebellar connectivity within and between microzones and provide hints to their genetic basis.

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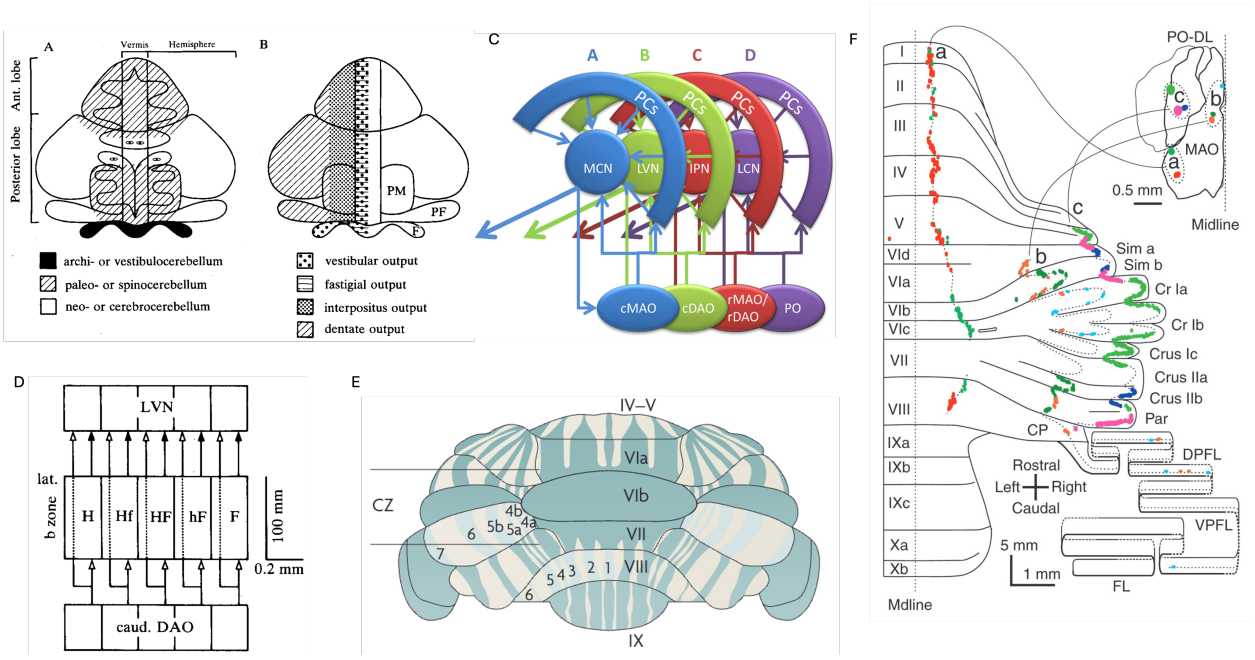

Figure 1: (A) Divisions of the cat cerebellar cortex on the basis of their inputs. (B) Divisions of one hemisphere of the cat cerebellum on the basis of the output to different cerebellar nuclei (C) The Four Main longitudinal zones of the Right Cerebellum. Each consists of a longitudinal zone of Purkinje cells in the cortex, along with their afferent neurons in the inferior olive and cerebellar nucleus targets. (D) Micro-Zones of the B zone of the Cat Vermis. (E) Aldolase C (Zebrin II) stripe pattern in cerebellar cortex. (F) Projections from spatially restricted regions in the inferior olive converge on long, narrow longitudinal stripes in the cerebellar cortex. A,B,D adapted from [9], C from [2], E from [1], and F from [14].

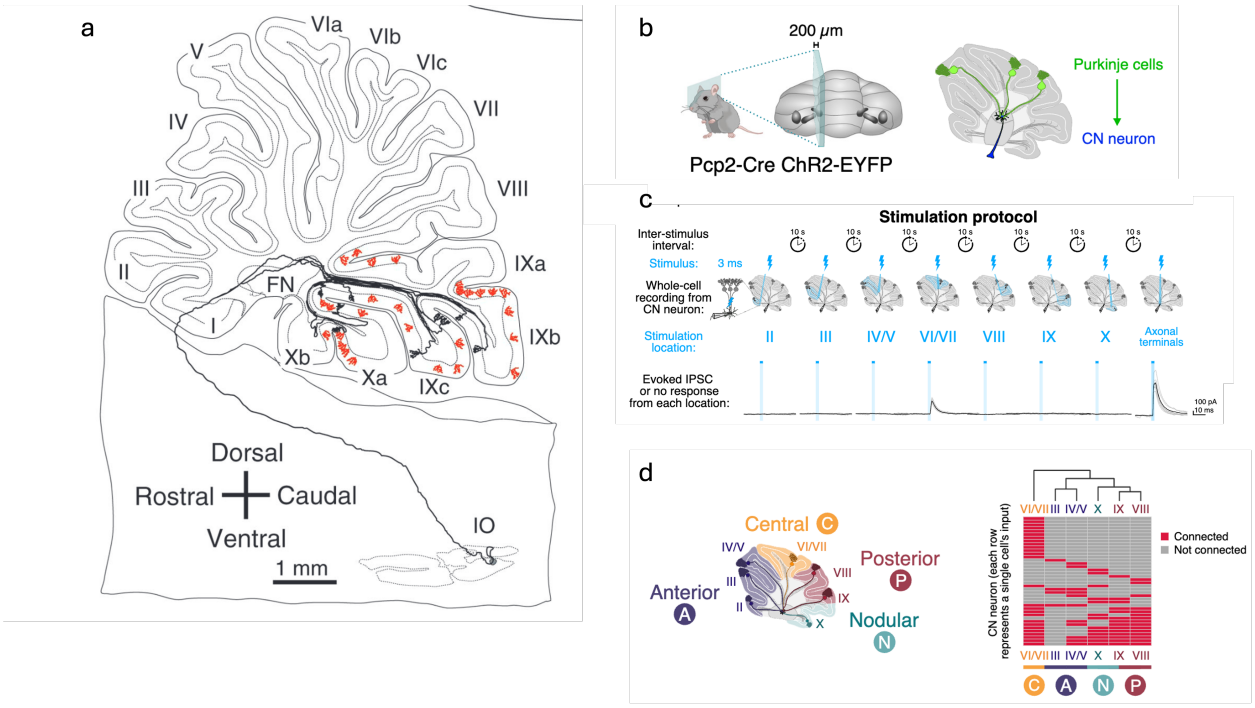

Figure 2: (a) Single-Axon Tracing Experiment from [14] shows that the climbing fibers originating from a single inferior olive axon are distributed across multiple lobules. (b, c, d) experiment from ref. [8]. (a) a slice of the cerebellar vermis of the mouse including a portion of the fastigial nucleus. (b) individual lobules were stimulated optogenetically and the resulting IPSCs measured in the fastigial nucleus neurons. (c) results show connectivity from single neurons in the fastigial nucleus to multiple transverse zones of the cortex.

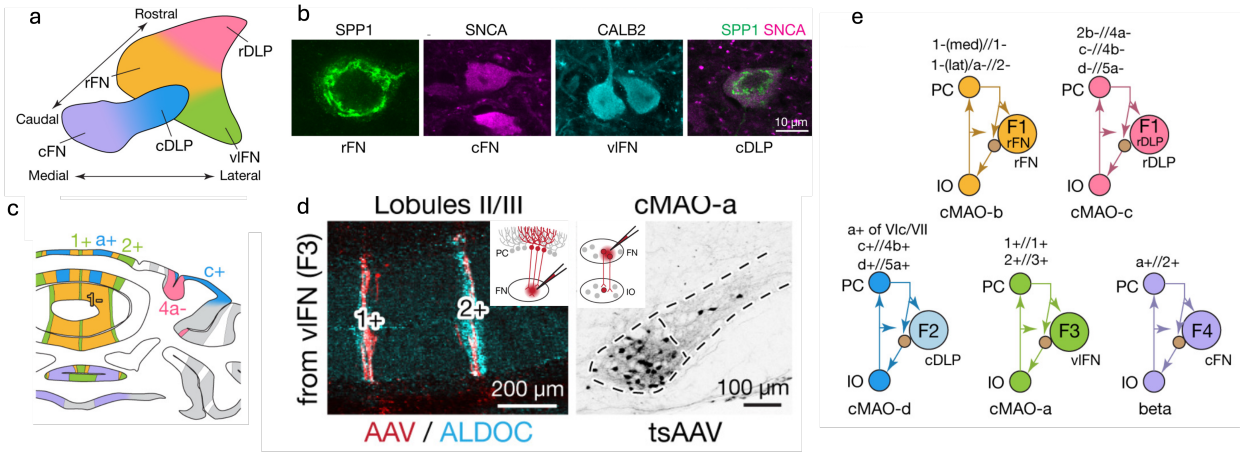

Figure 3: Summary of relevant experiments from [5]. (a) Subdivisions of the Fastigial Nucleus. (b) Genetic markers reliably stain neuronal subpopulations of the fastigial nucleus with distinct connectivity to the inferior olive and cerebellar cortex. (c) Subdivisions of the mouse cerebellar cortex based on alternating Aldolase C positive and negative longitudinal stripes (d) Left: retrograde tracer injections into the vlFN (F3) region of the fastigial nucleus labels two distinct stripes within lobules II/III of the cerebellar cortex. Right: anterograde injections into the vlFN (F3) label the cMAO-a subdivision of the inferior olive. (e) Fujita et al. suggest that the medial cerebellum of the mouse can be divided into five distinct zones containing particular portions of the cerebellar cortex, inferior olive, and Fastigial nucleus which form closed loops.